in each fraction is stained with Coomassie blue (FIG. 22A). Identical fractions are separated and transferred to PVDF for detection of autoantigens by probing with serum from a human TBI subject (FIG. 22B). The overlapping bands on the stained gel are excised and subjected to sequence analysis. Sequence analyses reveal that the autoantibodies present in human serum post-TBI recognize GFAP.

[00184] Antigen competition experiments confirm that autoantibodies present in human serum post-TBI recognize GFAP and Tau. Human brain lysates (300 μg) are probed with serum from three human TBI subjects alone or pre-incubated with of varying concentrations of GFAP (2.6 μg; 10 μg (Banyan Biomarkers)) or Tau (2.6 μg or 10 μg (Cytoskeleton Co.)). The presence of 2.5 μg of GFAP reduces the ability of GFAP specific autoantibodies to recognize GFAP present in brain lysates (FIG. 23). The signal is further reduced by 10 μg GFAP. Similarly, pre-incubation of serum with 10 μg Tau protein shows less antigen recognition than 2.6 μg Tau indicating the presence of Tau specific autoantibodies in human post-TBI serum (FIG. 23).

[00185] Autoantibodies to GFAP preferentially recognize GBDPs. Gels are loaded with human brain lysates and intact purified recombinant GFAP and probed with either an anti-GFAP (FIG. 24A) antibody, human post-TBI serum containing autoantibodies to GFAP (FIG. 24B), and or gels are stained with Coomassie blue (FIG. 23C). Autoantibodies show much greater recognition of GBDPs naturally derived from brain lysates than intact recombinant GFAP.

Example 9:

[00186] Detection of autoantibodies in studies of human TBI. Serum samples are obtained from human subjects following TBI at various timepoints. Table 10 illustrates the number of patients with samples provided at three different phases of TBI: acute + subacute phase (injury to day 10); severe TBI subacute phase (day 1 to day 30); and severe TBI –chronic phase (>1 mo post TBI).

[00187] **Table 10:**

	Cases	Auto-Antibody Positive	Percent (%)	95% CI
Normal	41	7	17.7	0.0821-0.3158
Severe TBI (Day 1-10) acute + subacute phase	28	21	75	0.5639-0.8758
Severe TBI (Day 1, Day 7, Day 30) –subacute phase	10	6	60	03116-0.8329
Severe TBI (1 mo. To 3 yr. post injury)- chronic phase	28	21	75	0.5639-0.8758

[00188] Confidence intervals are established by modified Wald methods. Overall, the number of TBI patients presenting with autoantibodies in their serum is much greater than normal controls.

[00189] The intensity of autoantibodies is measured by western blot using the procedures described in Example 8. An intensity level of 5 is scored the highest level of autoantibodies with an intensity level of zero representing no detectable autoantibody by the western blotting procedures. Healthy controls are compared to study samples obtained by Banyan Biomarkers, UP, and Italy study hosts. The majority of TBI patients had detectable levels of autoantibodies in their serum (FIG. 25).

Example 10:

[00190] An additional study of human subjects diagnosed with TBI is performed to analyze the correlation of the presence of autoantibodies with survival. Serum samples are obtained from twenty human subjects with severe (GCI score of 3-5) or mild (GCI score of 6-15) TBI. Table 11 illustrates the characteristics of subjects in the study.

[00191] **Table 11:**

TBI (HU-SZ)	
n	20
Age, years Mean (SD)	52± 21
Range	24-86
F/M, n (%)	3/17 (15/85)
Ethnicity	
Not Hispanic or Latino	20
Race	
Caucasian	20
Best GCS	
3-5	10
6-15	9
Mechanism of injury:	
Motor vehicle	7
Motor cycle	1
Fall	11
Other	1

[00192] Overall, 6 (30 percent) of subjects are negative for autoantibodies in their serum. Seventy percent show positive autoantibody development with 10 percent showing highly positive autoantibody levels as illustrated in Table 12.

[00193] **Table 12:**

Intensity	Frequency	Percent
Negative	6	30
Positive(1-4)	12	60
Highly Positive (5)	2	10

[00194] **Table 13:**

Survival 6 months	Frequency	Percent
Favourable Outcome	5	27.78
Unfavourable Outcome	13	72.22

[00195] Overall, more male and female subjects present autoantibodies than not (FIG. 26A). Of subjects less than 56 years of age more present with autoantibodies than those who do not. For subjects from 57 to 71 years and 72 to 84 years two subjects present with autoantibodies and two subjects do not (FIG. 26B).

[00196] The presence of autoantibodies is relatively greater in subjects with a GCS score of 6-15 although more subjects with a GSC score of 3-5 are also autoantibody positive (FIG. 27). Interestingly, the subjects with unfavorable outcome (death <6 mo) show a higher propensity for the development of autoantibodies in serum than do subjects with a favorable outcome (FIG. 28). This indicates that the presence of autoantibodies correlates with survival.

[00197] The level of autoantibodies on the 5 point intensity scale correlates with the levels of GFAP and UCHL1 in serum taken within 24 hours of TBI. Serum samples from the human subjects taken within 24 hours after TBI are also analyzed for the presence and level of UCHL1, GFAP, and SBDP145 by ELISA essential as described in Example 2. These levels are then plotted against the intensity of autoantibodies. As depicted in FIG. 29 increased levels of GFAP and UCHL1 correlate with increased intensity of autoantibodies. GFAP correlates with a Pearson correlation coefficient of 0.72 and a P-value of 0.0009. UCHL1 correlates with a Pearson correlation of 0.56 and a P-value of 0.02.

[00198] Similar correlations are observed in CSF taken from human subjects within 24 hours after TBI. The level of GFAP correlates to later development of autoantibodies with a Pearson correlation of 0.47 and a P-value of 0.07. The levels of UCHL1 and autoantibody intensity correlate with a Pearson correlation of 0.56 and a P-value of 0.02. The level of SBDP145 also correlates with autoantibody intensity demonstrating a statistically significant Pearson correlation of 0.62 and a P-value of 0.01. (FIG. 30) Thus, the level of autoantibodies correlates with the level of soluble biomarker proteins in both serum and CSF in human subjects.

[00199] The relative abundance of autoantibodies in the subjects' serum is measured by western blot by similar techniques to those described in Example 9 with a maximal intensity level of 5. Overall, female subjects show a higher autoantibody intensity than do males (FIG. 31A). Among those with a favorable outcome (survival >6 mo.) male subjects have a higher

autoantibody intensity than do females (FIG. 31B). Overall, while female subjects show reduced mortality, they also show higher autoantibody intensity and while males show higher mortality, the autoantibody intensity is lower (FIG. 31C). These data suggest that there is an inverse gender correlation between autoantibody intensity and outcome.

[00200] When the average intensity is correlated with survival independent of gender, there is no observable correlation between autoantibody intensity and survival of greater than 6 months (FIG. 32A). Thus, intensity functions as a predictor of outcome only on a gender dependent level. The presence of autoantibodies independent of their intensity also functions to predict outcome on a gender dependent level. Among female subjects, the presence of autoantibodies correlated with increased survival even thought the intensity is lower on average (FIG. 32B). Female non-survivors are less likely to show the presence of autoantibodies, yet the intensity is higher (FIG. 32B). Thus, among female study subjects the presence of low levels of autoantibodies suggests improved outcome relative to the presence of high levels of autoantibodies. Among male study subjects, while fewer study subjects with a favorable outcome show the presence of autoantibodies, those with a higher intensity are more likely to survive (FIG. 32C). Non-survivor males are more likely to have autoantibodies present, but their intensity is lower (FIG. 32C). Overall, the frequency and intensity of autoantibodies correlate with outcome in a gender dependent manner.

[00201] The intensity of autoantibodies does trend higher with the severity of TBI as measured by GCS score (FIG. 33). Subjects with a GCS value of 3 show much higher autoantibody intensity than do subjects with a higher GCS score of 7. A reducing trendline with increasing GCS score suggests that subjects with greater autoantibody intensity are more likely to have reduced GCS score and greater TBI severity.

[00202] **Example 11:**

[00203] Serum or CSF levels of nucleic acid biomarkers as a measure of neuronal injury. Just as proteins enter the CSF and plasma compartments after organ or brain injury, so do RNA and DNA. Cells undergoing apoptosis or necrosis release nucleic acid biomarkers into the blood or CSF (FIG. 34). The severity and site of injury is identifiable by appropriate real-time PCR assays to quantify the amount of cell type specific RNAs. Examples of neural-specific RNAs include beta III tubulin, UCHL1, GFAP, and synaptophysin.

[00204] Whole blood samples obtained from human subjects of the study of Example 7 are used to determine the level of nucleic acids encoding GFAP or UCHL1 therein. FIG. 35 illustrates the presence of increased serum GFAP and UCHL1 protein levels in subjects with

severe TBI. To determine whether RNA encoding GFAP or UCHL1 is present in serum, analyses of serum by techniques similar to that described by Rainer, TH, et al., *Clin Chem*, 2003; 49:562-560, are used. Briefly, 10-mL of subject blood is withdrawn from the antecubital vein of each subject upon admission, collected into tubes containing EDTA, and double centrifuged at 3000x*g* for 15 min. Alternatively or in addition, the plasma is filtered to remove cellular material by centrifugation through a 0.2 micrometer filter. The supernatant cell free plasma is transferred into plain polypropylene tubes containing RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics) and stored at -80 °C pending further processing.

[00205] Poly(A)$^+$ mRNA is extracted using the mRNA Isolation Kit for Blood and Bone Marrow (Roche Diagnostics) according to the manufacturer's protocol. cDNA is synthesized and amplified in a 50-μL PCR reaction using the Qiagen One Step RT-PCR reagent set (Qiagen). In brief, the mRNA in 6 μL of plasma is reverse-transcribed with specific primers for GFAP or UCHL1, using One Step RT-PCR enzyme mixture (Qiagen). The resulting cDNA is measured by Taqman assay using primers and probes for GFAP: forward primer – ACATCGAGATCGCCACCTACA (SEQ ID NO: 45); reverse primer – GTCTGCACGGGAATGGTGAT (SEQ ID NO: 46); and labeled probe – AGCTGCTAGAGGGCGAGGAGAACCG (SEQ ID NO: 47) using an annealing temperature of 60 °C as per the protocol of Pattyn, F, et al, *Nucleic Acids Research*, 2003; 31(1):122-123. For detection of UCHL1 the primers an probes are: forward primer – ACTGGGATTTGAGGATGGATCAG (SEQ ID NO: 48); reverse primer – GCCTTCCTGTGCCACGG (SEQ ID NO: 49); labeled probe – AATGAGGCCATACAGGCAGCCCATG (SEQ ID NO: 50) using an annealing temperature of 60 °C. Overall, the presence of mRNA for both GFAP and UCHL1 are present in cell free plasma from subjects following TBI.

[00206] **Example 12:**

[00207] The presence of miRNAs related to TBI in humans is detected in human serum one day following TBI. Serum collected, made cell free by centrifugation at 3000x*g* for 15 min and moving through a 0.2 micrometer filter, and used to prepare total RNA. The total RNA (5 μg) is size fractionated (b300 nucleotides) by using a YM-100 Microcon centrifugal filter (Millipore). The collected small RNAs (b300 nt) are 3′-extended with a poly(A) tail using poly (A) polymerase. An oligonucleotide tag is ligated to the poly (A) tail for later fluorescent dye staining. The small RNAs are hybridized overnight on a μParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies). Each detection probe on the chip consists of a

chemically modified nucleotide coding segment complementary to target human miRNA sequences.

[00208] The miRNA sequences that show at least a two-fold increase or decrease in serum from TBI subjects versus serum from human control subjects are presented in Tables 14 and 15.

[00209] **Table 14:** Upregulated miRNA detected in human serum following TBI.

S14_hsa_miRNA name	S14_hsa_miRNA_Sequence	SEQ ID NO:	S14_hsa_miRNA_MIMAT	Fold Change Up > 2 Serum TBI / Serum Ctrl (Log2)
hsa-miR-744	UGCGGGGCUAGGGCUAACAGCA	51	MIMAT0004945	2.22
hsa-miR-762	GGGGCUGGGGCCGGGGCCGAGC	52	MIMAT0010313	2.27
hsa-miR-711	GGGACCCAGGGAGAGACGUAAG	53	MIMAT0012734	2.41
hsa-miR-484	UCAGGCUCAGUCCCCUCCCGAU	54	MIMAT0002174	2.46
hsa-miR-765	UGGAGGAGAAGGAAGGUGAUG	55	MIMAT0003945	2.58
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU	56	MIMAT0000063	2.61
hsa-miR-663	AGGCGGGGCGCCGCGGGACCGC	57	MIMAT0003326	2.90
hsa-miR-30c-1*	CUGGGAGAGGGUUGUUUACUCC	58	MIMAT0004674	2.94
hsa-let-7i	UGAGGUAGUAGUUUGUGCUGUU	59	MIMAT0000415	2.95
hsa-miR-1246	AAUGGAUUUUUGGAGCAGG	60	MIMAT0005898	3.02
hsa-miR-1275	GUGGGGGAGAGGCUGUC	61	MIMAT0005929	3.88
hsa-miR-1290	UGGAUUUUUGGAUCAGGGA	62	MIMAT0005880	4.43
hsa-miR-93	CAAAGUGCUGUUCGUGCAGGUAG	63	MIMAT0000093	4.68
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	64	MIMAT0004748	4.75
hsa-miR-1202	GUGCCAGCUGCAGUGGGGGAG	65	MIMAT0005865	5.90
hsa-miR-198	GGUCCAGAGGGGAGAUAGGUUC	66	MIMAT0000228	8.51
hsa-miR-320c	AAAAGCUGGGUUGAGAGGGU	67	MIMAT0005793	11.13
hsa-miR-320a	AAAAGCUGGGUUGAGAGGGCGA	68	MIMAT0000510	11.86
hsa-miR-320b	AAAAGCUGGGUUGAGAGGGCAA	69	MIMAT0005792	12.83
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU	70	MIMAT0000064	14.07
hsa-miR-24	UGGCUCAGUUCAGCAGGAACAG	71	MIMAT0000080	14.81
hsa-miR-320d	AAAAGCUGGGUUGAGAGGA	72	MIMAT0006764	14.91
hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG	73	MIMAT0002177	16.85
hsa-miR-425	AAUGACACGAUCACUCCCGUUGA	74	MIMAT0003393	18.11
hsa-miR-92a	UAUUGCACUUGUCCCGGCCUGU	75	MIMAT0000092	25.52
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU	76	MIMAT0000062	26.23

hsa-miR-25	CAUUGCACUUGUCUCGGUCUGA	77	MIMAT0000081	29.86
hsa-miR-483-5p	AAGACGGGAGGAAAGAAGGGAG	78	MIMAT0004761	30.87
hsa-miR-30d	UGUAAACAUCCCCGACUGGAAG	79	MIMAT0000245	33.21
hsa-miR-185	UGGAGAGAAAGGCAGUUCCUGA	80	MIMAT0000455	92.20
hsa-miR-223	UGUCAGUUUGUCAAAUACCCCA	81	MIMAT0000280	102.06
hsa-miR-451	AAACCGUUACCAUUACUGAGUU	82	MIMAT0001631	137.35
hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	83	MIMAT0000069	172.25

[00210] **Table 15:** Downregulated miRNA detected in human serum following TBI.

S14_hsa_miRNA name	S14_hsa_miRNA_Sequence	SEQ ID NO:	S14_hsa_miRNA_MIMAT	Fold Change Down> 2 Serum TBI / Serum Ctrl (Log2)
hsa-miR-675*	CUGUAUGCCCUCACCGCUCA	84	MIMAT0006790	0.27
hsa-miR-1910	CCAGUCCUGUGCCUGCCGCCU	85	MIMAT0007884	0.28
hsa-miR-1181	CCGUCGCCGCCACCCGAGCCG	86	MIMAT0005826	0.29
hsa-miR-1972	UCAGGCCAGGCACAGUGGCUCA	87	MIMAT0009447	0.30
hsa-miR-1229	CUCUCACCACUGCCCUCCCACAG	88	MIMAT0005584	0.30
hsa-miR-634	AACCAGCACCCCAACUUUGGAC	89	MIMAT0003304	0.31
hsa-miR-449b*	CAGCCACAACUACCCUGCCACU	90	MIMAT0009203	0.31
hsa-miR-885-5p	UCCAUUACACUACCCUGCCUCU	91	MIMAT0004947	0.35
hsa-miR-1825	UCCAGUGCCCUCCUCUCC	92	MIMAT0006765	0.35
hsa-miR-532-3p	CCUCCCACACCCAAGGCUUGCA	93	MIMAT0004780	0.35
hsa-miR-1224-3p	CCCCACCUCCUCUCUCCUCAG	94	MIMAT0005459	0.37
hsa-miR-1260	AUCCCACCUCUGCCACCA	95	MIMAT0005911	0.41
hsa-miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU	96	MIMAT0000446	0.41
hsa-miR-1233	UGAGCCCUGUCCUCCCGCAG	97	MIMAT0005588	0.42
hsa-miR-636	UGUGCUUGCUCGUCCCGCCCGCA	98	MIMAT0003306	0.42
hsa-miR-1228	UCACACCUGCCUCGCCCCCC	99	MIMAT0005583	0.43
hsa-miR-197	UUCACCACCUUCUCCACCCAGC	100	MIMAT0000227	0.43
hsa-miR-1226	UCACCAGCCCUGUGUUCCCUAG	101	MIMAT0005577	0.43
hsa-miR-1296	UUAGGGCCCUGGCUCCAUCUCC	102	MIMAT0005794	0.43
hsa-miR-328	CUGGCCCUCUCUGCCCUUCCGU	103	MIMAT0000752	0.44
hsa-miR-1976	CCUCCUGCCCUCCUUGCUGU	104	MIMAT0009451	0.45
hsa-miR-574-3p	CACGCUCAUGCACACACCCACA	105	MIMAT0003239	0.46

hsa-miR-766	ACUCCAGCCCCACAGCCUCAGC	106	MIMAT0003888	0.46
hsa-miR-1280	UCCCACCGCUGCCACCC	107	MIMAT0005946	0.46
hsa-miR-1470	GCCCUCCGCCCGUGCACCCCG	108	MIMAT0007348	0.46
hsa-miR-125b-1*	ACGGGUUAGGCUCUUGGGAGCU	109	MIMAT0004592	0.46
hsa-miR-1227	CGUGCCACCCUUUUCCCCAG	110	MIMAT0005580	0.47
hsa-miR-615-3p	UCCGAGCCUGGGUCUCCCUCUU	111	MIMAT0003283	0.48
hsa-miR-1225-3p	UGAGCCCCUGUGCCGCCCCCAG	112	MIMAT0005573	0.48
hsa-miR-1285	UCUGGGCAACAAAGUGAGACCU	113	MIMAT0005876	0.49

[00211] The identified sequences are subjected to a cluster analysis as depicted in FIG. 36.

[00212] Additional miRNAs with associated localizations are listed in Tables 16 and 17.

[00213] **Table 16:** Examples of brain specific or brain enriched miRNAs.

MicroRNA Name	Brain region localization	Other characteristics
miR-92b	Ventral and dorsal subpallium, pallium, thalamus (dorsal (DT) and ventral (VT) , hypothalamus, retectum, tegmentum and hindbrain as well as in the tectal proliferative zone , rhombic lip and retinal ciliary marginal zone	Neuronal precursors and stem cells
miR-124	Larval brain and retina	Proliferation to differentiation
miR-124	Mature neuron	
miR-222	telecephalon	
miR-135	Larval brain	
miR-9	Telencephalic,diencephalic and tectal periventricular proliferative zones as well as the mature neuron	
miR-183	Retinal photoreceptors, peripheral sensory neuromasts, olfactory sensory neurons and hair cells of the ear.	
miR-34,	Habenular cells of the adult brain	
miR-218a	Motor neurons	

Seven Human brain-specific miRNAs: miR-9, -124a, -124b, -135, -153, -183,-219

Seven human brain-enriched miRNAs: miR-9*, -125a, -125b, -128, -132, -137, -139

[00214] **Table 17:** miRNAs with putative targets and functions.

miRNA	Putative mRNA targets	Function/disease
rno-miR-130a	synaptotagmin VI, CRMP-2	polyQ disease
rno-miR-140*	**CRMP-2**, CAPN1	TBI, MCAO
rno-miR-145	CRMP-2, insulin receptor substrate-1 (IRS-1)	MCAO, colon cancer
rno-miR-135a	**Synaptotagmin-1**, Smad5	Brain enriched, Colorectal Cancer
miR-124	CAPN1, 2, 6,	Brain specific, neurogenesis
rno-miR-34b	**MBP**, synaptotagmin IX, Rho-kinase, p53	Apoptosis/chronic lymphocytic leukemia
rno-miR-19b	SYTL1, calpastatin , synapsin I, CRMP-1, syntaphilin, ATXN1	MCAO, TBI, polyQ disease

[00215] The presence of miRNA associated with regulation of GFAP is also measured in cell free plasma samples as described above. The miRNA-125b is increased in subjects with degenerative neuronal conditions. Pogue, AI, et al, *Neurosci. Lett.*, 2010; 476:18-22. Using the same primers and probes to detect miRNA-125b, increased levels of this miRNA are detected in cell free plasma from subjects following TBI. Briefly, total RNA from the cell free plasma is extracted with miRNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA purity is determined by spectrophotometric determination at 260/280 nm and 260/230 nm. An absorbance ration of 260/280nm greater than 1.8 is usually considered an acceptable indicator of RNA purity. 10 ng of total RNA from each sample is then reverse-transcribed to cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and miRNA specific primers (Applied Biosystems). The presence of the miRNAs identified by Dharap, A, and Vemuganti, R, *J. Neurochem.*, 2010; 113:1685–1691 are detected in cell free plasma in TBI subjects as well as in an independent set of subjects following ischemic stroke. Also, the levels of the miRNAs identified by Lei, P, et al., *Brain Research*, 2009; 1284:191-201 are examined for presence in cell free plasma.

Example 13:

[00216] Sequence of human GFAP (SEQ ID NO: 114)

```
  1 MERRRITSAA RRSYVSSGEM MVGGLAPGRR LGPGTRLSLA RMPPPLPTRV DFSLAGALNA
 61 GFKETRASER AEMMELNDRF ASYIEKVRFL EQQNKALAAE LNQLRAKEPT KLADVYQAEL
121 RELRLRIDQL TANSARLEVE RDNLAQDLAT VRQKLQDETN LRLEAENNLA AYRQEADEAT
181 LARLDLERKI ESLEEEIRFL RKIHEEEVRE LQEQLARQQV HVELDVAKPD LTAALKEIRT
241 QYEAMASSNM HEAEEWYRSK FADLTDAAAR NAELLRQAKH EANDYRRQLQ SLTCDLESLR
301 GTNESLERQM REQEERHVRE AASYQEALAR LEEEGQSLKD EMARHLQEYQ DLLNVKLALD
361 IEIATYRKLL EGEENRITIP VQTFSNLQIR ETSLDTKSVS EGHLKRNIVV KTVEMRDGEV
421 IKESKQEHKD VM
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[00217] Patent documents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. These documents and publications are incorporated herein by reference to the same extent as if each individual document or publication was specifically and individually incorporated herein by reference.

[00218] The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. The following claims, including all equivalents thereof, are intended to define the scope of the invention.

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CLAIMS

1. A process for detecting a neurological condition comprising:

measuring a quantity of one or more neuron specific biomarkers in a biological sample, wherein the synthesis of said biomarker is altered following an injury; and

detecting a neurological condition based on a ratio of the quantity one or more of said biomarkers in said biological sample.

2. The process of claim 1 wherein said condition is selected from the group comprising:

brain injury, multiple-organ injury, stroke, neurodegenerative disease, including Alzeimer's disease, Pakinson disease and Chronic Traumatic Encephalopathy (CTE), or combinations thereof.

3. The process of claim 1 wherein said biomarker is an autoantibody directed toward a protein, wherein said protein is: GFAP; Tau; S100β; βIII-tubulin; Neuroflilament light, medium or heavy polypeptide (NF-L, -M and –H); V-type proton ATPase; Gamma-enolase (NSE); vimentin; endophilin-A1; Microtubule-associated protein 2 (MAP-2); alpha-internexin; neuroserpin; neuromodulin; synaptotagmin-1; Voltage-gated potassium channel; collapsin response mediator proteins (CRMP-1 to 5); αII-spectrin; neurofascin; MBP; ubiquitin carboxyl-terminal esterase; Poly (ADP-ribose) polymerase (PARP); breakdown products thereof; derivatives thereof; or combinations thereof.

4. The process of claim 1 wherein said biomarker is a nucleic acid encoding at least a portion of a protein, wherein said protein is GFAP; αII-spectrin; an αII-spectrin breakdown product; neurofascin; MBP; MAP2; ubiquitin carboxyl-terminal esterase; a ubiquitin carboxyl-terminal hydrolase; a neuronally-localized intracellular protein; MAP-tau; C-tau; Poly (ADP-ribose) polymerase (PARP); a collapsin response mediator protein; breakdown products thereof, derivatives thereof, or combinations thereof.

5. The process of claim 1 wherein said biomarker is a at least a portion of a miRNA that regulates the expression of a protein, wherein said protein is GFAP; αII-spectrin; an αII-spectrin breakdown product; neurofascin; MBP; MAP2; ubiquitin carboxyl-terminal esterase; a

ubiquitin carboxyl-terminal hydrolase; a neuronally-localized intracellular protein; MAP-tau; p53; SYTL1; calpastatin; Poly (ADP-ribose) polymerase (PARP); CAPN1, 2, or 6; IRS-1; SMAD5; a collapsin response mediator protein; synaptotagmin-1 or -9; Rho kinase; synapsin 1; syntaphilin; ATXN1; derivatives thereof; or combinations thereof.

6. The process of claims 1 or 5 wherein said miRNA is one of SEQ ID NOs: 51-113.

7. The process of claims 1 or 5 wherein said miRNA is one of SEQ ID NOs: 51-60 or 84-93.

8. The process of claims 1 or 5 wherein said miRNA is miR-9, miR-34, miR-92b, miR-124a, miR-124b, miR-135, miR-153, miR-183, miR-219, miR-222, miR-125a, miR-125b, miR-128, miR-132, miR-135, miR-137, miR-139, miR-218a, or combinations thereof.

9. The process of claim 1 wherein said biomarker is a breakdown product of GFAP or Tau.

10. The process of claim 9 wherein said breakdown product of GFAP is formed by cleavage at Asn59, Thr383, or both, in SEQ ID NO: 114.

11. The process of claims 1 or 9 wherein said biomarker is a breakdown product of human Tau (SEQ ID NO: 11) from cleavage C-terminal to amino acid 25, 44, 129, 157, 229, 421, or combinations thereof.

12. The process of claim 1 or 9 wherein biomarker is a breakdown product of rat Tau (SEQ ID NO: 5) from cleavage C-terminal to amino acid 43, 120, 220, 370, 412, or combinations thereof.

13. The process of claim 1 wherein said ratio is the concentration of said biomarker following said injury relative to the concentration of said biomarker in a control subject.

14. The process of claim 1 wherein said ratio is positive.

15. The process of claims 1, 3, 4, 5, 6, or 9 wherein said biological sample is blood, serum, plasma, CSF, urine, saliva or tissue.

16. The process of claim 1 wherein said injury is induced by a compound.

17. A process of diagnosing a traumatic brain injury in a subject comprising:

obtaining a biological sample from a subject following an impact;

measuring the quantity of one or more biomarkers of a neurological condition in said biological sample, wherein said biomarker is an autoantibody, miRNA, or protein breakdown product; and

diagnosing a traumatic brain injury based on the altered quantity of said biomarker in said biological sample relative to a control subject.

18. The process of claim 17 wherein said biomarker is: a GFAP breakdown product; an autoantibody directed to GFAP or a GFAP break down product; a nucleic acid encoding at least a portion of GFAP; or a miRNA sequence that regulates the expression of GFAP.

19. The process of claim 17 wherein said biomarker is: a Tau breakdown product.

20. The process of claim 17 wherein said autoantibody binds: GFAP; neuroflilament light; medium and heavy polypeptide (NF-L, -M and –H); V-type proton ATPase; Gamma-enolase (NSE); vimentin; endophilin-A1; Microtubule-associated protein 2 (MAP-2); alpha-internexin; neuroserpin; neuromodulin; synaptotagmin-1; voltage-gated potassium channel; collapsin response mediator proteins (CRMP-1 to 5); or a breakdown product thereof.

21. The process of claim 17 wherein said biomarker is at least a portion of a miRNA sequence that regulates the expression of GFAP; a ubiquitin carboxyl-terminal esterase; a ubiquitin carboxyl-terminal hydrolase; β-III tubulin; or synaptophysin.

22. The process any one of claims 17-21 wherein said biological sample is: whole blood; plasma; serum; CSF; urine; saliva; or tissue.

23. The process of claims 17 or 21 wherein said biological sample is cell free plasma.

24. A detection agent to a breakdown product of GFAP, said detection agent binding a portion of human GFAP within 10 amino acids of the cleavage site at amino acid position 59 or 383 in SEQ ID NO: 114.

25. A detection agent to a breakdown product of GFAP, said detection agent binding at least a portion of SEQ ID NOs 139, 140, 141, or 142.

26. A detection agent to a breakdown product of human Tau, said detection agent binding a human Tau (SEQ ID NO: 11) amino sequence within ten amino acids of the cleavage site at amino acid position 25, 44, 129, 157, 229, or 421.

27. The detection agent of claim 26 that binds an amino acid sequence within 10 amino acids C-terminal to Ser129, within 10 amino acids N-terminal to Val229, within 10 amino acids N-terminal to Asp421, within 10 amino acids C-terminal to Lys44, within 10 amino acids N-terminal to Ser129, within 10 amino acids C-terminal to amino acid position 25, within 10 amino acids N-terminal to 25, within 10 amino acids N-terminal to 157, within 10 amino acids C-terminal to 157, within 10 amino acids N-terminal to Lys44, within 10 amino acids C-terminal to Val229, within 10 amino acids C-terminal to Asp421.

28. The detection agent of claim 26 that binds an amino acid sequence that is at least a portion of SEQ ID NOs: 20, 21, 24, 25, 26, 27, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, or 44.

29. A detection agent to a breakdown product of human Tau that binds at least a portion of SEQ ID NO: 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, or 138.

30. A detection agent to a breakdown product of rat Tau, said detection agent binding a rat Tau (SEQ ID NO: 5) amino sequence within ten amino acids of the cleavage site at amino acid position 43, 121, 229, 38, or 412.

31. The detection agent of claim 30 wherein said detection agent binds an amino acid sequence within 10 amino acids N-terminal to Asp421, within 10 amino acids N-terminal to

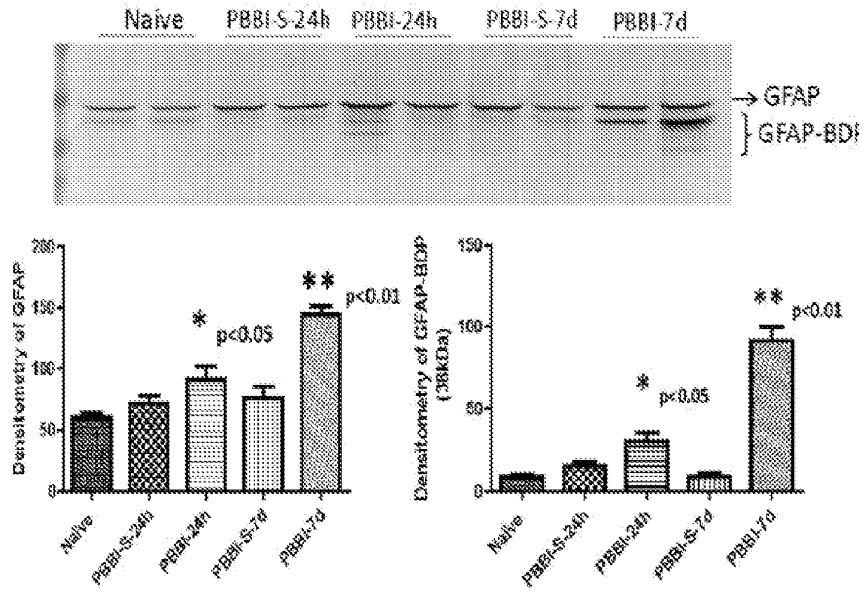
Val220, within 10 amino acids C-terminal to Ser120, within 10 amino acids C-terminal to Lys43, or within 10 amino acids C-terminal to Arg370.

30. The detection agent of claim 30 that binds an amino acid sequence that is at least a portion of at least one of SEQ ID NOs: 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 38, 39, 40, or 41.

31. A detection agent to a breakdown product of rat Tau that binds at least a portion of SEQ ID NO: 115, 116, 117, 118, 119, 120, 121, 122, 123, or 124.

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EX-1045
2426

A


Naive
PBBI-S-24h
PBBI-24h
PBBI-S-7d
PBBI-7d
GFAP
GFAP-BDI
Densitometry of GFAP
Densitometry of GFAP-BDP (38kDa)
*
p<0.05
**
p<0.01

B

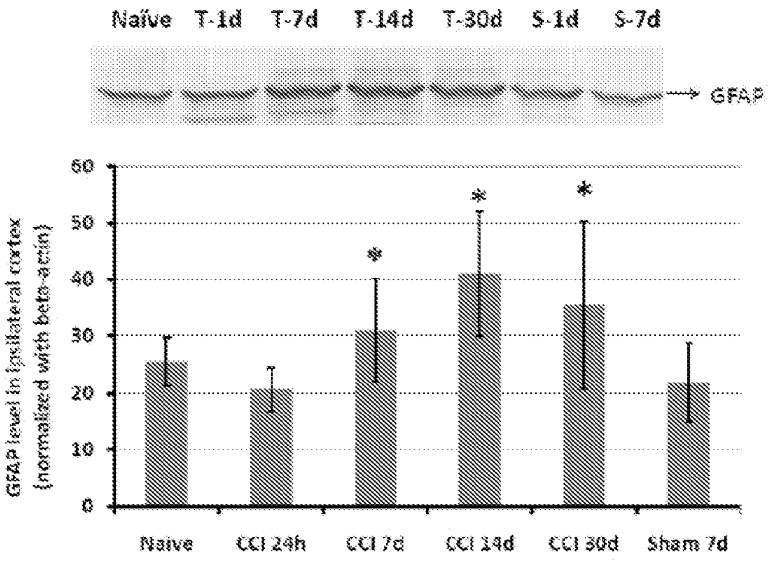

Naive
T-1d
T-7d
T-14d
T-30d
S-1d
S-7d
GFAP
GFAP level in ipsilateral cortex (normalized with beta-actin)
Naive
CCI 24h
CCI 7d
CCI 14d
CCI 30d
Sham 7d

FIG. 1

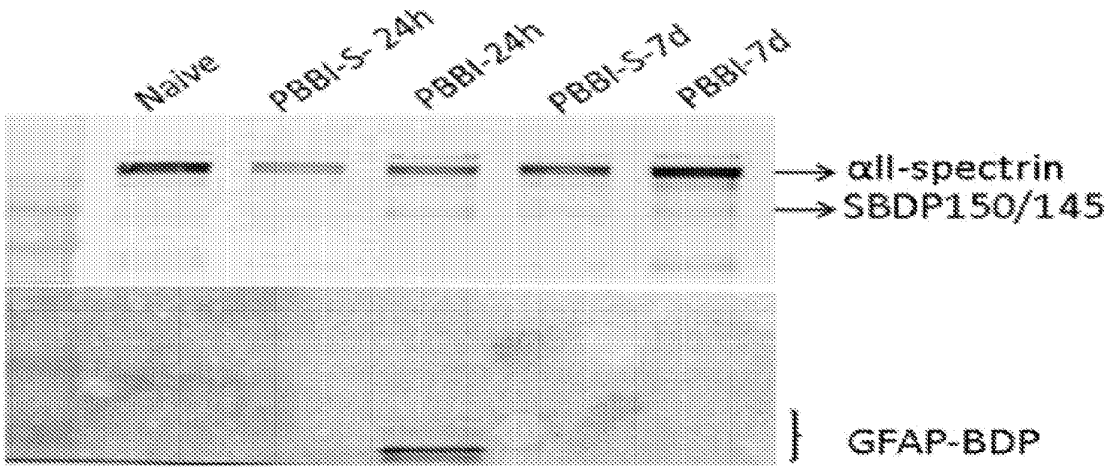
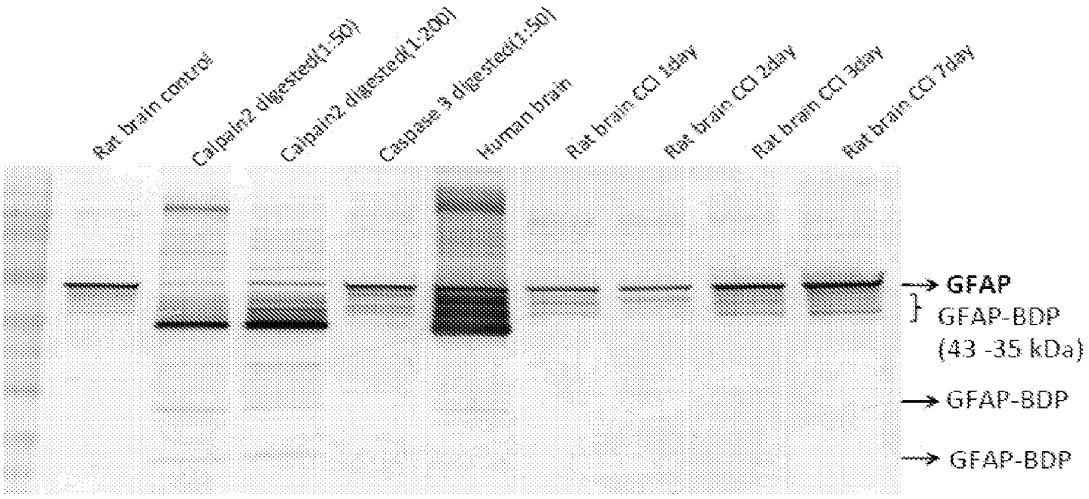

Naive
PBBI-S- 24h
PBBI-24h
PBBI-S-7d
PBBI-7d
αII-spectrin
SBDP150/145
GFAP-BDP

FIG. 2

A


Rat brain control
Calpain2 digested(1:50)
Calpain2 digested(1:200)
Caspase 3 digested(1:50)
Human brain
Rat brain CCI 1day
Rat brain CCI 2day
Rat brain CCI 3day
Rat brain CCI 7day
GFAP
GFAP-BDP
(43 -35 kDa)
GFAP-BDP
GFAP-BDP

B

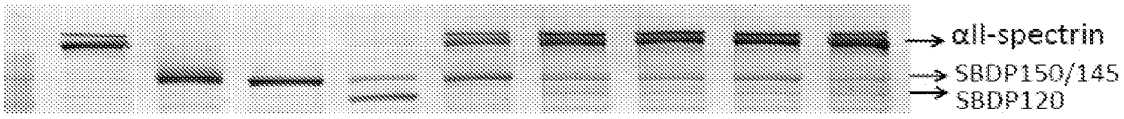

αII-spectrin
SBDP150/145
SBDP120

FIG. 3B

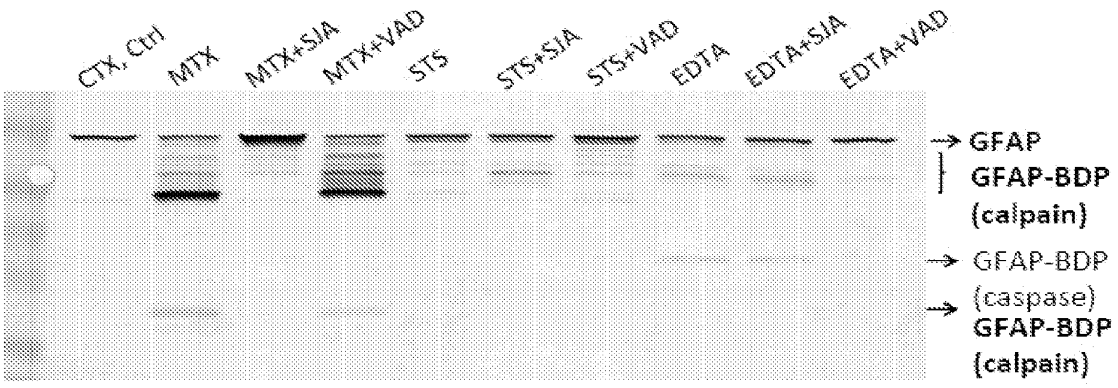

CTX, Ctrl
MTX
MTX+SJA
MTX+VAD
STS
STS+SJA
STS+VAD
EDTA
EDTA+SJA
EDTA+VAD
GFAP
GFAP-BDP
(calpain)
GFAP-BDP
(caspase)
GFAP-BDP
(calpain)

FIG. 4

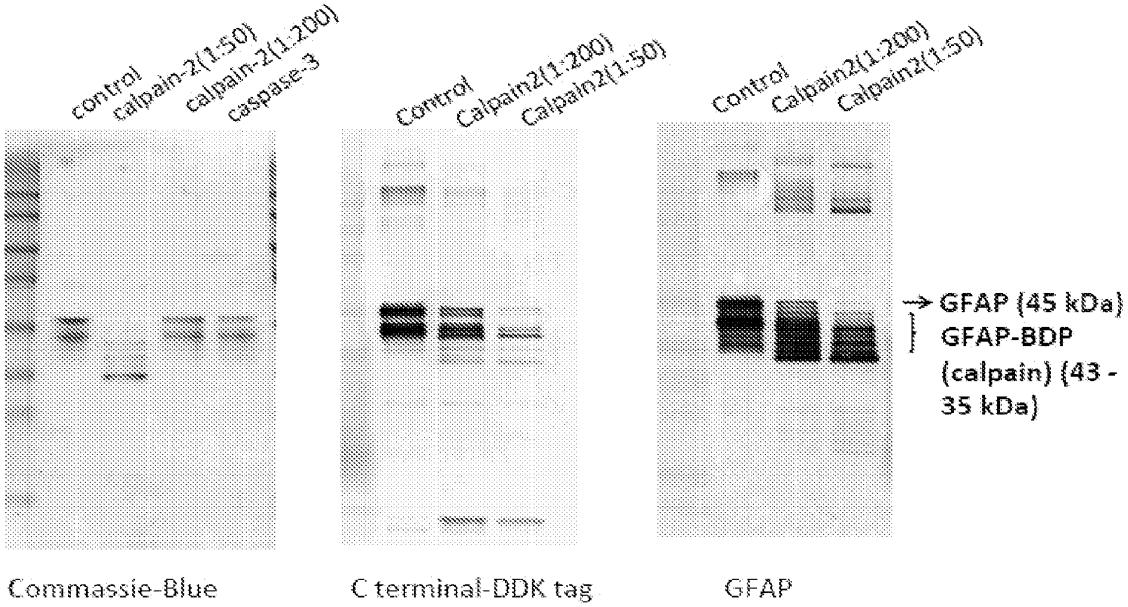

control
calpain-2(1:50)
calpain-2(1:200)
caspase-3
Control
Calpain2(1:200)
Calpain2(1:50)
Control
Calpain2(1:200)
Calpain2(1:50)
GFAP (45 kDa)
GFAP-BDP
(calpain) (43 -
35 kDa)
Commassie-Blue
C terminal-DDK tag
GFAP

FIG. 5

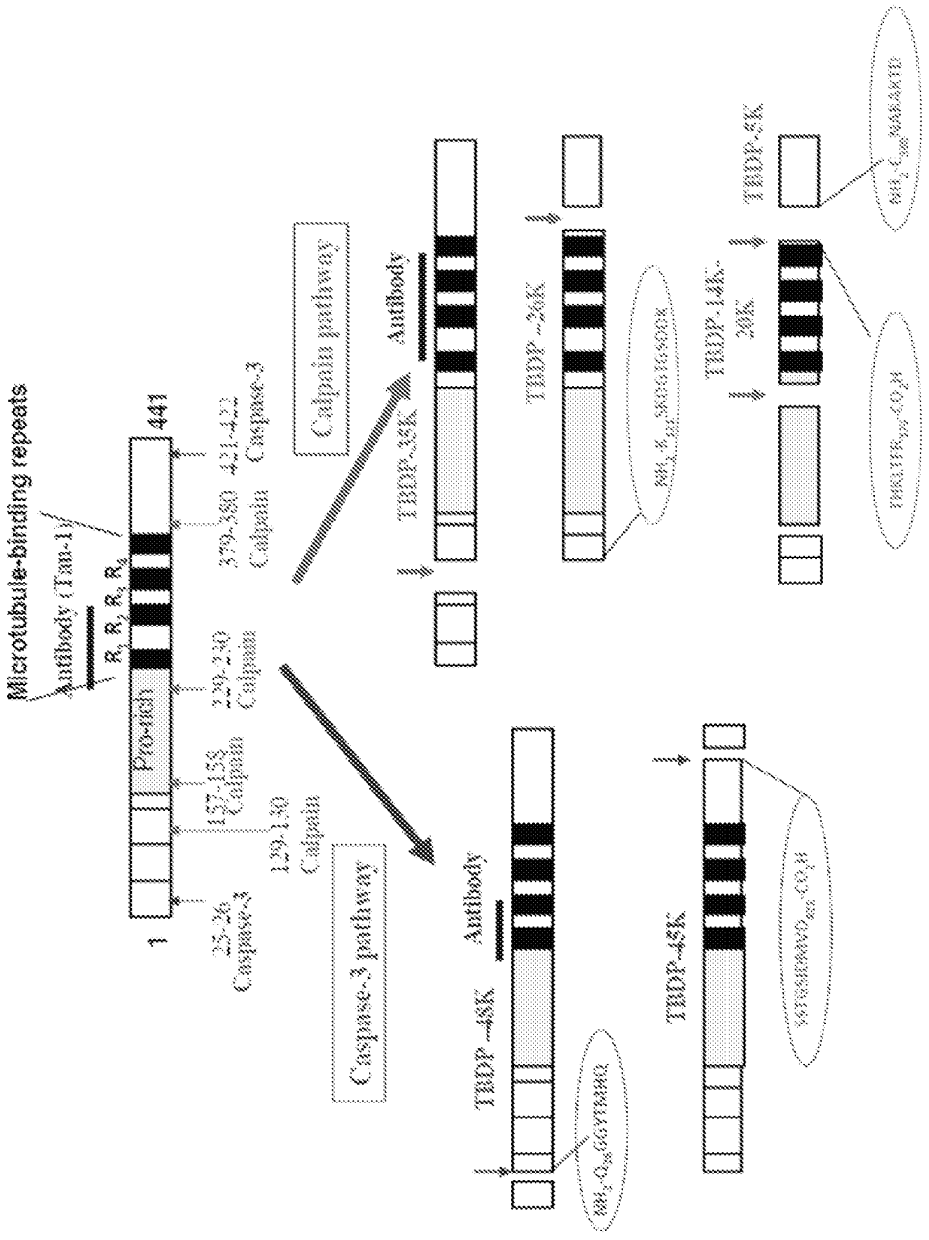

Microtubule-binding repeats
Antibody (Tau-1)
R1 R2 R3 R4
Pro-rich
1
441
25-26 Caspase-3
129-130 Calpain
157-158 Calpain
229-230 Calpain
379-380 Calpain
421-422 Caspase-3
Calpain pathway
Caspase-3 pathway
Antibody
TBDP-35K
TBDP-48K
TBDP-26K
TBDP-14K-20K
TBDP-5K

FIG. 6

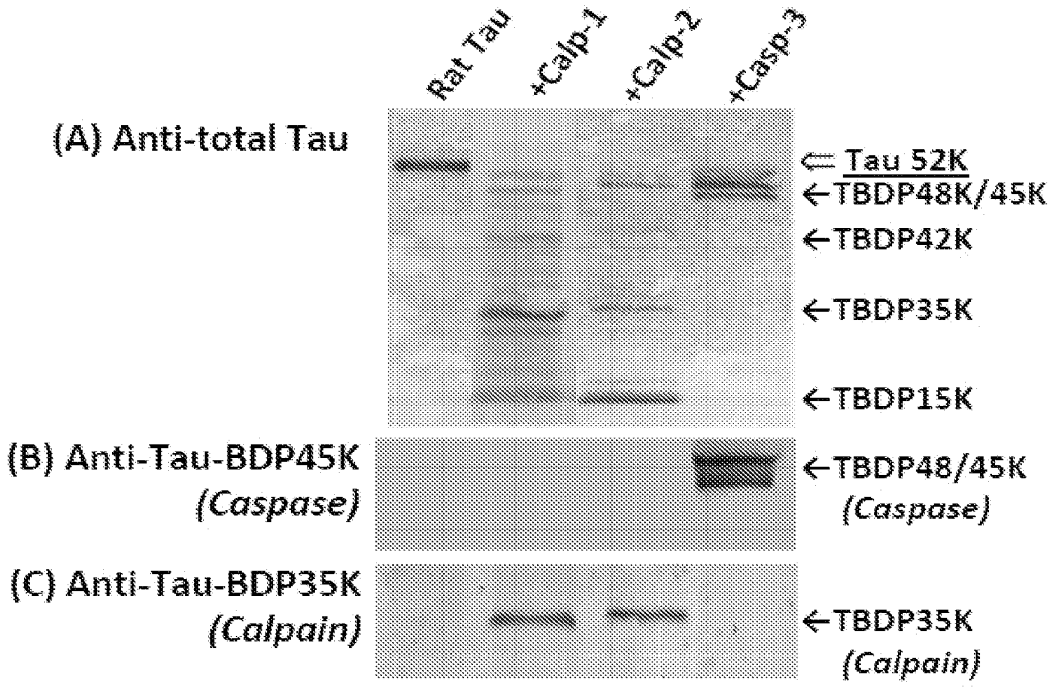
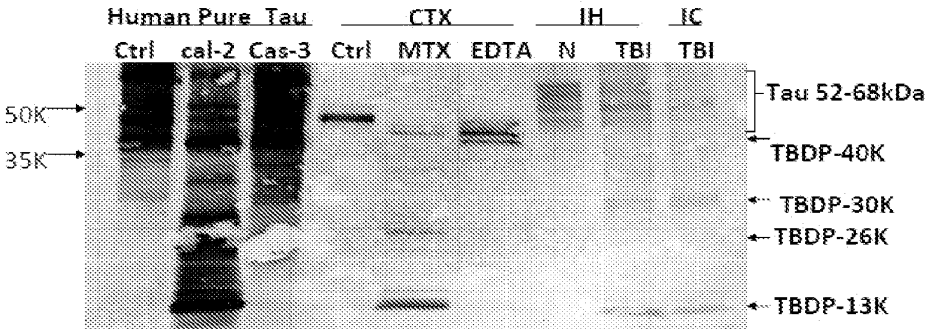
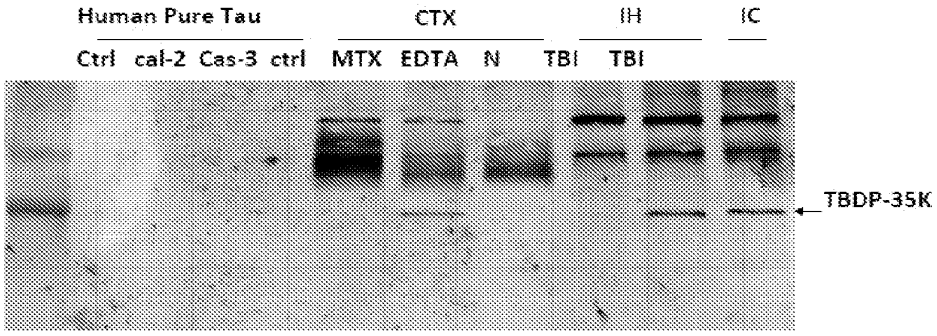

Rat Tau
+Calp-1
+Calp-2
+Casp-3
(A) Anti-total Tau
⇐ Tau 52K
←TBDP48K/45K
←TBDP42K
←TBDP35K
←TBDP15K
(B) Anti-Tau-BDP45K
(Caspase)
←TBDP48/45K
(Caspase)
(C) Anti-Tau-BDP35K
(Calpain)
←TBDP35K
(Calpain)

FIG. 7

A


Human Pure Tau
CTX
IH
IC
Ctrl
cal-2
Cas-3
Ctrl
MTX
EDTA
N
TBI
TBI
50K
35K
Tau 52-68kDa
TBDP-40K
TBDP-30K
TBDP-26K
TBDP-13K

B


Human Pure Tau
CTX
IH
IC
Ctrl
cal-2
Cas-3
ctrl
MTX
EDTA
N
TBI
TBI
TBDP-35K

C

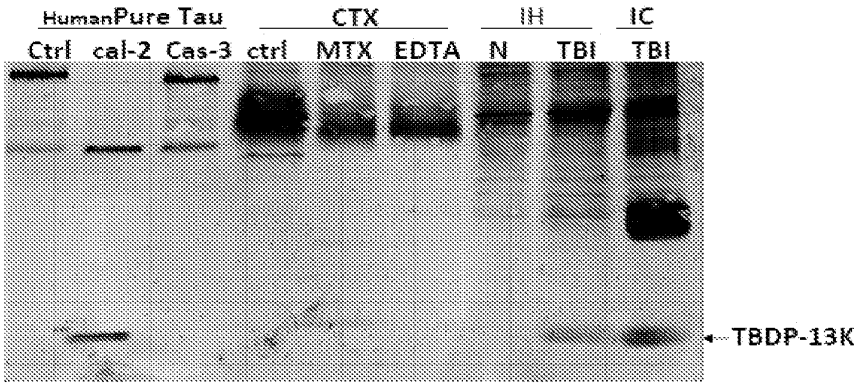

HumanPure Tau
CTX
IH
IC
Ctrl
cal-2
Cas-3
ctrl
MTX
EDTA
N
TBI
TBI
TBDP-13K

FIG. 8

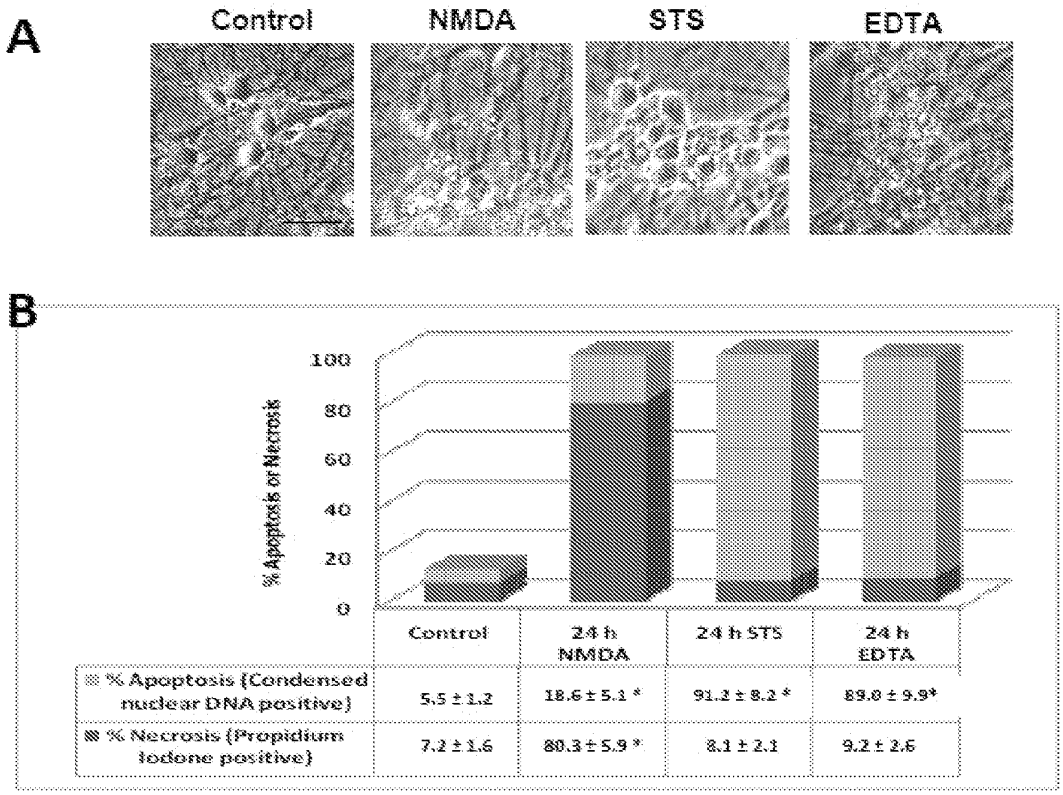

A
Control
NMDA
STS
EDTA
B
% Apoptosis or Necrosis
100
80
60
40
20
0
Control
24 h NMDA
24 h STS
24 h EDTA
% Apoptosis (Condensed nuclear DNA positive)
5.5 ± 1.2
18.6 ± 5.1 *
91.2 ± 8.2 *
89.0 ± 9.9*
% Necrosis (Propidium Iodone positive)
7.2 ± 1.6
80.3 ± 5.9 *
8.1 ± 2.1
9.2 ± 2.6

FIG. 9

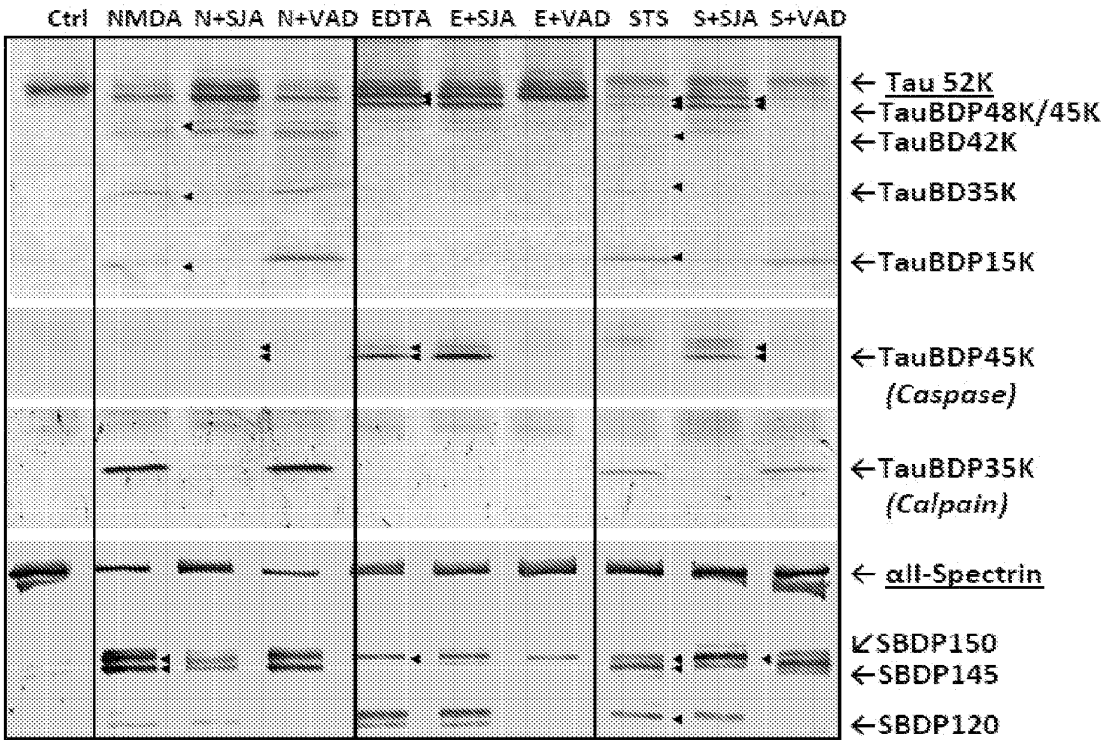

Ctrl
NMDA
N+SJA
N+VAD
EDTA
E+SJA
E+VAD
STS
S+SJA
S+VAD
← Tau 52K
←TauBDP48K/45K
←TauBD42K
←TauBD35K
←TauBDP15K
←TauBDP45K
(Caspase)
←TauBDP35K
(Calpain)
← αII-Spectrin
↙SBDP150
←SBDP145
←SBDP120

FIG. 10

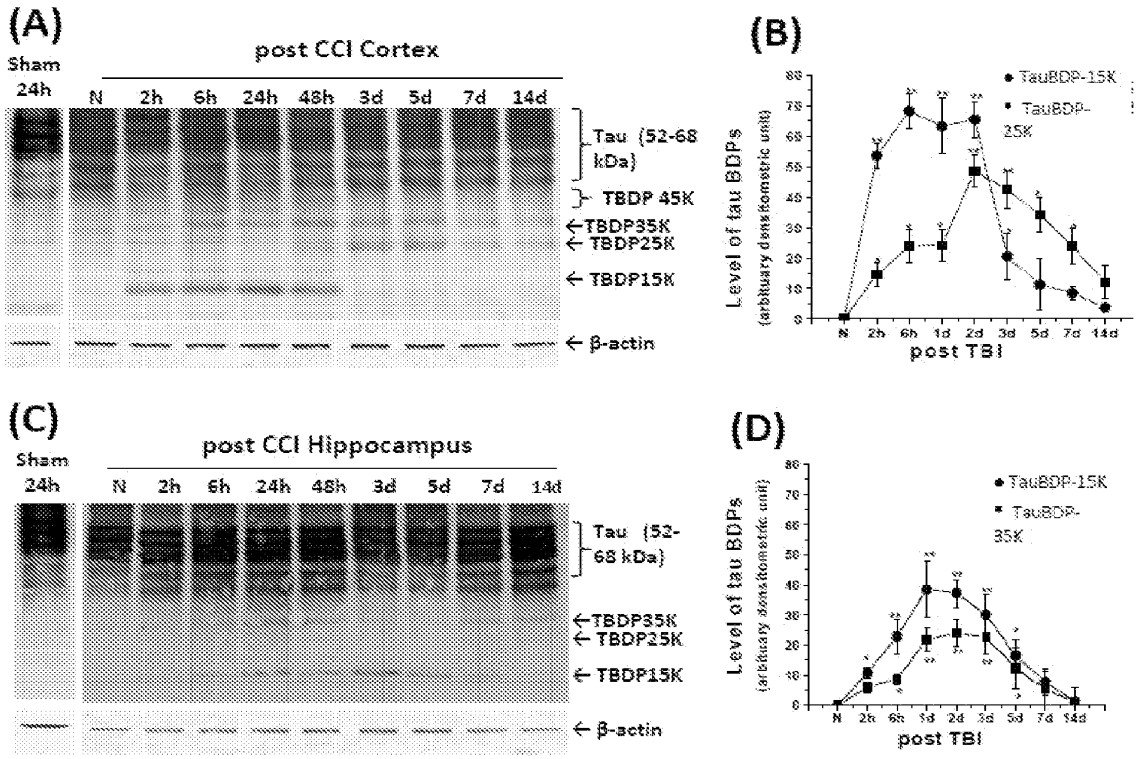

(A)
Sham 24h
post CCI Cortex
N 2h 6h 24h 48h 3d 5d 7d 14d
Tau (52-68 kDa)
TBDP 45K
←TBDP35K
← TBDP25K
← TBDP15K
← β-actin
(B)
TauBDP-15K
TauBDP-25K
Level of tau BDPs
post TBI
(C)
Sham 24h
post CCI Hippocampus
N 2h 6h 24h 48h 3d 5d 7d 14d
Tau (52-68 kDa)
←TBDP35K
← TBDP25K
← TBDP15K
← β-actin
(D)
TauBDP-15K
TauBDP-35K
Level of tau BDPs
post TBI

FIG. 11

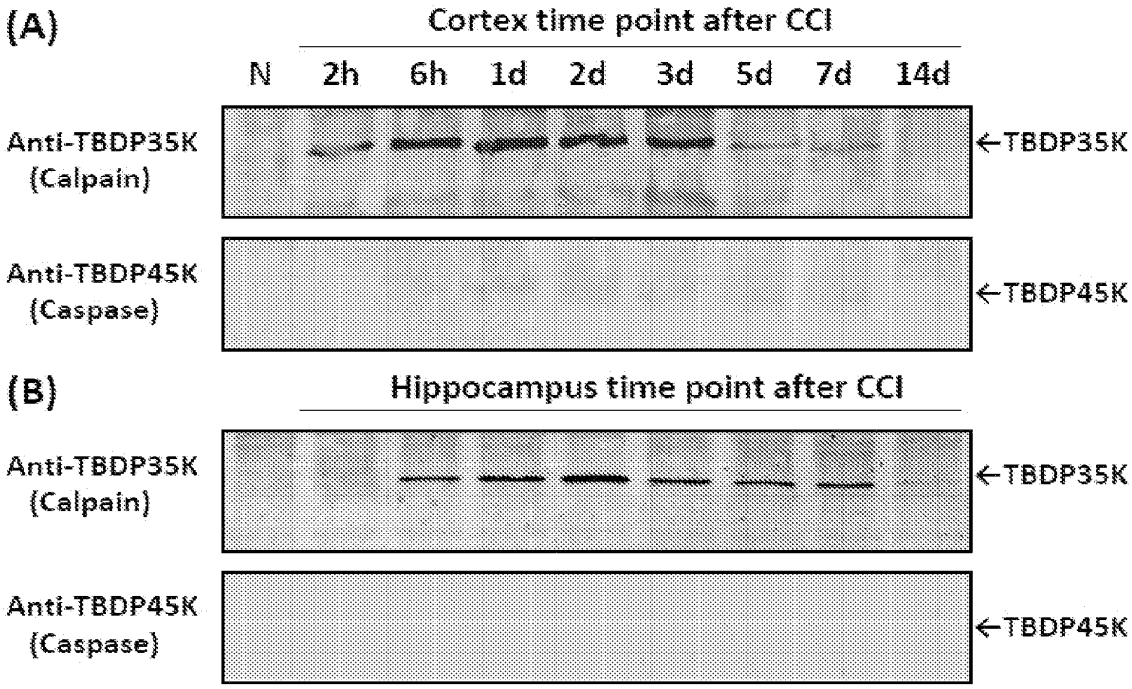

(A)
Cortex time point after CCI
N
2h
6h
1d
2d
3d
5d
7d
14d
Anti-TBDP35K
(Calpain)
←TBDP35K
Anti-TBDP45K
(Caspase)
←TBDP45K
(B)
Hippocampus time point after CCI
Anti-TBDP35K
(Calpain)
←TBDP35K
Anti-TBDP45K
(Caspase)
←TBDP45K

FIG. 12

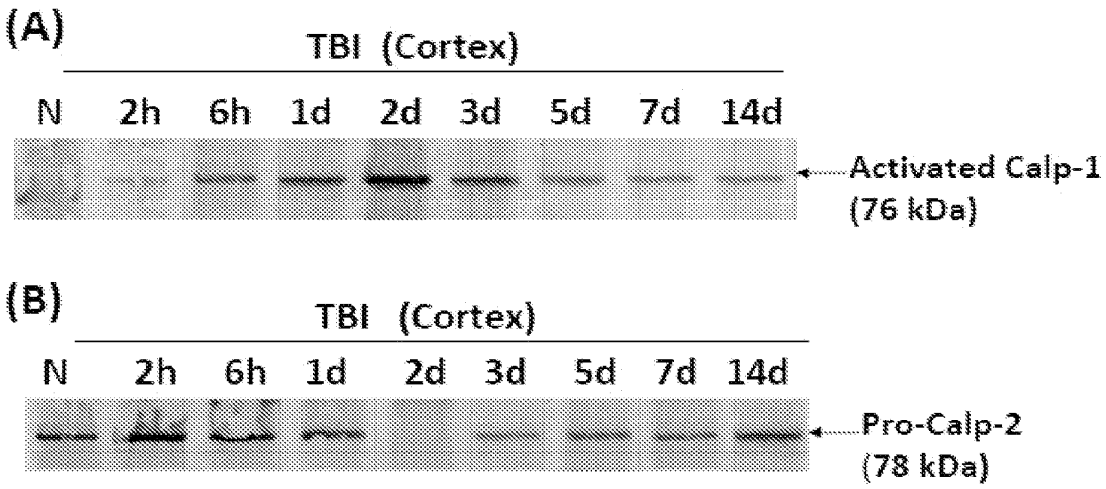

(A)
TBI (Cortex)
N
2h
6h
1d
2d
3d
5d
7d
14d
Activated Calp-1
(76 kDa)
(B)
TBI (Cortex)
N
2h
6h
1d
2d
3d
5d
7d
14d
Pro-Calp-2
(78 kDa)

FIG. 13

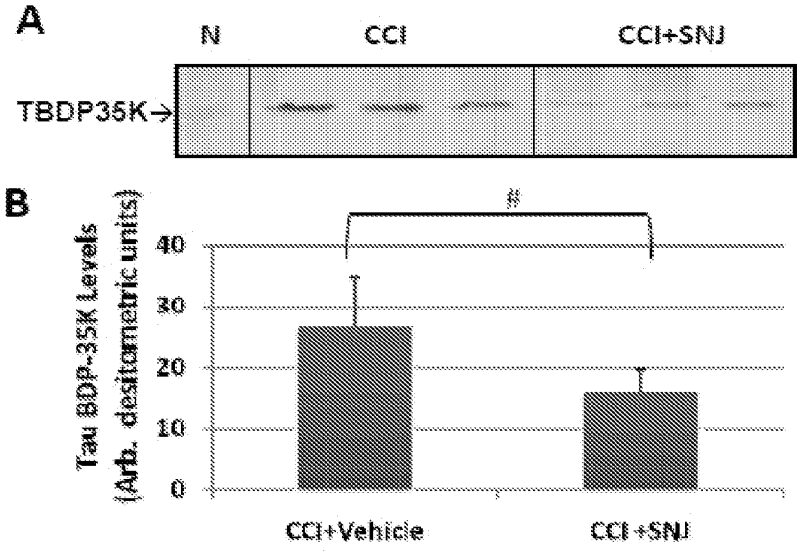
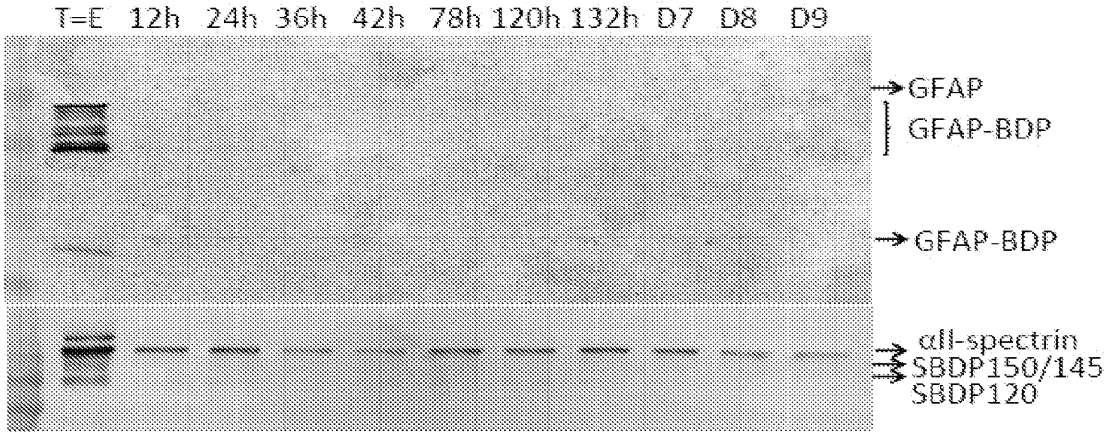

A
N
CCI
CCI+SNJ
TBDP35K
B
Tau BDP-35K Levels
(Arb. densitometric units)
#
40
30
20
10
0
CCI+Vehicle
CCI+SNJ

FIG. 14

(A)


T=E 12h 24h 36h 42h 78h 120h 132h D7 D8 D9
GFAP
GFAP-BDP
GFAP-BDP
αII-spectrin
SBDP150/145
SBDP120

(B)

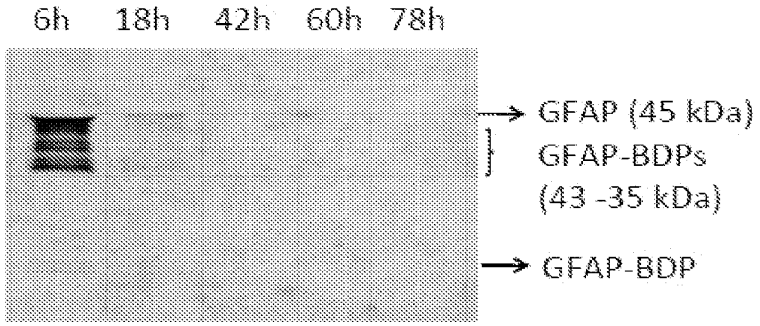

6h 18h 42h 60h 78h
GFAP (45 kDa)
GFAP-BDPs
(43 -35 kDa)
GFAP-BDP

FIG. 15

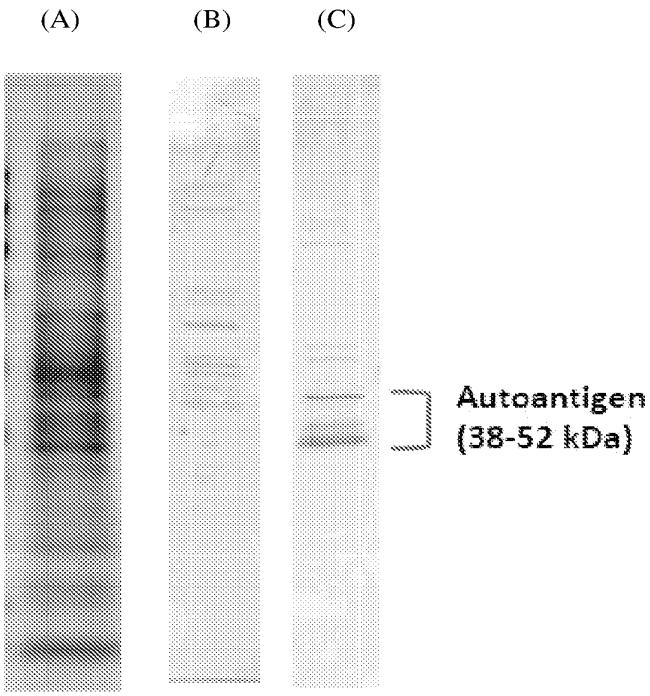


FIG. 16

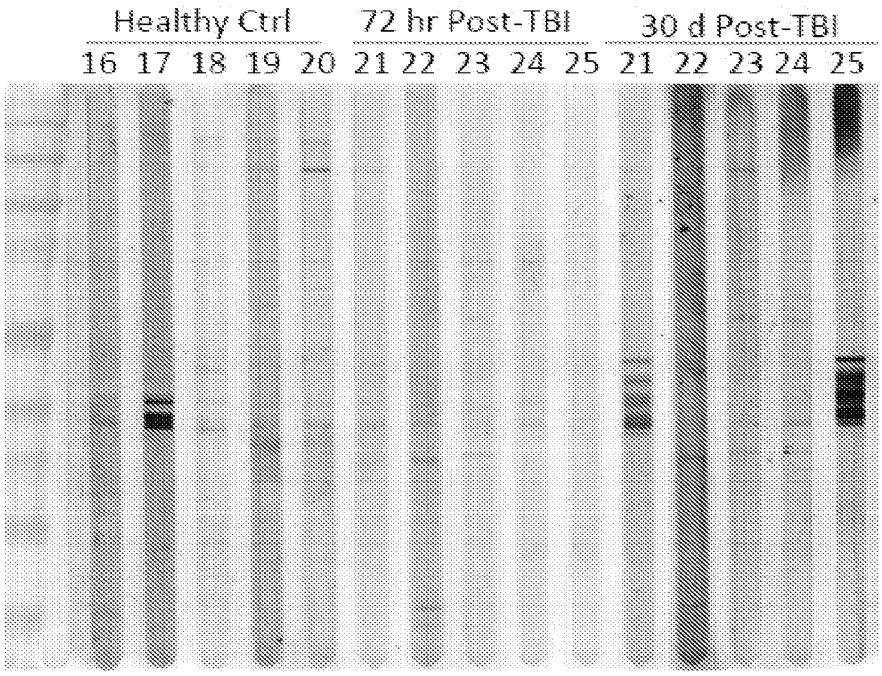
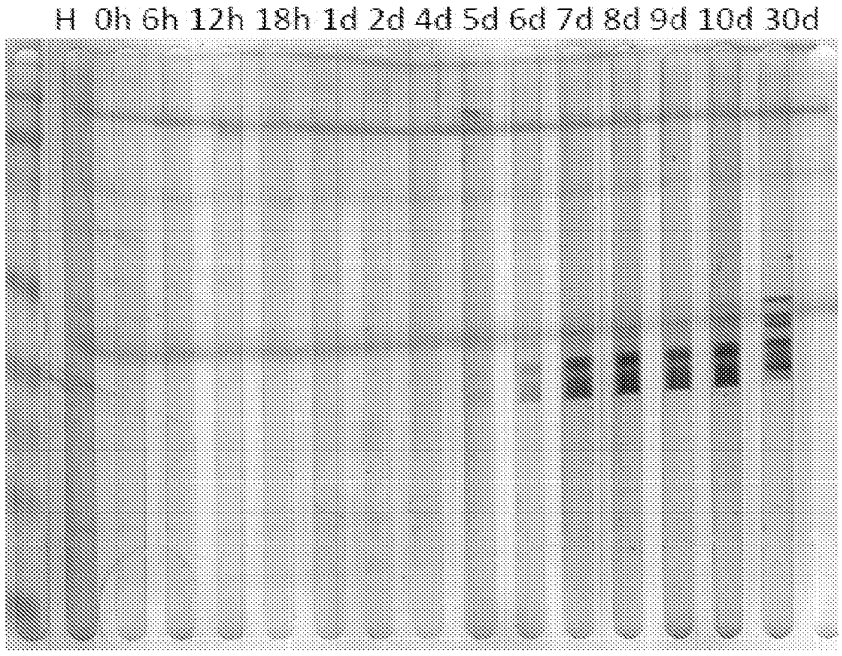

Healthy Ctrl
72 hr Post-TBI
30 d Post-TBI
16 17 18 19 20 21 22 23 24 25 21 22 23 24 25

FIG. 17

(A)


H 0h 6h 12h 18h 1d 2d 4d 5d 6d 7d 8d 9d 10d 30d

(B)

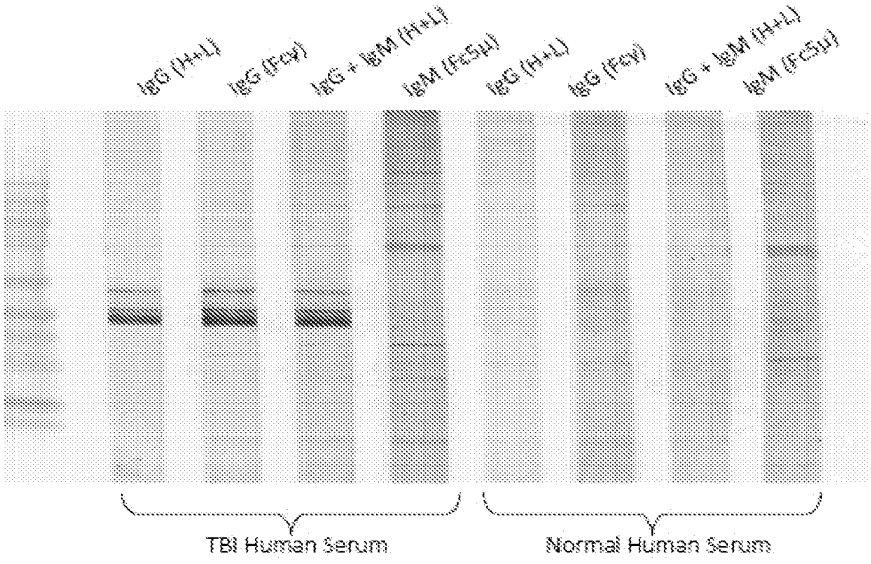

IgG (H+L)
IgG (Fcγ)
IgG + IgM (H+L)
IgM (FcSμ)
IgG (H+L)
IgG (Fcγ)
IgG + IgM (H+L)
IgM (FcSμ)
TBI Human Serum
Normal Human Serum

FIG. 18

(A)

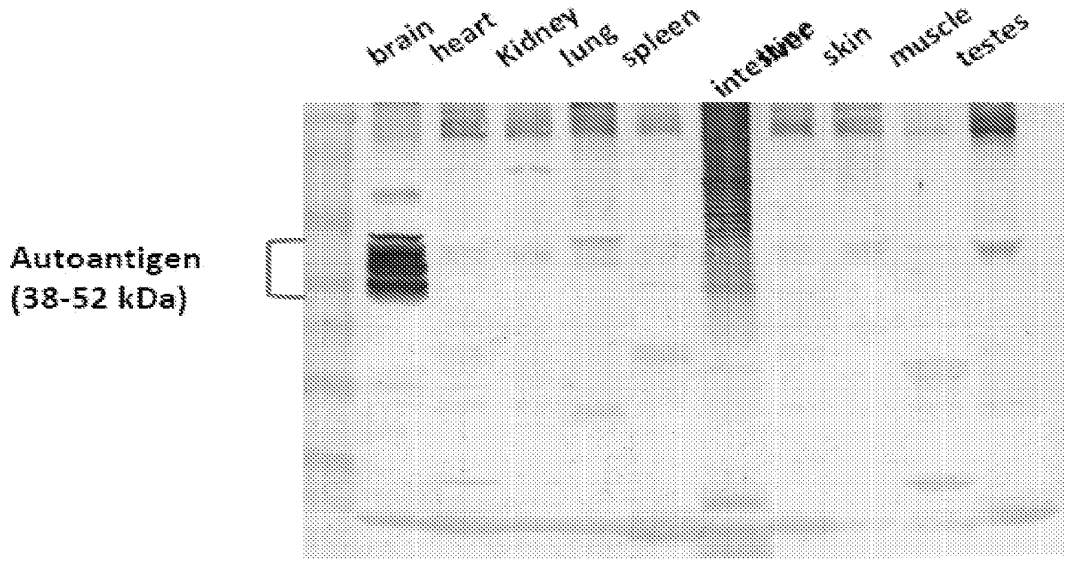

brain
heart
Kidney
lung
spleen
skin
muscle
testes
Autoantigen
(38-52 kDa)

FIG. 19

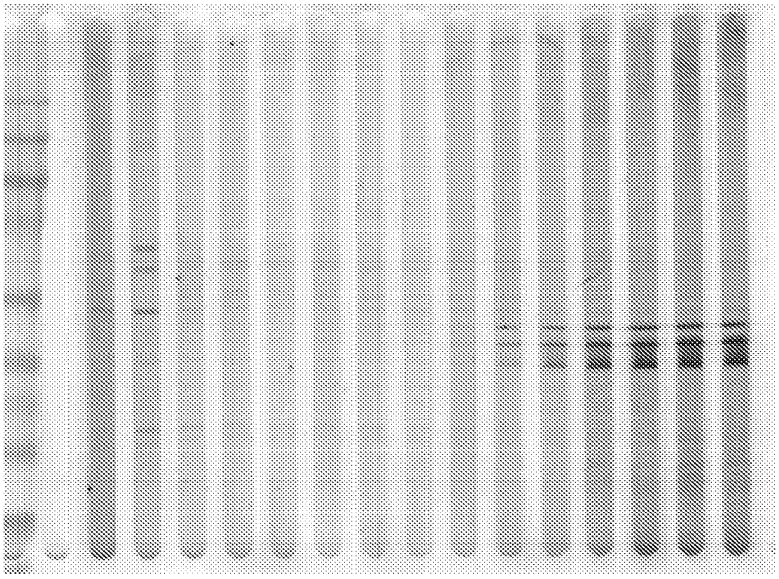


FIG. 20

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
2444

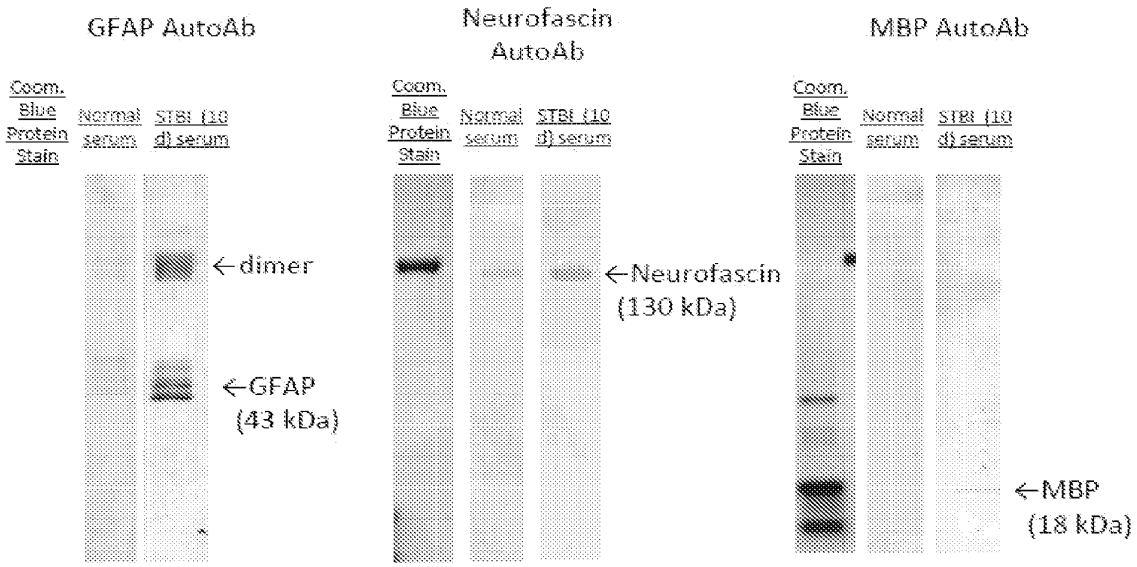
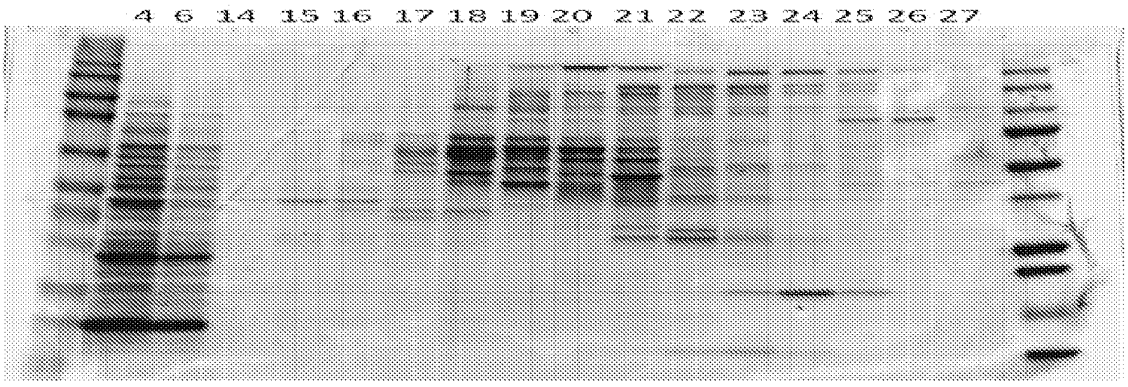

GFAP AutoAb
Coom. Blue Protein Stain
Normal serum
STBI (10 d) serum
←dimer
←GFAP (43 kDa)
Neurofascin AutoAb
Coom. Blue Protein Stain
Normal serum
STBI (10 d) serum
←Neurofascin (130 kDa)
MBP AutoAb
Coom. Blue Protein Stain
Normal serum
STBI (10 d) serum
←MBP (18 kDa)

FIG. 21

(A)



(B)

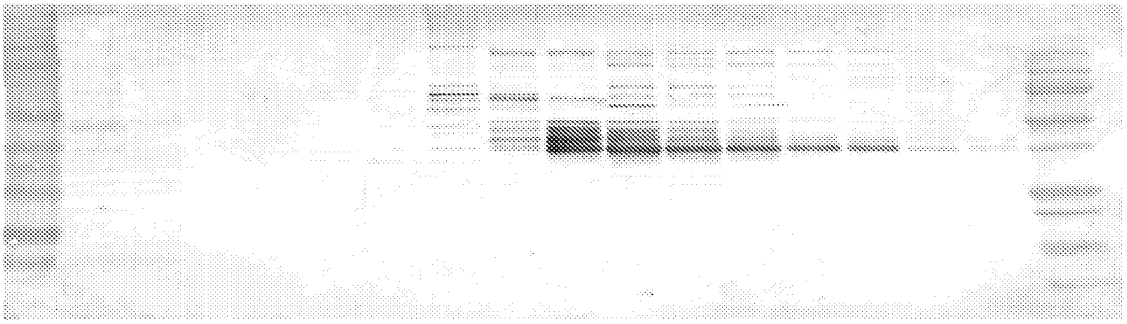
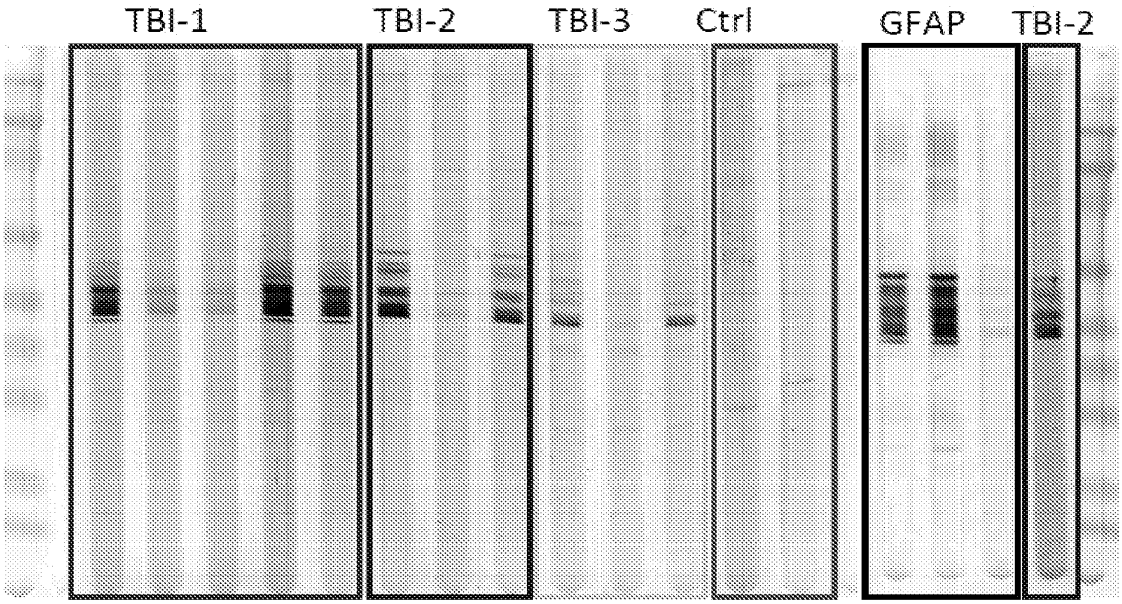
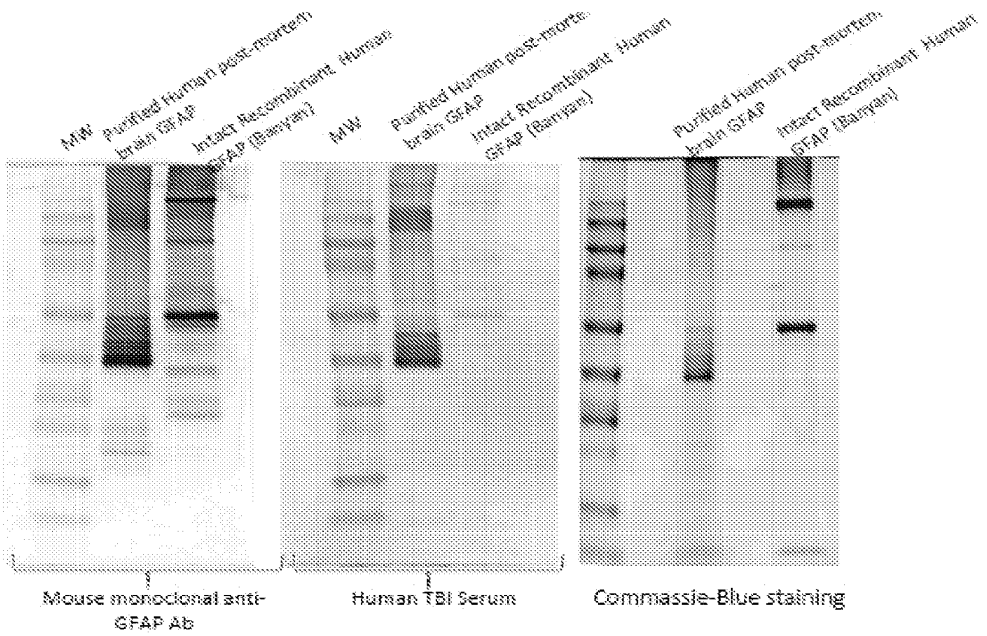


FIG. 22



Lane No.	Detail
1	TBI-1 serum
2	TBI-1 serum with 2.6ug purified human GFAP protein
3	TBI-1 serum with 10ugGFAPprotein
4	TBI-1 serum with 2.6ugTau*
5	TBI-1 serum with 10ugTau
6	TBI-2 serum
7	TBI-2 serum with 10ug GFAP
8	TBI-2 serum with 10ugTau
9	TBI-3 serum
10	TBI-3 serum with 10ugGFAP
11	TBI-3 serum with 10ugTau
12	Normal serum
13	Normal serum
14	Anti-GFAP * dilution: 1:10,000
15	Anti-GFAP dilution: 1:5,000
16	Anti-GFAP dilution: 1:5,000+10ug GFAP protein*
17	TBI-2 serum only

FIG. 23


MW
Purified Human post-mortem brain GFAP
Intact Recombinant Human GFAP (Banyan)
MW
Purified Human post-morte brain GFAP
Intact Recombinant Human GFAP (Banyan)
Purified Human post-mortem brain GFAP
Intact Recombinant Human GFAP (Banyan)
Mouse monoclonal anti-GFAP Ab
Human TBI Serum
Commassie-Blue staining

(A) (B) (C)

FIG. 24

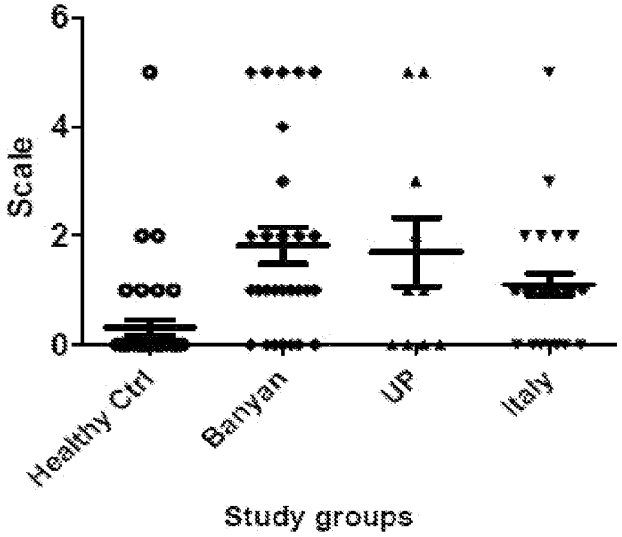
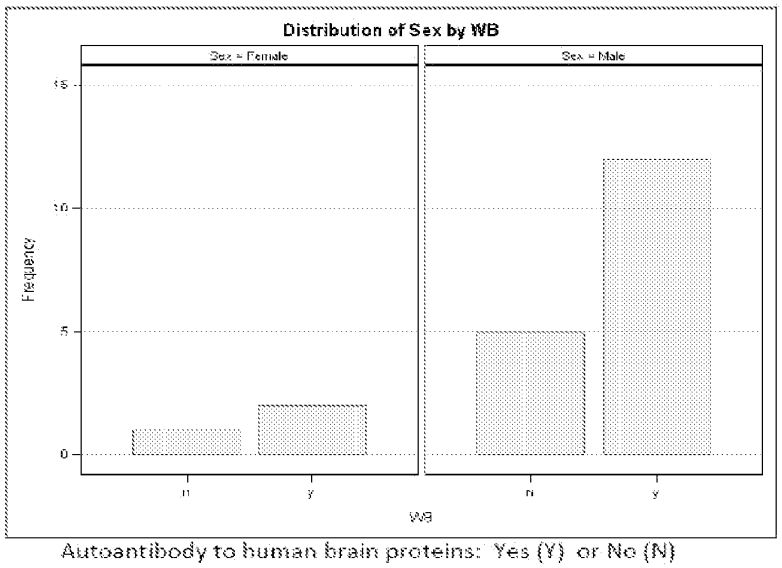


FIG. 25

(A)


Distribution of Sex by WB
Sex = Female
Sex = Male
Frequency
WB
n
y
Autoantibody to human brain proteins: Yes (Y) or No (N)

(B)

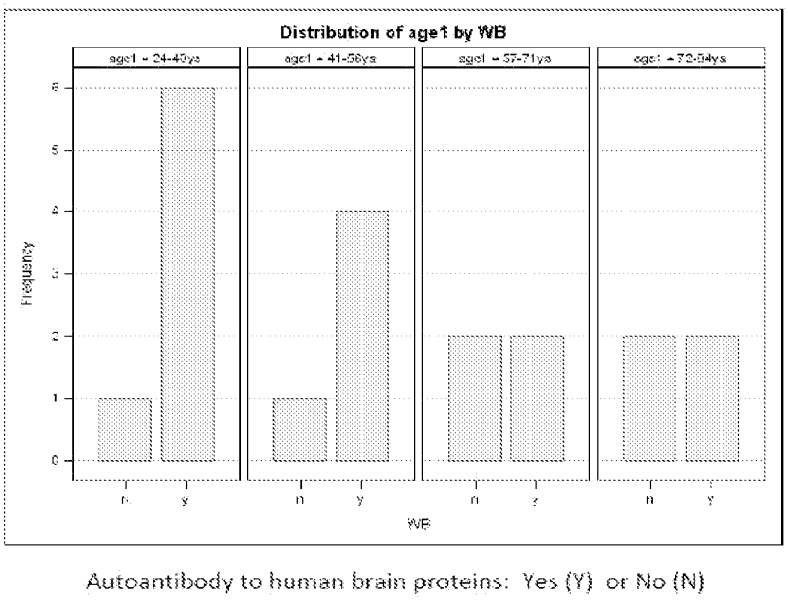
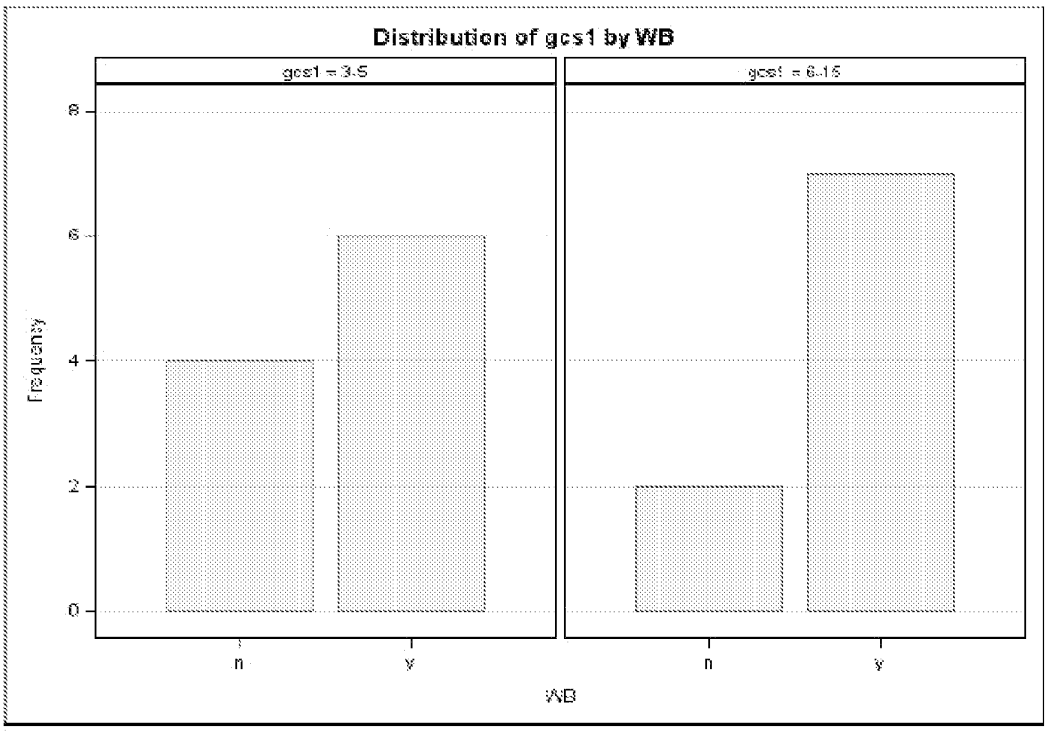
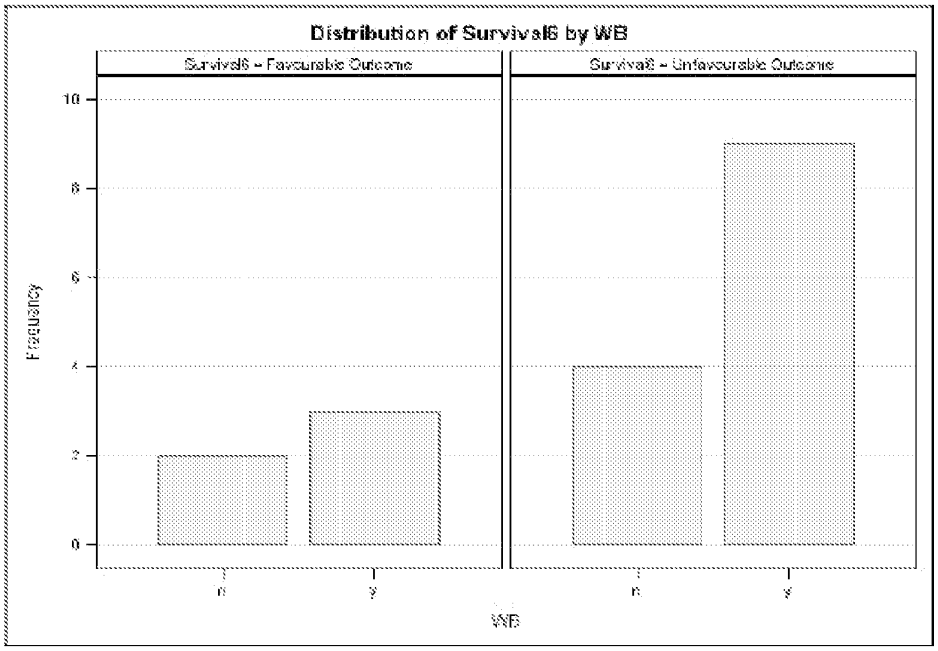

Distribution of age1 by WB
age1 = 24-40ys
age1 = 41-56ys
age1 = 57-71ys
age1 = 72-84ys
Frequency
WB
n
y
Autoantibody to human brain proteins: Yes (Y) or No (N)

FIG. 26



Autoantibody to human brain proteins: Yes (Y) or No (N)

FIG. 27



Autoantibody to human brain proteins: Yes (Y) or No (N)

FIG. 28

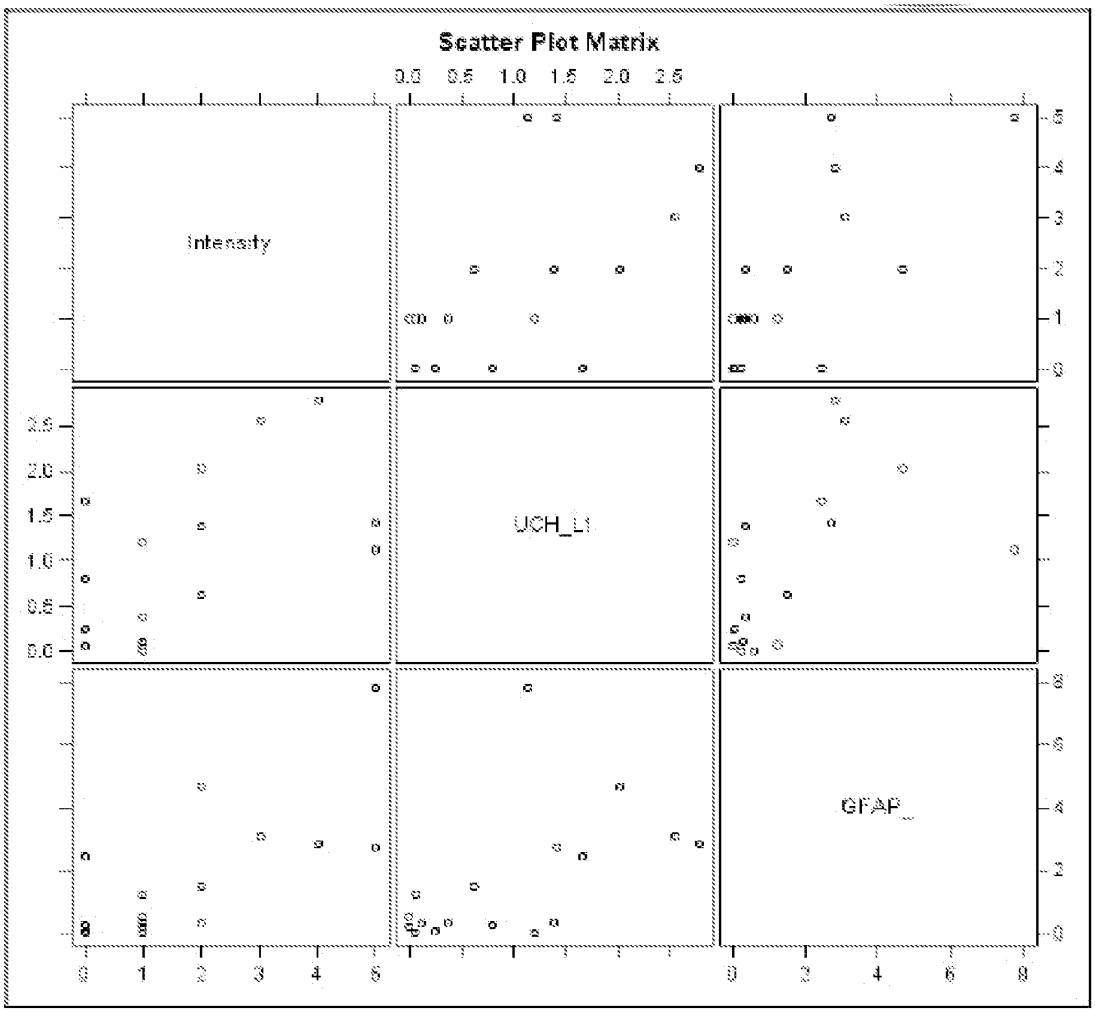


FIG. 29A

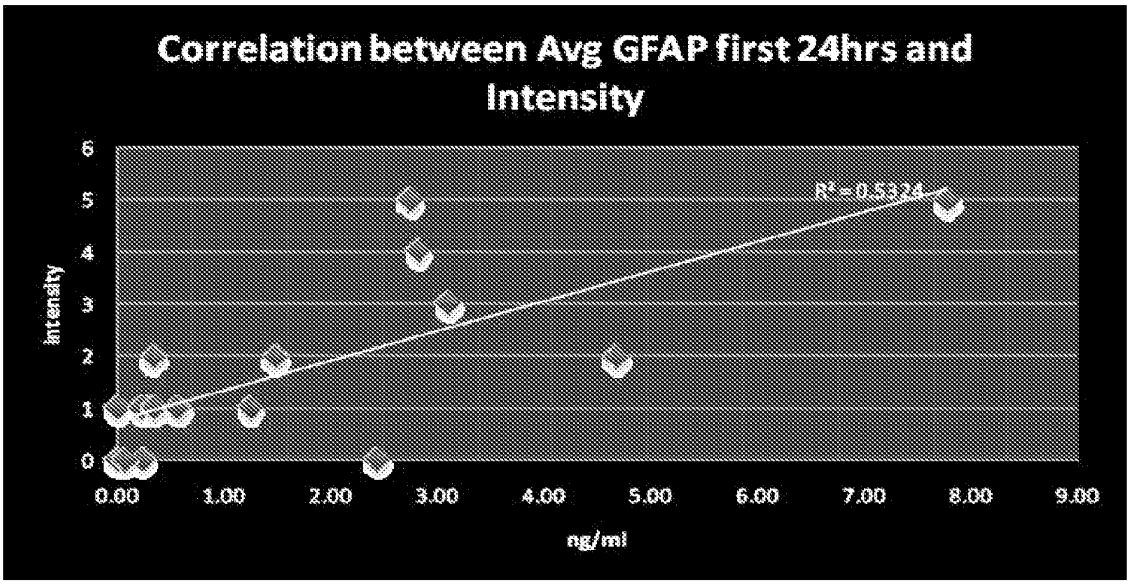

Correlation between Avg GFAP first 24hrs and Intensity
Intensity
ng/ml
R² = 0.5324
0
1
2
3
4
5
6
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00

FIG. 29B

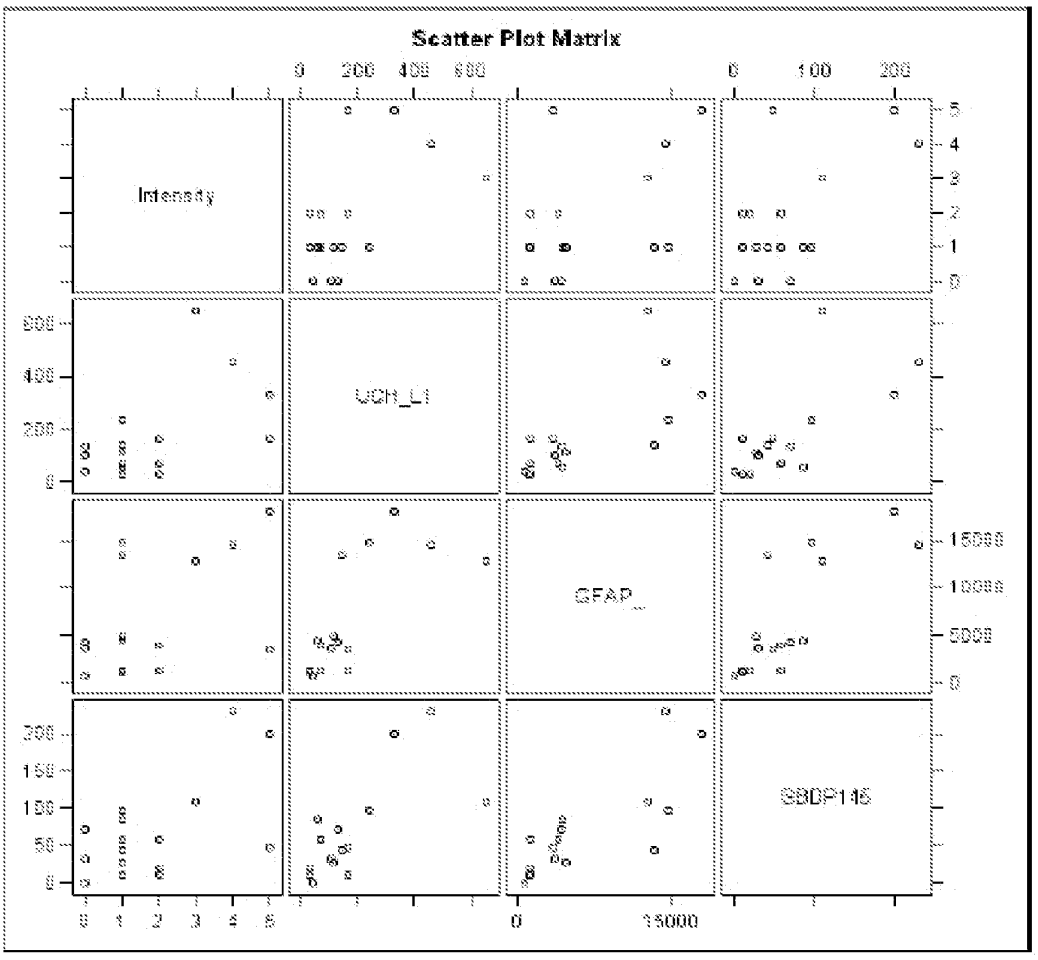
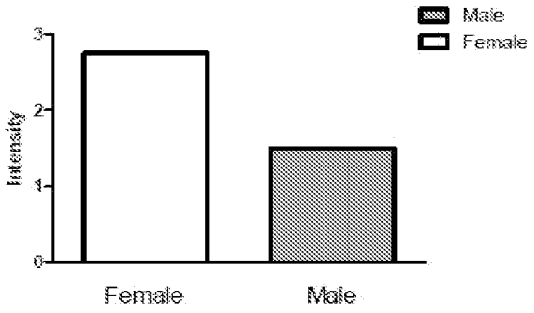


FIG. 30

(A)


Male
Female
Intensity
0
1
2
3
Female
Male

(B)


Female
Male
Intensity
0
2
4
6
Survivors
Not-Survivors

(C)

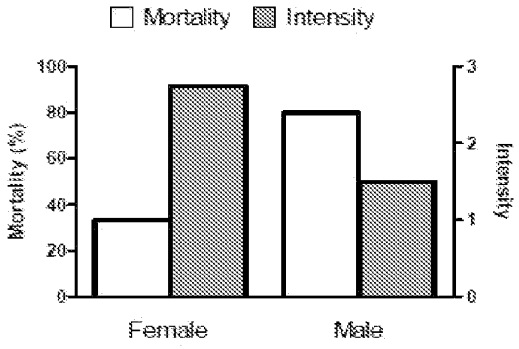
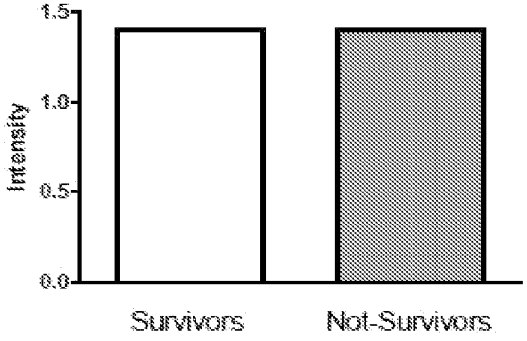
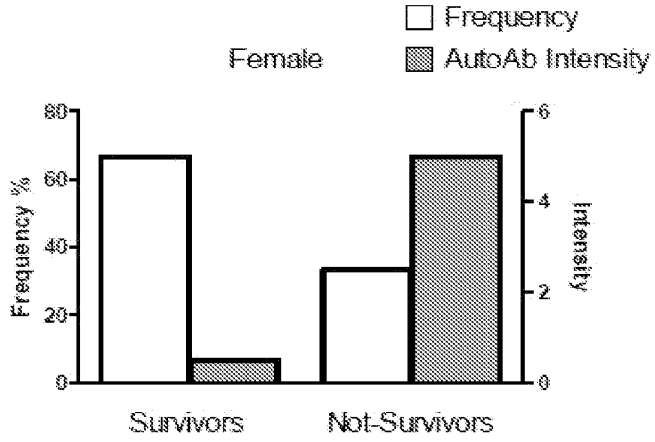

Mortality
Intensity
Mortality (%)
0
20
40
60
80
100
Intensity
0
1
2
3
Female
Male

FIG. 31

(A)


1.5
1.0
0.5
0.0
Intensity
Survivors
Not-Survivors

(B)


Frequency
Female
AutoAb Intensity
80
60
40
20
0
Frequency %
6
4
2
0
Intensity
Survivors
Not-Survivors

(C)

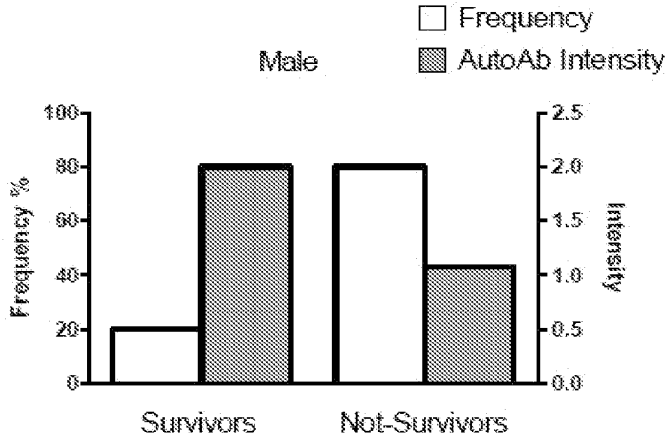

Frequency
Male
AutoAb Intensity
100
80
60
40
20
0
Frequency %
2.5
2.0
1.5
1.0
0.5
0.0
Intensity
Survivors
Not-Survivors

FIG. 32

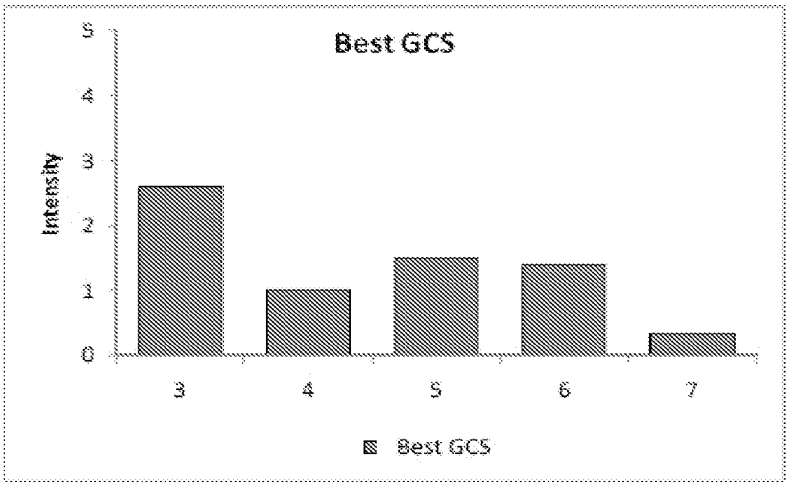


FIG. 33

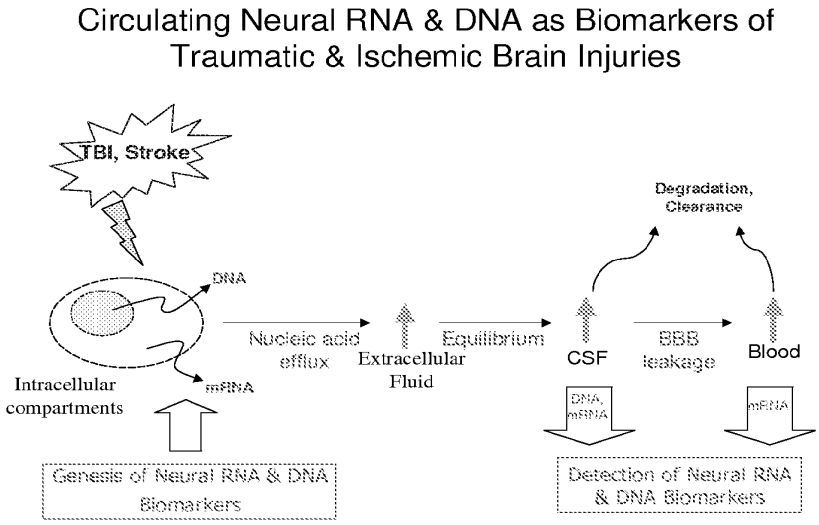

Circulating Neural RNA & DNA as Biomarkers of Traumatic & Ischemic Brain Injuries
TBI, Stroke
DNA
mRNA
Intracellular compartments
Nucleic acid efflux
Extracellular Fluid
Equilibrium
CSF
Degradation, Clearance
BBB leakage
Blood
DNA, mRNA
mRNA
Genesis of Neural RNA & DNA Biomarkers
Detection of Neural RNA & DNA Biomarkers

FIG. 34

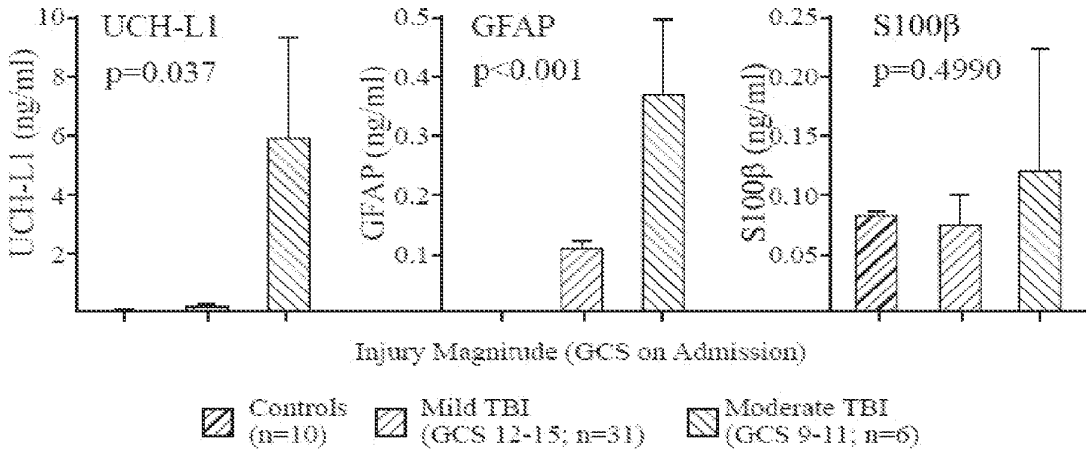

UCH-L1
p=0.037
GFAP
p<0.001
S100β
p=0.4990
UCH-L1 (ng/ml)
GFAP (ng/ml)
S100β (ng/ml)
Injury Magnitude (GCS on Admission)
Controls (n=10)
Mild TBI (GCS 12-15; n=31)
Moderate TBI (GCS 9-11; n=6)

FIG. 35

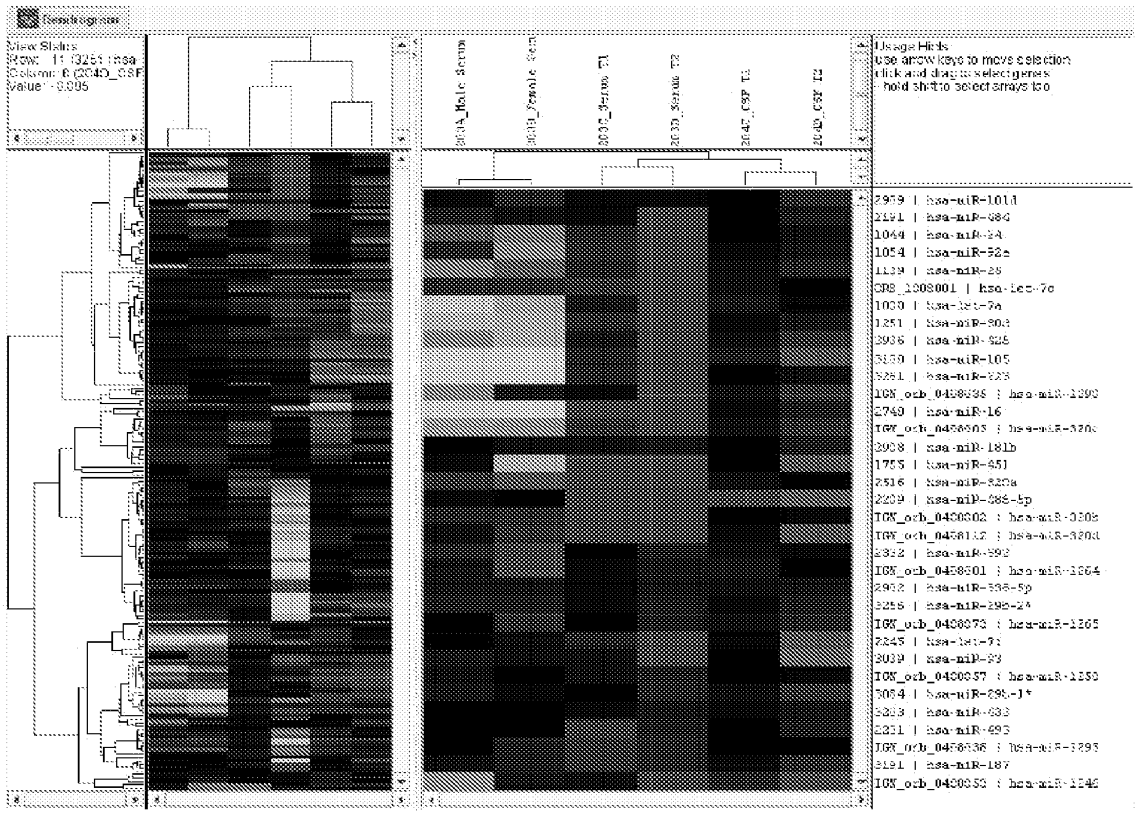

Usage Hints
use arrow keys to move selection
2979 | hsa-miR-101d
2191 | hsa-miR-484
1049 | hsa-miR-24
1054 | hsa-miR-92a
1139 | hsa-miR-26
CRS_1308001 | hsa-let-7c
1000 | hsa-let-7a
1251 | hsa-miR-30b
2936 | hsa-miR-425
3159 | hsa-miR-105
3281 | hsa-miR-425
IGN_ocb_0458695 | hsa-miR-1290
2749 | hsa-miR-16
2908 | hsa-miR-181b
1755 | hsa-miR-451
2516 | hsa-miR-320a
2209 | hsa-miR-486-5p
2312 | hsa-miR-592
2902 | hsa-miR-338-5p
3256 | hsa-miR-296-3p
2845 | hsa-let-7i
2039 | hsa-miR-99
3054 | hsa-miR-29b-1*
2231 | hsa-miR-493
3191 | hsa-miR-187

FIG. 36

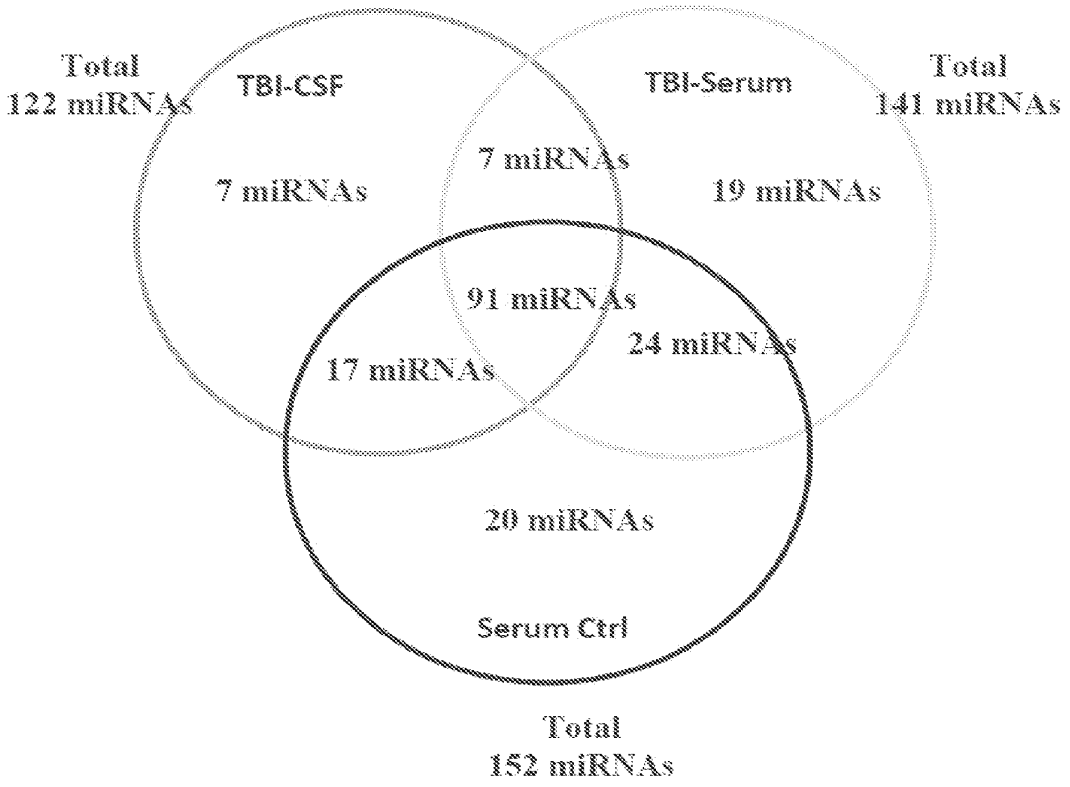

Total
122 miRNAs
TBI-CSF
TBI-Serum
Total
141 miRNAs
7 miRNAs
7 miRNAs
19 miRNAs
91 miRNAs
24 miRNAs
17 miRNAs
20 miRNAs
Serum Ctrl
Total
152 miRNAs

FIG. 37

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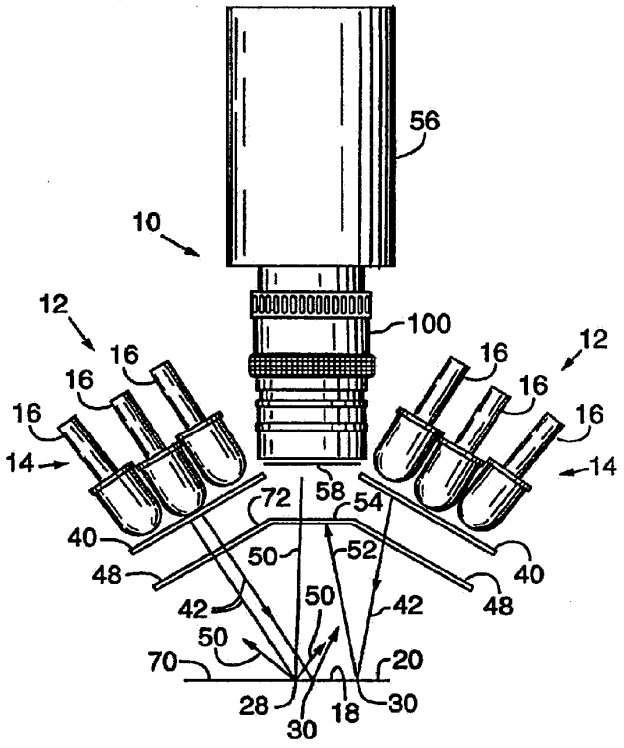
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(57) Abstract

A scribe mark reader (10) uses a source of circularly polarized light and a holographic beam-shaping optical element (40) to uniformly illuminate an area of a substrate (20) that includes a scribe mark (18). Light incident on the substrate at a scribe mark is predominantly scattered, whereas light incident on the substrate at a processed area (30) of the wafer surface between the pits (28) of the scribe mark is predominantly specularly reflected. The phase-reversed, specularly reflected light from the processed area is blocked by a circular analyser that passes light scattered from the scribe mark. Light passing the polarizer can then be used to form an image of the scribe mark. The intensity of the images formed are sufficiently consistent that the automatic gain control of the CCD camera can be used and the images formed can be readily interpreted by optical character recognition software.



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EX-1045
2464

SCRIBE MARK READER

Technical Field

This invention relates to illuminating and forming a light pattern of an identifying mark on a substrate and, in particular, to illuminating and imaging scribe marks on a semiconductor wafer.

Background of the Invention

Semiconductor wafers used in the manufacture of integrated circuits are often marked with identifying information to facilitate in-process tracking during their production. The identifying marks, known as scribe marks, typically comprise a series of characters, bar codes, or other two-dimensional codes, each of which is formed from small circular depressions or "pits" in the substrate. The pits are typically formed by using a laser to melt a small area of the substrate, the melted material being removed in subsequent etching steps. Because integrated circuits should not be formed on the scribe marks, they are typically positioned near the unused outer edge of the wafer by the primary fiducial to maximize the wafer surface area available for forming integrated circuits.

Using a human operator to manually read the marks directly from the wafers is labor intensive and susceptible to error. Moreover, the marks are not always accessible to the operator during wafer processing. Using an imaging system that presents an enlarged image of the

mark to the operator increases the accuracy of the observation, but the process is still labor intensive and susceptible to error because of poor image quality. Efficient wafer fabrication requires that the marks be reliably machine-readable by automated process equipment. Some marks, such as bar codes, are readable essentially only by a machine.

Automated reading of a mark is a three-step process. A camera typically forms an image of the scribe mark, and the image is converted into a computer-compatible digital format. The digitized image is then interpreted, for example, by optical character recognition software to determine letters, numbers, bar codes, or other symbols in the digitized image. For the mark to be interpreted by the software, the digitized image must be relatively clear, i.e., there must be adequate contrast between the background and the image of the mark.

It has been difficult to form a clear image of the scribe marks for several reasons. Because typical scribe marks comprise a group of relatively shallow depressions in the substrate, the marks are of the same color as the substrate background and are, therefore, hard to differentiate. The pits of shallow or "soft" marks, which are used to reduce contamination during wafer processing, can have a depth of only one micron. Moreover, the substrates are typically highly polished and, therefore, reflect into the camera a large amount of light that tends to obscure the mark. Light from the processed areas on the water surface around the pits of the scribe mark is process "noise" that tends to obscure the light "signal" from the scribe mark.

During wafer fabrication, layers are formed on the wafers using a variety of materials having a wide range of reflectivity and other optical properties.

Illumination levels required to achieve an image having adequate contrast between the background and the mark can vary over three orders of magnitude, thereby requiring frequent gain adjustments to the charge-coupled device ("CCD") camera. The automatic gain control on CCD cameras reacts too slowly to such a wide range of brightnesses and, if used, unacceptably delays processing. Manual gain adjustments or sequenced changes in the illumination level can be used to compensate for the variation in image intensity, but such methods also slow production. Moreover, manual adjustments require an operator to continuously monitor the scribe mark reader.

The optical properties of the wafer surfaces vary not only from wafer to wafer but also across the surface of an individual wafer. Wafers can exhibit an "edge bead," i.e., an abrupt change in contrast and reflectivity of the surface as a result of a layer, such as a metal or oxide coating, not extending to the outer periphery of the wafer. The edge bead can appear across the scribe mark itself, producing artifacts in an image formed of the mark. Imperfect forming or etching of the layers can also cause thickness variations in the layers, especially at the edges of the wafers where the thickness variations are observable as annular rings. The coloration and nonuniform reflectivity of such rings, located at the edge of the wafer where the scribe marks are also located, interfere with reading the scribe marks by producing artifacts in the image. Furthermore, materials used to treat or coat wafers, particularly photoresist, can accumulate in the depressions of the scribe marks, further obscuring the mark by affecting the optical properties of the substrate surface and scribe marks.

The difficulty in forming a clear image of the

mark is exacerbated by a lack of spatial uniformity in the intensity of the light illuminating the mark. A bright light source is required to provide sufficient light to form a useful image. Bright light sources, however, have "hot spots," i.e., very bright areas in the field of illumination that cause the image of the scribe mark to asymmetrically characterize the mark, thereby making the image more difficult to interpret. Although conventional diffusers can remove second order nonuniformities in the light source, they are incapable of removing first order hot spots.

Because the problems described above make it difficult to form a clear image of the scribe marks, the optical character recognition software often cannot interpret the poor quality image it receives. Scribe mark readers typically require, therefore, frequent intervention from human operators, either to adjust the optical elements to improve the image or to manually read the mark or significant investment by semiconductor manufacturers to improve or add additional process steps to improve the scribe readability.

Summary of the Invention

An object of the present invention is, therefore, to provide an apparatus and a method for forming a light pattern representing a mark on a substrate.

Another object of this invention is to provide an apparatus and a method for illuminating a mark on a substrate with light having a spatial intensity distribution essentially free of significant local nonuniformities.

A further object of this invention is to provide such a method and apparatus that can form a light pattern capable of being interpreted by optical character

recognition software with a minimum amount of human intervention and number of errors.

The present invention is a method and an apparatus for illuminating and forming an image of marks on a surface and forming an easily interpreted light pattern representative of the mark. The method and apparatus are particularly adapted to illuminating and imaging scribe marks on a semiconductor wafer surface.

A portion of a substrate that includes a scribe mark is uniformly illuminated, for example, by using a light source that includes a beam-shaping optical element positioned between a solid-state light emitter and the substrate. The beam-shaping optical element typically forms light from one or more emitters into a circularly or elliptically shaped beam having a spatial intensity essentially free of significant local nonuniformities. Depending on the configuration of the system, light from the emitter is reflected by or transmitted through the beam-shaping optical element toward the substrate.

Much of the light incident upon the processed areas of the substrate, i.e., areas of the substrate other than the pits that comprise the scribe mark, is specularly reflected. Light incident upon the pits comprising the scribe marks is primarily scattered. It is possible to preferentially attenuate light propagating from the scribe mark or light propagating from the processed area of the substrate around the scribe mark by using the different polarization characteristics of the scattered and reflected light components. Either of these light components can be used to form a relatively high contrast light pattern representative of the scribe mark, with process contributions from the other component reduced. The light pattern is then detected and interpreted.

In one preferred embodiment, a portion of the

substrate that includes a scribe mark is uniformly illuminated using a holographic optical element ("HOE") beam-shaping diffuser and circularly polarized light. Light propagating from the substrate passes through a circular polarizer to attenuate specularly reflected light, i.e., light that is not from the scribe mark. Light scattered from the scribe mark passes through the circular polarizer with less attenuation to form a light pattern representative of the scribe mark that is detected, typically in an imaging system using a CCD camera. A field stop is typically used to further reduce background light entering the camera.

The light pattern formed, therefore, consists primarily of light scattered by the scribe mark, with a minimum of process noise from other areas of the substrate. The range of light intensities of the images thus formed is sufficiently narrow to allow effective use of the automatic gain control of the camera. The high contrast between the image of the scribe mark and the background produces a consistently machine-readable image without frequent manual adjustments.

In a second preferred embodiment, a portion of the substrate that includes a scribe mark is uniformly illuminated using a reflection mode HOE beam-shaping diffuser. Light reflected from areas of the substrate around the scribe mark is specularly reflected into the CCD camera, whereas light scattered from the scribe mark is scattered away from the camera. An image of the scribe mark is formed, therefore, as dark dots against a bright background.

Additional objects and advantages of the present invention will be apparent from the following detailed description of preferred embodiments thereof, which proceeds with reference to the accompanying drawings.

Brief Description of the Drawings

Fig. 1 is a schematic representation of a preferred embodiment of a scribe mark reader incorporating the present invention.

Fig. 2 is a side elevation view of the scribe mark reader of Fig. 1.

Fig. 3 is a plan view of the substrate of Fig. 1, showing a typical scribe mark.

Fig. 4 is an enlarged view of the scribe mark shown of Fig. 3.

Fig. 5 shows a further enlarged single character of the scribe mark of Fig. 4.

Fig. 6 shows light incident on a portion of the substrate that includes a pit and a processed area.

Fig. 7 is a diagram showing the spatial relationship between the image of a scribe mark and a field stop masking a part of the CCD camera.

Fig. 8 is a graph of the intensity distribution of light transmitted through the HOE diffuser shown in Fig. 1.

Fig. 9 is an enlarged front elevation view of the bracket shown in Fig. 1 that holds the polarizers.

Fig. 10 is an oblique plan view of the bracket of Fig. 9 as seen by looking in the direction of lines 10.

Fig. 11 is a schematic representation of the optical elements of the embodiment of Fig. 1.

Fig. 12 is a schematic representation of a second preferred embodiment of a scribe mark reader of the present invention.

Detailed Description of Preferred Embodiments

Figs. 1 and 2 show a first preferred embodiment of a scribe mark reader 10 of the present invention. A light source 12 comprising two banks 14 of multiple lamps 16 emits light to illuminate a scribe mark 18 on a

substrate 20, such as a semiconductor wafer upon which integrated circuits are in the process of being fabricated. Fig. 3 shows a plan view of a typical scribe mark 18 on substrate 20; Fig. 4, which is an enlarged view of the scribe mark 18 of Fig. 3, shows that a typical scribe mark consists of a series of characters 26; and Fig. 5 shows that typical characters 26 consist of pits 28 surrounded by relatively flat, processed areas 30 of substrate 20.

Light from each bank 14 (Figs. 1 and 2) passes through a beam-shaping optical element 40, such as a refractive, diffractive, reflective, or hybrid micro-lens array. A preferred beam-shaping element comprises a holographic optical element ("HOE") such as a light-shaping diffuser composed of multiple micro-lenses (not shown). The micro-lenses of each HOE diffuser combine and shape the light from the multiple lamps 16 of each bank 14 into a single beam of light having a spatial intensity distribution essentially free of significant local nonuniformities and preferably having an elliptical cross-section to conform somewhat to the shape of the series of characters 26 of scribe mark 18.

Individual rays of light 42 of the shaped beam pass through a circular polarizer 48 before striking a portion of substrate 20 that includes mark 18. Fig. 6 shows that light 42 incident on substrate 20 at a pit 28 is primarily scattered as scattered light 50. Light 42 incident on substrate 20 at positions other than a pit 28, i.e., processed area 30, is primarily specularly reflected as reflected light 52. Upon specular reflection, the direction or "handedness" of the circular polarization of reflected light 52 is reversed compared to that of incident light beam 42 and scattered light 50.

Scattered light 50 and reflected light 52

propagate from substrate 20 and are incident on a circular polarizer 54 (Fig. 1). Reflected light 52 is attenuated by polarizer 54 because the direction of polarization of reflected light 52 is reversed from that of light beam 42. Scattered light 50, which has different polarization characteristics from those of reflected light 52, is transmitted through polarizer 54 with significantly less attenuation than that of reflected light 52. Polarizer 54 thus is used to preferentially attenuate light reflected by processed area 30 relative to light scattered by pits 28 of scribe mark 18.

A detector, such as a CCD camera 56, is positioned to receive light passing through polarizer 54. A field stop 58 (Figs. 1 and 7) positioned between polarizer 54 and CCD camera 56 has opaque portions 60 that further reduce background light entering camera 56 while providing a window 62 through which light from scribe mark 18 can pass. Opaque portions 60 typically mask approximately the upper and lower thirds of the field of view of camera 56, leaving a field of view just sufficient to form an image of scribe mark 18. Field stop 58 is not in the focal plane of the camera 56, so camera 56 does not form a sharp image of the field stop that could be misinterpreted as part of scribe mark 18. Camera 56 thus forms a light pattern representative of scribe mark 18 from light that includes a minimum of extraneous light, or process noise, from processed areas 30 of substrate 20.

Because the image of the scribe mark 18 appears as a bright field against a relatively dark background, and because field stop 58 and polarizer 54 greatly decrease the range of light intensity in the dark background, the automatic gain control of camera 56 can be efficiently used. The set point for differentiating between a background and a foreground pixel changes

significantly less than in prior art scribe mark readers allowing the automatic gain control to enhance the entire image. The increased consistency in light levels also allows the integrator bandwidth to be increased, thereby reducing processing time.

The light pattern or image of scribe mark 18 formed by the present invention is, therefore, clearer and more easily interpreted by optical character recognition software or by a person observing the displayed image on a video screen. Use of the automatic gain control of camera 56 eliminates the need for manual adjustment to the camera gain or the intensity of the illumination. It will be understood that the light pattern need not be displayed as an observable image but can be detected and analyzed electronically as a collection of data points.

The components of reader 10 are positioned close together to create a compact package for the convenience of mounting onto a wafer processing system. Reader 10 is positioned as close to scribe mark 18 as possible to reduce the required light intensity of light source 12. In one embodiment, reader 10 is mounted approximately four inches from substrate 20 and polarizer 48 is mounted in contact with beam-shaping optical element 40. The exact dimensions of the system are not considered to be critical, and the dimensions for any particular configuration can easily be determined.

Preferred lamps 16 of light source 12 provide high intensity illumination of scribe mark 18 while consuming minimal power. Suitable lamps include untinted, nondiffused, solid-state lamps that use a highly optimized light-emitting diode ("LED") material, such as aluminum gallium arsenide, incandescent lamps, and lasers. Such lamps provide excellent light output efficiency over a range of electrical currents and exhibit superior current

handling capability. For example, one commercially available lamp has a 4° viewing angle and provides an on-axis luminous intensity value of approximately 36 candelas.

Each bank 14 of lamps 16 includes three lamps 16. Banks 14 are positioned symmetrically, one on each side of camera 56, in a single plane 66 (Fig. 2) that also includes camera 56. Banks 14 are also positioned as nearly as possible and symmetrically with respect to scribe mark 18. Plane 66 is preferably oriented at an angle 68 to a plane 69 normal to a top surface 70 of substrate 20 to specularly reflect much of light 52 away from polarizer 54 and camera 56. Such an orientation is preferred because polarizer 54 does not completely block all incident light propagating from processed area 30.

Lamps 16, although sufficiently bright, typically provide unacceptably nonuniform illumination across scribe mark 18 due to local nonuniformities, such as hot spots. Such nonuniform illumination can produce artifacts in the image of scribe mark 18 formed by camera 56. Beam-shaping optical element diffusers 40 positioned between lamps 16 and substrate 20 transform the nonuniform illumination of lamps 16 into a shaped, light beam 42 having a spatial intensity distribution essentially free of significant local nonuniformities. Significant local nonuniformities are relatively abrupt changes in light intensity over a spatial scale comparable to that of the scribe marks and of a magnitude comparable to the difference in intensity of the scribe mark image and the background light. Significant local nonuniformities interfere with the ability of the reader to interpret the scribe marks.

It will be understood that the intensity of the beam after passing through beam-shaping optical element 40

typically decreases smoothly away from its center as shown in Fig. 8 and that the beam may have minor local variations that do not interfere with interpreting the scribe marks. Beams formed by beam-shaping optical element 40 do not carry images of hot spots or other artifacts. The index of refraction of each beam-shaping optical element 40 typically contains micro-variations that form randomly oriented micro-lenses (not shown) across its surface. The micro-lenses precisely shape and homogenize the resultant light beam to produce an output beam of the desired shape. Beam-shaping optical elements that produce variously shaped beams are available. The shape of the beam should approximate that of the string of characters 26 comprising scribe mark 18. Preferred beam-shaping optical elements in the form of HOE diffusers that form an elliptically shaped beam are commercially available under the name "Light Shaping Diffuser™" from Physical Optics Corporation, Torrance, California.

Each beam-shaping optical element diffuser 40 shapes light 42 into a beam that provides maximum illumination on the scribe mark 18 area while minimizing illumination of the surrounding area that would produce stray light that can enter camera 56 and obscure the image of scribe mark 18.

Polarizer 48 is positioned between beam-shaping optical element diffuser 40 and substrate 20. Figs. 9 and 10 show respective front and oblique plan views of a bracket 72 used to mount polarizers 48 and polarizer 54. Angle 73 is preferably approximately 58°.

Fig. 11 shows that polarizer 48 is preferably of a circularly polarizing type, typically comprising a linear polarizer 74 having a transmission axis 76 and a quarter-wave retarder 78 with mutually perpendicular respective fast and slow axes 80 and 82. Unpolarized

light 42 enters linear polarizer 74 and is linearly polarized in the direction of transmission axis 76. Axes 80 and 82 of quarter-wave retarder 78 are oriented at 45° to transmission axis 76 to convert the linearly polarized light to circularly polarized light by phase shifting mutually perpendicular components of the light by 90°. It will be understood that elliptically polarized light can also be used in the invention by substituting a retarder that retards more or less than $\lambda/4$ to phase shift mutually perpendicular components of the light by a phase angle of other than 90°.

Circularly polarized, spatially uniform light 42 illuminates a portion of substrate 20 that includes scribe mark 18. Figs. 4 and 5 show that scribe mark 18 typically consists of alpha-numeric characters 26 formed by multiple pits 28, each pit consisting of a small depression in substrate 20. Scribe mark 18 could also consist of a bar code or other two-dimensional codes formed by pits 28. Pits 28 are typically made by melting with repeated laser pulses a small area of substrate 20. The melted substrate material is removed during a subsequent processing step to leave a pit-like depression. The illuminated surface areas of substrate 20 around scribe mark 18 include processed areas 30 and pits 28.

Light 42 incident on pit 28 of substrate 20 is scattered as scattered light 50 with predominately random polarization. A significant portion of incident light beam 42 that strikes processed areas 30 is specularly reflected. Specular reflection causes a 180° phase shift that reverses the direction of the circular polarization of reflected light 52 compared to that of incident light 42. (Fig. 11 shows light 42 incident on one side of substrate 20 and light 50 and 52 emerging on the opposite side. It will be understood that such a depiction is for

purposes of illustration only and that light is not transmitted through substrate 20.)

Specularly reflected light 52 and scattered light 50 travel to polarizer 54, which is typically a circular polarizer comprising a quarter-wave retarder 90 having mutually orthogonal respective fast and slow axes 92 and 94 a linear polarizer 96 having a transmission axis 98 oriented at 45° to fast and slow axes 92 and 94.

Quarter-wave retarder 90 converts circularly polarized specularly reflected light 52 into linearly polarized light having a polarization direction perpendicular to transmission axis 98. Reflected light 52 is, therefore, blocked by linear polarizer 96. Scattered light 50, which includes a significant randomly polarized component, remains randomly polarized after passing through quarter-wave retarder 90 and, therefore, passes through linear polarizer 96 to a greater extent than does specularly reflected light 52. Thus, linear polarizer 96 selectively attenuates light reflected from processed area 30, thereby reducing the amount of background light that reaches CCD camera 56.

Polarizer 54 can be the same optical element as polarizer 48 because the desired polarization characteristics are identical. Alternatively, two separate optical elements can be used. Because light 52 reflected by substrate 20 is blocked and light 50 scattered by scribe mark 18 is transmitted to CCD camera 56, the image of the scribe mark 18 appears bright against a dark background. Field stop 58 further reduces the brightness of the dark background. The image of scribe mark 18 is magnified by a lens 100 to more completely fill the field of view of CCD camera 56, thereby facilitating interpretation of the image by optical character recognition software or an observer.

It will be understood that polarizer 54 does not completely block all light from processed areas 30. Plane 66 containing camera 56 and lamp banks 14 is, therefore, canted at slight angle 68 away from plane 69 normal to the top surface of substrate 20 to reduce the amount of reflected and scattered light from processed areas 30 that enters camera 56. As the magnitude of angle 68 is increased, the intensity of light entering camera 56 from processed area 30 is reduced, but so is the intensity of light 50 entering camera 56 from scribe mark 18. An angle 68 of 20° was found to significantly reduce reflected light 52 yet provide sufficient scattered light 50 to camera 56, although the exact angle 68 chosen is not considered to be critical.

Fig. 12 shows an alternative preferred embodiment of a scribe mark reader 106 of the invention. A light source 108 comprises an array of LEDs and a diffuser embedded in a matrix. Light 112 propagating from each LED is optically scattered to form an evenly illuminated light emitting surface yielding a near-Lambertian radiation pattern having a luminous intensity of approximately 100 mcd and a dominant wavelength of 637 nm. The geometry of light source 108 is carefully chosen to ensure proper illumination over the field of view of camera 56.

Light 112 is reflected by a HOE reflector 114 that consists of randomly oriented micro-lenses which form light 112 into a precisely shaped beam essentially free of significant local nonuniformities. Light 112 provides spatially uniform illumination in the field of view of camera 56 to provide a high coupling efficiently between light source 108 and camera 56. Baffles 116 pass light that will strike substrate 20 at angles causing specularly reflected light to be reflected into camera 56. Suitable

HOE reflectors are commercially available from Physical Optics Corporation, Torrance, California. The purpose of the optical elements between light 108 and camera 56 is to provide a spatially uniform, nearly collimated beam of light to reflect from substrate 20 and into camera 56 sufficient light to form an image while minimizing extraneous light reaching camera 56.

Light 112 incident on processed areas 30 of substrate 20 is specularly reflected as reflected light 118 into camera 56. Light 112 incident on scribe mark 18 is scattered as scattered light 130 throughout a solid angle at a Lambertian distribution. Only the small portion of scattered light 132 that is scattered nearly along the optical axis 134 of camera 56 and within the focusing power of lens 100 enters camera 56. Because the intensity at camera 56 of light 118 reflected from the processed area is so much greater than the intensity of the light scattered from pits 28 of the scribe mark 18, the image formed by camera 56 shows scribe marks 18 as dark areas against a bright background. Lens assembly 100, as in the first embodiment, ensures that the magnified scribe image fills the field of view of camera 56.

It will be obvious that many changes may be made to the above-described details of the invention without departing from the underlying principles thereof. For example, the system could be configured to use either reflective or transmissive HOEs and to operate as a dark field or light field system. The polarization states of the light propagating from substrate 20 can be used to preferentially attenuate light from scribe mark 18 or processed areas 30 without using beam-shaping optical element 40, such as by using a conventional diffuser to reduce nonuniformities in the illumination of the scribe

mark. Similarly, beam-shaping optical element 40 can be used without also using the polarization states to selectively attenuate light propagating from substrate. The scope of the present invention should, therefore, be determined only by the following claims.

Claims

1. A system for forming a light pattern representative of a scribe mark on a substrate, comprising:

a source of unpolarized lightfor illuminating a portion of the substrate including the scribe mark;

a detector for receiving light propagating from the substrate and forming a light pattern representative of the scribe mark, the source and detector defining an optical path therebetween;

a single polarizer positioned along the optical path, the polarizer filtering light propagating from the substrate to the detector to attenuate process noise, thereby allowing the detector to form an interpretable light pattern of the scribe mark.

2. The system of claim 1 in which the single polarizer is positioned along the optical path to polarize light propagating from the light source to the substrate and to filter light propagating from the substrate to the detector to eliminate multiple polarizer misalignments that would reduce the intensity of light entering the detector.

3. The system of claim 2 in which the polarizer is of a circularly polarizing type.

4. The system of claim 2 in which light propagating from the light source has an angle of reflection from the substrate and in which the detector is oriented at an angle other than the angle of reflection so that the amount of specularly reflected light entering the detector is reduced.

5. The system of claim 2 in which the source of unpolarized light includes a beam-shaping optical element that shapes the light into a beam having a shape related to that of the scribe mark and having a spatial intensity

distribution substantially free of significant local nonuniformities.

6. The system of claim 5 in which the polarizer is of a circularly polarizing type and in which light propagating from the light source has an angle of reflection from the substrate and in which the detector is oriented at an angle other than the angle so that the amount of specularly reflected light entering the detector is reduced.

7. The system of claim 2 further comprising a field stop for blocking light not required for forming the light pattern representative of the scribe mark.

8. A method of forming a light pattern representative of a scribe mark on a substrate, comprising:

providing a source of unpolarized light;

illuminating with light from the light source an area of the substrate that includes the scribe mark;

preferentially attenuating light propagating from the scribe mark or light propagating from a substrate portion around the scribe mark by passing though a polarizer the light propagating from the substrate and using the different polarization characteristics of the light propagating from the scribe mark and the light propagating from the substrate portion around the scribe mark; and

forming a light pattern representative of the scribe mark from light propagating from the scribe mark or from light propagating from the substrate portion around the scribe mark.

9. The method of claim 8 further comprising passing the unpolarized light before it strikes the substrate through the same polarizer used to preferentially attenuate light propagating from the

substrate, thereby increasing the amount of light available to form the light pattern by eliminating light loss caused by misalignment of multiple polarizers.

10. The method of claim 9 in which providing a source of unpolarized light includes passing light through a beam-shaping optical element to form the light into a beam having a shape related to that of the scribe mark and having a spatial intensity distribution substantially free of significant local nonuniformities.

11. The method of claim 9 in which preferentially attenuating light includes passing light propagating from the substrate through the polarizer to preferentially attenuate light that is not propagating from the scribe mark.

12. The method of claim 9 in which light propagating from the substrate includes specularly reflected light and in which preferentially attenuating light includes passing light propagating from the substrate through the polarizer to preferentially attenuate the specularly reflected light.

13. The method of claim 12 in which:

passing though a polarizer the light propagating from the substrate includes passing the light through a polarizer of the circularly polarizing type;

light propagating from the light source has an associated angle of reflection from the substrate and forming a light pattern includes forming a light pattern using light propagating from the substrate at an angle other than the angle of reflection to reduce the amount of specularly reflected light interfering with the formation of the light pattern representative of the scribe mark; and

providing a source of light includes passing light through a beam-shaping optical element to form the

light into a beam having a shape related to that of the scribe mark and having a spatial intensity distribution substantially free of significant local nonuniformities.

14. The method of claim 9 in which passing the unpolarized light through a polarizer includes passing the unpolarized light through a circular polarizer and in which preferentially attenuating light includes passing light propagating from the substrate through the circular polarizer to preferentially attenuate light that is not propagating from the scribe mark.

15. The method of claim 14 in which preferentially attenuating the light includes passing the light through a field stop to block light that is not useful for forming the light pattern.

16. The method of claim 9 in which passing the unpolarized light through the polarizer includes passing the unpolarized light through an elliptical polarizer and in which preferentially attenuating light includes passing light propagating from the substrate through the elliptical polarizer to preferentially attenuate light that is not scattered by the scribe mark.

17. The method of claim 9 in further comprising forming a digital representation of the light pattern and electronically interpreting the digital representation of light pattern.

18. A method of illuminating a scribe mark on a substrate for use in a scribe mark reader, comprising:

providing a light source that produces light characterized by a spatial intensity distribution;

modifying the spatial intensity distribution by using a beam-shaping optical element to provide a light beam having a shape related to that of the scribe mark and a spatial intensity distribution essentially free of significant local nonuniformities; and

illuminating with the light beam a portion of the substrate including the scribe mark.

19. The method of claim 18 in which the light source comprises multiple light emitters.

20. The method of claim 18 in which providing a light source includes providing a light source that produces unpolarized light and further comprising polarizing the light produced by the light source.

21. The method of claim 20 in which polarizing the light includes circularly polarizing the light.

22. The method of claim 18 in which modifying the spatial intensity distribution includes using a holographic optical element to reflect light emitted by the light emitter.

23. The method of claim 18 in which modifying the spatial intensity distribution includes transmitting through a holographic optical element light emitted by the light emitter.

24. A method of forming a light pattern representative of a scribe mark on a substrate, comprising:

illuminating the scribe mark by the method of claim 18; and

forming a light pattern representative of the scribe mark from light propagating from the scribe mark or from light propagating from the substrate portion around the scribe mark.

25. The method of claim 24 further comprising preferentially attenuating light propagating from the scribe mark or light propagating from a substrate region around the scribemark by passing light propagating from the substrate through a polarizer, and using the different polarization characteristics of the light propagating from the scribe mark and the light propagating from the

substrate region aound the srcibe mark.

26. The method of claim 25 in which illuminating the substrate includes illuminating the substrate using polarized light.

27. The method of claim 26 in which preferentially attenuating light includes passing the light through a polarizer.

28. The method of claim 27 in which illuminating the substrate and preferentially attenuation the light include passing light through the same polarizer.

29. The method of claim 27 in which illuminating the substrate and preferentially attenuation the light include passing light through different polarizers.

30. The method of 24 in which the light beam has an angle of reflection from the substrate and in which forming a light pattern representative of the scribe mark includes using light reflected at the angle of reflection from the substrate portion around the scribe mark, so that points in the light pattern corresponding to the substrate portion around the scribe mark appear relatively light and points in the light pattern corresponding to the scribe mark, which scatters light at angles other than the angle of reflection, appear relatively dark.

31. The method of 24 in which the light beam has an angle of reflection from the substrate and in which forming a light pattern representative of the scribe mark includes using light primarily scattered from the scribe mark at angles other than the angle of reflection, points in the light pattern corresponding to the scribe mark appearing relatively light and points in the light pattern corresponding to the substrate portion around the scribe appearing relatively dark.

32. The method of claim 31 in which modifying the spatial intensity distribution includes using a holographic optical element to reflect light emitted by the light emitter.

33. A light source for illuminating a scribe mark on a substrate, comprising:

a light emitter producing light having a spatial intensity distribution; and

a beam-shaping optical element that modifies the spatial intensity distribution of the light to produce a light beam having a shape related to that of the scribe mark and being substantially fee of significant local nonuniformities in the illumination of the scribe mark.

34. The light source of claim 33 in which the beam-shaping optical element comprises multiple micro-lenses.

35. The light source of claim 33 in which the beam-shaping optical element reflects light propagating from the light emitter toward the substrate.

36. The light source of claim 33 in which the light emitter emits unpolarized light.

37. The light source of claim 33 in which the beam-shaping optical element refracts light propagating from the light emitter toward the substrate.

38. The light source of claim 33 in which the light emitter includes multiple light emitters.

39. The light source of claim 33 in which the light emitter emits unpolarized light and further comprising a polarizer for polarizing light from the light emitter.

40. The light source of claim 39 in which the polarizer is of the circularly polarizing type.

FIG. 1

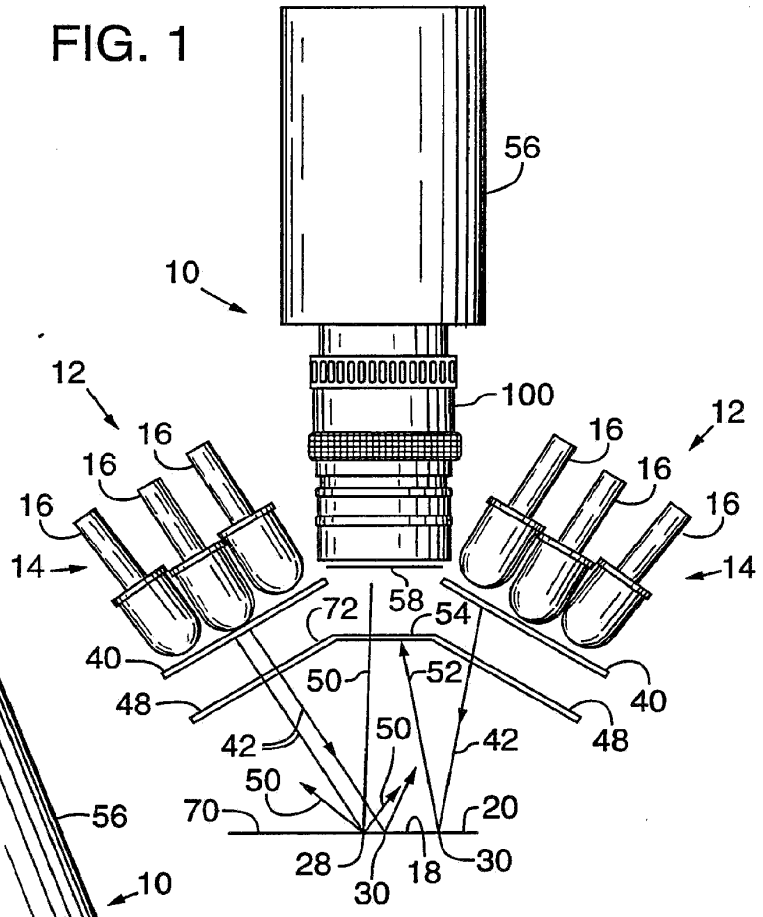
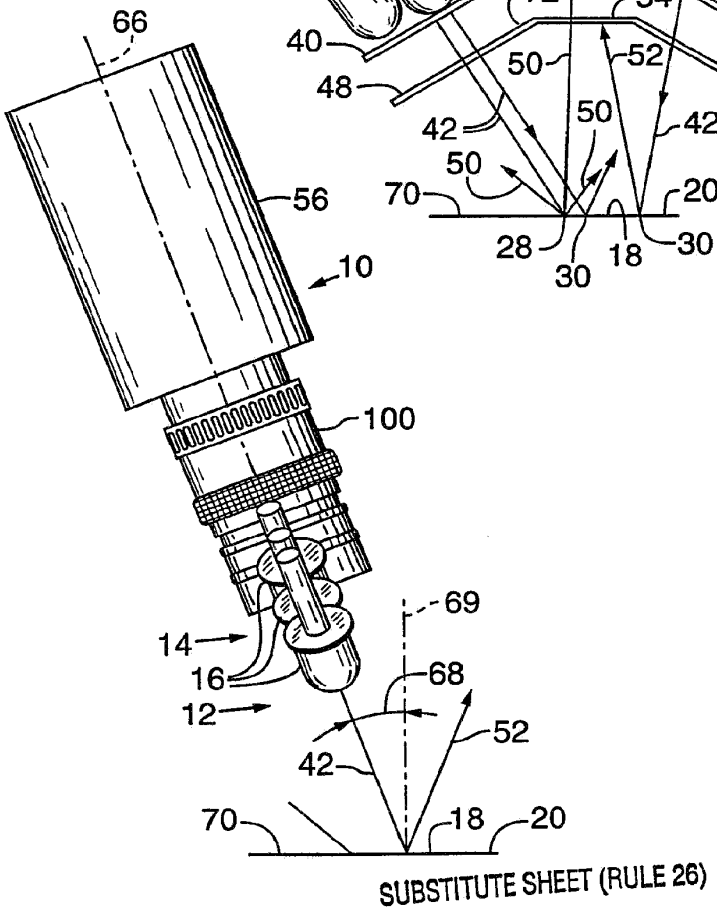


FIG. 2



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FIG. 3

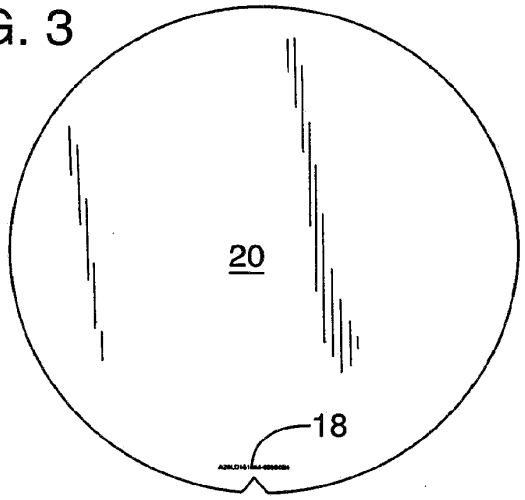


FIG. 4

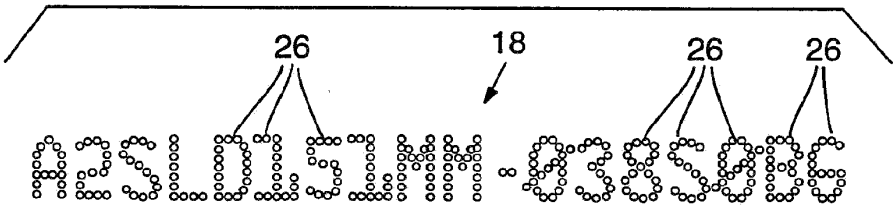
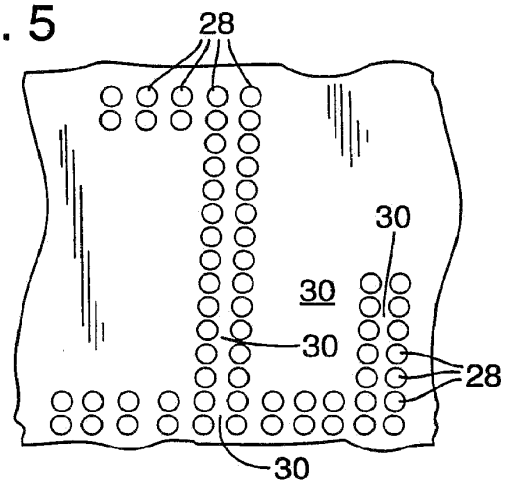


FIG. 5



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FIG. 6

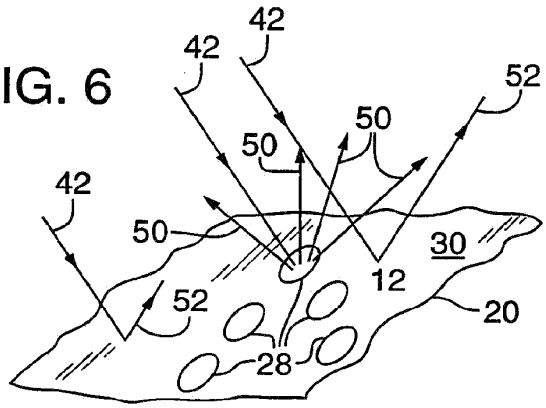


FIG. 7

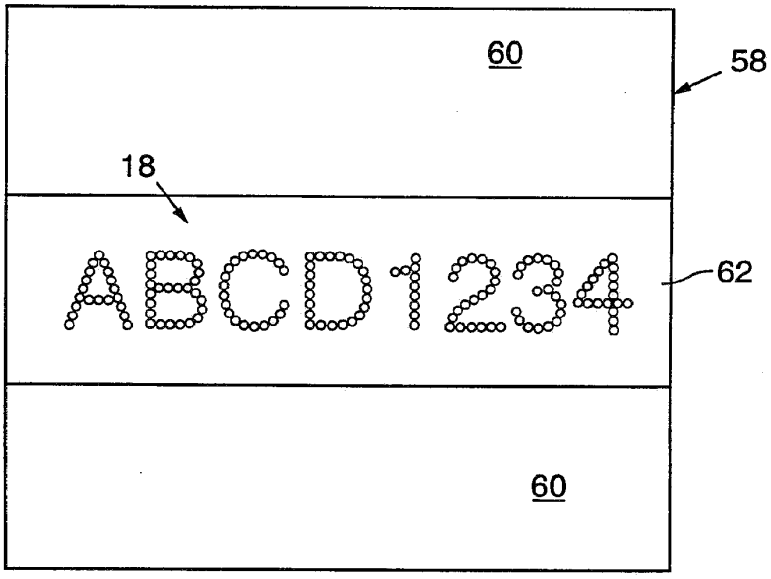
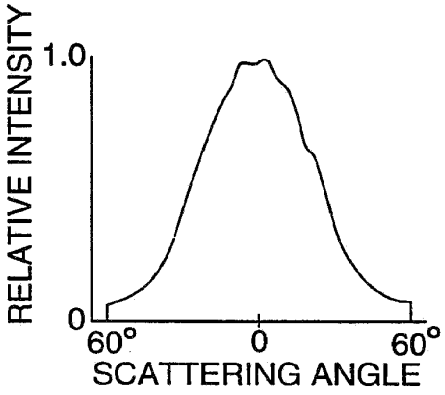


FIG. 8



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FIG. 9

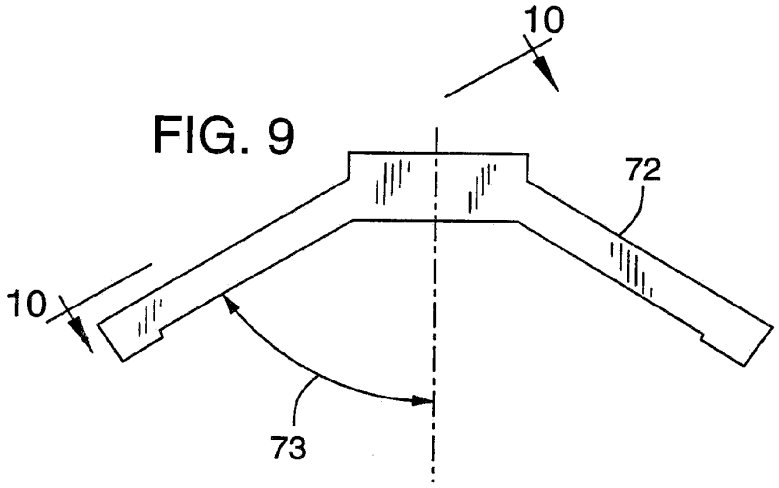


FIG. 10

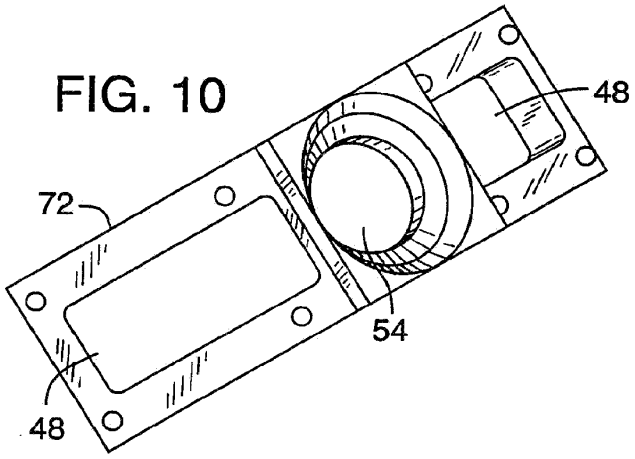
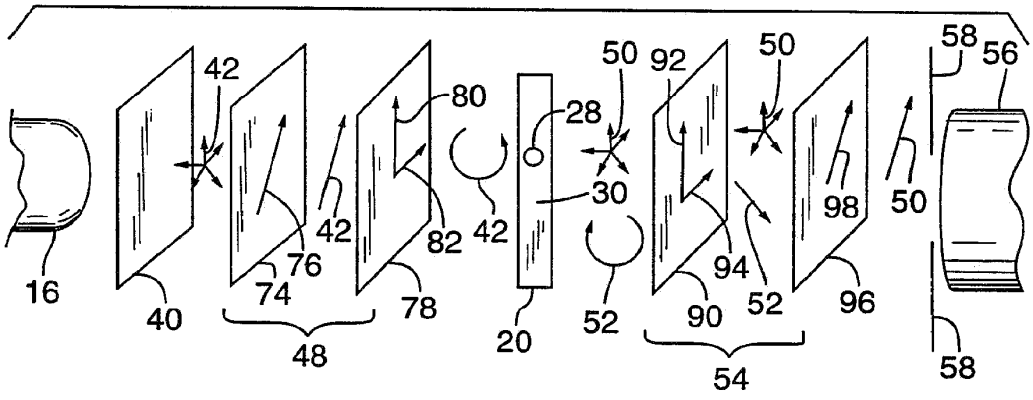
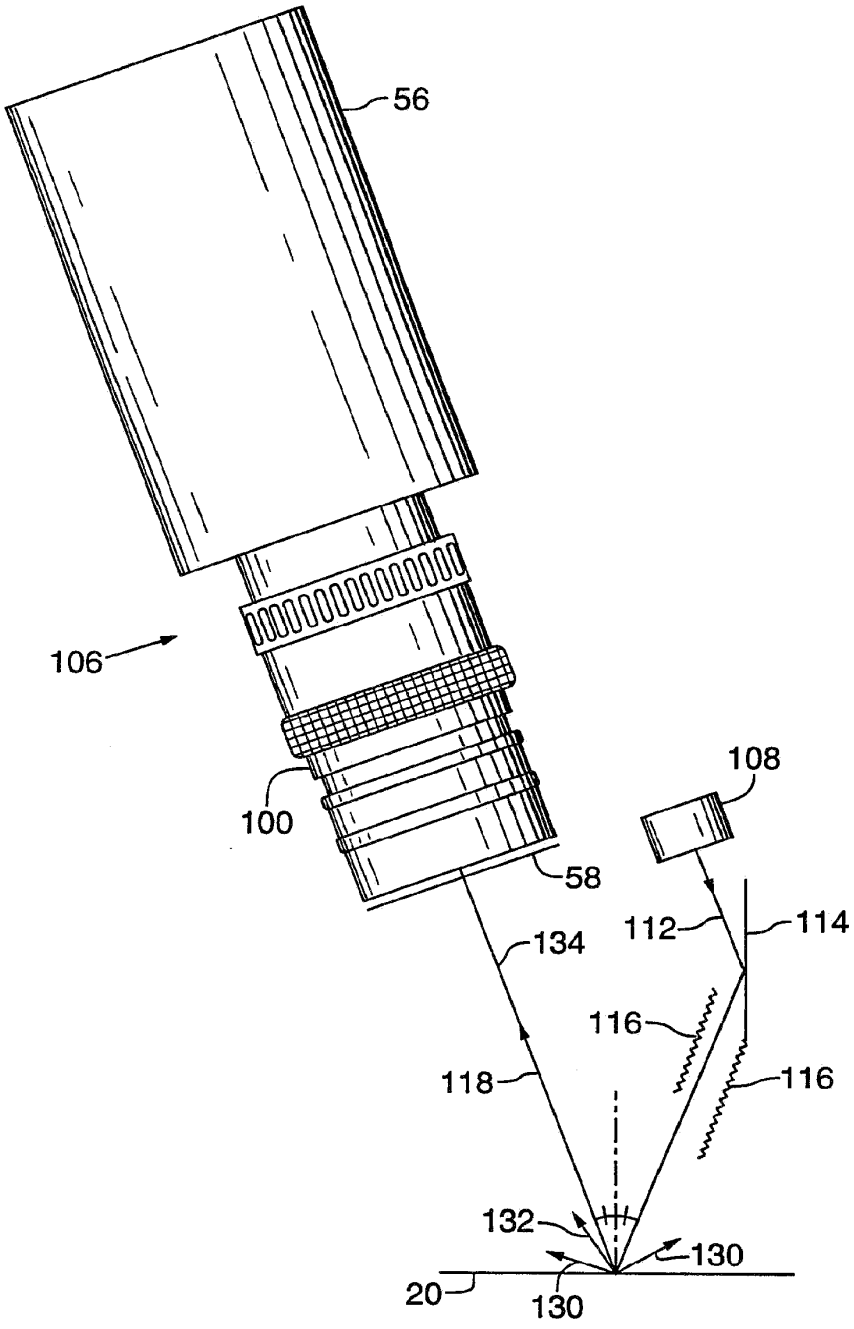


FIG. 11



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FIG. 12



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(57) Abstract

The invention relates to the fabrication and use of biosensors comprising a plurality of optical fibers each fiber having attached to its "sensor end" biological "binding partners" (molecules that specifically bind other molecules to form a binding complex such as antibody-antigen, lectin-carbohydrate, nucleic acid-nucleic acid, biotin-avidin, etc.). The biosensor preferably bears two or more different species of biological binding partner. The sensor is fabricated by providing a plurality of groups of optical fibers. Each group is treated as a batch to attach a different species of biological binding partner to the sensor ends of the fibers comprising that bundle. Each fiber, or group of fibers within a bundle, may be uniquely identified so that the fibers, or group of fibers, when later combined in an array of different fibers, can be discretely addressed. Fibers or groups of fibers are then selected and discretely separated from different bundles. The discretely separated fibers are then combined at their sensor ends to produce a high density sensor array of fibers capable of assaying simultaneously the binding of components of a test sample to the various binding partners on the different fibers of the sensor array. The transmission ends of the optical fibers are then discretely addressed to detectors - such as a multiplicity of optical sensors. An optical signal, produced by binding of the binding partner to its substrate to form a binding complex, is conducted through the optical fiber or group of fibers to a detector for each discrete test. By examining the addressed transmission ends of fibers, or groups of fibers, the addressed transmission ends can transmit unique patterns assisting in rapid sample identification by the sensor.

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HIGH DENSITY ARRAY FABRICATION AND READOUT METHOD FOR A FIBER OPTIC BIOSENSOR

This invention was made in part with government support under Grant (or Contract) No. CA45919 awarded by the National Institutes of Health. The government has certain rights in this invention.

This invention relates to the fabrication and use of biosensors comprising biological "binding partners" (molecules that specifically bind other molecules to form a binding complex such as antibody-antigen, lectin-carbohydrate, nucleic acid-nucleic acid, biotin-avidin, *etc.*) linked to optical fibers. The invention provides improved methods for attachment of various biomolecules to solid surfaces, particularly optical fibers. Batches of optical fibers are mass processed with the same species of binding partner, singulated from their particular batch, and regrouped with like optical fibers from other batches having other species of binding partners.

This invention was made with the Government support under Grant No. CA 45919, awarded by the National Institute of Health and under Grant No. DE-AC03-76SF0098, awarded by the Department of Energy. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Biosensors are sensors that detect chemical species with high selectivity on the basis of molecular recognition rather than the physical properties of analytes. *See, e.g., Advances in Biosensors*, A.P.F. Turner, Ed. JAI Press, London, (1991). Many types of biosensing devices have been developed in recent years, including enzyme electrodes, optical immunosensors, ligand-receptor amperometers, and evanescent-wave probes. Updike and Hicks, *Nature*, 214: 986 (1967), Abdel-Latif *et al., Anal. Lett.*, 21: 943 (111988); Giaever, *J. Immunol.*, 110: 1424 (1973); Sugao *et al. Anal. Chem.*, 65: 363 (1993), Rogers *et al. Anal. Biochem.*. 182: 353 (1989).

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Biosensors comprising a biological "binding molecule" attached to an optical fiber are well known in the prior art, most typically as evanescent wave detectors (*see*, for example, U.S. Patent 4,447,546 to Hirschfeld and U.S. Patents 4,582,809 and 4,909,990 to Block *et al.*). In order to maximize sensitivity and selectivity such biosensors typically utilize a single species of biological binding molecule affixed to the face of the sensor.

Such "single-species" biosensors are limited in that they have no inherent means to correct or calibrate for non-specific binding. Thus, they must be calibrated against an external standard. In addition, they are limited to the detection of a single analyte.

Biosensors comprising two or more species of biological binding partners overcome these limitations. A "multi-species" biosensor in principle permits simultaneous detection of as many different types of analytes as there are species of biological binding partner incorporated into the sensor. In addition, comparison of the amounts of a single analyte binding to multiple species of binding partner provides a measure of non-specific binding and thus acts as an intrinsic control for measurement variability introduced by non-specific binding.

In addition, the inclusion of fibers bearing biological binding partners specific for various analytes known to create a background signal in a particular assay provides a means for simultaneously measuring and substracting out the background signal. The provision of a multiplicity of fibers bearing different species of binding partner allows the detection of a multiplicity of moieties contributing to a background, or other, signal and the dissecting out of the contribution of each moiety to that signal.

To be most useful, a multi-species biosensor requires that the sensor provide a separate signal characterizing binding of analytes to each of the various species of binding partner comprising the probe. Thus each species of binding partner must be individually "addressed".

In addition, a "sensor face" (the surface bearing the biological binding partners) that has a relatively small surface area will facilitate measurement of small sample volumes as less sample material will be required to fully immerse the sensor face. A small surface area detector will also prove advantageous for use in *in vitro* measurements. Preparation of a detector bearing a large number of different biological

binding partners that occupies a small area may be viewed as the preparation of a high density array of biological binding partners.

The creation of high density arrays of biological binding partners where each species of binding partner is uniquely addressed presents formidable fabrication problems. One of the most successful approaches, to date, is the large scale photolithographic solid phase synthesis pioneered by Affymax Inc. (*see, e.g.*, Fodor *et al. Science* 251: 767-773 (1991) and U.S. Patent No. 5,143,854). In this approach arrays of peptides or nucleic acids are chemically synthesized on a solid support. Different molecules are simultaneously synthesized at different predetermined locations on the substrate by the use of a photolithographic process that selectively removes photolabile protecting groups on the growing molecules in particular selected locations of the substrate. The resulting array of molecules is "spatially addressed". In other words the identity of each biological molecule is determined by its location on the substrate.

The photolithographic approach, however, is limited to molecules that can be chemically synthesized. Thus, it is typically restricted to peptides shorter than about 50 amino acids and nucleic acids shorter than about 150 base pairs. In addition, the photolithographic approach typically produces such arrays on a planar substrate (*e.g.* a glass slide) and provides no intrinsic mechanism by which a signal produced by the binding of a particular biological binding partner may be transmitted.

U.S. Patent No. 5,250,264 to Walt *et al.* discloses a sensor comprising a fiber optic array using a "plurality of different dyes immobilized at individual spatial positions on the surface of the sensor." Each dye is capable of responding to a different analyte (*e.g.*, pH, O_2, CO_2, *etc.*) and the sensor as a whole is capable of providing simultaneous measurements of multiple analytes.

Although the sensor disclosed by Walt *et al.* is not a biosensor, the reference describes a means of fabricating a sensor bearing a plurality of uniquely addressed "detection moieties". In Walt *et al.* optical fibers are first assembled to form a bundle. Transmission ends of a fiber or group of fibers of are then specifically illuminated. Each illuminated fiber transmits the light to its respective sensor end where the light "photopolymerizes" a sensor dye mixture causing the dye to bind to the sensor end. This process is repeated with different fibers for different photopolymerized dyes. This repetition continues until a sensor array is constructed.

This approach suffers from the limitations that it requires photopolymerizable sensor dyes and thus is limited in the number of different species per probe by the number of different dye type. In addition, this reference provides no means for attaching uniquely addressed biological molecules (*e.g.* peptides, nucleic acids, antibodies) to the sensor. Thus Walt *et al.* provide no means for the fabrication of biosensors.

SUMMARY OF THE INVENTION

The present invention provides a novel means for fabricating biosensors comprising a plurality of biological "binding partners" (molecules that specifically bind other molecules to form a binding complex such as antibody-antigen, lectin-carbohydrate, nucleic acid-nucleic acid, biotin-avidin, *etc.*) linked to optical fibers. In particular the method provides a means of preparing a high density array of biological binding partners where each species of binding partner is uniquely addressed. In contrast to certain methods in the prior art the biological binding partners utilized in the present invention are not limited to chemically synthesized oligonucleotides or peptides, but rather include nucleic acids, antibodies, proteins, lectins and other binding partners derived from cells, tissues or organisms in their native state or otherwise modified through the methods of recombinant DNA technology.

The invention also provides improved methods for the attachment of various biomolecules (*e.g.*, nucleic acids) to solid surfaces. The methods involve the use of a matrix solution comprising the biomolecule of interest and an organic or inorganic matrix polymer. Typically, the matrix solution is monophasic solution comprising both the matrix polymer (*e.g.*, nitrocellulose) in an appropriate solvent (*e.g.*, DMSO) and an aqueous solution of the nucleic acid or other biomolecule. The matrix solutions of the invention can be used for attachment of nucleic acids and other biopolymers to any solid surface commonly used as a solid surface in nucleic acid hybridization, immunoassays and the like. Exemplary solid surfaces include glass (*e.g.*, optic fibers, slides, and beads), plastics, quartz, and the like.

In particular, the biosensors of the present invention comprise a multiplicity of optical fibers bundled together to form an optical fiber array. The sensor end of each optical fiber or group of optical fibers comprising the optical fiber array bear

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a particular species of biological binding partner. Optical signals produced by binding of an analyte to a biological binding partner are conducted along the respective optical fibers to a transmission end which may be attached to a detector. Detection of the signal from the fibers corresponding to each species of biological binding partner provides a simultaneous measurement of the binding of a multiplicity of analytes.

The present invention provides a method of fabrication of fiber optic biosensors. The method involves providing a multiplicity of optical fibers which are grouped into a plurality of separate fiber groups or batches. Each fiber has a sensor end and a transmission end and the fibers in each group are oriented so that the sensor ends are commonly aligned. Each group of fibers is then treated to attach a single species of biological binding partner to the sensor ends of the constituent fibers. Alternatively, a multiplicity of species of biological binding partner may be attached to each group as long as the multiplicity of species of biological binding partners attached to one fiber group is different than the multiplicity of species attached to the other fiber groups.

Fibers or groups of fibers are then selected and discretely separated from their respective batches. One or more of the discretely separated fibers from each group are then recombined at their sensor ends with other fibers from other batches to produce an optical fiber array. The sensor ends may be arranged in a substantially planar orientation or may be tiered to form a tiered sensor face. The optical fiber array contains fibers capable of assaying simultaneously the binding of components of a test sample to the various binding partners on the different fibers of optical fiber array.

The batch identity of each fiber is maintained during the bundling process, preferably at or adjacent to the transmission end of the fibers. These transmission ends are then discretely addressed to detectors - such as a multiplicity of optical sensors. The location and spatial array of the transmission ends corresponding to particular biological binding partners are distinct from one another and known.

Thus, the invention provides for the fabrication of a high density array of biological binding partners in which each binding partner is uniquely addressed. An optical signal, produced by binding of the binding partner to its substrate to form a binding complex, is conducted through the optical fiber or group of fibers to a detector for each discrete test. Thus, binding of a molecule to a particular biological binding partner is specifically detectable. By examining the addressed transmission ends of

fibers, or groups of fibers, the addressed transmission ends can transmit unique patterns assisting in rapid sample identification of analytes by the sensor.

In one embodiment, the fiber optics might bear nucleic acid binding partners to which nucleic acids in the test sample might hybridize. As used herein, the terms "nucleic acid" or "nucleic acid molecule" refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

In one particularly preferred embodiment, the biosensor comprises a plurality of fibers, each fiber including an sensor end and a transmission end, the sensor end of at least one first fiber having attached a first biological binding partner and the sensor end of at least one second fiber having attached a second biological binding partner, a transmission array having first and second positions addressing the transmission ends of the first and second fibers, means for addressing the transmission ends of the first and second fibers to the transmission array, optical interrogation means adjacent the transmission ends for examining the comparative attachment of analytes at the sensor ends of the fibers. The sensor ends of the first and second fibers may be arranged to form a tiered sensor face. The first and second binding partners may be nucleic acids, for example, DNA and cDNA. The nucleic acids may be mapped to specific regions on one or more human chromosomes or may be expressed sequences such as cDNA or mRNA. In a particularly preferred embodiment, the target nucleic acids are about 1,000 to 1,000,000 nucleotides in complexity.

Nucleic acid bearing arrays are particularly useful in Comparative Genomic Hybridization (CGH) assays to detect chromosomal abnormalities; in particular increases or decreases in copy number of particular chromosomal regions. In one example of this approach, a first collection of (probe) nucleic acids is labeled with a first label, while a second collection of (probe) nucleic acids is labeled with a second label. A biosensor, as described above, is one in which the biological binding partners are target nucleic acids. (As used herein the term "target nucleic acids" typically refers to nucleic acids attached to the optical fibers comprising the fiber optic array, while "probe nucleic acids" are those nucleic acids free in solution that hybridize with the target nucleic acids.) The ratio of hybridization of the nucleic acids is determined by the ratio

of the two (first and second) labels binding to each fiber in the array. Where there are chromosomal deletions or multiplications, differences in the ratio of the signals from the two labels will be detected. Identification of the specific optical fibers in the array giving rise to these ratios will indicate the nucleic acid sequence the probe bears and thus the nucleic acid sequence that is altered.

The target nucleic acids (the nucleic acids attached to the optical fibers) may include DNA and cDNA and may be mapped to specific regions in human chromosomes. In addition, the target nucleic acids are preferably about 1,000 to 1,000,000 nucleotides in complexity. The complexity of the sequence complementary to the target nucleic acid is preferably less than 1% of the total complexity of the sequences in the sample.

The first and second labels are preferably fluorescent labels. In a particularly preferred embodiment, the first probe nucleic acids comprise mRNA or cDNA from a test cell and the second probe nucleic acids comprise mRNA or cDNA from a reference cell. In another preferred embodiment, the first probe nucleic acids are from a test genome and the second probe nucleic acids are from a reference genome. The test genome may comprise nucleic acids from fetal tissue or from a tumor.

According to one aspect of the invention, arrays of optical fibers are disclosed where the interrogating end of each fiber in the array comprises a multiplicity of biological "binding partners." Each binding partner is attached to one or more optical fibers specifically addressed or identified at the transmitting end as being connected to the particular binding partner. With the transmission ends properly addressed and interrogated, measurement occurs.

In one specific embodiment, arrays of optical fibers bearing nucleic acid molecules are disclosed. These optical fibers at their interrogating ends have specific nucleic acids such as nucleic acids having a certain minimum length (*e.g.* 400 bp), or being derived from particular libraries (*e.g.* evenly spaced along a particular chromosome or representing a particular gene).

It is an advantage of the disclosed apparatus and process that the constructed array can be tailored to rapid screening of extensive arrays of biological binding partners. Using already identified information, arrays can be assembled which can simultaneously and rapidly survey samples nucleic acid variations across entire genomes. For example, a fiber optic sensor bearing 30,000 target nucleic acids, each containing 100kb of genomic DNA could give complete coverage of the human genome.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration showing a group of fibers with the sensor ends bound together for joint treatment to attach a binding partner and the transmission ends discretely marked to enable the fibers of the illustrated group to be distinguished from fibers of similar groups when subsequent separation of the fibers later occurs from the group.

Figure 2 illustrates a plurality of differing batches of treatment solution with the sensor ends of the group of fibers of Figure 1 being immersed for treatment in one of the fiber batches;

Figure 3 illustrates differing groups of previously treated fibers lying side-by-side with fibers being singulated from each group for gathering into a common high density array.

Figure 4 illustrates an assembled high density array at the sensor and transmitting ends only with disposition of the sensor ends in a tiered disposition for transillumination by interrogating light and the detector ends identified and discretely addressed to a sensor array, the sensor array here illustrated having a corresponding array of condensing lenses for relaying fiber illumination to a detector surface;

Figure 5 illustrates a comparative genomic hybridization process being carried out with two samples for comparison having been previously tagged with differing fluorophores and being added together in a common batch.

Figures 6A and 6B illustrate an expanded detail of the common batch of Figure 5 being transilluminated at the tiered sampling fibers at the sensor end of the array for the excitation of fluorophores attached to the binding partners without undue direct illumination of the fibers themselves.

Figures 7A and 7B illustrate a detector that may be used in conjunction with an optical fiber array or with any other array of light sources (*e.g.* an array of hybridized fluorescent probes).

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is a marked improvement in fiber optic biosensors, methods for fabricating biosensors, and methods for performing qualitative and quantitative measurements of biological molecules using a unique fiber optic biosensor. In particular, the present invention provides for a novel method of construction of a biosensor comprising a high-density array of biological binding partners.

The biosensors of the present invention generally comprise a bundle of coalligned optical fibers. Each individual optical fiber or group of fibers within the biosensor bears a single species of biological binding partner. As used herein biological binding partners are molecules that specifically recognize and bind other molecules thereby forming a binding complex. Typical binding complexes include, but are not limited to, antibody-antigen, lectin-carbohydrate, nucleic acid-nucleic acid, biotin-avidin, receptor-receptor ligand, etc.

The terms "specifically recognize and bind" refers to the binding of a biological binding partner to a particular molecule and to no other molecule to which the biological binding partner is normally exposed. In the case of nucleic acids, specific binding is by hybridization and the terms "specific hybridization" or "specifically hybridizes with" refers to hybridization in which a probe nucleic acid binds substantially to target nucleic acid and does not bind substantially to other nucleic acids present in the biosensor under defined stringency conditions. One of skill will recognize that relaxing the stringency of the hybridizing conditions will allow sequence mismatches to be tolerated. The degree of mismatch tolerated can be controlled by suitable adjustment of the hybridization conditions.

The term "species", as used herein, refers to a biological binding partner capable of specifically binding a particular target molecule. Thus, for example, biological binding partners may both be nucleic acids, but if they have different nucleotide sequences, so that they specifically hybridize to different molecules, they are

considered different species. Similarly, two antibodies specific for different epitopes are considered different species.

In a preferred embodiment, the biosensor bears two or more different species of biological binding partner. The use of two or more species of binding partner permits the simultaneous detection of two or more analytes in a test sample with the number of detectable analytes limited only by the number of different biological binding partners present on the biosensor. The biosensor may optionally include additional optical fibers lacking biological binding partners. These additional fibers may bear moieties for the detection of various physical parameters of the test sample, such as temperature or pH, or alternatively may lack any moiety and simply serve as an optical conduit for visualization thereby serving as an endoscope for guiding the insertion of the biosensor probe in various *in vivo* applications.

A biosensor bearing a plurality biological binding partners permits the simultaneous assay of a multiplicity of analytes in a sample. In addition, the measurement of binding of a single analyte to a number of different species of biological binding partners provides a control for non-specific binding. A comparison of the degree of binding of different analytes in a test sample permits evaluation of the relative increase or decrease of the different analytes. Finally, because of the small cross-sectional area of optical fibers, the bundling together into an optical array a number of optical fibers, each bearing a different biological binding partner, provides an effective mechanism for the fabrication of high density arrays of biological binding partners for suitable for a wide variety of *in vivo* and *in vitro* assays.

I. Organization of the Biosensor.

The unique fiber optic biosensor of the present invention, its organization, its construction and its component parts are illustrated by Figures **1-6** respectively. Each discrete fiber optic biosensor is comprised of a plurality of fiber optic strands **10** disposed coaxially along their lengths to form a single, discrete construction. The biosensor thus comprises an optical fiber array **14**, the smallest common repeating unit within which is a single fiber optical strand **10**.

A preferred fiber optical biosensor is illustrated by Figure **4.** As seen therein an individual fiber optical strand **10** comprises a single optical fiber having a rod-like shaft and two fiber ends designated a sensor end **11** and a transmission end **12** each

of which provides a substantially planar end surface. The optical fiber strand **10** is typically composed of glass or plastic and is a flexible rod able to convey light energy introduced at either of its ends **11, 12**. Such optical fibers **10** are conventionally known and commercially available. Alternatively, the user may himself prepare optical fibers in accordance with the conventional practices and techniques reported by the scientific and industrial literature. Accordingly, the optical fiber **10** is deemed to be conventionally known and available as such.

It will be appreciated that Figures **1-6** are illustrations in which the features have been purposely magnified and exaggerated beyond their normal scale in order to provide both clarity and visualization of extreme detail. Typically, the conventional optical fiber has a cross section diameter of 5-500 micrometers and is routinely employed in lengths ranging between centimeters (*e.g.* in the laboratory) to kilometers (*e.g.* in field telecommunications). Typically, however, when utilized in a biosensor, the optical fibers will preferably range in length from centimeters to about a meter.

Although the optical fiber **10** is illustrated via Figures **1-4** as a cylindrical extended rod having substantially circular end surfaces, there is no requirement or demand that this specific configuration be maintained. To the contrary, the optical fiber may be polygonal or asymmetrically shaped along its length, provide special patterns and shapes at the sensor end or transmission end and need not present an end surface that is substantially planar. Nevertheless, in a preferred embodiment, the optical fiber is substantially cylindrical.

Each optical fiber **10** may be individually clad axially along its length. The cladding may be any material which has a lower refractive index and prevents the transmission of light energy photons from the optical fiber **10** to the external environment. The cladding may thus be composed of a variety of different chemical formulations including various glasses, silicones, plastics, cloths, platings and shielding matter of diverse chemical composition and formulation. Methods of cladding including deposition, extrusion, painting and covering are scientifically and industrially available and any of these known processes may be chosen to meet the requirements and convenience of the user.

The user has a variety of choices at his discretion regarding the configuration of the sensor end **11** of the optical fiber **10**. As indicated above, the sensor end **11** may present a surface that is substantially planar and smooth. Alternatively the sensor end **11** may provide an end surface which is essentially convex or concave.

It will be appreciated that the range and variety of dimensional and configurational variation of the optical fiber **10** is limited only by the user's ability to subsequently dispose and immobilize a biological binding partner on the intended sensor end **11** of the strand. The use of concave or convex sensor ends **11** will provide greater surface area upon which to immobilize a biological binding partner thereby increasing efficiency (the signal to noise ratio per optical fiber) of the biosensor.

While the single repeating component of the fiber optic biosensor is the individual optical fiber **10**, it is the aggregation of a plurality of such fibers to form a discrete optical fiber array **14** that permits the simultaneous detection of a multiplicity of analytes. When the optical fibers are aggregated to form a discrete optical fiber array **14**, the coalligned sensor ends **11** of the fibers are aggregated to form a sensor face **13**. A typical biosensor is illustrated in Figure **4** and Figures **6A** and **6B** in which the sensor face **13** appears in exaggerated, highly simplified views without regard to scale. The optical fiber array **14** comprises a unitary rod-like collective body forming a sensor face **13** and a and a transmission face **15**.

In practice, it is estimated that there are typically 1000-3000 fiber optical strands in a conventional imaging fiber of 0.5 mm diameter and nearly 1 million strands per square millimeter. The total number of individual fiber optic strands forming the optical fiber array **14** of the present invention will be approximately as great; the total number varying with the cross-sectional diameter of each optical fiber, the pattern of packing of the individual optical fibers in the collective body, and the thickness of cladding material, when employed. It will be appreciated that a 1 square millimeter biosensor, containing nearly 1 million strands in which groups of about 33 optical fibers are each labeled with a different species of biological binding partner will produce a sensor face **13** comprising approximately 30,000 different species of biological binding partner in 1 square millimeter. As explained above, such a sensor could provide nucleic acid biological binding partners covering the entire human genome at 10 megabase intervals.

The sensor face **13** need not be arranged as a planar surface. Rather, the individual optical fibers may be "tiered" so as to protrude from the optical array varying distances. This will maximize the exposure of each optical fiber sensor end **11** both to the sample fluid and to a transilluminating light source **19** as shown in Figures **6A** and **6B**.

In a preferred embodiment, the sensor ends **11** of the optical fibers comprising the optical fiber array **14** will be bundled together in a random or haphazard pattern to form the sensor face **13**. Alternatively, the placement of the sensor ends **11** may be highly ordered with the sensor end of each fiber occupying a specific predetermined location in the sensor face **13**. As indicated above, the sensor ends **11** of the optical fibers **10** forming the sensor face **13** or the optical fiber array **14** have attached a biological binding partner.

Each optical fiber or group of fibers comprising the optical fiber array **14** may bear a different species of biological binding molecule. Although the use of a single species of biological binding partner per optical fiber or group of fibers is preferred, alternatively, each optical fiber or group of fibers may bear a multiplicity of biological binding partners as long as that multiplicity differs from the biological binding partners or multiplicity of biological binding partners present on other fibers or groups of fibers comprising the optical fiber array **14**. The fibers bearing like species of binding partner may be physically grouped together thereby producing distinct regions of the sensor face **13** characterized by the presence of a particular biological binding partner or alternatively the fibers bearing different binding partners may be intermingled, the sensor face **13** presenting a relatively uniform or haphazard or random distribution of species of biological binding partners.

The transmission face **15** of the optical array may present a substantially planar optical array lacking any further attachments. However, in a preferred embodiment, the transmission face **15** will be permanently or removeably attached to a detector **20**, as illustrated in Figure **4**. The detector may comprise one or more lenses for focusing and enhancement of an optical signal transmitted along the optical fibers comprising the optical fiber array **14**. The detector may additionally comprise a device for transforming the optical signal into a digital or analog electrical signal. Preferred detectors include phototubes (photomultipliers) or charge coupled devices (CCDs). A

single photomultiplier or CCD element may be arranged to measure the aggregate signal provided by the entire transmission face **15** of the biosensor. Alternatively, a CCD (or other) camera may be focused at the transmission face of the biosensor to simultaeously read signals from all of the optical fibers while permitting individual evaluation of the signal from each fiber or group of fibers. In another embodiment, multiple CCD elements or phototubes are used to each detect a signal representing binding of a single species of biological binding partner present at the sensor face **13** of the biosensor. Thus the detector is preferably arranged to read the signal from single optical fibers **10** or from groups of optical fibers where all of the optical fibers **10** in a group bear the same species of biological binding partner.

In addition to detecting optical signals from a fiber optic array, the detector **20**, may be generally used to amplify and detect optical signals from any array of light sources. Thus, for example, the array of light sources may be an array of fluorescent spots as due to hybridization of fluorescently labeled probes hybridized to arrays of target nucleic acids. Similarly, the array may be of fluorescently labeled antibodies bound to an array of proteins to which the antibodies bind, or conversely fluorescently labeled proteins bound to an array of antibodies.

In a preferred embodiment suitable for such applications, illustrated in Figures **7A** and **7B**, the detector **20** may comprise a compound objective lens **31** that consists of an array of single lenses **32**. The single lenses are spaced so that each lens is focused on a location **34** in the array where fluorescence is to be measured. The detector may optionally include a beam splitter **35** a second lens **36** an optical filter **37** and a detection device such as a camera **38**. The beam splitter is then used to direct an excitation illumination **39** upon the array of light sources. The resulting fluorescence at each spot is then focused through the compound objective lens **31** optionally focused by a second lens, optionally filtered by an optical filter and then detected either visually or by a detection means such as a camera.

The compound objective may be cast, pressed, etched, or ground out of glass, plastic, quartz, or other materials well known as suitable for lens manufacture. The compound lens may be formed as a single piece, or alternatively may be assembled by attaching together simple lenses to form a compound objective.

II. Fabrication of the Biosensor

Figures 1-4 illustrate a method of fabrication of a biosensor comprising a plurality of optical fibers bearing biological binding partners. In general the method involves providing a plurality of optical fibers with each fiber having a sensor end and a transmission end with a particular species of biological binding partner attached to the sensor end of each fiber. Fibers with differing binding partners are combined to form an optical fiber array wherein said fibers have commonly aligned sensor ends for simultaneous assay of a sample. The transmission ends of the combined discrete fibers are addressed for interrogation to produce the fiber optic sensor.

Figure 1, illustrates a particularly preferred embodiment that details one method of providing the optical fibers with attached biolgocial binding partners. A plurality of optical fibers **10** are provided, each fiber having a sensor end **11** and a transmission end **12**. The fibers are arranged together to form a plurality of fiber groups or bundles **16**, as shown in Figure **2**, with the fibers in each bundle disposed coaxially alongside each other with the sensor ends **11** of each fiber commonly aligned at the same end of the bundle. The fibers comprising each bundle may be optionally marked **17** to permit their identification when subsequently removed from the bundle.

As shown in Figure **2**, each bundle of fibers is separately treated to attach a particular species of biological binding partner **18** to the sensor ends **11** of the optical fibers comprising the particular bundle. Many methods for immobilizing biological binding partners **18** on a variety of solid surfaces are known in the art. In general, the desired component may be covalently bound or noncovalently attached through nonspecific binding.

In preparing the sensor end **11** for attachment of the binding partner, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, proteins (*e.g.*, bovine serum albumin) or mixtures of macromolecules (*e.g.*, Denhardt's solution) can be employed to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a biological binding partner and the surface of the sensor end **11** is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and

used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like.

Covalent linkage of the binding partner to the sensor end may be direct or through a covalent linker. Generally linkers are either hetero- or homo-bifunctional molecules that contain two or more reactive sites that may each form a covalent bond with the respective binding partner. Linkers suitable for joining biological binding partners are well known to those of skill in the art. For example, biological binding partners may be joined by a peptide linker, by a straight or branched chain carbon chain linker, or by a heterocyclic carbon. Heterobifunctional cross linking reagents such as active esters of N-ethylmaleimide have been widely used. See, for example, Lerner *et al. Proc. Nat. Acad. Sci. (USA)*, 78: 3403-3407 (1981) and Kitagawa *et al. J. Biochem.*, 79: 233-236 (1976), which are incorporated herein by reference.

The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. Proteins, for example, may be joined to linkers or to functional groups on the sensor end **11** by coupling through their amino or carboxyl termini, or through side groups of various constituent amino acids. Thus, coupling through a disulfide linkage to a cystein is common.

Similarly, methods for immobilizing nucleic acids by introduction of various functional groups to the molecules is known (*see, e.g.*, Bischoff *et al.*, *Anal. Biochem.* 164:336-344 (1987); Kremsky *et al.*, *Nuc. Acids Res.* 15:2891-2910 (1987) which are incorporated herein by reference). Modified nucleotides can be placed on the target using PCR primers containing the modified nucleotide, or by enzymatic end labeling with modified nucleotides.

Preferred methods of immobilizing the nucleic acids and other biomolecules solid surfaces involve the use of a matrix solution comprising the nucleic acid of interest and an organic or inorganic matrix polymer. Typically, the matrix solution is monophasic solution comprising both the matrix polymer and the nucleic acid or other biomolecule. In some embodiments, the matrix solution is prepared by mixing the matrix polymer in an appropriate solvent and an aqueous solution of the biomolecule. Exemplary solvents for the matrix polymer useful for this purpose include organic polar solvents such as DMSO, DMF and tetramethylene sulfone, as well as acetonitrile/water solutions, and Bligh and Dyer monophase solutions.

Alternatively, a diphasic solution comprising a concentrated aqueous solution of the desired nucleic acid or other biomolecule and the matrix polymer in an organic nonpolar solvent such as xylene, toluene, or chloroform can be used. In these embodiments, the diphasic solution must be sonicated or otherwise emulsified to ensure contact between the matrix polymer and the nucleic acid.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the matrix polymer material in the solution. Illustrative polymers which are preferably of low fluorescence, include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like.

The matrix solutions of the invention can be used for attachment of nucleic acids and other biopolymers to any solid surface commonly used as a solid surface in nucleic acid hybridization, immunoassays and the like. Exemplary solid surfaces include glass (*e.g.*, optic fibers, slides, and beads), plastics, quartz, and the like.

A particularly preferred method of attaching biological molecules such as nucleic acids to the sensor end of an optic fiber or other solid surface is by mixing a solution of nitrocellulose in DMSO with the biological molecule or with a highly concentrated aqueous solution of molecule. The resulting matrix solution is spotted on the sensor end of the fiber and dried. The ratio of nitrocellulose to biological molecule is preferably adjusted to ensure that the biological molecule is capable of specifically binding with its binding partner (*e.g.*, a complementary nucleic acid) when attached to the surface. In the case of nucleic acids, the ratio of matrix polymer (*e.g.*, nitrocellulose) to nucleic acid is typically between about 1:10 and about 2:1 by weight, usually between about 1.5:10 to about 1:1 by weight.

An exemplary protocol is a follows. A stock solution consisting of about 2 g of nitrocellulose dissolved in 50 ml DMSO is diluted about 100:1 with DMSO. The diluted solution is then mixed with an appropriate amount of nucleic acid such that the final ratio of nitrocellulose to nucleic acid is in the range noted above. For instance, 4 μl of the diluted DMSO solution (comprising 1.6 μg nitrocellulose) can be mixed with 10 μg of DNA. Alternatively, the DMSO solution can be mixed with a concentrated aqueous solution of the DNA to achieve the desired ratio. The nucleic acid need not be

labelled or be absolutely pure and the aqueous solution may contain proteins such as restriction enzymes. The matrix solution containing the nitrocellulose and the nucleic acid is heated to denature the nucleic acid, if required, and applied to a clean, acid washed surface. The spots are then heated for about 0 to about 60 minutes at about 70°C to dry the solution.

Referring to Figure 3, after the biological binding partners **18** are attached to the sensor faces **11** of the optical fibers **10** comprising each bundle, individual fibers, or groups of fibers, are separated from each bundle. In Figure 3, only four fiber bundles F_1 - F_4 are illustrated. In the process of being separated from each of the respective fiber bundles F_1 - F_4 are individual fibers 10_a - 10_d. These respective fibers are being regrouped into optical fiber array **14**.

The individual fibers or groups of fibers, may be marked prior to separation from the original bundle to facilitate identification of the binding partner bound to a particular fiber or group during later assembly steps. The separated fibers, or groups of fibers, are recombined with fibers or groups of fibers, separated from different bundles to form an optical fiber array **14** comprising a plurality of fibers or groups of fibers where each fiber or group of fibers bears a different species of biological binding partner.

Figure 4 illustrates that members of the optical fiber array **14** are oriented such that the sensor ends **11** of all of the constituent optical fibers **10** are commonly aligned at the same end of the optical fiber array **14** thereby forming a sensor face **13**. The fibers may be arranged in a substantially planar configuration or tiered, as illustrated in Figures **6A** and **6B**.

The fibers may be bundled at the sensor face **13** in a substantially random or haphazard manner with the relative location of a the sensor end **11** of a particular fiber within the sensor face **13** being determined by chance. Alternatively, the fibers may be positioned within the fiber array in a highly ordered manner such that the location of any particular optical fiber **10** in the sensor face **13** is predetermined.

The transmission ends **12** of the optical fibers comprising the optical fiber array **14** are addressed to permit interrogation and detection of binding events to the biological binding partners attached to the sensor face **13**. Addressing is accomplished by any of a number of means well known to those of skill in the art. In a preferred

embodiment, the transmission ends **12** of individual optical fibers, or groups of optical fibers all bearing the same species of biological binding partner, are spatially addressed. This comprises localizing the optical fibers or bundles of optical fibers at fixed locations relative to the other optical fibers or bundles of optical fibers comprising the optical fiber array **14**, see *e.g.* Figure **4**. Most typically this may be accomplished by attaching the fiber array to a fiber optic connector and ferrule (*e.g.* see AMP, Inc. Harrisburg, PA).

Alternatively, the transmission ends **12**, may be addressed by attaching the transmission end of each optical fiber **10** or bundle of optical fibers bearing a particular biological binding partner to an individual detector. Each detector is subsequently known to be associated with a particular biological binding partner and there is no need to preserve a fixed spatial relationship between any of the transmission ends **12**.

Detection of a signal from the biosensor (optical array) may be accomplished by visual inspection of the transmission face **15** of the optical fiber array **14** or by the use of one or more detectors **20**. As indicated above, the transmission face may be permanently or removably attached to a single optical lens or system of multiple optical lenses. The lens or lenses may be arranged to focus an optical signal from the entire transmission face **15** or from selected subregions of the transmission face. In a preferred embodiment, lenses will be arranged to each focus an optical signal from the portion of the transmission face **15** corresponding to a single biological binding partner. In the extreme case, the signal for each optical fiber comprising the optical fiber array **14** will be individually focused.

Again with a lens or lens system present, the signal may be simply detected visually. However, in a preferred embodiment, the use of detectors is contemplated. Preferred detectors are devices that convert an optical signal into a digital or analog electrical signal. Typically detectors are of two general types: phototubes and charge coupled devices (CCDs). A single photomultiplier or CCD element may be utilized to measure the aggregate signal provided by the entire transmission face **15** of the biosensor. More preferably, however, multiple CCD elements or phototubes are used to each detect a signal representing binding of a single species of biological binding partner present at the sensor face **13** of the optical fiber array **14**.

The detector system may be employed with a computerized data acquisition system and analytical program. In this embodiment, providing a fully

automated, computer controlled processing apparatus and measurement system, the data obtained from the biosensor is processed into immediately useful information. By using such fully automated, computerized apparatus and analytical systems, not only are a variety of different measurements made and diverse parameters measured concurrently within a single fluid sample, but also many different fluid samples may be analyzed individually seriatim for detection of multiple analytes of interest concurrently -- each individual fluid sample following its predecessor in series.

II. Methods of Use

A variety of *in vitro* measurements and analytical determinations may be made using a fiber optic biosensor prepared in accordance with the present invention. *In vitro* applications and assay techniques may be performed concurrently using one or multiple fluid samples. Each concurrently conducted measurement or determination for different analytes of interest is made individually, accurately and precisely. The observed results are then correlated and/or computed to provide precise information regarding a variety of different parameters or ligands individually.

The fiber optic biosensor of the present invention may also be employed in a variety of different *in vivo* conditions with both humans and animals. The present invention provides accurate and precise measurements and determinations using a single discrete fiber optic biosensor rather than the conventional bundle of different sensors joined together for limited purposes. The present invention thus provides a minimum-sized diameter sensor for *in vivo* catheterization: a minimum intrusion into the bloodstream or tissues of the living subject for assay purposes, and a minimum of discomfort and pain to the living subject coupled with a maximum of accuracy and precision as well as a multiplicity of parameter measurement in both qualitative and/or quantitative terms.

The biosensor of the present invention may be utilized for the detection of a wide variety of analytes, depending on the particular biological binding partner selected. As indicated above, biological binding partners are molecules that specifically recognize and bind other molecules thereby forming a binding complex. Typical binding complexes include, but are not limited to, antibody-antigen, lectin-carbohydrate, nucleic acid-nucleic acid, biotin-avidin, receptor-receptor ligand, *etc.* Either member of the

binding complex may be used as the biological binding member attached to the sensor end **11** of the optical fibers comprising the biosensor. Thus, for example, where it is desired to detect an antibody in a sample, the corresponding antigen may be attached to the sensor end. Conversely, where it is desired to detect the antigen in the sample, the antibody may be attached to the sensor end.

The selection of binding partners for a particular assay is well known to those of skill in the art. Typically, where proteins are to be detected, antibodies are most preferred as the biological binding partner. Where enzymatic substrates are to be detected, enzymes are preferred biological binding partners, and where nucleic acids are to be detected, nucleic acid binding partners are most preferred. Thus, for example, fiber optic biosensors have been described that utilize enzymes such as xanthine oxidase and peroxidase to detect hypoxanthine and xanthine (Hlavay *et al.*, *Biosensors and Bioelectronics*, 9(3): 189-195 (1994), that use alkaline phosphatase to detect organophosphorous-based pesticides (Gao *et al. Proceedings -- Lasers and Electro-Optics Society, Annula Meeting*, 8(4): abstract 20782 (1994), and that use antibodies or DNA binding proteins (Anderson, *et al.*, *Fiber Optic Medical and Fluorescent Sensors and Applications*, *Proc. S.P.I.E.* 1648: 39-43 (1992).

Of course the biosensor may be designed to simultaneously detect several different classes of analyte. Thus the sensor may bear a combination of two or more different classes of biological binding partner. The sensor face **13** will bear one or more binding partners selected, for example, from the group consisting of nucleic acids, proteins, antibodies, carbohydrates, biotin, avidin, and lectins.

In the simplest application, the biosensor of the present invention may be utilized to detect a single analyte in a test sample. The test sample may be *in vivo*, in culture, or *in vitro*. The assay may register simple presence or absence of the analyte or may quantify the amount of analyte present in the sample.

The assay may be run in either a direct or a competitive format. In a direct format, the amount of analyte is determined directly measuring the analyte bound to the biological binding partner. In a competitive format, a known analyte is present in the sample and the test analyte is detected by its ability to compete the known analyte from the biological binding partners present on the sensor face **13**.

In a preferred method of use, the optical fibers **10** comprising the optical fiber array **14** conduct an optical signal indicative of the binding between a biological binding partner on the sensor face **11** and the analyte in the sample. The optical signal may be produced by a number of means known to those of skill in the art. Typically the optical signal is generated by a fluorescent, luminescent, or colorimetric label present at the sensor end **11** of the optical fiber **10**. Typically the concentration of label at the sensor end of the optical fiber is a function of the concentration of analyte that specifically binds to the biological binding partner present on that sensor end.

Methods of providing a label whose concentration is a function of the amount of an analyte specifically associated with a biological binding partner are well known to those of skill in the art. In the simplest approach, the analyte itself is labeled. Binding of the analyte to the binding partner then brings the label into proximity with the sensor end **11** to which the binding partner is attached. Alternatively, a labeled "blocking" analyte may be provided in the test sample or pre-bound to the biosensor. Displacement of the labeled "blocking" analyte by the unlabeled test analyte in the sample produces a reduction of label bound to the sensor end where the reduction is proportional to the concentration of unlabeled analyte in the test sample.

Other approaches may use a second biological binding partner that itself is labeled. The first biological binding partner attached to the sensor end binds and thereby immobilizes the analyte. The second, labelled binding partner then binds to the analyte immobilized on the sensor end thereby bringing the label in close proximity to the sensor end where it may be detected.

Luminescent labels are detected by measuring the light produced by the label and conducted along the optical fiber. Luminescent labels typically require no external illumination.

In contrast colorimetric or fluorescent labels typically require a light source. Colorimetric labels typically produce an increase in optical absorbance and/or a change in the absorption spectrum of the solution. Colorimetric labels are measured by comparing the change in absorption spectrum or total absorbance of light produced by a fixed light source. In the present invention, the change in light absorbance or absorption spectrum is preferably detected through the optical fibers comprising the biosensor. The change in absorbance, or absorption spectrum, may be measured as a change in

illumination from an absolutely calibrated light source, or alteratively may be made relative to a second "reference light source". The light source may be external to the biosensor or may be provided as an integral component. In one embodiment, some of the constituent optical fibers will conduct light from the signal and/or reference source to the sensor face. For maximum sensitivity the light used to measure absorbance, or absorption spectrum, changes will be directed directly at the sensor face of the biosensor.

Fluorescent labels produce light in response to excitation by a light source. The emitted light, characteristically of a different (lower) wavelength than the excitation illumination, is detected through the optical fiber to which the fluorescent label has become bound.

The excitation illumination may be provided by an integral component of the biosensor or by a separate light source according to a number of methods well known to those of skill in the art. Evanescent wave systems involve introducing a light beam at the transmission end **12** of the optical fiber. This light beam is conducted along the fiber until it reaches the sensor end **11** of the fiber where it generates in the test solution an electromagnetic waveform known as the evanescent wave component. The evanescent wave component may be sufficient to excite a fluorophore and produce a fluorescent signal. (See, for example U.S. Pat. No. 4,447,546 and U.S. Pat. No. 4,909,990 which are incorporated herein by reference).

In another embodiment, the excitation illumination is provided external to the biosensor. It is particularly preferred that the illumination be provided as a "transillumination" normal to the sensor ends **11** of the optical fibers (see, *e.g.* Figure **4**). This provides an increased signal to noise ratio as, in this configuration, most of the excitation illumination will not be conducted along the optical fibers. The individual optical fibers **10** comprising the biosensor may be tiered, for example, as shown in Figures **6A** and **6B**, to prevent individual fibers from shadowing each other when transilluminated.

To optimize a given assay format one of skill can determine sensitivity of fluorescence detection for different combinations of optical fiber, sensor face configuration, fluorochrome, excitation and emission bands and the like. The sensitivity for detection of analyte by various optical fiber array configurations can be readily determined by, for example, using the biosensor to measure a dilution series of

fluorescently labeled analytes. The sensitivity, linearity, and dynamic range achievable from the various combinations of fluorochrome and biosensor can thus be determined. Serial dilutions of pairs of fluorochromes in known relative proportions can also be analyzed to determine the accuracy with which fluorescence ratio measurements reflect actual fluorochrome ratios over the dynamic range permitted by the detectors and biosensor.

Use in Comparative Genomic Hybridization

In a particularly preferred embodiment, the biosensor of the present invention will be used in a Comparative Genomic Hybridization (CGH) assay. Comparative genomic hybridization (CGH) is a recent approach used to detect the presence and identify the chromosomal location of amplified or deleted nucleotide sequences. (*See,* Kallioniemi *et al.*, *Science* 258: 818-821 (1992), WO 93/18186, and copending application U.S.S.N. 08/353,018, filed on December 9, 1994, which are incorporated herein by reference).

In the traditional implementation of CGH, genomic DNA is isolated from normal reference cells, as well as from test cells (*e.g.*, tumor cells). The two nucleic acids (DNA) are labelled with different labels and then hybridized *in situ* to metaphase chromosomes of a reference cell. The repetitive sequences in both the reference and test DNAs may be removed or their hybridization capacity may be reduced by some means such as an unlabeled blocking nucleic acid (*e.g.* Cot-1). Chromosomal regions in the test cells which are at increased or decreased copy number can be quickly identified by detecting regions where the ratio of signal from the two DNAs is altered. For example, those regions that have been decreased in copy number in the test cells will show relatively lower signal from the test DNA than the reference compared to other regions of the genome. Regions that have been increased in copy number in the test cells will show relatively higher signal from the test DNA.

In one embodiment, the present invention provides a CGH assay in which the biosensor of the present invention replaces the metaphase chromosome used as the hybridization target in traditional CGH. Instead, the biological binding partners present on the biosensor are nucleic acid sequences selected from different regions of the genome. The biosensor itself becomes a sort of "glass chromosome" where hybridization

of a nucleic acid to a particular binding partner is informationally equivalent to hybridization of that nucleic acid to the region on a metaphase chromosome from which the biological binding partner is derived. In addition, nucleic acid binding partners not normally contained in the genome, for example virus nucleic acids, can be employed.

More particularly, in a CGH assay, the biosensor may be utilized in methods for quantitatively comparing copy numbers of at least two nucleic acid sequences in a first collection of nucleic acid molecules relative to the copy numbers of those same sequences in a second collection, as illustrated in Figure **5**. The method comprises labeling the nucleic acid molecules in the first collection **25** and the nucleic acid molecules in the second collection **26** with first and second labels, respectively thereby forming at least two collections of nucleic acid probes. The first and second labels should be distinguishable from each other.

As used herein, the term "probe" is thus defined as a collection of nucleic acid molecules (either RNA or DNA) capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through hydrogen bond formation. The probes are preferably directly or indirectly labelled as described below. They are typically of high complexity, for instance, being prepared from total genomic DNA or mRNA isolated from a cell or cell population.

The probes **30** thus formed are contacted, either simultaneously or serially, to a plurality of target nucleic acids, present on the sensor face **13** of the biosensor of array **14** under conditions such that nucleic acid hybridization to the target nucleic acids can occur. Here a tranilluminating light source 19 is utilized. After contacting the probes to the target nucleic acids the amount of binding of each probe, and ratio of the binding of the probes is determined for each species of target nucleic acid. Typically the greater the ratio of the binding to a target nucleic acid, the greater the copy number ratio of sequences in the two probes that bind to nucleic acid. Thus comparison of the ratios of bound labels among target nucleic acid sequences permits comparison of copy number ratios of different sequences in the probes.

In a preferred embodiment, the sequence complexity of each target nucleic acid in the biosensor is much less than the sequence complexity of the first and second collections of labeled nucleic acids. The term "complexity"is used here according to standard meaning of this term as established by Britten *et al. Methods of Enzymol.*

29:363 (1974). *See, also* Cantor and Schimmel *Biophysical Chemistry: Part III* at 1228-1230 for further explanation of nucleic acid complexity.

The methods are typically carried out using techniques suitable for fluorescence in situ hybridization. Thus, the first and second labels are usually fluorescent labels.

To inhibit hybridization of repetitive sequences in the probes to the target nucleic acids, unlabeled blocking nucleic acids (e.g., Cot-1 DNA) can be mixed with the probes. Thus, the invention focuses on the analysis of the non-repetitive sequences in a genome. However, use of repetitive sequences as targets on teh biosensor and omiting the blocking nucleic acids would permit relative copy number determinations to be made for repetitive sequences.

In a typical embodiment, one collection of probe nucleic acids is prepared from a test cell, cell population, or tissue under study; and the second collection of probe nucleic acids is prepared from a reference cell, cell population, or tissue. Reference cells can be normal non-diseased cells, or they can be from a sample of diseased tissue that serves as a standard for other aspects of the disease. For example, if the reference probe is genomic DNA isolated from normal cells, then the copy number of each sequence in that probe relative to the others is known (*e.g.*, two copies of each autosomal sequence, and one or two copies of each sex chromosomal sequence depending on gender). Comparison of this to a test probe permits detection in variations from normal. Alternatively the reference probe may be prepared from genomic DNA from a primary tumor which may contain substantial variations in copy number among its different sequences, and the test probe may prepared from genomic DNA of metastatic cells from that tumor, so that the comparison shows the differences between the primary tumor and its metastasis. Further, both probes may be prepared from normal cells. For example comparison of mRNA populations between normal cells of different tissues permits detection of differential gene expression that is a critical feature of tissue differentiation. Thus in general the terms test and reference are used for convenience to distinguish the two probes, but they do not imply other characteristics of the nucleic acids they contain.

Target nucleic acids

The target nucleic acids comprising the biological binding partners attached to the sensor ends **11** of the optical fibers **10** and the probes may be, for example, RNA, DNA, or cDNA. The nucleic acids may be from any organism. The nucleic acids may be genomic sequences which correspond to specific regions of a chromosome or may be expressed sequences, such as cDNA or mRNA. Usually the nucleic acid in the target sequences and the probes are from the same species.

The "target nucleic acids" comprising biological binding partners typically have their origin in a defined region of the genome (for example a clone or several contiguous clones from a genomic library), or correspond to a functional genetic unit, which may or may not be complete (for example a full or partial cDNA). The target nucleic acids can also comprise inter-*Alu* or Degenerate Oligonucleotide Primer PCR products derived from such clones. If gene expression is being analyzed, a target element can comprise a full or partial cDNA.

The target nucleic acids may, for example, contain specific genes or, be from a chromosomal region suspected of being present at increased or decreased copy number in cells of interest, *e.g.*, tumor cells. The target nucleic acid may also be an mRNA, or cDNA derived from such mRNA, suspected of being transcribed at abnormal levels.

Alternatively, target nucleic acids may comprise nucleic acids of unknown significance or location. The array of such target nucleic acids comprising the sensor face **13** of a biosensor of the present invention could represent nucleic acids derived from locations that sample, either continuously or at discrete points, any desired portion of a genome, including, but not limited to, an entire genome, a single chromosome, or a portion of a chromosome. The number of target elements and the complexity of the nucleic acids in each would determine the density of sampling. For example an biosensor bearing 300 different species of target nucleic acid (biological binding partners), each target nucleic acid being DNA from a different genomic clone, could sample the entire human genome at 10 megabase intervals. An array of 30,000 elements, each containing 100kb of genomic DNA could give complete coverage of the human genome.

Similarly, an array of target nucleic acids comprising nucleic acids from anonymous cDNA clones would permit identification of those that might be differentially expressed in some cells of interest, thereby focusing attention on study of these genes.

In some embodiments, previously mapped clones from a particular chromosomal region of interest are used as targets. Such clones are becoming available as a result of rapid progress of the worldwide initiative in genomics.

Mapped clones can be prepared from libraries constructed from single chromosomes, multiple chromosomes, or from a segment of a chromosome. Standard techniques are used to clone suitably sized fragments in vectors such as cosmids, yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs) and P1 phage.

While it is possible to generate clone libraries, as described above, libraries spanning entire chromosomes are also available commercially. For instance, chromosome-specific libraries from the human and other genomes are available from Clonetech (South San Francisco, CA) or from The American Type Culture Collection (*see*, *ATCC/NIH Repository of Catalogue of Human and Mouse DNA Probes and Libraries*, 7th ed. 1993).

If necessary, clones described above may be genetically or physically mapped. For instance, FISH and digital image analysis can be used to identify and map the locations on a chromosome to which specific cosmid inserts hybridize. This method is described, for instance, in Lichter *et al.*, *Science*, 247:64-69 (1990). The physically mapped clones can then be used to more finally map a region of interest identified using CGH or other methods.

One of skill will recognize that each target nucleic acids may be selected so that a number of nucleic acids of different length and sequence represent a particular region on a chromosome. Thus, for example, a the sensor face **13** of the biosensor may bear more than one copy of a cloned piece of DNA, and each copy may be broken into fragments of different lengths. One of skill can adjust the length and complexity of the target sequences to provide optimum hybridization and signal production for a given hybridization procedure, and to provide the required resolution among different genes or genomic locations. Typically, the target sequences will have a complexity between about 1 kb and about 1 Mb.

Preparation of probe nucleic acids

As with target nucleic acids (those attached to the fiber optic sensor), a wide variety of nucleic acids can be used as probe nucleic acids in the methods of the present invention. The probes may be comprise, for example, genomic DNA representing the entire genome from a particular organism, tissue or cell type or may comprise a portion of the genome, such as a single chromosome.

To compare expression levels of a particular gene or genes, the probe nucleic acids can be derived from mRNA or cDNA prepared from an organism, tissue, or cell of interest. For instance, test cDNA or mRNA, along with mRNA or cDNA from normal reference cells, can be hybridized to an array of target nucleic acids on the sensor comprising clones from a normalized cDNA library. In addition, probes made from genomic DNA from two cell populations can be hybridized to a target cDNA array to detect those cDNAs that come from regions of variant DNA copy number in the genome.

The methods of the invention are suitable for comparing copy number of particular sequences in any combination of two or more populations of nucleic acids. One of skill will recognize that the particular populations of sample nucleic acids being compared is not critical to the invention. For instance, genomic or cDNA can be compared from two related species. Alternatively, levels of expression of particular genes in two or more tissue or cell types can be compared. As noted above, the methods are particularly useful in the diagnosis of disease.

Standard procedures can be used to isolate nucleic acids (either DNA or mRNA) from appropriate tissues (*see*, *e.g.*, Sambrook, *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1985)). Conventional methods for preparation of cDNA from mRNA can also be used.

The particular cells or tissue from which the nucleic acids are isolated will depend upon the particular application. Typically, for detection of abnormalities associated with cancer, genomic DNA is isolated from tumor cells. For prenatal detection of disease, fetal tissue will be used.

If the tissue sample is small, so that a small amount of nucleic acids is available, amplification techniques such as the polymerase chain reaction (PCR) using degenerate primers can be used. For a general description of PCR, *see*, *PCR Protocols*,

Innis *et al.* eds. Academic Press, 1990. In addition, PCR can be used to selectively amplify sequences between high copy repetitive sequences. These methods use primers complementary to highly repetitive interspersed sequences (*e.g.*, *Alu*) to selectively amplify sequences that are between two members of the *Alu* family (*see*, Nelson *et al.*, *Proc. Natl. Acad. Sci. USA* 86:6686 (1989)).

CGH, at the cytogenetic level, facilitates the search for disease genes by identifying regions of differences in copy number between a normal and tumor genome, for example. For instance, CGH studies have been applied to the analysis of copy number variation in breast cancer (*see*, *e.g.*, Kallioniemi *et al. Proc. Natl. Acad. Sci. USA* 91:2156-2160 (1994)).

In CGH, the resolution with which a copy number change can be mapped is on the order of several megabases. With the present invention the resolution is a function of the length of the genomic DNA segments comprising the target nucleic acid sequences and the difference in map position between neighboring clones. Resolution of more than a factor of 10 better than with standard CGH can be achieved with the present invention. This improved localization will facilitate efforts to identify the critical genes involved in a disease, and permit more sensitive detection of abnormalities involving a small region of the genome, such as in microdeletion syndromes.

Labeling nucleic acid probes

As noted above, the nucleic acids which are hybridized to the target nucleic acids are preferably labeled to allow detection of hybridization complexes. The nucleic acid probes used in the hybridization described below may be detectably labeled prior to the hybridization reaction. Alternatively, a detectable label may be selected which binds to the hybridization product. As noted above, the target nucleic acid array is hybridized to two or more probe nucleic acids, either simultaneously or serially. Thus, the probes are each preferably labeled with a separate and distinguishable label.

The particular label or detectable group attached to the probe nucleic acids is selected so as to not significantly interfere with the hybridization of the probe to the target sequence. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of nucleic acid hybridizations and in general most any label useful in such methods can be applied to the present invention. Thus a label is any composition detectable by

spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means.

However, preferred labels produce an optical signal. Thus, particularly useful labels in the present invention include fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like) and labels that produce a colorimetric signal such as various enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA).

The nucleic acids can be indirectly labeled using ligands for which detectable anti-ligands are available. For example, biotinylated nucleic acids can be detected using labeled avidin or streptavidin according to techniques well known in the art. In addition, antigenic or haptenic molecules can be detected using labeled antisera or monoclonal antibodies. For example, N-acetoxy-N-2-acetylaminofluorene-labelled or digoxigenin-labelled probes can be detected using antibodies specifically immunoreactive with these compounds (*e.g.*, FITC-labeled sheep anti-digoxigenin antibody (Boehringer Mannheim)). In addition, labeled antibodies to thymidine-thymidine dimers can be used (Nakane *et al. ACTA Histochem. Cytochem.* 20:229 (1987)).

Generally, labels which are detectable in as low a copy number as possible, thereby maximizing the sensitivity of the assay, and yet be detectable above any background signal are preferred. A label is preferably chosen that provides a localized signal, thereby providing spatial resolution of the signal from each target element.

The labels may be coupled to the DNA in a variety of means known to those of skill in the art. In a preferred embodiment the probe will be labeled using nick translation or random primer extension (Rigby, *et al. J. Mol. Biol.*, 113: 237 (1977) or Sambrook, *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1985)).

Hybridization of labeled nucleic acids to targets

The copy number of particular nucleic acid sequences in two probes are compared by hybridizing the probes to one or more target nucleic acid arrays (biosensors). The hybridization signal intensity, and the ratio of intensities, produced by the probes on each of the target elements is determined. Typically the greater the ratio of the signal intensities on a target nucleic acid the greater the copy number ratio of sequences in the two probes that bind to that target sequence. Thus comparison of the

signal intensity ratios among target elements permits comparison of copy number ratios of different sequences in the probes.

Standard hybridization techniques are used to probe a target nucleic acid array. Suitable methods are described in references describing CGH techniques (Kallioniemi *et al.*, *Science* 258: 818-821 (1992) and WO 93/18186). Several guides to general techniques are available, *e.g.*, Tijssen, *Hybridization with Nucleic Acid Probes*, Parts I and II (Elsevier, Amsterdam 1993). For a description of techniques suitable for *in situ* hybridizations *see*, Gall *et al. Meth. Enzymol.*, 21:470-480 (1981) and Angerer *et al.* in *Genetic Engineering: Principles and Methods* Setlow and Hollaender, Eds. Vol 7, pgs 43-65 (plenum Press, New York 1985).

Generally, nucleic acid hybridizations utilizing the biosensors of the present invention comprise the following major steps: (1) prehybridization treatment to increase accessibility of target DNA, and to reduce nonspecific binding; (2) hybridization of the mixture of nucleic acids to the nucleic acid targets on the biosensor; (3) posthybridization washes to remove nucleic acid fragments not bound in the hybridization and (4) detection of the hybridized nucleic acid fragments. The reagent used in each of these steps and their conditions for use vary depending on the particular application.

In some applications it is necessary to block the hybridization capacity of repetitive sequences. A number of methods for removing and/or disabling the hybridization capacity of repetitive sequences are known (*see*, *e.g.*, WO 93/18186).

For instance, bulk procedures can be used. In many genomes, including the human genome, a major portion of shared repetitive DNA is contained within a few families of highly repeated sequences such as *Alu*. These methods exploit the fact that hybridization rate of complementary sequences increases as their concentration increases. Thus, repetitive sequences, which are generally present at high concentration will become double stranded more rapidly than others following denaturation and incubation under hybridization conditions. The double stranded nucleic acids are then removed and the remainder used in hybridizations. Methods of separating single from double stranded sequences include using hydroxyapatite or immobilized complementary nucleic acids attached to a solid support. Alternatively, the partially hybridized mixture can be used and the double stranded sequences will be unable to hybridize to the target.

Alternatively, unlabeled sequences which are complementary to the sequences whose hybridization capacity is to be inhibited can be added to the hybridization mixture. This method can be used to inhibit hybridization of repetitive sequences as well as other sequences. For instance, "Cot-1" DNA can be used to selectively inhibit hybridization of repetitive sequences in a sample. To prepare Cot-1 DNA, DNA is extracted, sheared, denatured and renatured to a $C_0t \sim 1$ (for description of reassociation kinetics and C_0t values, *see*, Tijssen, *supra* at pp 48-54). Because highly repetitive sequences reanneal more quickly, the resulting hybrids are highly enriched for these sequences. The remaining single stranded (*i.e.*, single copy sequences) is digested with S1 nuclease and the double stranded Cot-1 DNA is purified and used to block hybridization of repetitive sequences in a sample. Although Cot-1 DNA can be prepared as described above, it is also commercially available (BRL). Reassociation to large C_0t values will result in blocking DNA containing reptitive sequences that are present at lower copy number.

Analysis of detectable signals from hybridizations

Standard methods for detection and analysis of signals generated by labeled probes can be used. In particular, the optical signal produced by binding of a labeled probe to a particular binding partner will be carried along the optical fibers **10**, to which that binding partner is attached. As indicated above, the optical signal may be visualized directly or transduced into an analog or digital electronic signal by means of a detector **20**. To facilitate the display of results and to improve the sensitivity of detecting small differences in fluorescence intensity, a detector and a digital signal analysis system is preferably used. The detector may be equipped with one or more filters to pass the emission wavelengths while filtering out excitation wavelengths thereby increasign the signal to noise ratio. The use of filters will also facilitate distinguishing between binding events involving the two, or more, differently labeled probes. Such detector/filter/signal processing systems are well known to those of skill in the art.

WHAT IS CLAIMED IS:

1. A process for attaching a biological binding partner to a solid surface using a matrix polymer, the process comprising the steps:

forming a matrix solution comprising the nucleic acid and a matrix polymer;

contacting the matrix solution with the solid surface, thereby attaching the biological binding partner such that it can specifically recognize and bind a second molecule.

2. A process for attaching a biological binding partner to a solid surface using a matrix polymer according to claim 1, wherein:

the step of forming the matrix solution comprises contacting an aqueous solution comprising the biological binding partner with a second solution comprising the matrix polymer.

3. A process for attaching a biological binding partner to a solid surface using a matrix polymer according to claim 1, wherein:

the biological binding partner is a nucleic acid and is attached to the solid surface such that the nucleic acid is capable of specifically hybridizing with a complementary nucleic acid.

4. A process for attaching a biological binding partner to a solid surface using a matrix polymer according to claim 1, wherein:

the solid surface is a glass slide.

5. A process for attaching a biological binding partner to a solid surface using a matrix polymer according to claim 1, wherein:

the solid surface is a sensor end of an optical fiber.

6. A process for attaching a biological binding partner to a solid surface using a matrix polymer according to claim 1, wherein:

the matrix polymer is nitrocellulose.

7. A process for attaching a biological binding partner to a solid surface using a matrix polymer according to claim 1, wherein:

the second solution comprises nitrocellulose dissolved in DMSO.

8. A process for attaching a biological binding partner to a solid surface using a matrix polymer according to claim 1, further comprising the step of drying the matrix solution after contacting the matrix solution with the solid surface.

9. A process for constructing a fiber optic bundle for sensing a plurality of biological binding partners within a sample, the process comprising the steps of:

providing a plurality of optical fibers with each fiber having a sensor end and a transmission end wherein each fiber has attached to its sensor end a species of biological binding partner, the binding partner being attached to the sensor end of the optical fiber using a matrix polymer, such that the biological binding partner can specifically recognize and bind a second molecule.;

combining said fibers with differing binding partners to form an optical fiber array wherein said fibers have commonly aligned sensor ends for simultaneous assay of a sample; and,

addressing the transmission end of the combined discrete fibers for interrogation to produce the fiber optic sensor for the sample.

10. The process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

the matrix polymer is nitrocellulose.

11. The process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

the biological binding partner is a nucleic acid and is attached to the sensor end such that the nucleic acid is capable of hybridizing to a complementary nucleic acid.

12. The process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein said providing step comprises:

providing a plurality of optical fibers with each fiber having a sensor end and a transmission end;

placing the plurality of optical fibers in a plurality of fiber groups, each fiber group with commonly aligned sensor ends for simultaneous treatment;

providing a plurality of differing batches, each batch comprising a single species of biological binding partner suitable for attachment under treatment to the commonly aligned sensor ends of the optical fibers of a selected group of optical fibers;

placing and treating the commonly aligned sensor ends of differing fiber groups in differing batches with differing biological binding partners to produce a plurality of groups of optical fibers with the sensor ends of the optical fibers of each group having the same species of biological binding partner and the sensor ends of optical fibers of differing groups having differing species of biological binding partners; and

separating fibers from each group of fibers thereby providing a plurality of optical fibers with each fiber having a sensor end and a transmission end wherein each fiber has attached to its sensor end a species of biological binding partner.

13. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

the combining separated fibers with differing binding partners with commonly aligned sensor ends includes randomly gathering the sensor ends.

14. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

the combining separated fibers with differing binding partners with commonly aligned sensor ends includes assembling said sensor ends to form a tiered sensor face.

15. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

the combining separated fibers with differing binding partners with commonly aligned sensor ends includes assembling said sensor ends to form a planer sensor face.

16. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

the addressing the transmission end of the recombined discrete fibers for interrogation includes addressing the transmission ends of each of the discrete fibers to an optical array.

17. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

transmission ends of the optical fibers of each group having similar binding partners have similar markings for distinguishing the transmission ends corresponding to binding partners.

18. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 9, wherein:

the providing a plurality of batches of differing binding partners includes batches of nucleic acid binding partners to which nucleic acids in the sample might hybridize.

19. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 18, wherein:

the nucleic binding partners each correspond to specific regions on chromosomes.

20. A process for constructing a fiber optic bundle for sensing a plurality of binding partners within a sample according to claim 18, wherein:

the nucleic binding partners each correspond to expressed sequences.

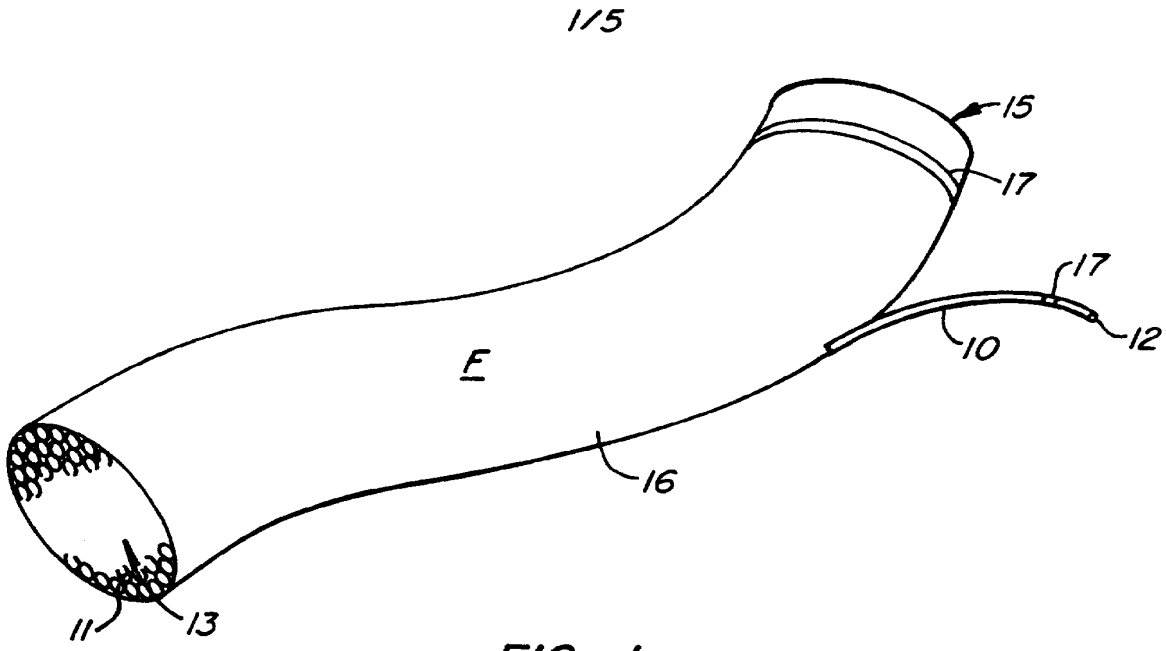

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FIG. 1.

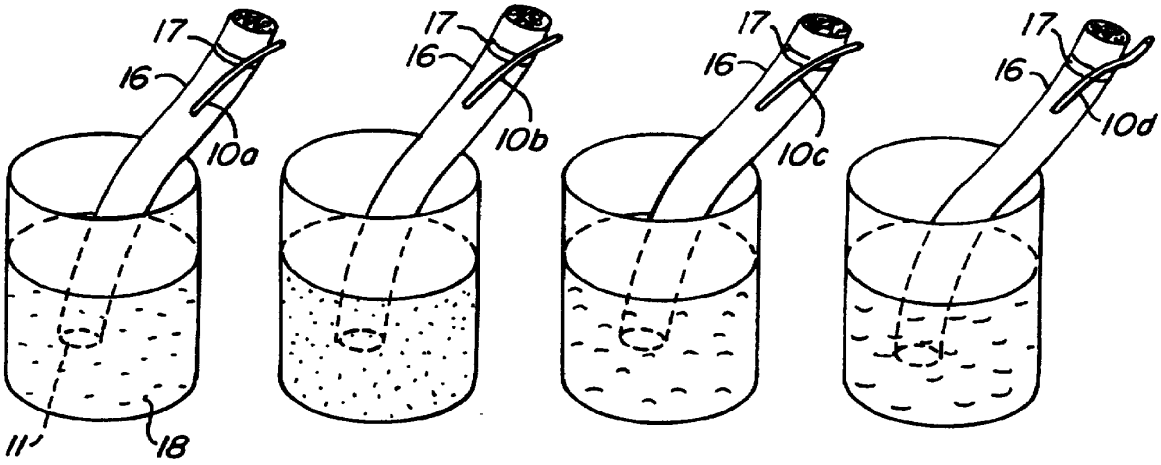

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FIG. 2.

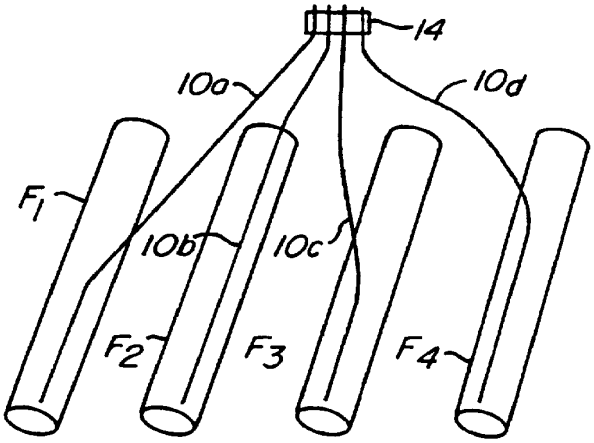


FIG. 3.

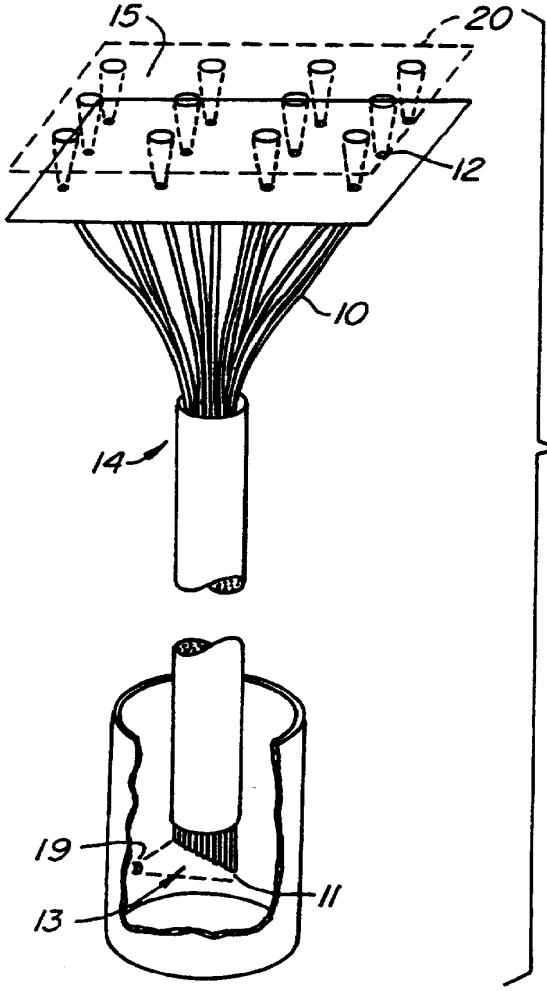


FIG. 4.

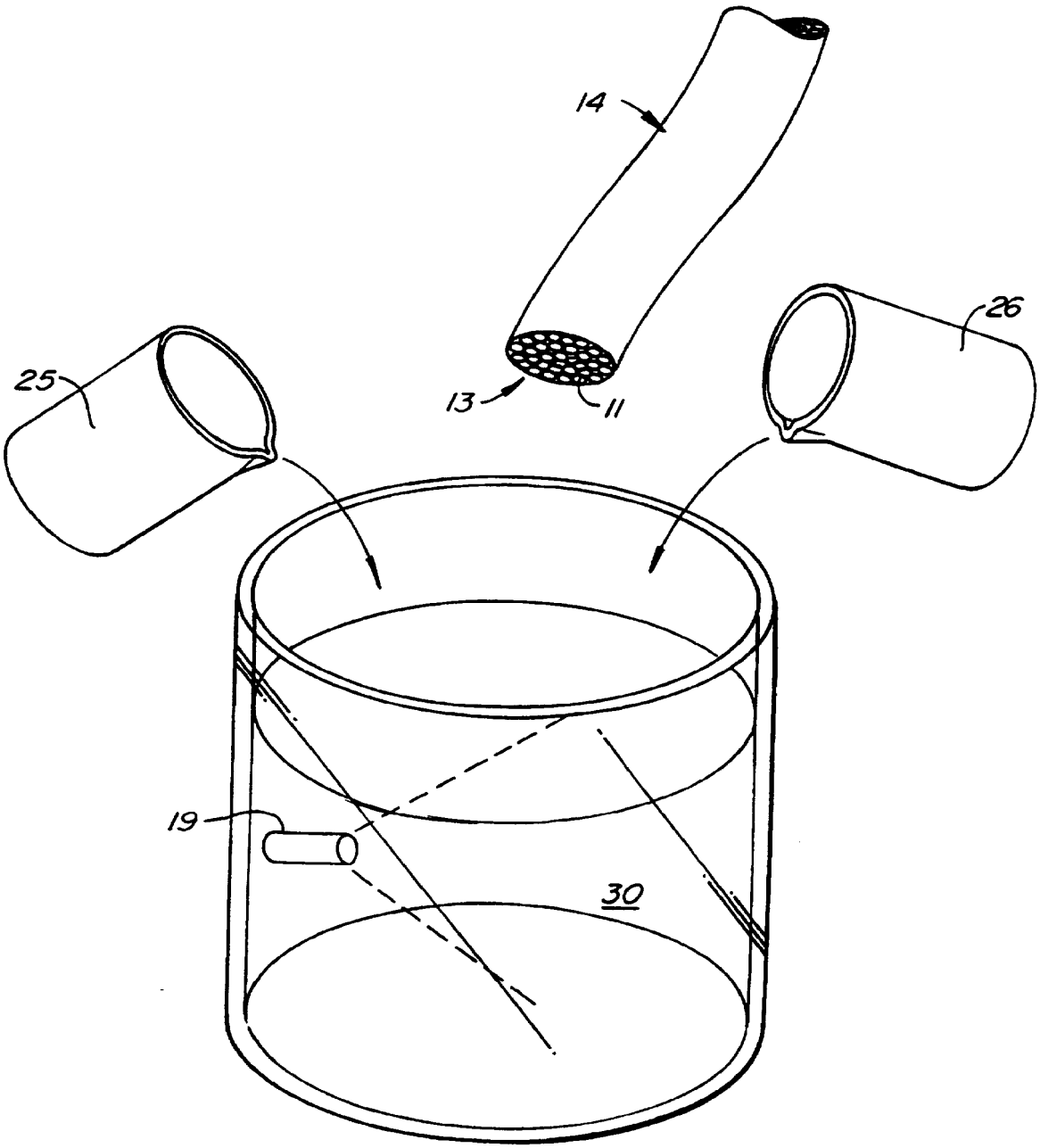


FIG. 5.

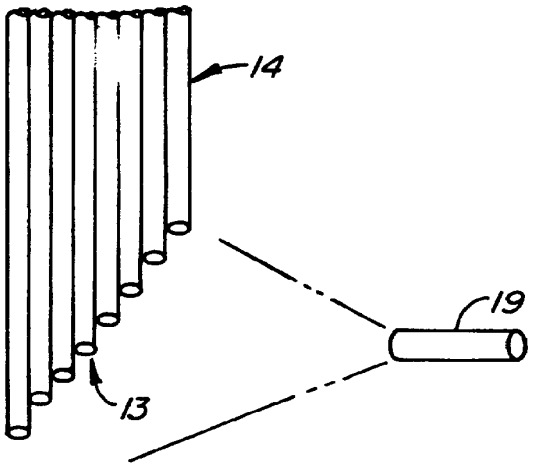


FIG. 6A.

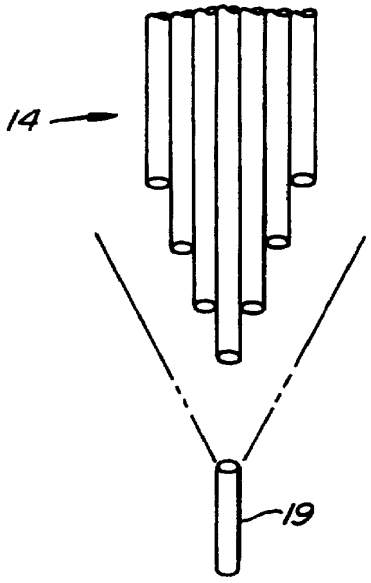


FIG. 6B.

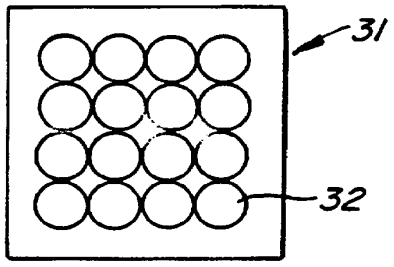


FIG. 7A.

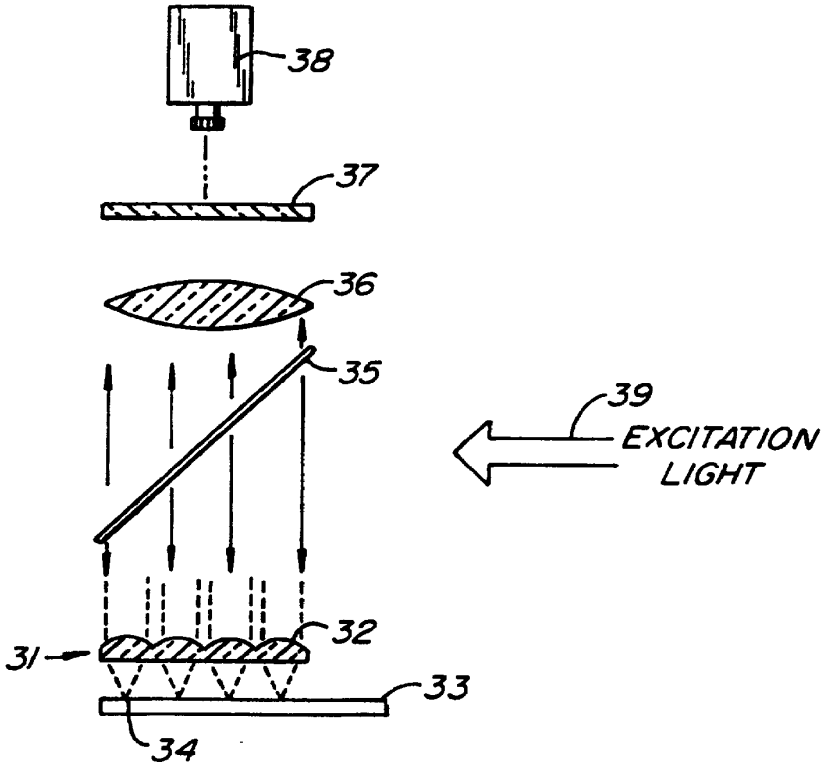


FIG. 7B.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01052

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 91.1, 91.2; 65/409; 422/82.05, 82.06, 82.07, 82.08, 82.09; 436/172; 250/227.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,244,636 A (WALT et al) 14 September 1993, see entire document.	1, 2, 5, 8 ---- 3, 4, 6, 7, 9-20
Y	US H1398 A (CAMPBELL) 03 January 1995, see entire document.	1-20
A	US 5,304,492 A (KLINKHAMMER) 19 April 1994, see entire document.	1-20
Y	US 5,082,630 A (PARTIN et al) 21 January 1992, see entire document.	1-20
Y	US 5,166,990 A (RICCITELLI et al) 24 November 1992	14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search 13 MARCH 1997	Date of mailing of the international search report 13 MAY 1997
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01052

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,449,625 A (KOBAYASHI et al) 12 September 1995, see entire document.	1-20
A	US 5,260,029 (HOSOI et al) 09 November 1993, see entire document.	1-20
Y	GRAHAM et al. Gene probe assays on a fibre-optic evanescent wave biosensor. Biosensors & Bioelectronics. 1992. Vol. 7. pages 487-493, see entire document.	1-20
Y	BARNARD et al. A fibre-optic chemical sensor with discrete sensing sites. Nature. 26 September 1991. Vol. 353. pages 338-340, see entire document.	1-20
Y	LIPSON et al. Multifiber, multiwavelength, fiber optic fluorescence spectrophotometer. IEEE Trans. Biomed. Engineer. 09 September 1992. Vol. 39. No. 9. pages 886-892, see entire document.	1-20
Y	MCCURLEY et al. An optical biosensor using a fluorescent swelling sensing element. Biosensors & Bioelectronics. 1994. Vol. 9. pages 527-533, see entire document.	1-20
Y	BLUM et al. Luminescence fiber optic biosensor. Anal. Lett. 1988. Vol. 21. No. 5. pages 717-726, see entire document.	1-20
Y	PIUNNO et al. Fiber-optic based DNA sensor for fluorometric nucleic acid determination. August 1995. Anal. Chem. Vol. 67. No. 15. pages 2635-2643, see entire document.	1-20

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INTERNATIONAL SEARCH REPORT

Inte. .ional application No.
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A. CLASSIFICATION OF SUBJECT MATTER:
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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS USPAT, MEDLINE, BIOSIS, CAPLUS, WPIDS
search terms: biosensor, sensor, fiber, optic, address, DNA, RNA, nucleic, oligonucleotide, polynucleotide, photolithography, bundle, matrix, support, surface, polymer, nitrocellulose, tiered

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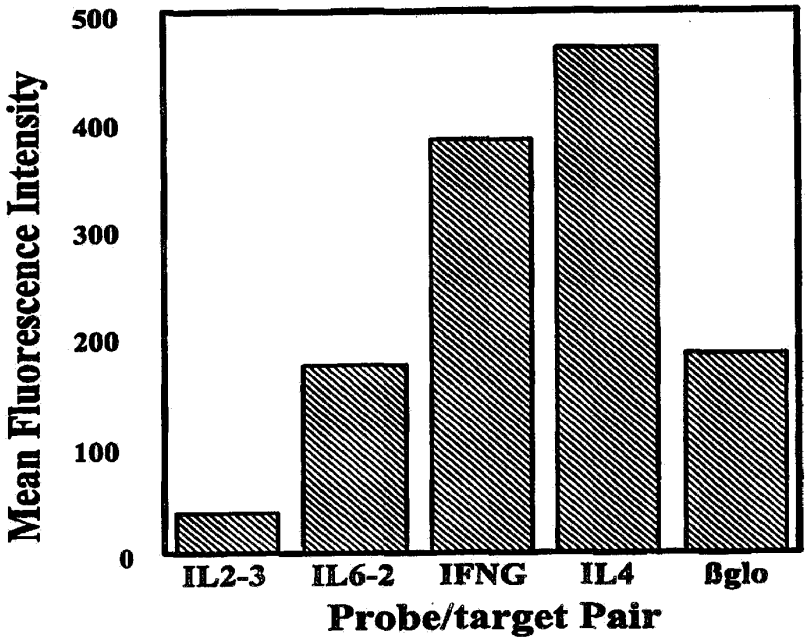
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(54) Title: FIBER OPTIC BIOSENSOR FOR SELECTIVELY DETECTING OLIGONUCLEOTIDE SPECIES IN A MIXED FLUID SAMPLE

(57) Abstract

The present invention provides biosensors, apparatus and methods for selectively detecting at least one complementary oligonucleotide target specie in a fluid sample containing a mixture of different oligonucleotide fragments. One preferred embodiment of the biosensor is as a unitary fiber optic array having an in–situ hybridization zone comprising not less than one specie of single stranded oligonucleotide disposed as individual deposits in aligned organization upon multiple strand end faces at differing spatial positions on the distal array end surface. In this manner, a collective of deployed, single specie, multiple fixed probes are presented for selective in–situ hybridization on–demand with at least one mobile complementary target specie ultimately bearing a joined identifying label. The biosensor provides for optical detection of in–situ hybridization on the distal end surface via the presence of the concomitantly disposed joined identifying label at the differing spatial positions.



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FIBER OPTIC BIOSENSOR FOR SELECTIVELY DETECTING OLIGONUCLEOTIDE SPECIES IN A MIXED FLUID SAMPLE

RESEARCH SUPPORT

The research for the present invention was supported by a grant from the National Institutes Of Health under contract grant GM 48142.

FIELD OF THE INVENTION

The present application is concerned generally with apparatus and methods for the analysis of genes and gene expression; and is particularly directed to the construction and use of a fiber optic biosensor able to detect selectively one or multiple DNA, RNA, or PNA oligonucleotide fragments concurrently.

BACKGROUND OF THE INVENTION

In less than twenty years, the field of molecular genetics, including the specialty of genetic engineering, has revolutionized the science of biology as a whole and is in the process of restructuring medicine in both diagnostic and therapeutic applications. Not only are individual genes now being isolated and characterized, but also extensive research studies as to how genes function and are regulated in-vivo are being actively pursued. Moreover, many techniques for manipulating and modifying genes have been reported and are today becoming widespread in use and diverse in application. Merely exemplifying the many authoritative texts and published articles presently available in the literature regarding genes, gene manipulation and genetic analysis are the following: Gene Probes for Bacteria (Macario and De Macario, editors) Academic Press Inc. 1990; Genetic Analysis. Principles Scope and Objectives by John R.S.Fincham, Blackwell Science Ltd., 1994; Recombinant DNA Methodology II (RayWu, editor), Academic Press, 1995; Molecular Cloning, A Laboratory Manual (Maniatis, Fritsch, and Sambrook, editors), Cold Spring Harbor Laboratory,1982; PCR (Polymerase Chain Reaction), (Newton and Graham, editors), Bios Scientific Publishers, 1994; and the many references individually cited within each of these publications.

Among the many innovative ideas and novel techniques generated by molecular genetic research studies has been the generation of nucleic acid probes for identifying the existence of specific genes, the products of gene expression, and the presence of mutations

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in one or more genes. By definition a nucleic acid probe is a DNA or RNA oligonucleotide fragment or peptide nucleic acid (PNA) of known base sequence. Existing as a single-stranded segment of base codons, a nucleic acid probe which will bind to a complementary base sequence of nucleic acids which is the analyte of interest for any purpose. Thus, the oligonucleotide probe, via its selective binding capability, is employed to detect and identify individual gene fragments or nucleic acid sequences present in viruses, bacteria, and other cells serving as samples for scientific, research or medical interest.

In general, any DNA, RNA, or PNA sequential fragment (obtained from any source and regardless of whether the sequence is naturally occurring or synthetically prepared) must meet two essential criteria in order to be truly useful as an oligonucleotide probe. First, the oligonucleotide probe sequence must be as specific as possible for the intended complementary target sequence; and, preferably, bind exclusively with only the complementary target sequence with little or no cross-reaction. Secondly, the oligonucleotide probe must be able to distinguish among closely related nucleic acid base sequences having a substantial degree of homology as well as be able to bind selectively with varying types and sources of nucleic acid fragments having the complementary target sequence as part of its composition. Thus, the size or length of the oligonucleotide probe and the repetitive nature of or copy number for the complementary target sequence will meaningfully affect not only the specificity, but the sensitivity of the probe for detection purposes.

The technique employing an oligonucleotide probe for selective binding to a complementary target sequence is generally termed "hybridization". However, the development of hybridization based assays for the identification of specific genes and gene expression products has been severely limited to date because of major difficulties in: (a) isolating highly specific nucleic acid sequences for use as oligonucleotide probes; (b) developing assay formats that are sufficiently rapid and simple in order to identify even one complementary target sequence in a fluid mixture containing many varieties of different single-stranded oligonucleotides in admixture; and (c) devising non-radioactive detection systems that provide a desired level of sensitivity. Thus, several types of DNA (or RNA, or PNA) hybridization assay formats have come into prevalent use.

Four hybridization assay formats are commonly employed today. Each of these hybridization detection formats suffers from relatively poor sensitivity, although various target sequence amplification techniques (such as PCR) have also been developed to

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reduce the severity of this problem. The four most commonly used types of hybridization assay formats are: the Southern blot technique; the dot or spot blot technique; in-situ hybridization; and sandwich hybridization assays. As with the selection of an appropriate oligonucleotide probe, the choice of a hybridization assay format often rests upon the degree of specificity and sensitivity that is required for the particular analysis; and upon the factors of speed, reliability, and ease of performance and interpretation of the assay result -- which varies markedly among the different assay formats.

In Southern blot assays, specimen DNA is isolated and purified prior to restriction endonuclease digestion; followed by separation of the digestion products by electrophoresis on an agarose gel, denaturation of the DNA in the gel, and transfer of the denatured DNA fragments to a solid matrix such as a nitrocellulose membrane. The DNA bound to the solid matrix is then hybridized in the presence of radioactively labeled DNA targets to establish homology between the probe and target DNA. Hybridization of the targets to the probes is detected by autoradiography and often requires several days or weeks of exposure. This format is thus often too lengthy and cumbersome for routine or large-scale analyses of many specimens.

The dot-blot procedure also requires that specimen DNA be isolated and purified before being denatured and applied to a suitable solid matrix (such as nitrocellulose). Hybridization to the matrix-bound DNA is then performed using probe-specific targets. The hybridization of target DNA to the probe DNA is detected either by auto-radiography or by visual inspection using non-radioactive detection procedures. The spot-blot assay format is similar except that specimens or specimen lysates are directly applied to the solid matrix without prior extraction of their DNA. Although this assay format allows many different samples to be processed at one time, these assays are often limited to high background noise that complicates the interpretation of results and is also subject to lengthy time of processing for each sample to be evaluated.

The in-situ hybridization technique intends that the DNA or RNA in the cells of a fixed tissue section or fixed culture cell be hybridized to DNA probes directly on a microscope slide. The results are determined by microscopy if non-radioactive detection systems are used and by autoradiography if radioisotopes are employed for the targets. This assay format can detect the presence of only a few copies of the target DNA sequence to be hybridized. The conventional in-situ hybridization assay is not suitable for screening large numbers of specimens due to the need to separate and remove extraneous cellular

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materials from the sample prior to addition of the labeled target.

Lastly, the sandwich hybridization assay requires that at least two different specific probes hybridize to the target DNA of interest, rather than just one probe alone. In this format, the first probe (the capture sequence) is bound to a solid support and is allowed to bind (capture) the specimen DNA. A second probe (the signaling probe) with a sequence that is adjacent or close to the capture sequence on the target DNA molecule is then allowed to hybridize to the support-bound target DNA. This signaling probe can be labeled with either radioactive or non-radioactive labels; and the removal of non-specific cellular material in the first step of the procedure enhances the specificity of the hybridization assay by reducing the effects of contaminating tissue or debris.

More recently however, the value of using immobilized, spatially distinguishable, hybridization probes for concurrent analyses of multiple gene sequences has been recognized and resulted in the development of miniaturized hybridization assays using solid matrix assays [Southern, E.M., Trends in Genetics 12: 110-115 (1996)]. Thus, hybridization using said matrix arrays have been performed on glass surfaces [Maskos, U. and E.M. Southern, Nuc. Acids. Res. 20: 1679-1684 (1992); Guo et al., Nuc. Acid. Res. 22: 5456-5465 (1994)]; on microtiter plates [Kalakowski et al., Anal. Chem. 68: 1197-1200 (1996); Nikiforov et al., Nuc. Acids Res. 22: 4167-41 75 (1994); Rasumussen et al., Anal. Biochem. 198:138-142 (1991)]; on plastic sheets [Matson et al., Anal. Biochem. 224:110-116 (1995)]; on thin polymer gels [Khrapko et al., J. DNA Seq. Map. 1: 375-388 (1991)]; and using semiconductor devices [Eggers et al., Bio Techniques 17: 51 6-524 (1994); Kreiner, T, Am. Lab.: 39-43 (1996)]. In addition, the desire for using non-radioactive means for detection have caused a surge of interest in means for detection of hybridization on solid matrix supports which employ fluorescence [Kumke et al., Anal. Chem. 67: 3945-3951 (1995); Piunno et al., Anal. Chim. Acta. 288: 205-214 (1994)]; chemiluminescence [Ito et al., J. Neurosci. Methods 59: 265-271 (1995); Nguyen et al., Biosen. Bioelectron. 7: 487-493 (1995)]; evanescent wave technology [Graham et al., Biosen. Bioelectron. 7: 487-493 (1992); Strachan et al., Lett. App. Microbiol. 21: 5-9 (1995); Watts et al., Anal. Chem. 67: 4283-4289 (1995)]; confocal microscopy [Fodor et al., Nature (London) 364: 555-556 (1993)]; light scattering [Stimpson et al., Proc. NatI. Acad. Sci. USA 92: 6379-6383 (1995)]; electrochemistry [Milland et al., Anal. Chem. 66: 2943-2948 (1994); Pandey et al Anal. Chem. 66:1236-1241(1994) Hashimoto et al., Anal. Chim. Acta. 286: 219-224 (1994)]; and surface resonance phenomena [Yamaguchi et al.,

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Anal. Chem. 65:1925-1927 (1993)].

Despite these recent innovations using probes immobilized on solid matrix arrays the major obstacles and limitations of hybridization methods generally continue to restrict and contain the currently available techniques and formats. These demands and limitations include a requirement for a large sample volume; an inability to perform multiple analyses concurrently in real time; a requirement for a relatively high concentration of target DNA (the complementary target sequence) in the fluid sample; an inability to detect multiple species concurrently; relatively slow kinetics for hybridization to occur between the target sequences and the immobilized probes within the assay format; and a dependence upon lengthy assays. Moreover, despite the use of new in-vitro amplification techniques such as the polymerase chain reaction procedure, the problems of assay sensitivity, lengthy times for analysis, the quantum of background signal noise, and the inability to detect more than one target nucleic acid sequence at a time remain as recurring handicaps and continuing obstacles for each of these techniques. It will be recognized and appreciated by persons working in this field today, therefore, that the development of a unique biosensor which overcomes and eliminates most, if not all, of these major limitations and procedural hindrances would be seen as a major advance and unforeseen improvement in this art.

SUMMARY OF THE INVENTION

The present invention has multiple aspects and formats. A first aspect provides an optical biosensor for selectively detecting an oligonucleotide specie in a fluid sample, said biosensor comprising:

a clad optical fiber strand of determinable configuration and dimensions which presents two strand end faces as discrete optic surfaces for introduction and conveyance of light energy;

an oligonucleotide in-situ hybridization zone comprising one specie of single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber strand, said deposit of single stranded oligonucleotide within said hybridization zone serving as a deployed, single specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light

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energy absorbing dye of known spectral characteristic, and

(b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probe at said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said strand end face surface.

A second aspect defines an optical biosensor for selectively detecting at least one oligonucleotide specie in a fluid sample, said biosensor comprising:

a bundled array comprising a plurality of individually clad, fiber optic strands disposed co-axially along their lengths and having two discrete array ends each of which is formed of multiple strand end faces, said bundled array being of determinable configuration and dimensions and said two discrete array ends presenting two optic array surfaces for introduction and conveyance of light energy; and

at least one oligonucleotide in-situ hybridization zone comprising a plurality of single stranded oligonucleotide species disposed as individual specie deposits in aligned organization at differing spatial positions on one zone of said optic array end surface of said bundled array, the differing spatial positioning for each deposit of single stranded oligonucleotide specie in aligned organization within said hybridization zone serving as deployed, single specie, multiple fixed probes suitable for selective in-situ hybridization on-demand with its mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction products formed by a complementary oligonucleotide target specie with said multiple fixed probes at said differing spatial positions within said hybridization zone are optically detectable via the spectral characteristics of said identifying label concomitantly disposed at said differing spatial positions.

A third aspect of the present invention provides an optical biosensor for selectively detecting a plurality of individual oligonucleotide species concurrently in a fluid sample containing a mixture of different oligonucleotide species, said biosensor comprising:

a preformed, unitary fiber optic array comprising a plurality of individually clad, fiber optic strands disposed co-axially along their lengths and having two discrete optic

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array ends each of which is formed of multiple strand end faces, said preformed unitary fiber optic array being of determinable configuration and dimensions and said two discrete optic array ends presenting two discrete optic array surfaces for introduction and conveyance of light energy; and

at least one oligonucleotide in-situ hybridization zone comprising a plurality of single-stranded oligonucleotide species disposed as individual specie deposits in aligned organization upon multiple strand end faces at differing spatial positions on one of said discrete optic fiber array surfaces of said unitary fiber optic array, the differing spatial positioning for each deposit of single-stranded oligonucleotide specie in aligned organization within said in-situ hybridization zone serving as a collective of deployed, specie specific, fixed probes suitable for selective in-situ hybridization on-demand with its mobile complementary oligonucleotide target specie in a fluid mixture containing alternative mobile complementary target species,

(a) wherein such hybridized complementary oligonucleotide target species as are hybridized in-situ each ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with its collective of multiple fixed probes at said differing spatial positions within said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said differing spatial positions, each alternative in-situ hybridized oligonucleotide reaction product formed being concurrently optically detectable via the spectral characteristics of each joined identifying label at differing spatial positions within said hybridization zone.

BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more easily understood and better appreciated when taken in conjunction with the accompanying drawing, in which:

Fig. I is an overhead view of an individually clad, optical fiber strand;

Figs. 2A and 2B are views of the proximal and distal surfaces of the fiber optical strand of Fig. 1;

Figs 3A and 3B are alternative constructions of the optical end surface for the optical fiber strand of Fig. 1;

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Fig. 4 is an overhead view of a bundled array of single core, optical fiber strands;

Figs. 5A and 5B are views of the distal and proximal end surfaces for the bundle of fibers of Fig 4;

Fig. 6 is an overhead view of a preformed, unitary fiber optic array using the optical fiber strand of Fig. 1;

Fig. 7 is a view of the intended distal array end surface of the unitary fiber optic array of Fig. 6;

Fig. 8 is a view of the intended proximal array end surface of the unitary fiber optic array of Fig. 6;

Fig. 9 is a frontal view of an illumination source able to provide light energy at precise spatial positions concurrently;

Figs. 10-15 illustrate the manipulative steps performed during the disposition of oligonucleotide probes at precise spatial positions on the distal array end surface of Fig. 8.

Fig. 16 is a schematic diagram of the apparatus comprising the biosensor

Fig. 17 is a schematic diagram of a fiber optic biosensor detection apparatus and system;

Figs. 18A and 18B are white light and background fluorescent images viewed through the proximal end of the biosensor apparatus;

Fig. 19 is a graph showing the background-subtracted mean fluorescence intensities obtained with fixed IL4 probes on the distal end of the biosensor;

Fig. 20 is a graph showing the plot of background-subtracted mean fluorescence as a function of time using a sensor having a β-glo probe and a 1.0 kM f3-glo target solution;

Fig. 21 is a graph showing the plot of background-subtracted mean fluorescence as a function of time using a sensor having a β-glo probe and an 0.1 kM f3-glo target solution;

Figs. 22A - 22F are fluorescent images from a biosensor apparatus after immersion in a IL2 target, a IL4 target, an IL6 target, a β-glo target, an IFNG target, a IL4 and IFNG and β-glo targets;

Fig. 23 is a graph illustrating the background-subtracted mean fluorescence signals as a function of the probe/target pair of Fig. 22;

Fig. 24 is a graph showing the plot of hybridization competition between labeled and unlabeled formats of the same target nucleic acid sequence;

Fig. 25 is a graph showing the fluorescence intensity of the poly(dA) matrix upon

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repeated hybridization to and dehybridization from a poly(dT)-FITC target;

Fig. 26 is a graph showing the fluorescence intensity of a poly(dA)/acrylamide biosensor to varying concentrations of poly(dT)-F ITC;

Fig. 27 is a graph showing the calibration curve of the data of Fig. 25;

Fig. 28 is a graph showing the calibration curve of a poly(dA) matrix biosensor after in-situ hybridization with a poly(dT)-biotin target;

Fig. 29 is a graph showing the melting curves of a DNA biosensor after hybridization to a Δ target at 28°C

Figs. 30A and 30B are fluorescent images from the DNA biosensor apparatus after in-situ hybridization with a Δ-FITC target at 28°C and 50°C respectively;

Fig. 31 is a graph showing the fluorescence intensity over time for in-situ hybridization of a 196 nM Δ target to a H-ras wt./H-ras Δ matrix biosensor;

Fig. 32 is a graph showing the calibration of a DNA biosensor to a Δ-FITC target at 54°C and

Fig. 33 is a fluorescence image from a DNA biosensor apparatus after a 20 minute hybridization time to a biotinylated Δ PCR amplicon at 54°C followed by a 5 minute labeling reaction with streptavidin-FITC.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a fiber optic biosensor comprising at least one fiber optic strand; can comprise a bundled array of single core fibers joined together; and preferably comprises a preformed, unitary array of fibers -- these biosensor types having at least one and desirably several, oligonucleotide probes immobilized at individual and differing spatial positions on the distal end surface. The immobilized oligonucleotide probe(s) do not bear any identifying label of any kind. Rather, when the distal strand end surface or, alternatively, the distal array end surface supporting the immobilized probes are placed into reactive contact with a fluid sample containing at least one complementary target oligonucleotide segment bearing a joined identifying label, an in-situ hybridization will occur; and the reaction product of the in-situ hybridization (formed by a complementary oligonucleotide target collectively with the immobilized probe at each of the differing spatial positions on the distal end surface) can be detected and monitored by observing the fluorescence or reflected color of the joined identifying label that accompanied or is the consequence of hybridization at that specific spatial position. Equally important, because

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the hybridization is specific between the oligonucleotide base sequence of the spatially immobilized probe with a complementary target base sequence, the detection of fluorescent or reflected light energy from the joined label at a predetermined spatial position on the distal end surface will demonstrate and evidence the occurrence of a specific and selective in-situ hybridization.

A number of different major advantages and unique capabilities are thus provided by the biosensor comprising the present invention. Among these advantages and capabilities are the following.

1. The present invention provides a biosensor which offers a choice of formats using a single optical fiber strand; or a bundled array of single core fibers joined together; or, alternatively, a plurality of optical fiber strands disposed co-axially along their lengths as a preformed unitary fiber optic array. The single optical strand format will provide one oligonucleotide hybridization zone and a single specie fixed probe suitable for selective in-situ hybridization with one mobile complementary oliognucleotide target specie. The bundled array of fibers format provides multiple hybridization zones and multiple single specie fixed probes for selective in-situ hybridization with one or a plurality of different mobile complementary oligonucleotide species. The alternative unitary fiber optic array format provides collectives of multiple and different fixed sets of specie-specific probes, each set of fixed probes being collectively suitable for selective in-situ hybridization with a different mobile complementary oligonucleotide target specie. The unitary fiber optic array format and the bundled array of fibers format are thus able to detect multiple and diverse types of mobile oligonucleotide target species simultaneously or concurrently in one sample and a single test; in comparison, single optical fiber strand format is limited to the detection of a single oligonucleotide target specie per sample and test.

2. The present invention, in all formats, is able to detect one specific target nucleic acid segment in a fluid mixture of different oligonucleotide fragments; and, in two formats, is able to detect a plurality of different complementary target oligonucleotide species in a single fluid sample containing a mixture of many different nucleic acid sequence fragments. The specificity of the in-situ hybridization reaction depends primarily upon the choice of oligonucleotide probe employed. Thus, when probes are chosen which have a known base sequence and having a recognized or specified binding capability, the hybridization is highly selective and specific for a single complementary nucleic acid target sequence; and only selective binding to that one complementary species will occur

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despite the presence of other oligonucleotides in the fluid sample. Similarly, multiple and different in-situ hybridization reactions can concurrently occur at different spatial positions on the distal array end surface using a variety of alternative fixed probes. Each fixed probe is species specific; is fixed at a predetermined spatial position on the distal end surface; and will hybridize in-situ on-demand with its intended complementary target specie.

3. The present invention provides a biosensor which is extraordinarily rapid in providing evaluations and results based on in-situ hybridization reactions. Typically, the biosensor will provide optical detection of a labeled complementary target specie in a fluid sample in less than ten (10) minutes time and depending on the chosen components of the detection system, may provide optical detection of multiple target species concurrently or simultaneously within the same time duration, approximately ten minutes or less. The present invention thus provides a speed of detection which is unmatched by any previously known format or conventional technique.

4. The present invention provides an extremely high level of sensitivity for in-situ hybridization reaction. As the experimental data provided hereinafter reveals, even a simple embodiment of the biosensor and its supporting apparatus can optically detect 10 nM of a target oligonucleotide segment via the spectral characteristics of a joined identifying label. The apparatus and method of optical detection yields very little background signal as noise; and it is expected that as little as 0.01-0.1 nM will be detectable under optimum operating conditions. Accordingly, the traditional requirement for high concentrations of DNA (or RNA, or PNA) test sample in order that an accurate and reliable measurement be made is no longer necessary or required. Also, the absolute amounts of DNA (or RNA, or PNA) needed for detection are small due to the extremely small size of the sensor.

5. The present invention will provide high specificity of in-situ hybridization reactions in a fluid sample when used at ambient room temperatures generally, or at elevated temperatures. No special or unique environmental considerations or demands are necessary in order to use the present invention; to the contrary, the sole requirement is that the distal end surface bearing the specie-specific fixed probes be placed in reactive contact with a fluid sample suspected of carrying the complementary target base sequence.

6. The present invention requires only a very small fluid sample volume in order for optical determinations to be made and accurate results to be obtained. If necessary, a few nanoliters of fluid may be employed for detection purposes. It is generally desirable,

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however, that a 1-2 microliter volume or greater be employed as the fluid sample. This low volume feature for the fluid sample may be maintained so long as there is sufficient liquid volume to cover the distal end surface effectively such that the fixed probe(s) immobilized on the distal surface may come into reactive contact with the contents of the fluid sample itself. If this minimal requirement is met and satisfied by the user, the true volume of the fluid sample is irrelevant and inconsequential.

7. The present invention also allows the user the ability to monitor the hybridization process over time in-situ without physical separation of the apparatus from the fluid sample undergoing evaluation. Thus, assuming the user employs a very rapid reaction time for analysis (less than 10 minutes), continuous observation and optical detection of the ongoing hybridization process over the 10 minutes can be made for one or for all of the differing spatial positions for the fixed probes immobilized on the distal end surface. Thus as hybridization occurs at each fixed probe location on the end surface, the presence of a bound label joined directly or indirectly to the complementary target specie will be detected via the spectral characteristics of the light energy absorbing dye employed as an identifying label. The user may thus monitor the ongoing reaction in real time as it actually proceeds in-situ; and may determine even more quickly whether or not the complementary target specie does in fact exist within that fluid sample.

8. The biosensor of the present invention allows the user not only to detect rapidly the presence of the complementary target base segment in a fluid sample, but also permits the user to quantify in proportional degree the concentration of the complementary target specie actually present in the fluid sample volume. A semi-quantitative estimate is based on a calibrated detection of the signal intensity emanating from the fixed probes undergoing in-situ hybridization with the fluid sample. In addition, a competitive assay in which a fixed amount of added labeled target specie is displaced proportionately by an unknown quantity of unlabeled target in a test sample will also provide quantitative results. A quantitative estimate which is both reliable and reproducible is rarely otherwise available without much more rigorous experiments and analytical test conditions.

For easier comprehension and a better appreciation of the features and attributes of the present invention, the detailed disclosure herein will be presented in separate sections seriatim. The order of presentation will be: a detailed description of the unitary fiber optic array comprising the biosensor; the DNA (or RNA, or PNA) oligonucleotide(s) which may be employed as probes; a preferred means for immobilizing the oligonucleotide probes at

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differing spatial positions on the distal array end surface; the supporting apparatus desirably employed with the biosensor for detecting in-situ hybridization reactions on-demand; a description of complementary nucleic acid segments as target species; the manner of identifying the resulting in-situ hybridized reaction product; and a series of experiments and empirical results demonstrating some of the major advantages and unusual benefits provided by the present invention. Each of these will be described in detail below.

Also, since the present invention is definable in multiple formats and may be employed in different modes for a variety of divergent purposes and applications, the subject matter as a whole which is the present invention will be presented and described individually as component parts and then collectively as assemblies in order that the prospective user may more quickly recognize and appreciate their major differences and distinctions in comparison to the conventionally known systems.

I. The Organization And Construction Of The Biosensor

The unique biosensor may be prepared in three different formats. The simplest format employs a single optical fiber strand; and presents a single oligonucleotide in-situ hybridization zone disposed on the distal end surface comprising a single specie of single-stranded DNA (or RNA, or PNA) disposed as a fixed probe for selective hybridization with one mobile complementary target specie. A more elaborate format is the bundled array of single core fibers joined together. This format type presents an in-situ hybridization zone; and allows the user a choice of employing either a single specie or multiple species of DNA (or RNA or PNA) as fixed probes for selective in-situ hybridization. The alternative and most sophisticated format employs: a preformed, unitary fiber optic array comprised of a plurality of individually clad fiber optical strands disposed co-axially along their lengths; and at least one oligonucleotide in-situ hybridization zone comprising not less than one specie of single-stranded DNA (or RNA, or PNA) disposed as a plurality of individual deposits in aligned organization upon multiple fiber optical strand end faces at differing spatial positions and which serve as a collective of multiple fixed probes for specie selective in-situ hybridization on-demand. Each format will be described in detail.

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A. The Format Choices

The individually clad, optical fiber strand

A typical optical fiber strand is illustrated by Figs. 1 and 2A and 2B. As seen therein, an individual optical fiber strand 10 is comprised of a single optical fiber 12 having a rod-like shaft 14 and two fiber ends 16, 18, each of which provides a substantially planar end surface. The intended distal surface 20 at the fiber end 16 is illustrated by Fig. 2A, while the intended proximal surface 22 at the fiber end 18 is illustrated within Fig. 2B. It will be recognized and appreciated that the terms "proximal" and "distal" are relative and interchangeable until the strand is ultimately positioned in an apparatus. The optical fiber 12 is composed typically of glass or plastic; and is a flexible rod able to convey light energy introduced at either of its ends 16 and 18. Such optical fibers 12 are conventionally known and commercially available. Alternatively, the user may himself prepare individual optical fibers in accordance with the practices and techniques reported in the scientific and industrial literature. Accordingly, the optical fiber 12 is deemed to be conventionally known and available as such.

It will be appreciated that Figs. 1-2 are illustrations in which the features have been purposely magnified and exaggerated beyond their normal scale in order to provide both clarity and extreme detail. Typically, the conventional optical fiber has a cross section diameter of 5-500 micrometers; and is routinely employed in lengths ranging between meters (in the laboratory) to kilometers (in field telecommunications). Moreover, although the optical fiber 12 is illustrated via Figs. 1-2 as a cylindrical extended rod having substantially circular proximal and distal end surfaces, there is no requirement or demand that this specific configuration be maintained. To the contrary, the optical fiber may be polygonal or asymmetrically shaped along its length; provided with special patterns and shapes at the proximal and/or distal faces; and need not present an end surface which is substantially planar. Nevertheless, for best efforts, it is presently believed that the substantially cylindrical rod-like optical fiber having planar end surfaces is most desirable.

Each optical fiber 12 is desirably, but not necessarily, individually clad axially along its length by cladding 26. This cladding 26 is composed of any material with a lower refractive index than the fiber core and prevents the transmission of light energy photons from the optical fiber 12 to the external environment. The cladding material 26 may thus be composed of a variety of radically different chemical formulations including various glasses, silicones, plastics, platings, and shielding matter of diverse chemical composition

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and formulation. The manner in which the optical fiber 12 is clad is also inconsequential and of no importance to the present invention. Many methods of deposition, extrusion, painting and covering are scientifically known and industrially available; and any of these conventionally known processes may be chosen to meet the requirements and convenience of the user. Moreover, the quantity of cladding employed need only be that minimal amount which effectively prevents light energy conveyed by the optical fiber 12 from escaping into the ambient environment. It will be recognized and appreciated therefore, that the depth of cladding 26 as appears within Figs. 1 and 2 respectively is greatly exaggerated and purposely thickened in appearance in order to show the general relationship; and is without scale or precise ratios between the cladding 26 and the optical fiber 12.

It will also be recognized that the configuration of the cladding 26 as shown by Figs. 1 and 2 has been shaped as a circular coating to illustrate a preferred embodiment only. For reasons as will become clear subsequently, it is desirable that the cladding 26 take form in regular geometric form such as a round or circular shape. The illustrated configuration, however, is merely a preferred embodiment of the cladding 26 as it extends co-axially along the length of the optical fiber 12. For purposes of added clarity also, Fig. 1 reveals the individually clad, optical fiber strand 10 is partial cross-section to demonstrate the relationship between the optical fiber core 12 and the cladding 26 which is coextensive along its length.

The user also has a variety of choices at his discretion regarding the configuration of the "distal" end 16 of the optical fiber 12 as shown by Figs. 3A and 3B; however, both ends of the strand must be the same -- i.e., if the "distal" end is cylindrical then the "proximal" end must be also. As seen in Fig. 3A, the "distal" end 16 is substantially cylindrical in shape and desirably presents a surface 20 which is substantially planar and smooth. A possible, but less desirable, alternative is shown by Fig. 3B, in which the distal end 30, nevertheless provides a very different end surface for the optical fiber 12. The surface 32 includes a depression or well 34 which extends into the substance of the optical fiber 12 at a depth typically of several micrometers. Although the well 34 appears substantially circular within Fig. 3B, oval or other rotund configured depressions may also be employed as fits the needs or convenience of the use. Similarly, the void volume of the well 34 from its greatest depth to the proximal surface 32 may also be varied.

It will be recognized and appreciated as well that the range and variety of

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dimensional and configurational divergence for the strand end is limited only by the user's ability to subsequently dispose and immobilize an oligonucleotide of known composition/formulation of controlled thickness on the intended distal surface of the optical fiber 12. In some embodiments, a greater depth of deposit on the surface of the distal end surface may be highly desirable; nevertheless, for most general assay purposes, both quantitative and qualitative, the intendeddistal surface illustrated within Fig. 3A as a substantially planar and smooth surface is deemed to be most suitable and desirable.

For general construction of the single strand sensor and for most purposes and applications of the improved optical detecting system and procedures described hereinafter, it is desirable to employ the individually clad, fiber optical strand illustrated by Figs. 1, 2A, 2B in preference to a bare, unsheathed strand. Clearly, the optical fiber strand is unable to transmit light energy photons to any other entity due to the cladding material 26 which forms a sheath. This sheath, having a refractory index less than the strand core, also prevents loss of light energy photons into the general environment. Accordingly, the potential for photon loss, distortion, or other optical error is minimized and reduced. For these reasons, the individually clad optical fiber mode of construction is preferable to the use of bare optical fiber strands in order to achieve greater precision and accuracy.

The bundled array of single core fibers

It will be noted that the bundling of individual single core fibers into an organized array can be made either before or after one oligonucleotide specie has been deposited and immobilized on the distal end surface of each optical fiber strand forming the bundled array of fibers. A detailed and empirically evaluated bundled array of fibers prepared in advance and used herein as a biosensor is presented by the data of Experimental Series A comprising Experiments 1-5 respectively.

For illustrative purposes and ease of understanding, however, the detailed description in this section will limit itself to a bundled array of fibers which do not yet have any oligonucleotide specie immobilized onto their individual strand end faces. In addition, the number of individual, single core, optical fibers employed in a bundled array format has been intentionally limited to only three strands. It will be recognized and appreciated, however, any small quantity of single core optical fiber strands ranging in number from not less than 2 to about 20 fibers can be combined together as a single discrete bundle, the exact number of single core strands being bundled into an array

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depending greatly upon the size of the core strands.

The bundling of individual, single core, optical fiber strands into an organized array is a conventionally known technique and is well established in this field. A number of different bundling formats are well known which combine prepared individual single strand sensors as an integrated bundle for a specific purpose. Merely exemplifying the conventionally known range of articles using the bundled array of fibers format are European Patent Application 88105676.6; and U.S. Patent Nos. 5,047,627 and 4,999,306, as well as the references cited within each of these.

Accordingly, a typical bundled array of single core optical fibers is illustrated by Figs. 4 and 5 respectively. As seen therein, the bundled array 40 formed of single core fibers appears as a substantially triangular-shaped integrated article having a distal end face 42 and a proximal end face 44; and is comprised of three different and distinct, single core, optical fiber strands 50, 60, and 70 which are disposed co-axially along their lengths to form the integral bundle. Each of the individual strands 50, 60, and 70 is a single optical fiber strand having a distal strand end face 52, 62, 72 and a proximal strand end face 54, 64, 74. Also, each strand has a single optical core 56, 66, and 76 which is individually covered and surrounded by cladding material 58, 68, and 78.

By way of example, as shown in Figs. 4 and 5, three single core fibers 50, 60, 70 are shown joined together as a configured bundle using epoxy adhesives which are optically dense and using such additional cladding material as is necessary as a bulk filler to form an integral fiber bundle 40 configured in substantially triangular form. A sheath 41 may optionally be used to protect the unexposed length 43 of the bundle. Note that each optical fiber strand in the bundled array 40 has been spatially oriented and intentionally placed into a triangular configuration such that fiber strand 50 lies at the apex while fiber strands 60 and 70 lie at the base of the shaped bundle. Once placed and immobilized into this triangular organizational format, the spatial positioning for each of the three single core fibers 50, 60, and 70 within the bundled array 40 is always maintained.

Furthermore, Fig. 5A shows that the distal ends 52, 62, 72 of each respective single core strand 50, 60 and 70 each individually extends for a short distance (typically several centimeters) beyond the protective sheath 41. This is a desirable feature which facilitates the subsequent immobilization of one oligonucleotide specie to the distal strand end of each face 52, 62, and 72 respectively. In comparison, Fig. 5B shows that the proximal end face 44 of the bundled array 40 is coextensive with the proximal strand end faces 54, 64,

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and 74 of the single core fibers 50, 60 and 70 respectively.

The unitary array

A typical preformed fiber optic array, its organization and construction, and its component parts are illustrated by Figs. 6-8 respectively. Each discrete unitary fiber optic array is a preformed composite comprised of a plurality of individually clad, fiber optical strands disposed coaxially along their lengths. The smallest common repeating unit within the preformed array is thus a single optical fiber strand. The manner in which these optical fiber strands are prepared and the manner in which these prepared optical strands are joined collectively into an organized optic array are conventionally known, but is fundamental to a proper understanding and use of the alternative format.

It is recognized also that the unitary array of optical fibers has been used previously as a major component in other inventions and is described in earlier issued patents. Such usage is exemplified by U.S. Patent Nos. 5,244,636; 5,244, 813; 5,250,264; and 5,298,741; the texts of which are individually expressly incorporated by reference herein.

The unitary fiber optic array 100 appears in exaggerated, highly simplified views without regard to scale within Fig. 6. The preformed array is composed of a plurality of individually clad, fiber optical strands which collectively lie coaxially along their respective lengths as the discrete, unitary optic array 104 of fixed and determinable configuration and dimensions. The optic array 104 has a unitary, rod-like collective body 106 and intended distal and proximal collective ends 108,110 formed of multiple strand end faces. The intended distal collective end 108 provides a substantially planar and smooth optic array surface 114. The topographical surface 116 is the result of fusing the clad of each fiber optical strand 102 collectively with a fiber material 118 such that the fusion is drawn and appears as a discrete, unitary array. In this manner, the exterior surface 116 of the collective array body 106 may be configured and dimensioned as an assembly in an acceptable manner and useful manner. It will be recognized and appreciated also that a substantially cylindrical configuration and topography is maintained and presented by the unitary imaging fiber optic array 100 merely as one preferred embodiment. Any other regular or irregular configuration and design may be achieved and employed to satisfy the individual user's needs or desires.

For purposes of clarity and ease of understanding Figs. 7 and 8 present a very limited and greatly reduced number of individually clad, fiber optical strands 102 present

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within the preformed optical array 104. A total of only 120 individually clad, fiber optical strands are seen to comprise the optical array 104 in greatly magnified and scale-exaggerated views. Moreover, the relationship of the optical array surface 112 (the intended distal end) with respect to the other optical array surface 114 (the intended proximal end) becomes simplified and more-readily appreciated when using this limited number of 120 optical fiber strands. In practice and reality, however, it is estimated that typically there are 2000-3000 optical fiber strands in a conventional array of 200 μM diameter. Thus the true total number of individually clad, fiber optic strands forming the unitary imaging fiber optic array will typically be in the thousands and vary substantially with the cross-sectional diameter of each optical fiber and the thickness of the cladding material employed when constructing the optical fiber strands themselves.

The construction, coherent organization, and positional alignment within a typical fiber optic unitary array is revealed by Figs. 6-8. For descriptive purposes only, each of the individually clad, optical fiber strands is presumed to be linearly straight in position and has been arbitrarily assigned an identifying number S1-S120 as shown via Figs. 7 and 8. The intended distal optic array end surface 112 of Fig. 7 shows that each of the individual optical fiber strands S1-S120 can be identified and distinguished from its adjacently disposed neighbor as well as from any other optical fiber strand within the preformed array 104 by a set of spatial positioning coordinate numbers for the strand end faces. The intended distal optical array surface 112 may be arbitrarily divided into two axial directions as is illustrated by Fig. 7. The exact location of the S1 strand is thus identifiable by the numerical coordinates "XII D" showing the strand end face. Similarly, the exact spatial positioning and strand end face of the S72 fiber is designated as "VIM." In this manner, the individual spatial position and strand end faces for each optical fiber strand S1-S120 is thus completely locatable and identifiable using the coordinate numeral labeling system.

The other optic array end surface 114 (the intended proximal end surface) allows for a similar mode of identification (presuming straight linear alignment of strands) by spatial positioning of each individual optical strand -- again as a result of using dual-axis numerical coordinates as seen in Fig. 8. Accordingly, fiber end strand end face S1 is located at numerical position "12d", and fiber S72 is identifiable, locatable, and distinguishable from all other fibers at the optic array surface by its individual numerical coordinates "6m". In this manner, the precise and exact position of each individually clad

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optical fiber strand and strand end faces on each of the discrete optic array surfaces 112,114 can be located, identified, and specified via a series of two different numerical coordinates. The intended distal and proximal optic array surfaces are thus completely identifiable and distinguishable as per individual fiber optical strand 102 despite its presence in the preformed collective body 106 of the unitary fiber optical array 100.

It will be appreciated also that the overall organization of the individually clad, optical fiber strands 102 within the unitary array 100 of Figs. 6-8 is as aligned, parallel, strands which maintain their relative organizational positioning in a coherent, consistently aligned manner over the entire length of the collective body 106. This is deemed to be the most desirable and most easily constructable organization scheme for the preformed optical fiber array of the present invention.

Although this highly organized, coherent, and rigidly aligned collective construction is deemed to be most desirable, this high degree of organizational alignment is not an absolute requirement for each and every embodiment using an unitary optical array. Alternative manufacturing practices allow for a more random disposition of the individually clad, optical fiber strands disposed coaxially along their lengths. Although less desirable, a partially random disposition and a completely random alignment of the optical fiber will also result in a unitary collective body of optical fibers and in proximal and distal collective ends which provide two discrete optical array surfaces. It will be recognized therefore that while the individually clad, optical fiber strands may lie adjacent one another at one end, they may deviate and meander through the length of the array such that their position relative to one another may vary substantially in part or in whole -- thereby creating semi-coherent or incoherent positional alignments which vary in the randomness of their organizational construction. There is no requirement that the positioning of the intended proximal end of one strand be aligned and/or identical with the positioning of the intended distal end within the unitary optic array.

The entirety of the construction for the unitary optical fiber array (whether uniformly coherent, semi-random, or completely randomly organized) provides a means of introducing light energy photos of any determinable wavelength at one optic array surface with the knowledge that the light energy will exit at the other optic array surface. Therefore, by using the preferred completely coherent and rigidly maintained parallel alignment of strands illustrated by Figs. 7 and 8 (the intended distal and proximal optic array end surfaces respectively) of a unitary fiber optic array, the user may introduce light

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energy to a portion or all of the optic array end surface 114 and have accurate knowledge and confidence that the light energy would be conveyed by the fiber strands and exit from the other optic array end surface 112. Conversely, were light energy introduced to the optic array end surface 112, the light energy would be conveyed by the optical fibers of the array and will exit from the other optic array end surface 114.

In addition, the topography of the unitary optic array end surfaces 112 and 114 will vary with the nature of the end faces for the individual optical fibers strands comprising the array. Thus, if the optical fiber strand end faces conform to that illustrated by Fig. 3A, then the array end surface will present a substantially planar and smooth topography. Alternatively, however, if the optical fiber end faces forming the array are exemplified by Fig. 3B; then the unitary array end surface will appear as a collective of wells or depressions, each well extending into the collective substance of the array end surface at a set depth (typically of a few micrometers). The topography of the unitary array end would then present a pitted and crater-like surface for the immobilization of oligonucleotides as fixed probes.

It will also be recognized that the user may chose to introduce light energy to only a specific spatial location on the optic array end surface 114 -- for example, only to fibers S1, S7 and S8 -- and have accurate knowledge and confidence that the light energy would be conveyed only by those three optical fiber strands and exit from numerical positions "XIID", "XIC", and "XID" alone on the optic array end surface 112. No other light energy would appear from any other spatial position from the optic array surface 112. Similarly, were light energy of specific wavelength introduced at the optic array surface 112 via fibers S107, S108, and S115, respectively, the use can accurately predict and identify that the light energy will be conveyed by only these three optical fibers; and will exit only at the optic array surface 114 of numerical coordinate position numbers 2c, 2d, and 1d respectively and from no other spatial positions on this optic array surface. In this manner, not only does one have knowledge of the individual spatial positioning of each optical fiber strand in the preformed array but also one has the ability to identify and precisely locate light energy photons emerging from individual optical fiber strands within the whole of the optic array surface in a practical and reliable mode.

Accordingly, the critical and essential requirements of any optical fiber array construction allows and demands the capability for precise spatial positional introduction and conveyance of light energy via different fiber optical strands within the collective body

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of the preformed, unitary fiber optical array. This capability to introduce light energy photons at precise spatial positions at one optic array of a unitary array; to convey the introduced light energy along the length of only a few fiber optical strands; and to control the exit of the conveyed light energy at a second, precisely known, spatial position on the other optic array surface of the unitary array is a hallmark of the singular fiber optic sensor presented herein.

B. Oligonucleotides Immobilized As One Or As Multiple Fixed Probes

The construction of the biosensor intends that at least one specie of single-stranded DNA, or RNA, or PNA, fragment be deployed and immobilized on the distal end surface of a single optical fiber strand; or on the distal end surface of at least one single core strand comprising an organized bundled array of optical fibers; or on the distal array end surface of a unitary array at differing spatial positions -- and thus serve as one or more deployed fixed probes suitable for selective in-situ hybridization on-demand. Each specie of DNA (or RNA, or PNA) fragments is disposed as an individual deposit on at least one strand end face or as multiple independent deposits in aligned organization on multiple strand end faces at one or at many different spatial positions on the distal optic end surface. Each oligonucleotide specie deposit on an optical fiber strand end face, therefore, serves as one fixed probe immobilized at a predetermined spatial position; and in each of the array formats, the multiple fixed probes serve collectively as many singular and different reaction zones acting repetitiously and in common for in-situ hybridization with a mobile complementary nucleic acid target (directly or indirectly bearing a joined identifying label).

The nature of the oligonucleotide fragment

The oligonucleotide sequence chosen for use as a probe may be obtained from any source; prepared or purified in any manner; vary in size or length without meaningful limit; and be used as a genetic tool for any research purpose, for a diagnostic goal or therapeutic application, or for any other result involving genetic manipulation. The chosen nucleic acid sequence may be DNA, or RNA, or a peptide nucleic acid (PNA) in composition; may be endogenously or exogenously derived; may be naturally occurring or synthetically prepared; may be composed of a minimum number of nucleic acid bases; or be composed of many thousands of nucleotides in sequence. For example, the user may thus employ

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genomic or chromosomal DNA, plasmid DNA, and/or a cloned or replicated DNA which is representative of a marker or is unique for a virus, a bacteria, a particular cell type, or a cytoplasmic or nuclear zone within a cell. Similarly, the oligonucleotide or peptide nucleic acid fragment utilized may represent or comprise RNA in its many forms (such as messenger or transfer, or mitochondrial RNA) as it exists in nature or is synthetically produced. All of these diverse origins and sources of nucleic acid sequences are conventionally known; and many techniques for their individual preparation, isolation, and purification as single-stranded nucleic acid chains of known composition, sequence, and specific binding capabilities are available in the published literature today.

Regardless of the particulars regarding the nucleic acid sequence or fragment chosen for use as an oligonucleotide probe, two essential requirements must be met and satisfied in every instance. First, the nucleic acid segment should be of known composition and sequence order, and thus bind selectively and specifically with a particular complementary target sequence primarily, if not exclusively. The degree of selectivity and hybridization for the oligonucleotide probe will thus vary with the specificity of its nucleic acid sequence and the degree of homology permitted among different and competing complementary target species. Thus, the more controlled the nucleic acid sequence of the probe, the more selective and specific the binding with one complementary target specie. Second, the oligonucleotide probe must bind with its intended target at each and every instance and occasion where the complementary target species is presented for reactive contact. Thus, although the complementary target sequence may exist in single or low copy number and/or be encased in a larger-sized fragment containing non-complementary sequences, the oligonucleotide probe should nevertheless bind to the target portion of these larger mobile fragments when they come into reactive contact with the probe. Such binding capacity provides both specificity and sensitivity for the biosensor as a whole.

C. Oligonucleotide Probe Immobilization

When depositing the individual DNA or RNA oligonucleotide specie to be used as a probe on the end surface of a single optical fiber strand or at precisely spatially positioned locations on one optical array end surface, it is necessary that the oligonucleotide(s) remain immobilized at the single or the different spatial positions assigned to each of them individually without migrating towards any other position. Multiple methods of oligonucleotide probe deposition and immobilization are

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conventionally known and are suitable for use in making an embodiment of the present invention. Thus, one may prepare a specific formulation comprising one specie of DNA (or RNA, or PNA) bases in sequence and dispose the formulation at a specific spatial position and location on the optic end surface.

Among the conventional practices of deposition a variety of generally applicable polymerization processes are known, including thermal techniques, ionization methods, plasma methods, and electroinitiation procedures. These different methodologies are exemplified by the following publications, the text of each being expressly incorporated by reference herein. Thermal methods: Graham et al., J. Org. Chem. 44: 907 (1979); Stickler and Meyerhoff, Makromal. Chem. 179: 2729(1978); and Brand et al., Makromol. Chem. 181: 913(1980). Ionization methods: A. Chapiro, Radiation Chemistry of Polymer Systems, Chapter IV, Wiley-Interscience, Inc., New York, 1962; JE. Wilson, Radiation Chemistry of Monomers, Polymers, and Plastics, chapters 1-5, Marcel Dekker New York, 1974. Plasma methods: Yasuda, W. and T.S. Hsu, J. Polym. Sci. Polym., Chem. Ed. 15: 81(1977); Tibbett et al., Macromolecules 10: 674 (1977). Electroinitiation method: Pistoria, G. and O. Bagnarelli, J. Polm. Sci. Polm, Chem. Ed. 17: 1001 (1979); and Philips et al., J. Polym. Sci. Polym. Chem. Ed. 15: 1563 (1977).

One method of oligonucleotide probe disposition and immobilization preferred for use with unitary fiber optic arrays is the process known as photoactivaction; and employs one or more photoactivated monomer preparations in admixture with one species of oligonucleotide as a photopolymerizable formulation [as described in Munkholm et al., Anal. Chem. 58: 1427 (1986) and Jordan et al., Anal. Chem. 59: 437 (1987)]. Such monomer preparations typically comprise solutions of several monomers in admixture and a concentration of the chosen DNA or RNA oligonucleotide specie. A representative listing of different monomer compositions suitable for preparing an admixture which subsequently can be photopolymerized are given by Table 1 below.

It will be appreciated that the listing of Table I are merely representative of the many different substances which can be usefully employed in admixture with a specie of oligonucleotide. In addition, the scientific and industrial literature provides many alternative monomer preparations and admixtures which are also suitable for use in making the present invention. Accordingly, all of these conventionally known monomer preparations are considered to be within the scope of the present invention.

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Table I

A. Monomers

acrylamide
N,N-methylene bis (acrylamide)
hydroxyethylmethacrylate
EGDMA
vinyl acetate
N-(3-aminopropyl) meth-acrylamide
hydrochloride [Kodak, Inc.]
N-acryloxy succinimide

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II. A Preferred Method Of Making A Biosensor

To demonstrate a most desirable method of making the biosensor comprising the present invention; and as a demonstration of the effectiveness for making optical determinations using the fully constructed fiber optic sensor, a detailed description of the manipulative steps for making a sensor able to hybridize in-situ with and consequently detect a mobile complementary oligonucleotide target specic bearing an identifying dye label is presented. It will be expressly understood, however, that the detailed description which follows hereinafter is merely illustrative and representative of the many different kinds of biosensors utilizing a unitary fiber optic array which can be made having one or more individual species of oligonucleotides deposited as multiple fixed probes at precise spatial positions on the optical array end surface, each disposed species-selective fixed probe being able to react with and individually hybridize with a labeled, mobile complementary target specie of oligonuclcotide which is of interest in a fluid sample.

Surface silanization

Initially, fiber optic array similar to that illustrated by Figs. 6-8 respectively was obtained from commercial sources [Applied Fiber Optics, Inc., Southbridge, Mass.]. One optical array surface was submerged in a acetone 10% solution of 3-(trimethoxysilyl) propyl-methacrylate dispersed in dry acetone and allowed to soak for 2 hours duration. After silanization, this optical array surface was rinsed first with dry acetone and then with distilled water.

Light Source

A fiber optic connector and ferrule [AMP, Inc., Harrisburg, Pa.] were modified to physically secure the fiber optic array to a fiber optic cable able to transport light energy of varying wavelengths to precise spatial positions on the distal array surface of the imaging fiber optic array. The exterior surface of one representative lighting cable is illustrated in an enlarged view by Fig. 9.

An inspection of the lighting cable of Fig. 9 reveals (in an exaggerated, highly oversimplified view for purposes of clarity) that the individual light sources via coordinated numerals correspond precisely to the spatial positions of Figs. 7 and 8; and are directly aligned with individual fiber optical strands S1-S120 (which also are precisely

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positioned spatially and identifiable via linear coordinates). Thus, light originating from source L1 will be introduced only to fiber S1 spatially positioned at coordinate number "12a"; similarly, light energy emanating from source L85 will be introduced only to that precise spatial position on the proximal optical array end surface identifiable as fiber S85 at coordinates "4a". In this manner, only predetermined and pre-chosen fiber optical strands will receive light energy of determinable wavelengths for a specified duration; at a time desired by the user alone; and no other optical fiber strand will receive any light energy whatsoever other than those strands located at a precise spatial position on the surface of the optic array surface. By purposeful choosing, therefore, of which light sources on the lighting cable are to be employed, the user may introduce light energy at will to only pre-chosen, precise spatial positions and only to those few fiber optical strands known to be present at precisely that location alone on the optical array surface.

In most practical use instances, however, the lighting cable of Fig. 9 will not be employed because of its limitations. Recognizing that the typical cross-sectional diameter of a single fiber optical strand is only 2-20 micrometers; and recognizing further that the specie of oligonucleotide to be deposited precisely at a known spatial position on the optic array surface will desirably provide and encompass a surface area greater than the diameter of a single fiber strand; then clearly it is impractical and functionally unnecessary to employ only a lighting cable of such limited one-to-one correspondence as that shown by Fig.9.

In actual practice, therefore, a lighting apparatus having a pinhole in a filter holder which allows fine focusing and precise placement of light is employed in the making of the sensor. This pinhole apparatus has only one light source of illumination rather than a cable having multiple light sources; and the single pinhole acts as a light source to introduce focused light energy to several dozen individually clad, optical fiber strands simultaneously -- all the simultaneously illuminated strands being adjacently positioned within the imaging fiber optic array at precisely known spatial positions. In this manner, the single pinhole light source corresponds to and aligns with multiple fiber strands simultaneously; and permits the deposition of an oligonucleotide and monomer admixture over multiple strand faces simultaneously. The advantages and benefits of using the single source of focused lighting are that a controlled volume of admixture is precisely deposited at the pre-chosen spatial position on the optic array surface with minimal time and labor.

The lighting cable of Fig. 9, although completely operational for its intended

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purpose, is often far too cumbersome for practical use; is provided only as a representative article to demonstrate the principle of introducing light energy to a precise location on the proximal optic array surface; and is used merely to illustrate the method and the manner in which the dye becomes photopolymerized and precisely positioned at a pre-chosen location on the distal optic array surface. Having illustrated both the principle and the intended results, it will be recognized and appreciated that any lighting source of any correspondence with the fiber optical strands of the fiber optic array will serve so long as the disposed oligonucleotide specie deposits are spatially separate and spatially distinguishable from one another on the optic array surface.

Photopolymerization

The manipulations performed during photopolymerization are illustrated via Figs. 10-15 respectively. For descriptive purposes only, the general magnified and oversimplified construction of the optic array surface of Fig. 7 and the lighting cable of Fig. 9 will again be used.

As seen within Figs. 10-15, a fiber optical connector 130 and illumination source 140 provide the capability for illuminating specific areas of one optic array surface of the imaging fiber optic array described previously. Thus, the light energy photons emanating from the surface of the illumination source 140 of Fig. 10 are produced by only light sources L23, L24 and L34 respectively. Only light energy at those precise spatial positions is directed towards the proximal optic array surface 114 of the unitary fiber optical array 100. Consequently, as shown via Fig. 10 only those fiber optical strands located at spatial position coordinates 10k, 101, and 9k respectively receive the light energy photons provided by the illumination source 140. Then as illustrated by Fig. 11, only those corresponding individually clad fiber optical strands S23, S24 and S34 convey the introduced light energy through the body of the unitary fiber optical array 100; and the light exits at the distal optic array end surface 112 only at precise spatial positions (that is, solely at coordinate numbers XK, XL, and IXK as seen within Fig. 7 above. It will be recognized and appreciated that no other spatial positions on the distal array end surface 112 are illuminated during this manipulation.

As the light energy photons emerge from the distal array end surface 112 at only the precise spatial positions indicated by Fig. 11, the optic array end surface 112 lies submerged in a prepared first monomer admixture. The light employed at only this precise

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spatial positions was pre-chosen to be at a set wavelength for photopolymerization and the optic array end surface was allowed to react with the first monomer preparation for approximately 30 seconds duration. The reactive contact between the first monomer admixture and the light energy initiated a photopolymerization reaction on the distal array end surface and caused a deposition and an immobilization of the first polymer zone only at those illuminated spatial positions. Thus, at the end of the allotted reaction time for photopolymerization, a discrete volume 150 of a polymer was deposited and immobilized solely on the distal optic array surface solely at spatial positions XK, XIK, and IXK. No other fiber strands were illuminated; no other fiber strands conveyed any light energy whatsoever; and no polymerization or deposition occurred at any other spatial positions. This is illustrated by Fig.13.

After the first polymerization was completed, the illumination source 140 was then used again to illuminate the light positions corresponding to light position L15, L16, an L27. Light energy from only these positions introduced light energy photons precisely to the proximal optic array end surface 114 only at coordinate positions l0b, l0c, and 9c. This caused the introduced light energy photons to be conveyed solely by fibers SIS, S16 and S27. No other fiber strands were illuminated and no other fiber strands conveyed any light energy whatsoever. This is illustrated by Figs. 13 and 14.

Consequently, as appears in Fig. 14, light energy photons carried by only these individually clad, fiber optical strands (S15, S16, and S27) cause the light to be conveyed and to exit from the distal optic array end surface 112 only at coordinate position numbers XB, XC, and IXC. The optic array surface was then immersed in a prepared second monomer mixture and the light energy allowed to react with the prepared mixture for a predetermined duration. During this reaction time, photopolymerization proceeded and the second zone was deposited solely at those spatial positions which were illuminated. In this manner, the multiple deposits of different polymer zones became immobilized by photopolymerization at only those precisely illuminated locations identifiable by the coordinate numbers XB, XC and IXC. At the end of the allotted time for reactive contact, the distal optic array end surface of the imaging fiber optic array was removed from the second monomer admixture and revealed the deposition of an immobilized deposit of a second polymer matrix at the precise spatial positions identifiable precisely by coordinate numbers XB, XC and IXC. A discrete polymerized cone-shaped deposit 160 of the second oligonucleotide specie is seen extending from the distal optic array end surface as

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illustrated by Fig. 15.

It will again be recognized and appreciated that under typical conditions the size of fiber core diameter in the fiber optic array so overwhelmingly exceeds the amount of corresponding cladding material that there is no effective separation between the fiber strands during the photopolymerization process. Thus the photopolymerization of the mixture at only the pre-chosen and illuminated spatial fluid positions results in the deposition of a single, unitary continuous volume large enough in surface area to encompass and cover multiple fiber end faces on the distal optic array surface. The presence of the cladding within the fiber optic array thus does not interfere with or hinder the continuity of the deposition. The result is both true and constant regardless of what specific process for depositing oligonucleotide species is employed and whether or not the favored photopolymerization technique is used.

The practitioner ordinarily skilled in this field will by now also recognize that there is no requirement or demand that an illumination fiber or cable as such be employed in this photoactivated method for making the sensor. To the contrary, one merely needs to introduce pinpoints of light into separate portions of areas of the imaging fiber optic array for photopolymerization to proceed. Thus, for example, one could achieve equivalent effects using lenses and/or lasers. Accordingly, any conventionally known means or manner of introducing light is deemed to be within the scope of the present invention.

The results of the completed photopolymerization process are illustrated by Fig. 15 in which the polymerized first oligonucleotide specie deposit 150 and the polymerized second oligonucleotide specie deposit 160 are individually located and identifiable at precise spatial positions on the distal optic array end surface. It will also be recognized that much of the distal optic array surface 112 remains unencumbered and unobscured; and that were additional light introduced at the proximal array end surface 114 at any of the unobscured strand spatial positions, such light photons would be conveyed and would exit from the distal optic array end surface 112 as unencumbered light energy which does not affect or influence the discrete deposits 150,160 positioned separately nearby.

III. The Optical Sensing Apparatus And Instrumentation System

In order to be effectively employed, the prepared biosensor is combined with optical apparatus and instrumentation and is utilized as a system to detect and identify one or more specific analytes or peptide nucleic acids of interest. A generalized and

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representative optical apparatus and instrumentation system which is conventionally available and preferably employed is illustrated by Fig.16.

Sensor measurements may be performed using the apparatus shown schematically by Fig. 16 in the following manner: White light from an excitation source 200 (such as an arc lamp) is collimated; focused by a lens 201; is passed through an excitation filter 202; and is focused on an optic sensor 205 via a 10X microscope objective 204. The optic sensor 205 is held in an xyz-micropositioner 206 which allows for fine focusing. Excitation light is transmitted and illuminates each thin film sensing receptor unit in the array of the sensor which individually fluoresces in proportion to analyte concentration. The returning fluorescence light is reflected 90° by the dichroic filter 203; desirably, but optimally passed through a beam splitter cube 208; filtered at an appropriate emission wavelength by emission filter wheel 210; and then is detected by the CCD camera 220. Ratiometric measurements are obtained by monitoring fluorescence while switching between two excitation filters 202 using the emission filer wheel 210. The CCD camera typically contains a photosensitive element and may be coupled to an electronic intensifier; which in turn is connected to a computer having a Video Frame Grabber graphic card that digitizes and processes the video image. Visual imaging is achieved by using a CCD video camera to collect the light which is reflected 90° by the beam splitter cube. Illumination for visual imaging purposes is achieved either by rotating the excitation filter wheel to an empty position (using neutral density filters as necessary); or by illuminating the sample and its environs at the distal end of the sensor with an independent light source.

The optic sensing apparatus and instrumentation system shown by Fig. 16 detects fluorescence either as light intensity or as light wavelengths -- that is, a spectral response generated by and released by a deposited probe from a single optical fiber strand end surface; or from at least one strand end face in a bundled array of single core fibers; or from at least one individual spatial position on the distal array end surface of the unitary array after initial illumination with light energy of a pre-determined wavelength. The light energy emitted or reflected from each fixed probe position individually is collected using a CCD video camera using standard frame grabbing technology and image processing capabilities. Each spectral response detected as emerging light energy by the detector of the CCD is recorded; and the pattern of fluorescence or color is shown either as energy wavelength or as light intensity pixels on the detector representing the spatial dimension. By definition, a pixel is a picture element --a sensitive region -- which determines light

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intensity and/or light energy quantum.

IV. The Mobile Complementary Nucleic Acid Target Sequence

The analyte of interest to be detected optically using the biosensor and supporting apparatus is at least one mobile complementary oligonucleotide target specie bearing an identifying label joined directly or indirectly. The complementary target specie represents a sequence of nucleotides which corresponds as the complementary base sequence to the nucleic acids comprising the fixed oligonucleotide probe. It is expected and envisioned that the mobile complementary target specie will bind selectively to and hybridize in-situ with the nucleic acid sequence of the probe; and thereby generate a reaction product which is the result of specific and selective binding between only the fixed probe specie and the complementary target specie.

The present invention also intends and expects that an in-situ hybridization reaction will occur between each oligonucleotide specie deposit immobilized at one position or at multiple and differing spatial positions as fixed probes, each type of specie deposit reacting selectively with one mobile complementary oligonucleotide target specie (while other specie specific probes react concurrently with another alternative type of mobile complementary target specie). In this manner, different kinds of individually labeled complementary target species will react with only their counterpart and corresponding specie-specific fixed probes on at least one and preferably at several individual and differing spatial positions on the distal array end surface. Thus, at the user's choice and option, the array formats of the biosensor may be utilized to selectively detect only one mobile target species or a plurality of different target species intermixed in a fluid sample.

There are therefore only three requirements for each specie of complementary nucleic acid target to be detected using the present invention. These are: that the complementary target specie be mobile; that the complementary target specie ultimately bear a directly or indirectly joined identifying label; and that the complementary target specie be present in a fluid sample placed into reactive contact with the distal end of the biosensor. It will be noted and appreciated also that while there is no requirement as such, it is often desirable that the individual complementary oligonucleotide target specie be separated, isolated, purified or semi-purified before being placed into reactive contact in order for an effective in-situ hybridization to occur. Thus, the fluid sample often may comprise a mixture of different materials including not only multiple nucleic acid

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sequenced fragments, some of which are the complementary target specie for hybridization; but also may contain some extraneous matter, such as cellular debris or unrelated pharmacologically active molecules which typically are present only as incidental remnants from an earlier extraction, reaction, or preparation process. As a general rule, therefore, the fewer the number of extraneous chemical entities in the fluid sample, the faster the kinetics of in-situ hybridization will proceed.

The light energy absorbing dyes useful as joined identifying labels

At least one light energy absorbing dye is bound initially or becomes linked subsequently to each mobile complementary oligonucleotide target species as an identifying label. If desired, more than one dye reagent can be employed as a joined identifying label.

Each light energy absorbing dye formulation or composition will be bound directly or becomes linked indirectly to the one specie or to multiple different species of oligonucleotides intended for use as complementary targets. Moreover, each dye will then show evidence of its presence by either absorbing and reflecting a portion of the light energy; or, alternatively, by absorbing light energy and then subsequently emitting light energy of a different wavelength in return. Such reflected or emitted light energy is intended to be conveyed from the distal end surface; and such conveyed light will emerge from the proximal end surface for detection and measurement.

The various dyes which may be bound initially or linked subsequently to a chosen oligonucleotide fragment as a joined identifying label are all conventionally known and often commercially available. The present invention intends that all the commonly useful properties and capabilities of the various classes of light energy absorbing dyes be employed directly and indirectly, and as needed or desired for the specific use or application. Merely illustrative of the many different dyes are those fluorophores, indirect (secondary) labels, and interchelators listed below within Tables 2, 3, and 4 respectively.

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Table 2

Compounds	Excitation Wavelength (range or maximum)	Fluorescence emission range (max)
A. Fluorophores		
Eosin	520-530 nm	530-580 nm (550 nm)
TRITC-amine	555 nm	570-619 nm (590 nm)
Quinine	320-352 nm	381 -450 nm
Fluorescein W	488-496 nm	530 nm
Acridine yellow	464 nm	500 nm
Lissamine Rhodamine	567 nm	580 nm
B Sulfonyl Chloride		
Erythroscein	504 nm	560 nm
Ruthenium (tris, bipyridium)	460 nm	580 nm
Texas Red	591 nm	612 nm
Sulfonyl Chloride		
B-phycoerythin	545, 565 nm	575 nm
Nicotinamide adenine	340 nm	435 nm
dinucleotide (NADH)		
Flavin adenine	450 nm	530 nm
dinucleotide (FAD)		
Carboxy	587 nm	640 nm
Seminaphthorhydafluor		
Naphthofluorescein	594 nm	663 nm
Carboxy Fluorescein (Fam)	495 nm	520 nm
BODIPY*		
JOE*		
TAMRA*	540 nm	564 nm

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ROX*	567 nm	591 nm
B. Fluorescent Antibody Conjugates		
Protein A fluorescein conjugates	480 nm	520 nm
Anti-Atrazine fluorescein Conjugates	480 nm	520 nm
digoxin-Anti-digoxin Texas Red Conjugates	590 nm	615 nm

* Trademarks of Molecular Probes Inc. (Eugene, OR)

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Table 3:

Secondary Label Pairs (Labels include but are not limited to those mentioned in Table 2)	
Biotin	Labeled avidin / streptavidin
Protein A	Labeled IgG
Digoxin	Labeled anti-digoxin
Enzymes such as:	
alkaline phosphatase	diphosphate derivatives ELF-97 substrates (enzyme activated)
β-glucuronidase	glucuronide galactopyranoside
Horseradish Peroxidase	

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Table 4:

Intercalators

ethidium bromide;

Cy-5;

Ru $(byp)_2$ MCCP;

Hoechst 33258 (bis-benzimide);

Cyanine dyes*

- TOTO®
- YOYO®
- BOBO™
- POPO™
- SYBR I®

* Trademarks of Molecular Probes Inc. (Eugene, OR)

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It will be recognized and appreciated also that the available range, variety, and diversity of light energy absorbing dyes, dye formulations, and dye mixtures is not dependent upon a single light source or light energy supply in order to be effective. Although light energy of determinable wavelengths is desirably provided by electrical light sources -- that is, light emitting diodes (LEDs), lasers, laser diodes and filament lamps whose bands of light energy are typically controlled and selected by filters, diffraction gratings, polarized filters; or alternatively broken into various broad wavelengths of light energy via prisms, lenses, or other optical/spectral articles, these are not exclusively the only source of useful light energy. Clearly, in various applications and circumstances other less typical light energy sources will also be useful. Accordingly, neither the true source, nor the nature of light energy photons, nor the manner in which they are conveyed or otherwise caused to be created is of importance or consequence.

In addition, the dye label individually may comprise a pair of specifically binding materials such as the chemical compounds listed within Table 3 for subsequent reactive contact and indirect juncture of an identifying label. Thus each dye label individually may in fact be formulated as a composite comprising light emitting dye in part; and include a variety of receiving elements which are able to interact as specific binding partners for joining the light energy dye label subsequently. Exemplifying some multiple formulations and combinations are those described below and used experimentally hereinafter.

Methods for preparing a mobile complementary target specie bearing an identifying label

A range of different preparation methods and processes are conventionally known and available in the published scientific literature for creating a mobile complementary oligonucleotide target specie of known base sequence joined directly to an identifying label as a conjugate. Representative of and exemplifying these direct attachment procedures are the following: Agrawal et al., Nucleic Acids Res. 14: 6227-6245 (1986); Smith et al., Nucleic Acids Res. 13: 2399-2412 (1985); Cardullo et al., Proc. Natl. Acad. Sci. USA 85: 8790-8794 (1988); J Fluorescence 1:135 (1991).

An alternative preparation strategy and procedure is exemplified by incorporation of biotin, in the form of a biotinylated nucleotide (such as biotin-d UTP) into the nucleic acid structure using conventional procedures such as nick translation or tailing [Rigby et al., J. Mol. Biol. 113: 237 (1977); Lobban, P.E. and A.D. Kiser, J. Mol. Biol. 78: 453 (1973)]. The selective binding of the biotinylated targeted to the fixed probes in the in-situ

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hybridization reaction may then proceed in the absence of the dye label itself. Subsequently a conjugate complex constituted of avidin or streptavidin (proteins with a high affinity for binding to biotin) covalently are linked to a fluorescent or color reflecting dye ligand is then added as a dye label complex to the reaction fluid after hybridization has occurred; and the selective binding capability for the paired agents will then cause the identifying dye label to be joined via the avidin (or streptavidin) indirectly to each biotinylated target species wherever it is found. Qualitative and quantitative optical detection of the hybridized target specie can therefore be made on the basis of the spectral characteristics of the ultimately and indirectly joined identifying label. The listing of Table 3 provides other alternative pairs of specific binding agents suitable for use.

If desired, a biotinylated complementary target specie may be also prepared using polymerase chain reaction processing. The biotinylated amplified product obtained by PCR methodology may be used immediately or purified before being placed into reactive contact with the biosensor. Also, any of the other pairs listed in Table 3 may be substituted for use in the PCR method.

In addition, the user may optionally employ the technology known as enzyme-labeled fluorescence (ELF) signal amplification to provide a joined identifying label. This technology is described in detail by U.S. Patent Nos. 5,136,906 and 5,443, 986, the texts of which are expressly incorporated by reference herein. In brief, an enzyme such as alkaline phosphatase is attached directly or is linked subsequently to the complementary oligonucleotide target species. The substrate for the enzyme's catalytic activity is one which provides an intense fluorescent signal and also demonstrates a very large Stoke's shift. Such substrates have been shown to be highly detectable labels when used with conventional in-situ hybridization method. See for example: Am.J. Human Genet. Suppl. 55, A271, Abstract #1588 (1994); FASEB J. 8: A1444, Abstract #1081 (1994); and Mol. Biol. of the Cell Suppl. 4, 226a, Abstract #1313 (1993).

The user is thus given the option of preparing the complementary oligonucleotide target specie in several ways. The target specie nucleic acid sequence may be directly and immediately bound to an identifying dye label (such as those of Table 2) if desired. Alternatively, the complementary oligonucleotide target specie or species may be prepared as molecules having a receiving element such as biotin. The biotinylated target specie is allowed to hybridize in-situ with the fixed probes on the distal end surface of the biosensor; and then a prepared specific binding partner (such as avidin or streptavidin

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complex) bearing an identifying dye label as a component part can then be added to the reaction fluid after hybridization is completed -- thereby causing the identifying label to be joined subsequently as well as indirectly to the immobilized hybridized reaction product. Finally, the intercalators (such as those listed within Table 4), may be used in unmodified form to label double-stranded, hybridized reaction products without any prior intermediate agent. The target species thus remains unlabeled throughout the entire hybridization process; the intercalators then will bind directly to the double-stranded reaction product upon reactive contact.

V. Identifying The Resulting, Species-Specific, In-Situ Hybridization Oligonucleotide Reaction Product

The optical biosensor of the present invention intends and requires that a species-specific, in-situ hybridized reaction product result as the consequence of a selective reactive contact between a mobile complementary oligonucleotide target specie; a subsequently linked or directly joined identifying dye label; and one or more specie selective oligonucleotide probes deployed either on the distal end surface of a single optical fiber strand or at differing fixed spatial positions upon one array end surface of a unitary fiber optic array. Two features are essential for the biosensor: (a) The individual spatial positioning of the individual specie deposits as multiple fixed probes deployed on the distal end surface of the biosensor allows for a single or many different and distinct specie-specific, in-situ hybridizations to occur with a minimal volume of a mobile complementary oligonucleotide target specie having an initially existing or subsequently joined identifying label; and (b) the predetermined and fixed, individual spatial positioning(s) of the deployed, single specie, fixed probe(s) upon the discrete optic end surface utilize the initially or ultimately joined identifying label to identify the occurrence of a species-specific hybridization in-situ. Also each biosensor format relies upon spatial resolution as a means by which to differentiate and distinguish among the alternatively positioned hybridized complementary target species concurrently immobilized at many differing spatial locations on the optic array surface. In the fiber optic array format and in the bundle of fibers format, therefore, it is the combination of fixed spatial positionings for in-situ hybridization at the differing chosen locations and the spatial resolution capability to separate and distinguish the joined identifying dye label among the differing fixed surface positionings (and the different, hybridization reactions occurring concurrently)

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which avoids and eliminates random intermixing of individual light energy photons traveling to and from an identifying label concomitantly joined to each in-situ hybridized reaction product formed on the optic array surface of the biosensor.

Thus, within the in-situ hybridization zone on the distal end surface, each identifying label directly or indirectly joined to a hybridized complementary oligonucleotide target specie becomes, in turn, concomitantly immobilized and disposed only at individual probe locations fixed on the optic array end surface and the presence of a joined identifying label held at any one or more fixed spatial positions can only be the secondary consequence of a specie-specific in-situ hybridization reaction having occurred at that spatial position. Each immobilized identifying dye label will then show evidence of its presence at that precise spatial position by either absorbing and reflecting a portion of the light energy or absorbing light and then subsequently emitting light energy of a different wavelength in return. Such reflected or emitted light energy is conveyed via one or more individual fiber optic strands in aligned position with the immobilized dye itself. Such conveyed light will emerge from the other optic end surface only at precisely located spatial positions; and thus be distinguishable as such from other light energy conveyed by any other fiber optical strands via the precise spatial positioning and the spatial resolution of the emerging light at the optic array surface. In this manner, the conventional limitations and demands of single channel optical fibers are eliminated since the strands within the fiber optical array retain the spatial positioning for each of the disposed dye labels. Thus, the traditional requirement for spectral resolution is removed due to the ability by the fiber optical array to resolve each of the dye labels spatially.

VI. Experiments And Empirical Data

Experimental Series A

Single optical fiber strand preparation

The distal and proximal faces of several single optical fiber strands are polished and cleaned. Each strand's distal end was silanized in 10% (amino) propyltriethoxysilane in acetone (v/v). The single core fiber strands were removed after 2 hours, rinsed with acetone, and then air dried for 30 minutes. The single core strands were placed in a 1.25% gluteraldehyde solution in 0.02 M phosphate buffer (pH 6.8) for 30 minutes. The single

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core fiber strands then were rinsed with distilled water and placed in 3% polyethyleneimine (PEI) in 0.02 M phosphate buffer (pH 6.8). Finally, the single core strands were air dried for 1 hour, rinsed, and stored in distilled water until being functionalized individually with oligonucleotides.

Bundled optical fiber array preparation

To make a bundled optical fiber array, seven functionalized 200p.M diameter single core fiber strands were bundled together. The distal end surface of each fiber strand was first functionalized as described herein with a different cytokine oligonucleotide as a fixed probe - the bundling of seven individual strands creating a multi-target sensing array. The proximal ends of the bundled optical fiber array were epoxied into a 1 mm stainless steel tube (A) and placed into the fiber chuck of the epifluorescence imaging system as shown in Fig. 17. The bundled optical fiber array was three feet long in length and was used for remote sensing (B). The protective rubber tubing is removed from the distal end of the bundled arrays to enable individual functionalization (C).

Oligonucleotide preparation:

10 nmoles of 5'-amino-terminal oligonucleotide were dissolved in 90 μL of 0.1 sodium borate buffer (SBB). Oligonucleotide activation was initiated by adding 5 nmoles of cyanuric chloride in 10 μl of acetonitrile. The reaction proceeded at room temperature for 1 hour. Unreacted cyanuric chloride was removed by three cycles of centrifugal ultrafiltration (3000 d MW cutoff, Microcon 3, Amicon) and diluted with 200 μL of 0.1 M SBB. The activated primers were recovered in approximately 50 μL of 0.1 M SBB and stored at 4°C. Primers were used within one month of activation.

Single core fiber sensor preparation:

The modified distal end of each single core strand was washed by dipping several times in five-2 ml changes of 0.1 M SBB. The distal strand tips were immersed in a 10 μL solution of 150 μL cyanuric chloride-activated oligonucleotide in 0.1 M SBB for 1-2 hours. The fiber strand tips were then immersed in 200 μL of 90% DMSO, 10% 1 M SBB buffer pH 8.3, 0.1 M succinic anhydride for 1 hour at room temperature. The fiber tips were washed (as described above) with 2 changes of 0.1 M SBB, 5 changes of TE (10 mM Tris-HCl pH 8.3, 1mM EDTA) containing 0.1 M NaCl and 0.1% SDS. Fibers tips were

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stored in the washing buffer until use.

Fluorescence measurements:

Fluorescence measurements were acquired with a modified Olympus epifluorescence microscope / charged coupled device camera described previously herein.

Primary PCR reaction:

A 176 base pair region from a human IL-4 cDNA plasmid clone [Yokota et al., Proc. Natl. Acad. Sci. USA 83: 5894-5898 (1986)] was amplified using primers IL4-U (5-CATCGTTAGCTTCTCCTGA-3') and IL4-L (5'-AAAGTTTTGATGATCTCCTGTA-3') generating a double-stranded PCR product. Conditions for the reaction were 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Tween-20, each primer at 0.5 μM dNTPs, 2 U AmpliTaq polymerase, 10^4 copies of BamHI-cleaved pcd-hIL-4. Thermocycling parameters were 35 cycles consisting of 10 seconds at 94°C, 10 seconds at 55°C, and 30 seconds at 72°C.

Secondary PCR reaction:

The double-stranded primary amplification products were internally labeled during an asymmetric PCR step using fluorescein-labeled dGTP. Conditions for the reaction were 10 mM Tris-HCl pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$ 0.5% Tween-20; 1 μM unlabeled primer IL-4 L; 50 μM each dATP, dCTP, dTTP; 25 μM dGTP; 25 μM dGTP; 25 μM fluorescein-labeled dGTP. After asymmetric amplification, the reaction was extracted with phenol and isobutanol. Primers and nucleotides were removed by two cycles of centrifugal ultrafiltration (Microcon 30, Amicon 400 μL TE per wash). Samples were recovered from the filtration unit in 50 μL of TE.

PCR Product Assay

The array distal end was placed in 5 μL of 16 nM labeled PCR product in buffer (TE containing 0.1% SDS and 0.35 M NaCl) for 20 minutes then rinsed with buffer solution (TE containing 0.1 % SDS and 0.35 M NaCl). Signal was acquired while the biosensor array was in buffer solution.

Experiment 1: The Biosensor

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To create a DNA biosensor array, probes specific for human cytokine mRNA sequences were immobilized on the tips of single core optical fibers. Cytokines are powerful immune system hormones whose expression is stimulated in response to inflammatory stimuli or infection. For this reason, there is widespread interest in the regulation of the cytokine gene expression. The sequences of the cytokine probe and targets used are shown in Table E1.

To make a bundled optical fiber array, seven functionalized 200 μM diameter single core fibers were combined and bundled together using epoxy. The distal end surface of each optical fiber strand was first functionalized as described above with a different cytokine oligonucleotide as a fixed probe; and then the bundling of seven functionalized strands created a multi-target sensing array. The proximal ends of the bundled optical fiber array were then epoxied into a 1 mm stainless steel tube and placed into the fiber chuck (A) of the epifluorescence imaging system as shown in Fig. 17. The bundled array was three feet long in length and was used for remote sensing (B). The protective rubber tubing is removed from the distal end of the bundled array to enable individual functionalization (C).

The bundled array of optical fibers was then tested by placing the distal end of the bundled strand sensor into a solution containing one or more 5'-fluorescein-labeled cytokine sequences. The bundled array was removed from the target solution after incubation for 5 minutes and rinsed with buffer solution (TE, 0.1 % SDS, 0.1 M NaCl). The bundled array was then placed in buffer solution and fluorescence signals were acquired (excitation wavelength 490 nm emission wavelength 530 nm). Signal analysis was performed in less than 30 seconds with commercially available software (IP Lab Spectrum). The results are shown by Fig. 22.

The images of the bundled array biosensor in buffer solution as acquired by a CCD camera are shown by Figs. 18A and 18B. The image with white light transmitted through the distal end of the array is demonstrated by Fig. 18A. In comparison, a background fluorescence image at 530 nm taken with 490 nm excitation in buffer solution is shown by Fig. 18B. While there is some light transmitted through the cladding, there is no fluorescence observed across the distal end surface of the bundled array.

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Table E1

Probe Sequences	*Target Sequences*
β-glo(+) (segment of human β-globin)	β-glo(+)-CF
5' -(NH_2-$(CH_2)_{6-}$)DTT TTT TTT TCA ACT TCA TCC ACG TTC ADD-3'	5' -Fluorescein-TG AAC GTG GAT GAS GTT G-3'
IFNG (interferon gamma 1)	IFNG-CF
5' -(NH_2-$(CH_2)_{6-}$)T12-TGG FTT CTC TGG GCT GTT ACT-3'	5' -Fluorescein-AG TAA CAG CCA AGA GAA CCC AAA-3'
IL2 (interleukin-2)	IL2-CF
5' -(NH_2-$(CH_2)_{6-}$)T12-TA CAA GAA TCC CAA ACT CAC CAG-3'	5' -Fluorescein-CT GGT GAG TTT GGG ATT CTT GTA-3'
IL4 (interleukin 4)	IL4-CF
5' -(NH_2-$(CH_2)_{6-}$)T12-CC AAC TGC TTC CCC CTC TGT-3'	5' -Fluorescein-AC AGA GGG GGA AGC AGT TGG-3'
1L6 (interleukin-6)	IL6-CF
5'-(NH_2-$(CH_2)_{6-}$)T12-GT TGG GTC AGG GGT GGT TAT T-3'	5'-Fluorescein-AA TAA CCA CCC CTG ACC CAA C-3'

Experiment 2:

Background-subtracted mean fluorescence intensities were then obtained with IL-4 probe on the sensor. The bundled strand biosensor array was tested in different target solutions employing the sequences of probes and targets shown in Table El. The biosensor was placed in a target solution for 5 minutes; rinsed with buffer solution (TE containing 0.1% SDS and 0.1 mM NaCI); and fluorescent images were then acquired with the fiber tip in buffer. The result is shown by Fig. 19.

The bundled strand array was dipped in 90% formamide in TE between each test to remove any hybridized target and to regenerate the sensor. The IL-4 target solution contained flurescein labeled oligonucleotide complementary to the IL-4 probe on the sensor. After hybridization and read-out, the sensor was regenerated and used to test the IL-2 and IFNG-target solutions containing fluorescein labeled targets that are not complementary to the IL-4 probe. As seen in Fig. 19, these targets do not hybridize to the sensor. The second IL-4 hybridization afforded a signal close to the original test.

Plots of background subtracted mean fluorescence at 530 nm were then taken with 490 nm excitation as a function of time using a 500 μm diameter single core sensor with β-glo probe. A kinetic study using 1 μM β-glo target solution is shown by Fig. 20. Also, a kinetic study using 0.1 μM β-glo target solution is shown by Fig. 21. The sensor was placed in each of the β-glo target solutions for a given time; rinsed with buffer solution (TE containing 0.1 % SDS and 0.1 M NaCI); and a fluorescence signal was acquired with the sensor in buffer solution. After data acquisition, the fiber optic array biosensor was placed back in the target solution for an allotted time, rinsed, and examined in buffer. Once the kinetic data was acquired, the hybridized target was removed with 90% formamide in TE to regenerate the sensor.

Fluorescent images using the fiber optic biosensor array were acquired in buffer solution after treatment with IL2 target (A); IL4 target (B); IL6 target (C); β-glo target (D); IFNF target (E); and a mixture of IL4, IFNG and β-glo targets. The biosensor array distal end was placed sequentially in a fluid (A)-(F) using a 1 μM solution in TE containing 0.1% SDS and 0.1 M NaCI for 5 minutes; and was then rinsed with buffer solution (TE containing 0.1%

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SDS and 0.1 M NaCl). Hybridized oligonucleotides were removed after each immersion and optical detection using a 90% formamide solution in TE after each analysis. As shown by Fig. 22, the specificity of each probe-target hybridization (A)-(F) was confirmed. The signals obtained in buffer before hybridization were subtracted from the signals obtained after hybridization. High intensities are signified with white light.

The background subtracted mean fluorescence signals from Fig. 22 are shown in graph form by Fig. 23. Note: When a combination of oligonucleotide targets was placed in solution, the signal obtained was comparable to the signal obtained when a single target was in the solution. Figs. 22 and 23 in combination reveal that high signals were only observed on those fibers carrying probes complementary to the added target, demonstrating that hybridization to the fiber is also highly specific.

Experiment 4:

Hybridization competition between labeled and unlabeled variations of the same target sequences were performed using the biosensor array. Increasing concentrations of unlabeled targets were added to 1-μM solutions of a labeled identical target. The optical fiber biosensor was placed in the mixture of labeled and unlabeled target solution for 10 minutes; rinsed with buffer solution; and the background-subtracted fluorescence image was acquired while the distal array tip was in the buffer solution. The distal tip was rinsed with 90% formamide in TE between test samples to remove the hybridized target. The results are shown by Fig. 24; the fluorescence decrease was directly proportional to increasing concentration of sample target.

Experiment 5:

A practical application of the fiber optic biosensor is shown by the assay of cDNA samples generated by reverse-transcription PCR (RT-PCR) [Egger, et al J Clin. Microbiol. 33: 142 (1995)]. A 176 base pair fragment containing the IL-4 probe sequence (Table EI) was amplified from a cloned IL-4 cDNA target, and internally labeled with fluorescein using an asymmetrical PCR procedure [Cronin et al., Human Mutation 7: 244-255 (1996)]. The cytokine sensor array was dipped directly into the purified PCR reaction mixture. Using 5 μL of a 16 nm PCR target solution the sensor gave a clear, detectable, specific signal after 20

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minutes.

Optimization of the system

Immobilized probe concentrations limit the amount of complementary target that can hybridize to the probe and generate a signal. The feasibility of detecting a fluorescein labeled target in-situ hybridization to the immobilized probe on the distal surface was tested first using a 500 μm diameter single core fiber with an immobilized IL-4 probe (Table E-1). The tips of the array (surface area 0.002 cm^2) were placed in the target solution, which can be as small as 3 μL in a 0.4 mL eppendorf tube. The detection system consists of a modified epifluorescence microscope with the optics optimized to couple with an optical fiber.

The detection system parameters were also optimized. Increasing the acquisition time improved the signal however, the increased exposure time caused photobleaching and adversely affected subsequent sensor use. A two second acquisition time was found to be optimal in maximizing the detection while minimizing photobleaching of the fluorescein-labeled target. This fiber optic biosensor system has a detection limit of 10 nM.

Characterization of the sensor

The sensor's specificity was evaluated by placing the sensor in complementary and non-complementary-labeled target solutions. The fluorescent signal increases upon exposure to the complementary labeled target. The complete absence of signal when the biosensor was placed in a non-complementary labeled targets confirmed the hybridization specificity (Fig. 19).

Lengthy incubation times are frequently a concern in hybridization experiments utilizing immobilized probes. The optical fiber biosensor described here shows excellent hybridization kinetics. Hybridization is 85% complete after 1 minute using a 1-μM target solution (Fig. 20). With a 0.1-μM target solution, hybridization is 90% complete after 15 minutes (Fig. 21). The 10-nM target solution required 10 minutes to generate a clear signal (data not shown).

Fiber biosensors can be regenerated repeatedly by dipping the distal array tip in 90% formamide in TE buffer (10mM Tris-HCl pH 8.3, 1mM EDTA) for 10 seconds at room

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temperature. The biosensor then gives an original comparable signal for all subsequent analyses with alternative complementary oligonucleotide target (Fig. 19). Heating the formamide solution to 45°C was also effective and did not compromise sensor integrity.

The biosensor offers significant advantages for hybridization analysis. The optical fiber array serves as the hybridization support and also facilitates sensitive, quantitative fluorescent detection of in-situ hybridization using a very low sample volume. Multiple synthetic oligonucleotide probes were covalently immobilized and fixed on one end of a 200 μm diameter optical fiber.

The fiber optic biosensor and methodology demonstrates a fast reproducible, highly sensitive and durable system for the specific identification of DNA sequences. Fiber optic biosensor arrays enable simultaneous detection of multiple DNA sequences with reduced assay time and increased convenience. Complete analysis of multiple DNA sequences can be accomplished in under 5 minutes. Biosensors can be prepared in advance and be stored at 40°C; these maintain their sensing capabilities for many months. The biosensor's small size also offers the ability to perform hybridization analysis on large numbers of target sequences using extremely small sample volumes. The biosensor is also useful for performing in-situ hybridization analyses in settings where conventional hybridization methods would be difficult, if not impossible, to implement.

Experimental Series B

Experimental Protocol:

The approach to DNA immobilization involves the site-selective photodeposition of an acrylamide and N-acryloxysuccinimide copolymer. The distal face of a fiber optic imaging array is first functionalized with 3-trimethoxysilylpropylmethacrylate to attach the photopolymerizable acrylate to the glass and surface allowing for the covalent attachment of polymer matrices to the distal face. The proximal array end surface of the unitary array is placed on a photodeposition system which allows for site-positioned illumination of the proximal array end. The distal end of the functionalized array is then dip coated with a thin film of an acrylamide/acryloxysuccinimide prepolymer containing a photoinitiator. The prepolymer is then photopolymerized by light illumination through the fiber for a fixed time

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and excess prepolymer is removed by rinsing with ethanol. After the polymer matrix is deposited, the distal end is placed in a solution of a 5'-amino-terminated oligonucleotide. As the polymer then hydrates, the oligonucleotide reacts with the succinimidyl ester residue, thereby covalently immobilizing the oligonucleotide. The residual reactivity of the esters is capped by placing the distal end in a 1 mM ethanolamine buffer solution, pH 8.5. The process is subsequently repeated to immobilize other oligonucleotide probes. Once fabricated, the DNA sensor array is connected to a modified epifluorescence microscope (Olympus) with computer-controlled excitation and emission filter wheels and a frame-transfer charge coupled device (CCD) camera (Photometrics) as previously described herein.

Experiment 6

Initial studies were performed using a poly (dA) sensor array comprised of both an immobilized poly (dA) polymer matrix and a control acrylamide polymer matrix, which was not copolymerized with N-acryloxysuccinimide, using a poly (dT)-FITC target. The biosensor was placed directly in a dilute solution of poly (dT)-FITC. During hybridization of poly (dT)-FITC to the poly (dA) polymer matrix, poly-FITC concentrates in the polymer matrix resulting in an increase in fluorescence over the background solution fluorescence. The acrylamide matrix thus serves as a control for non-specific absorption of target. After hybridization to the poly (dT)-FITC, the poly (dA) biosensor array could be regenerated by immersing in 65°C buffer for 15 minutes after hybridization. This procedure completely dehybridized the probe/target duplex. Table E2 lists the oligonucleotide probes used in this study along with the complementary target sequences.

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Table E2:

Probe Name (code)	Sequence 5'-3'	Sequence 5'-3'	Target Name (code)
(p(dA))	H2N-$(a)_{18}$	FITC-$(T)_{18}$ Biotin-$(T)_{18}$	(p(dT)-FITC) (p(dT)-biotin)
H-ras wild type (H-ras Wt.)	H2N-CCGGCGGTGT	FITC-ACACCGCCGG	Wild type target (Wt-FITC target)
H-ras mutant (H-ras Δ)	H2N-GCCGTCGGTGT	FITC-ACACCGACGGC	Mutant target (Δ-FITC target)
		5'-labeled biotin amplicon containing ACACCGACGGC	PRC amplicon Δ PCR 109 bp

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Fig. 25 shows the fluorescence increase of the poly (dA) matrix upon repeat hybridizations to poly (dT)-FITC; and shows three repeat hybridizations after regenerating the poly (dA) sensor array with 65°C buffer. The signal increases are essentially the same for all three hybridizations when the decrease in solution fluorescence is taken into account as measured with the acrylamide matrix control. The plot demonstrates that sensor regeneration occurs in less than 10 seconds, simplifying and speeding up the testing procedure.

The poly (dA) biosensor array was then tested for its time response to various concentrations of poly (dT)-FITC. This data is shown by Fig. 26. The sharp decrease between standards represents the biosensor regeneration with 65°C buffer.

Other determinations were then made. Fig. 27 plots the initial hybridization rate versus poly (dT)-FITC concentration. The poly (dA) sensor array shows a linear response from 1.3-130 nM with an R^2 value of 0.99 demonstrating that the rate of hybridization is directly proportional to the concentration of the target oligonucleotide. This linearity indicates that the response time of the DNA sensor array is diffusion controlled; and that the oligonucleotide probes are immobilized on the surface of the polymer matrix; allowing for solution-type kinetics.

Experiment 7:

A different labeling procedure was developed to increase the array's sensitivity and expand its generality. Presently PCR samples for fluorescence analysis must be labeled after amplification. However, PCR samples can be biotinylated during the amplification which decreases the time to analysis and minimizes sample contamination. The detection procedure involves hybridization with a biotinylated nucleic acid target followed by label juncture with streptavidin-FITC.

Accordingly, calibration of the poly (dA) matrix with poly (dT)-Biotin; and hybridization was carried out for 20 minutes at 0.2, 2.0 and 19.6 nM followed by development with streptavidin-FITC. The results are shown by Fig. 28 which plots the mean fluorescence intensity of the poly (dA) matrix versus the log of poly (dT)-biotin concentration. As in a diffusion controlled system, the fluorescence intensity is shown to be linear with the log poly (dT)-biotin concentration from 0.2-20 nM. This system has a detection limit approximately an

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order of magnitude lower in target concentration for the same in-situ hybridization time. This improvement is due to the multiple FITC labels on each streptavidin molecule and the absence of photobleaching during hybridization that otherwise occurs with FITC-labeled oligonucleotides. The technique has a detection limit of 0.2 nM which is approximately an order of magnitude lower than other DNA biosensors.

Experiment 8:

In order to test if the DNA biosensor could distinguish single point mutations, an array comprised of multiple H-ras wild type probes (H-ras Wt.) and multiple mutant probes with a single base difference (H-ras Δ) was fabricated. A FITC-labeled mutant (Δ-FITC) target was hybridized to the DNA sensor array at 28°C in low stringency buffer, 2X SSPE. The Δ-FITC target is the perfect complement for the immobilized H-ras Δ. The low stringency buffer condition was insufficient to distinguish the non-complementary oligonucleotide target at 28°C.

As temperature is often utilized to distinguish non-complementary targets, thermal studies were then undertaken to determine the melt characteristics of the duplexes. The distal array end of the biosensor was immersed in buffer solution and the temperature was raised while monitoring fluorescence. Fig. 29 shows the melting curves of the DNA sensor array after hybridization at 28°C with the Δ-FITC target. The plot shows that T_m for the H-ras Wt./Δ-FITC target duplex occurs at approximately 42°C while the T_m for the perfect complement duplex H-ras/Δ-FITC target occurs at 55°C. The data shows that if hybridization is performed at approximately 54°C, only the complementary target will hybridize.

Figs. 30A and 30B respectively show fluorescence images of the DNA sensor array after hybridization with the Δ-FITC target at 28°C and 54°C, A and B respectively. The images indicate that the DNA sensor array can distinguish a point mutation when hybridization is conducted at 54°C. Images were acquired at 490 nm.

The DNA sensor array was then calibrated with the Δ-FITC target by the same procedure used for the poly (dA) sensor array and poly (dT)-FITC with the exception that hybridizations were carried out at 54°C. Fig. 31 shows hybridization data for the DNA sensor

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array to 196 nM Δ-FITC target at 54°C. The H-ras Wt. matrix showed no response to the H-ras Δ-FITC target. Fig. 32 is a plot of the Δ target calibration curve for the data of Fig. 31 which demonstrates the sensitivity of the sensor in the concentration range 20 196 nM.

Experiment 9:

In order to test the array's ability to distinguish point mutations of amplified DNA, a PCR sample containing the A target sequence was obtained. The target sample, biotinylated during PCR amplification, was first determined and diluted; allowed to hybridize to the DNA sensor array for 20 minutes at 54°C; washed and then labeled by juncture to streptavidin-FITC.

Fig. 33 shows a fluorescence image of the DNA sensor array after a 20 minute hybridization to a biotinylated Δ PCR amplification at 54°C, followed by a 5 minute streptavidin-FITC development. The image was acquired at 490 nm excitation, 530 nm emission. The image of Fig. 33 demonstrates that the biosensor can distinguish single base mutations in amplified DNA.

A fiber optic DNA sensor array has been fabricated by photodeposition of amine-reactive polymer matrices on an imaging fiber optic array; and 5'-aminoterminal oligonucleotides have been covalently immobilized as multiple fixed probes through amide bond formation with the succinimidyl ester residues of the polymer matrices. This fiber optic DNA sensor array is capable of simultaneously monitoring multiple hybridization events. The DNA sensor array also has the added advantage of simultaneously evaluating a fluid mixture of target oligonucleotides with multiple kinds of probe oligonucleotides with real-time monitoring. The DNA sensor array can be utilized to identify a single point mutation of Ras oncogene PCR product; and the sensor optically detected point mutations at DNA concentrations of 0.2 - 196 nM following a 20 minute hybridization. Lower concentrations of target oligonucleotides could be detected by hybridizing for longer times. The biosensor's small size (350 μm o.d.) and the small volume of the individual array elements (20 pL) enable sub-microliter sample volumes to be analyzed, increasing the value of the sensor.

The present invention is not to be restricted in form nor limited in scope except by the claims appended hereto.

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What we claim is:

1. An optical biosensor for selectively detecting an oligonucleotide specie in a fluid sample, said biosensor comprising:

a clad optical fiber strand of determinable configuration and dimensions which presents two strand end faces as discrete optic surfaces for introduction and conveyance of light energy;

an oligonucleotide in-situ hybridization zone comprising one specie of single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber strand, said deposit of single stranded oligonucleotide within said hybridization zone serving as a deployed, single-specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probe at said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said strand end face surface.

2. An optical biosensor for selectively detecting at least one oligonucleotide species in a fluid sample, said biosensor comprising:

a bundled array comprising a plurality of individually clad, optical fiber strands disposed co-axially along their lengths and having two discrete array ends each of which is formed of multiple strand end faces, said bundled array being of determinable configuration and dimensions and said two discrete array ends presenting two optic array surfaces for introduction and conveyance of light energy; and

at least one oligonucleotide in-situ hybridization zone each comprising one specie of single-stranded oligonucleotide disposed as individual specie deposits in aligned organization

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upon a single strand end face at differing spatial positions on one of said optic array end surfaces of said bundled array, the differing spatial positionings for each deposit of single-stranded oligonucleotide specie in aligned organization within said hybridization zone serving as deployed, single-specie, multiple fixed probes suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie.

(a) wherein such complementary oligonucleotide target specie as is hybridized bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said multiple fixed probes at said differing spatial positions within said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said differing spatial positions.

3. An optical biosensor for selectively detecting a plurality of oligonucleotide species concurrently in a fluid sample containing a mixture of different oligonucleotide species, said biosensor comprising:

a preformed, unitary fiber optic array comprising a plurality of individually clad, optical fiber strands disposed co-axially along their lengths and having two discrete optic array ends each of which is formed of multiple strand end faces, said preformed unitary fiber optic array being of determinable configuration and dimensions and said two discrete optic array ends presenting two discrete optic array surfaces for introduction and conveyance of light energy; and

at least one oligonucleotide in-situ hybridization zone comprising a plurality of single stranded oligonucleotide species disposed as individual specie deposits in aligned organization upon multiple strand end faces at differing spatial positions on one of said discrete optic fiber array surfaces of said unitary fiber optic array, the differing spatial positionings for each deposit of single stranded oligonucleotide specie in aligned organization within said in-situ hybridization zone serving as a collective of deployed, specie specific, fixed probes suitable for selective in-situ hybridization on-demand with its mobile

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complementary oligonucleotide target specie in a fluid mixture containing alternative mobile complementary target species,

(a) wherein such complementary oligonucleotide target species as are hybridized in-situ each ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction products formed by a complementary oligonucleotide target specie with one collective of multiple fixed probes deployed at said differing spatial positions within said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at each of said differing spatial positions, each of the alternative in-situ hybridized oligonucleotide reaction products formed being concurrently optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at each of said differing spatial positions within said hybridization zone.

4. The optical biosensor as recited in claim 1, 2, or 3 further comprising

means for placing said hybridization zone of said biosensor into reactive contact with a fluid sample;

means for introducing light energy to a discrete optic surface of said biosensor such that said biosensor conveys said introduced light and illuminates at least a portion of said in-situ hybridization zone; and

means for detecting light energy emerging from said illuminated in-situ hybridization zone of said biosensor, said detected emerging light energy serving as an optical determination of at least one complementary oligonucleotide target specie in the fluid sample.

5. An optical method for selectively detecting at least one oligonucleotide specie in a fluid sample containing a mixture of oligonucleotide species, said optical method comprising the steps of:

obtaining a biosensor comprised of a clad optical fiber strand of determinable configuration and dimensions which presents two strand end faces as discrete optic surfaces

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for introduction and conveyance of light energy;

providing an oligonucleotide in-situ hybridization zone comprising one specie of single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber strand, said deposit of single stranded oligonucleotide within said hybridization zone serving as a deployed, single-specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probe at said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said strand end face surface.

placing said in-situ hybridization zone of said biosensor into reactive contact with a fluid sample containing a mixture of mobile oligonucleotide species;

permitting hybridization to occur in-situ between said fixed probe of said biosensor and such complementary oligonucleotide target species as are present in the fluid sample;

joining an identifying label to the complementary oligonucleotide target specie hybridized in-situ;

introducing light energy to a discrete optic array surface of said biosensor such that said individually clad fiber optical strand conveys said introduced light energy and illuminates said fixed probe within said in-situ hybridization zone of said biosensor; and

detecting light energy emerging from said illuminated fixed probe within said in-situ hybridization zone of said biosensor, said detected emerging light serving as an optical detection of a specific complementary oligonucleotide target specie in the fluid sample.

6. An optical method for selectively detecting at least one oligonucleotide specie in a fluid sample containing a mixture of oligonucleotide species, said optical method comprising the steps of:

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obtaining a biosensor comprised of a bundle of clad optical fiber strands of determinable configuration and dimensions which presents a bundle of two strand end faces as discrete optic surfaces for introduction and conveyance of light energy;

providing an oligonucleotide in-situ hybridization zone comprising one specie of single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber strand bundle array, said deposit of single stranded oligonucleotide within said hybridization zone serving as a deployed, single-specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probes at said hybridization zones is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said strand end face surfaces;

placing said fixed probe within said in-situ hybridization zone of said biosensor into reactive contact with a fluid sample containing a mixture of mobile oligonucleotide species each bearing a joined identifying label;

permitting hybridization to occur in-situ between said fixed probe of said biosensor and such complementary oligonucleotide target species bearing a joined identifying label as are present in the fluid sample;

introducing light energy to one of said discrete optic array surface of said biosensor such that said individually clad fiber optical strand conveys said introduced light energy and illuminates said fixed probe within said in-situ hybridization zone of said biosensor; and

detecting light energy emerging from said illuminated fixed probe within said in-situ hybridization zone of said sensor, said detected emerging light serving as an optical detection of a specific complementary oligonucleotide target specie in the fluid sample.

7. An optical method for selectively detecting at least one oligonucleotide specie in a fluid

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sample containing a mixture of oligonucleotide species, said optical method comprising the steps of:

obtaining a biosensor comprised of

an integrated optic array comprising a plurality of individually clad, optical fiber strands disposed co-axially along their lengths and having two discrete optic array ends each of which is formed of multiple strand end faces, said integrated optic array being of determinable configuration and dimensions and said two discrete array ends presenting two optic array surfaces for introduction and conveyance of light energy, and

at least one oligonucleotide in-situ hybridization zone comprising not less than one specie of single-stranded oligonucleotide disposed as individual specie deposits in aligned organization upon multiple strand end faces at differing spatial positions on one of said discrete optic array surfaces of said integrated optic array, the differing spatial positionings for each deposit of single stranded oligonucleotide specie in aligned organization within said hybridization zone serving as deployed, single specie, multiple fixed probes suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probes deployed at said differing spatial positions within said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said differing spatial positions;

placing said in-situ hybridization zone of said biosensor into reactive contact with a fluid sample containing a mixture of mobile oligonucleotide species;

permitting hybridization to occur in-situ between said multiple fixed probes of said biosensor and such complementary oligonucleotide target species as are present in the fluid sample;

joining an identifying label to the complementary oligonucleotide target specie

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hybridized in-situ;

introducing light energy to a discrete optic array surface of said biosensor such that a plurality of individually clad fiber optical strands convey said introduced light energy concurrently and illuminate at least a portion said multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said biosensor; and

detecting light energy emerging from said illuminated multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said biosensor, said detected emerging light energy serving as an optical detection of at least one specific complementary oligonucleotide target specie in the fluid sample.

8. An optical method for selectively detecting at least one oligonucleotide specie in a fluid sample containing a mixture of oligonucleotide species, said optical method comprising steps of:

obtaining a biosensor comprised of

an integrated optic array comprising a plurality of individually clad, optical fiber strands disposed co-axially along their lengths and having two discrete optic array ends each of which is formed of multiple strand end faces, said integrated optic array being of determinable configuration and dimensions and said two discrete array ends presenting two optic array surfaces for introduction and conveyance of light energy, and

at least one oligonucleotide in-situ hybridization zone comprising one single stranded oligonucleotide species disposed as individual specie deposits in aligned organization at differing spatial positions on one of said discrete optic fiber array surfaces of said integrated optic array, the differing spatial positionings for each deposit of single stranded oligonucleotide specie in aligned organization within said in-situ hybridization zone serving as deployed, single strand, multiple fixed probes suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, species selective, in-situ hybridized oligonucleotide reaction

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product formed by a complementary oligonucleotide target specie with said fixed probes deployed at said differing spatial positions within the hybridization zone of said biosensor is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said differing spatial positions;

placing said multiple fixed probes within said in-situ hybridization zone of said biosensor into reactive contact with a fluid sample containing a mixture of mobile oligonucleotide species each bearing a joined identifying label;

permitting hybridization to occur in-situ between said multiple fixed probes of said biosensor and such complementary oligonucleotide target species bearing a joined identifying label as are present in the fluid sample;

introducing light energy to one of said discrete optic array surfaces of said biosensor such that a plurality of individually clad fiber optical strands convey said introduced light energy concurrently and illuminate at least a portion of said multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said biosensor; and

detecting light energy emerging from said illuminated multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said sensor, said detected emerging light energy serving as an optical detection of at least one complementary oligonucleotide target specie in the fluid sample.

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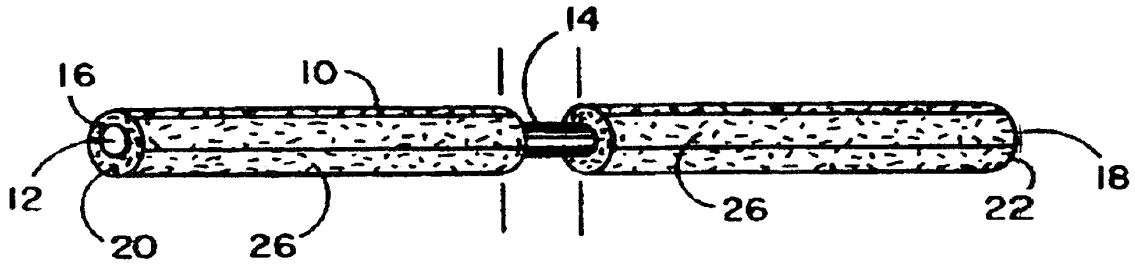


FIG. 1

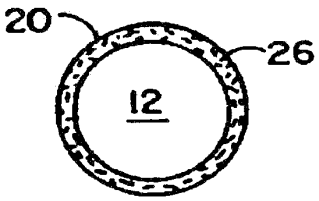


FIG. 2A

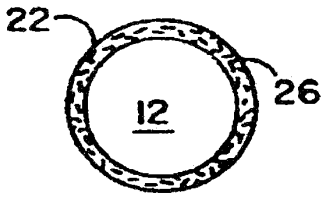


FIG. 2B

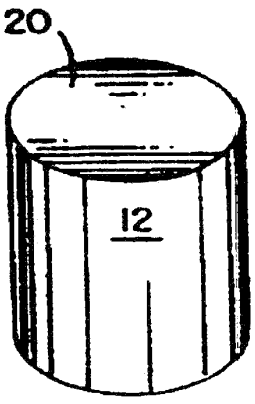


FIG. 3A

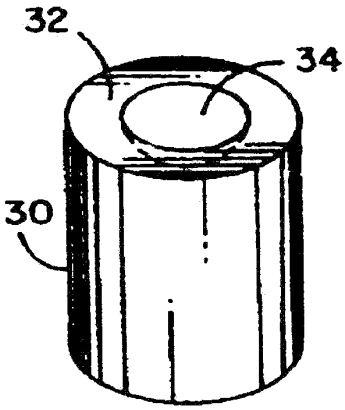


FIG. 3B

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FIG. 4

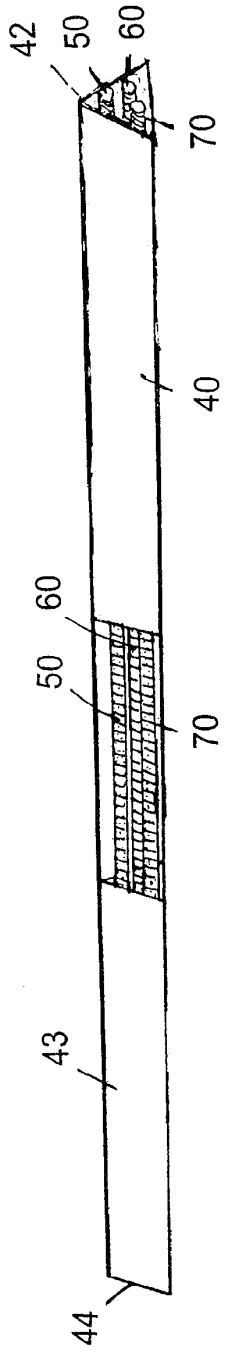


FIG. 5B

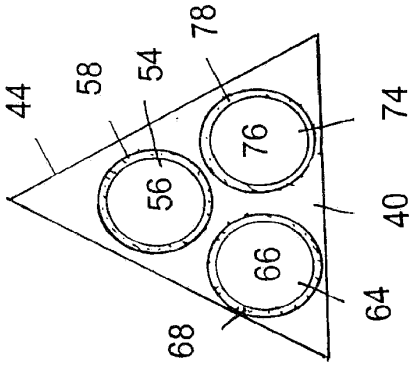
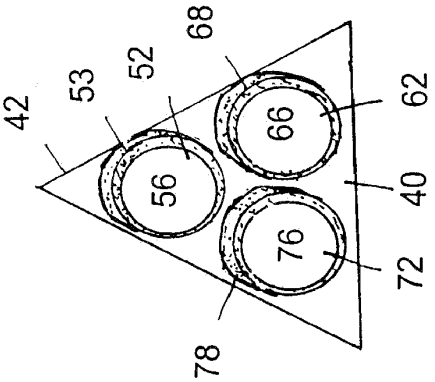


FIG. 5A



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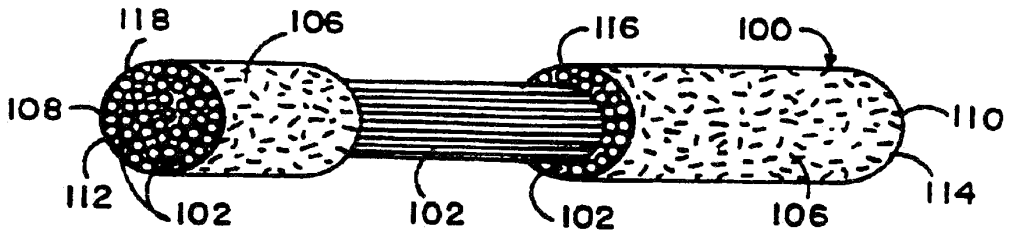


FIG. 6

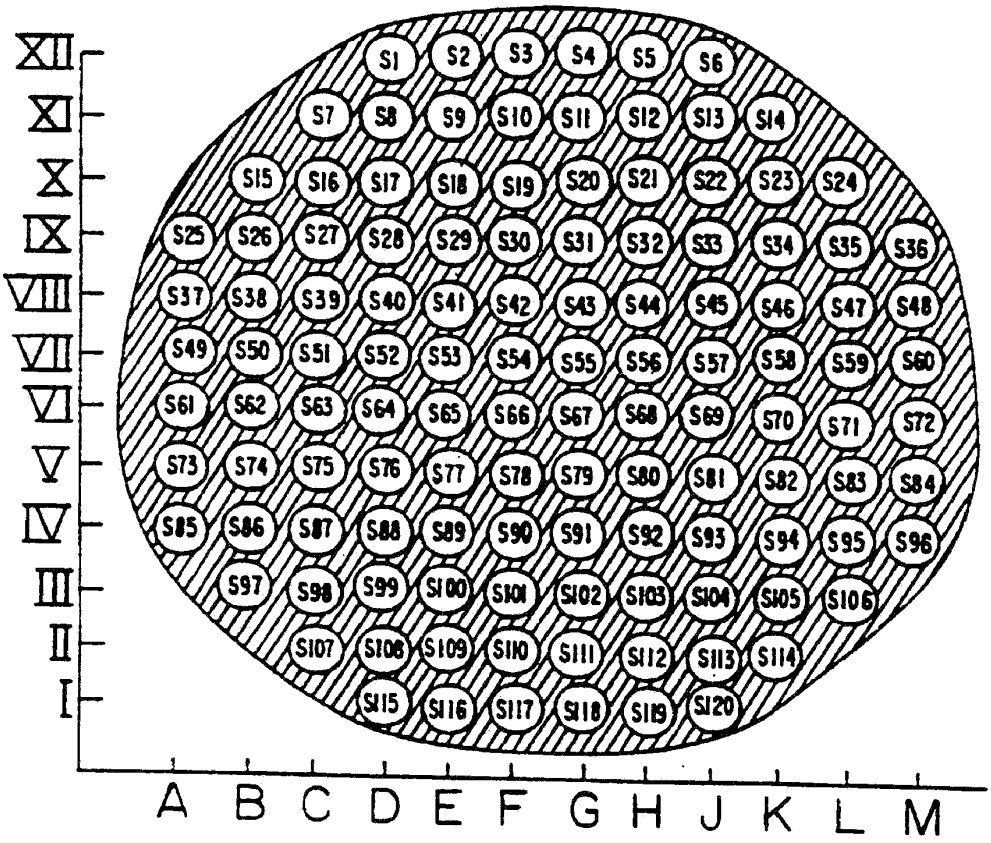


FIG. 7

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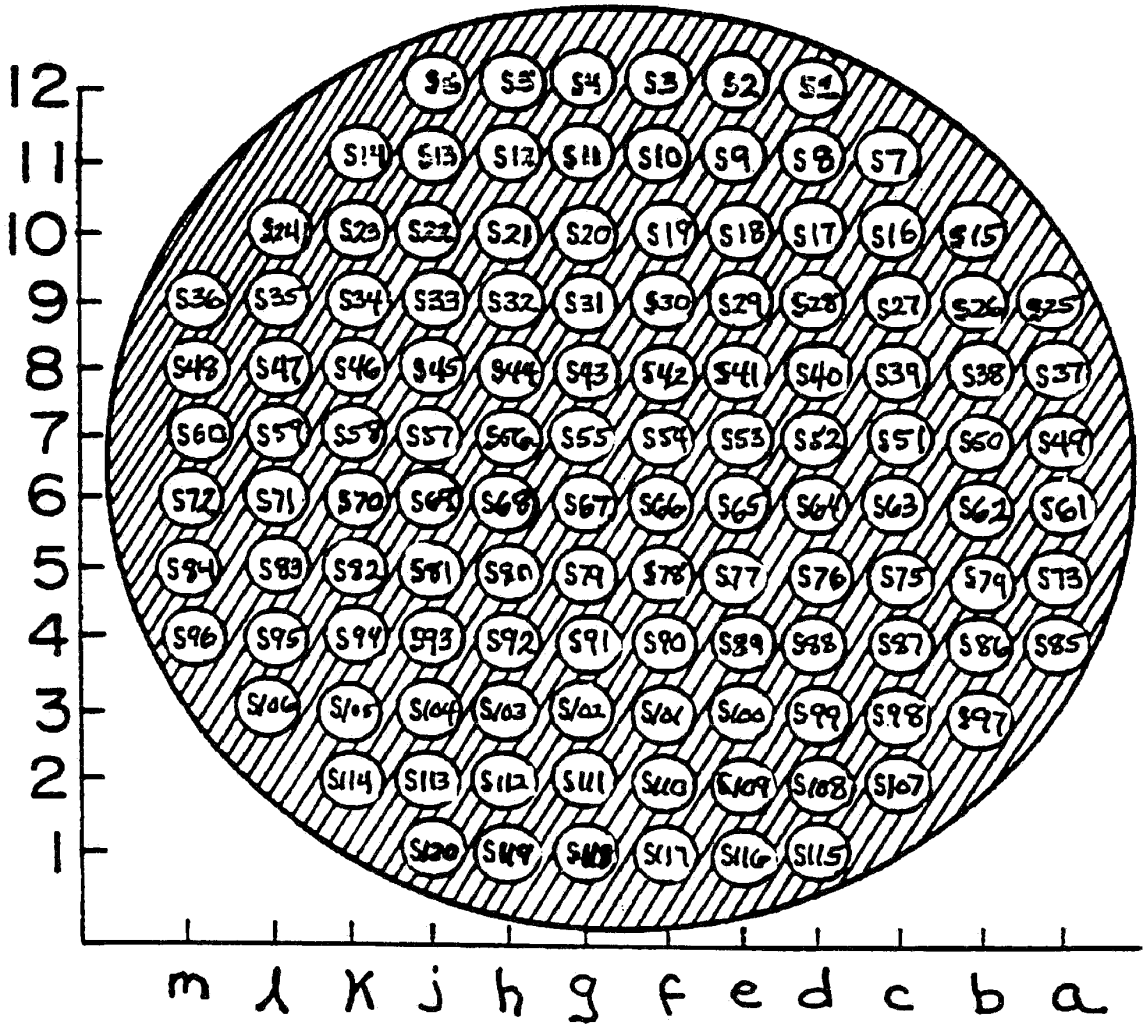


FIG. 8

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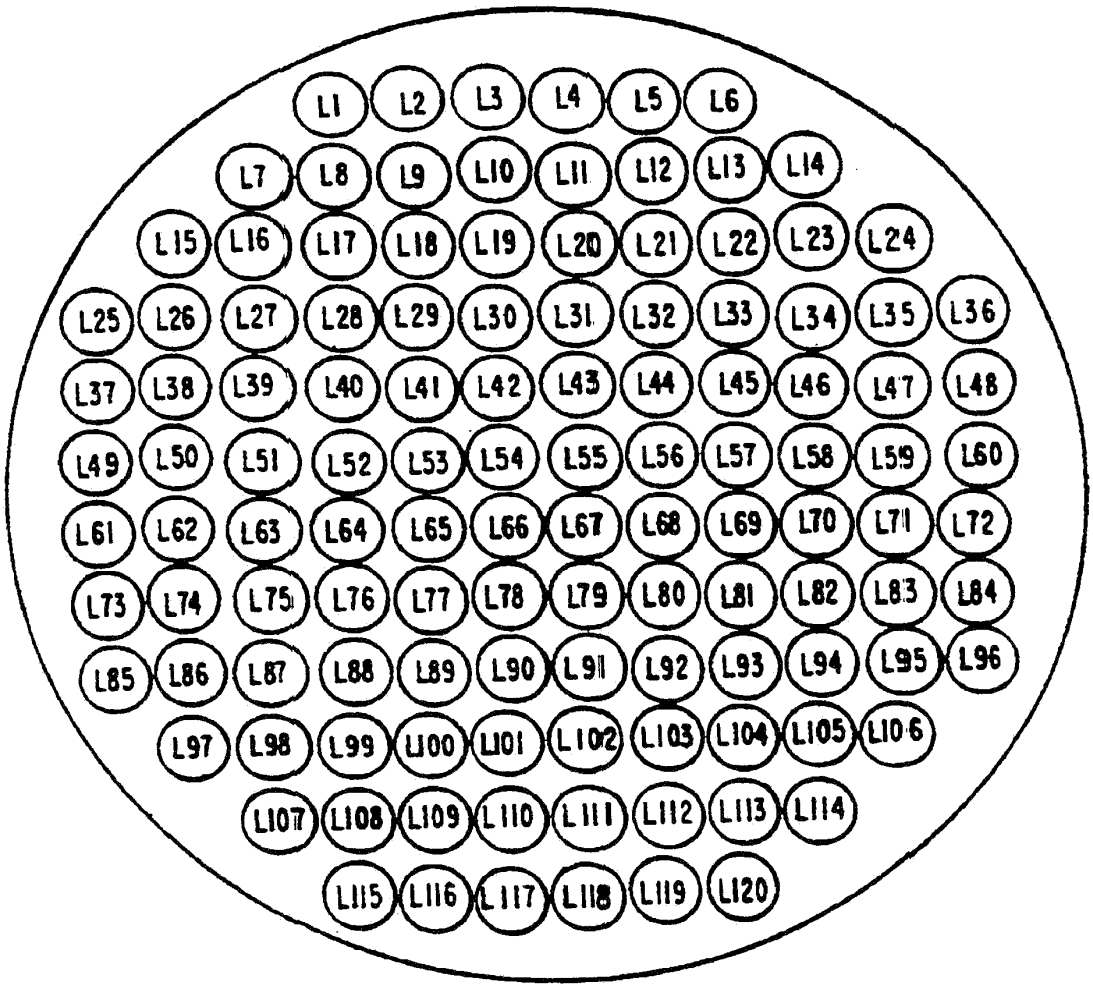


FIG. 9

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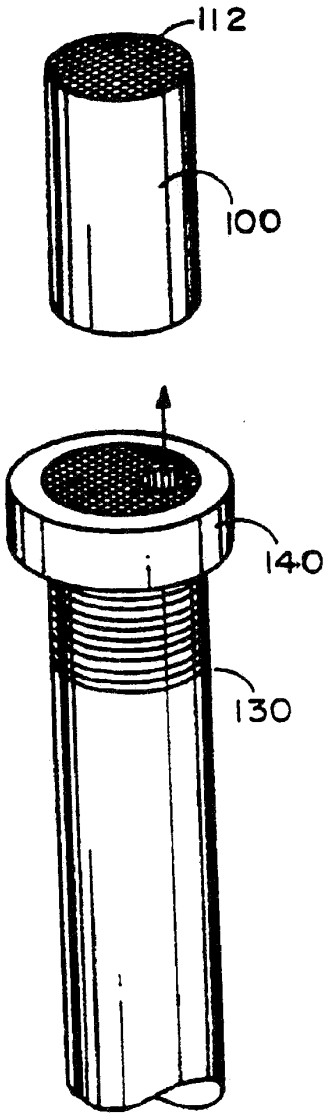


FIG. 10

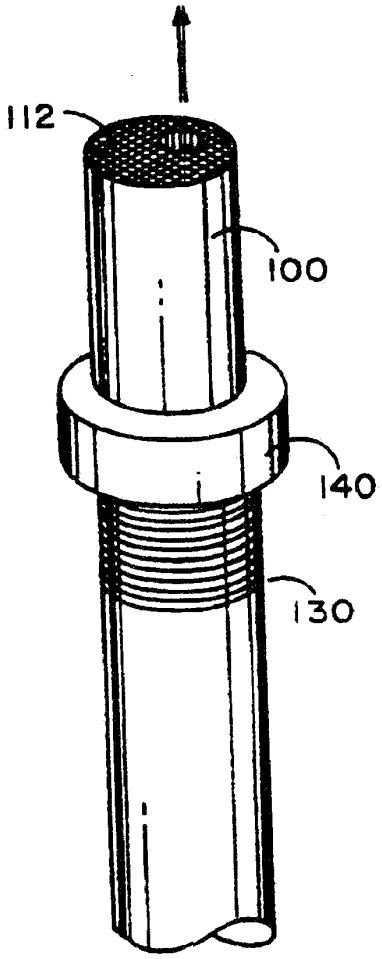


FIG. 11

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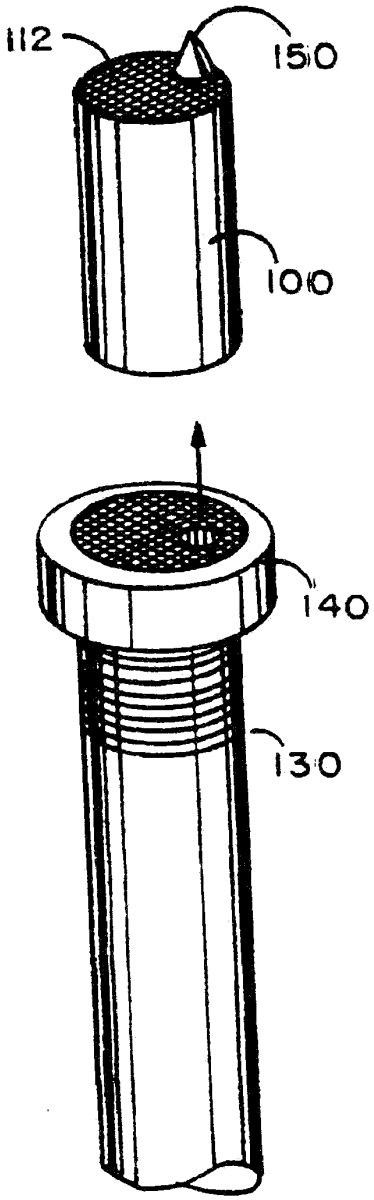


FIG. 12

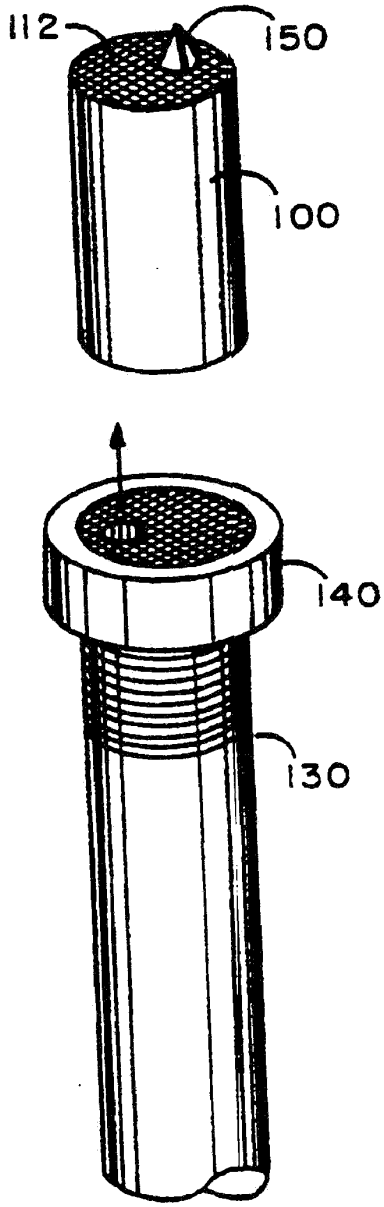


FIG. 13

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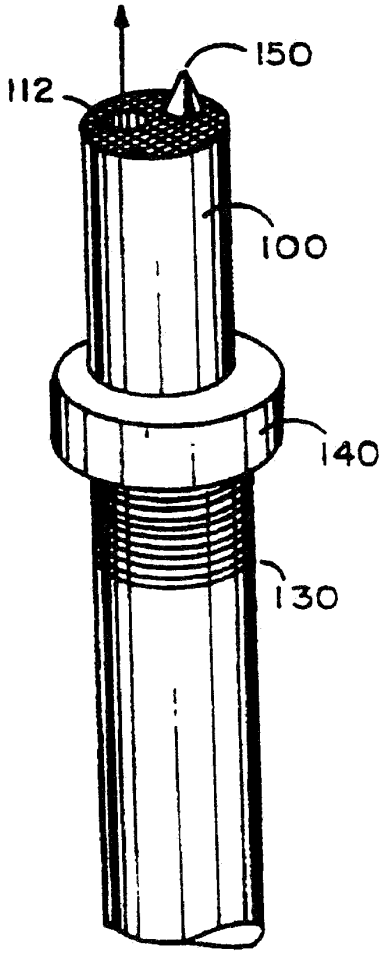


FIG. 14

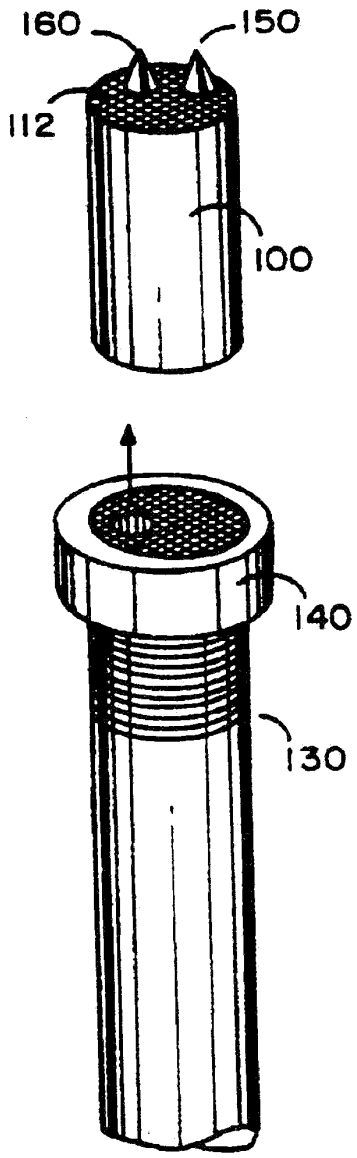


FIG. 15

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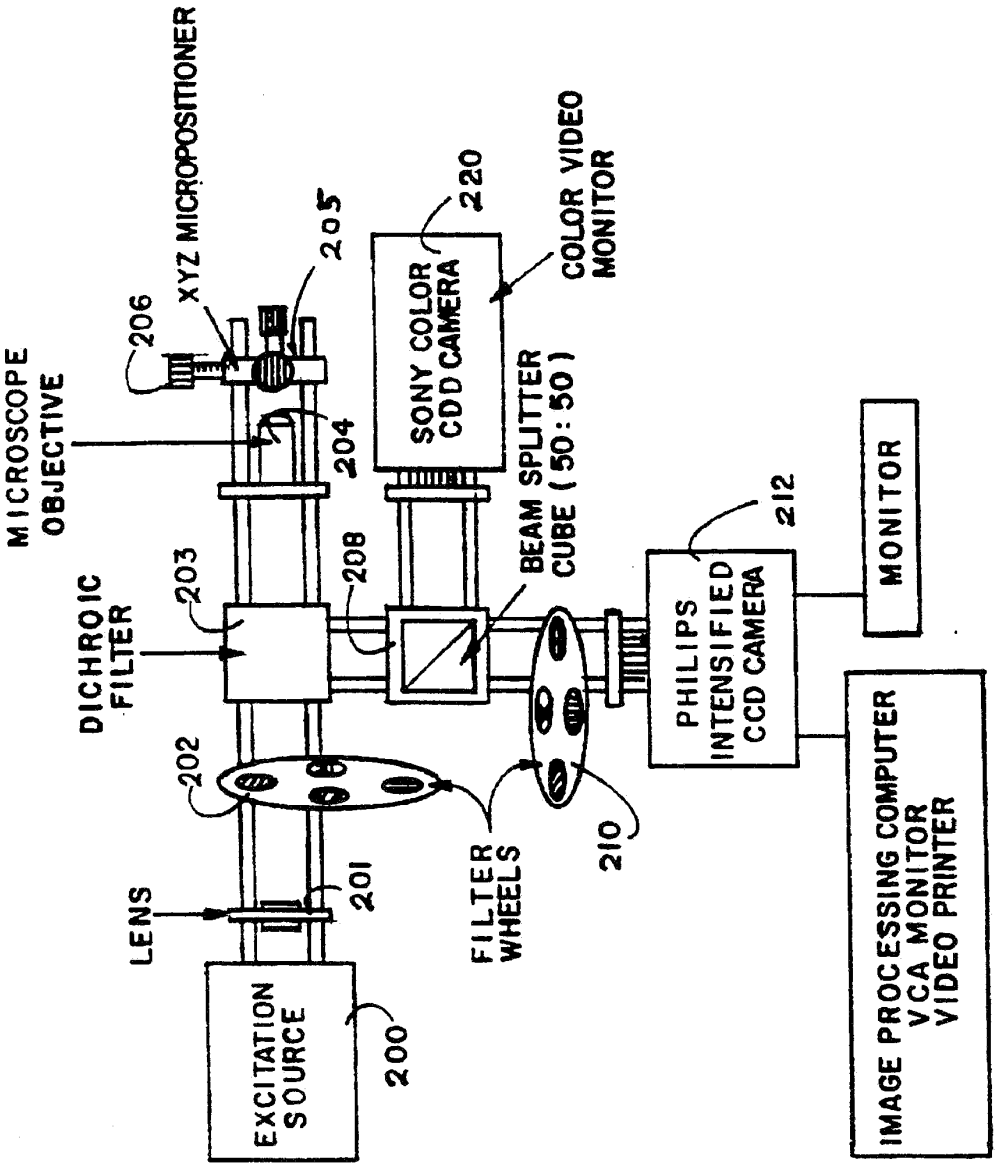


FIG. 16

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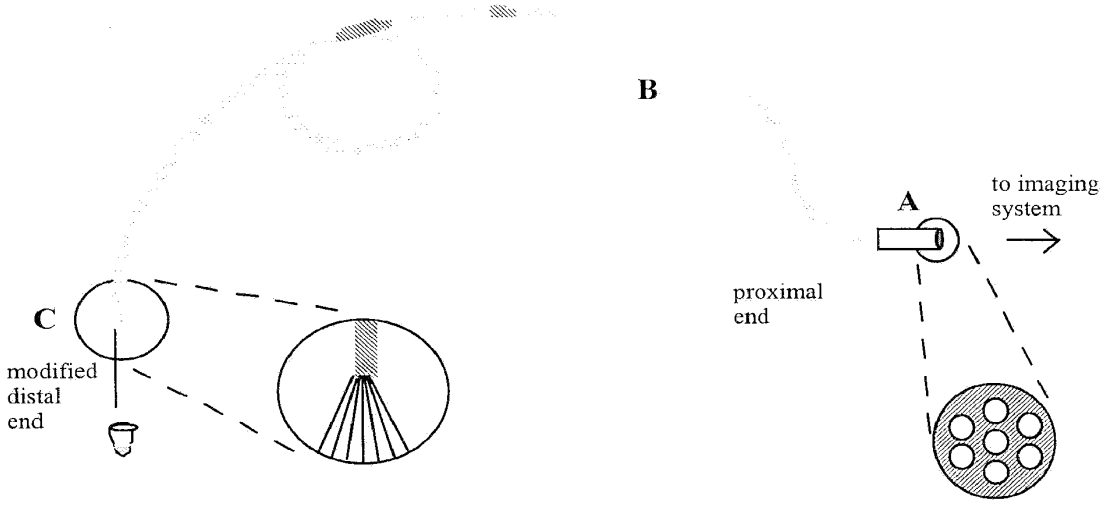


FIG. 17

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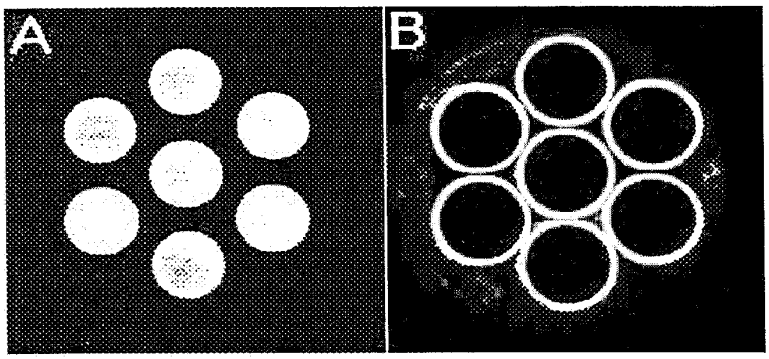


FIG. 18

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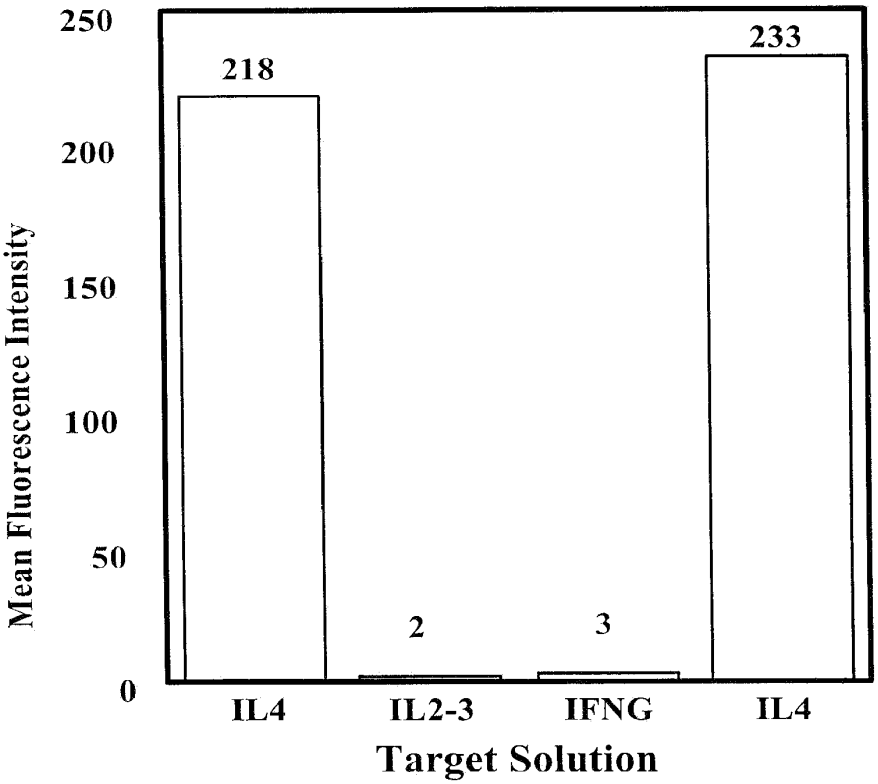


FIG. 19

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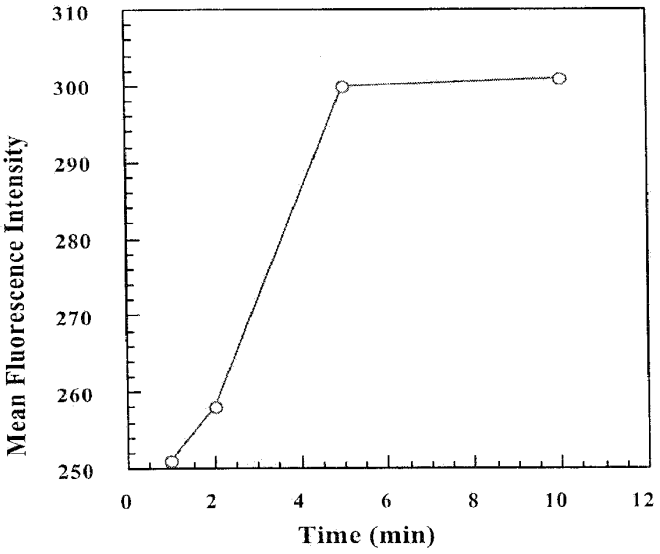


FIG. 20

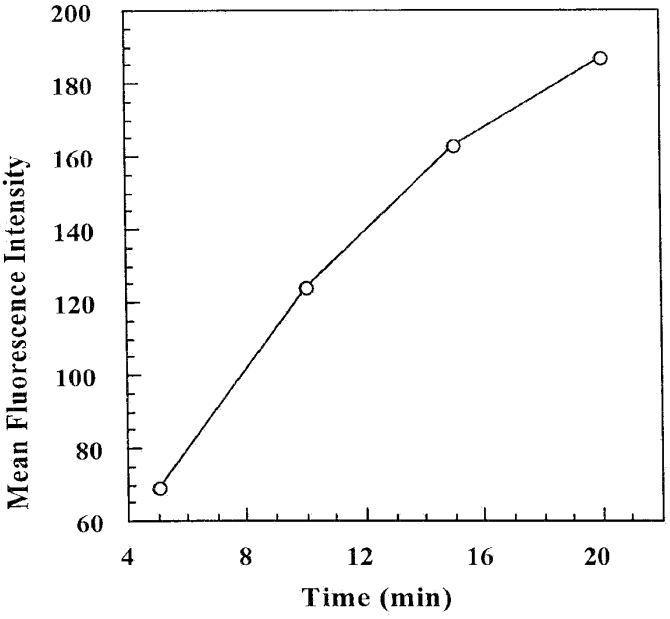


FIG. 21

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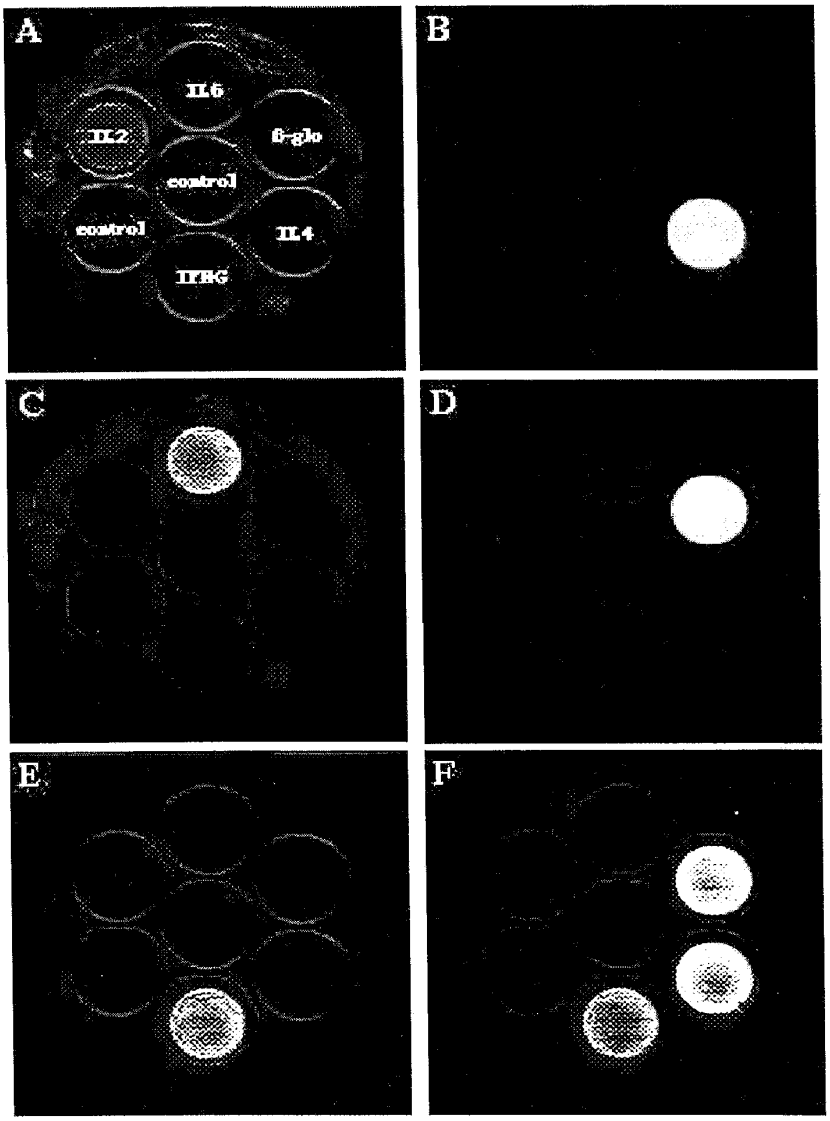


FIG. 22

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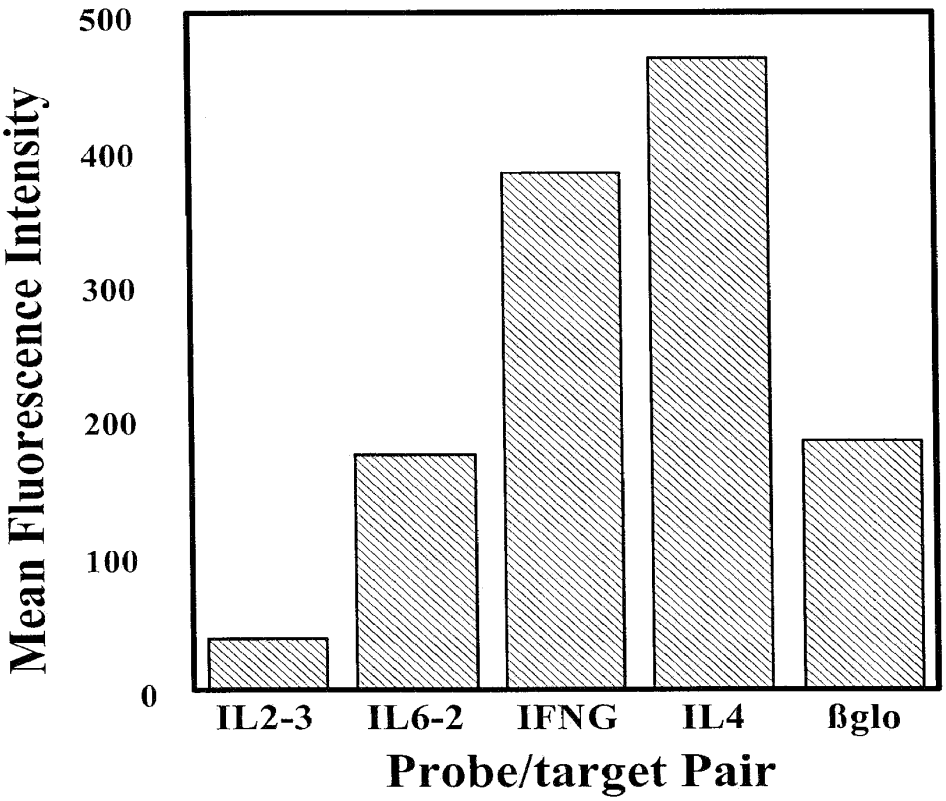


FIG. 23

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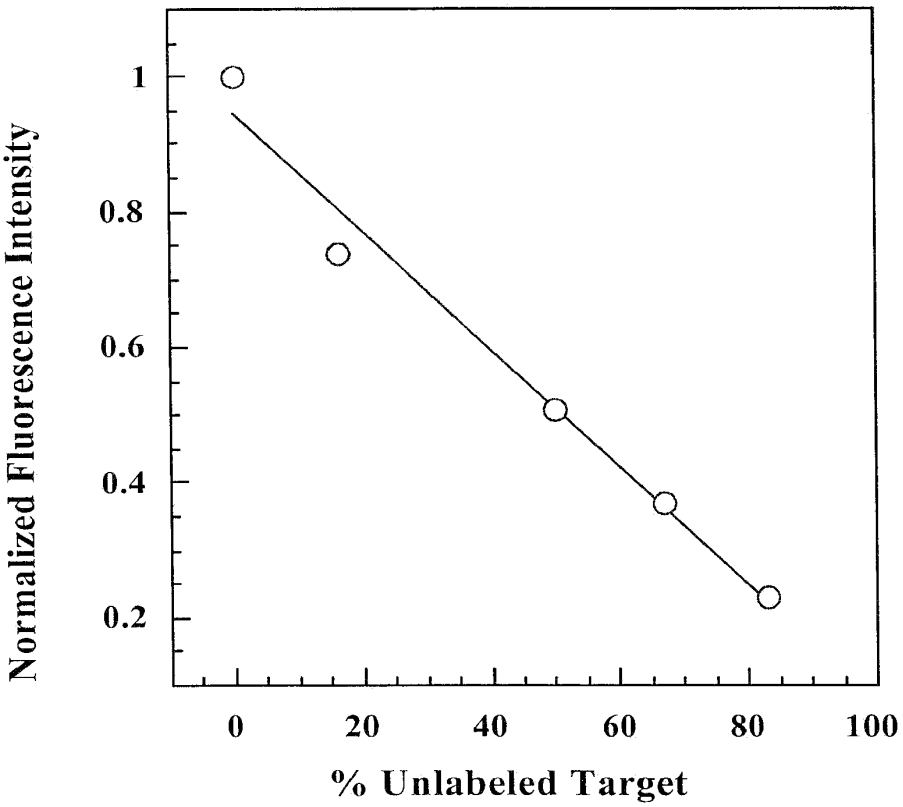


FIG. 24

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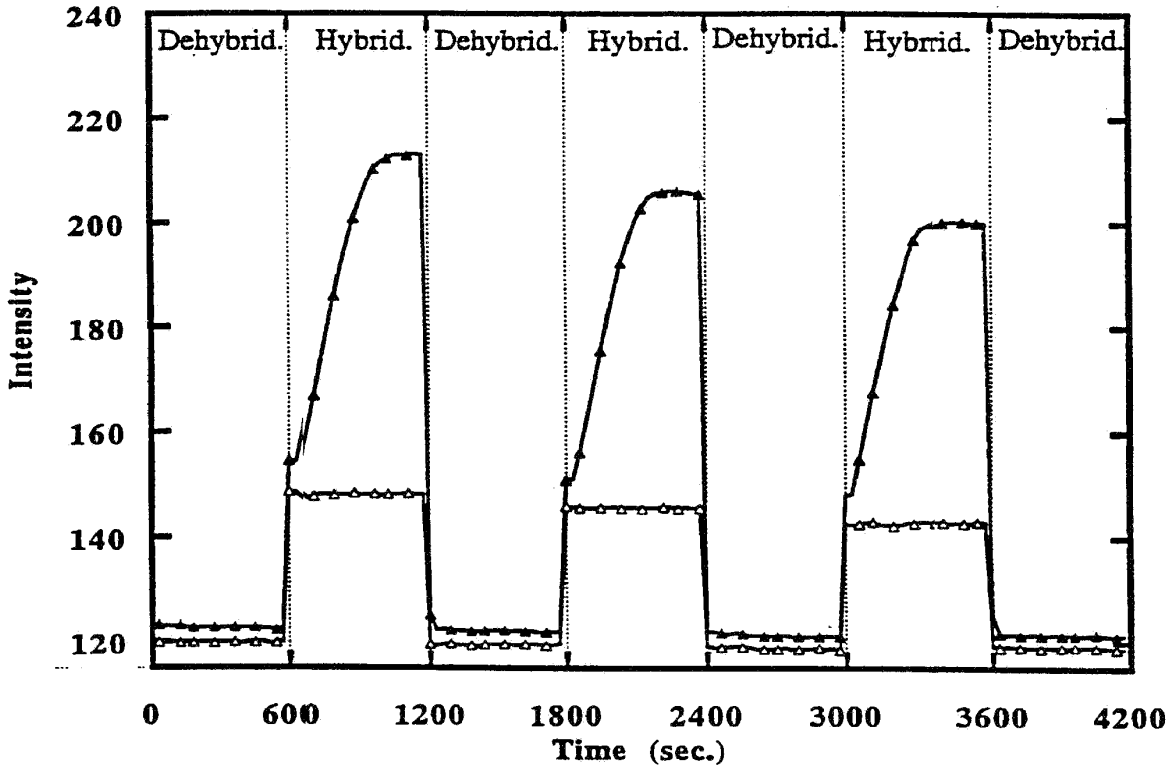


FIG. 25

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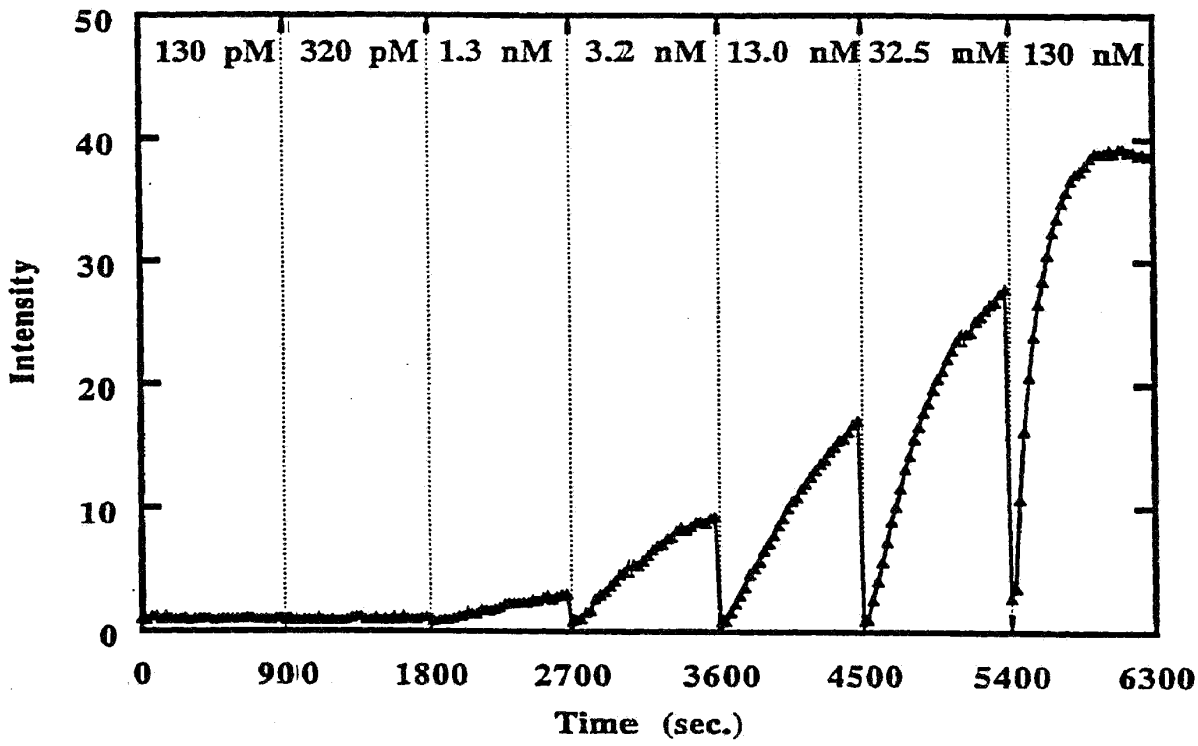


FIG. 26

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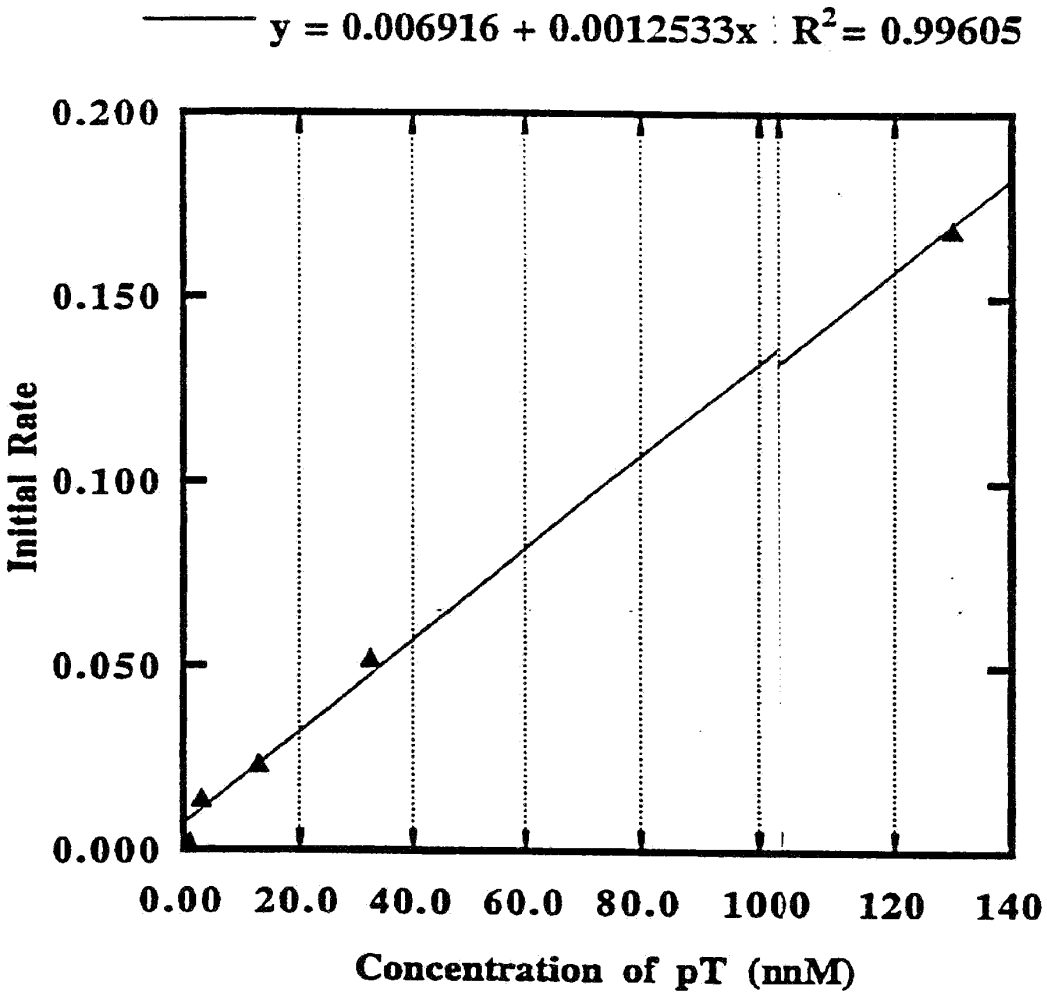


FIG. 27

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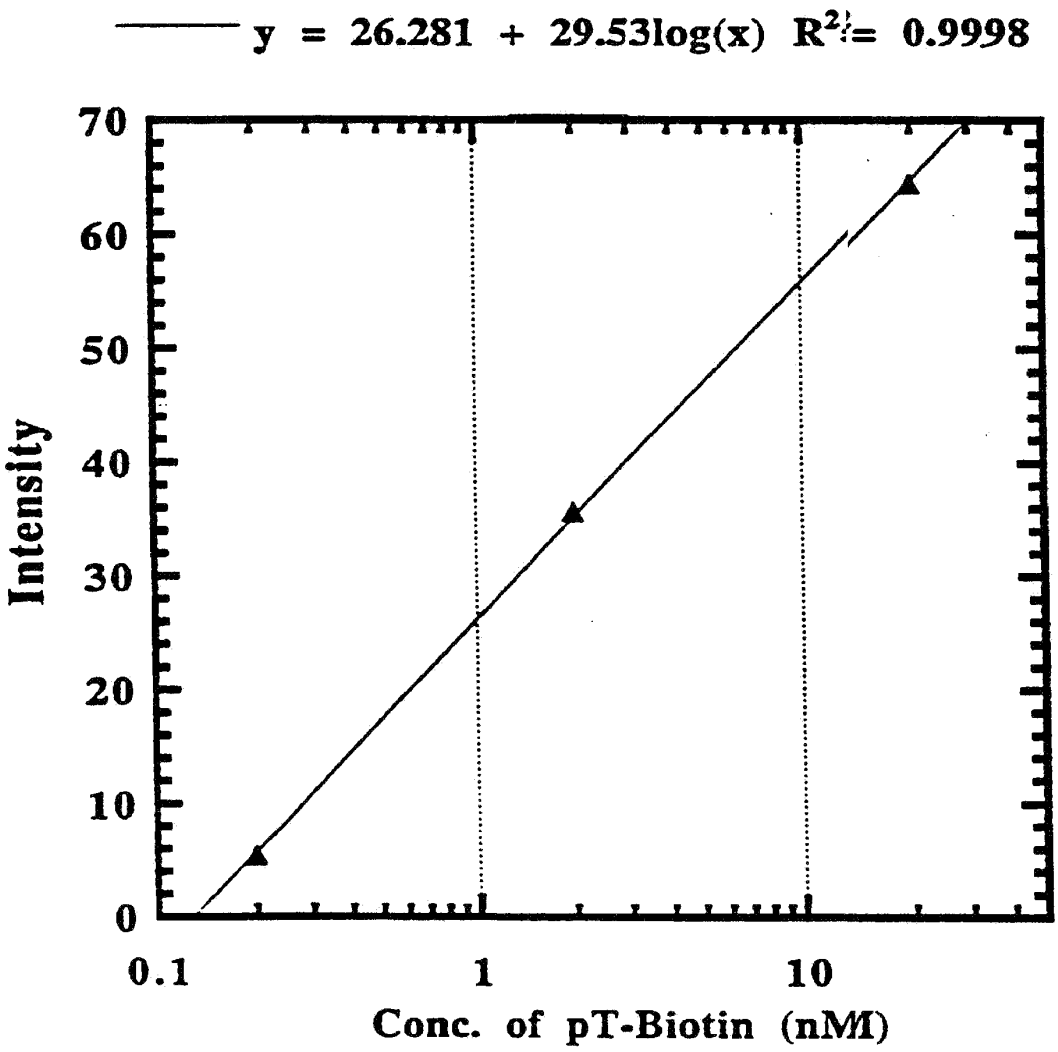


FIG. 28

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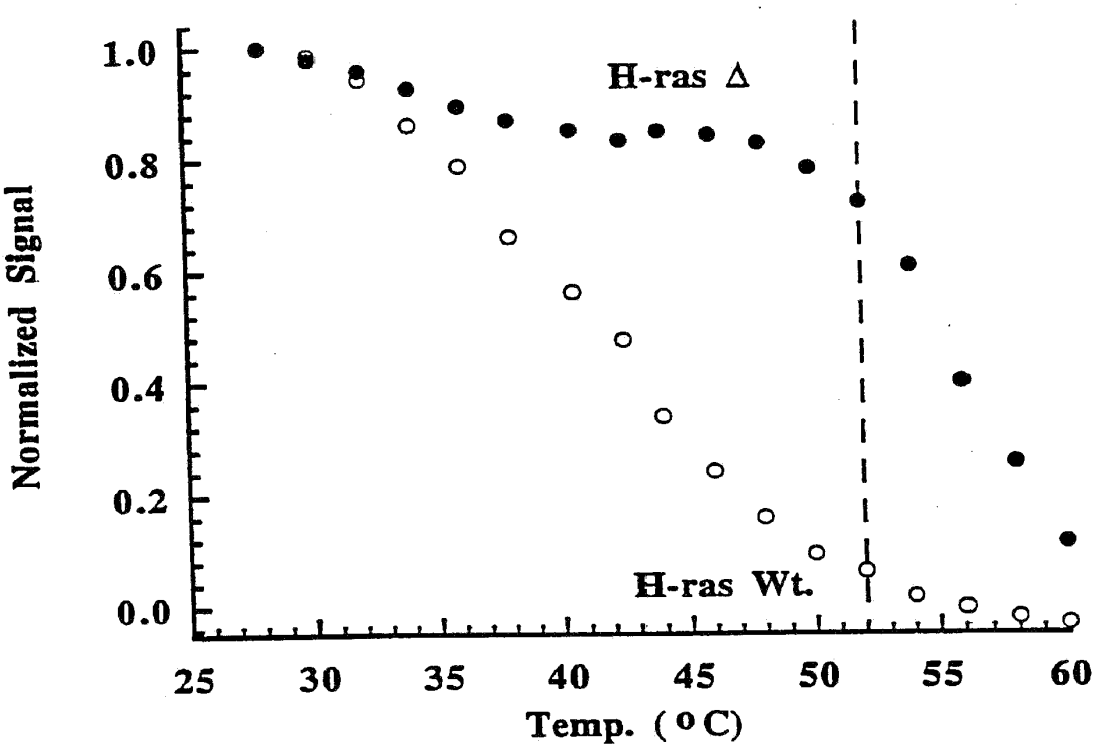
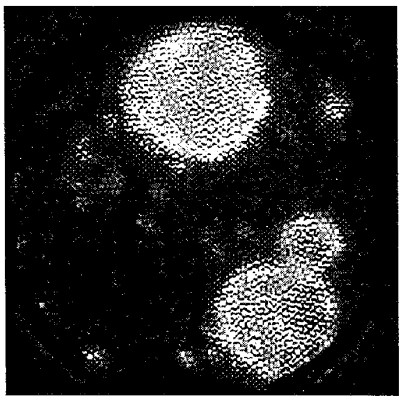


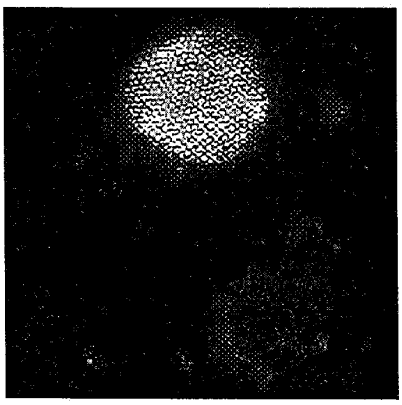
FIG. 29

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28° C

FIG. 30A



54° C

FIG. 30B

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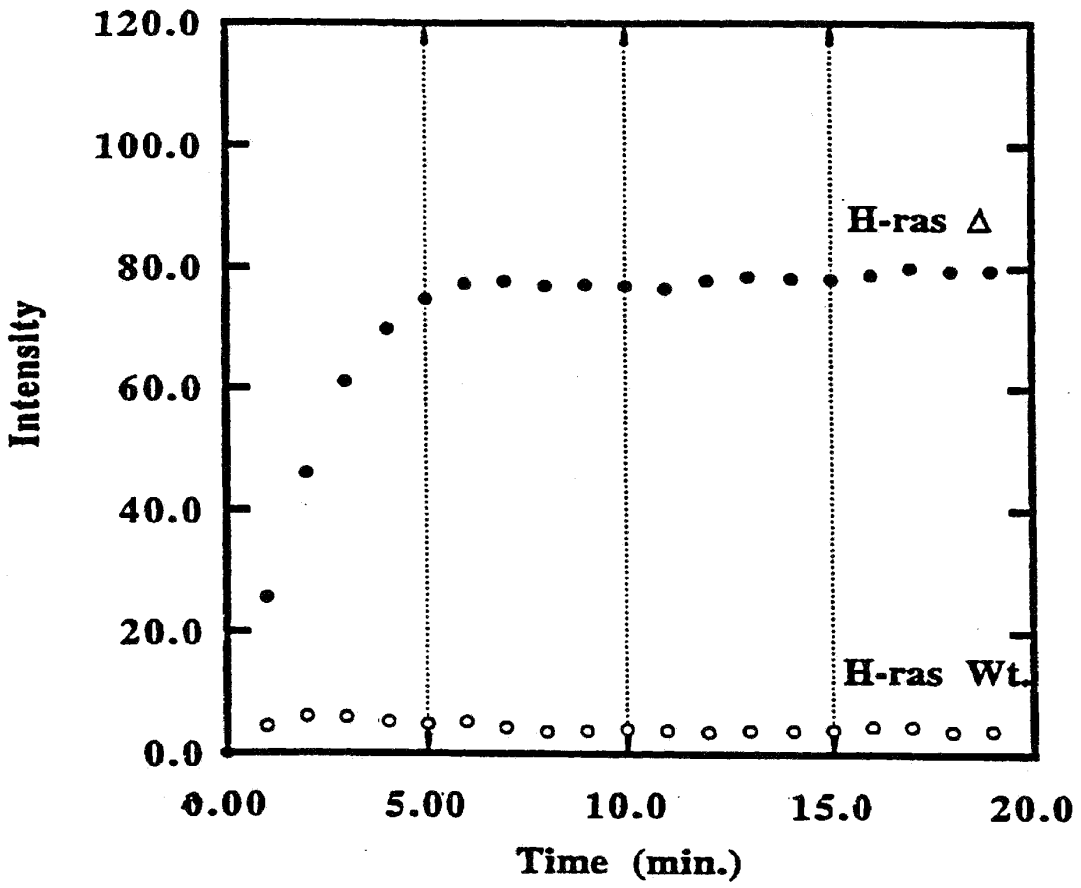


FIG. 31

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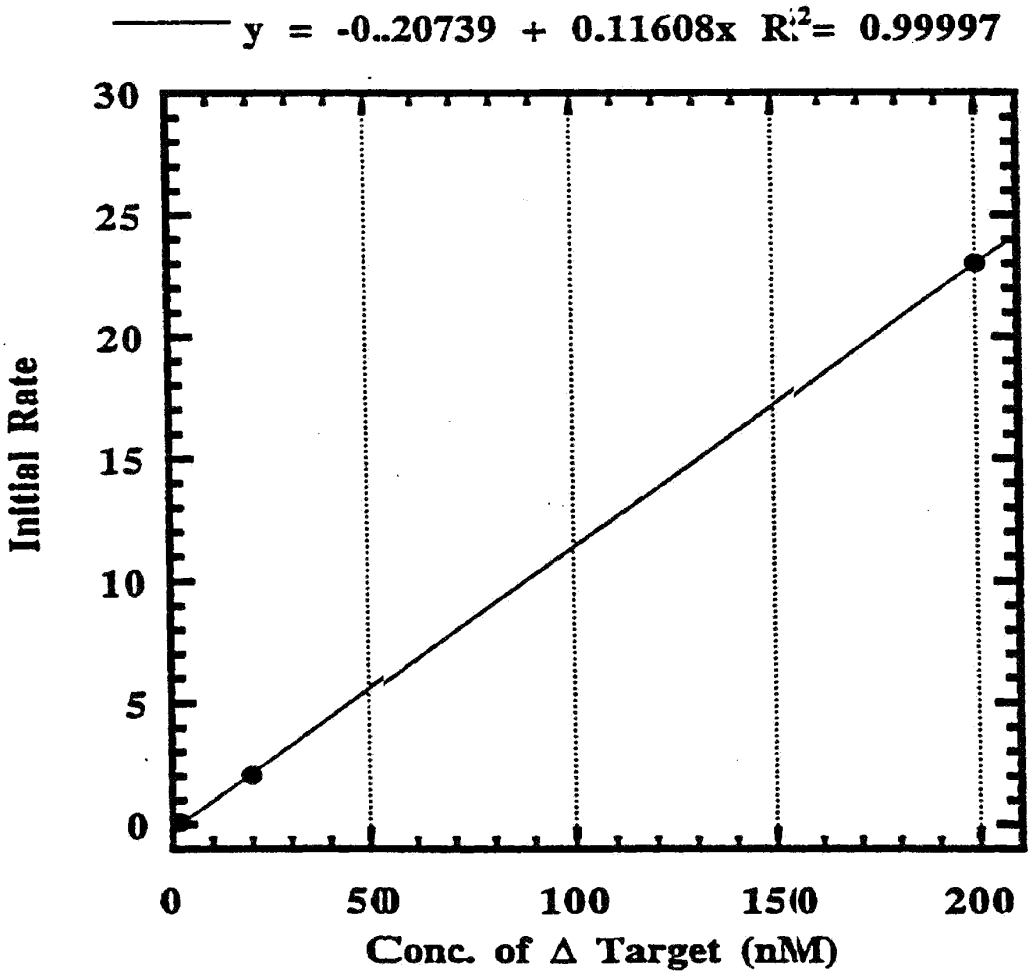


FIG. 32

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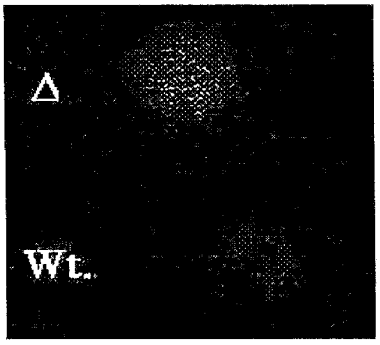


FIG. 33

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(71) Applicant *(for all designated States except US)*: TRUSTEES OF TUFTS COLLEGE [US/US]; Tufts University, Ballou Hall, Medford, MA 02155 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WALT, David, R. [US/US]; 4 Candlewick Close, Lexington, MA 02178 (US). TAYLOR, Laura [US/US]; 15A Bradbury Avenue, Medford, MA 02155 (US).

(74) Agent: CREEHAN, R., Dennis; P.O. Box 750070, Arlington Heights, MA 02175-0070 (US).

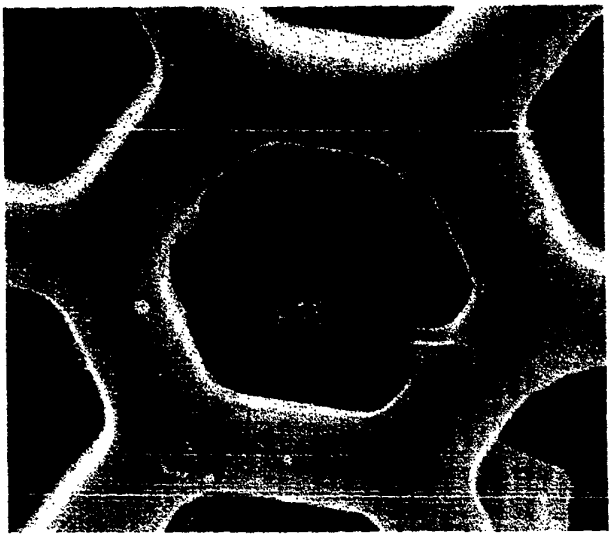
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
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(54) Title: BIOSENSOR ARRAY COMPRISING CELL POPULATIONS CONFINED TO MICROCAVITIES

(57) Abstract

A biosensor, sensor array, sensing method and sensing apparatus are provided in which individual cells or randomly mixed populations of cells, having unique response characteristics to chemical and biological materials, are deployed in a plurality of discrete sites in a substrate. In a preferred embodiment, the discrete sites comprise microwells formed at the distal end of individual fibers within a fiber optic array. The biosensor array utilizes an optically interrogatable encoding scheme for determining the identity and location of each type in the array and provides for simultaneous measurements of large numbers of individual cell responses to target analytes. The sensing method utilizes the unique ability of cell populations to respond to biologically significant compounds in a characteristic and detectable manner. The biosensor array and measurement method may be employed in the study of biologically active materials, in situ environmental monitoring, monitoring of a variety of bioprocesses, and for high throughput screening of large combinatorial chemical libraries.



Scanning Electron Micrograph (SEM) of a single NIH 3T3 mouse fibroblast cell in an etched microwell. Each well has a diameter of 7μm, a depth of 5μm, and a volume of ~145fL.

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BIOSENSOR ARRAY COMPRISING CELL POPULATIONS CONFINED TO MICROCAVITIES

FIELD OF THE INVENTION

The present invention is generally concerned with biosensors, biosensor arrays, and sensing apparatus, and sensing methods for the analysis of chemical and biological materials. More particularly, the invention is directed to biosensors, biosensor arrays, sensing apparatus and sensing methods which employ cells and mixed populations of cells for analysis of chemical and biological materials.

BACKGROUND OF THE INVENTION

It is generally recognized that important technical advances in chemistry, biology and medicine benefit from the ability to perform microanalysis of samples in minute quantities. However, making analytical measurements on minute quantities has long been a challenge due to difficulties encountered with small volume sample handling, isolation of analytes, and micro-analysis of single-cell physiology.

Nanoliter, picoliter, and femtoliter volume studies have been explored in a range of applications involving in vitro and in vivo cellular investigations [R.M. Wightman, et al., *Proc. Natl. Acad. Sci. U.S.A.* 88:10754(1991); R.H.Chow, et al. *Nature* 356:60(1992); T.K. Chen, et al. *Anal. Chem.* 66:3031(1994); S.E. Zerby, et al., *Neurochem.* 66:651(1996); P.A. Garis, et al. *J.Neurosci.* 14:6084(1994); G.Chen, et al., *J.Neurosci.* 15:7747(1995)], electrochemistry [R.A. Clark, et al., *Anal. Chem.* 69(2):259(1997)], matrix-assisted laser desorption- ionization mass spectrometry [S. Jespersen, et al., *Rapid Commun. Mass Spectrom.* 8:581(1994)], micro-column liquid chromatography [I.A.Holland, et al., *Anal.Chem.* 67:3275(1995); M.D. Oates, et al., *Anal. Chem.* 62:1573(1990)], micro-titration [M. Gratzl, et al *Anal.Chem.* 65:2085(1993); C.Yi, et al., *Anal.Chem.* 66:1976(1994)], and capillary electrophoresis [M.Jansson, et al., *J.Chromatogr.* 626:310(1992); P.Beyer Hietpas, et al. *J.Liq.Chromatogr.* 18:3557(1995)].

Clark, et al. [*Anal.Chem.* 69(2):259(1997)] has disclosed a method for fabricating picoliter microvials for electrochemical microanalysis using conventional photolithographic masking and photoresist techniques to transfer mold polystyrene microvials on silicon wafer templates. These

microvials typically exhibit non-uniformity in size and shape due to the difficulty in controlling the resist etching of the molding surface and the transfer molding process.

Park, et al. [*Science* 276:1401(1997)] has disclosed a modified lithographic method for producing arrays of nanometer-sized holes using polystyrene-polybutadiene, ordered, diblock copolymers as masks in reactive ion etching of silicon nitride. This multi-step method is capable of producing arrays of picoliter-sized holes which are typically 20 nanometers in diameter and 20 nanometers deep with a spacing of 40 nanometers. Hole densities of up to 10^{11} holes/cm^2 are disclosed. The range of sizes and spacings of the holes produced by this method is limited by the size of the copolymer microdomains. Uniformity of hole size and spacing is difficult to maintain with this method due to difficulties in controlling the etching method employed to form the holes.

Deutsch, et al. [*Cytometry* 16:214(1994)] have disclosed a porous electroplated nickel microarray comprised of micron-sized conical holes in blackened nickel plate. Hole sizes range from a 7 um upper diameter to a 3 um lower diameter with an 8 um depth. The array is used as a cell carrier for trapping individual cells while studying the responses of individual cells to changes in their microenvironment. In U.S. Patent 4,772540, Deutsch, et al., have also disclosed a method for making such an array using a combined photoresist and electroplating technique.

Corning Costar Corp. (Acton, Ma) produces a commercial microwell array for miniaturized assays under the trademark PixWell™. These arrays are made from microformed glass plates and comprise 40 um diameter by 20 um deep tapered wells with a well density of 4356 wells/cm^2.

Microwell arrays have particular utility in the study of living cells. In cell research, the measurement of responses of individual cells to changes or manipulations in their local environment is desirable. Any method or device designed for such studies must provide for the capability of maintaining cell viability, identifying the location of individual cells, and correlating response measurements with individual cells.

Due to the availability of viable fluorescent probes for intracellular studies, fluorescence measurements of living cells have significant utility in the study of cell functions. Thus fluorescence optical measurements are often utilized in cell studies where three generic methods of cell measurement are available, comprising bulk measurements of cell populations, dynamic measurements of cell populations or individual cells, and static measurements of individual cells.

The characteristics of an entire cell population as a whole can be studied with bulk measurements of sample volumes having a plurality of cells. This method is preferred where cell populations are very homogeneous. A generally recognized limitation of this method is the presence of background fluorescence which reduces the sensitivity of measurements and the inability of distinguishing differences or heterogeneity within a cell population.

Flow cytometry methods are often employed to reduce problems with background fluorescence which are encountered in bulk cell population measurements [M.R.Gauci, et al., *Cytometry* 25:388(1996); R.C.Boltz, et al., *Cytometry* 17:128(1994)]. In these methods, cell fluorescence emission is measured as cells are transported through an excitation light beam by a laminar flowing fluid. Flow cytometry methods may be combined with static methods for preliminary sorting and depositing of a small number of cells on a substrate for subsequent static cell measurements [U.S. Patent No. 4,009,435 to Hogg, et al.; Kanz, et al., *Cytometry* 7:491(1986); Schildkraut, et al., *J.Histochem Cytochem* 27;289(1979)].

Gauci, et al., disclose a method where cell size, shape and volume is measured by light scattering and fluorescent dyes are utilized to determine protein content and total nucleic acid content of cells. This method further provides for counting and sizing various cells at a rate of approximately 100 cells per second.

Flow cytometry techniques are generally limited to short duration, single measurements of individual cells. Repetitive measurements on the same cell over time are not possible with this method since typical dwell times of a cell in the excitation light beam are typically a few microseconds. In addition, the low cumulative intensity from individual cell fluorescence emissions during such short measurement times reduces the precision and limits the reliability of such measurements.

Regnier, et al., [*Trends in Anal.Chem.* 14(4):177(1995)] discloses an invasive, electrophoretically mediated, microanalysis method for single cell analysis. The method utilizes a tapered microinjector at the injection end of a capillary electrophoresis column to pierce an individual cell membrane and withdraw a sample of cytoplasm. The method measures cell contents, one cell at a time. The method is generally limited to the detection of easily oxidized species.

Hogan, et al., [*Trends in Anal.Chem.* 12(1):4(1993)] discloses a microcolumn separation technique which may be utilized in combination with either a conventional gas chromatograph-mass spectrometer, micro thin layer chromatography or high pressure liquid manipulation of small cellular

volumes. The sensitivity of the method is limited and may require pre-selection of target compounds for detection.

Static methods are generally the preferred method for measurements on individual cells. Measurement methods range from observing individual cells with a conventional optical microscope to employing laser scanning microscopes with computerized image analysis systems [see L. Hart, et al., *Anal. Quant. Cytol. Histol.* 12:127(1990)]. Such methods typically require the attachment of individual cells to a substrate prior to actual measurements. Problems are typically encountered in attaching single cells or single layers of cells to substrates and in maintaining cells in a fixed location during analysis or manipulation of the cell microenvironment. Additionally, repetitive measurements on individual cells typically require physically indexing the location of individual cells and providing a mechanism for scanning each cell sequentially and returning to indexed cell locations for repeated analysis of individual cells.

Huang, et al., [*Trends in Anal. Chem.,* 14(4)158(1995)] discloses a static electrochemical method and electrode for monitoring the biochemical environment of single cells. The method requires fabrication and manual positioning of a microelectrode reference and working electrode within the cell. The method has been used to detect insulin, nitric oxide and glucose inside single cells or external to the cells. The method is generally limited to the study of redox reactions within cells.

Ince, et al. [*J.Immunol. Methods* 128:227(1990)] disclose a closed chamber device for the study of single cells under controlled environments. This method employs a micro-perfusion chamber which is capable of creating extreme environmental conditions for cell studies. Individual cells are held in place by two glass coverslips as various solutions are passed through the chamber. One limitation of the method is the difficulty in eliminating entrapped gas bubbles which cause a high degree of autofluorescence and thus reduces the sensitivity of measurements due to background fluorescence.

In an attempt to overcome the limitations encountered with conventional static methods, Deutsch, et al., [*Cytometry* 16:214(1994)] and Weinreb and Deutsch, in U.S. Patent Nos. 4,729,949, 5,272,081, 5,310,674, and 5,506,141, have disclosed an apparatus and method for repetitive optical measurements of individual cells within a cell population where the location of each cell is preserved during manipulation of the cell microenvironment.

A central feature of the apparatus disclosed by Deutsch, et al., is a cell carrier, comprising a two dimensional array of apertures or traps which are conical-shaped in order to trap and hold individual cells by applying suction. The cell carrier is typically fabricated by the combined electroplating-photoresist method disclosed in U.S. Patent 4,772540 to Deutsch, et al. The purpose of the cell carrier is to provide a means for maintaining the cells in fixed array locations while manipulating the cell environment. Individual cells are urged into cell carrier holes by suction and the wells are subsequently illuminated with a low-intensity beam of polarized light that reads back-emitted polarization and intensity. Measurements are compared when two different reagents are sequentially reacted with the cells. The method as disclosed requires two separate cell carriers for both a baseline control and analyte measurement.

The method and device of Deutsch, et al., have been employed by pathologists in diagnostic tests to determine the health and viability of cell samples taken from patients. The method and device have been applied to both cancer screening [Deutsch, et al., *Cytometry* 16:214(1994), *Cytometry* 23:159(1996), and *European J. Cancer* 32A(10):1758(1996)] and rheumatoid arthritis [Zurgil, et al., *Isr.J.Med.Sci.* 33:273(1997)] in which fluorescence polarization measurements are used to differentiate lymphocytes of malignant versus healthy cells based on changes in the internal viscosity and structuredness of the cytoplasmic matrix induced by exposure to tumor antigen and mitogens.

The method and device disclosed by Deutsch, et al., requires employment of a scanning table driven by three stepping motors and a computer control system for mapping, indexing and locating individual cells in the cell carrier. The use of such mechanical scanning methods introduces limitations in reproducibility and accuracy of measurements due to conventional mechanical problems encountered with backlash and reproducible positioning of individual cell locations for repeated measurements. In addition, mechanical scanning of the entire array prolongs the measurement time for each cell in the array.

The method disclosed by Deutsch, et al., is further limited by the use of fluorescence polarization measurements which have certain intrinsic limitations due to the significant influence of various optical system components on polarization as the fluorescence emission response is passed from the cell carrier to optical detectors. Birindelli, et al. [European J. Cancer 33(8):1333(1997)], has also identified limitations in this method due to fluctuations in electropolarisation values which require taking averages of at least three measurement scans for each condition so as to obtain reliable

measurements. In addition, for cell studies, polarization measurements are generally limited to cell responses which produce sufficient changes in cytoplasm viscosity to produce a detectable change in polarization. Since not all cell responses are accompanied by detectable viscosity changes, the method is further limited to the cell activities which create such viscosity changes in the cytoplasm.

Zare, et al., [*Science* 267:74(1995); *Biophotonics International*, March-April, p17 (1995)] discloses a biosensor system based on the response of living cells to complex biological materials fractionated by a microcolumn separation technique. Cells which were positioned on a glass cover slip were treated with a fluorescent probe and subsequently shown to be sensitive to a series of biological compounds including acetylcholine, bradykinin, and adenosine triphosphate as well as changes in intracellular calcium levels.

Yeung, et al. [*Acc. Chem. Res.* 27:409(1994)] has reviewed a number of methods for single cell response studies and has observed a significant variation and heterogeneity within cell populations based on analyte measurements. For example, the reference discloses a capillary electrophoresis method for exposing cells to biologically reactive compounds, extracting the intracellular fluid of individual cells produced in response to such compounds, and identifying analytes from migration times in the capillary column. Other fluorescence-based assays are also disclosed. Significant cell-to-cell variations and heterogeneity in individual cell responses within a cell population were observed which differences could provide a means for discriminating between biological and chemical compounds in contact with individual cells.

McConnell, et al. [*Science*, 257:1906(1992)], disclose a microphysiometer device known as the "Cytosensor" which uses a light addressable potentiometer sensor to measure the rate at which cells acidify their environment. This sensor acts as miniaturized pH electrode for monitoring cell responses which produce detectable changes in local pH. The disclosed device is limited to the measurement of proton excretions from cells and thus is only capable of detecting acidic cell responses to analytes.

U.S. Patent No. 5,177,012 to Kim, et al., disclose a biosensor for the determination of glucose and fructose. The biosensor is produced by treating whole cells with an organic solvent and immobilizing the treated cells residue on a support to form a whole cell membrane which is applied to a pH electrode.

U.S. Patent No. 5,690,894 to Pinkel, et al., discloses a biosensor which employs biological "binding partners", materials such as nucleic acids, antibodies, proteins, lectins and other materials derived from cells, tissues, natural or genetically-engineered organisms. These agents are used in conjunction with a fiber optic array where each species of binding partners is uniquely addressed by a group of fibers within the fiber optic bundle which is coupled to an optical detector. The array was designed for screening of extensive arrays of biological binding partners.

While many of the prior art methods provide for the analysis of either single cells or populations of cells and some of these methods provide for monitoring cell responses to target analytes, none of the disclosed methods provides for employing large populations of monocultures or mixed populations of living cells for simultaneously monitoring the responses of individual cells to biological stimuli produced by chemical and biological analytes. Thus there is a need for a biosensor array and method which efficiently utilizes the ability of populations of living cells to respond to biologically significant compounds in a unique and detectable manner. Since the selectivity of living cells for such compounds has considerable value and utility in drug screening and analysis of complex biological fluids, a biosensor which makes use of the unique characteristics of living cell populations would offer distinct advantages in high throughput screening of combinatorial libraries where hundreds of thousands of candidate pharmaceutical compounds must be evaluated. In addition, such a sensor would be useful in monitoring bioprocesses and environmental pollution where the enhanced sensitivity of living cells to their environment can be exploited.

SUMMARY OF THE INVENTION

In general, the invention provides for a biosensor, a biosensor array, a biosensor sensing system and sensing methods for the analysis of chemical and biological materials. More particularly, the invention provides for biosensors and biosensor arrays, sensing apparatus and sensing methods which employ living cells and mixed populations of living cells for analysis of chemical and biological materials.

The biosensor array of the present invention comprises either a monoculture of living cells or randomly mixed populations of living cells wherein each individual cell in the array is positioned on a substrate at an optically-addressable, discrete site which accomodates the size and shape of individual cells. In one embodiment, the discrete site comprises a microwell or microcavity which is

preformed to accommodate the size and shape of the individual cells. The biosensor array sensing method relies on the well known fact that individual cells, which are chemically or biologically stimulated by the presence of a biological or chemical material in the cell environment, will respond by producing a change in the cell or cellular environment which can be optically interrogated and detected within the cell itself or from an indicator compound, for example, a fluorophore, chromophore or dye, either attached to the cell, taken up in the cell, or added to the local cell environment. The biosensor of the present invention thus capitalizes on the ability of living cells to respond to biologically significant compounds. Since the selectivity of living cells for such compounds has considerable value and utility in drug screening and analysis of complex biological fluids, the biosensor of the present invention offers distinct advantages to high throughput screening of combinatorial libraries where hundreds of thousands of candidate compounds must be evaluated.

As will be appreciated by one skilled in the art, a variety of substrate materials and substrate configurations may be employed with the biosensor array of the present invention. Any substrate or material that can be adapted to provide discrete sites that are appropriate for the attachment or association of individual cells and is accessible for optical interrogation of the cell array and detection of cell responses would be particularly useful as an array substrate. Candidate substrate materials include, but are not limited to, glasses, polymers, ceramics, metals, and composites formed from these materials. Substrate surface geometries may be planer, spherical, concave, convex, and textured. Preferably, a suitable substrate would be configured so as to provide for optical interrogation of the array and detection of cell responses to analytes of interest.

In a preferred embodiment, the biosensor array of the present invention is incorporated into a fiber optic array which serves as a substrate for cell placement. By "fiber optic array" or other grammatical equivalents herein is meant a plurality of individual fibers or fiber strands that may be either grouped together as discrete, individual fibers in a fiber bundle or, alternatively, joined along their axial dimensions as a preformed unitary fiber optic array. The individual fibers in such an array may be arranged in a uniform or coherent configuration, for example as in an imaging fiber, or may be randomly oriented and incoherent. When a fiber optic array is employed as a sensor substrate, the distal end of each fiber in a fiber optic bundle or fiber optic array is chemically etched so as to create a cavity or microwell. A schematic diagram of the biosensor array concept of the present invention is shown in Fig. 1. In a preferred embodiment, individual living cells of either a monoculture of cells or

mixed populations of cell lines are deployed in the microwells. The microwells are formed by anisotropic etching of the cores of the individual fiber in the fiber bundle or fiber array. The microwells are formed by controlling the etching process so as to remove a centralized core portion of the individual fiber strands while leaving the surrounding cladding intact. The resultant etched cavity is dimensioned for accommodating an individual cell. By selecting a fiber optic bundle or fiber optic array whose individual fiber cores are appropriately sized and by careful control of the etching conditions, the diameter and depth of the microwells can be controlled and adjusted over any convenient dimension range so as to match the size of any desired cell type.

In one embodiment, either discrete substrate cites or the interior surfaces of the microwells may be coated with a thin film of biologically compatible material such as collagen, fibronectin, polylysine, polyethylene glycol, polystyrene, or a metal such as gold, platinum or palladium. In an alternative embodiment, an indicator compound, for example, a fluorophore, a chromophore or dye, may be attached to the microwell surface for detecting cell responses to chemical or biological stimulation.

By incorporating a biosensor into an optically interrogatable substrate or a fiber optic array, the innovation of the biosensor of the present invention is in providing for optical coupling of individual cells located at discrete substrate cites or microwells with discrete detector elements, CCD cameras, or individual optical fibers in a fiber optic array or bundle that are in optical communication with such devices. By "optical coupling", "optical communication", or "optical cooperation" or other grammatical equivalents herein is meant the capability of either optically stimulating individual cells within the biosensor array with excitation light or optically interrogating the optical response of individual cells within the array to analytes, by conveying light to and from individual cells located at discrete cites within the array using either conventional optical train elements or optical fibers. Since typical fiber optic arrays contain thousands of discrete individual fiber strands, the invention thus provides for the individual optical coupling and interrogation of thousands of cells within an array, thereby providing for a large number of independent cell response measurements for each cell population within an array. Due to both the number of cell populations available and the correspondingly large number of individual cells within each cell population, a significant innovation of the present invention is in providing for the summing and amplification of the characteristic optical response signatures of

multiple independent measurements taken from cells within each cell population, thereby improving the detection limit and sensitivity of the biosensor.

An additional innovation of the present invention is that, by deploying a large number of cell populations within the array, and providing a large number of individual cells in each population, the discriminating capabilities of the biosensor array toward biological or chemical analytes is significantly enhanced by providing for thousands of cell responses from a large number of cell populations. This feature directly mimics the actual behavior of the human olfactory system where the combined signals from thousands of receptor cells, in each grouping of nearly a thousand different receptor cell types found in the epithelium layer, none of which are particularly sensitive in themselves, lead to a highly amplified sensory response to odors [see Kauer, et al, *Trends Neurosci.* 14:79(1991). One embodiment of the present invention thus mimics the evolutionary scent amplification process found in the human olfactory system in order to significantly enhance biosensor array sensitivity to analytes by summing the low-level responses of a large number of cells in the biosensor array. By summing the responses from a number of cells at low analyte concentrations, a substantial improvement in signal-to-noise ratio can be achieved and a corresponding reduction in the detection limit of the biosensor array is obtained.

A unique feature of the biosensor array of the present invention is that each of the individual cells and cell populations in the array may be encoded for maintaining cell type identity and location where randomly mixed populations of cells are employed. Cells may be encoded prior to disposing them in the microwells or, alternatively, following placement in the microwells. The invention provides for either encoding randomly mixed individual cells and cell populations with a fluorophoric or chromophoric dye compound or, alternatively, using self-encoded cells which are either naturally fluorescing or genetically engineered to fluoresce. Although cell populations may be randomly mixed together, this innovative feature provides for the identity and location of each cell type to be determined via a characteristic optical response signature when the cell array is either illuminated by excitation light energy or, alternatively, subjected to biological stimuli.

In one embodiment, cells or cell populations may be self-encoded by selecting cell populations, such as green fluorescent protein mutants, which exhibit either chemiluminescence, bioluminescence, or whose optical response to biological stimuli yield a unique detectable fluorescence signal. Other cell populations may be employed where cells within a population yield a

unique temporal optical response to stimuli. Either naturally occurring of genetically engineered cell lines may be utilized.

In various alternative embodiments, cells may be encoded with dye compounds which are attached to cells, taken up by cells or provided in the local cell environment. Examples of useful encoding dyes include fluorophores, chromophores, stains or a dye compounds. For example, conventional cell fluorophore probes such as fluoresceins, rhodamines, naphthalimides, phycobiliproteins, nitrobenzoxadiazole may be utilized. A particularly useful reference for selecting appropriate encoding dyes is R.P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (6^{th} ed.), Molecular Probes Inc.(Eugene, OR, 1996) which is herein incorporated by this reference.

When dye compounds are employed for encoding, cell populations within the biosensor array may be readily decoded by exciting the array with excitation light and indexing cells types within randomly dispersed populations by their response to excitation. In one embodiment, a single fluorophoric or chromophoric material or dye is used for encoding the cells. In an alternative embodiment, two or more encoding materials or dyes may be used to encode cell populations and the optical response intensity ratios for the dyes, produced by exposure to excitation light energy, are employed to encode and identify members of the cell population with the array. In an alternative embodiment, cells may be decoded by excitation light when exposed to a common analyte. In another embodiment, encoded cells may be decoded by their response to a generic cell activator using either a pH or Ca^{+2} indicator.

The innovative cell encoding feature of the present invention overcomes certain limitations of prior art devices by eliminating the need for mechanically scanning the array, mechanically indexing the location of cells, and mechanically positioning the array for measurements of individual cells within the array. The invention thus provides for rapid, simultaneous measurements of all cells and cell populations within the array without the need to mechanically scan the array to acquire a series of sequential measurements for each cell. Thus monitoring and measuring the responses of all cells in the array occurs simultaneously without a prolonged delay between the first cell measurement and last cell measurement. The ability to measure all cell responses simultaneously thus provides for the capability to monitor both short term cell response and long term cell response. This innovative feature thus enables the monitoring of rapid biologically significant cell processes and cell responses

on a short time scale. In addition, the ability to simultaneously measure cell responses over a short time scale enables the measurement of individual cell and cell population response rates to changes in the biosensor array environment. This feature thus provides for additional discriminating response information which is useful for detecting biological or chemical analytes.

The biosensor array of the present invention can employ either naturally occurring cells and cell populations or genetically engineered cell lines. Virtually any cell type and size can be accommodated by matching the cell size to individual optical fiber optic core diameters and etching conditions. In one embodiment, NIH 3T3 mouse fibroblast cells were employed. In alternative embodiments, other cells types such as *e. coli* bacteria, staphylococcus bacteria, myoblast precursors to skeletal muscle cells, neutrophil white blood cells, lymphocyte white blood cells, erythroblast red blood cells, osteoblast bone cells, chondrocyte cartilage cells, basophil white blood cells, eosinophil white blood cells, adipocyte fat cells, invertebrate neurons (*Helix aspera*), mammalian neurons, or adrenomedullary cells, may be utilized as well. Any cell type or mixtures of cell population types may also be employed providing the microwell can accommodate the individual cell size.

The optical responses of individual cells and cell populations to chemical or biological stimuli are typically interrogated and detected by coupling individual cells with appropriate indicators which may be either fluorophores, chromophores, stains or a dye compounds. For example, conventional cell fluorophore probes such as fluoresceins, rhodamines, naphthalimides, phycobiliproteins, nitrobenzoxadiazole may be utilized. Alternatively, permeant or impermeant cell membrane potential indicators, ion indicators, reactive oxygen indicators and pH indicators may be employed. A particularly useful reference for selecting appropriate indicators is R.P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (6^{th} ed.), Molecular Probes Inc.(Eugene, OR, 1996) which is herein incorporated by this reference. Any suitable indicator or combinations of indicators may be utilized provided the indicator does not compromise cell response. In a variety of alternative embodiments, indicators may be either incorporated directly into the cell, for example by attachment to the cell membrane, by absorption or injection into the cell cytoplasm, or added to the cell external environment, such as a fluid contained within the microwells. In an alternative embodiment, indicators may be attached to the surface of the microwells.

Individual cells or arrays of cells and cell populations may be optically interrogated and cell responses to analytes may be measured by conventional optical methods and instrumentation that

are known to those skilled in the art. Cells may be optically interrogated with any suitable excitation light energy source, such as arc lamps, lasers, or light emitting diodes, that are capable of producing light at an appropriate wavelength for exciting dye indicators that may be employed for encoding cell populations or for responding to analytes of interest. The optical responses of individual cells or cell populations may be monitored and measured with any suitable optical detection means, including, but not limited to film or conventional optical detectors, such as photoresistors, photomultiplier tubes, photodiodes, or charge coupled device (CCD) cameras. In a preferred embodiment, CCD cameras are employed to capture fluorescent images of the biosensor array for detecting responses of each cell and various cell subpopulations to analytes. In this embodiment, both individual cell responses and a captured image of the array response may be employed for detecting analytes.

In summary, the biosensor array and sensing method of the present invention offers many distinct advantages in overcoming the limitations of prior art devices. The sensor arrays are easily fabricated from commercially available optical imaging fibers to yield a cost effective, high density, precisely formed, biosensor array without requiring any sophisticated machining or forming process. Since optical fibers and fiber optic arrays are available in a wide variety of fiber core diameters, most cell types and sizes may be accommodated in by the device and method of the present invention. In addition, cells can be readily dispersed into the microwell array in random fashion with no need for physical indexing or scanning to locate individual cells or cell populations due to the innovative cell encoding technique. Sensing methods and sensing systems which employ the biosensor and sensor array of the present invention avoids many of the limitations in manipulating cells encountered with prior art devices. Once cells are placed within the microwells of the array, conventional imaging systems and methods which employ an imaging camera and conventional optics, can monitor the response of thousands of cells simultaneously, eliminating requirements for mechanical scanning mechanisms. Analysis of measurement data is further facilitated by implementing commercially available imaging software to process images of the biosensor array using pattern recognition techniques combined with neural network and other statistical methods.

The biosensor array and sensing method of the present invention may be employed for a number of useful analytical applications where individual cells, which are chemically or biologically stimulated by the presence of a biological or chemical material in the local cell environment, will respond to their environment by producing an optically detectable response either due to the

presence of an appropriate indicator or due to the characteristic optical response of particular cell types which exhibit either natural or genetically-engineered chemiluminescence or bioluminescence. The biosensor array and method of the present invention thus capitalizes on the ability of living cells to respond to biologically significant compounds. Since the selectivity of living cells for such compounds has considerable value and utility in drug screening and analysis of complex biological fluids, the biosensor of the present invention offers distinct advantages to high throughput screening of combinatorial libraries where hundreds of thousands of candidate compounds must be evaluated.

The above and other features of the invention, including various novel details of construction and methods, and other advantages, will now be more particularly described with reference to the accompanying drawings and claims. It will be understood to one skilled in the art that the particular apparatus and method embodying the invention are shown by way of illustration and not as a limitation of the invention. The principles and features of the invention may be employed in various and numerous embodiments without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is pointed out with particularity in the appended claims. Other features and benefits of the invention can be more clearly understood with reference to the specification and the accompanying drawings in which:

Fig. 1 is a schematic diagram of the biosensor array concept of the present invention;

Figs. 2a-b are atomic force microscope photomicrographs of a microwell array used in a biosensor array of the present invention;

Fig. 3 is a schematic diagram of the method for depositing cells in microwells used for a biosensor array of the present invention;

Fig. 4 is an SEM photomicrograph of a single NIH 3T3 mouse fibroblast cell in a microwell;

Fig. 5 is a schematic representation of a method for establishing cell viability of cell populations within the biosensor array of the present invention;

Fig. 6 is a characteristic fluorescence image pattern identifying the location of a cells which test positively for cell viability in a biosensor array of the present invention;

Fig. 7 shows the temporal response of the combined fluorescence intensity of the viable cell population identified in Fig. 6;

Fig. 8 is a fluorescence optical image of an encoded cell population within a biosensor array of the present invention;

Fig. 9 is a schematic block diagram of the measurement system used for optical measurements of the microwell sensor array;

Fig. 10 is a plot of average BCECF fluorescence for nine biosensor array cells over time; and

Figs. 11a-b compares the viability of cells inserted in microwells both with and without a pretreatment for filling the microwells with culture media.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Fabrication of Microwell Arrays

The present invention provides array compositions comprising at least a first substrate with a surface comprising individual sites. By "array" herein is meant a plurality of cells in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different cells to many millions can be made, with very large fiber optic arrays being possible. Generally, the array will comprise from two to as many as a billion or more, depending on the size of the cells and the substrate, as well as the end use of the array, thus very high density, high density, moderate density, low density and very low density arrays may be made. Preferred ranges for very high density arrays are from about 10,000,000 to about 2,000,000,000 (all numbers herein are per cm2), with from about 100,000,000 to about 1,000,000,000 being preferred. High density arrays range about 100,000 to about 10,000,000, with from about 1,000,000 to about 5,000,000 being particularly preferred. Moderate density arrays range from about 10,000 to about 100,000 being particularly preferred, and from about 20,000 to about 50,000 being especially preferred. Low density arrays are generally less than 10,000, with from about 1,000 to about 5,000 being preferred. Very low density arrays are less than 1,000, with from about 10 to about 1000 being preferred, and from about 100 to about 500 being particularly preferred. In some embodiments, the compositions of the invention may not be in array format; that is, for some embodiments, compositions comprising a single cell may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

In addition, one advantage of the present compositions is that particularly through the use of fiber optic technology, extremely high density arrays can be made. Thus for example, because cells frequently may be 200 fm or less and very small fibers are known, it is possible to have as many as 250,000 or more (in some instances, 1 million) different fibers and cells in a 1 mm2 fiber optic bundle, with densities of greater than 15,000,000 individual cells and fibers (again, in some instances as many as 25-50 million) per 0.5 cm2 obtainable.

By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of cells and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, TeflonJ, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not themselves appreciably fluorescese.

Generally the substrate is flat (planar), although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the cells in a porous block of plastic that allows sample access to the cells and using a confocal microscope for detection. Similarly, the cells may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred substrates include optical fiber bundles as discussed below, and flat planar substrates such as glass, polystyrene and other plastics and acrylics.

In a preferred embodiment, the substrate is an optical fiber bundle or array, as is generally described in U.S.S.N.s 08/944,850 and 08/519,062, PCT US98/05025, and PCT US98/09163, all of which are expressly incorporated herein by reference. Preferred embodiments utilize preformed unitary fiber optic arrays. By "preformed unitary fiber optic array" herein is meant an array of discrete individual fiber optic strands that are co-axially disposed and joined along their lengths. The fiber strands are generally individually clad. However, one thing that distinguished a preformed unitary array from other fiber optic formats is that the fibers are not individually physically manipulatable; that

is, one strand generally cannot be physically separated at any point along its length from another fiber strand. In general, the discussion herein is focused on fiber optic arrays, although as will be appreciated by those in the art, other substrates as described above may be used in any embodiment described herein.

At least one surface of the substrate is modified to contain discrete, individual sites for later association of cells. These sites may comprise physically altered sites, i.e. physical configurations such as wells or small depressions in the substrate that can retain the cells, such that a cell can rest in the well, or the use of other forces (magnetic or compressive), or chemically altered or active sites, such as biologically or chemically functionalized sites, electrostatically altered sites, hydrophobically/hydrophilically functionalized sites, spots of adhesive, etc.

The sites may be a pattern, i.e. a regular design or configuration, or randomly distributed. A preferred embodiment utilizes a regular pattern of sites such that the sites may be addressed in the X-Y coordinate plane. "Pattern" in this sense includes a repeating unit cell, preferably one that allows a high density of cells on the substrate. However, it should be noted that these sites may not be discrete sites. That is, it is possible to use a uniform surface of adhesive or chemical functionalities, for example, that allows the attachment of cells at any position. That is, the surface of the substrate is modified to allow attachment of the cells at individual sites, whether or not those sites are contiguous or non-contiguous with other sites. Thus, the surface of the substrate may be modified such that discrete sites are formed that can only have a single associated cell, or alternatively, the surface of the substrate is modified and cells may go down anywhere, but they end up at discrete sites.

In a preferred embodiment, the surface of the substrate is modified to contain microwells, i.e. depressions in the surface of the substrate. This may be done as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, pressing, casting, molding, microetching, electrolytic deposition, chemical or physical vapor deposition employing masks or templates, electrochemical machining, laser machining or ablation, electron beam machining or ablation, and conventional machining. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the substrate.

In a preferred embodiment, physical alterations are made in a surface of the substrate to produce the sites. In a preferred embodiment, the substrate is a fiber optic bundle and the surface of the substrate is a terminal end of the fiber bundle, as is generally described in 08/818,199 and

09/151,877, both of which are hereby expressly incorporated by reference. In this embodiment, wells are made in a terminal or distal end of a fiber optic bundle comprising individual fibers. In this embodiment, the cores of the individual fibers are etched, with respect to the cladding, such that small wells or depressions are formed at one end of the fibers. The required depth of the wells will depend on the size of the cells to be added to the wells.

Generally in this embodiment, the cells are non-covalently associated in the wells, although the wells may additionally be biologically or chemically functionalized as is generally described below, cross-linking agents may be used, or a physical barrier may be used, i.e. a film or membrane over the cells.

In a preferred embodiment, the surface of the substrate is modified to contain biologically or chemically modified sites, that can be used to attach, either covalently or non-covalently, the cells of the invention to the discrete sites or locations on the substrate. "Chemically modified sites" in this context includes, but is not limited to, the addition of a pattern of chemical functional groups including amino groups, carboxy groups, oxo groups and thiol groups, that can be used to attach cells which generally also contain corresponding reactive functional groups on their surfaces; the addition of a pattern of adhesive that can be used to bind the cells, (either by prior chemical functionalization for the addition of the adhesive or direct addition of the adhesive); the addition of a pattern of charged groups (similar to the chemical functionalities) for the electrostatic attachment of the cells, i.e. when the cells comprise charged groups opposite to the sites; the addition of a pattern of chemical functional groups that renders the sites differentially hydrophobic or hydrophilic, such that the addition of similarly hydrophobic or hydrophilic cells under suitable experimental conditions will result in association of the cells to the sites on the basis of hydroaffinity. Alternatively, biological modifications include the use of binding ligands or binding partner pairs, including, but not limited to, antigen/antibody pairs, enzyme/substrate or inhibitor pairs, receptor-ligand pairs, carbohydrates and their binding partners (lectins, etc.).

In a preferred embodiment, an array of micrometer-sized wells is created at the distal face of an optical imaging fiber by a selective etching process which takes advantage of the difference in etch rates between core and cladding materials. This process has been previously disclosed by Pantano, et al., *Chem. Mater.* 8:2832 (1996), and Walt, et al., in U.S. Patent Application 08/818,199. The etch

reaction time and conditions are adjusted to achieve desired control over the resultant microwell size and volume. Microwells are thus sized to accommodate a single cell of any desired cell type.

The sensor array design can accommodate a variety of cell sizes and configurations utilizing either commercially available optical fibers and fiber optic arrays or custom made fibers or fiber arrays. The major requirement in selecting candidate fibers or fiber optic arrays for fabricating sensors is that the individual fibers have etchable cores.

In one embodiment, the interior surfaces of the microwells may be coated with a thin film or passivation layer of biologically compatible material, similar to the biological modifications of the substrate as outlined above. For example, materials known to support cell growth or adhesion may be used, including, but not limited to, fibronectin, any number of known polymers incldiuing collagen, polylysine and other polyamino acids, polyethylene glycol and polystyrene, growth factors, hormones, cytokines, etc. Similarly, binding ligands as outlined above may be coated onto the surface of the wells. In addition, coatings or films of metals such as a metal such as gold, platinum or palladium may be employed. In an alternative embodiment, an indicator compound, for example, a fluorophore, a chromophore or dye, may be attached to the microwell surface for detecting cell responses to chemical or biological stimulation.

The method of fabricating microwells can be adapted to any fiber size so as to accommodate a wide range of cell sizes for incorporation into appropriately sized microwells. For example, optical fibers having core diameters ranging from 1.6 to 100 um are commercially available from either Galileo Electro-Optics Corp. (Sturbridge, MA) or Edmund Scientific (Barrington, NJ). In addition, larger sizes are available by custom order. Thus, appropriately sized fibers can be utilized to study such diverse cell sizes as *E. coli*, with a typical cell dimension of 0.7 to 1.5 um, and mammalian neurons, with a cell dimension of up to 150 um.

In one embodiment, a fiber optic array, having a 1 mm outer diameter and 7 um individual fiber core diameters, available from Galileo Electro-Optics Corp. (Sturbridge, MA), was utilized. One end of the fiber optic array was polished using a series of aluminum oxide lapping films 12, 9, 3, 1, 0.3 um available from Mark V Lab (East Granby, CT). The fibers were then sonicated for approximately 1 minute to remove any residue from the polishing procedure. The etching procedure was performed by submerging the distal face of the fiber at a right angle in 700 uL of a buffered hydrofluoric acid solution, comprising 100 uL hydrofluoric acid (50%), 0.2g ammonium fluoride, and 600 uL of

deionized water, for approximately 65 seconds. The fiber was then rinsed with deionized water and sonicated for 1 minute to remove any salts that may have been formed during the etching procedure. In this embodiment, the etch reaction time was tailored such that the well size was 7 microns in diameter, 3 microns in depth, and approximately 90 fL in volume. Both scanning electron microscopy (SEM) and atomic force microscopy (AFM) may be utilized to characterize etched microwells. In Fig. 2a, a typical microwell array formed by the etching procedure is shown in an AMF photomicrograph (Digital Instruments Nanoscope IIIa, Santa Barbara, Ca). In Fig. 2, an oblique view of the microwell array is provided. As shown in these figures, the microwells formed by this process are extremely uniform due to the uniform characteristic structure of the fiber optic array.

B. Selection of Cell Types

Virtually any cell type and size can be accommodated in fabricating the sensor of the present invention by matching the cell size to individual optical fiber optic core diameters. Virtually any naturally occurring or genetically engineered (i.e. containing exogeneous nucleic acid) eukaryotic or procaryotic cell type may be used, with plants, invertebrates, bacteria and mammalian cells, including, but not limited to, green fluorescent protein mutants, primate, rodent and human cells and cell lines being preferred, as well as mixtures of cell types.

In one embodiment, NIH 3T3 mouse fibroblast cells were employed. These cells are typically 15-20 um in size. Other cells types such as *E. coli* bacteria, 1 x 3 um, staphylococcus bacteria, approximately 1 um, myoblast precursors to skeletal muscle cells, 15-20 um, neutrophil white blood cells, 10 um, lymphocyte white blood cells, 10 um, erythroblast red blood cells, 5 um, osteoblast bone cells, 15-20 um, chondrocyte cartilage cells, 15-20 um, basophil white blood cells, 10 um, eosinophil white blood cells, 10 um, adipocyte fat cells, 20 um, invertebrate neurons (*Helix aspera*), 125 um, mammalian neurons, 4-140 um, or adrenomedullary cells, 13-16 um, melanocytes, 20 um, epithelial cells, 20 um, or endothelial cells , 15-20 um, may be utilized as well. Additional other suitable cell types include, but are not limited to, tumor cells of all types (particularly melanoma, myeloid leukemia, carcinomas of the lung, breast, ovaries, colon, kidney, prostate, pancreas and testes), cardiomyocytes, endothelial cells, epithelial cells, lymphocytes (T-cell and B cell) , mast cells, eosinophils, vascular intimal cells, hepatocytes, leukocytes including mononuclear leukocytes, stem cells such as haemopoetic, neural, skin, lung, kidney, liver and myocyte stem cells, osteoclasts, chondrocytes and other connective tissue cells, keratinocytes, melanocytes, liver cells, kidney cells,

and adipocytes. Suitable cells also include known research cells, including, but not limited to, Jurkat T cells, NIH3T3 cells, CHO, COS, etc. A particularly useful source of cell lines may be found in *ATCC Cell Lines and Hybridomas* (8th ed., 1994), *Bacteria and Bacteriophages* (19th ed., 1996), *Yeast* (1995), *Mycology and Botany* (19th ed., 1996), and *Protists: Algae and Protozoa* (18th ed., 1993), available from American Type Culture Co. (Rockville, MD), all of which are herein incorporated by reference.

C. Dispersion of Cells in Microwells

Once the microwell size is tailored to accommodate specific cell sizes, the next step in creating an array of cells is to randomly disperse cells into the microwells. Fig. 3 is a schematic diagram showing the method for dispersing and depositing cells in the microwell array. Cell populations are conventionally cultured with growth media which matches cell needs. Culture media is formulated according to either recipes provided by cell line providers, journal articles or reference texts. A particularly useful reference for media preparation is *ATCC Quality Control Methods for Cell Lines* (2nd ed.), American Type Culture Co. (Rockville, MD) which is herein incorporated by reference. After culturing, cells are typically trypsinized using aseptic techniques to remove them from the cell culture dish and suspend them in growth media.

In one embodiment, NIH 3T3 mouse fibroblast cells, available from American Type Culture Collection (Rockville, MD) were utilized. The cells were randomly dispersed into the wells by removing the adhered cells from the culture dish with a proteolytic enzyme, such as Trypsin-EDTA (0.05% Trypsin, 0.53 mM EDTA.4Na), available from ; Gibco BRL (Grand Island, NY], using aseptic techniques and placing the cells into a single-cell suspension in growth media, such as Dulbecco's Modified Eagle Medium with 1% PenicillinStreptomycin, 1% L-Glutamine-200mM, and 10% fetal calf serum, available from Gibco BRL.

To disperse cells into the wells, approximately 1.5 ml of the cell suspension was concentrated by centrifugation at 2000 RPM for 3.5 minutes. The supernatant is drawn off and the centrifuge tube was tapped to resuspend the cells. The cell suspension was then added to a 1.5 mm diameter capillary tube. Prior to adding cells to the microwell array, the end of the fiber optic array which contains the microwells was sonicated under vacuum in cell media for approximately 15 minutes to flush and fill the microwells with media. The microwell end of the fiber was then inserted into the capillary tube and secured in place with a thin strip of laboratory film. The capillary tube/fiber set-up

was then incubated in the vertical position for 1-2 hours allowing the suspended cells to settle into the wells and adhere to the well bottom. The length of time required to fill the microwells is dependent only on the amount of time required for the cells to adhere to the microwell bottom. Excess cells which were not accommodated by a well were wiped away with a cotton swab wet with growth media. Fig. 4 is an SEM photomicrograph of a single cell dispersed in an etched microwell.

Once they are positioned within the microwells, cells with typically attach to microwell surfaces within 1-2 hours by protein contact. Cell culture media in the microwells may be periodically replenished by exposing the distal surface of the fiber optic array to fresh media and allowing nutrients to diffuse into the microwell cavities. Typically, cells will divide every twelve to fifteen hours. While the size of the microwells tends to confine individual cells, the array will accommodate limited cell splitting over time. Microwell volume will restrict cell splitting due to the well know cell phenomenon of contact inhibition when cells are touching.

D. Establishing Cell Viability

In one embodiment, the cell viability in the wells was investigated using a pH indicator, 2'-7'-bis-(2carboxyethyl)-5-(and-6-)-carboxyfluorescein (BCECF-AM) which has an excitation wavelength of 505 nm and an emission wavelength of 535 nm and is available from Molecular Probes (Eugene, OR). The acetoxymethyl (AM) ester form of BCECF is non-fluorescent in solution. The BCECF-AM is cell membrame permeant and passively enters the cell where, once inside the cell, the lipophilic blocking groups are cleaved by non-specific esterases resulting in an increase in fluorescent intensity. This increase in fluorescent intensity is indicative of the cell viability. Fig. 5 is a schematic representation of one embodiment of a method used for establishing cell viability in a sensor array.

For viability tests, an array of encoded cells previously dispersed in microwells was immersed in a 1 uM solution of BCECF-AM for 1 minute. The cell array was removed and rinsed thoroughly to remove any residual dye. Fluorescent images of cell responses were acquired every 30 seconds using a 1.0 second acquisition time to monitor the increase in fluorescent intensity due to the pH of healthy cells. Prior to viability measurements, a cell location template was generated through excitation of the encoded cells, using the encoding method described herein, and the locations of individual cells was superimposed on viability measurement images in order to identify the location of healthy cells within the array. A fluorescence image of the encoded cells is shown in Fig. 6. In Fig. 7, the fluorescent intensity response of the cells indicates a gradual increase as the lipophilic groups are

cleaved from the BCECF-AM dye within the cell. The fluorescent intensity response of the wells which do not contain cells was negligible.

In a alternative embodiment, BCECF-Dextran, available from Molecular Probes, was used for cell viability measurements. A 0.1 uM solution of the dye was added to cell media contained within array microwells. BCECF requires excitation at two wavelengths, 490 nm and 440 nm, and the ratio of the emitted light intensity at 530 nm for each wavelength is proportional to pH. This dye is conjugated with a large Dextran group to prevent entry into the cell through the cell membrane. Thus, BCECF-Dextran can monitor decreases in pH within the external cell environment due to cell metabolism. Fig. 10 shows the average response of nine cells over time where the pH of the cell environment gradually decreases due to cell metabolism.

In another embodiment a commercial cell viability assay, LIVE / DEAD® from Molecular Probes (Eugene, OR), was employed. This assay provides a two-color fluorescence cell viability assay based on intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the enzymatic conversion of the cell-permeant non-fluorescent calcein AM to fluorescent calcein, with an excitation wavelength at 495nm and an emission wavelength at 515nm. Dead cells are distinguished by binding ethidium homodimer (EthD-1), with an excitation wavelength at 495nm and an emission wavelength at 635nm, to nucleic acids which is accompanied by a 40-fold increase in fluorescent intensity. EthD-1 is excluded by the intact plasma membranes of living cells. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually non-fluorescent before interacting with cells.

In a typical procedure, a working solution of 2 uM calcein AM and 4uM EthD-1 was prepared in serium-free medium. An array of NIH 3T3 mouse fibroblast cells was cultured at the distal face of a microwell array. The proximal face of the imaging fiber was focused on the imaging system. The cell array at the distal face of the imaging fiber was placed in serium-free medium without dye. The cell array was excited at 495nm and emission values from a population of 25 cells were acquired for 300ms at 515nm (Live) and 635nm (Dead). These values serve as background reading for these measurements. The cell array was then placed in the dye solution and the cell array was excited at 495nm and emission values from 25 cells were acquired for 300ms at 515nm (Live) and 635nm (Dead) every minute for 30 minutes. All measurements were performed at room temperature.

Average fluorescent intensity for the live and dead cell wavelengths are plotted versus time after subtracting the background fluorescence measurement at each emission wavelength.

This assay was used to evaluate pretreatment methods for filling the microwells of a biosensor arrray with culture media prior to inserting cells. By comparing the viability of cells placed in microwells after various treatments, it was determined that, in a preferred embodiment, sonicating the mircrowell array under vacuum for 15 minutes prior to insertion of cells improves viability by ensuring that the microwells are filled with culture media. In Fig. 11a, the average fluorescent intensity for dead cells and live cells is plotted with time where no pretreatment was utilized. In Fig. 11b, results are plotted for a pretreated microwell array. Comparison of the two figures demonstrates the advantage of prefilling the microwells with culture media prior to cell insertion. In this example, when the microwell array was not sonicated prior to adding the cells the cells displayed immediate cell death. In this example, when the microwells were sonicated under vacuum to fill the arrays with culture media, the cells remained viable for approximately 20 minutes.

E. Encoding Cell Populations

A unique feature of the biosensor array of the present invention is that cells within each cell population are individually encoded for maintaining cell identity within the array when randomly mixed populations of cells are employed. Cells may be encoded with a single fluorophore or chromophore dye or ratios of such dyes. Alternatively, cells may be encoded by either injecting a non-toxic fluorescing compound into the cell cytoplasm or by employing natural or genetically-engineered cells lines which exhibit chemiluminescence or bioluminescence , such as green fluorescent protein mutants. Although a plurality of cell populations may be randomly mixed in the biosensor array, the identity and location of each cell type is determined via a characteristic optical response signature when the array is illuminated by excitation light energy. Cells may be encoded prior to disposing them in the microwells or, alternatively, following placement in the microwells. . In one embodiment, a single fluorophoric or chromophoric material or dye is used for encoding the cells. In an alternative embodiment, two or more encoding materials or dyes may be used to encode cell populations and the ratio of the optical response light intensity from each material or dye, produced by exposure to excitation light energy, is used to identify the positions of individual cells within the cell population and locate them in the array. In various alternative embodiments, cells may be encoded with dye compounds which are attached to cells, taken up by cells or provided in the local cell environment.

A wide variety of fluorophores, chromophores, stains or a dye compounds may be used for encoding cells. Encoding dyes may be permeant or impermeant to the cell membrane. Impermeant dyes may be conjugated with acetoxymethyl ester to allow take up by cells. In one embodiment, conventional conjugate or reactive cell membrane stains, cell tracers, or cell probes such as fluoresceins, rhodamines, eosins naphthalimides, phycobiliproteins, nitrobenzoxadiazole may be utilized. In other embodiments, cyanine dyes, such as SYTO® (Molecular Probes), amine-reactive dyes, thiol-reactive dyes, lipopilic dyes, and DNA intercalators, such as acridine orange, may be employed. In one embodiment, fluorogenic or chromogenic enzyme substrates may be taken up by the cells, processesed by intracellular enzymes, such as glycosidases, phosphatases, luciferase, or chloramphenicol acetyltransferase, and provide encoding for cell populations. In an alternative embodiment, cell organelle dye probes may be employed for encoding. In one embodiment, cell membrane probes such as carbocyanines and lipophilicaminostyrls may be utilized for encoding.

By way of example, Tables 1 and 2 provide a partial listing of a various types of dyes and their corresponding excitation and emission wavelengths which have utility for encoding cell populations in sensor arrays of the present invention. In addition, a particularly useful reference for selecting other types of encoding dyes is R.P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (6th ed.), Molecular Probes Inc.(Eugene, OR, 1996).

Cell encoding eliminates the need for mechanically scanning the array, mechanically indexing the location of cells, and mechanically positioning the array for measurements of individual cells. In addition, by encoding all cells within the array, simultaneous measurements of the entire array are possible since the responses of individual cells are readily associated with a specific cell population type because each cell is encoded. Encoding of cells thus provides for rapid, simultaneous measurements of all cells and cell populations within the array without the need to mechanically scan the array to acquire a series of sequential measurements for each cell. Since monitoring and measuring the responses of all cells in the array occurs simultaneously, without a prolonged delay between the first cell measurement and last cell measurement, the biosensor array of the present invention provides the capability to monitor both short term and long term cell responses to biological stimuli. The biosensor array of the present invention thus has the unique ability for measuring both cell responses and response rates to analytes. This feature provides

TABLE 1

1. Plasma Membrane Stains

PKH22 green fluorochrome	551ex/567em
PHK67 green fluorochrome	490ex/502em
PKH26 red fluorochrome	551ex/567em

lipophilic carbocyanines

DiI - orange fluorescence
DiO - green fluorescence
DiD - red flurescence
FM 1-43 - green fluorescence 510ex/626em
FM 4-64 - red fluorescence 515ex/640em
RH 414 - orange fluorescence 532ex/716em

lipophilic dextrans

tetramethylrhodamine dextrans (10,000 MW) 470ex/500em
fluorescein dextran (10,000 MW) 495ex/530em

2. Tracker Probes

Blue 7-amino-4-chloromethyl coumarin 354ex/466em
4-chloromethyl-6,8-difluoro-7-hydroxy coumarin 371ex/464em
4-chloromethyl-7-hydroxy coumarin 372ex/470em
Green 5-chloromethyl fluorescein diacetate 492ex/516em
8-chloromethyl BODIPY 522ex/528em

Yellow-Green
5-chloromethyleosin diacetate 524ex/544em

Orange
5-(and 6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine 540ex/566em

3. Amine-Reactive Probes

fluorescein isothiocyanate 494ex/519em
carboxyfluorescein succinimidyl esters 504ex/529em
carboxyeosin succinimidyl esters 492ex/517em

additional discriminating response information which is useful for detecting biological or chemical analytes of interest.

While a number of encoding dyes and methods are available, in one embodiment, external fluorescent cell membrane labels , PKH67 with an excitation wavelength of 490 nm and an emission wavelength of 502 nm, and PKH26 with an excitation wavelength of 551 nm and an emission wavelength of 567 nm, were utilized. PKH26 and PKH67 are part of a family of dyes manufactured by Zynaxis Cell Science (Malvern, PA), sold under the trademark Zyn-Linker® (Phanos Technologies Inc.), produced by the method of U.S. Patent 5,665,328 to Hogan, et al., and available from Sigma (St.Louis, Mo). In this embodiment, both dyes were applied to suspended fibroblast cells to encode the cells prior to placement in the sensor array. Encoded cells were placed in microwells filled with growth media. The encoded cells were then illuminated with excitation light transmitted through the fiber optic array and the resulting fluorescent emission response of cells was collected through the same fiber and passed to the detection system. Fig. 6 shows a typical fluorescence optical image of a PKH26 encoded cell population in a biosensor array of the present invention. Fig. 8 shows a typical fluorescence image of a PKH67 encoded cell population in a biosensor array. Note that only those microwells containing encoded cells yields a fluorescent signal upon excitation at the encoding dye wavelength.

In a typical encoding procedure, external or internal encoding labels are applied to suspended cell populations prior to placement in the sensor array. In one embodiment, an external label is applied to the cell by first washing the suspended cells with serum-free media and then centrifuging the cells into a loose pellet at 2000 RPM for 5 minutes. The supernatant is drawn off and the centrifuge tube is tapped to resuspend the cells. Approximately 1 mL of Diluent C, available as dye kit from Sigma, was added to the cells and the tube was inverted to mix. Immediately prior to encoding, a solution of 4×10^{-6} M Diluent C dye was prepared. The suspended cells are added to the dye solution, mixed by pipetting the cell/dye solution, and incubated for 5 minutes. To stop the encoding reaction, 2 mL of fetal calf serum was added to the cells, incubated for 1 minute, and then diluted by adding 4 mL growth media. The cells are then centrifuged at 2000 RPM for 10 minutes to remove the cells from the staining solution. The supernatant was drawn off and the cells are transferred to a new tube. Finally, the cells are subjected to a minimum of three washings by adding 10 mL growth media, centrifuging, and resuspending the cells.

Blue Fluorescent and Nonfluorescent Probes		Green Fluorescent Probes		Yellow and Orange Fluorescent Probes		Red Fluorescent Probes	
Cat #	Probe	Cat #	Probe	Cat #	Probe	Cat #	Probe
L-6868	Lucigenin †	A-1372	Nonyl acridine orange	D-288	4-Di-1-ASP (DASPMI)	M-7512	MitoTracker Red CMXRos *
		D-273	DiOC$_6$(3)	D-308	2-Di-1-ASP (DASPMI)	T-3168	JC-1 ‡
		D-303	DiOC$_5$(5)	D-426	DASPEI		
		D-378	DiOC$_4$(3) (for plant mitochondria)	M-7510	MitoTracker Orange CMTMRos *		
		M-7502	MitoFluor Green	R-634	Rhodamine 6G		
		M-7514	MitoTracker Green FM *	R-648	Rhodamine B, hexyl ester		
		R-302	Rhodamine 123	T-639	Tetramethylrosamine		
		S-7529	SYTO 18 yeast mitochondrial stain	T-668	Tetramethylrhodamine, methyl ester		
		T-3168	JC-1 ‡	T-669	Tetramethylrhodamine, ethyl ester		
		D-632	Dihydrorhodamine 123	D-633	Dihydrorhodamine 6G	M-7513	MitoTracker Red CM-H$_2$XRos *
				D-638	Dihydrotetramethylrosamine		
				M-7511	MitoTracker Orange CM-H$_2$TMRos *		
D-1552	DAMP §	L-7526	LysoTracker Green DND-26	D-113	Dansyl cadaverine	L-7528	LysoTracker Red DND-99
H-7599	Hydroxystilbamidine ¶	L-7542	LysoTracker Green Br	L-7527	LysoTracker Yellow DND-68		
L-7525	LysoTracker Blue DND-22 ¶						
L-7537	LysoTracker biotin §						
L-7538	LysoTracker biotin/DNP §						
L-7532	LysoSensor Blue DND-192 ¶	L-7534	LysoSensor Green DND-153	A-1301	Acridine orange	N-3246	Neutral red
L-7533	LysoSensor Blue DND-167 ¶	L-7535	LysoSensor Green DND-189	L-7545	LysoSensor Yellow/Blue DND-160 ‡		
L-7545	LysoSensor Yellow/Blue DND-160 ‡						
		B-7447	BODIPY FL brefeldin A	B-7449	BODIPY 558/568 brefeldin A		
		D-272	DiOC$_5$(3)	D-282	DiIC$_{18}$(3)		
		D-273	DiOC$_6$(3)	D-384	DiIC$_{18}$(3)		
				R-648	Rhodamine B, hexyl ester		
				R-634	Rhodamine 6G		
				T-668	Tetramethylrhodamine, methyl ester		
				T-669	Tetramethylrhodamine, ethyl ester		
B-7450	Brefeldin A	B-7447	BODIPY FL brefeldin A	B-7449	BODIPY 558/568 brefeldin A	D-3521	BODIPY FL C$_5$-ceramide ‡
		D-3521	BODIPY FL C$_5$-ceramide ‡			D-7540	BODIPY TR ceramide ‡
		D-3522	BODIPY FL C$_5$-sphingomyelin			D-7546	BODIPY FL Br$_2$C$_5$-ceramide ‡
		D-7546	BODIPY FL Br$_2$C$_5$-ceramide ‡				
		N-1154	NBD C$_6$-ceramide				
		N-3524	NBD C$_6$-sphingomyelin				

* Aldehyde-fixable probe. † Chemiluminescent probe. ‡ Dual emission spectrum. § Nonfluorescent probe. ¶ Blue fluorescent probe.

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To demonstrate the encoding method with PKH26 and PKH67 dyes, encoded NIH 3T3 mouse fibroblast cells were dispersed in microwells in sensor array and the location of encoded cells within the array was determined by excitation of the cells at a wavelength of 551 nm. Upon excitation, the encoded cells form a characteristic, detectable fluorescence pattern within the sensor array where the pattern provides a locational template for cell viability measurements using BCECF and an excitation wavelength of 505 nm. The excitation and emission wavelengths of the PKH26 encoding label and the BCECF-AM dye are sufficiently separated so that there is no interference between the two dyes. Fig. 6 is a characteristic fluorescence image pattern identifying the location of PKH26 encoded cells in the biosensor array. A similar procedure was followed for encoding the mouse fibroblast cells with PKH67 where a corresponding characteristic fluorescence image pattern is shown in Fig. 8.

In addition to encoding individual cell populations with a single unique dye, each cell population within the array may be encoded with unique dye ratios which yield a characteristic fluorescent intensity ratio in a distinct wavelength range. A range of dye ratios may be employed with two or more dye combination for producing a series of encoding ratios useful for a identifying a large number of cell populations. The fluorescent intensity ratio, produced by a specific dye ratio which is used for encoding a cell population, can be distinguished by taking the mean intensity minus the background intensity at each emission wavelength and then dividing the two values to obtain the range for that particular ratio.

In one embodiment, two separate populations of NIH 3T3 mouse fibroblast cells were encoded by labeling cell membranes with either a 1:5 or a 5:1 ratio of PHK67 and PHK26 dyes. Each cell population comprised approximately 125 encoded cells. The cell populations were illuminated at 490 nm and 551 nm excitation wavelengths and emitted fluorescent intensity ratios were measured at 502 nm and 567 nm. Measurements of the average intensity ratio for each dye ratio were made over a two day period. The initial average intensity ratio for the 1:5 PKH67/PKH26 ratio was 0.0863 and the ratio for the 5:1 dye ratio was 0.7014. The final average intensity ratio for the 1:5 ratio was 0.2655 and for the 5:1 ratio it was 0.9090 indicating that the ratios are reasonably stable, even where cell splitting has occurred, and that dye ratio encoded cell populations remain distinguishable with time. Thus, dye ratios can provide a useful alternative encoding mechanism for

identifying and locating cell populations which are randomly dispersed on a biosensor array of the present invention.

F. Indicator Dyes

The optical responses of individual cells and cell populations to chemical or biological stimuli are typically interrogated and detected by coupling individual cells with appropriate indicators which may be either fluorophores, chromophores, stains or a dye compounds. Any suitable indicator or combinations of indicators may be utilized provided the indicator does not compromise cell response.

For example, conventional cell fluorophore probes such as fluoresceins, rhodamines, naphthalimides, phycobiliproteins, nitrobenzoxadiazole may be utilized. Alternatively, permeant or impermeant cell membrane potential indicators, ion indicators, reactive oxygen indicators and pH indicators may be employed. By way of example a number of indicators which would have particular utility for the biosensor array of the present invention are listed in Tables 3 through 8 together with their characteristic excitation and emission wavelengths. A particularly useful reference for selecting appropriate indicators is R.P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (6^{th} ed.), Molecular Probes Inc.(Eugene, OR, 1996).

In one embodiment, indicators may be incorporated directly into the cell by attachment to the cell membrane, by absorption into the cell cytoplasm by membrane permeant indicators, or by microinjection into the cell cytoplasm. In an alternative embodiment, ultrafine fluorescing microspheres, such as Molecular Probes FluoSpheres™ , are ingested by the cells and are employed as indicators. In another environment, indicators are added to the culture media fluid contained within the microwells. In an alternative embodiment, indicators may be attached to the surface of the microwells by a conventional

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TABLE 3

Parent Fluorophore	pH Range	Typical Measurement
SNAFL indicators	7.2–8.2	Excitation ratio 490/540 nm or emission ratio 540/630 nm
SNARF indicators	7.0–8.0	Emission ratio 580/640 nm
HPTS (pyranine)	7.0–8.0	Excitation ratio 450/405 nm
BCECF	6.5–7.5	Excitation ratio 490/440 nm
Fluoresceins and carboxyfluoresceins	6.0–7.2	Excitation ratio 490/450 nm
Oregon Green dyes	4.2–5.7	Excitation ratio 510/450 nm or excitation ratio 490/440 nm
Rhodols (including NERF dyes)	4.0–6.0	Excitation ratio 514/488 nm or excitation ratio 500/450 nm
LysoSensor probes	3.5–8.0 *	Excitation ratio 340/380 nm †

* Depends on pK_a of selected probe; see Table 23.2 for pK_a of each LysoSensor probe. † Applies to L-7545 only. Other LysoSensor probes allow single excitation and emission measurements only; see Table 23.2 for wavelengths.

Table 23.2 Summary of the pH response of our LysoSensor probes.

Cat #	LysoSensor Probe	Abs/Em * (nm)	pK_a †	Useful pH Range †
L-7532	LysoSensor Blue DND-192	374/424	7.5	6.5–8.0
L-7533	LysoSensor Blue DND-167	373/425	5.1	4.5–6.0
L-7534	LysoSensor Green DND-153	442/505	7.5	6.5–8.0
L-7535	LysoSensor Green DND-189	443/505	5.2	4.5–6.0
L-7545	LysoSensor Yellow/Blue DND-160	384/540 ‡ 329/440 §	4.2	3.0–5.0

* Absorption (Abs) and fluorescence emission (Em) maxima, at pH 5; values may vary somewhat in cellular environments. † All pK_a values were determined *in vitro*; values may be different in cells. ‡ at pH 3; § at pH 7.

Table 23.4 Reactive pH indicator dyes.

pH Indicator	Preferred Reactive Form
BCECF	BCECF (B-1151, see Section 23.2) *
Carboxyfluorescein	5-(and-6)-carboxyfluorescein, succinimidyl ester (C-1311, see Section 1.3)
Cl-NERF	Cl-NERF (C-6831, see Section 23.3) *
Dichlorofluorescein	2',7'-dichlorofluorescein-5-isothiocyanate (D-6078, see Section 1.5)
Dimethylfluorescein	5-(and-6)-carboxy-4',5'-dimethylfluorescein (C-366, see Section 23.2) *
DM-NERF	DM-NERF (D-6830, see Section 23.3) *
Naphthofluorescein	5-(and-6)-carboxynaphthofluorescein, succinimidyl ester (C-653)
Oregon Green 488	Oregon Green 488 carboxylic acid, succinimidyl ester (O-6147, O-6149)
Oregon Green 500	Oregon Green 500 carboxylic acid, succinimidyl ester (O-6136)
Oregon Green 514	Oregon Green 514 carboxylic acid, succinimidyl ester (O-6139)
Rhodol Green	Rhodol Green carboxylic acid, succinimidyl ester (R-6108)
SNAFL-1	5-(and-6)-carboxy SNAFL-1, succinimidyl ester (C-3061)
SNAFL-2	5-(and-6)-carboxy SNAFL-2, succinimidyl ester (C-3062)
SNARF-1	5-(and-6)-carboxy SNARF-1 (C-1270, see Section 23.2) *

* Carboxylic acids require activation with EDAC/NHSS before reaction with amines (see Section 1.1).

conjugation to dextrans. However, coupling of pH indicators to these relatively inert polysaccharides changes several other properties of the dyes:

- The conjugates have high water solubility and must be loaded into cells by microinjection, patch-clamp techniques, scrape loading, endocytosis, liposome fusion or comparable techniques (see Table 15.1 in Chapter 15).

TABLE 4

Table 23.3 Molecular Probes' pH indicator dextrans, in order of decreasing pK_a

Dye	Cat #	pK_a*	Measurement Wavelengths	Application Notes
SNAFL	D-3301, D-3302	~7.8	Excitation ratio 490/540 nm detected at 580 nm Emission ratio 540/630 nm excited at 514 nm	• Acidic form has the higher quantum yield (see Figure 23.7) • Fluorescence increases in acidic organelles
SNARF	D-3303, D-3304	~7.5	Emission ratio 580/640 nm excited at 514 or 488 nm	• Best conjugate for ratiometric emission measurements, with spectra similar to carboxy SNARF-1 (see Figure 23.6)
HPTS	D-7179	~7.0	Excitation ratio ~470/~450 nm detected at 530 nm	• Spectra of dextran conjugate are identical to HPTS, relative to free dye
BCECF	D-1878, D-1879, D-1880	~7.0	Excitation ratio 490/440 nm detected at 530 nm	• Best conjugate for ratiometric excitation measurements, with spectra similar to BCECF (see Figure 23.8)
Fluorescein	D-1821, D-1823, D-1844, D-1890, D-1905	~6.4	Excitation ratio 490/450 nm detected at 520 nm	• Fluorescence is strongly quenched upon uptake into acidic organelles (see Figure 23.2B)
Fluorescein and tetramethylrhodamine	D-1950, D-1951	~6.4	Excitation ratio 495/555 nm detected at 580 nm	• Conjugate incorporating both pH sensitive and pH insensitive fluorescent dyes (see Figure 23.11)
Rhodol Green	D-7148, D-7150	~5.6	Excitation ratio 500/450 nm detected at 530 nm	• High photostability • Most useful below pH 6 (see Figure 23.15)
DM-NERF	D-3319, D-3320	~5.4	Excitation ratio 510/450 nm detected at 540 nm	• Useful at a higher pH than CI-NERF dextrans (see Figure 23.12)
Oregon Green 488	D-7170, D-7172	~4.7	Excitation ratio 494/440 nm detected at 520 nm	• Good photostability • Optimum pH sensitivity range between that of DM-NERF and CI-NERF
Oregon Green 514	D-7174, D-7176	~4.7	Excitation ratio 510/450 nm detected at 530 nm	• Excellent photostability • Optimum pH sensitivity range between that of DM-NERF and CI-NERF (see Figure 23.11)
CI-NERF	D-3324, D-3325	~3.8	Excitation ratio 510/450 nm detected at 540 nm	• Useful at a lower pH than DM-NERF dextrans (see Figure 23.12)

* pK_a values are those determined for the free dyes. Actual values for dextran conjugates may differ by up to ~0.3 pH units and may vary between production lots. † Ratiometric emission measurements at 530/580 nm (with excitation at 495 nm) are also possible in principle, however the response may be complicated by fluorescence resonance energy transfer.

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TABLE 5

Table 22.1 *Summary of fluorescent Ca^{2+} indicators available from Molecular Probes*

Ca^{2+} Indicator	Salt *	AM Ester †	Dextran ‡	Mode §	K_d (nM) ¶	Notes
Bis-fura	B-6810			Ex 340/380	370	1
BTC	B-6790	B-6791		Ex 400/480	7000	2
Calcium Green-1	C-3010	C-3011, C-3012	C-6765, C-3713, C-3714, C-6766	Em 530	190	3,4
Calcium Green-2	C-3730	C-3732		Em 535	550	3,5
Calcium Green-5N	C-3737	C-3739		Em 530	14,000	3
Calcium Orange	C-3013	C-3015		Em 575	185	2
Calcium Orange-5N	C-6770	C-6771		Em 580	20,000	2
Calcium Crimson	C-3016	C-3018	C-6824, C-6825	Em 615	185	2
Fluo-3	F-1240, F-3715	F-1241, F-1242		Em 525	390	3,4
Fura 2	F-1200, F-6799	F-1201, F-1221, F-1225	F-6764, F-3029, F-3030	Ex 340/380	145	2
Fura Red	F-3019	F-3020, F-3021		Ex 420/480	140	2,6,7
Indo-1	I-1202	I-1203, I-1223, I-1226	I-3032, I-3033	Em 405/485	230	2
Mag-fura-2	M-1290	M-1291, M-1292		Ex 340/380	25,000	2
Mag-fura-5	M-3103	M-3105		Ex 340/380	28,000	2
Mag-indo-1	M-1293	M-1295	M-6907, M-6908	Em 405/485	35,000	2,8
Magnesium Green	M-3733	M-3735		Em 530	6000	3
Oregon Green 488 BAPTA-1	O-6806	O-6807	O-6798, O-6797	Em 520	170	3
Oregon Green 488 BAPTA-2	O-6808	O-6809		Em 520	580	3,9
Oregon Green 488 BAPTA-5N	O-6812	O-6813		Em 520	20,000	3
Quin-2	Q-1287	Q-1288, Q-1289		Em 495	60	2,10
Rhod-2	R-1243	R-1244, R-1245		Em 570	570	2
Texas Red–Calcium Green			C-6800	Em 535/615	370	11

* Catalog number for cell-impermeant salt. † Catalog number for cell-permeant AM ester. ‡ Catalog number for dextran conjugate. § Measurement wavelengths (in nm), where Ex = Fluorescence excitation and Em = Fluorescence emission. Indicators for which a pair of wavelengths are listed have dual-wavelength ratio-measurement capability. ¶ Ca^{2+} dissociation constant; measured at Molecular Probes *in vitro* at 22°C in 100 mM KCl, 10 mM MOPS pH 7.2, unless otherwise noted. K_d values depend on temperature, ionic strength, p and other factors, and are usually higher *in vivo*. Because indicator dextrans are intrinsically polydisperse and have variable degrees of substitution, lot-specific K_d values are printed on val in most cases.

1. Similar Ca^{2+}-dependent fluorescence response to fura-2 but ~75% greater molar absorptivity. 2. AM ester form is fluorescent (a major potential source of error in Ca^{2+} measurements 3. AM ester form is nonfluorescent; 4. Calcium Green-1 is more fluorescent than fluo-3 in both Ca^{2+}-bound and Ca^{2+}-free forms. Magnitude of Ca^{2+}-dependent fluorescence increase is greater for fluo-3; 5. Larger Ca^{2+}-dependent fluorescence increase than Calcium Green-1; 6. Can also be used in combination with fluo-3 for dual-wavelength ratio measurements. Ex = 488 nm, Em = 530/670 nm (Cytometry 17, 135 (1994); Cell Calcium 14, 359 (1993)). 7. Mag-Fura Red has similar spectral properties, with K_d for Ca^{2+} = 17 µM; 8. K_d determine 100 mM KCl, 40 mM HEPES, pH 7.0 at 22°C (Biochem Biophys Res Comm 177, 184 (1991)); 9. Larger Ca^{2+}-dependent fluorescence increase than Oregon Green 488 BAPTA-1 10. K_d determined in 120 mM KCl, 20 mM NaCl, pH 7.05 at 37°C (Meth Enzymol 172, 230 (1989)); 11. This indicator consists of Ca^{2+}-sensitive Calcium Green-1 and Ca^{2+}-insensitive Texas Red dyes linked to the same dextran.

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TABLE 6

O_2 Indicato

1,3-diphenylisobenzofuran - singlet oxygen in membranes and in cells
Ab - 409 Em - 476

rubrene

9,10-diphenylanthracene Ab - 391 Em - 405

trans-1-(2'-Methoxyvinyl)pyrene - excimer detection
Ab - 362 Em - 401

Nitrite Indicators

2,3-diaminonaphthalene 10nM - 10μM detection
Ab - 340 Em - 377

NO Indicators

luminol Ab 355 Em - 411

dihydrorhodamine - NO reacts w/ superoxide or hydrogen peroxide to produce peroxynitrite anion which oxidizes the dye
Ab - 289 Em - none

Ca^{2+} Indicators

Bis-fura	340/380		
Calcium Green	Em 530	- visible light excitation	
Fura 2	340/380	uv excitable	ratiometric Ca^{2+} indicator
Indo 1	405/485	uv excitable	ratiometric Ca^{2+} indicator
Fluo-3	Em 525	visible light excitation	
Rhod-2	Em 570	visible light excitation	

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TABLE 7

Mg^{2+} Indicators

Mag-Fura-2	340/380	UV excitable
Mag-Fura-5	340/380	
Mag-Indo-1	405/485	
Magnesium Green	475/Em 530	
Magnesium Orange	545ex	

Zn^{2+} Indicators

Newport Green 506/535

TSQ N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide

-used to localize Zn^{2+} pools in the CNS. 334/385

Cu^{+} Indicators

Phen Green 492/517

-also detects Fe^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+}, Ni^{2+} at submicromolar concentrations

Na^{+} Indicators

SBFI Ab339/Em 585 - UV ex

SBFO 354/575

Sodium Green 506/535 - visible light ex. -employs crown ether

K^{+} Indicators

PBFI Ab 336/Em 557

Cl^{-} Indicators

SPQ	344/443	follow Cl^{-} transport w/ ms time resolution
MQAE	350/460	high fluorescence quantum yield

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TABLE 8

Fc OxyBurst Green
" Orange 550/574 } detects oxidative burst that occurs in phagoracuoles

Dichlorodihydrofluorescein Diacetate (H2DCFDA) em 504/529 ex - monitors oxidative activity - cell permeant

Dihydrorhodamine 123 em 507/529 ex - investigates reactive oxygen intermediates produced by phagocytes.

Fura Red™ - calcium indicator - ~~measures oxidative bursts and~~ calcium fluxes

Dihydroethidium em 518/605 ex — respiratory burst in phagocytes.

Aequorin em 430/465 ex — Bioluminescent Ca^{2+} indicator

silanization treatment for bonding the indicator to the glass surface. In one embodiment, natural or genetically engineered cell lines which exhibit chemiluminescence, bioluminesence, or color changes when stimulated are used alone without need for a separate indicator since they produce intrinsic optical responses. Examples of such cells include green fluorescent protein mutants. In other embodiments, cells which express enzymes are employed with a reagent which enables an optical response, such as the generation of a fluorescent product. For example when luciferase is expressed by a cell in the presence of luciferen, the cell produces a fluorescence response when biologically stimulated.

In alternative embodiments, multiple indicators may be employed within a cell array, within a cell population, or within individual cells so as to provide for simultaneous monitoring of cell responses to a variety of different chemical or biological materials. Where two or more indicators are utilized, dyes with spectral separation of narrow spectral bandwidths are particularly useful, for example Molecular Probes BODIPY™ dyes.

G. Optical Measurements

Individual cells and populations of cells within the biosensor array of the present invention may be optically interrogated and cell responses may be monitored by employing conventional optical components known to one of ordinary skill in the art. Where external optical stimulation of cells is required to elicit an optical response, conventional light sources such as arc lamps, photodiodes, or lasers may be employed for excitation light energy. Cell responses may be monitored by conventional detectors such as photomultiplier tubes, photodiodes, photoresistors or charge coupled device (CCD) cameras. Conventional optical train components, such as lenses, filters, beam splitters, dichroics, prisms and mirrors may be employed to convey light to an from such light sources and detectors either to discrete substrate sites or through optical fiber strands to microwells that contain individual cells. The principal requirement for any particular optical apparatus configuration that is employed in optical measurements is that the combination of optical components provide for optically coupling the cells in the array to detectors and light sources. While a particular apparatus configuration that was employed in experimental optical measurements is described below, other configurations may also be employed which are functionally equivalent and appropriate suited for a particular measurement requirement.

The instrumentation used for fluorescence measurements is a modified Olympus (Lake Success, NY) epifluorescence microscope which was converted from a vertical to a horizontal configuration. A schematic diagram of the measurement system 100 is shown in Fig. 9. White light from a 75W xenon lamp 110 is collimated by a condensing lens 112, passed through an excitation filter 120, reflected by a dichroic mirror 130, and focused onto the proximal end 210 of an imaging fiber 200 with a microscope objective 140. A neutral density filter 122 may be employed for adjustment of excitation light intensity. The imaging fiber 200 is precisely positioned by an x-y micropositioner 150, available from Spindler and Hoyer (Milford, MA), and a microscope z-translation stage 152. Excitation light is transmitted through the fiber 200 to the biosensor array 300 at the distal fiber end 212. The emitted fluorescence light from the biosensor array is returned through the fiber 200, through the dichroic mirror 130, filtered through an emission filter 160, and detected by a PXL™ Photometrics (Tuscon, AZ) charge coupled device (CCD) camera 170. A magnification lens 165 may be employed if necessary. Data is processed on an Apple Power Macintosh 440 (Sunnyvale, CA) desktop computer 180 using IPLab 3.0 image processing software, commercially available from Signal Analytics (Vienna, VA).

While a bench-top measurement system was utilized for these measurements, in one embodiment, a compact portable measurement system may be assembled from conventional optical and electronic components.

H. Biosensor Array Applications

The biosensor, biosensor array, sensing apparatus and sensing method of the present invention can be applied to a large variety of conventional assays for screening and detection purposes. The biosensor may be configured for virtually any assay and offers a distinct advantage for high throughput screening where a plurality of encoded cell populations, which have utility in particular assays or are genetically engineered cell to provide unique responses to analytes, may be employed in a single sensor array for conducting a large number of assays simultaneously on a small sample. The biosensor array thus provides both for tremendous efficiencies in screening large combinatorial libraries and allows conduction of a large number of assays on extremely small sample volumes, such as biologically important molecules synthesized on micron sized beads. The biosensor of the present invention can be applied to virtually any analyte measurements where there is a detectable cell response to the analyte due to biological stimulation.

The biosensor array and method of the present invention utilizes the unique ability of living cell populations to respond to biologically significant compounds in a characteristic and detectable manner. Since the selectivity of living cells for such compounds has considerable value and utility in drug screening and analysis of complex biological fluids, a biosensor which makes use of the unique characteristics of living cell populations offers distinct advantages in high throughput screening of combinatorial libraries where hundreds of thousands of candidate pharmaceutical compounds must be evaluated. In addition, such a biosensor and sensing method can be utilized for either off-line monitoring of bioprocesses or in situ monitoring of environmental pollutants where the enhanced sensitivity of living cells to their local environment can be exploited.

Thus, the present invention provides methods for detecting the responses of individual cells to analytes of interest. By "analyte of interest" or "target analyte" or "candidate bioactive agent" or "candidate drug" or grammatical equivalents herein is meant any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc., to be tested for the ability to directly or indirectly altering a cellular phenotype, including its optical properties. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level of detection.

Analytes encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Analytes comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial,

fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

In a preferred embodiment, the candidate bioactive agents are naturally occuring proteins or fragments of naturally occuring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eucaryotic proteins may be made for screening in the methods of the invention. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

The peptides may be digests of naturally occuring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of nucleic acid binding domains, the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

As described above generally for proteins, nucleic acid candidate bioactive agents may be naturally occuring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eucaryotic genomes may be used as is outlined above for proteins.

"Nucleic acids" in this context includes both DNA and RNA, and nucleic acid analogs including PNA.In a preferred embodiment, the candidate bioactive agents are organic chemical moieties, a wide variety of which are available in the literature.

While the examples below provide a variety of specific assays which may be useful in configuring and employing the biosensor array and method of the present invention, they are not intended to limit either the scope of applications envisioned or the broad range of sensing methods which can be employed with a plurality of cell populations with the biosensor of the present invention.

In one embodiment, the biosensor array can be employed for remotely monitoring redox states of individual cells or cell populations in bioprocesses. For example, NADH dependent fluorescence can be measured in bacteria, fungi, plant or animal cells. NAD(P) /NAD(P)H can be measured to monitor changes from aerobic to anaerobic metabolism in fermentation processes using the method disclosed by Luong, et al., in Practical Fluorescence, G. Guilbault ed., Marcel Dekker (New York, 1990).

Alternatively, the biosensor array may be employed for in situ monitoring of cellular processes in response to environmental contaminants by incorporating the method disclosed by Hughes, et al., *Analytica Chimica Acta* 307:393(1995) to provide for distinguishable cell population responses within an array. In this method, micron-sized spheres, impregnated with a fluorophore and modified on the surface with a fluorogenic enzyme probe, are ingested by cells and enzymatic activity occurs at the sphere surface, producing a detectable fluorescent signal.

In yet another embodiment, the biosensor array can be employed with genetically engineered bioluminescent bacteria for in situ monitoring and optical sensing of metallic compounds. For example, cell population responses to antimonite and arsenite may be utilized by incorporating the method disclosed in Ramanathan, et al., *Anal Chem.* 69:3380(1997) into cell populations within the biosensor array. With this method, cell plasmid regulates the expression of bacterial luciferase depending on the metal concentration.

In another embodiment, the cell populations within the biosensor array can be encoded with ATP dependent luminescent proteins, for example firefly luciferase, which are injected into rat hepatocytes for pathological studies according to the method disclosed by Koop, et al., *Biochem. J.* 295:165(1993). These cells exhibit a decrease in cytoplasmic ATP when exposed to pathological insults and changes in fluorescence directly relate to the extent of metabolic poisoning in the cell.

In one embodiment, the cell populations within the biosensor array can be encoded with green fluorescent protein [see T. Gura, *Science* 276:1989(1997); Niswender, et al., *J. Microscopy* 180(2):109(1995); Cubitt, et al., *TIBS* 20:448(1995); Miyawaki, et al., *Nature* 388:882(1997)]. Several genetically-engineered mutants of GFP are available which have distinguishable fluorescence emission wavelengths. These proteins have additional utility as fluorescing indicators of gene expression and Ca^{+} levels within cells.

In an additional embodiment, the biosensor array can be used in measurements of cell proliferation by in situ monitoring of calcium levels and calcium oscillations in single cells using fluorescent markers, such as aequorin or fura-2, according to the method disclosed by Cobbold, et al., *Cell Biology* 1:311(1990).

As will be appreciated by those in the art, the assays of the invention may be run in a wide variety of ways and for a wide variety of purposes. For example, the cells may be used as a detection system for a particular analyte; the cells undergo a characteristic optically detectable change in the presence of a particular analyte. Alternatively, the cells may be used to screen drug candidate libraries for the ability to alter a cellular phenotype that is optically detectable. For example, the expression of a therapeutically relevant cell surface receptor may be increased such that the receptor can now bind a fluorescent ligand; similarly a therapeutically relevant enzyme may now be activated such that a fluorescent reaction product is generated. In this way any modulation, incluing both increases and descreases, may be monitored. Similarly, the use of reporter genes such as green fluorescent proteins and derivatives thereof facilitates high throughput screening for relevant analyte interactions, through the use of inducible promoters, for example.

Generally, in a preferred embodiment, a candidate bioactive agent is added to the cells prior to analysis, and the cells allowed to incubate for some period of time. By "administration" or "contacting" herein is meant that the candidate agent is added to the cells in such a manner as to allow the agent to act upon the cell, whether by uptake and intracellular action, or by action at the cell surface. In some embodiments, nucleic acid encoding a proteinaceous candidate agent (i.e. a peptide) may be put into a viral construct such as a retroviral construct and added to the cell, such that expression of the peptide agent is accomplished; see PCT US97/01019, hereby expressly incorporated by reference.

Once the candidate agent has been administered to the cells, the cells can be washed if desired and are allowed to incubate under preferably physiological conditions for some period of time.

The reactions outlined herein may be accomplished in a variety of ways, as will be appreciated by those in the art. Components of the reaction may be added simultaneously, or sequentially, in any order. In addition, the reaction may include a variety of other reagents may be included in the assays. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal detection, and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used.

In general, at least one component of the assay is labeled. By "labeled" herein is meant that the compound is either directly or indirectly labeled with a label which provides a detectable signal, e.g. radioisotope, fluorescers, enzyme, antibodies, particles such as magnetic particles, chemiluminescers, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

Once the assay is run, the data is analyzed to determine the experimental outcome, i.e. either the presence or absence of a target analyte, the effect of a candidate agent on a cellular phenotype, etc. This is generally done using a computer.

In this way, bioactive agents are identified. Compounds with pharmacological activity are able to alter a cellular phenotype. The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host, as previously described. The agents may be administered in a variety of ways, orally, parenterally e.g., subcutaneously, intraperitoneally, intravascularly, etc. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up

compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

1. A biosensor for detecting the response of individual cells to at least one analyte of interest comprising:

a) a substrate comprising a plurality of discrete sites; and

b) a plurality of cells each dispersed at one of said discrete sites, wherein each cell is encoded with at least one optically interrogatable material.

2. The biosensor of claim 1 wherein said substrate comprises a fiber optic array comprising a plurality of fibers, each fiber having a distal end and a proximal end, and said discrete sites comprise a plurality of microwells each formed on an end surface ofsaid distal end, and each one of said plurality of cells is dispersed in one of said microwells, wherein each of said cells is optically coupled and in optical
communication with a distal end of at least one of said fibers in said array.

3. The biosensor of claims 1 or 2 wherein said sites have an internal diameter ranging from about 1 um to about 200 um, a depth ranging from about 0.25 um to about 200 um, and a volume ranging from about 1 fL to about 5 nanoliters.

4. The biosensor of claims 2, or 3 wherein said fibers further comprise a fiber core having a diameter ranging from about 1 um to about 200 um.

5. The biosensor of claims 1, 2, 3 or 4 wherein a surface of said sites are coated with a biologically compatible material.

6. The biosensor of claims 1, 2, 3, 4, or 5 wherein an indicator compound is attached or inserted into at least one of said cells.

7. The biosensor of claims 1, 2, 3, 4, 5, or 6 wherein said plurality of cells comprises at least two cell populations.

8. The biosensor of claim 7 wherein each cell population is encoded withat least one optically interrogatable materials selected from the groupconsisting of a dye, a fluorophore, a chromophore, a chemiluminescent compound and a bioluminescent compound.

9. An apparatus for detecting the response of individual cells to at least one analyte of interest comprising:

a) a biosensor array comprising:

i) a substrate comprising a plurality of discrete sites; and

ii) a plurality of cells each dispersed at one of said discrete sites, wherein each cell is encoded with at least one optically interrogatable material.

b) a detector means optically coupled to and in optical communication with said discrete sites on said substrate, said detector means capable of detecting an optical response of said cells dispersed in said discrete sites to an analyte.

10. The apparatus of claim 9 wherein

said substrate comprises a fiber optic array comprising a plurality of fibers, each fiber having a distal end and a proximal end;

said discrete sites comprise a plurality of microwells each formed on an end surface ofsaid distal end of said fibers;

each one of said plurality of cells is dispersed in one of said microwells; and

said detector means is optically coupled to and in optical communication with a proximal end of said fibers for detecting an optical response of said cells at said distal fiber ends.

11. An apparatus according to claims 9 or 10, further comprising an excitation light energy means coupled to an end of said fibers.

12. An apparatus according to claims 9, 10 or 11 further comprising an image capturing means for capturing images of detected optical responses from a plurality of said cells.

13. The apparatus of claim 12 wherein said image capturing means comprises a charge coupled device or CCD camera.

14. A method for detecting the response of individual cells to at least one analyte of interest comprising:

a) providing a biosensor array comprising:

i) a substrate comprising a plurality of discrete sites; and

ii) a plurality of cells each dispersed at one of said discrete sites, wherein each cell is encoded with at least one optically interrogatable material.

b) contacting said biosensor array with an analyte of interest; and

c) detecting an optical response of said cells.

15. The method of claim 14 wherein

a) said providing a biosensor array comprises:

i) a substrate comprising a fiber optic array comprising a plurality of fibers, each fiber having a distal end and a proximal end;

ii) said discrete sites comprise a plurality of microwells each formed on an end surface of said distal end of said fibers;

iii) each one of said plurality of cells is dispersed in one of said microwells; and

b) said detecting comprises providing a detecting means optically coupled to and in optical communication with a proximal end of said fibers for detecting an optical response of said cells at said distal fiber ends.

16. A method according to claims 14 or 15 wherein said analyte of interest comprises an optically detectable label.

17. A method according to claims 14, 15, or 16 wherein said biosensor is contacted with a plurality of analytes.

18. A method of making a biosensor array for detecting the response of individual cells to at least one analyte of interest comprising:

a) providing a substrate comprising a plurality of discrete sites; and

b) contacting said substrate with a plurality of cells sucht that each cell is dispersed at one of said discrete sites.

19. A method according to claim 18 wherein

a) said providing a substrate comprises a fiber optic array comprising

i) a plurality of fibers, each fiber having a distal end and a proximal end; and

ii) a plurality of microwells each formed on an end surface of said distal end; and

b) said contacting comprises contacting said fiber optic array with a plurality of cells such that each cell is inserted into one of said microwells.

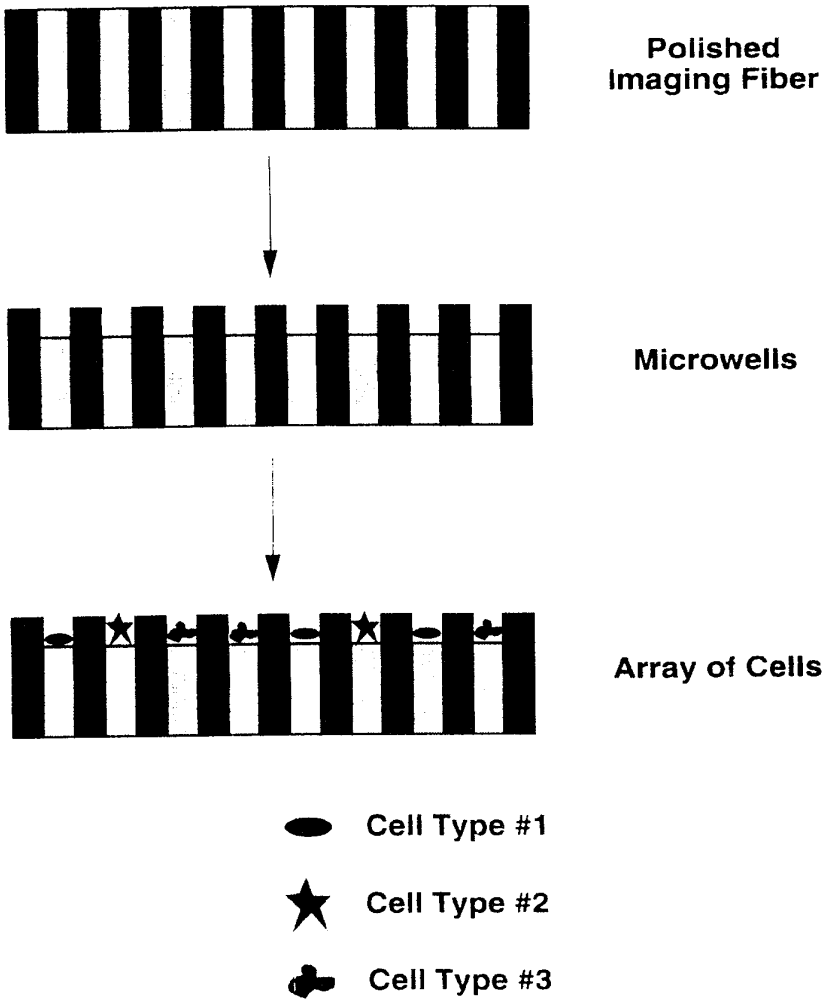
20 A method according to claims 18 or 19 wherein said plurality of cells comprises at least two cell populations.

21. A method according to claim 18, 19, or 20 wherein each cell population is encoded with at least one optically interrogatable material selected from the group consisting of a dye, a fluorophore, a chromophore, a chemiluminescent compound and a bioluminescent compound.

22. A method according to claim 21 further comprising indexing the location of individual cells in said array by emitted light energy produced by illuminating said encoded cells with excitation light energy.

Fig. 1

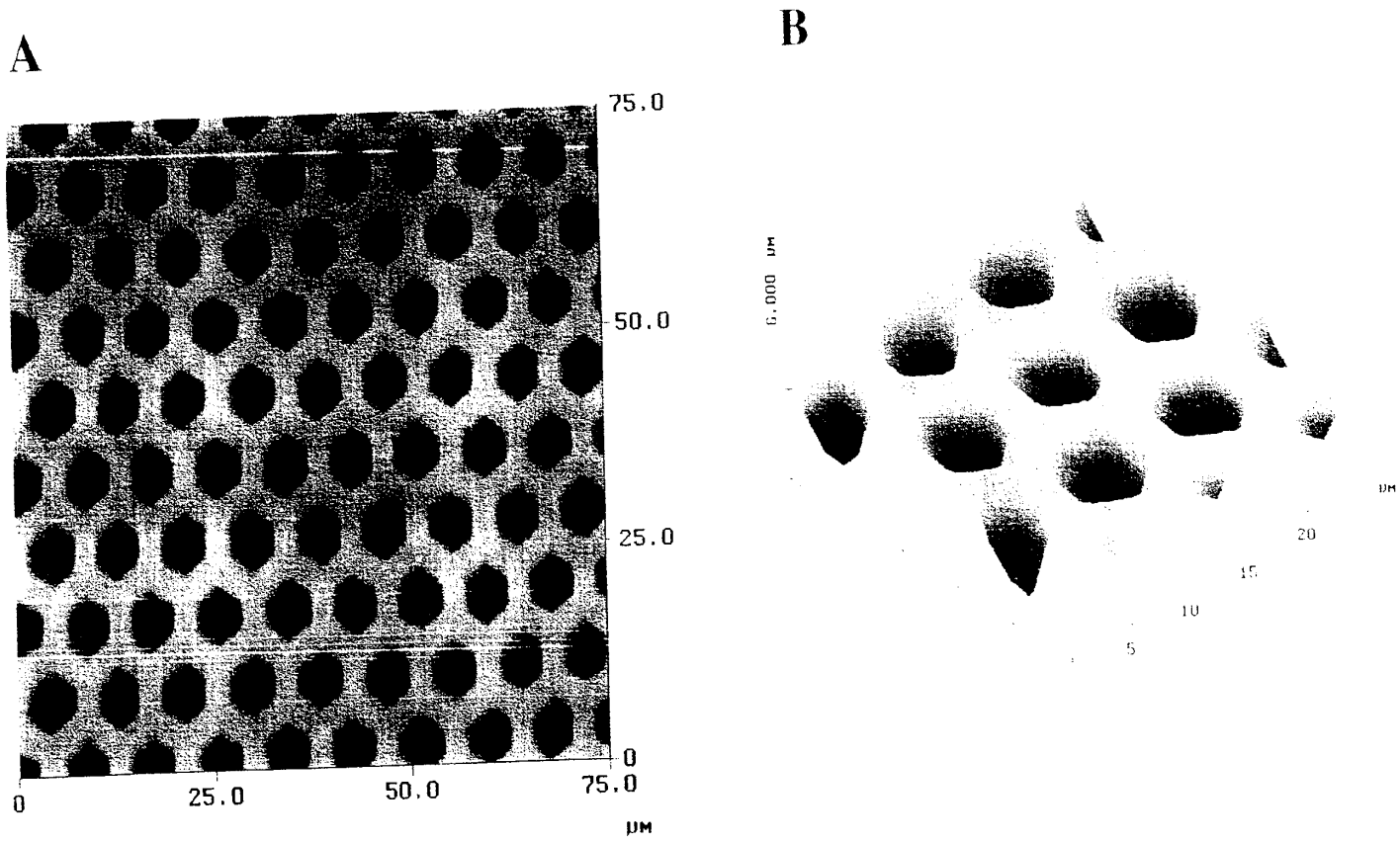
Cells in Wells Concept



Schematic concept of an optical sensor array of cultured cells. A polished optical imaging fiber is chemically etched to produce an array of micron-sized wells at the distal face of the fiber. Cells suspended in media above the array of wells settle into the wells and adhere to the well bottom. The wells are etched such that each well accommodates one cell. Differences in core diameters and etch times can accommodate a wide variety of cell types and sizes.

Figs 2a-b

Fiber Optic Microwell Array

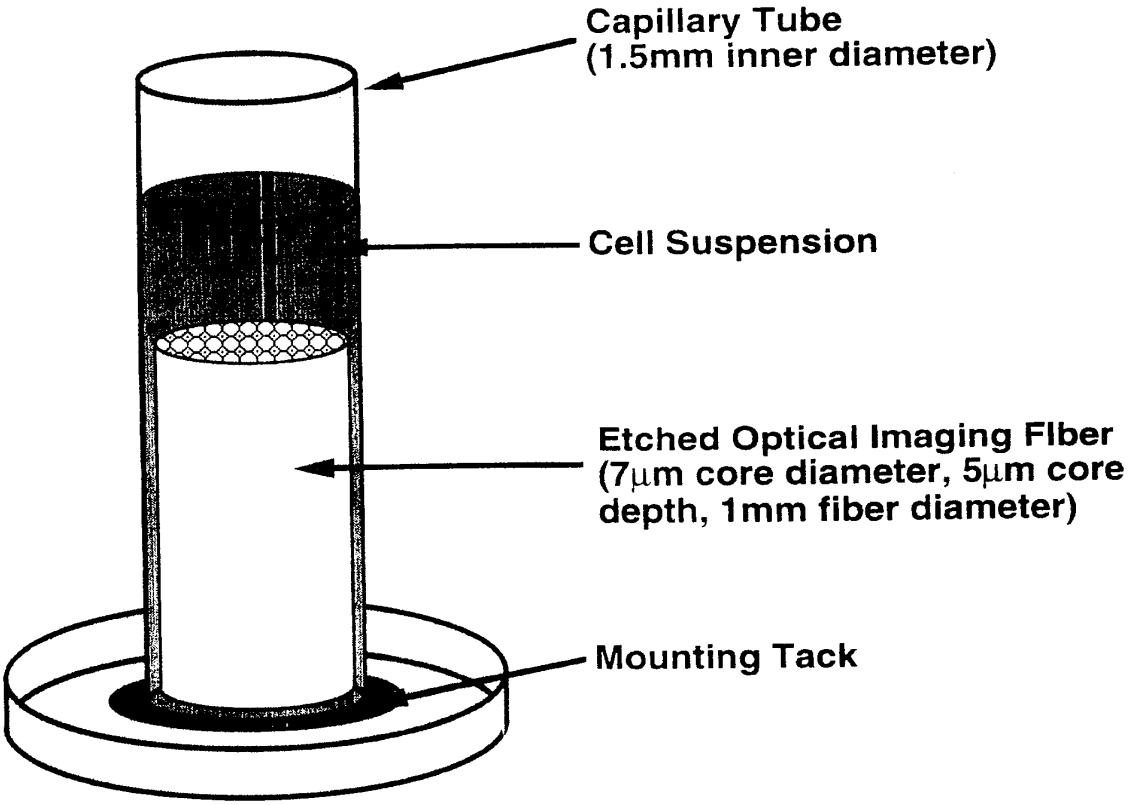


Atomic Force Micrograph (AFM) images of an (A) 75μm x 75μm 2-D array and a (B) 25μm x 25μm 3-D array of etched microwells. An array of micron-sized wells is formed at the distal tip of an optical imaging fiber by a wet-etch process that takes advantage of differences in core and clad etch rates.

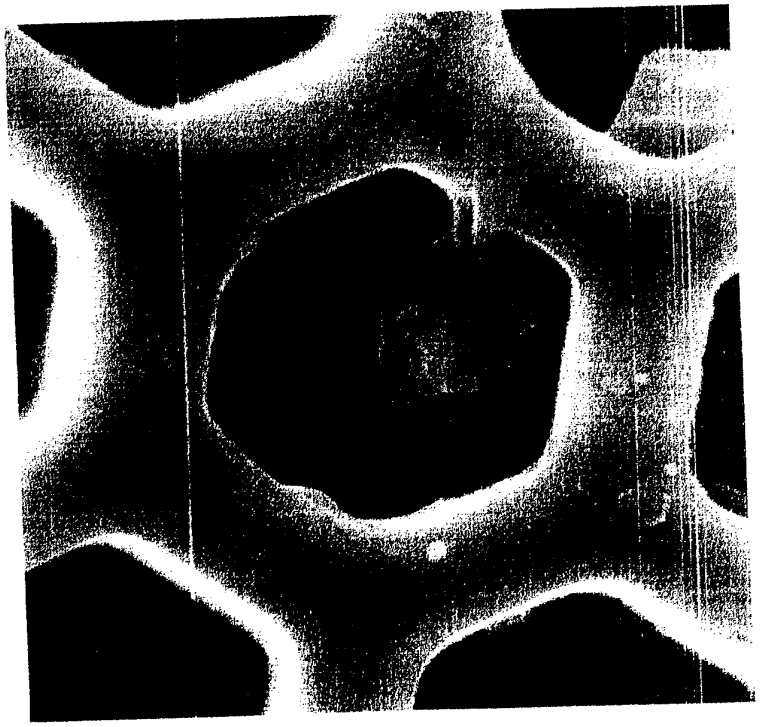
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Cells in Wells Set-up

Fig.3



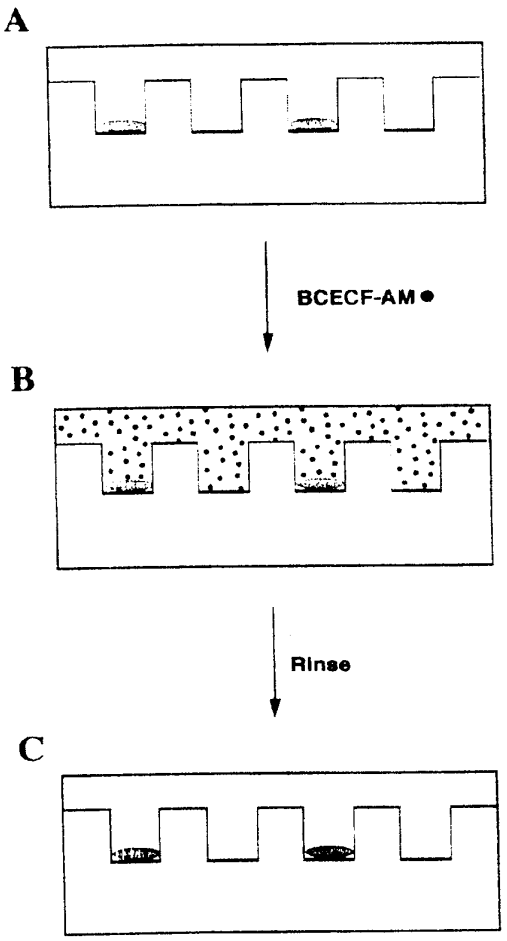
Cells are deposited into wells at the distal face of an optical imaging fiber. Cells are trypsanized using aseptic technique to remove them from the cell culture dish and suspended in growth media. A portion of the cell suspension is concentrated using centrifugation and added to a 1.5mm diameter capillary tube. The etched face of the fiber id inserted into the tube and secured in place with a thin strip of laboratory film. the tube/fiber set-up is then incubated in the vertical position for 12-15 hours for cell adhesion to occur.



Scanning Electron Micrograph (SEM) of a single NIH 3T3 mouse fibroblast cell in an etched microwell. Each well has a diameter of 7μm, a depth of 5μm, and a volume of ~145fl.

Fig. 4

Fig. 5



Schematic representation of the response of cells adhered in wells to the acetoxymethyl (AM) ester form of the fluorscein derivative, bis-carboxyethyl bis-carboxy fluorscein (BCECF). The array of cells (A) is incubated in a solution of BCECF-AM (B) and then rinsed thoroughly. The non-fluorescent BCECF-AM passively enters the cell. Once inside the cell, the lypophillic blocking groups are cleaved by the non-specific esteraces resulting in an increase in fluorescent intensity (C).

Fig. 6

PKH26 Labeled NIH 3T3 Mouse Fibroblast Cells (551nm / 567nm)

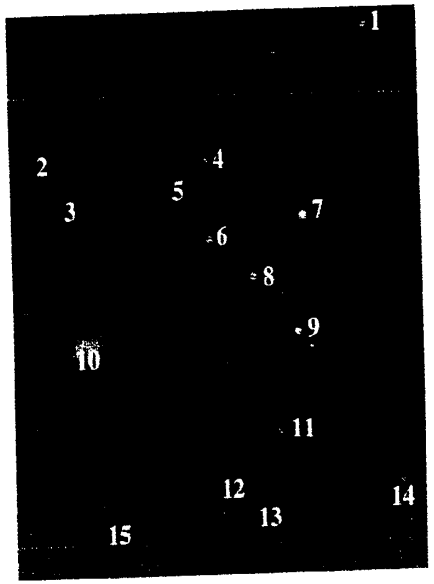
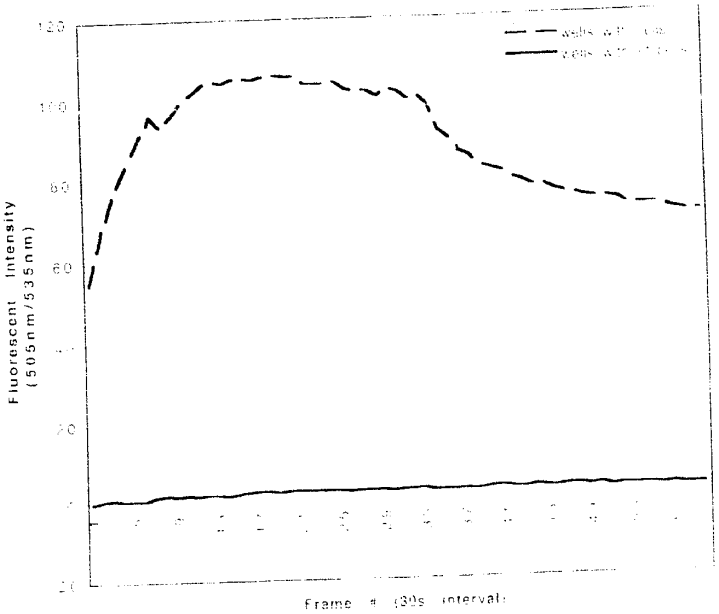


Fig. 7

Average Response of 15 Cells to 0.001μM BCECF-AM (after 1 minute incubation)

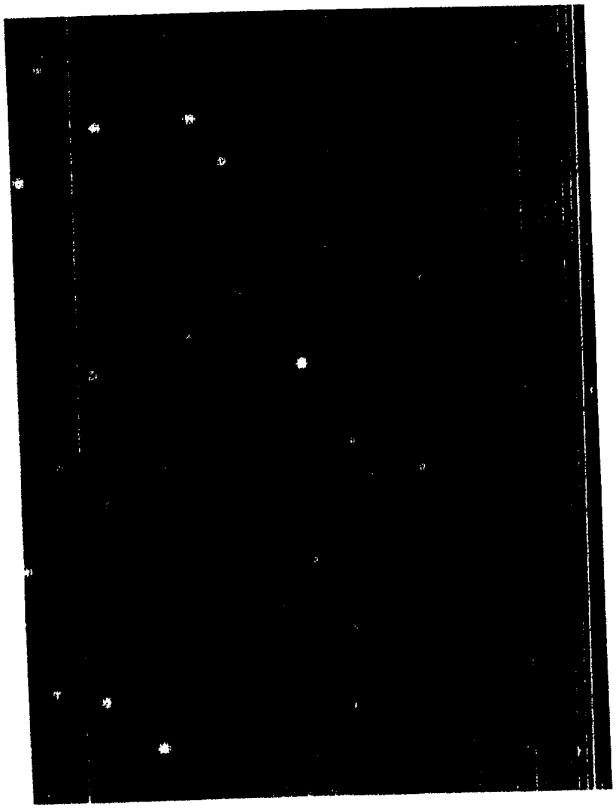


Fluorescently labeled (551ex, 567em) NIH 3T3 mouse fibroblast cells were deposited in wells at the distal face of an optical imaging fiber. The location of the adhered cells is pin-pointed by exciting at a wavelength compatible with the cell label. The fifteen cells within the designated region of interest form a characteristic pattern which will act as a locational template for fluorescence measurements at the excitation wavelength of BCECF (505ex, 535em).

An array of adhered cells in wells was immersed in a 0.001mM solution of BCECF-AM, prepared in serum-free media, for 1 minute. The cell array was removed and rinsed thoroughly with serum-free media to remove any residual dye. Fluorescent images (lex 505 nm, lem 535 nm) were acquired every 30 seconds using a 1.0 second acquisition time. The locational template generated by the fluorescent cell label was transferred to the measurement images in order to locate wells which contain cells. The fluorescent intensity response of the cells shows a gradual increase as the lypophillic groups are cleaved from the BCECF-AM dye within the cell. The fluorescent intensity response of the wells which do not contain cells is negligible.

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Fig. 8



Fluorescently labeled (lex 490 nm, lem 502 nm) NIH 3T3 mouse fibroblast cells were cultured in wells etched at the distal face of a 1mm diameter optical imaging fiber. Excess cells (those not accommodated by a well) were wiped away with a cotton swab wet with growth media. The cells were then excited through the fiber. Cell packing densities can be obtained on the order of ~10^7.

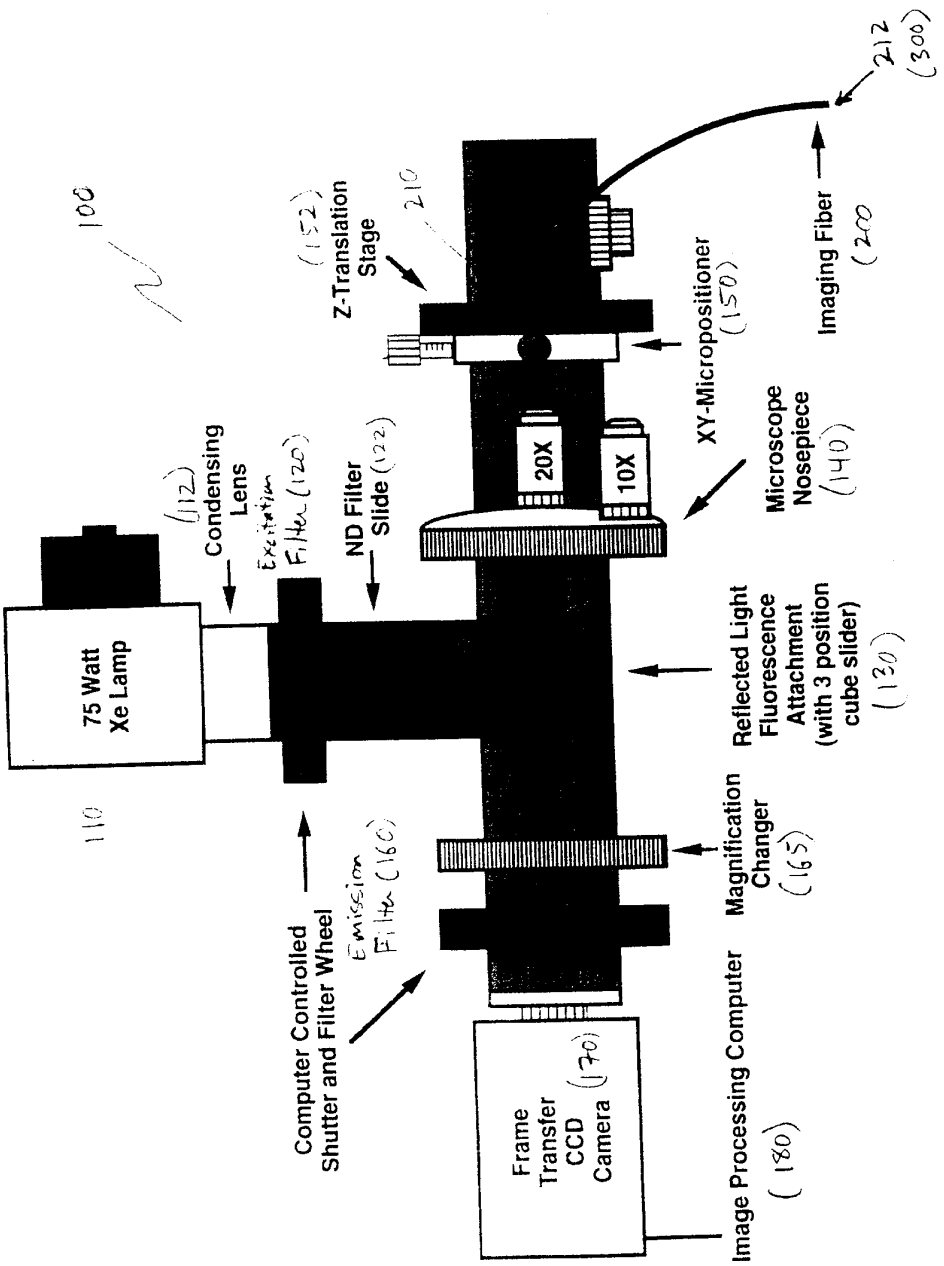

Instrumentation: Modified Fluorescence Microscope
100
75 Watt Xe Lamp
110
(112) Condensing Lens
Excitation Filter (120)
ND Filter Slide (122)
(152) Z-Translation Stage
210
20X
10X
XY-Micropositioner (150)
Microscope Nosepiece (140)
Imaging Fiber (200)
212 (300)
Reflected Light Fluorescence Attachment (with 3 position cube slider) (130)
Magnification Changer (165)
Emission Filter (160)
Computer Controlled Shutter and Filter Wheel
Frame Transfer CCD Camera (170)
Image Processing Computer (180)

Fig. 9

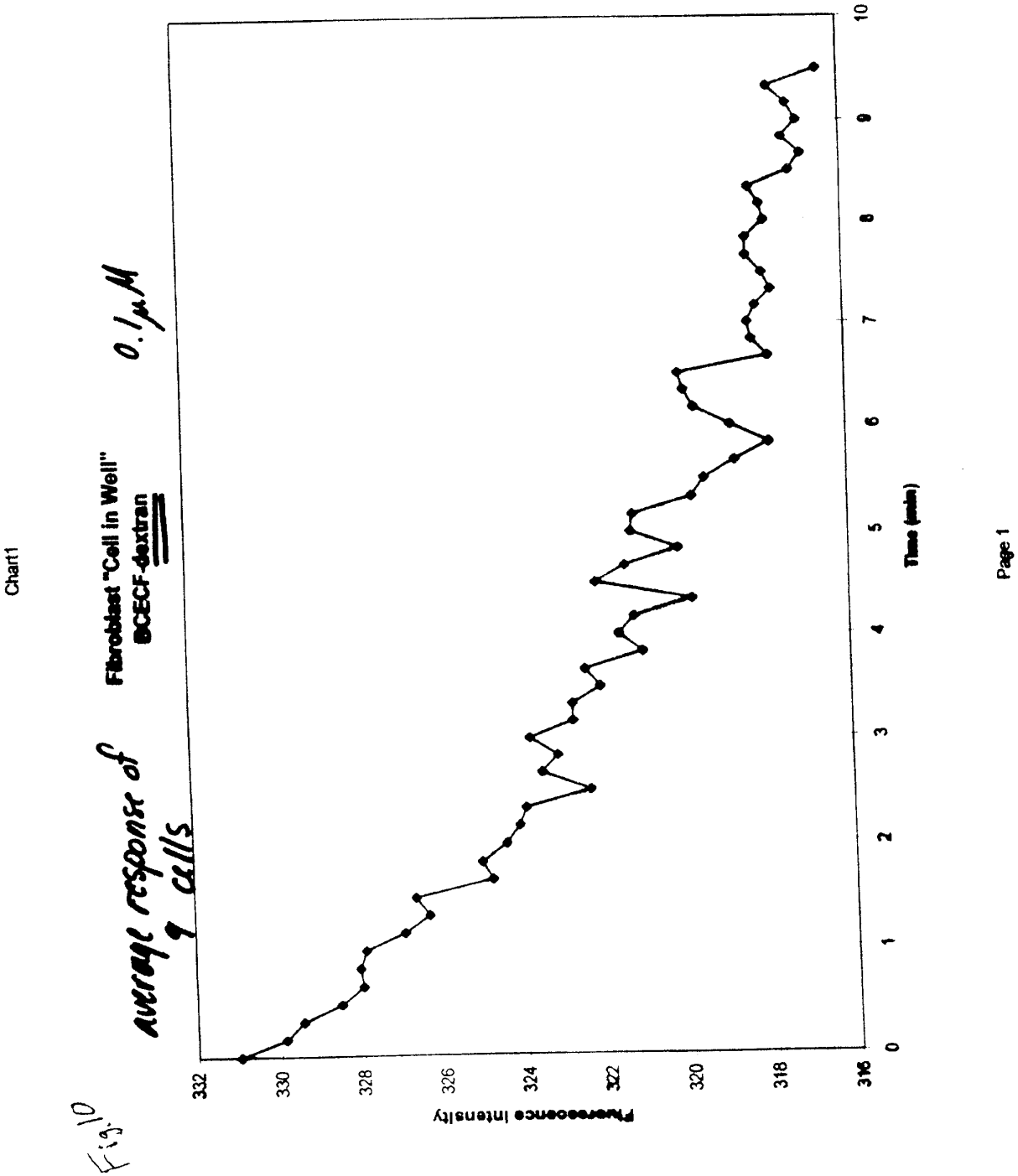

Chart1
Average response of 9 cells
Fibroblast "Cell in Well"
BCECF-dextran
0.1μM
Fluorescence Intensity
Time (min)
332
330
328
326
324
322
320
318
316
0
1
2
3
4
5
6
7
8
9
10
Page 1

Fig. 10

Cell Viability in Etched Microwells

(average response of 25 cells)

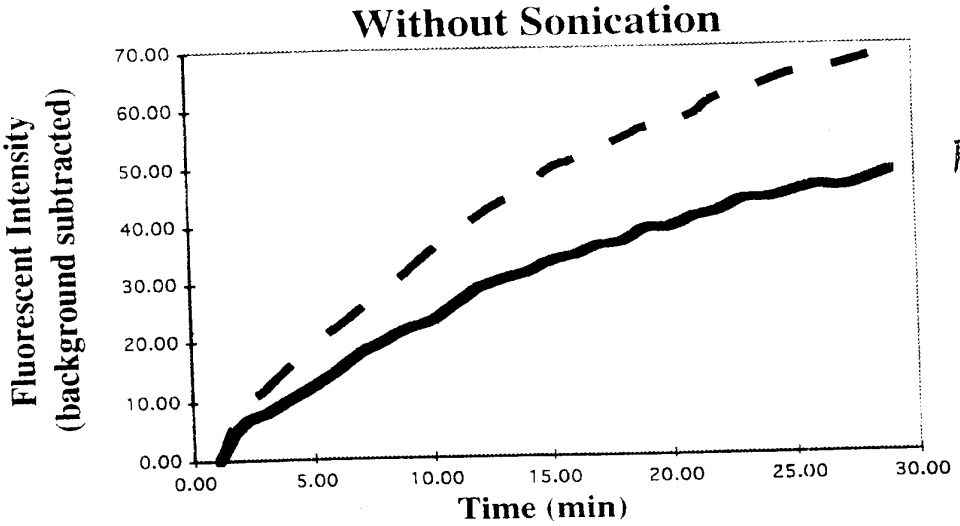


Fig 11a

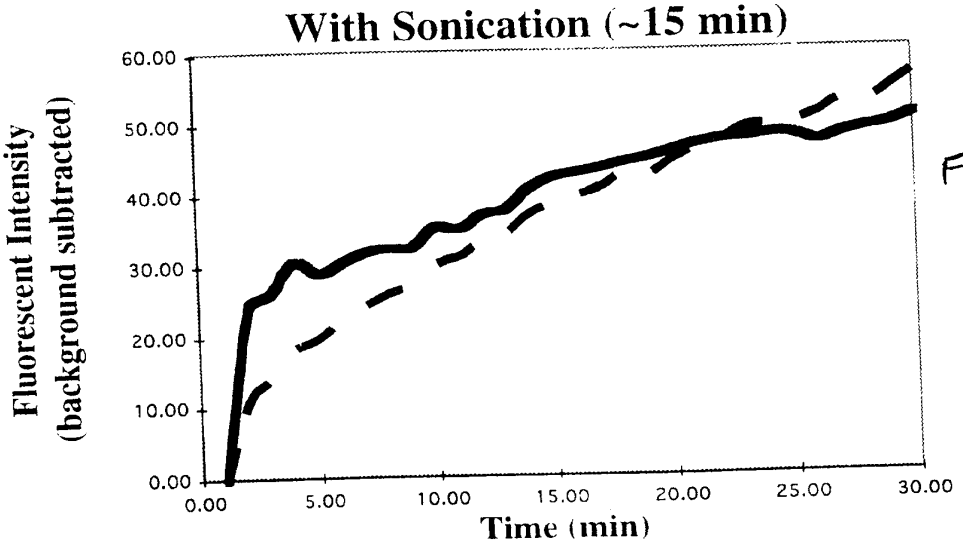
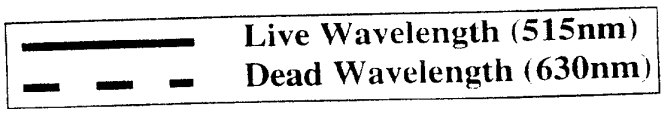


Fig. 11b



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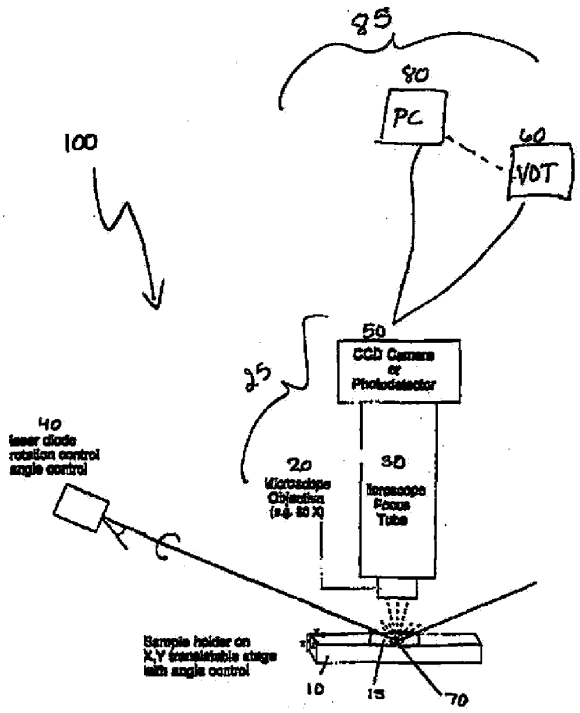
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(54) Title: ENUMERATION METHOD OF ANALYTE DETECTION

(57) Abstract

This invention is directed to an optically–based method and system for analyte detection using solid phase immobilization, specific analyte labels adapted for signal generation and corresponding processes for the utilization thereof. The enumeration detection method disclosed herein narrows the area for signal observation, thus, improving detectable signal to background ratio. The system is comprised of a platform/support for immobilizing a sample stage having a labeled sample (analyte complex) bound thereto, a radiation source, an optical apparatus for generating and directing radiation at said sample and a means for data collection and analysis. Upon engagement of the system, the sample generates a signal capable of differentiation from background signal, both of which are collected and imaged with a signal detector that generated a sample image to a data processing apparatus. Said apparatus receives signal measurements and, in turn, enumerates individual binding events. Generated signal may be increased via selected mass enhancement. The invention, enumeration assay methodology detecting individual binding events, may be used, for example, in analyses to detect analyte or confirm results in both research, commercial and point of care applications.



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ENUMERATION METHOD OF ANALYTE DETECTION

FIELD OF THE INVENTION

This invention relates to the general fields of molecular biology, biochemistry, microbiology and biological research, specifically, to detection of analytes, and more specifically, to an enumeration assay method and system for the detection of individual binding events. The present invention enables the detection of low concentrations of specific molecules of interest (analytes) using solid phase immobilization and optical signals capable of generating, detecting and measuring mass changes.

BACKGROUND AND PRIOR ART

Improving the lower limit of detection -- the threshold of detection of chemical sensitivity -- has been a primary objective of ligand binding assay development since its inception. It has long been recognized that optical detection methods defined by the relationship between various optical interactions with mass on a solid phase, in particular ellipsometry, are capable in principle of providing a high level of sensitivity for standard binding reactions when compared to alternative signal generation methods, for example, enzyme/substrate interaction, fluorescent emission, radioactive emission and color emission. It has also been recognized that mass could be added to the binding complex in order to amplify the optical signal generated. It has been demonstrated that large amounts of mass can be successfully conjugated to the binding complex to this end. An example of this method is provided by the optical ellipsometric immunoassay (OpTest™, DDx, Inc.), a detection system for molecular and microscopic scale events, that measures interactions between

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biological samples and light.

The prior art discloses several imaging methods for the detection of analytes. U.S. Patent no. 5,599,668 to Stimpson et al., entitled Light Scattering Optical Waveguide Method for Detecting Specific Binding Events, discloses a DNA-hybridization imager that detects the scattering of light directed into a waveguide, using labeled microspheres (beads) and visually monitors binding by video imaging. The waveguide device is required as a solid phase and imaging is achieved with a CCD camera and frame grabber software.

Allen et al., U.S. Patent no. 5,488,567, entitled Digital Analyte Detection System is directed to the digital detection of the presence of analyte particles based upon illumination thereof. Distinct pixel regions of the sample are illuminated and the emitted signal detected.

A novel optical biosensor system is taught in *A Biosensor Concept Based on Imaging Ellipsometry for Visualization of Biomolecular Interactions* (Jin et al. (1995) Anal. Biochem. 232:69. The biosensor system utilizes specificities of biomolecular interactions in combination with protein patterned surfaces and imaging ellipsometry and a CCD camera to collect data.

The general use of imaging ellipsometry in conjunction with a CCD camera and framegrabber board is disclosed in *Performance of a Microscopic Imaging Ellipsometer* (Beaglehole (1988) Rev. Sci. Instrum. 59(12):2557. No type of life science or biological system application of the imaging is suggested.

A Method for Detecting the Presence of Antibodies using Gold-Labeled Antibodies and Test Kit are taught in U.S. Patent no. 5,079,172 to Hari et al. This methodology is directed to detecting labeled microparticles using

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microscopy, for example, an electron microscope imaging system.

Chemical and biochemical analysis involving the detection and quantitization of light occurs in a variety of situations. One application is the detection of analytes for the determination of the presence or amount of a particular analyte. In many assays for analytes, the concern lies with either absorption or emission of light radiation (e.g., fluorescence or chemiluminescence). In such cases, a sample is irradiated and the effect of the sample on the transmitted or emitted light is detected. In the case of emitted light resulting from irradiation, non-analyte molecules may also emit light creating relatively high background noise and resulting in the introduction of substantial error in measurement. Additional systematic errors may also collectively contribute to the noise associated with measurement.

The quality of chemical measurements involving light can be defined in terms of the ratio of a suitable measurement of the optical signal from a sample due to the presence of analyte to the noise variation inherent within the system. The source of noise that may affect the results may come from anywhere within the optical path, including the sample, the signal source, detector variation and environmental interference. However, these variations are not necessarily inherent, and may also include externally imposed or induced variations. In general, efforts to augment this signal to noise (S/N) ratio have centered on improving the sensitivity of a measurement apparatus so as to reduce the "detection limit" associated with a particular analyte. The detection limit refers to the analyte concentration within a sample above which the signal attributable to the presence of analyte is such that a desired S/N ratio is achieved. In

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practice, this detection limit is ascertained by conducting an experimental procedure designed to elicit an optical signal related to analyte concentration. Specifically, data relating to signal and noise intensity is plotted in the form of a calibration curve for a range of analyte concentrations, thereby enabling straightforward determination of the detection limit.

The determination of concentration in unknown samples is then effected by comparing the signal obtained experimentally from the unknown with the calibration curve. A typical unit of concentration in chemical measurements is moles/liter [i.e., Molarity (M)], where a mole is defined as Avogadro's number (6.0225×10^{23}). Unfortunately, even the most sensitive conventional experimental techniques have detection limits on the order of about one femtomolar (fM), or nearly one billion analyte particles per liter.

Measurements in which concentration is determined by reference to a calibration curve may be characterized as being inherently "analog" rather than "digital". That is, a signal correlated with analyte concentration is initially produced by the measurement device. The calibration curve is then consulted to obtain an approximation of the analyte concentration. Since the calibration curve is continuous as a function of concentration, the concentration derived from the calibration curve generally is not an integer. In contrast, digital measurement data are often embodied in binary (i.e., two-level) signals that unequivocally represent specific integers. Accordingly, a fundamental difference between analog and digital modes of measurement is that the addition of a single additional analyte to a sample analyzed using analog means cannot be unambiguously detected. Although dramatic improvements have been

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made in the accuracy of chemical measurements, such advancements have been based on the fundamentally analog concepts of increasing signal and reducing noise.

In molecular samples involving low levels of analyte concentration a digital measurement methodology affords at least two advantages: no calibration curve reference and detection of single molecules in a sample. Enumeration methodologies are useful in samples where the analyte concentration is sufficiently low that statistical noise accompanying each binary measurement value remains less than the difference between successive integers. Accordingly, it is an object of the present invention to provide an optical technique for determining low levels of analyte concentration by means of an intrinsically digital measurement scheme adapted for individual binding event detection.

To date, development in the prior art has been directed to imaging of an area of binding, as opposed to distinct video pixels (an array of digitized picture elements) or individual binding sites. The various problems of the prior art are overcome by the present invention. Shortcomings of the prior art include, for example, limitation to emission based reaction detection, averaging and/or detecting reactions over an area or plurality of pixels and the necessity of both signal producing and non-producing areas and distribution determination. The present invention overcomes these drawbacks by providing an integrated system and methodology for analyte detection through enumeration of individual binding events. While prior art is suitable for qualitative and limited quantitative determination, none of the prior art can be easily and efficiently

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used in the accurate enumeration of individual analyte binding events, nor does it teach the enhanced performance characteristics disclosed herein. The present invention provides improved enumeration sensitivity and accuracy, thereby obviating the herein-described prior art.

A prior art search failed to reveal any references disclosing the present invention or making it obvious to one of ordinary skill in the art. Furthermore, combinations of the disclosures of the referenced prior art would not teach the present invention nor would such a combination make the invention obvious. No reference teaches, or suggests, the novel characteristics or combinations employed in the instant detection of solid-phase bound analyte on a molecule-by-molecule basis. The methods disclosed herein are useful, for example, for the solid phase detection of biological markers where the frequency, density or distribution of binding events is below the detectable threshold of conventional immunoassay, DNA probe and immuno-chromatographic detection methodologies.

SUMMARY OF THE INVENTION

The instant invention is based on novel methods of analyte detection as a means for detection of specific molecules using solid phase immobilization and optical signal generation. More specifically, this invention comprises the use of optical signals and detectors capable of detecting and measuring mass changes resulting in analyte detection. This method further relates to commercial applications for automating detection and interfacing with existing assay methodologies, therefore lending itself to commercial applications, for

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example, high throughput pharmaceutical screening and point-of-care detection. That is, this invention is directed to the solid phase, optical detection and enumeration of individual binding events mediated by specific binding interactions.

This invention is defined by analyte solid phase immobilization, a signal generator, a signal carrier including optical pathways, a means of signal detection and novel data analysis. It encompasses a method for improving the detectability of individual binding events by utilizing a narrow optical beam size or by parsing or dividing a larger beam into smaller virtual beams using a diode array or a charged-coupled device (CCD) detector. The use of various optical signals and physical amplification elements is discussed herein.

In its broadest embodiment, the invention is directed to a method and system for solid phase, optical detection and enumeration of individual target analyte binding events comprising the steps of: immobilizing an analyte complex on a reflective or transmissive substrate directly from solution, said complex comprising a target analyte complexed with at least one signal generator element conjugated to at least one secondary analyte specific binding element; reflecting or transmitting electromagnetic radiation from or through the substrate having the analyte complex immobilized thereon; capturing a signal generated from said reflecting or transmitting of electromagnetic radiation; and, analyzing the signal for the presence and/or amount of analyte present.

More specifically, a system and method for digitally detecting the presence of analyte particles within a sample is disclosed herein. Each analyte complex is disposed to generate an optically detectable response upon

stimulation (e.g., illumination) in a known manner. Furthermore, signal generators may be passive or active. Passive signal generators include those that interact with, but do not process, illumination, e.g., absorption, scattering. Active signal generators are those that actively transform photonic energy through a change in state, i.e., fluorescence, chemiluminescence and plasmon resonance. For stimulation or illumination, the digital analyte detection system includes optical apparatus for illuminating a multiplicity of distinct pixel regions within the sample so as to induce each of the analyte complexes included therein to generate an optical signal, i.e., photons. As discussed herein, Stimpson et al. and Allen et al. employ the use of CCDs and pixels for detection purposes. In the instant invention, the pixel regions are dimensioned such that the number of analyte complexes included within each region is sufficiently small that the aggregate optical signal generated by each region is less than a maximum detection threshold, preferably, 1 particle per pixel or multiple pixels per particle.

The digital detection system further includes apparatus for measuring the optical signal generated from each pixel region. A data processing network receives the optical signals, quantifies the signals, and based on the measurements, counts the number of analyte particles within each pixel region so as to determine the number of analyte particles within the sample.

The detection techniques of the present invention can be used for detecting a wide variety of analytes. As used herein, the term "optical response" is intended to collectively refer to the signal generation from a single analyte complex, however induced. In addition, the term "generated signal" as

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used herein corresponds to a measurement of the optical responses detected from a particular pixel or pixel region. The assay sample medium is preferably a solid phase bound analyte complex in which detectable label not bound to an analyte may be removed through conventional washing procedures.

In a preferred embodiment the analyte particles within each pixel region are measured individually based on discrete signal units providing optical responses substantially above a background noise level. The magnitude of each optical response is required to be large enough to allow the particular photodetection apparatus employed to discriminate between optical responses and ambient background noise. One or more optical responses of a signal unit may be associated with a single analyte particle, but the number of units will be substantially identical for each analyte particle. For the most part, the number of signal units per analyte complex will be more than one.

The assay sample medium often has low concentrations of analyte, generally at picomolar or less, frequently femtomolar or less. Assay volumes are usually less than about 100 µl, frequently less than 10 µl and may be 1 µl or less. It is desirable to match the CCD pixels to the signal generator label, ranging in size from 50 nm to 5 microns, such that the labels can be individually detected. The actual size of the CCD pixels is irrelevant in that this is accomplished through magnifying optics.

Assays normally involve specific binding pairs, where by specific binding pairs it is intended that a molecule has a complementary molecule, where the binding of the elements of the specific binding pair is at a substantially higher affinity than random complex formation. The elements of a

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specific binding pair can be referred to as "ligands" and "receptors." Generally receptors are immobilized to the solid phase to capture, or immobilize, the analyte of interest (the "ligand") from a fluid sample. Thus, specific binding pairs may involve haptens and antigens (referred to as "ligands") and their complementary binding elements, such as antibodies, enzymes, surface membrane protein receptors, lectins, etc. (generally known as "receptors"). Specific binding pairs may also include complementary nucleic acid sequences, both naturally occurring and synthetic, either RNA or DNA, where for convenience nucleic acids will be included within the concept of specific binding elements comprising ligands and receptors.

In carrying out the assay, a conjugate of a specific binding element and a detectable and discrete label is involved. Methods of preparing these conjugates are well known, and are, therefore, not discussed herein. Depending upon the analyte, various protocols may be employed, which may be associated with commercially available reagents or such reagents which may be modified.

Other features and advantages of the instant invention will become apparent from the following detailed description, taken in conjunction with the accompanying figures, that illustrate by way of example, the principles of the instant invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the determination of mass per unit volume or equivalent thereof in standard immunoassay methodology;

Figure 2 depicts optical averaging occurring over an assay area;

Figure 3 depicts the highly non-homogeneous assay area integration;

Figure 4 illustrates the statical reduction to insignificance when low numbers of binding events are averaged over a large assay area;

Figure 5 shows small bean ellipsometry or scatterometry provide higher relative signal for discreet binding events;

Figure 6 illustrates the methodological approach for surface resolution, thereby approximating discreet binding event identification;

Figure 7 illustrates laser determination of aggregate response;

Figure 8 depicts scanning micro-laser configuration for the determination of individual cellular scale readings;

Figure 9 illustrates relative size in relation to detection;

Figure 10 depicts CCD and/or diode array beam employed to parse the laser beam into discreet signals;

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Figure 11 illustrates the variability of optical signals useful for detection and resolution purposes;

Figure 12 shows examples of optical signal formats: past, current and prophetic;

Figure 13 illustrates the scale of potential scanning micro-laser configurations; and

Figure 14 depicts optical enhancement potential.

Figure 15 depicts the preferred instrumentation embodiment of the instant invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed. The general principles and conditions for analyte detection, manipulations (hybridization and amplification), and optics (lasers and ellipsometry) are well known in the art. The instant invention describes a novel method of detection for individual binding events.

One skilled in the art recognizes that the instant invention, as disclosed herein, may be performed in a broad range of samples. Such samples include, for example, biological samples derived from agriculture sources, bacterial and

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viral sources, and from human or other animal sources, as well as other samples such as waste or drinking water, agricultural products, processed foodstuff and air. The present invention is useful for the detection of low numbers of immobilized specific molecules.

The present invention is embodied in a method employing optical signals and detectors capable of detecting and measuring mass changes in a sample assay area. Regardless of the specific application of the instant invention, the methodology details are calculated according to protocols well known in the art, as well as those disclosed herein. Further, the refinement of said necessary calculations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them without undue experimentation.

This application references and specifically discusses the use of ellipsometry as the optical method; this convention is for convenience only. It is understood that this methodology applies to a range of optical signal types, including those referenced in Figure 12. It is specifically envisioned that the performance of a variety of optical methods will be substantially improved by adopting the general approach described herein. In particular, scattering methods form the basis of one class of instruments that is distinct from ellipsometry. Other effects such as absorption, refractive index change, and diffraction are used within an essentially similar optical configuration, and may provide particular result benefits. In application, the defining of the optical signal format drives the choice of appropriate immobilization surfaces and suitable data analysis methods for the purpose of distinguishing individual

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binding events. Thus, the attributes of the immobilization system and data analysis system are contingent upon the attributes of the selected optical signal format. The purpose of the optical signal format (the conjunction of a signal carrier, signal generator and signal detector) is to cause and detect a signal. The ability to distinguish the signal caused by the signal generator label from the signal caused by the background platform upon which the system is run, the solid phase, is fundamental to the optical signal format.

Definitions helpful in understanding the specification and claims are included throughout the instant disclosure. The definitions provided herein should be borne in mind when these terms are used in the following examples and throughout the instant application. The disclosures made herein are limited, for simplicity and convenience, to assays directed to the addition of mass (e.g. ligand binding assays), and reference is made to immunoassay methods. However, the same principles of optical signal detection generally apply to systems where mass is removed from the system (e.g. lytic or dissociation assays), and this invention is, thus, applicable to assays measuring mass change and derivatives thereof. Furthermore, this invention is directed to both transmission- and reflection-based solid phase assays.

Those skilled in the art readily recognize the present invention is broadly applicable in the areas of art described herein. The following examples and detailed descriptions serve to explain and illustrate the present invention. Said examples are not to be construed as limiting of the invention in anyway. Various modifications are possible within the scope of the invention.

The advent of small bead conjugation, beads ranging in diameter from

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25 nm to 20 microns, opened the way to a new form of signal detection. That signal detection is described in the present application, and hereinafter referred to as the enumeration method. The instant invention enables the detection of individual binding events. The principle being to narrow the size, actual or virtual, of the area observed for signal, thereby improving the ratio of true signal to background signal, while concurrently using selected mass enhancement elements to increase the signal generated. Only strong signal generators are able to be detected for individual events occurring at the molecular level. On the scale of detectability typical for optical thin film systems, the addition of mass ranging 50 nm to 5 microns generate a high signal, allowing individual binding event detection. Further, certain macromolecules or cellular bodies are large enough that they may be detected without additional mass enhancement, i.e., without secondary labels or reagents. The present invention, thus, solves the problem of detection of low concentrations of specific molecules of interest (analytes) using solid phase immobilization and optical signals capable of detecting and measuring mass changes.

In one embodiment, such mass changes are additively achieved or mediated by analyte complexing or binding via steric, shape mediated or other non-covalent, interactions with a ligand binding pair. Examples of such interactions include antigen-antibody binding, nucleic acid (DNA, RNA, PNA) binding, and other specific macromolecular (protein, glycoprotein, or carbohydrate binding) interactions. Alternatively, mass change is subtractively achieved through specific enzymatic, chemical or other specific dissociating or

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lytic agents. Examples of assay systems utilizing specific binding or lytic interactions suitable for mass change analysis include, for example, immunoassay, hybridization assay, protein binding assay and enzyme activity assay.

Alternate embodiments of this invention include secondary reagents used to amplify or differentiate the optical signal associated with the binding or lytic event through specific enhancement or alteration of that signal. Such enhancement involves the addition of simple mass to a complexing event, or the generation of a differentiable type of signal from a specific species or process. Alternatively, such enhancement involves the alteration of one or more of the elements of the binding or lytic event generating a differentiable optical signal, or the enhancement initiates a detectable self-assembly or aggregation process.

In solid phase assay of the type described herein, results are typically derived from a statistical distinction between the assay signal and the background noise. This type of assay is typically performed utilizing macro-scale volumes (> 1 µl) of a liquid sample or suspension. Similarly, the immobilization area typically used for this type of assay is also at the macro-scale (> 1000 microns). These assays detect and/or quantify the target analyte through detection and measurement of signal generated by large numbers of binding or lytic events. The signals generated by tens of thousands to hundreds of millions of discrete binding or lytic events are aggregated, typically through the interaction of all of the events with a single optical signal path providing a single result. One reason for this traditional approach is that the binding or lytic events to be detected occur on a molecular scale, and thus large numbers

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of events are required to create a detectable signal. Additionally, this large number of events creates a statistically meaningful basis for the result.

A clear limitation of this traditional approach is evidenced in the case of very low concentrations of analyte. The signal generated by sparse binding events must be great enough to be distinguished against the background noise. Alternatively, the signal generated must be differentiable against the field of negative signal caused by averaging the change in signal over the entire surface area of the reaction zone. In solid phase assays, the signal strength of this field is, thus, a function of the volume of sample or the area of the reactive surface. In these cases, the signal generated by sparse binding or lytic events incorporates the signal generated by the much larger unaffected region of the test area. In the case of very low concentration analytes this has the effect of creating a very small difference between a positive and a negative signal, in turn, limiting the lower level of detection that is achievable.

The instant invention is a solid phase detection method and system for biological markers where the frequency, density or distribution of the binding events is far below that which is detectable by traditional immunoassay, DNA probe, immuno-chromatographic or other ligand binding methods.

Immobilization

Solid phase methods are well known in the art of assay development as a means of separating, or capturing, an analyte of interest ("ligand" or "analyte") from a multi-component fluid sample. Solid phase assays require a capture material ("receptor") that is immobilized onto the solid phase that binds

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specifically to the analyte of interest, forming a ligand-receptor complex.

The ligand and receptor bind specifically to each other, generally through non-covalent means such as ionic and hydrophobic interactions, Vanderwaal's forces and hydrogen bonding. Certain ligand-receptor combinations are well known in the art and can include, for example, immunological interactions between an antibody or antibody Fab fragment and its antigen, hapten, or epitope; biochemical binding of proteins or small molecules to their corresponding receptors; complementary base pairing between strands of nucleic acids.

Solid phase immobilization of receptor material is well known in the are. General classes of immobilization include, for example, but are not limited to adsorption, covalent attachment, and linker-mediated. Adsorptive binding is generally non-specific and relies on the non-covalent interactions between the solid phase and the capture material. Covalent binding refers to linking of the capture material to the solid phase via the formation of a chemical bond. Linker mediated immobilization involves the specific use of secondary molecules and/or macromolecules attached to the surface and capture material that interact specifically to form a bound structure. Immobilization methods are generally chosen so that the capture material retains its specificity for binding to the analyte of interest.

Once the capture material is immobilized to the solid phase, the solid support is reactive to analyte binding ("reactive surface"). Before the addition of a fluid sample containing the analyte of interest, it may be necessary to treat the reactive surface with additional materials to prevent ("block") the non-

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specific binding ("NSB") of non-analyte components of the fluid sample to be tested. Typical blocking materials include, for example, proteins such as casein and bovine serum albumin, detergents, and long-chain polymers.

Typically, the chosen receptor is immobilized to a solid phase. A test solution containing the analyte of interest comes in contact with the immobilized receptor whereby a ligand-receptor complex is formed on the solid phase. Once this complex is formed, all other components of the test solution are removed, usually by rinsing the solid phase. The analyte bound to the solid phase may be additionally complexed with a mass amplifying agent through a secondary specific receptor binding to form an analyte complex. This complex may be formed either in the fluid sample containing the analyte before the sample contacts the reactive surface, or after the analyte is bound to the reactive surface. After binding of the analyte or analyte complex to the reactive surface is complete, this binding can be measured by any of several means.

Substrates useful for creating the disclosed solid phase binding platform include all reflective and transmissive materials suitable for optical or "near optical" wavelength reading. Suitable substrates include, for example, those substrates that provide sufficiently consistent or precise interactions with light in order to yield consistent and meaningful results. To that end, the use of highly absorptive surfaces or attachment layers may create optical contrast in the scattering applications disclosed herein.

Optical Signal Format

The Optical Signal Format of the instant invention is comprised of at

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least a signal carrier, a signal generator and a signal detector.

Optical Signal Format: Signal Generator

The present invention specifically relates to a method for altering the ratio of signal to non-signal surface area, allowing for more sensitive results. Also, this invention uses specific labels selected to interact with specific optical beam types to create an enhanced, differentiable or amplified signal.

The traditional goal of a binding assay method is the determination of mass per unit volume (e.g., ng/ml) or equivalent (e.g., IU). See Figure 1. A solid phase is typically used as a separation platform to isolate an analyte from other elements of a sample and from excess reagents. For certain types of assays, the signal generator remains attached to the binding complex, and thus is read from the solid phase (e.g., optical methods as discussed *infra*, or fluorescence). The mass of analyte found in the volumetric sample is converted to mass immobilized on the solid phase in a proportional manner.

The signal generator, as used herein, is that component of the invention that interacts with a signal carrier to create a signal. Key to this concept is the known, specific and predictable interaction between the two. A signal generator element includes material which may be used to specifically label, amplify, distinguish, mark or generate a detectable signal associated with the immobilized target analyte, thus differentiating binding from the absence thereof.

Limitations on selection of a signal generator are driven by the selection of signal carrier, secondary reagent conjugation specificity, target analyte, and

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physical, chemical and/or electrical reactions. Within these limitations, a plethora of signal generators exists. These include, for example, material adding significant mass to the analyte complex, self-assembling, aggregating, enzymatic or chemically active materials, film-forming materials, materials generating optical signatures or distinctive optical properties, i.e., high refractive index, chiral properties, high absorption, high levels of scatter. Furthermore, multiple signal generators may be employed to create discrete signals for different binding events.

Light Scattering Labels

The signal generator component of the scattering embodiments disclosed herein may be referred to as a light-scattering label. A light scattering label is a molecule or a material, often a particle, which causes incident light to be scattered elastically, i.e. substantially without absorbing the light energy. Exemplary labels include metal and non-metal labels such as colloidal gold or selenium; red blood cells; and dyed polymer particles and microparticles (beads) made of latex, polystyrene, polymethylacrylate, polycarbonate or similar materials. The size of such particulate labels ranges from 10 nm to 10 μm, typically from 50 to 900 nm, and preferably 50 nm - 5 microns. The larger the particle, the greater the light scattering effect, but this is true of both bound and bulk solution particles. Suitable particle labels are available from Bangs Laboratories, Inc and Fishers.

In the present invention, the label is attached to either a secondary receptor ("labeled secondary receptor") that binds specifically to the analyte of

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interest, or to an analog of the analyte ("labeled analog"), depending on the format of the assay. For a competitive assay format, the labeled analog specifically binds with the reactive surface in competition with the analyte of interest. For a direct sandwich assay format, the labeled secondary receptor is specific for a second epitope on the analyte. This permits the analyte to be "sandwiched" between the immobilized receptor and the labeled secondary receptor. In an indirect sandwich assay format, the secondary receptor is also specific for a second epitope on the analyte and is labeled with a material that specifically binds an additional light scattering label. For example, once an analyte is captured by the reactive surface, a biotinylated antibody may be used to sandwich the analyte, and an avidinated light scattering label is used for signal generation.

Regardless of the assay format, the receptor or analog must be attached to the light scattering label to form a "labeled conjugate." As with the immobilization of the capture ligands to the solid phase, the light scattering labels may be covalently bonded to the receptor or analog, but this is not essential. Physical adsorption is also suitable. In such case, the attachment to form the labeled conjugate needs only to be strong enough to withstand forces in certain subsequent assay steps, such as washing or drying.

In the preferred embodiment, signal generators are conjugated to binding reagents, which in turn, allow specific interaction with the target analyte, analyte complex or immobilized capture material. Such signal generators include, for example, beads and microparticles and colloidal metals, as discussed previously. Signal generators may also include self-assembling and

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synthetic polymers, glass, silica, silial compounds, silanes, liquid crystals or other optically active materials, macromolecules, nucleic acids, catalyzed, auto-catalyzed or initiated aggregates, and endogenous or exogenous sample components. Useful binding reagents generally include antibodies, antigens, specific binding proteins, carbohydrates, lectins, lipids, enzymes, macromolecules, nucleic acids and other specific binding molecules.

Optical Signal Format: Signal Carrier

Signal carriers useful in the instant invention are optical and near-optical pathways. These pathways interact with a signal generator such that single event detection is possible. Either monochromatic or multiple wavelength electromagnetic radiation reflected from or transmitted through the sample may be used to detect a change in signal.

Optical Signal Format: Signal Detection

Historically, the effect of the use of a single optical beam for reading the surface, e.g., a laser beam, is the production of a single result representing the mass change effects of all binding events within the assay area. Where a large beam is presented to the immobilized mass and the result is integrated by a single detector, the effective result is the same.

As shown in Figure 2, the historically idealized model for this method is the optical averaging occurring over a statistically significant or an entire assay area; represented by an approximately normal distribution of binding events over the assay area. In virtually all actual cases, the binding distribution over

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the assay area is highly non-homogeneous. See Figure 3. An advantage of the current optical ellipsometric read method employing a single large beam and single detector, hereinafter referred to as OTER™ (DDx, Inc.), is that it inherently integrates all of the binding events within the assay area without regard to distribution, aggregating countless individual binding events into a single average result.

A disadvantage of this method derives from that same optical averaging effect. As depicted in Figure 4, in those cases in which the target analyte is comprised of small molecular size particles or in which there are sparse binding events, this method tends to cause results to be statistically reduced to insignificance when averaged over this relatively large assay area. Consequently, results that involve very low concentration positives are indistinguishable from negative results against background noise or variability of the assay system.

One embodiment of the instant invention involves a novel microbiological use of ellipsometric methodologies, that is, the determination of individual binding events via enumeration. This method solves the signal averaging problem by dividing the surface being analyzed into a large number of discrete "local" detection areas. Any signal generated within such a local reading zone is averaged over a much smaller area or field, and thus is "diluted" against an otherwise negative background to a much smaller extent.

For low concentration analytes this method generates numerous local results for any given test surface, most of which report negative results. However, in those cases where positive binding has occurred, the local reaction

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zone reports a very high positive signal; the averaging over the entire area has not diluted the positive signal. Thus, a non-integrated result profile is generated thereby reporting discrete positive results over a total test area that may be by in large negative, while allowing for much larger individual signals to be generated for local positive events.

The enumeration methodology, thus, allows for extremely sensitive assay procedures, including the determination of individual binding events. An obvious application of this method (as referenced in Figure 5) is in microbiology for the detection of low numbers of microorganisms. The ability to detect individual cells or clusters of cells (colony forming units) enables the elimination of time consuming culture steps. This is particularly important for those pathological organisms for which the presence of even a single organism must be considered a positive result. That is, a zero-tolerance level. Another useful application of the instant invention is in hybridization assays, wherein the reaction product exists in extremely small quantities. In this case, individual binding event detection eliminates the need for cumbersome amplification techniques, for example, PCR, NASBA and SDA. All assay systems having clinically relevant thresholds of detection below those readily achieved by traditional assay methods benefit from this invention.

The enumeration principle is illustrated in Figure 5 using a small beam diameter, to provide a local reading area. This beam provides a vastly higher relative signal for discrete binding events, as averaged over a much smaller spot area. More specifically, a collimated beam of light is scanned over a test piece in a raster (X-Y) fashion. The beam, outside diameter (OD) approximately 20

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microns, scans over a cell or group of cells evidencing drastic changes in the reflected light properties as received at the detector. The amplitude of those changes depends on, for example, the size of the optical beam and/or the size of the cell or cell groups. In particular, a cell that is small in comparison to the beam will be difficult to detect above general noise associated with background light and detector amplification. The closer the beam OD and cell size approach each other, the larger the optical property changes. Practical light sources for application of the instant invention include a beam having an OD ranging approximately from 0.650-1.550 microns, i.e., laser diodes. Laser diodes are compact in size and utilize small diameter lenses to manipulate light, thus, facilitating variable equipment dimensions, for example, bench top, lap top and hand held equipment. Moreover, a CCD detector could result in a significant improvement in sensitivity and shorten assay run time. A fundamental difference between the OTER and enumeration approaches, thus, is the optical pathway employed.

A signal detector, in general, must be receptive at the wavelength of the signal carrier and must be configured to receive the system information. Signal detectors may include CCD cameras, single silicon detectors and diode array detectors. An ellipsometer in conjunction with CCD looks at the entire reaction zone and breaks it up into areas. Thus, there is a need to eliminate the negative areas and sum the positive areas. The invention disclosed herein magnifies a spot on the reaction zone and breaks that spot into areas, looking for individual binding events, e.g., beads, cells, colony forming units. Figure 6 depicts topological resolution of the surface evidencing enumeration of individual

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binding events.

It is, in fact, because the binding events are not integrated over the surface that this method is used to approximate individual or discrete binding event identification. Key to practicing the enumeration method, is the ability to segment, parse or segregate discrete areas of signal for highly focused readings, thereby, increasing the ability to discriminate a positive from a negative result. Signal parsing may take place either within the carrier aspect or the detector aspect of the invention. These results are displayed as a series of discrete signal values and compared to a predetermined cut-off point, thereby determining positive binding events within any local read zone. In this manner individual binding events are enumerated on the surface, with a resolution determined by the size of the read zone. To change the relative aspect ratios of the true signal versus background signal or noise involves changing the amount of background over which any true signal is averaged. A constant signal, averaged over a progressively smaller background signal becomes progressively more distinct, until individual signal generators are readily enumerated.

Figures 7 and 8 compare the differences between the current OTER instrument configuration and one of the enumeration capable instrument configurations. The intersecting beam in the OTER configuration has a surface area of approximately 13 square millimeters ($Pi*r^2=SA\ (mm^2) = 3.14159 \times 2^2 = 12.6566\ mm^2$) over which any positive binding events are averaged. Signal parsing by the use of a much smaller diameter beam is illustrated in Figure 8 (i.e., 20 μm). The beam is scanned across the surface, taking discrete local readings over the same total surface area. In this example, the reaction zone is

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2 mm in diameter, and the scanning beam is 20 μm in diameter. Using standard conversions (see Figure 9), the total reaction zone surface area is 3,141,590 μm^2, while the small scanning beam reads 314.159 μm^2 at each local zone. With 100 discrete measurements along the diameter, a 20 μm beam makes 10,000 discrete readings withing the reaction zone.

An inherent signal is generated by each binding event. That signal is not altered by the reduction of the reading zone. Each event generates the same response locally as it would in the OTER configuration. However, the area over which this signal is averaged is reduced 10,000 times, thus, effectively amplifying the signal against the background by 10,000 times in the enumeration system. This change represents an enormous increase in the ability to differentiate a positive result from a negative result, effectively improving the lower limit of detection (chemical sensitivity or threshold of detection) of the assay method by 10,000 times.

Figure 9 represents preliminary calculations as to the limits of detection possible using the OTER and the enumeration approaches. The specific number and examples chosen are not significant to the disclosure, and should not be interpreted as limiting its scope. Rather, they are included herein as an example of the sensitivity differences possible between the two systems. Enumeration is able to detect a single binding event, and as few as 100 binding events generate a clearly enumerable positive result over the system and biological noise. The probable limit of detection for an unamplified OTER system under comparable circumstance is 2×10^6 cfu/ml. The addition of mass to the system via amplification does not result in substantial improvement of sensitivity due to the

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pervasive effect of area averaging.

Signal parsing may also take place at the detector. Through the detector system, an aggregate signal may be divided into discrete information pathways correlating to discrete areas on the test-piece using a broad or large beam width. For example, a CCD or diode array detector may be used in this manner. In cases such as this, the parsed signals must be kept discrete and proportional through the detection and reporting process; magnification, focus and carrier:detector position control are methods for keeping information commensurate throughout the system. The use of a monolithic or single crystal diode detector requires signal to be divided into suitable small units within the signal carrier.

An alternative embodiment to the small beam scanning approach is the use of a CCD or diode array to read and parse the laser beam into smaller discrete signals. The object of this embodiment remains the determination of small spot response within the large beam spot area. However, in this case the definition of the small read zone (local result) is not provided by the diameter of the intersecting beams, but by the arrangement of the detector receiving the beam. Further, the detector, such as a photo diode array, CCD or other non-integrating signal receiver, receives the information contained in the large beam, and preserves this information as smaller local results for processing. This effectively creates a large number of virtual beams, defined by the path that the light intersecting the array as a specific detection point has taken, all operating simultaneously. The aggregate signal for all virtual beams equals the large beam signal -- each virtual beam references only a limited surface area -- and

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the results are not integrated together.

An advantage of this method is that it is rapid (parallel signal processing). The scanning approach is a serial process in which each reading is made in sequence. Additionally, the technical challenges of producing this embodiment are substantially less than those involved in the development of a small beam laser and an accurate scanning control mechanism.

As discussed *supra*, a variety of optical signals may be used within this system. The specific optical signal is selected to provide the appropriate level of information, based upon the nature of the material to be detected, and the resolution desired. The examples provided herein use ellipsometry and scatterometry, see Figure 11. However, a variety of optical methods will be substantially improved by adopting the general concepts and methodologies described herein. In particular, effects such as absorption, refractive index change, chiral effects and diffraction may be used within essentially similar optical configurations. Figure 12 lists possible optical signal types, thus, displaying the range of methods amenable to the enumeration approach. It is neither limiting nor intended to comprise a complete listing thereof.

Mass enhancement labels can play a central role in the practice of the enumeration method at high sensitivities. Figures 13 and 14 illustrate, proportionally, the aspect ratio or relative height:width:breadth of various size materials that may be used as signal generators. As is diagramed in these figures, organisms at the cellular scale generate very significant signal without amplification within the system. In comparison, the thin attachment layer represented along the bottom of the reading zone surface creates a clearly

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distinguishable signal with the current OTER format. The signals generated by mass contained in the much larger objects used as labels significantly improve sensitivity.

Additionally, for either the scanning (small beam) or the array (virtual beam) approach as discussed, a substantial improvement in signal detectability is possible using unique characteristics of optically based mass detection systems. Particular properties of any given mass enhancement label may be used to alter the optical signal based upon its physical characteristics, including its effect on optical characteristics: refractive index, scatter, chiral effect, general adsorption, wavelength specific adsorption and diffraction.

Use of selected labels to induce unique or distinct optical effects creates an improved ability to discriminate the signal generated by the binding of label to the complex from that created by surface background or in the absence of specific binding events. This operates through the creation of an enhanced or attenuated apparent signal over that which would be created by normal materials.

Figure 14 specifically provides an example of this type of effect through the use of high refractive index material in an ellipsometric format. Because the change in polarization state detected by ellipsometry is caused by two distinct factors (absolute mass and refractive index) the use of a high refractive index material as the mass enhancement label effectively increases the apparent mass detected by the ellipsometer, thus, further amplifying the signal from the binding event.

Any number of optical interactions with specific types of material

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designed to amplify or enhance the strength of the signal, or to create a unique signal type, are envisioned and are included herein by reference.

Detection of scattered light (scatterometry) may occur visually or by photoelectric means. For visual detection the eye and brain of an observer perform the image processing steps that result in the determination of scattering or not at a particular situs. The terms "situs" and "site" refer, herein, to the area covered by one ligand. Scattering is observed when the situs appears brighter than the surrounding background. If the number of sites are small, perhaps a dozen or less, the processing steps can be effected essentially simultaneously. If the number of sites is large (a few hundred or more) a photoelectric detection system is desired.

Photoelectric detection systems include any system that uses an electrical signal which is modulated by the light intensity at the situs. For example, photodiodes, charge coupled devices, photo transistors, photoresistors and photomultipliers are suitable photoelectric detection devices. Preferably, detector arrays (pixels) correspond to the array of sites on the reactive surface for signal parsing, some detectors corresponding to non-situs portions. More preferred, however, are digital representations of the reactive surface such as those rendered by a charge coupled device (CCD) camera in combination with available frame grabbing and image processing software. The image processing techniques preferred in the instant invention are derived from Image-Pro® Plus for Windows™ (Media Cybermetrics).

A CCD camera or video camera forms an image of the entire reactive surface, including all label and non-label areas, and feeds this image to a frame

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grabber card of a computer. The image is converted by the frame grabber to digital information by assigning a numerical value to each pixel. The digital system may be binary (e.g. bright=1 and dark=0) but an 8-bit gray scale is preferred, wherein a numerical value is assigned to each pixel such that a zero (0) represents a black image, and two hundred and fifty-five (255) represents a white image, the intermediate values representing various shades of gray at each pixel.

Data Analysis

The digital information may be displayed on a monitor, or stored in RAM or any storage device for further manipulation, such as imaging printing and archiving. Image processing software, such as Image Pro Plus for Windows (IPP), is used to analyze the digital information and determine the boundaries or contours of each situs, and the value of intensity at each situs. IPP is commercially available software for digital image acquisition, processing and analysis. IPP automatically counts and measures objects within an image, after which it sorts and classified the objects by specific characteristics, including, for example: angles, area, length, width, diameter radius perimeter, area or aspect ratios, color, position, optical density and hole areas. IPP is also able estimate the number of objects contained within a cluster of objects.

IPP may be programmed to perform a specific series of functions and analyses in order to differentiate true aanalyte complex particles form other particles or optical features, e.g., dust, non-specific binding, solid phase anomolies, masking. That is to say, the object mearurement characteristics

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discussed herein may be used to create signal:non-signal filters.

Often, the image will require ehnhancement to improve the software's ability to enumerate individual binding events. Enhancement techniques may include, for example, brightness:contract adjustment and spatial:morphological filtering. More specifically, there are three basic categories of image enhancement: intensity index modification, spatial filtering and image frequency manipulation.

Modification of the intensity index is directed to a change in the way intensity values of each pixel are interpreted. Aspects of the intensity index include, for example, birghtness, contract, gamma correction, thresholding, background flattening, background subtraction and intensity equalizatoin.

Spatial filtering techniques analyze and process an image in small regions of pixels. Specifically, by reducing or increasing the rate of change that occurs in the intesntiy transitons within an image. This filtering includes convolution (linear) and non-convolution (non-linear).

Manipulation of the image frequencies is directed to the elimination of periodic or coherent noise in an image by converting the image to a set of frequencies, and editng out the frequencies causing the noise problem. A common technique used for this is the Fourier Transform.

It is envisioned that the digigtal image processing funcions necessary may be consolidated into a laboratory-basded intrument adapted for and capable of semi- and/or automatically performing all sofware-based steps of enumeration. It is not an essential element of the invention to display the ssurface image. It is essential only that the software image processing is

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performed entirely with the datea provided by the digitization of the image.

The inventive clustering process as described in U.S. Patent no. 5,329,461 may be adapted for utilization in a variety of applications to spatially resolve and count discrete analyte particles or individual binding events in conjunction with the instant invention. For example, detection of analyte particles comprising a molecule and a labor or for rapid scanning to locate areas of interest within an image of a sample.

Instrumentation

Generally, a prepared test piece is secured to the sample stage and manually positioned such that the center of a test spot is aligned with the center of the objective lens. The test piece may be prepared to contain multiple test spots, therefore, to begin the test spot designated as 1, or first, is centered. Using the sample stage's translational capabilities, the detector is manually focused on the scattering particles. Next, the image produced by the light scattering is collected and saved. Finally, the sample stage is translated to two alternate locations, one each to the left and right of center, and image acquisition repeated at each location. Each generally herein-described step in the detection process may be repeated for any number of test spots contained on a test piece.

The instrument employed for the enumeration methodology disclosed herein consists of 3 defining modules: a sample stage, an optical signal format corresponding to the immobilized analyte complex, and a means for data collection and analysis. Each module is adapted for independent translation on

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at least 2 axises, thereby facilitating optimal optical effect, alignment and focus. The instrument and its modules, *in toto*, are fixed and stationary in relation to one another by standard attachment means to, for example, a solid, planar, horizontal platform. More specifically, as shown in Figure 15, the enumerator **100** is comprised of a means for data collection and analysis **85** consisting essentially of a computer **80** and video display terminal **60** functionally combined with a sample stage **10** and optical signal format consisting essentially of a signal carrier **40** and a signal detector **25** configured such that when a signal generator, such as a light scattering label, is irradiated, it is able to be detected by the enumerator **100**.

The sample stage **10** may be any planar stage or platform adapted for receiving and securing thereon a mounting jig **15** onto which a test piece **70** is secured to the mounting jig **15**. The test piece **70** may be secured by any suitable means, such as, double sided adhesive tape or a mechanical mounting means. Said stage **10** translates on at least an X-Y axis basis, and in the preferred embodiment, also possesses additional rotational and angle control. The test piece **70** is further comprised of test spots, prepared as described herein.

The optical signal format is comprised of a signal generator such as a light scattering label bound to a test spot as described herein, a signal carrier **40** and a signal detector **25**. In the preferred embodiment the signal carrier **40** is an electromagnetic radiation source, and more preferably, a laser diode adaptively mounted to possess both rotational and angular control. The signal detector **25**, an integrally combined microscope focus tube **30** and objective **20**

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functionally combined with a photodetector, and preferably a CCD camera **50** are disposed, by any standard mounting means, vertically above the sample stage **10**. The signal detector **25** is functionally combined by standard means with the data collection and analysis means **85** comprised of a PC **80** and video display terminal **60**, each of which is accordingly appointed with appropriate software and electronics.

In use, the PC **80** and video display terminal **60**, and signal carrier **40** are powered on and allowed to warm up for at least 30 minutes. While the unit is warming up, the test piece **70** is adhered to the mounting jig **15**, which in turn, is secured to the sample stage **10** directly and vertically below the signal detector **25**. The test spot on the test piece **70** that has the target analyte bound thereto is then centered, aligned and focused between the signal detector **25** and the signal carrier **40**. The enumerator **100** is engaged, an image acquired and exhibited and/or stored accordingly. The test piece **70** is realigned for additional image capture to the left and right of the test spot, as described herein. Engagement of the enumerator **100** and image capture is repeated in a similar manner for each of the test spots on the test piece **70**.

Prior to engagement of the enumerator **100**, the appropriate software preparation is performed. For example, subfolders, default settings and macros are setup.

Generally, light scattered by surface-bound microspheres is collected and magnified by a microscope objective lens and focused onto a CCD array, e.g. 640 x 480 pixels. CCD signal output is fed to both a black and white monitor and a data translation frame grabber such as Data Translation DT3155 high

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accuracy scientific frame grabber (Data Translation, Inc.). Image acquisition and analysis of the image formed by scattered light is accomplished with software adapted for and/or specifically directed to such function, for example, Image Pro Plus (Media Cybernetics).

Data analysis that includes discrimination and counting of scattering objects within an image is performed by software designed for such a purpose. Customized functions adapted into such software via, for example, macro programs, include exclusion of non-binding events from the object count by filtering, image intensity averaging and binary filtering. An example of a macro adapted for use in the preferred embodiment of the invention includes: transformation of bright scattering objects into a standard 3 x 3 cross; application of a watershed filter to the resulting cresses to separate scattered objects; determination of mean image intensity and the standard deviation of that mean; determination of a lower limit intensity threshold for a binary filter based on the mean image intensity; application of binary filter with threshold values of lower limit; and, automatic count of resulting objects having a mean diameter, for example, less than 10 pixels. The number of objects counted for each image is averaged over the three images produced for each test spot -- center, left and right.

Example 1: Specific Binding Assay

Preparation of Whole Wafer Test Pieces. The test pieces used are commercially available 5' silicon (Si) wafers. Thin layer polyurethane coated wafers are produced using standard spin-coating procedures to lay the

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polyurethane on the reflective surface of the wafer. Briefly, the wafers are prepared by addition of 500 µl of a thoroughly mixed 1.25% solution of Polymedica M1020 Polyurethane (Polymedica, Inc.) in N,N-dimethylacetamide (DMAC) (Sigma Chemical Co.) To the center of a silicon wafer (Addison Engineering) spinning at 5000 rpm. The wafer is air dried and then baked at 70° C for 16-20 hours. Next, a 10 circle by 10 circle pattern is applied to the non-reflective wafer surface using a 3.5" x 3.5" rubber stamp coated with RTV 108 silicone rubber adhesive sealant (GE Silicones, Inc.). The resulting circular outlines serve as a means to isolate each circular polyurethane coated test spot (~0.25" diameter). The adhesive is cured at ambient room temperature for approximately 24 hours prior to use in assay.

Adsorption of Streptavidin Coated Microspheres to a Biotinylated Surface. Each of the polyurethane coated wafer test spots are coated with 20 µl of a 1 µg/ml of biotinylated bovine serum albumin (BSA) (Sigma Chemical Co.), or alternatively a non-biotinylated BSA for use as a negative control. The wafer is incubated at 37° C for one hour in a 100% humidity chamber. After incubation, the wafers are rinsed 3 times with deionized water and dried with compressed air. Following BSA immobilization, the test spots are blocked with 30 µl of 3% BSA for 1 hour at 37° C, then rinsed 3 times with deionized water and dried with compressed air.

Streptavidin coated polystyrene microspheres (350 nm diameter) (Bangs Laboratories) are serially diluted in borate buffer (0.1 M, pH 8.5 + 0.01 % Tween-20), for resulting dilution ranging between 1:10 and 1:10,000. Next, 20 µl of each dilution is applied to the biotinylated and non-biotinylated test spots

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and the wafer incubated at 37° C for 1 hour, rinsed for 10 seconds with deionized water, compressed air dried and analyzed with the invention disclosed herein, the results of which are shown in Table I. These data show that light scattering labels bound to a surface can be detected and enumerated using the present invention; that streptavidin coated microspheres bind specifically to a biotinylated surface; and that the number of microspheres counted on the surfaces is dependent on the number applied to the surface.

Example 2: *Staphylococcal* Enterotoxin B (SEB) Detection Assay

Preparation of Whole Wafer Test Pieces. The test pieces used are commercially available 5' silicon (Si) wafers. Thin layer polyurethane coated wafers are produced using standard spin-coating procedures to lay the polyurethane on the reflective surface of the wafer. Briefly, the wafers are prepared by addition of 500 µl of a thoroughly mixed 1.25% solution of Polymedica M1020 Polyurethane (Polymedica, Inc.) In N,N-dimethylacetamide (DMAC) (Sigma Chemical Co.) To the center of a silicon wafer (Addison Engineering) spinning at 5000 rpm. The wafer is air dried and then baked at 70° C for 16-20 hours. Next, a 10 circle by 10 circle pattern is applied to the non-reflective wafer surface using a 3.5" on 3.5" rubber stamp coated with RTV 108 silicone rubber adhesive sealant (GE Silicones, Inc.). The resulting circular outlines serve as a means to isolate each circular Polyurethane coated test spot (~0.25" diameter). The adhesive is cured at ambient room temperature for approximately 24 hours prior to test spot mounting on test piece and use in assay.

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SEB Detection. A full sandwich assay is used for the detection of SEB in a sample buffer. The general protocol consists of coating capture antibody to individual test spots, blocking, adding different concentrations of SEB to the coated test spots, applying a biotinylated secondary reporting antibody, and labeling the bound secondary antibody with avidinated polystyrene microspheres.

Test wafers are coated with polyclonal ∝-SEB capture antibody by applying 20 µl of a 30 µg/ml (in 0.1 M PBS, pH 7.2) solution to each assay test spot. The wafer is incubated at 37° C for 1 hour to allow passive adsorption of the capture antibody to the polyurethane. After incubation, the wafer is rinsed 3 time with deionized water and dried with compressed air.

Following capture antibody immobilization, each test spot is blocked with 40 µl of a 3% BSA solution (0.1 M PBS, pH 7.2) to reduce non-specific protein adsorption from subsequent assay steps. The wafer is incubated at 37° C for 1 hour and subsequently rinsed 3 times with deionized water and dried with compressed air.

SEB samples are prepared by serial dilution of a 1 mg/ml stock into sample buffer (0.1 M PBS + 1% BSA + 0.01% Tween-2-, pH 7.2), with final toxin concentrations ranging from 0.1 ng/ml to 100 mg/ml. Buffer with no SEB is used as a negative control. Twenty µl of each of the dilutions and the negative control are applied to separate test spots across the wafer surface. The water is incubated at 37° C for 30 minutes then rinsed 3 times with deionized water and dried with compressed air.

Biotinylated ∝-SEB antibody is diluted to 4 µg/ml in sample buffer.

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Each test spot is coated with 20 µl of this secondary antibody dilution. The wafer is incubated at 37° C for 30 minutes then rinsed 3 times with deionized water and dried with compressed air.

Test spots are coated with 20 µl of a 1:100 dilution of streptavidin coated 350 nm diameter polystyrene microspheres in borate buffer (0.1 M, pH 8.5 + 0.01 % Tween-20). The wafer is incubated at 37° C for 30 minutes then each test spot is rinsed for 10 seconds, dried with compressed air and analyzed. The results of such analysis are shown in Table II. These data show that the present invention can be used to enumerate the binding of an antigen to a solid phase in a specific and quantitative manner. The lower limit of detection for this method is 550 pg/ml.

Data acquisition and analysis are performed as generally described herein. The wafer or test piece is mounted on a stage, positioned, focussed and images captured. Data analysis includes employing a macro program within Image Pro Plus.

While the above description contains many specificities, these specificities should not be construed as limitations on the scope of the invention, but rather exemplification of the preferred embodiment thereof. That is to say, the foregoing description of the invention is exemplary for purposes of illustration and explanation. Without departing from the spirit and scope of this invention, one skilled in the are can make various changes and modifications to the invention to adapt it to various usages and conditions. As such, these changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the claims. Thus, the scope of the

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invention should be determined by the appended claims and their legal equivalents, rather than by the examples provided herein.

ENSDOCID: <WO_____9958948A2_I_>

TABLE I

Specific Adsorption of Beads to Biotinylated Surfaces

Bead Dilution	# Objects: Biotinylated Surface	# Object: non-Biotinylated Surface
1:10	2263	201
1:100	2019	27
1:500	1375	9
1:1000	849	13
1:10,000	115	8

BNSDOCID: <WO_____9958948A2_I_>

TABLE II

SEB Detection Assay

SEB Concentration (ng/ml)	# Objects	Standard Deviation
0	62	5
0.1	72	12
0.5	121	10
1	203	51
10	906	281
100	1800	353

We claim:

1. A method for solid phase, optical detection and enumeration of individual target analyte binding events comprising the steps of:

Immobilizing an analyte complex on a reflective or transmissive substrate directly from solution, said complex comprising a target analyte complexed with at least one signal generator element conjugated to at least one secondary analyte specific binding element;

Reflecting or transmitting electromagnetic radiation from or through the substrate having the analyte complex immobilized thereon;

Capturing a signal generated from said reflecting or transmitting of electromagnetic radiation; and,

Analyzing the signal for the presence and/or amount of analyte present.

2. The method as defined in claim 1, wherein immobilizing the analyte complex is accomplished by adsorptive, covalent, steric, chemically mediated, linker, self assembling or force mediated binding to a solid phase or solid phase matrix.

3. The method as defined in claim 2, wherein immobilizing further comprises one or a plurality of intermediate layers disposed between said substrate and said analyte complex.

4. The method as defined in claim 1, wherein said target analyte is separated from a material or materials via immobilization.

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5. The method as defined in claim 1, wherein said analyte is complexed in liquid phase or solid phase.

6. The method as defined in claim 1, wherein said signal generator element is selected from the group consisting of self-assembling, aggregating, enzymatic, chemically active, film-forming and optically unique materials.

7. The method as defined in claim 6, wherein said signal generator element is selected from the group consisting of microparticles, colloidal metals or non-metals, polymers, glass, silial compounds, optically active materials, macromolecules and nucleic acid.

8. The method as defined in claim 6, wherein said signal generator element adds mass to said analyte complex.

9. The method as defined in claim 1, wherein a plurality of signal generator elements are complexed with said analyte complex creating a plurality of distinct signals indicative of distinct binding events.

10. The method as defined in claim 1, wherein said secondary binding element is selected from the group consisting of antibodies, antigens, macromolecules, nucleic acid and specific binding molecules.

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11. The method as defined in claim 1, wherein said electromagnetic radiation source is a laser diode.

12. A system for solid phase, optical detection and enumeration of target analyte individual binding events comprising:

a means for target analyte capture, said means comprising a substrate having an analyte complex immobilized thereon, said analyte complex comprising a target analyte complexed with at least one signal generator element conjugated to at least one secondary analyte specific binding element;

a signal carrier means consisting of electromagnetic radiation, said signal carrier having a known interaction with said signal generator and generating a detectable signal evidencing analyte binding event or events;

a signal capture means having an optical resolution element or elements and configured to receive information generated from the signal carrier; and,

a signal analyzing means, said means processing information generated by said signal capture means for qualitative and/or quantitative analyte detection.

13. The system as defined in claim 12, wherein said electromagnetic radiation is monochromatic wavelength within the range of 400 nm - 700 nm.

14. The system as defined in claim 12, wherein said electromagnetic radiation is multiple wavelength within the range of 400 nm - 700 nm.

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15. The system as defined in claim 12, wherein said resolution element magnifies, focuses and/or controls the signal carrier means.

16. The system as defined in claim 12, wherein said resolution element parses discrete signals from an aggregate signal generated from said signal generator.

17. The system as defined in claim 12, wherein said signal carrier means is selected from the group consisting of interference, diffraction, reflection, polarization, scattering, birefringence, absorption and refraction.

18. The system as defined in claim 12, wherein said analyte complex further comprises mass enhancement means for amplifying a signal for detection related to the presence of the target analyte.

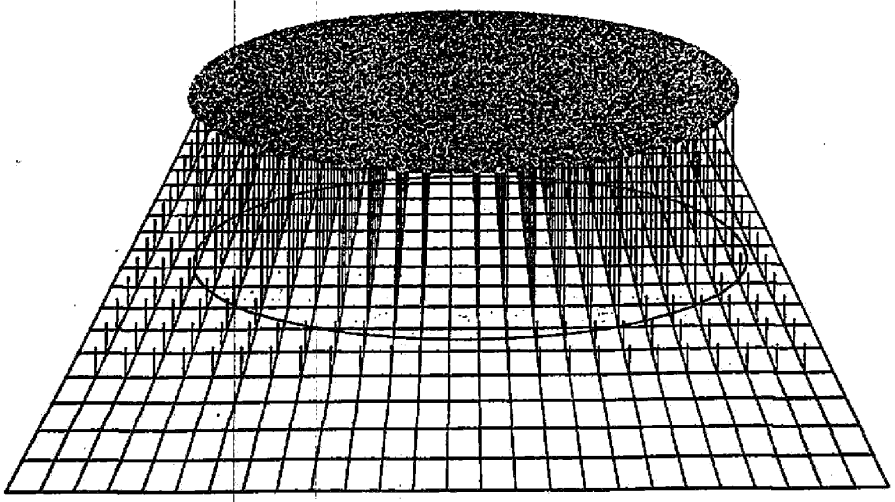
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Aggregate vs. Scanning Ellipsometry

Innovation Group

Standard Immunoassay Use

Determination of mass per unit volume (eg. ng/ml) or equivalent (eg. IU)



The effect of the use of a single large beam (in this case approx. 2mm) for reading the surface is the production of a single result representing the mass change effects of all binding events within the spot area.

Figure 1

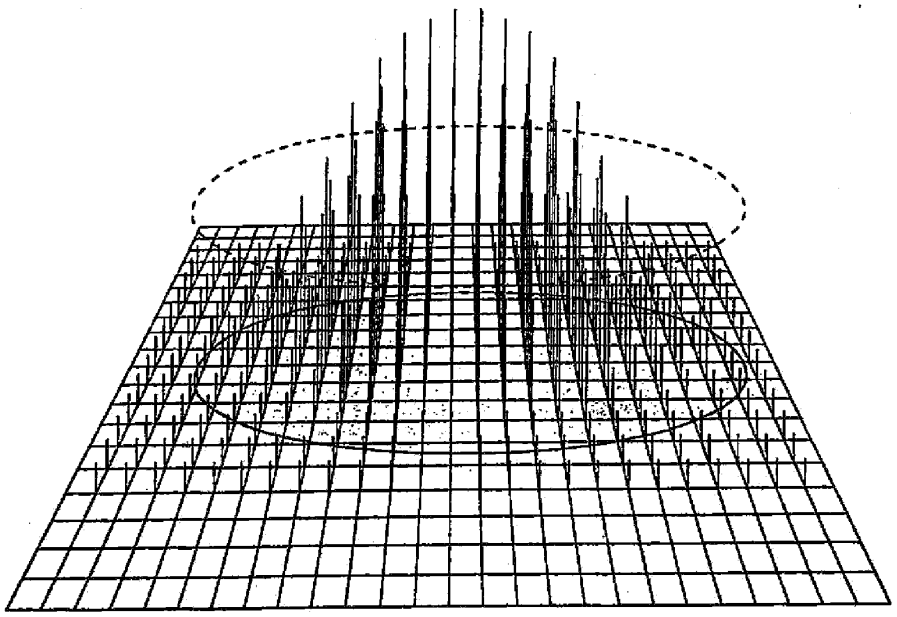
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Aggregate vs. Scanning Ellipsometry

Innovation Group

Standard Immunoassay Use

Determination of mass per unit volume (eg. ng/ml) or equivalent (eg. IU)



The idealized model for this method is the optical averaging occuring over the entire read area (in this case represented by an approx. normal distribution of binding events over the spot area).

Figure 2

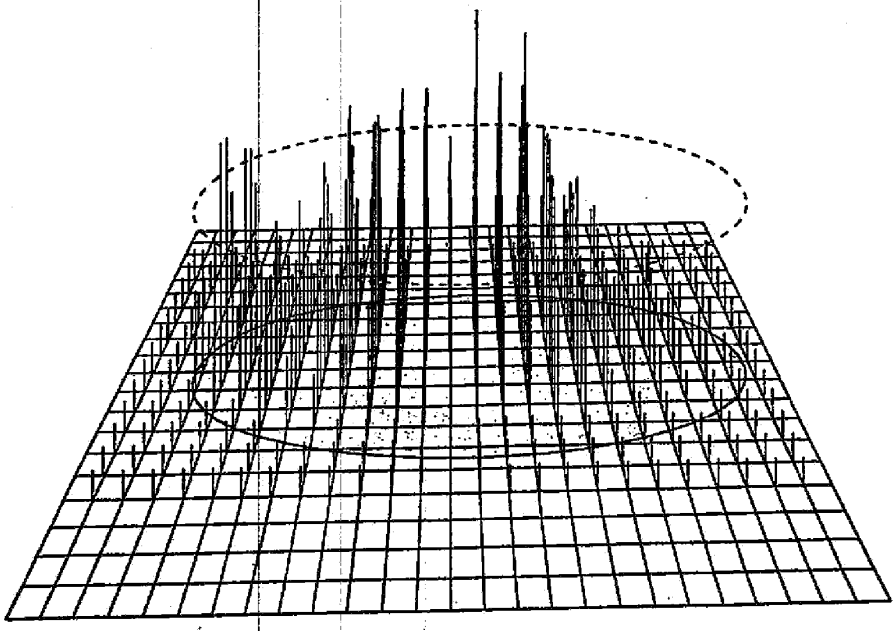
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Aggregate vs. Scanning Ellipsometry

Innovation Group

Standard Immunoassay Use

Determination of mass per unit volume (eg. ng/ml) or equivalent (eg. IU)



In virtually all cases the binding distribution over the spot area is actually highly inhomogenious. The advantage of this method is that it inherently integrates all of the binding events within the spot area, without regard to their distribution.

Figure 3

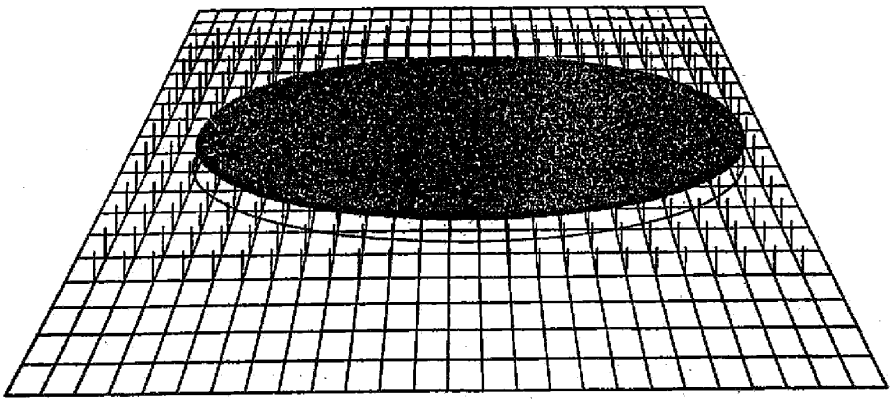
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Aggregate vs. Scanning Ellipsometry

Innovation Group

Standard Immunoassay Use

Determination of mass per unit volume (eg. ng/ml) or equivalent (eg. IU)



The disadvantage is that very small or very sparce binding events tend to be statistically reduced to insignificance when averaged over this relatively large spot area.

Figure 4

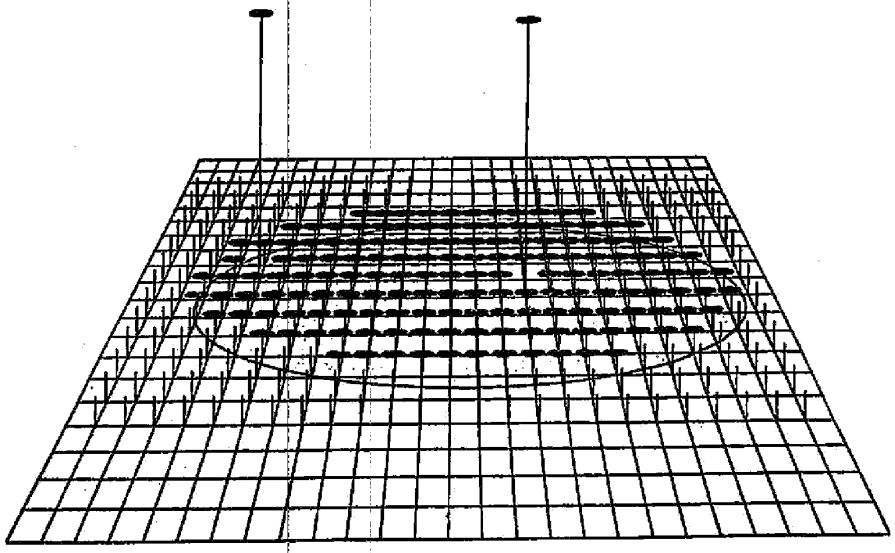
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Aggregate vs. Scanning Ellipsometry

Innovation Group

New Microbiological Use

Determination of individual binding events or CFUs



A scanning ellipsometric method or scatterometric method or both, when used with a very small beam diameter (in this case 20um) can provide a vastly higher relative signal for discreet binding events (that is as averaged over a much smaller spot area).

Figure 5

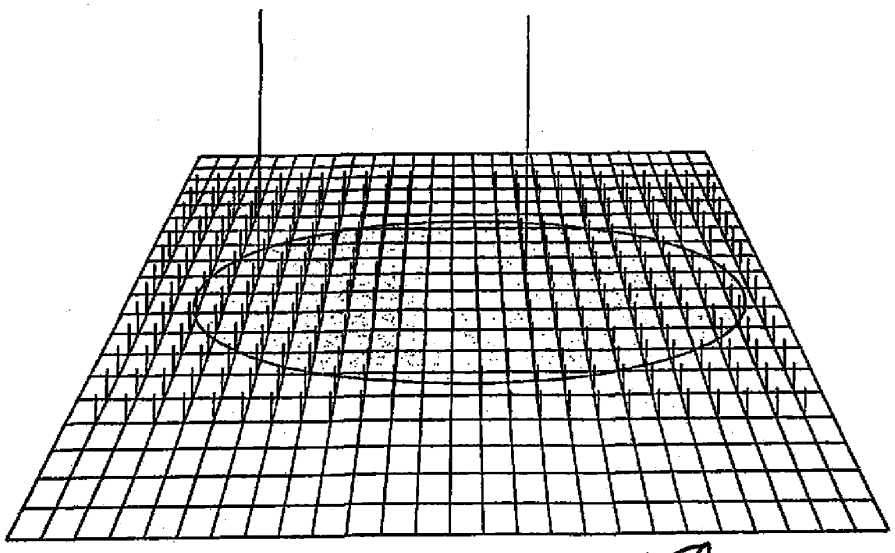
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Aggregate vs. Scanning Ellipsometry

Innovation Group

New Microbiological Use

Determination of individual binding events or CFUs



This approach allows for the surface to be resolved as a type of topology. It is, in fact, because the binding events are not integrated over the surface that this method can be used to approximate individual or discreet binding event identification (depending upon the diameter of the beam used).

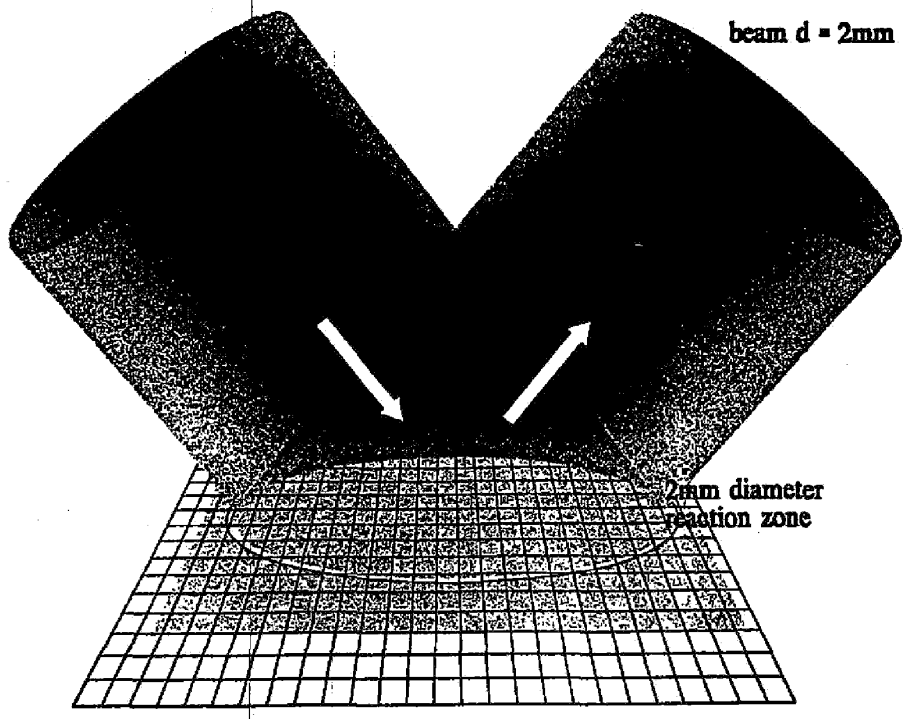
Figure 5

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Aggregate vs. Scanning Ellipsometry

Innovation Group

Current OTER Laser Configuration
Determination of aggregate response over the beam spot area



pi_{ct}^2 = SA (in mm^2) = 3.1459 x 1^2 = 3.1459 mm^2

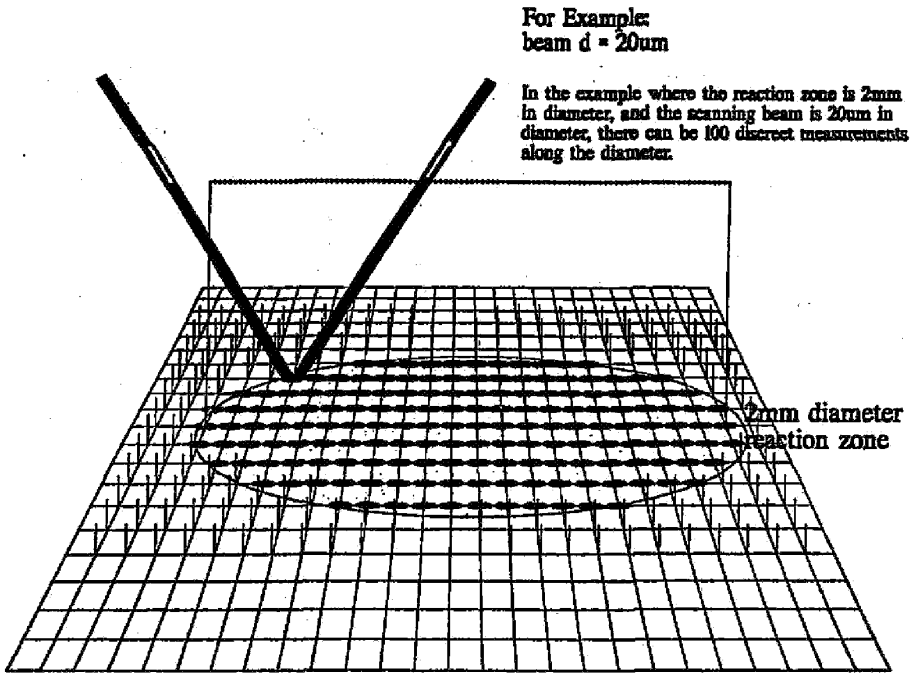
Figure 7

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Aggregate vs. Scanning Ellipsometry

Innovation Group

Scanning Micro-Laser Configuration
Determination of individual cellular-scale readings



10,000 A = 1um
1,000um = 1mm
Angstrom Unit = 3.937x10^-9 inch, 1x10^-10 meters, 1x10^-4, microns, 0.1 milli-micron (micro-millimeter).
Micron = 3.937x10^-5 inch, 0.039370 mil, 1x10^-6 meter, 0.001 millimeter, 1x10^4 Angstrom units.

1 mm^2 = 1,000,000 um^2 Reaction zone SA = 3,141,590 um^2 Scanning beam reads 314.159 um^2

Thus a 20um beam can make 10,000 discreet readings within the reaction zone

Figure 8

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Aggregate vs. Scanning Ellipsometry

How Big is Small ?

In the case where a single organism (1 um^3) is to be measured on a 2 mm^2 surface:

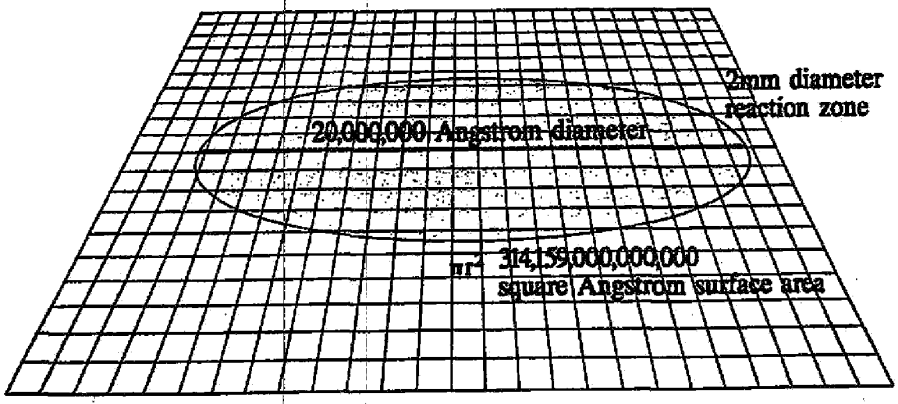
314,159,000,000,000 A^2 surface area of spot
78,500,000 A^2 surface area of organism
→ ratio of 4,000,000 A^2 : 1 A^2

10,000 A (height) / 4,000,000 = .00250 A (height) contribution across the spot

1 x 10^2 cells / .02ml = 5 x 10^3 cells / ml	→ .25 A (height) contribution across the spot
1 x 10^3 cells / .02ml = 5 x 10^4 cells / ml	→ 2.5 A (height) contribution across the spot
1 x 10^4 cells / .02ml = 5 x 10^5 cells / ml	→ 25 A (height) contribution across the spot
1 x 10^5 cells / .02ml = 5 x 10^6 cells / ml	→ 250 A (height) contribution across the spot
1 x 10^6 cells / .02ml = 5 x 10^7 cells / ml	→ 2500 A (height) contribution across the spot

probable unamplified detectability limit

With an amplification system that provided 2X mass, the system needs 2.5 x 10^6 cells / ml
With an amplification system that provided 5X mass, the system needs 1 x 10^6 cells / ml
With an amplification system that provided 10X mass, the system needs 5 x 10^5 cells / ml



10,000 A = 1um
1,000um = 1mm
Angstrom Unit = 3.937x10^-9 inch, 1x10^-10 meters, 1x10^-4, microns, 0.1 milli-micron (micro-millimeter)
Micron = 3.937x10^-5 inch, 0.039370 mil, 1x10^-6 meter, 0.001 millimeter, 1x10^4 Angstrom units.

Figure 9

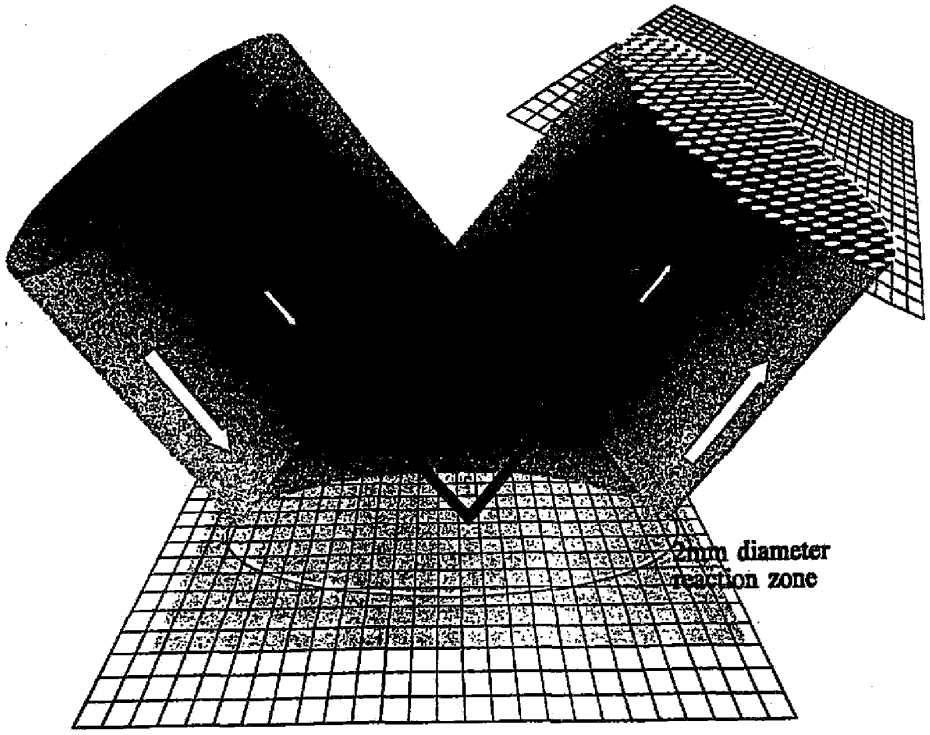
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Scanning vs. Array Ellipsometry

An alternative to the small beam "Scanning" approach is the use of a CCD or diode array to read and "parce" the larger laser beam into smaller discreet signals.

Determination of small spot response within the large beam spot area



This effectively creates a "virtual" beam (defined by the path that the light intersecting the array at a specific dection point has taken).

The aggregate signal for all virtual beams equals the large beam signal, but each virtual beam references only a limited surface area. The virtual beam approach may be subject to greater error than the small beam approach, due in part to the potential for signal mixing across the array, however it allows for a major increase in sensitivity over the large beam approach.

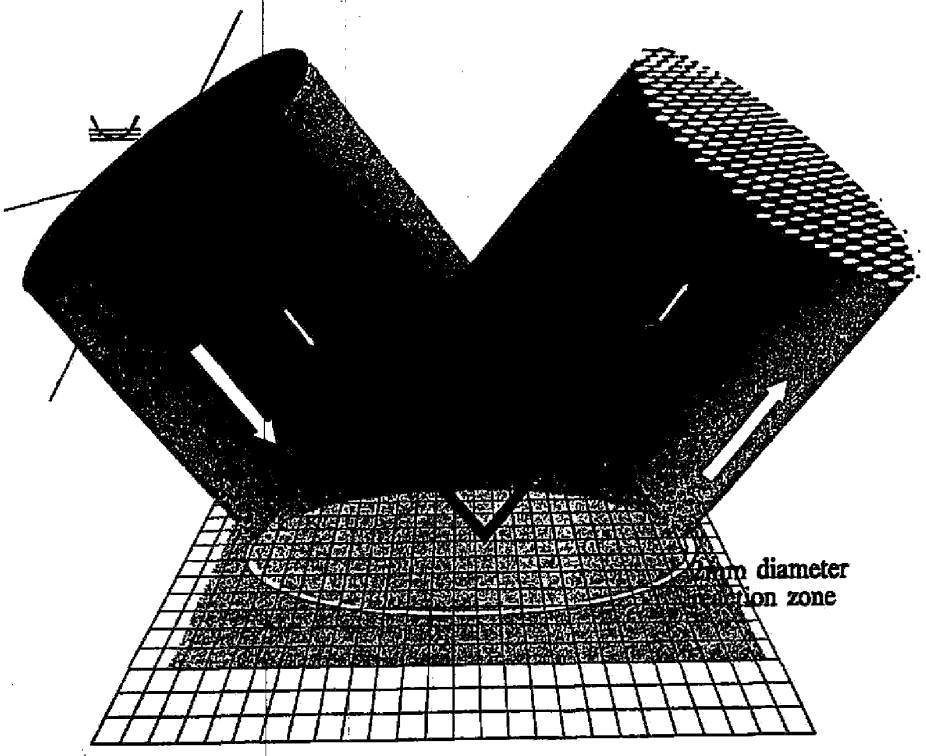
Figure 10

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Various Optical Signal Formats

Innovation Group

The specific optical signal can be selected so as to provide the appropriate level of information, based upon the nature or the material to be detected, and the resolution desired.



A variety of optical signals may be used within this system.

The examples provided in this discussion use ellipsometry as the example optical method. However it is expected that a variety of optical methods will be substantially improved by adopting the general approach described here. In particular we have demonstrated that scattering methods will form the basis of one class of instruments that is distinct from ellipsometry. Other effects such as absorption, refractive index change, chiral effects, and diffraction may be used within an essentially similar optical configuration, and may provide particular and significant benefits.

Figure 11

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Examples of Optical Signal Formats

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Principle	Label Type	Instrument	DDx Status
Scatter	polymer beads silica beads magnetic beads	scatterometry	demonstrated
Optical absorption	colloidal gold magnetic beads	reflectometry photometry	scheduled
Change in polarization state	polymer beads silica beads	ellipsometry (with compensator) polarimetry (wout compensator)	scheduled
Change in refractive index	high refractive index or optically active materials	ellipsometry (with compensator) polarimetry (wout compensator)	scheduled
Chiral effects	azio dyes chiral compounds		envisioned
Diffraction effects	patterned surface	interferometry	envisioned
Spectroscopic effects	wavelength selective materials	spectrometer	envisioned

Signal reception techniques might include:
single diode detector - e.g. scanning (small beam method)
diode array detector - e.g. array (virtual beam method)
CCD detector - e.g. array (virtual beam method)

Figure 12

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Aggregate vs. Scanning Ellipsometry

Innovation Group

Scanning Micro-Laser Configuration
Scale of potential elements

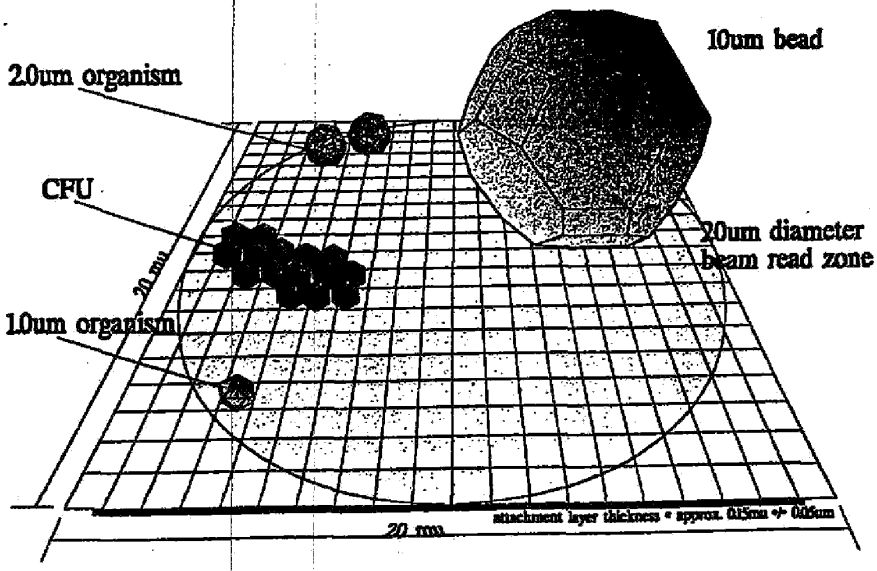


Figure 13

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Potential for Optical Enhancement

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For either the Scanning (small beam) or the Array (virtual beam) approach, a substantial improvement in signal delectability may be possible by using unique characteristics of optically based mass detection systems.

Properties of the mass enhancement label may alter the optical signal due to a number of physical characteristics, including:

- refractive index
- scatter
- chiral effect
- general adsorption
- wavelength specific adsorption
- diffraction

effectively creating an improved ability to discriminate the signal generated by the binding of the label to the complex from that created by surface background or in the absence of specific binding events. This may operate through the creation of an enhanced or attenuated "apparent" signal over that which would be created by "normal" materials.

Scale of normal vs. optically active elements @ 2X effect

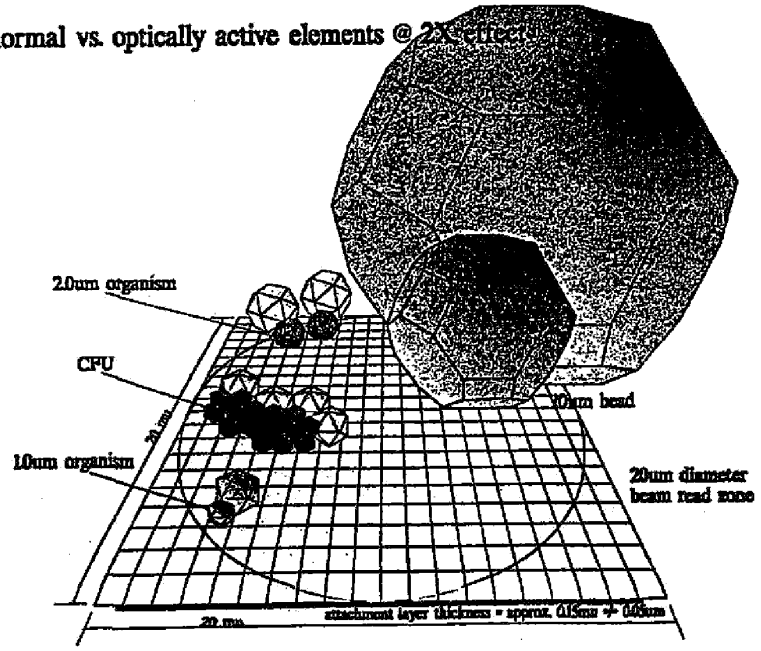


Figure 14

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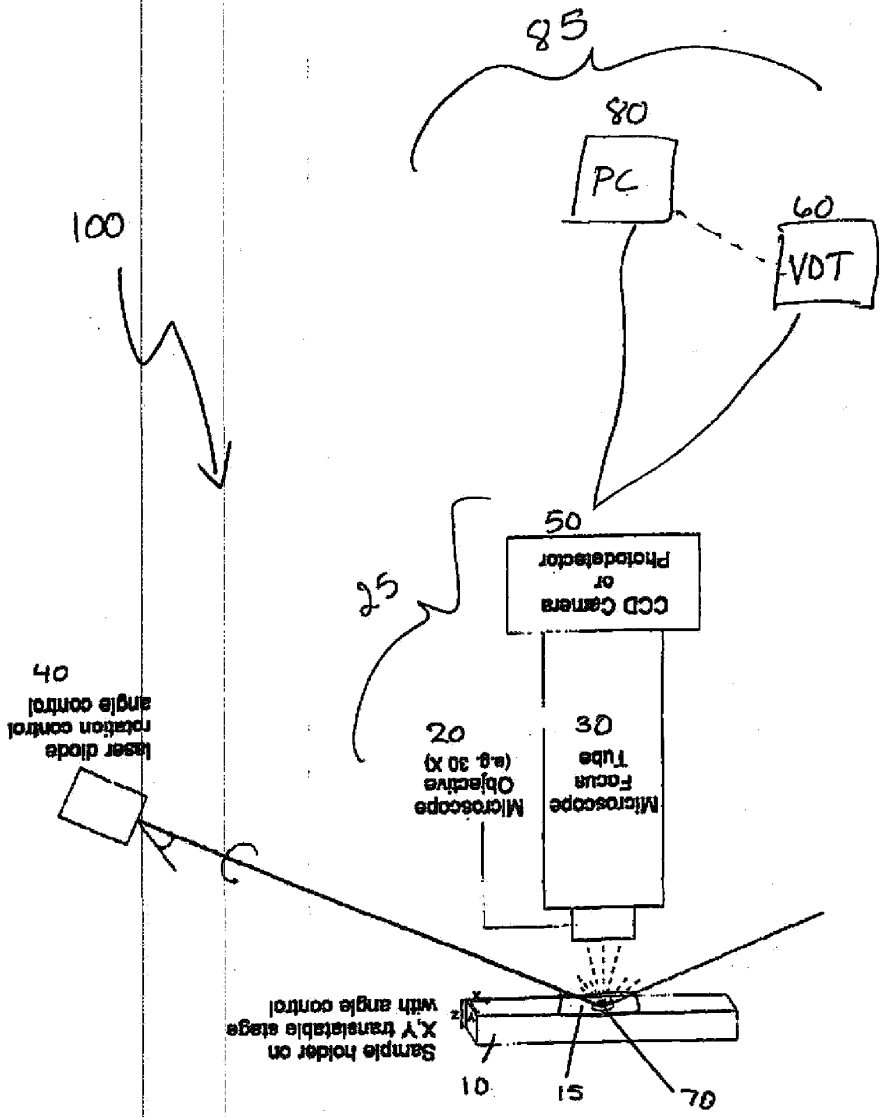


Fig. 15

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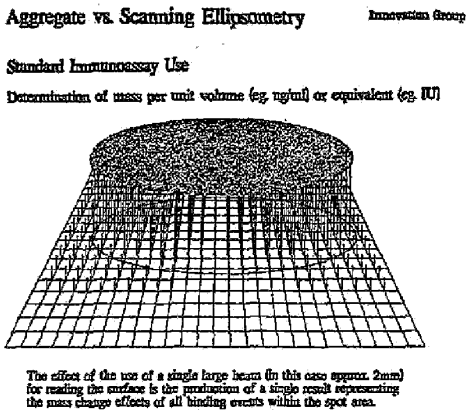
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[Continued on next page]

(54) Title: ENUMERATION METHOD OF ANALYTE DETECTION



The effect of the use of a single large beam (in this case approx. 2mm) for reading the surface is the production of a single result representing the mass change effects of all binding events within the spot area.

(57) Abstract: This invention is directed to an optically-based method and system for analyte detection using solid phase immobilization, specific analyte labels adapted for signal generation and corresponding processes for the utilization thereof. The enumeration detection method disclosed herein narrows the area for signal observation, thus, improving detectable signal to background ratio. The system is comprised of a platform/support for immobilizing a sample stage having a labeled sample (analyte complex) bound thereto, a radiation source, an optical apparatus for generating and directing radiation at said sample and a means for data collection and analysis. Upon engagement of the system, the sample generates a signal capable of differentiation from background signal, both of which are collected and imaged with a signal detector that generated a sample image to a data processing apparatus. Said apparatus receives signal measurements and, in turn, enumerates individual binding events. Generated signal may be increased via selected mass enhancement. The invention, enumeration assay methodology detecting individual binding events, may be used, for example, in analyses to detect analyte or confirm results in both research, commercial and point of care applications.

WO 99/58948 A3

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INTERNATIONAL SEARCH REPORT

rnational application No.
PCT/US99/10917

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,521,522 A (LUNDSTROM et al.) 04 June 1985 (04.06.85), col. 1, line 52-col. 2, line 45.	1-18
Y	US 4,508,832 A (CARTER et al.) 02 April 1985 (02.04.85), col. 5, line 30-col. 7, line 15.	1-18
A	US 5,017,009 A (SCHUTT et al.) 21 May 1991 (21.05.91), col. 3, line 10-col. 4, line 50.	1-18
A	US 4,876,208 A (GUSTAFSON et al.) 24 October 1989 (24.10.89), col. 2, lines 15-55.	1-18
A	US 5,496,701 A (POLLARD-KNIGHT) 05 March 1996 (05.03.96), col. 2, line 1-col. 3, line 14.	1-18
A	US 5,622,868 A (CLARKE et al.) 22 April 1997 (22.04.97), col. 2, line 1-col. 3, line 35.	1-18

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 4,805,623 A (JOBSIS) 21 February 1989 (21.02.89), col. 3, line 60-col. 7, line 68.	1-18
A	US 4,885,077 A (KARAKELLE et al.) 05 December 1989 (05.12.89), col. 2, line 43-col. 3, line 22.	1-18

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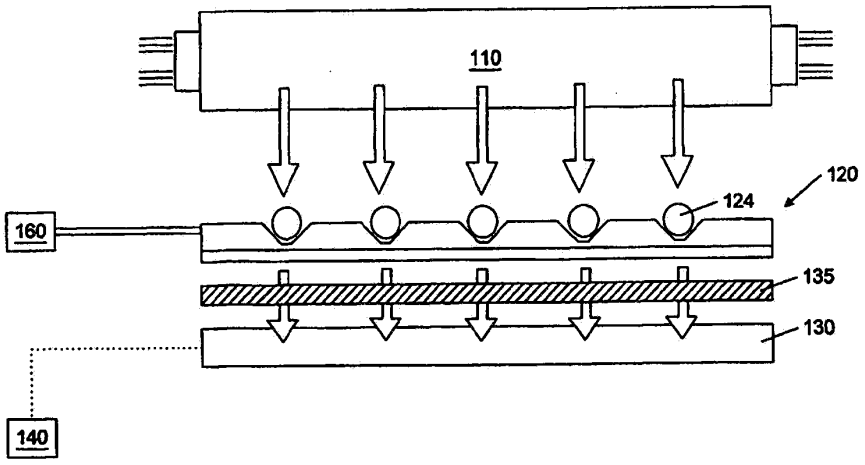
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: SENSOR ARRAYS FOR THE MEASUREMENT AND IDENTIFICATION OF MULTIPLE ANALYTES IN SOLUTIONS



(57) Abstract

A system for the rapid characterization of multi-analyte fluids, in one embodiment, includes a light source, a sensor array, and a detector. The sensor array is formed from a supporting member into which a plurality of cavitites may be formed. A series of chemically sensitive particles microspheres are, in one embodiment positioned within the cavities. The particles may be configured to produce a signal when a receptor coupled to the particle interacts with the analyte. Using pattern recognition techniques, the analytes within a multi-analyte fluid may be characterized.

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TITLE: SENSOR ARRAYS FOR THE MEASUREMENT AND IDENTIFICATION OF MULTIPLE ANALYTES IN SOLUTIONS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Research leading to this invention was federally supported, in part, by grant No. 1R01GM57306-01 entitled "The Development of an Electronic Tongue" from the National Institute of Health and the U.S. Government has certain rights to this invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a method and device for the detection of analytes in a fluid. More particularly, the invention relates to the development of a sensor array system capable of discriminating mixtures of analytes, toxins, and/or bacteria in medical, food/beverage, and environmental solutions.

2. Brief Description of the Related Art

The development of smart sensors capable of discriminating different analytes, toxins, and bacteria has become increasingly important for clinical, environmental, health and safety, remote sensing, military, food/beverage and chemical processing applications. Although many sensors capable of high sensitivity and high selectivity detection have been fashioned for single analyte detection, only in a few selected cases have array sensors been prepared which display solution phase multi-analyte detection capabilities. The advantages of such array systems are their utility for the analysis of multiple analytes and their ability to be "trained" to respond to new stimuli. Such on site adaptive analysis capabilities afforded by the array structures make their utilization promising for a variety of future applications. Array based sensors displaying the capacity to sense and identify complex vapors have been demonstrated recently using a number of distinct transduction schemes. For example, functional sensors based on Surface Acoustic Wave (SAW), tin oxide (SnO_2) sensors, conductive organic polymers, and carbon black/polymer composites have been fashioned. The use of tin oxide sensors, for example, is described in U.S. Patent No. 5,654,497 to Hoffheins et al. These sensors display the capacity to identify and discriminate between a variety of organic vapors by virtue of small site-to-site differences in response characteristics. Pattern recognition of the overall fingerprint response for the array serves as the basis for an olfaction-like detection of the vapor phase analyte species. Indeed, several commercial "electronic noses" have been developed recently. Most of the well established sensing elements are based on SnO_2 arrays which have been derivatized so as to yield chemically distinct response properties. Arrays based on SAW crystals yield extremely sensitive responses to vapor, however, engineering challenges have prevented the creation of large SAW arrays having multiple sensor sites. To our knowledge, the largest SAW device reported to date possesses only 12 sensor elements. Additionally, limited chemical diversity and the lack of understanding of the molecular features of such systems makes their expansion into more complex analysis difficult.

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Other structures have been developed that are capable of identifying and discriminating volatile organic molecules. One structure involves a series of conductive polymer layers deposited onto metal contacting layers. When these sensors are exposed to volatile reagents, some of the volatile reagents adsorb into the polymer layers, leading to small changes in the electrical resistance of these layers. It is the small differences in the behavior of the various sites that allows for a discrimination, identification, and quantification of the vapors. The detection process takes only a few seconds, and sensitivities of part-per-billion can be achieved with this relatively simple approach. This "electronic nose" system is described in U.S. Patent No. 5,698,089 to Lewis et al. which is incorporated by reference as if set forth herein.

Although the above described electronic nose provides an impressive capability for monitoring volatile reagents, the system possesses a number of undesirable characteristics that warrant the development of alternative sensor array systems. For example, the electronic nose can be used only for the identification of volatile reagents. For many environmental, military, medical, and commercial applications, the identification and quantification of analytes present in liquid or solid-phase samples is necessary. Moreover, the electronic nose systems are expensive (e.g., the Aromascan system costs about $50,000/unit) and bulky ($\geq 1 ft^3$). Furthermore, the functional elements for the currently available electronic nose are composed of conductive polymer systems which possess little chemical selectivity for many of the analytes which are of interest to the military and civilian communities.

One of the most commonly employed sensing techniques has exploited colloidal polymer microspheres for latex agglutination tests (LATs) in clinical analysis. Commercially available LATs for more than 60 analytes are used routinely for the detection of infectious diseases, illegal drugs, and early pregnancy tests. The vast majority of these types of sensors operate on the principle of agglutination of latex particles (polymer microspheres) which occurs when the antibody-derivatized microspheres become effectively "cross-linked" by a foreign antigen resulting in the attachment to, or the inability to pass through a filter. The dye-doped microspheres are then detected colorimetrically upon removal of the antigen carrying solution. However, the LATs lack the ability to be utilized for multiple, real time analyte detection schemes as the nature of the response intrinsically depends on a cooperative effect of the entire collection of microspheres.

Similar to the electronic nose, array sensors that have shown great analytical promise are those based on the "DNA on a chip" technology. These devices possess a high density of DNA hybridization sites that are affixed in a two-dimensional pattern on a planar substrate. To generate nucleotide sequence information, a pattern is created from unknown DNA fragments binding to various hybridization sites. Both radiochemical and optical methods have provided excellent detection limits for analysis of limited quantities of DNA. (Stimpson, D. I.; Hoijer, J. V.; Hsieh, W.; Jou, C.; Gardon, J.; Theriault, T.; Gamble, R.; Baldeschwieler, J.D. Proc. Natl. Acad. Sci. USA 1995, 92, 6379). Although quite promising for the detection of DNA fragments, these arrays are generally not designed for non-DNA molecules, and accordingly show very little sensitivity to smaller organic molecules. Many of the target molecules of interest to civilian and military communities, however, do not possess DNA components. Thus, the need for a flexible, non-DNA based sensor is still desired. Moreover, while a number of prototype DNA chips containing up to a few thousand different nucleic acid probes have been described, the existing technologies tend to be difficult to expand to a practical size. As a result, DNA chips may be prohibitively expensive for practical uses.

A system of analyzing fluid samples using an array formed of heterogeneous, semi-selective thin films

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which function as sensing receptor units is described in U.S. Patent No. 5,512,490 to Walt et al., which is incorporated by reference as if set forth herein. Walt appears to describe the use of covalently attached polymeric "cones" which are grown via photopolymerization onto the distal face of fiber optic bundles. These sensor probes appear to be designed with the goal of obtaining unique, continuous, and reproducible responses from small localized regions of dye-doped polymer. The polymer appears to serve as a solid support for indicator molecules that provide information about test solutions through changes in optical properties. These polymer supported sensors have been used for the detection of analytes such as pH, metals, and specific biological entities. Methods for manufacturing large numbers of reproducible sensors, however, has yet to be developed. Moreover, no methods for acquisitions of data streams in a simultaneous manner are commercially available with this system. Optical alignment issues may also be problematic for these systems.

A method of rapid sample analysis for use in the diagnostic microbiology field is also desirable. The techniques now used for rapid microbiology diagnostics detect either antigens or nucleic acids. Rapid antigen testing is based on the use of antibodies to recognize either the single cell organism or the presence of infected cell material. Inherent to this approach is the need to obtain and characterize the binding of the antibody to unique structures on the organism being tested. Since the identification and isolation of the appropriate antibodies is time consuming, these techniques are limited to a single agent per testing module and there is no opportunity to evaluate the amount of agent present.

Most antibody methods are relatively insensitive and require the presence of 10^5 to 10^7 organisms. The response time of antibody-antigen reactions in diagnostic tests of this type ranges from 10 to 120 minutes, depending on the method of detection. The fastest methods are generally agglutination reactions, but these methods are less sensitive due to difficulties in visual interpretation of the reactions. Approaches with slower reaction times include antigen recognition by antibody conjugated to either an enzyme or chromophore. These test types tend to be more sensitive, especially when spectrophotometric methods are used to determine if an antigen-antibody reaction has occurred. These detection schemes do not, however, appear to allow the simultaneous detection of multiple analytes on a single detector platform.

The alternative to antigen detection is the detection of nucleic acids. An approach for diagnostic testing with nucleic acids uses hybridization to target unique regions of the target organism. These techniques require fewer organisms (10^3 to 10^5), but require about five hours to complete. As with antibody-antigen reactions this approach has not been developed for the simultaneous detection of multiple analytes.

The most recent improvement in the detection of microorganisms has been the use of nucleic acid amplification. Nucleic acid amplification tests have been developed that generate both qualitative and quantitative data. However, the current limitations of these testing methods are related to delays caused by specimen preparation, amplification, and detection. Currently, the standard assays require about five hours to complete. The ability to complete much faster detection for a variety of microorganisms would be of tremendous importance to military intelligence, national safety, medical, environmental, and food areas.

It is therefore desirable that new sensors capable of discriminating different analytes, toxins, and bacteria be developed for medical/clinical diagnostic, environmental, health and safety, remote sensing, military, food/beverage, and chemical processing applications. It is further desired that the sensing system be adaptable to

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the simultaneous detection of a variety of analytes to improve throughput during various chemical and biological analytical procedures.

SUMMARY OF THE INVENTION

Herein we describe a system and method for the analysis of a fluid containing one or more analytes. The system may be used for either liquid or gaseous fluids. The system, in some embodiments, may generate patterns that are diagnostic for both the individual analytes and mixtures of the analytes. The system in some embodiments, is made of a plurality of chemically sensitive particles, formed in an ordered array, capable of simultaneously detecting many different kinds of analytes rapidly. An aspect of the system is that the array may be formed using a microfabrication process, thus allowing the system to be manufactured in an inexpensive manner.

In an embodiment of a system for detecting analytes, the system, in some embodiments, includes a light source, a sensor array, and a detector. The sensor array, in some embodiments, is formed of a supporting member which is configured to hold a variety of chemically sensitive particles (herein referred to as "particles") in an ordered array. The particles are, in some embodiments, elements which will create a detectable signal in the presence of an analyte. The particles may produce optical (e.g., absorbance or reflectance) or fluorescence/phosphorescent signals upon exposure to an analyte. Examples of particles include, but are not limited to functionalized polymeric beads, agarous beads, dextrose beads, polyacrylamide beads, control pore glass beads, metal oxides particles (e.g., silicon dioxide (SiO_2) or aluminum oxides (Al_2O_3)), polymer thin films, metal quantum particles (e.g., silver, gold, platinum, etc.), and semiconductor quantum particles (e.g., Si, Ge, GaAs, etc.). A detector (e.g., a charge-coupled device "CCD") in one embodiment is positioned below the sensor array to allow for the data acquisition. In another embodiment, the detector may be positioned above the sensor array to allow for data acquisition from reflectance of the light off of the particles.

Light originating from the light source may pass through the sensor array and out through the bottom side of the sensor array. Light modulated by the particles may pass through the sensor array and onto the proximally spaced detector. Evaluation of the optical changes may be completed by visual inspection or by use of a CCD detector by itself or in combination with an optical microscope. A microprocessor may be coupled to the CCD detector or the microscope. A fluid delivery system may be coupled to the supporting member of the sensor array. The fluid delivery system, in some embodiments, is configured to introduce samples into and out of the sensor array.

In an embodiment, the sensor array system includes an array of particles. The particles may include a receptor molecule coupled to a polymeric bead. The receptors, in some embodiments, are chosen for interacting with analytes. This interaction may take the form of a binding/association of the receptors with the analytes. The supporting member may be made of any material capable of supporting the particles, while allowing the passage of the appropriate wavelengths of light. The supporting member may include a plurality of cavities. The cavities may be formed such that at least one particle is substantially contained within the cavity.

In an embodiment, the optical detector may be integrated within the bottom of the supporting member, rather than using a separate detecting device. The optical detectors may be coupled to a microprocessor to allow evaluation of fluids without the use of separate detecting components. Additionally, a fluid delivery system may

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also be incorporated into the supporting member. Integration of detectors and a fluid delivery system into the supporting member may allow the formation of a compact and portable analyte sensing system.

A high sensitivity CCD array may be used to measure changes in optical characteristics which occur upon binding of the biological/chemical agents. The CCD arrays may be interfaced with filters, light sources, fluid delivery and micromachined particle receptacles, so as to create a functional sensor array. Data acquisition and handling may be performed with existing CCD technology. CCD detectors may be configured to measure white light, ultraviolet light or fluorescence. Other detectors such as photomultiplier tubes, charge induction devices, photo diodes, photodiode arrays, and microchannel plates may also be used.

A particle, in some embodiments, possess both the ability to bind the analyte of interest and to create a modulated signal. The particle may include receptor molecules which posses the ability to bind the analyte of interest and to create a modulated signal. Alternatively, the particle may include receptor molecules and indicators. The receptor molecule may posses the ability to bind to an analyte o f interest. Upon binding the analyte of interest, the receptor molecule may cause the indicator molecule to produce the modulated signal. The receptor molecules may be naturally occurring or synthetic receptors formed by rational design or combinatorial methods. Some examples of natural receptors include, but are not limited to, DNA, RNA, proteins, enzymes, oligopeptides, antigens, and antibodies. Either natural or synthetic receptors may be chosen for their ability to bind to the analyte molecules in a specific manner.

In one embodiment, a naturally occurring or synthetic receptor is bound to a polymeric bead in order to create the particle. The particle, in some embodiments, is capable of both binding the analyte(s) of interest and creating a detectable signal. In some embodiments, the particle will create an optical signal when bound to an analyte of interest.

A variety of natural and synthetic receptors may be used. The synthetic receptors may come from a variety of classes including, but not limited to, polynucleotides (e.g., aptamers), peptides (e.g., enzymes and antibodies), synthetic receptors, polymeric unnatural biopolymers (e.g., polythioureas, polyguanidiniums), and imprinted polymers. Polynucleotides are relatively small fragments of DNA which may be derived by sequentially building the DNA sequence. Peptides include natural peptides such as antibodies or enzymes or may be synthesized from amino acids. Unnatural biopolymers are chemical structure which are based on natural biopolymers, but which are built from unnatural linking units. For example, polythioureas and polyguanidiniums have a structure similar to peptides, but may be synthesized from diamines (i.e., compounds which include at least two amine functional groups) rather than amino acids. Synthetic receptors are designed organic or inorganic structures capable of binding various analytes.

In an embodiment, a large number of chemical/biological agents of interest to the military and civilian communities may be sensed readily by the described array sensors. Bacteria may also be detected using a similar system. To detect, sense, and identify intact bacteria, the cell surface of one bacteria may be differentiated from other bacteria, or genomic material may be detected using oligonucleic receptors. One method of accomplishing this differentiation is to target cell surface oligosaccharides (i.e., sugar residues). The use of synthetic receptors which are specific for oligosaccharides may be used to determine the presence of specific bacteria by analyzing for cell surface oligosaccharides.

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BRIEF DESCRIPTION OF THE DRAWINGS

The above brief description as well as further objects, features and advantages of the methods and apparatus of the present invention will be more fully appreciated by reference to the following detailed description of presently preferred but nonetheless illustrative embodiments in accordance with the present invention when taken in conjunction with the accompanying drawings in which:

FIG. 1 depicts a schematic of an analyte detection system;

FIG. 2 depicts a particle disposed in a cavity;

FIG. 3 depicts a sensor array;

FIG. 4A-F depicts the formation of a Fabry-Perot cavity on the back of a sensor array;

FIG. 5 depicts the chemical constituents of a particle;

FIG. 6 depicts the chemical formulas of some receptor compounds;

FIG. 7 depicts a plot of the absorbance of green light vs. concentration of calcium (Ca^{+2}) for a particle which includes an o-cresolphthalein complexone receptor;

FIG. 8 depicts a schematic view of the transfer of energy from a first indicator to a second indicator in the presence of an analyte;

FIG. 9 depicts a schematic of the interaction of a sugar molecule with a boronic acid based receptor.

FIG. 10 depicts various synthetic receptors;

FIG. 11 depicts a synthetic pathway for the synthesis of polythioureas;

FIG. 12 depicts a synthetic pathway for the synthesis of polyguanidiniums;

FIG. 13 depicts a synthetic pathway for the synthesis of diamines from amino acids;

FIG. 14 depicts fluorescent diamino monomers;

FIG. 15 depicts a plot of counts/sec. (i.e., intensity) vs. time as the pH of a solution surrounding a particle coupled to *o*-cresolphthalein is cycled from acidic to basic conditions;

FIG. 16 depicts the color responses of a variety of sensing particles to solutions of Ca^{+2} and various pH levels;

FIG. 17 depicts an analyte detection system which includes a sensor array disposed within a chamber;

FIG. 18 depicts an integrated analyte detection system;

FIG. 19 depicts a cross-sectional view of a cavity covered by a mesh cover;

FIG. 20 depicts a top view of a cavity covered by a mesh cover;

FIG. 21A-G depicts a cross-sectional view of a series of processing steps for the formation of a sensor array which includes a removable top and bottom cover;

FIG. 22A-G depicts a cross-sectional view of a series of processing steps for the formation of a sensor array which includes a removable top and a stationary bottom cover;

FIG. 23A-G depicts a cross-sectional view of a series of processing steps for the formation of a sensor array which includes a removable top;

FIG. 24A-D depicts a cross-sectional view of a series of processing steps for the formation of a silicon based sensor array which includes a top and bottom cover with openings aligned with the cavity;

FIG. 25A-D depicts a cross-sectional view of a series of processing steps for the formation of a photoresist based sensor array which includes a top and bottom cover with openings aligned with the cavity;

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FIG. 26A-E depicts a cross-sectional view of a series of processing steps for the formation of a plastic based sensor array which includes a top and bottom cover with openings aligned with the cavity;

FIG. 27A-D depicts a cross-sectional view of a series of processing steps for the formation of a silicon based sensor array which includes a top cover with openings aligned with the cavity and a tapered cavity;

FIG. 28A-E depicts a cross-sectional view of a series of processing steps for the formation of a photoresist based sensor array which includes a top cover with openings aligned with the cavity and a tapered cavity;

FIG. 29A-E depicts a cross-sectional view of a series of processing steps for the formation of a photoresist based sensor array which includes a top cover with openings aligned with the cavity and a bottom cover;

FIG. 30A-D depicts a cross-sectional view of a series of processing steps for the formation of a plastic based sensor array which includes a top cover with openings aligned with the cavity and a bottom cover;

FIG. 31 depicts a cross-sectional view of a schematic of a micropump;

FIG. 32 depicts a top view of an electrohydrodynamic pump;

FIG. 33 depicts a cross-sectional view of a sensor array which includes a micropump;

FIG. 34 depicts a cross-sectional view of a sensor array which includes a micropump and channels which are coupled to the cavities;

FIG. 35 depicts a cross-sectional view of a sensor array which includes multiple micropumps each micropump being coupled to a cavity;

FIG. 36 depicts a top view of a sensor array which includes multiple electrohydrodynamic pumps;

FIG. 37 depicts a cross-sectional view of a sensor array which includes a system for delivering a reagent from a reagent particle to a sensing cavity.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Herein we describe a system and method for the simultaneous analysis of a fluid containing multiple analytes. The system may be used for either liquid or gaseous fluids. The system may generate patterns that are diagnostic for both individual analytes and mixtures of the analytes. The system, in some embodiments, is made of a combination of chemically sensitive particles, formed in an ordered array, capable of simultaneously detecting many different kinds of analytes rapidly. An aspect of the system is that the array may be formed using a microfabrication process, thus allowing the system to be manufactured in an inexpensive manner.

SYSTEM FOR ANALYSIS OF ANALYTES

Shown in FIG. 1 is an embodiment of a system for detecting analytes in a fluid. The system, in some embodiments, includes a light source 110, a sensor array 120 and a detector 130. The light source 110 may be a white light source or light emitting diodes (LED). In one embodiment, light source 110 may be a blue light emitting diode (LED) for use in systems relying on changes in fluorescence signals. For colorimetric (e.g., absorbance) based systems, a white light source may be used. The sensor array 120, in some embodiments, is formed of a supporting member which is configured to hold a variety of particles 124. A detecting device 130 (e.g., a charge-coupled device "CCD") may be positioned below the sensor array to allow for data acquisition. In another embodiment, the detecting device 130 may be positioned above the sensor array.

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Light originating from the light source 110, in some embodiments, passes through the sensor array 120 and out through the bottom side of the sensor array. The supporting member and the particles together, in some embodiments, provide an assembly whose optical properties are well matched for spectral analyses. Thus, light modulated by the particles may pass through the sensor array and onto the proximally spaced detector 130. Evaluation of the optical changes may be completed by visual inspection (e.g., with a microscope) or by use of a microprocessor 140 coupled to the detector. For fluorescence measurements, a filter 135 may be placed between supporting member 120 and detector 130 to remove the excitation wavelength. A fluid delivery system 160 may be coupled to the supporting member. The fluid delivery system 160 may be configured to introduce samples into and out of the sensor array.

In an embodiment, the sensor array system includes an array of particles. Upon the surface and within the interior region of the particles are, in some embodiments, located a variety of receptors for interacting with analytes. The supporting member, in some embodiments, is used to localize these particles as well as to serve as a microenvironment in which the chemical assays can be performed. For the chemical/biological agent sensor arrays, the particles used for analysis are about 0.05 - 500 microns in diameter, and may actually change size (e.g., swell or shrink) when the chemical environment changes. Typically, these changes occur when the array system is exposed to the fluid stream which includes the analytes. For example, a fluid stream which comprises a non-polar solvent, may cause non-polar particles to change in volume when the particles are exposed to the solvent. To accommodate these changes, it is preferred that the supporting member consist of an array of cavities which serve as micro test-tubes.

The supporting member may be made of any material capable of supporting the particles, while allowing the passage of the appropriate wavelength of light. The supporting member is also made of a material substantially impervious to the fluid in which the analyte is present. A variety of materials may be used including plastics, glass, silicon based materials (e.g., silicon, silicon dioxide, silicon nitride, etc.) and metals. In one embodiment, the supporting member includes a plurality of cavities. The cavities may be formed such that at least one particle is substantially contained within the cavity. Alternatively, a plurality of particles may be contained within a single cavity.

In an embodiment, the supporting member may consist of a strip of plastic which is substantially transparent to the wavelength of light necessary for detection. A series of cavities may be formed within the strip. The cavities may be configured to hold at least one particle. The particles may be contained within the strip by a transparent cover which is configured to allow passage of the analyte containing fluid into the cavities.

In another embodiment, the supporting member may be formed using a silicon wafer as depicted in FIG. 2. The silicon wafer 210 may include a substantially transparent layer 220 formed on the bottom surface of the wafer. The cavities 230, in one embodiment, are formed by an anisotropic etch process of the silicon wafer. In one embodiment, anisotropic etching of the silicon wafer is accomplished using a wet hydroxide etch. Photolithographic techniques may be used to define the locations of the cavities. The cavities may be formed such that the sidewalls of the cavities are substantially tapered at an angle of between about 50 to 60 degrees. Formation of such angled cavities may be accomplished by wet anisotropic etching of <100> silicon. The term "<100> silicon" refers to the crystal orientation of the silicon wafer. Other types of silicon, (e.g., <110> and <111> silicon) may lead to steeper angled sidewalls. For example, <111> silicon may lead to sidewalls formed at about 90

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degrees. The angled sides of the cavities in some embodiments, serve as "mirror layers" which may improve the light collection efficiency of the cavities. The etch process may be controlled so that the formed cavities extend through the silicon wafer to the upper surface of transparent layer 220. While depicted as pyramidal, the cavities may be formed in a number of shapes including but not limited to, spherical, oval, cubic, or rectangular. An advantage to using a silicon wafer for the support member, is that the silicon material is substantially opaque to the light produced from the light source. Thus, the light may be inhibited from passing from one cavity to adjacent cavities. In this manner, light from one cavity may be inhibited from influencing the spectroscopic changes produced in an adjacent cavity.

The silicon wafer, in some embodiments, has an area of approximately 1 cm^2 to about 100 cm^2 and includes about 10^1 to about 10^6 cavities. In an embodiment, about 100 cavities are formed in a ten by ten matrix. The center to center distance between the cavities, in some embodiments, is about 500 microns. Each of the cavities may include at least one particle.

The transparent layer 220 may serve as a window, allowing light of a variety of wavelengths to pass through the cavities 230 and to the detector. Additionally, the transparent layer may serve as a platform onto which the individual particles 235 may be positioned. The transparent layer may be formed of silicon dioxide (SiO_2), silicon nitride (Si_3N_4) or silicon dioxide/silicon nitride multi-layer stacks. The transparent layer, in some embodiments, is deposited onto the silicon wafer prior to the formation of the cavities.

The cavities 230 may be sized to substantially contain a particle 235. The cavities are, in some embodiments, larger than a particle. The cavities are, in some embodiments, sized to allow facile placement and removal of the particle within the cavities. The cavity may be substantially larger than the particle, thus allowing the particle to swell during use. For example, a particle may have a size as depicted in FIG. 2 by particle 235. During use, contact with a fluid (e.g., a solvent) may cause the particle to swell, for example, to a size depicted as circle 236. In some embodiments, the cavity is sized to allow such swelling of the particle during use. A particle may be positioned at the bottom of a cavity using, e.g., a micromanipulator. After a particle has been placed within the cavity, a transparent cover plate 240 may be placed on top of the supporting member to keep the particle in place.

When forming an array which includes a plurality of particles, the particles may be placed in the array in an ordered fashion using the micromanipulator. In this manner, a ordered array having a predefined configuration of particles may be formed. Alternatively, the particles may be randomly placed within the cavities. The array may subsequently undergo a calibration test to determine the identity of the particle at any specified location in the supporting member.

The transparent cover plate 240, in some embodiments, is coupled to the upper surface of the silicon wafer 220 such that the particles are inhibited from becoming dislodged from the cavity. The transparent cover plate, in some embodiments, is positioned a fixed distance above the silicon wafer, as depicted in FIG. 2, to keep the particle in place, while allowing the entrance of fluids into the cavities. The transparent cover plate, in some embodiments, is positioned at a distance above the substrate which is substantially less than a width of the particle. The transparent cover plate may be made of any material which is substantially transparent to the wavelength of light being utilized by the detector. The transparent cover plate may be made of plastic, glass, quartz, or silicon dioxide/silicon nitride.

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In one embodiment, the transparent cover plate 240, is a thin sheet of glass (e.g., a microscope slide cover slip). The slide may be positioned a fixed distance above the silicon wafer. Support structures 241 (See FIG. 2) may be placed upon the silicon wafer 210 to position the transparent cover plate 240. The support structures may be formed from a polymer or a silicon based material. In another embodiment, a polymeric substrate is coupled to the silicon wafer to form the support structures 241 for the transparent cover plate 240. In an embodiment, a plastic material with an adhesive backing (e.g., cellophane tape) is positioned on the silicon wafer 210. After the support structures 241 are placed on the wafer the transparent cover plate 240 is placed upon the support structures. The support structures inhibit the transparent cover sheet from contacting the silicon wafer 200. In this manner, a channel is formed between the silicon wafer and the transparent cover plate which allow the fluid to pass into the cavity, while inhibiting displacement of the particle by the fluid.

In another embodiment, the transparent cover plate 240 may be fastened to the upper surface of the silicon wafer, as depicted in FIG. 3. In this embodiment, the fluid may be inhibited from entering the cavities 230 by the transparent cover plate 240. To allow passage of the fluid into the cavities, a number of channels 250 may be formed in the silicon wafer. The channels, in one embodiment, are oriented to allow passage of the fluid into substantially all of the cavities. When contacted with the fluid, the particles may swell to a size which may plug the channels. To prevent this plugging, the channels may be formed near the upper portion of the cavities, as depicted in FIG 3. The channels, in one embodiment, are formed using standard photolithographic masking to define the regions where the trenches are to be formed, followed by the use of standard etching techniques. A depth of the cavity may be such that the particle resides substantially below the position of the channel. In this way, the plugging of the channels due to swelling of the particle may be prevented.

The inner surfaces of the cavities may be coated with a material to aid the positioning of the particles within the cavities. In one embodiment, a thin layer of gold or silver may be used to line the inner surface of the cavities. The gold or silver layer may act as an anchoring surface to anchor particles (e.g., via alkylthiol bonding). In addition, the gold or silver layer may also increase the reflectivity of the inner surface of the cavities. The increased reflectance of the surface may enhance the analyte detection sensitivity of the system. Alternatively, polymer layers and self-assembled monolayers formed upon the inner surface of the cavities may be used to control the particle adhesion interactions. Additional chemical anchoring methods may be used for silicon surfaces such as those based on siloxane type reagents, which may be attached to Si-OH functionalities. Similarly, monomeric and polymeric reagents attached to an interior region of the cavities can be used to alter the local wetting characteristics of the cavities. This type of methodology can be used to anchor the particles as well as to alter the fluid delivery characteristics of the cavity. Furthermore, amplification of the signals for the analytes may be accomplished with this type of strategy by causing preconcentration of appropriate analytes in the appropriate type of chemical environment.

In another embodiment, the optical detector may be integrated within the bottom transparent layer 220 of the supporting member, rather than using a separate detecting device. The optical detectors may be formed using a semiconductor-based photodetector 255. The optical detectors may be coupled to a microprocessor to allow evaluation of fluids without the use of separate detecting components. Additionally, the fluid delivery system may also be incorporated into the supporting member. Micro-pumps and micro-valves may also be incorporated into the silicon wafer to aid passage of the fluid through the cavities. Integration of detectors and a fluid delivery system

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into the supporting member may allow the formation of a compact and portable analyte sensing system. Optical filters may also be integrated into the bottom membrane of the cavities. These filters may prevent short wavelength excitation from producing "false" signals in the optical detection system (e.g., a CCD detector array) during fluorescence measurements.

A sensing cavity may be formed on the bottom surface of the support substrate. An example of a sensing cavity that may be used is a Fabry-Perot type cavity. Fabry-Perot cavity-based sensors may be used to detect changes in optical path length induced by either a change in the refractive index or a change in physical length of the cavity. Using micromachining techniques, Fabry-Perot sensors may be formed on the bottom surface of the cavity.

Figures 4A-F depict a sequence of processing steps for the formation of a cavity and a planar top diaphragm Fabry-Perot sensor on the bottom surface of a silicon based supporting member. A sacrificial barrier layer 262a/b is deposited upon both sides of a silicon supporting member 260. The silicon supporting member 260 may be a double-side polished silicon wafer having a thickness ranging from about 100 μm to about 500 μm, preferably from about 200 μm to about 400 μm, and more preferably of about 300 μm. The barrier layer 262a/b may be composed of silicon dioxide, silicon nitride, or silicon oxynitride. In one embodiment, the barrier layer 262a/b is composed of a stack of dielectric materials. As depicted in FIG 4A, the barrier layer 262 a/b is composed of a stack of dielectric materials which includes a silicon nitride layer 271a/b and a silicon dioxide layer 272a/b. Both layers may be deposited using a low pressure chemical vapor deposition ("LPCVD") process. Silicon nitride may be deposited using an LPCVD reactor by reaction of ammonia (NH_3) and dichlorosilane ($SiCl_2H_2$) at a gas flow rate of about 3.5:1, a temperature of about 800 □C, and a pressure of about 220 mTorr. The silicon nitride layer 271a/b is deposited to a thickness in the range from about 100 Å to about 500 Å, preferably from 200 Å to about 400 Å, and more preferably of about 300 Å. Silicon dioxide is may be deposited using an LPCVD reactor by reaction of silane (SiH_4) and oxygen (O_2) at a gas flow rate of about 3:4, a temperature of about 450 □C, and a pressure of about 110 mTorr. The silicon dioxide layer 272a/b is deposited to a thickness in the range from about 3000 Å to about 7000 Å, preferably from 4000 Å to about 6000 Å, and more preferably of about 5000 Å. The front face silicon dioxide layer 272a, in one embodiment, acts as the main barrier layer. The underlying silicon nitride layer 271a acts as an intermediate barrier layer to inhibit overetching of the main barrier layer during subsequent KOH wet anisotropic etching steps.

A bottom diaphragm layer 264a/b is deposited upon the barrier layer 262a/b on both sides of the supporting member 260. The bottom diaphragm layer 264a/b may be composed of silicon nitride, silicon dioxide, or silicon oxynitride. In one embodiment, the bottom diaphragm layer 264 a/b is composed of a stack of dielectric materials. As depicted in FIG 4A, the bottom diaphragm layer 264a/b is composed of a stack of dielectric materials which includes a pair of silicon nitride layers 273a/b and 275a/b surrounding a silicon dioxide layer 274a/b. All of the layers may be deposited using an LPCVD process. The silicon nitride layers 273a/b and 275a/b have a thickness in the range from about 500 Å to about 1000 Å, preferably from 700 Å to about 800 Å, and more preferably of about 750 Å. The silicon dioxide layer 274a/b has a thickness in the range from about 3000 Å to about 7000 Å, preferably from 4000 Å to about 6000 Å, and more preferably of about 4500 Å.

A cavity which will hold the particle may now be formed in the supporting member 260. The bottom diaphragm layer 264b and the barrier layer 262b formed on the back side 261 of the silicon supporting member 260

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are patterned and etched using standard photolithographic techniques. In one embodiment, the layers are subjected to a plasma etch process. The plasma etching of silicon dioxide and silicon nitride may be performed using a mixture of carbontetrafluoride (CF_4) and oxygen (O_2). The patterned back side layers 262b and 264b may be used as a mask for anisotropic etching of the silicon supporting member 260. The silicon supporting member 260, in one embodiment, is anisotropically etched with a 40% potassium hydroxide ("KOH") solution at 80 □C to form the cavity. The etch is stopped when the front side silicon nitride layer 271a is reached, as depicted in FIG 4B. The silicon nitride layer 271a inhibits etching of the main barrier layer 272a during this etch process. The cavity 267 may be formed extending through the supporting member 260. After formation of the cavity, the remaining portions of the back side barrier layer 262b and the diaphragm layer 264b may be removed.

Etch windows 266 are formed through the bottom diaphragm layer 264a on the front side of the wafer. A masking layer (not shown) is formed over the bottom diaphragm layer 264a and patterned using standard photolithographic techniques. Using the masking layer, etch windows 266 may be formed using a plasma etch. The plasma etching of silicon dioxide and silicon nitride may be performed using a mixture of carbontetrafluoride (CF_4) and oxygen (O_2). The etching is continued through the bottom diaphragm layer 264a and partially into the barrier layer 262a. In one embodiment, the etching is stopped at approximately half the thickness of the barrier layer 262a. Thus, when the barrier layer 262a is subsequently removed the etch windows 266 will extend through the bottom diaphragm layer 264a, communicating with the cavity 267. By stopping the etching at a midpoint of the barrier layer, voids or discontinuities may be reduced since the bottom diaphragm is still continuous due to the remaining barrier layer.

After the etch windows 266 are formed, a sacrificial spacer layer 268a/b is deposited upon the bottom diaphragm layer 264a and within cavity 267, as depicted in FIG. 4C. The spacer layer may be formed from LPCVD polysilicon. In one embodiment, the front side deposited spacer layer 268a will also at least partially fill the etch windows 266. Polysilicon may be deposited using an LPCVD reactor using silane (SiH_4) at a temperature of about 650 □C. The spacer layer 268a/b is deposited to a thickness in the range from about 4000 Å to about 10,000 Å, preferably from 6000 Å to about 8000 Å, and more preferably of about 7000 Å. The preferred thickness of the spacer layer 268a is dependent on the desired thickness of the internal air cavity of the Fabry-Perot detector. For example, if a Fabry-Perot detector which is to include a 7000 Å air cavity between the top and bottom diaphragm layer is desired, a spacer layer having a thickness of about 7000 Å would be formed. After the spacer layer has been deposited, a masking layer for etching the spacer layer 268a (not shown) is used to define the etch regions of the spacer layer 268a. The etching may be performed using a composition of nitric acid (HNO_3), water, and hydrogen fluoride (HF) in a ratio of 25:13:1, respectively, by volume. The lateral size of the subsequently formed cavity is determined by the masking pattern used to define the etch regions of the spacer layer 268a.

After the spacer layer 268a has been etched, the top diaphragm layer 270a/b is formed. The top diaphragm 270a/b, in one embodiment, is deposited upon the spacer layer 268a/b on both sides of the supporting member. The top diaphragm 270a/b may be composed of silicon nitride, silicon dioxide, or silicon oxynitride. In one embodiment, the top diaphragm 270a/b is composed of a stack of dielectric materials. As depicted in FIG. 4C, the top diaphragm 270a/b is composed of a stack of dielectric materials which includes a pair of silicon nitride layers 283a/b and 285a/b surrounding a silicon dioxide layer 284a/b. All of the layers may be deposited using an LPCVD process. The silicon nitride layers 283a/b and 285a/b have a thickness in the range from about 1000 Å to about

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2000 Å, preferably from 1200 Å to about 1700 Å, and more preferably of about 1500 Å. The silicon dioxide layer 284a/b has a thickness in the range from about 5000 Å to about 15,500 Å, preferably from 7500 Å to about 12,000 Å, and more preferably of about 10,500 Å.

After depositing the top diaphragm 270a/b, all of the layers stacked on the bottom face of the supporting member (e.g., layers 268b, 283b, 284b, and 285b) are removed by multiple wet and plasma etching steps, as depicted in FIG. 4D. After these layers are removed, the now exposed portions of the barrier layer 262a are also removed. This exposes the spacer layer 268a which is present in the etch windows 266. The spacer layer 268 may be removed from between the top diaphragm 270a and the bottom diaphragm 264a by a wet etch using a KOH solution, as depicted in FIG. 4D. Removal of the spacer material 268a, forms a cavity 286 between the top diaphragm layer 270a and the bottom diaphragm layer 264a. After removal of the spacer material, the cavity 286 may be washed using deionized water, followed by isopropyl alcohol to clean out any remaining etching solution.

The cavity 286 of the Fabry-Perot sensor may be filled with a sensing substrate 290, as depicted in FIG. 4E. To coat the cavity 286 with a sensing substrate 290, the sensing substrate may be dissolved in a solvent. A solution of the sensing substrate is applied to the supporting member 260. The solution is believed to rapidly enter the cavity 286 through the etched windows 266 in the bottom diaphragm 264a, aided in part by capillary action. As the solvent evaporates, a thin film of the sensing substrate 290 coats the inner walls of the cavity 286, as well as the outer surface of the bottom diaphragm 264a. By repeated treatment of the supporting member with the solution of the sensing substrate, the thickness of the sensing substrate may be varied.

In one embodiment, the sensing substrate 290 is poly(3-dodecylthiophene) whose optical properties change in response to changes in oxidation states. The sensing substrate poly(3-dodecylthiophene) may be dissolved in a solvent such as chloroform or xylene. In one embodiment, a concentration of about 0.1 g of poly(3-dodecylthiophene)/mL is used. Application of the solution of poly(3-dodecylthiophene) to the supporting member causes a thin film of poly(3-dodecylthiophene) to be formed on the inner surface of the cavity.

In some instances, the sensing substrate, when deposited within a cavity of a Fabry-Perot type detector, may cause stress in the top diaphragm of the detector. It is believed that when a sensing polymer coats a planar top diaphragm, extra residual stress on the top diaphragm causes the diaphragm to become deflected toward the bottom diaphragm. If the deflection becomes to severe, sticking between the top and bottom diaphragms may occur. In one embodiment, this stress may be relieved by the use of supporting members 292 formed within the cavity 286, as depicted in FIG. 4F. The supporting members 292 may be formed without any extra processing steps to the above described process flow. The formation of supporting members may be accomplished by deliberately leaving a portion of the spacer layer within the cavity. This may be accomplished by underetching the spacer layer (e.g., terminating the etch process before the entire etch process is finished). The remaining spacer will behave as a support member to reduce the deflection of the top diaphragm member. The size and shape of the support members may be adjusted by altering the etch time of the spacer layer, or adjusting the shape of the etch windows 266.

In another embodiment, a high sensitivity CCD array may be used to measure changes in optical characteristics which occur upon binding of the biological/chemical agents. The CCD arrays may be interfaced with filters, light sources, fluid delivery and micromachined particle receptacles, so as to create a functional sensor array. Data acquisition and handling may be performed with existing CCD technology. Data streams (e.g., red, green, blue for colorimetric assays; gray intensity for fluorescence assays) may be transferred from the CCD to a

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computer via a data acquisition board. Current CCDs may allow for read-out rates of 10^5 pixels per second. Thus, the entire array of particles may be evaluated hundreds of times per second allowing for studies of the dynamics of the various host-guest interaction rates as well as the analyte/polymer diffusional characteristics. Evaluation of this data may offer a method of identifying and quantifying the chemical/biological composition of the test samples. CCD detectors may be configured to measure white light, ultraviolet light or fluorescence. Other detectors such as photomultiplier tubes, charge induction devices, photodiode, photodiode arrays, and microchannel plates may also be used. It should be understood that while the detector is depicted as being positioned under the supporting member, the detector may also be positioned above the supporting member. It should also be understood that the detector typically includes a sensing element for detecting the spectroscopic events and a component for displaying the detected events. The display component may be physically separated from the sensing element. The sensing element may be positioned above or below the sensor array while the display component is positioned close to a user.

In one embodiment, a CCD detector may be used to record color changes of the chemical sensitive particles during analysis. As depicted in FIG. 1, a CCD detector 130 may be placed beneath the supporting member 120. The light transmitted through the cavities is captured and analyzed by the CCD detector. In one embodiment, the light is broken down into three color components, red, green and blue. To simplify the data, each color is recorded using 8 bits of data. Thus, the data for each of the colors will appear as a value between 0 and 255. The color of each chemical sensitive element may be represented as a red, blue and green value. For example, a blank particle (i.e., a particle which does not include a receptor) will typically appear white. For example, when broken down into the red, green and blue components, it is found that a typical blank particle exhibits a red value of about 253, a green value of about 250, and a blue value of about 222. This signifies that a blank particle does not significantly absorb red, green or blue light. When a particle with a receptor is scanned, the particle may exhibit a color change, due to absorbance by the receptor. For example, it was found that when a particle which includes a 5-carboxyfluorescein receptor is subjected to white light, the particle shows a strong absorbance of blue light. The CCD detector values for the 5-carboxyfluorescein particle exhibits a red value of about 254, a green value of about 218, and a blue value of about 57. The decrease in transmittance of blue light is believed to be due to the absorbance of blue light by the 5-carboxyfluorescein. In this manner, the color changes of a particle may be quantitatively characterized. An advantage of using a CCD detector to monitor the color changes is that color changes which may not be noticeable to the human eye may now be detected.

The support array may be configured to allow a variety of detection modes to be practiced. In one embodiment, a light source is used to generate light which is directed toward the particles. The particles may absorb a portion of the light as the light illuminates the particles. The light then reaches the detector, reduced in intensity by the absorbance of the particles. The detector may be configure to measure the reduction in light intensity (i.e., the absorbance) due to the particles. In another embodiment, the detector may be placed above the supporting member. The detector may be configured to measure the amount of light reflected off of the particles. The absorbance of light by the particles is manifested by a reduction in the amount of light being reflected from the cavity. The light source in either embodiment may be a white light source or a fluorescent light source.

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CHEMICALLY SENSITIVE PARTICLES

A particle, in some embodiments, possess both the ability to bind the analyte of interest and to create a modulated signal. The particle may include receptor molecules which posses the ability to bind the analyte of interest and to create a modulated signal. Alternatively, the particle may include receptor molecules and indicators. The receptor molecule may posses the ability to bind to an analyte o f interest. Upon binding the analyte of interest, the receptor molecule may cause the indicator molecule to produce the modulated signal. The receptor molecules may be naturally occurring or synthetic receptors formed by rational design or combinatorial methods. Some examples of natural receptors include, but are not limited to, DNA, RNA, proteins, enzymes, oligopeptides, antigens, and antibodies. Either natural or synthetic receptors may be chosen for their ability to bind to the analyte molecules in a specific manner. The forces which drive association/recognition between molecules include the hydrophobic effect, anion-cation attraction, and hydrogen bonding. The relative strengths of these forces depend upon factors such as the solvent dielectric properties, the shape of the host molecule, and how it complements the guest. Upon host-guest association, attractive interactions occur and the molecules stick together. The most widely used analogy for this chemical interaction is that of a "lock and key". The fit of the key molecule (the guest) into the lock (the host) is a molecular recognition event.

A naturally occurring or synthetic receptor may be bound to a polymeric resin in order to create the particle. The polymeric resin may be made from a variety of polymers including, but not limited to, agarous, dextrose, acrylamide, control pore glass beads, polystyrene-polyethylene glycol resin, polystyrene-divinyl benzene resin, formylpolystyrene resin, trityl-polystyrene resin, acetyl polystyrene resin, chloroacetyl polystyrene resin, aminomethyl polystyrene-divinylbenzene resin, carboxypolystyrene resin, chloromethylated polystyrene-divinylbenzene resin, hydroxymethyl polystyrene-divinylbenzene resin, 2-chlorotrityl chloride polystyrene resin, 4-benzyloxy-2'4'- dimethoxybenzhydrol resin (Rink Acid resin), triphenyl methanol polystyrene resin, diphenylmethanol resin, benzhydrol resin, succinimidyl carbonate resin, p-nitrophenyl carbonate resin, imidazole carbonate resin, polyacrylamide resin, 4-sulfamylbenzoyl-4'- methylbenzhydrylamine-resin (Safety-catch resin), 2-amino-2-(2'-nitrophenyl) propionic acid-aminomethyl resin (ANP Resin), p-benzyloxybenzyl alcohol-divinylbenzene resin (Wang resin), p-methylbenzhydrylamine-divinylbenzene resin (MBHA resin), Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine linked to resin (Knorr resin), 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink resin), 4-hydroxymethyl-benzoyl-4'- methylbenzhydrylamine resin (HMBA-MBHA Resin), p-nitrobenzophenone oxime resin (Kaiser oxime resin), and amino-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine handle linked to 2-chlorotrityl resin (Knorr-2-chlorotrityl resin). In one embodiment, the material used to form the polymeric resin is compatible with the solvent in which the analyte is dissolved. For example, polystyrene-divinyl benzene resin will swell within non-polar solvents, but does not significantly swell within polar solvents. Thus, polystyrene-divinyl benzene resin may be used for the analysis of analytes within non-polar solvents. Alternatively, polystyrene-polyethylene glycol resin will swell with polar solvents such as water. Polystyrene-polyethylene glycol resin may be useful for the analysis of aqueous fluids.

In one embodiment, a polystyrene-polyethylene glycol-divinyl benzene material is used to form the polymeric resin. The polystyrene-polyethylene glycol-divinyl benzene resin is formed from a mixture of polystyrene 375, divinyl benzene 380 and polystyrene-polyethylene glycol 385, see FIG. 5. The polyethylene glycol portion of the polystyrene-polyethylene glycol 385, in one embodiment, may be terminated with an amine.

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The amine serves as a chemical handle to anchor both receptors and indicator dyes. Other chemical functional groups may be positioned at the terminal end of the polyethylene glycol to allow appropriate coupling of the polymeric resin to the receptor molecules or indicators.

The chemically sensitive particle, in one embodiment, is capable of both binding the analyte(s) of interest and creating a detectable signal. In one embodiment, the particle will create an optical signal when bound to an analyte of interest. The use of such a polymeric bound receptors offers advantages both in terms of cost and configurability. Instead of having to synthesize or attach a receptor directly to a supporting member, the polymeric bound receptors may be synthesized *en masse* and distributed to multiple different supporting members. This allows the cost of the sensor array, a major hurdle to the development of mass-produced environmental probes and medical diagnostics, to be reduced. Additionally, sensor arrays which incorporate polymeric bound receptors may be reconfigured much more quickly than array systems in which the receptor is attached directly tot he supporting member. For example, if a new variant of a pathogen or a pathogen that contains a genetically engineered protein is a threat, then a new sensor array system may be readily created to detect these modified analytes by simply adding new sensor elements (e.g., polymeric bound receptors) to a previously formed supporting member.

In one embodiment, a receptor, which is sensitive to changes in the pH of a fluid sample is bound to a polymeric resin to create a particle. That is, the receptor is sensitive to the concentration of hydrogen cations (H^+). The receptor in this case is typically sensitive to the concentration of H^+ in a fluid solution. The analyte of interest may therefore be H^+. There are many types of molecules which undergo a color change when the pH of the fluid is changed. For example, many types of dyes undergo significant color changes as the pH of the fluid medium is altered. Examples of receptors which may be used to monitor the pH of a fluid sample include 5-carboxyfluorescein and alizarin complexone, depicted in FIG. 6. Each of these receptors undergoes significant color changes as the pH of the fluid is altered. 5-carboxyfluorescein undergoes a change from yellow to orange as the pH of the fluid is increased. Alizarin complexone undergoes two color changes, first from yellow to red, then from red to blue as the pH of the fluid increases. By monitoring the change in color caused by dyes attached to a polymeric particle, the pH of a solution may be qualitatively and, with the use of a detector (e.g., a CCD detector), quantitatively monitored.

In another embodiment, a receptor which is sensitive to presence of metal cations is bound to a polymeric particle to create a particle. The receptor in this case is typically sensitive to the concentration of one or more metal cations present in a fluid solution. In general, colored molecules which will bind cations may be used to determine the presence of a metal cation in a fluid solution. Examples of receptors which may be used to monitor the presence of cations in a fluid sample include alizarin complexone and *o*-cresolphthalein complexone, see FIG. 6. Each of these receptors undergoes significant color changes as the concentration of a specific metal ion in the fluid is altered. Alizarin complexone is particularly sensitive to lanthanum ions. In the absence of lanthanum, alizarin complexone will exhibit a yellow color. As the concentration of lanthanum is increased, alizarin complexone will change to a red color. *o*-Cresolphthalein complexone is particularly sensitive to calcium ions. In the absence of calcium, *o*-cresolphthalein complexone is colorless. As the concentration of calcium is increased, *o*-cresolphthalein complexone will change to a blue color. By monitoring the change in color of metal cation sensitive receptors attached to a polymeric particle, the presence of a specific metal ion may be qualitatively and, with the use of a detector (e.g., a CCD detector), quantitatively monitored.

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Referring to FIG. 7, a graph of the absorbance of green light vs. concentration of calcium (Ca^{+2}) is depicted for a particle which includes an *o*-cresolphthalein complexone receptor. As the concentration of calcium is increased, the absorbance of green light increases in a linear manner up to a concentration of about 0.0006 M. A concentration of 0.0006 M is the solubility limit of calcium in the fluid, thus no significant change in absorbance is noted after this point. The linear relationship between concentration and absorbance allows the concentration of calcium to be determined by measuring the absorbance of the fluid sample.

In one embodiment, a detectable signal may be caused by the altering of the physical properties of an indicator ligand bound to the receptor or the polymeric resin. In one embodiment, two different indicators are attached to a receptor or the polymeric resin. When an analyte is captured by the receptor, the physical distance between the two indicators may be altered such that a change in the spectroscopic properties of the indicators is produced. A variety of fluorescent and phosphorescent indicators may be used for this sensing scheme. This process, known as Forster energy transfer, is extremely sensitive to small changes in the distance between the indicator molecules.

For example, a first fluorescent indicator 320 (e.g., a fluorescein derivative) and a second fluorescent indictor 330 (e.g., a rhodamine derivative) may be attached to a receptor 300, as depicted in FIG. 8. When no analyte is present short wavelength excitation 310 may excite the first fluorescent indicator 320, which fluoresces as indicated by 312. The short wavelength excitation, however, may cause little or no fluorescence of the second fluorescent indicator 330. After binding of analyte 350 to the receptor, a structural change in the receptor molecule may bring the first and second fluorescent indicators closer to each other. This change in intermolecular distance may allow the excited first indicator 320 to transfer a portion of its fluorescent energy 325 to the second fluorescent indicator 330. This transfer in energy may be measured by either a drop in energy of the fluorescence of the first indicator molecule 320, or the detection of increased fluorescence 314 by the second indicator molecule 330.

Alternatively, the first and second fluorescent indicators may initially be positioned such that short wavelength excitation, may cause fluorescence of both the first and second fluorescent indicators, as described above. After binding of analyte 350 to the receptor, a structural change in the receptor molecule may cause the first and second fluorescent indicators to move further apart. This change in intermolecular distance may inhibit the transfer of fluorescent energy from the first indicator 320 to the second fluorescent indicator 330. This change in the transfer of energy may be measured by either a drop in energy of the fluorescence of the second indicator molecule 330, or the detection of increased fluorescence by the first indicator molecule 320.

In another embodiment, an indicator ligand may be preloaded onto the receptor. An analyte may then displace the indicator ligand to produce a change in the spectroscopic properties of the particles. In this case, the initial background absorbance is relatively large and decreases when the analyte is present. The indicator ligand, in one embodiment, has a variety of spectroscopic properties which may be measured. These spectroscopic properties include, but are not limited to, ultraviolet absorption, visible absorption, infrared absorption, fluorescence, and magnetic resonance. In one embodiment, the indicator is a dye having either a strong fluorescence, a strong ultraviolet absorption, a strong visible absorption, or a combination of these physical properties. Examples of indicators include, but are not limited to, carboxyfluorescein, ethidium bromide, 7-dimethylamino-4-methylcoumarin, 7-diethylamino-4-methylcoumarin, eosin, erythrosin, fluorescein, Oregon Green 488, pyrene, Rhodamine Red, tetramethylrhodamine, Texas Red, Methyl Violet, Crystal Violet, Ethyl Violet, Malachite green,

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Methyl Green, Alizarin Red S, Methyl Red, Neutral Red, *o*-cresolsulfonephthalein, *o*-cresolphthalein, phenolphthalein, Acridine Orange, *B*-naphthol, coumarin, and *a*-naphthionic acid. When the indicator is mixed with the receptor, the receptor and indicator interact with each other such that the above mentioned spectroscopic properties of the indicator, as well as other spectroscopic properties may be altered. The nature of this interaction may be a binding interaction, wherein the indicator and receptor are attracted to each other with a sufficient force to allow the newly formed receptor-indicator complex to function as a single unit. The binding of the indicator and receptor to each other may take the form of a covalent bond, an ionic bond, a hydrogen bond, a van der Waals interaction, or a combination of these bonds.

The indicator may be chosen such that the binding strength of the indicator to the receptor is less than the binding strength of the analyte to the receptor. Thus, in the presence of an analyte, the binding of the indicator with the receptor may be disrupted, releasing the indicator from the receptor. When released, the physical properties of the indicator may be altered from those it exhibited when bound to the receptor. The indicator may revert back to its original structure, thus regaining its original physical properties. For example, if a fluorescent indicator is attached to a particle that includes a receptor, the fluorescence of the particle may be strong before treatment with an analyte containing fluid. When the analyte interacts with the particle, the fluorescent indicator may be released. Release of the indicator may cause a decrease in the fluorescence of the particle, since the particle now has less indicator molecules associated with it.

An example of this type of system is illustrated by the use of a boronic acid substituted resin 505 as a particle. Prior to testing, the boronic acid substituted resin 505 is treated with a sugar 510 which is tagged with an indicator (e.g., resorufin) as depicted in FIG. 9. The sugar 510 binds to the boronic acid receptor 500 imparting a color change to the boronic substituted resin 505 (yellow for the resorufin tagged sugar). When the boronic acid resin 505 is treated with a fluid sample which includes a sugar 520, the tagged sugar 510 may be displaced, causing a decrease in the amount of color produced by the boronic acid substituted resin 505. This decrease may be qualitatively or, with the use of a detector (e.g., a CCD detector), quantitatively monitored.

In another embodiment, a designed synthetic receptor may be used. In one embodiment, a polycarboxylic acid receptor may be attached to a polymeric resin. The polycarboxylic receptors are discussed in U.S. patent application serial no. 08/950,712 which is incorporated herein by reference.

In an embodiment, the analyte molecules in the fluid may be pretreated with an indicator ligand. Pretreatment may involve covalent attachment of an indicator ligand to the analyte molecule. After the indicator has been attached to the analyte, the fluid may be passed over the sensing particles. Interaction of the receptors on the sensing particles with the analytes may remove the analytes from the solution. Since the analytes include an indicator, the spectroscopic properties of the indicator may be passed onto the particle. By analyzing the physical properties of the sensing particles after passage of an analyte stream, the presence and concentration of an analyte may be determined.

For example, the analytes within a fluid may be derivatized with a fluorescent tag before introducing the stream to the particles. As analyte molecules are adsorbed by the particles, the fluorescence of the particles may increase. The presence of a fluorescent signal may be used to determine the presence of a specific analyte. Additionally, the strength of the fluorescence may be used to determine the amount of analyte within the stream.

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RECEPTORS

A variety of natural and synthetic receptors may be used. The synthetic receptors may come from a variety of classes including, but not limited to, polynucleotides (e.g., aptamers), peptides (e.g., enzymes and antibodies), synthetic receptors, polymeric unnatural biopolymers (e.g., polythioureas, polyguanidiniums), and imprinted polymers., some of which are generally depicted in FIG. 10. Natural based synthetic receptors include receptors which are structurally similar to naturally occurring molecules. Polynucleotides are relatively small fragments of DNA which may be derived by sequentially building the DNA sequence. Peptides may be synthesized from amino acids. Unnatural biopolymers are chemical structure which are based on natural biopolymers, but which are built from unnatural linking units. Unnatural biopolymers such as polythioureas and polyguanidiniums may be synthesized from diamines (i.e., compounds which include at least two amine functional groups). These molecules are structurally similar to naturally occurring receptors, (e.g., peptides). Some diamines may, in turn, be synthesized from amino acids. The use of amino acids as the building blocks for these compounds allow a wide variety of molecular recognition units to be devised. For example, the twenty natural amino acids have side chains that possess hydrophobic residues, cationic and anionic residues, as well as hydrogen bonding groups. These side chains may provide a good chemical match to bind a large number of targets, from small molecules to large oligosaccharides. Amino acid based peptides, polythioureas, and polyguanidiniums are depicted in FIG. 10.

Techniques for the building of DNA fragments and polypeptide fragments on a polymer particle are well known. Techniques for the immobilization of naturally occurring antibodies and enzymes on a polymeric resin are also well known. The synthesis of polythioureas upon a resin particle may be accomplished by the synthetic pathway depicted in FIG. 11. The procedure may begin by deprotection of the terminal tBoc protecting group on an amino acid coupled to a polymeric particle. Removal of the protecting group is followed by coupling of the rigid spacer 410 to the resulting amine 405 using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole hydrate (HOBT). The spacer group may inhibit formation of a thiazolone by reaction of the first amino acids with subsequently formed thioureas. After the spacer group is coupled to the amino acid, another tBoc deprotection is performed to remove the spacer protecting group, giving the amine 415. At this point, monomer may be added incrementally to the growing chain, each time followed by a tBoc deprotection. The addition of a derivative of the diamine 420 (e.g., an isothiocyanate) to amine 415 gives the mono-thiourea 425. The addition of a second thiourea substituent is also depicted. After the addition of the desired number of monomers, a solution of benzylisothiocyanate or acetic anhydride may be added to cap any remaining amines on the growing oligomers. Between 1 to 20 thioureas groups may be formed to produce a synthetic polythiourea receptor.

The synthesis of polyguanidiniums may be accomplished as depicted in FIG. 12. In order to incorporate these guanidinium groups into the receptor, the coupling of a thiourea with a terminal amine in the presence of Mukaiyama's reagent may be utilized. The coupling of the first thiourea diamine 430 with an amino group of a polymeric particle gives the mono-guanidinium 434. Coupling of the resulting mono-guanidinium with a second thiourea diamine 436 gives a di-guanidinium 438. Further coupling may create a tri-guanidinium 440. Between 1 to 20 guanidinium groups may be formed to produce a synthetic polyguanidinium receptor.

The above described methods for making polythioureas and polyguanidiniums are based on the incorporation of diamines (i.e., molecules which include at least two amine functional groups) into the oligomeric

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receptor. The method may be general for any compound having at least two amino groups. In one embodiment, the diamine may be derived from amino acids. A method for forming diamines from amino acids is shown in FIG. 13. Treatment of a protected amino acid 450 with borane-THF reduces the carboxylic acid portion of the amino acid to the primary alcohol 452. The primary alcohol is treated with phthalimide under Mitsunobu conditions (PPh$_3$/DEAD). The resulting compound 454 is treated with aqueous methylamine to form the desired monoprotected diamine 456. The process may be accomplished such that the enantiomeric purity of the starting amino acid is maintained. Any natural or synthetic amino acid may be used in the above described method.

The three coupling strategies used to form the respective functional groups may be completely compatible with each other. The capability to mix linking groups (amides, thioureas, and guanidiniums) as well as the side chains (hydrophobic, cationic, anionic, and hydrogen bonding) may allow the creation of a diversity in the oligomers that is beyond the diversity of receptors typically found with natural biological receptors. Thus, we may produce ultra-sensitive and ultra-selective receptors which exhibit interactions for specific toxins, bacteria, and environmental chemicals. Additionally, these synthetic schemes may be used to build combinatorial libraries of particles for use in the sensor array.

In an embodiment, the indicator ligand may be incorporated into synthetic receptors during the synthesis of the receptors. The ligand may be incorporated into a monomeric unit, such as a diamine, that is used during the synthesis of the receptor. In this manner, the indicator may be covalently attached to the receptor in a controlled position. By placing the indicator within the receptor during the synthesis of the receptor, the positioning of the indicator ligand within the receptor may be controlled. This control may be difficult to achieve after synthesis of the receptor is completed.

In one embodiment, a fluorescent group may be incorporated into a diamine monomer for use in the synthetic sequences. Examples of monomeric units which may be used for the synthesis of a receptor are depicted in FIG. 14. The depicted monomers include fluorescent indicator groups. After synthesis, the interaction of the receptor with the analyte may induce changes in the spectroscopic properties of the molecule. Typically, hydrogen bonding or ionic substituents on the fluorescent monomer involved in analyte binding have the capacity to change the electron density and/or rigidity of the fluorescent ring system, thereby causing observable changes in the spectroscopic properties of the indicator. For fluorescent indicators such changes may be exhibited as changes in the fluorescence quantum yield, maximum excitation wavelength, and/or maximum emission wavelength. This approach does not require the dissociation of a preloaded fluorescent ligand, which may be limited in response time by $k_{(off)}$). While fluorescent ligands are shown here, it is to be understood that a variety of other ligand may be used including colorimetric ligands.

In another embodiment, two fluorescent monomers for signaling may be used for the synthesis of the receptor. For example, compound 470 (a derivative of fluorescein) and compound 475 (a derivative of rhodamine), depicted in FIG. 14, may both be incorporated into a synthetic receptor. Compound 470 contains a common colorimetric/fluorescent probe that will, in some embodiments, send out a modulated signal upon analyte binding. The modulation may be due to resonance energy transfer to compound 475. When an analyte binds to the receptor, structural changes in the receptor may alter the distance between monomeric units 470 and 475. It is well known that excitation of fluorescein can result in emission from rhodamine when these molecules are oriented correctly. The efficiency of resonance energy transfer from monomers 470 to 475 will depend strongly upon the presence of

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analyte binding; thus, measurement of rhodamine fluorescence intensity (at a substantially longer wavelength than fluorescein fluorescence) may serve as an indicator of analyte binding. To greatly improve the likelihood of a modulatory fluorescein-rhodamine interaction, multiple rhodamine tags may be attached at different sites along a receptor molecule without substantially increasing background rhodamine fluorescence (only rhodamine very close to fluorescein will yield appreciable signal). This methodology may be applied to a number of alternate fluorescent pairs.

In an embodiment, a large number of chemical/biological agents of interest to the military and civilian communities may be sensed readily by the described array sensors including both small and medium size molecules. For example, it is known that nerve gases typically produce phosphate structures upon hydrolysis in water. The presence of molecules which contain phosphate functional groups may be detected using polyguanidiniums. Nerve gases which have contaminated water sources may be detected by the use of the polyguanidinium receptors described above.

In order to identify, sense, and quantitate the presence of various bacteria using the proposed micro-machined sensor, two strategies may be used. First, small molecule recognition and detection may be exploited. Since each bacteria possesses a unique and distinctive concentration of the various cellular molecules, such as DNA, proteins, metabolites, and sugars, the fingerprint (i.e., the concentration and types of DNA, proteins, metabolites, and sugars) of each organism is expected to be unique. Hence, the analytes obtained from whole bacteria or broken down bacteria may be used to determine the presence of specific bacteria. A series of receptors specific for DNA molecules, proteins, metabolites, and sugars may be incorporated into an array. A solution containing bacteria, or more preferably broken down bacteria, may be passed over the array of particles. The individual cellular components of the bacteria may interact in a different manner with each of the particles. This interaction will provide a pattern within the array which may be unique for the individual bacteria. In this manner, the presence of bacteria within a fluid may be determined.

In another embodiment, bacteria may be detected as whole entities, as found in ground water, aerosols, or blood. To detect, sense, and identify intact bacteria, the cell surface of one bacteria may be differentiated from other bacteria. One method of accomplishing this differentiation is to target cell surface oligosaccharides (i.e. sugar residues). Each bacterial class (gram negative, gram positive, etc.) displays a different oligosaccharide on their cell surfaces. The oligosaccharide, which is the code that is read by other cells giving an identification of the cell, is part of the cell-cell recognition and communication process. The use of synthetic receptors which are specific for oligosaccharides may be used to determine the presence of specific bacteria by analyzing for the cell surface oligosaccharides.

In another embodiment, the sensor array may be used to optimize which receptor molecules should be used for a specific analyte. An array of receptors may be placed within the cavities of the supporting member and a stream containing an analyte may be passed over the array. The reaction of each portion of the sensing array to the known analyte may be analyzed and the optimal receptor determined by determining which particle, and therefore which receptor, exhibits the strongest reaction toward the analyte. In this manner, a large number of potential receptors may be rapidly scanned. The optimal receptor may then be incorporated into a system used for the detection of the specific analyte in a mixture of analytes.

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It should be emphasized that although some particles may be purposefully designed to bind to important species (biological agents, toxins, nerve gasses, etc.), most structures will possess nonspecific receptor groups. One of the advantages associated with the proposed sensor array is the capacity to standardize each array of particles via exposure to various analytes, followed by storage of the patterns which arise from interaction of the analytes with the particles. Therefore, there may not be a need to know the identity of the actual receptor on each particle. Only the characteristic pattern for each array of particles is important. In fact, for many applications it may be less time consuming to place the various particles into their respective holders without taking precautions to characterize the location associated with the specific particles. When used in this manner, each individual sensor array may require standardization for the type of analyte to be studied.

On-site calibration for new or unknown toxins may also be possible with this type of array. Upon complexation of an analyte, the local microenvironment of each indicator may change, resulting in a modulation of the light absorption and/or emission properties. The use of standard pattern recognition algorithms completed on a computer platform may serves as the intelligence factor for the analysis. The "fingerprint" like response evoked from the simultaneous interactions occurring at multiple sites within the substrate may be used to identify the species present in unknown samples.

The above described sensor array system offers a number of distinct advantages over exiting technologies. One advantage is that "real time" detection of analytes may be performed. Another advantage is that the simultaneous detection of multiple analytes may be realized. Yet another advantage is that the sensor array system allows the use of synthetic reagents as well as biologically produced reagents. Synthetic reagents typically have superior sensitivity and specificity toward analytes when compared to the biological reagents. Yet another advantage is that the sensor array system may be readily modified by simply changing the particles which are placed within the sensor array. This interchangability may also reduce production costs.

EXAMPLES

1. The determination of pH using a chemically sensitive particle.

Shown in FIG. 15 is the magnitude of the optical signal transmitted through a single polymer particle derivatized with o-cresolphthalein. Here, a filter is used to focus the analysis on those wavelengths which the dye absorbs most strongly (i.e., about 550 nm). Data is provided for the particle as the pH is cycled between acid and basic environments. In acidic media (i.e., at times of 100-150 seconds and 180-210 seconds), the particle is clear and the system yields large signals (up to greater than 300,000 counts) at the optical detector. Between times of 0-100 and 150-180 seconds, the solution was made basic. Upon raising the pH (i.e., making the solution more basic), the particle turns purple in color and the transmitted green light is greatly diminished. Large signal reductions are recorded under such circumstances. The evolution of the signal changes show that the response time is quite rapid, on the order of 10 seconds. Furthermore, the behavior is highly reproducible.

2. The simultaneous detection of Ca^{+2}, Ce^{+3}, and pH by a sensor array system.

The synthesis of four different particles was accomplished by coupling a variety of indictor ligands to a polyethylene glycol-polystyrene ("PEG-PS") resin particle. The PEG-PS resin particles were obtained from Novabiochem Corp., La Jolla, Ca. The particles have an average diameter of about 130 μm when dry and about

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250 μm when wet. The indicator ligands of fluorescein, *o*-cresolphthalein complexone, and alizarin complexone were each attached to PEG-PS resin particles using a dicyclohexylcarbodiimide (DCC) coupling between a terminal resin bound amine and a carboxylic acid on the indicator ligand.

These synthetic receptors, localized on the PEG-PS resin to create sensing particles, were positioned within micromachined wells formed in silicon/silicon nitride wafers, thus confining the particles to individually addressable positions on a multicomponent chip. These wells were sized to hold the particles in both swollen and unswollen states. Rapid introduction of the test fluids can be accomplished using these structures while allowing spectrophotometric assays to probe for the presence of analytes. For the identification and quantification of analyte species, changes in the light absorption and light emission properties of the immobilized resin particles can be exploited, although only identification based upon absorption properties are discussed here. Upon exposure to analytes, color changes for the particles were found to be 90% complete within one minute of exposure, although typically only seconds were required. To make the analysis of the colorimetric changes efficient, rapid, and sensitive, a charge-coupled-device (CCD) was directly interfaced with the sensor array. Thus, data streams composed of red, green, and blue (RGB) light intensities were acquired and processed for each of the individual particle elements. The red, blue, and green responses of the particles to various solutions are graphically depicted in FIG. 16.

The true power of the described bead sensor array occurs when simultaneous evaluation of multiple chemically distinct bead structures is completed. A demonstration of the capacity of five different beads is provided in FIG. 16. In this case, blank, alizarin, o-cresol phthalein, fluorescein, and alizarin-Ce3+ complex derivatized beads serve as a matrix for subtle differentiation of chemical environments. The blank bead is simply a polystyrene sphere with no chemical derivatization. The bead derivatized with o-cresolphthalein responds to Ca+2 at pHs values around 10.0. The binding of calcium is noted from the large green color attenuation noted for this dye while exposed to the cation. Similarly, the fluorescein derivatized bead acts as a pH sensor. At pHs below 7.4 it is light yellow, but at higher pHs it turns dark orange. Interesting, the alizarin complexone plays three distinct roles. First, it acts as a proton sensor yielding a yellow color at pHs below 4.5, orange is noted at pHs between 4.5 and 11.5, and at pHs above 11.5 a blue hue is observed. Second, it functions as a sensor for lanthanum ions at lower pHs by turning yellow to orange. Third, the combination of both fluoride and lanthanum ions results in yellow/orange coloration.

The analysis of solutions containing various amount of Ca^{+2} or F^- at various pH levels was performed using alizarin complexone, *o*-cresolphthalein complexone, 5-carboxy fluorescein, and alizarin-Ce^{3+} complex. A blank particle in which the terminal amines of a PEG-PS resin particle have been acylated was also used. In this example, the presence of Ca^{+2} (0.1 M $Ca(NO_3)_2$) was analyzed under conditions of varying pH. The pH was varied to values of 2, 7, and 12, all buffered by a mixture of 0.04 M phosphate, 0.04 M acetate, and 0.04 M borate. The RGB patterns for each sensor element in all environments were measured. The bead derivatized with o-cresolphthalein responds to Ca^{+2} at pH values around 12. Similarly, the 5-carboxy fluorescein derivatized bead acts as a pH sensor. At pHs below 7.4 it is light yellow, but at higher pHs it turns dark orange. Interesting, the alizarin complexone plays three distinct roles. First, it acts as a proton sensor yielding a yellow color at pHs below 4.5, orange is noted at pHs between 4.5 and 11.5, and at pHs above 11.5 a blue hue is observed. Second, it functions as

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a sensor for lanthanum ions at lower pHs by turning yellow to orange. Third, the combination of both fluoride and lanthanum ions results in yellow/orange coloration.

This example demonstrates a number of important factors related to the design, testing, and functionality of micromachined array sensors for solution analyses. First, derivatization of polymer particles with both colorimetric and fluorescent dyes was completed. These structures were shown to respond to pH and Ca^{2+}. Second, response times well under 1 minute were found. Third, micromachined arrays suitable both for confinement of particles, as well as optical characterization of the particles, have been prepared. Fourth, integration of the test bed arrays with commercially available CCD detectors has been accomplished. Finally, simultaneous detection of several analytes in a mixture was made possible by analysis of the RGB color patterns created by the sensor array.

3. The detection of sugar molecules using a boronic acid based receptor.

A series of receptors were prepared with functionalities that associate strongly with sugar molecules, as depicted in FIG. 9. In this case, a boronic acid sugar receptor 500 was utilized to demonstrate the functionality of a new type of sensing scheme in which competitive displacement of a resorufin derivatized galactose sugar molecule was used to assess the presence (or lack thereof) of other sugar molecules. The boronic acid receptor 500 was formed via a substitution reaction of a benzylic bromide. The boronic acid receptor was attached to a polyethylene glycol-polystyrene ("PEG-PS") resin particle at the "R" position. Initially, the boronic acid derivatized particle was loaded with resorufin derivatized galactose 510. Upon exposure of the particle to a solution containing glucose 520, the resorufin derivatized galactose molecules 510 are displaced from the particle receptor sites. Visual inspection of the optical photographs taken before and after exposure to the sugar solution show that the boron substituted resin is capable of sequestering sugar molecules from an aqueous solution. Moreover, the subsequent exposure of the colored particles to a solution of a non-tagged sugar (e.g., glucose) leads to a displacement of the bound colored sugar reporter molecule. Displacement of this molecule leads to a change in the color of the particle. The sugar sensor turns from dark orange to yellow in solutions containing glucose. The particles were also tested in conditions of varying pH. It was noted that the color of the particles changes from dark orange to yellow as the pH is varied from low pH to high pH.

FURTHER IMPROVEMENTS

Shown in FIG. 17 is an embodiment of a system for detecting analytes in a fluid. In one embodiment, the system includes a light source 512, a sensor array 522, a chamber 550 for supporting the sensor array and a detector 530. The sensor array 522 may include a supporting member which is configured to hold a variety of particles. In one embodiment, light originating from the light source 512 passes through the sensor array 522 and out through the bottom side of the sensor array. Light modulated by the particles may be detected by a proximally spaced detector 530. While depicted as being positioned below the sensor array, it should be understood that the detector may be positioned above the sensor array for reflectance measurements. Evaluation of the optical changes may be completed by visual inspection (e.g., by eye, or with the aid of a microscope) or by use of a microprocessor 540 coupled to the detector.

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In this embodiment, the sensor array 522 is positioned within a chamber 550. The chamber 550, may be configured to allow a fluid stream to pass through the chamber such that the fluid stream interacts with the sensor array 522. The chamber may be constructed of glass (e.g, borosilicate glass or quartz) or a plastic material which is transparent to a portion of the light from the light source. If a plastic material is used, the plastic material should also be substantially unreactive toward the fluid. Examples of plastic materials which may be used to form the chamber include, but are not limited to, acrylic resins, polycarbonates, polyester resins, polyethylenes, polyimides, polyvinyl polymers (e.g., polyvinyl chloride, polyvinyl acetate, polyvinyl dichloride, polyvinyl fluoride, etc.), polystyrenes, polypropylenes, polytetrafluoroethylenes, and polyurethanes. An example of such a chamber is a Sykes-Moore chamber, which is commercially available from Bellco Glass, Inc., in New Jersey. Chamber 550, in one embodiment, includes a fluid inlet port 552 and a fluid outlet port 554. The fluid inlet 552 and outlet 554 ports are configured to allow a fluid stream to pass into the interior 556 of the chamber during use. The inlet and outlet ports may be configured to allow facile placement of a conduit for transferring the fluid to the chamber. In one embodiment, the ports may be hollow conduits. The hollow conduits may be configured to have an outer diameter which is substantially equal to the inner diameter of a tube for transferring the fluid to or away from the chamber. For example, if a plastic or rubber tube is used for the transfer of the fluid, the internal diameter of the plastic tube is substantially equal to the outer diameter of the inlet and outlet ports.

In another embodiment, the inlet and outlet ports may be Luer lock style connectors. Preferably, the inlet and outlet ports are female Luer lock connectors. The use of female Luer lock connectors will allow the fluid to be introduced via a syringe. Typically, syringes include a male Luer lock connector at the dispensing end of the syringe. For the introduction of liquid samples, the use of Luer lock connectors may allow samples to be transferred directly from a syringe to the chamber 550. Luer lock connectors may also allow plastic or rubber tubing to be connected to the chamber using Luer lock tubing connectors.

The chamber may be configured to allow the passage of a fluid sample to be substantially confined to the interior 556 of the chamber. By confining the fluid to a small interior volume, the amount of fluid required for an analysis may be minimized. The interior volume may be specifically modified for the desired application. For example, for the analysis of small volumes of fluid samples, the chamber may be designed to have a small interior chamber, thus reducing the amount of fluid needed to fill the chamber. For larger samples, a larger interior chamber may be used. Larger chambers may allow a faster throughput of the fluid during use.

In another embodiment, depicted in FIG. 18, a system for detecting analytes in a fluid includes a light source 512, a sensor array 522, a chamber 550 for supporting the sensor array and a detector 530, all enclosed within a detection system enclosure 560. As described above, the sensor array 522 is preferably formed of a supporting member which is configured to hold a variety of particles. Thus, in a single enclosure, all of the components of an analyte detection system are included.

The formation of an analyte detection system in a single enclosure may allow the formation of a portable detection system. For example, a small controller 570 may be coupled to the analyte detection system. The controller 570 may be configured to interact with the detector and display the results from the analysis. In one embodiment, the controller includes a display device 572 for displaying information to a user. The controller may also include input devices 574 (e.g., buttons) to allow the user to control the operation of the analyte detection

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system. For example, the controller may control the operation of the light source 512 and the operation of the detector 530.

The detection system enclosure 560, may be interchangeable with the controller. Coupling members 576 and 578 may be used to remove the detection system enclosure 560 from the controller 570. A second detection system enclosure may be readily coupled to the controller using coupling members 576 and 578. In this manner, a variety of different types of analytes may be detecting using a variety of different detection system enclosures. Each of the detection system enclosures may include different sensor arrays mounted within their chambers. Instead of having to exchange the sensor array for different types of analysis, the entire detection system enclosure may be exchanged. This may prove advantageous, when a variety of detection schemes are used. For example a first detection system enclosure may be configured for white light applications. The first detection system enclosure may include a white light source, a sensor that includes particles that produce a visible light response in the presence of an analyte, and a detector sensitive to white light. A second detection system enclosure may be configured for fluorescent applications, including a fluorescent light source, a sensor array which includes particles which produce a fluorescent response on the presence of an analyte, and a fluorescent detector. The second detection system enclosure may also include other components necessary for producing a proper detection system. For example, the second detection system may also include a filter for preventing short wavelength excitation from producing "false" signals in the optical detection system during fluorescence measurements. A user need only select the proper detection system enclosure for the detection of the desired analyte. Since each detection system enclosure includes many of the required components, a user does not have to make light source selections, sensor array selections or detector arrangement selections to produce a viable detection system.

In another embodiment, the individual components of the system may be interchangeable. The system may include coupling members 573 and 575 that allow the light source and the detector, respectively, to be removed from the chamber 550. This may allow a more modular design of the system. For example, an analysis may be first performed with a white light source to give data corresponding to an absorbance/reflectance analysis. After this analysis is performed the light source may be changed to a ultraviolet light source to allow ultraviolet analysis of the particles. Since the particles have already been treated with the fluid, the analysis may be preformed without further treatment of the particles with a fluid. In this manner a variety of tests may be performed using a single sensor array.

In one embodiment, the supporting member is made of any material capable of supporting the particles, while allowing the passage of the appropriate wavelength of light. The supporting member may also be made of a material substantially impervious to the fluid in which the analyte is present. A variety of materials may be used including plastics (e.g., photoresist materials, acrylic polymers, carbonate polymers, etc.), glass, silicon based materials (e.g., silicon, silicon dioxide, silicon nitride, etc.) and metals. In one embodiment, the supporting member includes a plurality of cavities. The cavities are preferably formed such that at least one particle is substantially contained within the cavity. Alternatively, a plurality of particles may be contained within a single cavity.

In some embodiments, it will be necessary to pass liquids over the sensor array. The dynamic motion of liquids across the sensor array may lead to displacement of the particles from the cavities. In another embodiment, the particles are preferably held within cavities formed in a supporting member by the use of a transmission electron microscope ("TEM") grid. As depicted in FIG. 19, a cavity 580 is formed in a supporting member 582.

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After placement of a particle 584 within the cavity, a TEM grid 586 may be placed atop the supporting member 582 and secured into position. TEM grids and adhesives for securing TEM grids to a support are commercially available from Ted Pella, Inc., Redding, CA. The TEM grid 586 may be made from a number of materials including, but not limited to, copper, nickel, gold, silver, aluminum, molybdenum, titanium, nylon, beryllium, carbon, and beryllium-copper. The mesh structure of the TEM grid may allow solution access as well as optical access to the particles that are placed in the cavities. FIG. 20 further depicts a top view of a sensor array with a TEM grid 586 formed upon the upper surface of the supporting member 582. The TEM grid 586 may be placed on the upper surface of the supporting member, trapping particles 584 within the cavities 580. As depicted, the openings 588 in the TEM grid 586 may be sized to hold the particles 584 within the cavities 580, while allowing fluid and optical access to cavities 580.

In another embodiment, a sensor array includes a supporting member configured to support the particles, while allowing the passage of the appropriate wavelength of light to the particle. The supporting member, in one embodiment, includes a plurality of cavities. The cavities may be formed such that at least one particle is substantially contained within the cavity. The supporting member may be configured to substantially inhibit the displacement of the particles from the cavities during use. The supporting member may also be configured to allow the passage of the fluid through cavities, e.g., the fluid may flow from the top surface of the supporting member, past the particle, and out the bottom surface of the supporting member. This may increase the contact time between the particle and the fluid.

Figures 21A-G depict a sequence of processing steps for the formation of a silicon based supporting member which includes a removable top cover and bottom cover. The removable top cover may be configured to allow fluids to pass through the top cover and into the cavity. The removable bottom cover may also be configured to allow the fluid to pass through the bottom cover and out of the cavity. As depicted in FIG. 21A, a series of layers may be deposited upon both sides of a silicon substrate 610. First removable layers 612 may be deposited upon the silicon substrate. The removable layers 612 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon dioxide 612 is deposited upon both surfaces of the silicon substrate 610. Upon these removable layers, covers 614 may be formed. In one embodiment, covers 614 are formed from a material that differs from the material used to form the removable layers 612 and which is substantially transparent to the light source of a detection system. For example, if the removable layers 612 are formed from silicon dioxide, the cover may be formed from silicon nitride. Second removable layers 616 may be formed upon the covers 614. Second removable layers 616 may be formed from a material that differs from the material used to form the covers 614. Second removable layers 616 may be formed from a material similar to the material used to form the first removable layers 612. In one embodiment, first and second removable layers 612 and 616 are formed from silicon dioxide and covers 614 are formed from silicon nitride. The layers are patterned and etched using standard photolithographic techniques. In one embodiment, the remaining portions of the layers are substantially aligned in the position where the cavities are to be formed in the silicon substrate 610.

After the layers have been etched, spacer structures may be formed on the sidewalls of the first removable layers 612, the covers 614, and the second removable layers 616, as depicted in FIG. 21B. The spacer structures may be formed from the same material used to form the second removable layers 616. In one embodiment, depositing a spacer layer of the appropriate material and subjecting the material to an anisotropic etch may form the

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spacer structures. An anisotropic etch, such as a plasma etch, employs both physical and chemical removal mechanisms. Ions are typically bombarded at an angle substantially perpendicular to the semiconductor substrate upper surface. This causes substantially horizontal surfaces to be removed faster than substantially vertical surfaces. During this etching procedure the spacer layers are preferably removed such that the only regions of the spacer layers that remain may be those regions near substantially vertical surfaces, e.g., spacer structures 618.

After formation of the spacer structures 618, cover support structures 620, depicted in FIG. 21C, may be formed. The cover support structures may be initially formed by depositing a support structure layer upon the second removable layer 616 and spacer structures 618. The support structure layer is then patterned and etched, using standard photolithography, to form the support structures 620. In one embodiment, the support structures are formed from a material that differs from the removable layers material. In one embodiment, the removable layers may be formed from silicon dioxide while the support structures and covers may be formed from silicon nitride.

Turning to FIG. 21 D, the second removable layers 616 and an upper portion of the spacer structures 618 are preferably removed using a wet etch process. Removal of the second removable layers leaves the top surface of the covers 614 exposed. This allows the covers to be patterned and etched such that openings 622 are formed extending through the covers. These openings 622 may be formed in the covers 614 to allow the passage of fluid through the cover layers. In one embodiment, the openings 622 are formed to allow fluid to pass through, while inhibiting displacement of the particles from the subsequently formed cavities.

After the openings 622 have been formed, the remainder of the first removable layers 612 and the remainder of the spacer structures 618 may be removed using a wet etch. The removal of the removable layers and the spacer structures creates "floating" covers 614, as depicted in FIG. 21E. The covers 614 may be held in proximity to the silicon substrate 610 by the support structures 620. The covers 614 may now be removed by sliding the covers away from the support structures 620. In this manner removable covers 614 may be formed.

After the covers 614 are removed, cavities 640 may be formed in the silicon substrate 610, as depicted in FIG. 21F. The cavities 640 may be formed by, initially patterning and etching a photoresist material 641 to form a masking layer. After the photoresist material 641 is patterned, the cavities 640 may be etched into the silicon substrate 610 using a hydroxide etch, as described previously.

After the cavities 640 are formed, the photoresist material may be removed and particles 642 may be placed within the cavities, as depicted in FIG. 21G. The particles 642, may be inhibited from being displaced from the cavity 640 by placing covers 614 back onto the upper and lower faces of the silicon substrate 610.

In another embodiment, a sensor array may be formed using a supporting member, a removable cover, and a secured bottom layer. FIGS. 22 A-G depict a series of processing steps for the formation of a silicon based supporting member which includes a removable top cover and a secured bottom layer. The removable top cover is preferably configured to allow fluids to pass through the top cover and into the cavity. As depicted in FIG. 22A, a series of layers may be deposited upon both sides of a silicon substrate 610. A first removable layer 612 may be deposited upon the upper face 611 of the silicon substrate 610. The removable layer 612 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon dioxide 612 is deposited upon the silicon substrate 610. A cover 614 may be formed upon the removable layer 612 of the silicon substrate 610. In one embodiment, the cover 614 is formed from a material that differs from the material used to form the removable layer 612 and is substantially transparent to the light source of a detection system. For example, if the removable

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layer 612 is formed from silicon dioxide, the cover layer 614 may be formed from silicon nitride. In one embodiment, a bottom layer 615 is formed on the bottom surface 613 of the silicon substrate 610. In one embodiment, the bottom layer 615 is formed from a material that is substantially transparent to the light source of a detection system. A second removable layer 616 may be formed upon the cover 614. Second removable layer 616 may be formed from a material that differs from the material used to form the cover layer 614. Second removable layer 616 may be formed from a material similar to the material used to form the first removable layer 612. In one embodiment, first and second removable layers 612 and 616 are formed from silicon dioxide and cover 614 is formed from silicon nitride. The layers formed on the upper surface 611 of the silicon substrate may be patterned and etched using standard photolithographic techniques. In one embodiment, the remaining portions of the layers formed on the upper surface are substantially aligned in the position where the cavities are to be formed in the silicon substrate 610.

After the layers have been etched, spacer structures may be formed on the side walls of the first removable layer 612, the cover 614, and the second removable layer 616, as depicted in FIG. 22B. The spacer structures may be formed from the same material used to form the second removable layer 616. In one embodiment, the spacer structures may be formed by depositing a spacer layer of the appropriate material and subjecting the spacer layer to an anisotropic etch. During this etching procedure the spacer layer is preferably removed such that the only regions of the spacer layer which remain may be those regions near substantially vertical surfaces, e.g., spacer structures 618.

After formation of the spacer structures 618, cover support structures 620, depicted in FIG. 22C, may be formed upon the removable layer 616 and the spacer structures 618. The cover support structures 620 may be formed by depositing a support structure layer upon the second removable layer 616 and spacer structures 618. The support structure layer is then patterned and etched, using standard photolithography, to form the support structures 620. In one embodiment, the support structures are formed from a material that differs from the removable layer materials. In one embodiment, the removable layers may be formed from silicon dioxide while the support structures and cover may be formed from silicon nitride.

Turning to FIG. 22 D, the second removable layer 616 and an upper portion of the spacer structures 618 may be removed using a wet etch process. Removal of the second removable layer leaves the top surface of the cover 614 exposed. This allows the cover 614 to be patterned and etched such that openings 622 are formed extending through the cover 614. These openings 622 may be formed in the cover 614 to allow the passage of fluid through the cover. In one embodiment, the openings 622 are formed to allow fluid to pass through, while inhibiting displacement of the particle from a cavity. The bottom layer 615 may also be similarly patterned and etched such that openings 623 may be formed extending thorough the bottom layer 615.

After the openings 622 and 623 are formed, the first removable layer 612 and the remainder of the spacer structures 618 may be removed using a wet etch. The removal of the removable layers and the spacer structures creates a "floating" cover 614, as depicted in FIG. 22E. The cover 614 may be held in proximity to the silicon substrate 610 by the support structures 620. The cover 614 may now be removed by sliding the cover 614 away from the support structures 620. In this manner a removable cover 614 may be formed.

After the cover 614 is removed, cavities 640 may be formed in the silicon substrate 610, as depicted in FIG. 22F. The cavities 640 may be formed by, initially patterning and etching a photoresist material 641 to form a

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masking layer. After the photoresist material 614 is patterned, the cavities 640 may be etched into the silicon substrate 610 using a hydroxide etch, as described previously.

After the cavities 640 are formed, the photoresist material may be removed and particles 642 may be placed within the cavities, as depicted in FIG. 22G. The particles 642, may be inhibited from being displaced from the cavity 640 by placing cover 614 back onto the upper face 611 of the silicon substrate 610. The bottom layer 615 may also aid in inhibiting the particle 642 from being displaced from the cavity 640. Openings 622 in cover 614 and openings 623 in bottom layer 615 may allow fluid to pass through the cavity during use.

In another embodiment, a sensor array may be formed using a supporting member and a removable cover. FIGS. 23A-G depict a series of processing steps for the formation of a silicon based supporting member which includes a removable cover. The removable cover is preferably configured to allow fluids to pass through the cover and into the cavity. As depicted in FIG. 23A, a series of layers may be deposited upon the upper surface 611 of a silicon substrate 610. A first removable layer 612 may be deposited upon the upper face 611 of the silicon substrate 610. The removable layer 612 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon dioxide 612 is deposited upon the silicon substrate 610. A cover 614 may be formed upon the removable layer 612. In one embodiment, the cover is formed from a material which differs from the material used to form the removable layer 612 and which is substantially transparent to the light source of a detection system. For example, if the removable layer 612 is formed from silicon dioxide, the cover 614 may be formed from silicon nitride. A second removable layer 616 may be formed upon the cover 614. Second removable layer 616 may be formed from a material that differs from the material used to form the cover 614. Second removable layer 616 may be formed from a material similar to the material used to form the first removable layer 612. In one embodiment, first and second removable layers 612 and 616 are formed from silicon dioxide and cover 614 is formed from silicon nitride. The layers formed on the upper surface 611 of the silicon substrate may be patterned and etched using standard photolithographic techniques. In one embodiment, the remaining portions of the layers formed on the upper surface are substantially aligned in the position where the cavities are to be formed in the silicon substrate 610.

After the layers have been etched, spacer structures 618 may be formed on the side walls of the first removable layer 612, the cover layer 614, and the second removable layer 616, as depicted in FIG. 23B. The spacer structures 618 may be formed from the same material used to form the second removable layer 616. In one embodiment, the spacers may be formed by depositing a spacer layer of the appropriate material upon the second removable layer and subjecting the material to an anisotropic etch. During this etching procedure the spacer layer is preferably removed such that the only regions of the spacer layer which remain may be those regions near substantially vertical surfaces, e.g., spacer structures 618.

After formation of the spacer structures 618, cover support structures 620, depicted in FIG. 23C, may be formed upon the removable layer 616 and the spacer structures 618. The cover support structure may be formed by initially depositing a support structure layer upon the second removable layer 616 and spacer structures 618. The support structure layer is then patterned and etched, using standard photolithography, to form the support structures 620. In one embodiment, the support structures 620 are formed from a material that differs from the removable layer materials. In one embodiment, the removable layers may be formed from silicon dioxide while the support structure and cover layer may be formed from silicon nitride.

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Turning to FIG. 23D, the second removable layer 616 and an upper portion of the spacer structures 618 may be removed using a wet etch process. Removal of the second removable layer leaves the top surface of the cover 614 exposed. This allows the cover 614 to be patterned and etched such that openings 622 are formed extending through the cover 614. These openings 622 may be formed in the cover 614 to allow the passage of fluid through the cover 614.

After the openings 622 are formed, the remainder of the first removable layer 612 and the remainder of the spacer structures 618 may be removed using a wet etch. The removal of the removable layers and the spacer structures creates a "floating" cover 614, as depicted in FIG. 23E. The cover 614 is preferably held in proximity to the silicon substrate 610 by the support structures 620. The cover 614 may now be removed by sliding the cover 614 away from the support structures 620. In this manner a removable cover 614 may be formed.

After the cover 614 is removed, cavities 640 may be formed in the silicon substrate 610, as depicted in FIG. 23F. The cavities 640 may be formed by initially depositing and patterning a photoresist material 641 upon the silicon support 610. After the photoresist material 614 is patterned, the cavities 640 may be etched into the silicon substrate 610 using a hydroxide etch, as described previously. The etching of the cavities may be accomplished such that a bottom width of the cavity 643 is less than a width of a particle 642. In one embodiment, the width of the bottom of the cavity may be controlled by varying the etch time. Typically, longer etching times result in a larger opening at the bottom of the cavity. By forming a cavity in this manner, a particle placed in the cavity may be too large to pass through the bottom of the cavity. Thus, a supporting member that does not include a bottom layer may be formed. An advantage of this process is that the processing steps may be reduced making production simpler.

After the cavities 640 are formed, the photoresist material may be removed and particles 642 may be placed within the cavities, as depicted in FIG. 23G. The particles 642, may be inhibited from being displaced from the cavity 640 by placing cover 614 back onto the upper face 611 of the silicon substrate 610. The narrow bottom portion of the cavity may also aid in inhibiting the particle 642 from being displaced from the cavity 640.

Figures 24A-d depict a sequence of processing steps for the formation of a silicon based supporting member which includes a top partial cover and a bottom partial cover. The top partial cover and bottom partial covers are, in one embodiment, configured to allow fluids to pass into the cavity and out through the bottom of the cavity. As depicted in FIG. 24A, a bottom layer 712 may be deposited onto the bottom surface of a silicon substrate 710. The bottom layer 712 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon nitride 712 is deposited upon the silicon substrate 710. In one embodiment, openings 714 are formed through the bottom layer as depicted in FIG. 24A. Openings 714, in one embodiment, are substantially aligned with the position of the cavities to be subsequently formed. The openings 714 may have a width that is substantially less than a width of a particle. Thus a particle will be inhibited from passing through the openings 714.

Cavities 716 may be formed in the silicon substrate 710, as depicted in FIG. 24B. The cavities 716 may be formed by initially depositing and patterning a photoresist layer upon the silicon substrate 710. After the photoresist material is patterned, cavities 716 may be etched into the silicon substrate 710 using a number of etching techniques, including wet and plasma etches. The width of the cavities 716 is preferably greater than the width of a particle, thus allowing a particle to be placed within each of the cavities. The cavities 716, in one

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embodiment, are preferably formed such that the cavities are substantially aligned over the openings 714 formed in the bottom layer.

After the cavities have been formed, particles 718 may be inserted into the cavities 716, as depicted in FIG. 24C. The etched bottom layer 712 may serve as a support for the particles 718. Thus the particles 718 may be inhibited from being displaced from the cavities by the bottom layer 712. The openings 714 in the bottom layer 712 may allow fluid to pass through the bottom layer during use.

After the particles are placed in the cavities, a top layer 720 may be placed upon the upper surface 717 of the silicon substrate. In one embodiment, the top layer 720 is formed from a material is substantially transparent to the light source of a detection system. The top layer may be formed from silicon nitride, silicon dioxide or photoresist material. In one embodiment, a sheet of photoresist material is used. After the top layer 620 is formed, openings 719 may be formed in the top layer to allow the passage of the fluid into the cavities. If the top layer 720 is composed of photoresist material, after depositing the photoresist material across the upper surface of the silicon substrate, the openings may be initially formed by exposing the photoresist material to the appropriate wavelength and pattern of light. If the top layer is compose of silicon dioxide or silicon nitride the top layer 720 may be developed by forming a photoresist layer upon the top layer, developing the photoresist, and using the photoresist to etch the underlying top layer.

Similar sensor arrays may be produced using materials other than silicon for the supporting member. For example, as depicted in FIG 25 A-D, the supporting member may be composed of photoresist material. In one embodiment, sheets of photoresist film may be used to form the supporting member. Photoresist film sheets are commercially available from E. I. du Pont de Nemours and Company, Wilmington, DE under the commercial name RISTON. The sheets come in a variety of sizes, the most common having a thickness ranging from about 1 mil. (25 μm) to about 2 mil. (50 μm).

In an embodiment, a first photoresist layer 722 is developed and etched such that openings 724 are formed. The openings may be formed proximate the location of the subsequently formed cavities. Preferably, the openings have a width that is substantially smaller than a width of the particle. The openings may inhibit displacement of the particle from a cavity. After the first photoresist layer 720 is patterned and etched, a main layer 726 is formed upon the bottom layer. The main layer 720 is preferably formed from a photoresist film that has a thickness substantially greater than a typical width of a particle. Thus, if the particles have a width of about 30 μm, a main layer may be composed of a 50 μm photoresist material. Alternatively, the photoresist layer may be composed of a multitude of photoresist layers placed upon each other until the desired thickness is achieved, as will be depicted in later embodiments.

The main photoresist layer may be patterned and etched to form the cavities 728, as depicted in FIG. 25B. The cavities, in one embodiment, are substantially aligned above the previously formed openings 724. Cavities 728, in one embodiment, have a width which is greater than a width of a particle.

For many types of analysis, the photoresist material is substantially transparent to the light source used. Thus, as opposed to a silicon supporting member, the photoresist material used for the main supporting layer may be substantially transparent to the light used by the light source. In some circumstances, the transparent nature of the supporting member may allow light from the cavity to migrate, through the supporting member, into a second cavity. This leakage of light from one cavity to the next may lead to detection problems. For example, if a first

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particle in a first cavity produces a fluorescent signal in response to an analyte, this signal may be transmitted through the supporting member and detected in a proximate cavity. This may lead to inaccurate readings for the proximately spaced cavities, especially if a particularly strong signal is produced by the interaction of the particle with an analyte.

To reduce the occurrence of this "cross-talk", a substantially reflective layer 730 may be formed along the inner surface of the cavity. In one embodiment, the reflective layer 730 is composed of a metal layer which is formed on the upper surface of the main layer and the inner surface of the cavity. The metal layer may be deposited using chemical vapor deposition or other known techniques for depositing thin metal layers. The presence of a reflective layer may inhibit "cross-talk" between the cavities.

After the cavities 728 have been formed, particles 718 may be inserted into the cavities 728, as depicted in FIG. 25C. The first photoresist layer 722 may serve as a support for the particles 718. The particles may be inhibited from being displaced from the cavities by the first photoresist layer 722. The openings 724 in the first photoresist layer 722 may allow fluid to pass through the bottom layer during use.

After the particles 728 are placed in the cavities 728, a top photoresist layer 732 may be placed upon the upper surface of the silicon substrate. After the cover layer is formed, openings 734 may be formed in the cover layer to allow the passage of the fluid into the cavities.

In another embodiment, the supporting member may be formed from a plastic substrate, as depicted in FIG. 26A-D. In one embodiment, the plastic substrate is composed of a material which is substantially resistant to the fluid which includes the analyte. Examples of plastic materials which may be used to form the plastic substrate include, but are not limited to, acrylic resins, polycarbonates, polyester resins, polyethylenes, polyimides, polyvinyl polymers (e.g., polyvinyl chloride, polyvinyl acetate, polyvinyl dichloride, polyvinyl fluoride, etc.), polystyrenes, polypropylenes, polytetrafluoroethylenes, and polyurethanes. The plastic substrate may be substantially transparent or substantially opaque to the light produced by the light source. After obtaining a suitable plastic material 740, a series of cavities 742 may be formed in the plastic material. The cavities 740 may be formed by drilling (either mechanically or with a laser), transfer molding (e.g., forming the cavities when the plastic material is formed using appropriately shaped molds), or using a punching apparatus to punch cavities into the plastic material. In one embodiment, the cavities 740 are formed such that a lower portion 743 of the cavities is substantially narrower than an upper portion 744 of the cavities. The lower portion 743 of the cavities may have a width substantially less than a width of a particle. The lower portion 743 of the cavities 740 may inhibit the displacement of a particle from the cavity 740. While depicted as rectangular, with a narrower rectangular opening at the bottom, it should be understood that the cavity may be formed in a number of shapes including but not limited to pyramidal, triangular, trapezoidal, and oval shapes. An example of a pyramidal cavity which is tapered such that the particle is inhibited from being displaced from the cavity is depicted in FIG. 25D.

After the cavities 742 are formed, particles 718 may be inserted into the cavities 742, as depicted in FIG. 26B. The lower portion 743 of the cavities may serve as a support for the particles 718. The particles 718 may be inhibited from being displaced from the cavities 742 by the lower portion 743 of the cavity. After the particles are placed in the cavities 740, a cover 744 may be placed upon the upper surface 745 of the plastic substrate 740, as depicted in FIG. 26C. In one embodiment, the cover is formed from a film of photoresist material. After the cover

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744 is placed on the plastic substrate 740, openings 739 may be formed in the cover layer to allow the passage of the fluid into the cavities.

In some circumstances a substantially transparent plastic material may be used. As described above, the use of a transparent supporting member may lead to "cross-talk" between the cavities. To reduce the occurrence of this "cross-talk", a substantially reflective layer 748 may be formed on the inner surface 746 of the cavity, as depicted in FIG. 26E. In one embodiment, the reflective layer 748 is composed of a metal layer which is formed on the inner surface of the cavities 742. The metal layer may be deposited using chemical vapor deposition or other techniques for depositing thin metal layers. The presence of a reflective layer may inhibit cross-talk between the cavities.

In another embodiment, a silicon based supporting member for a sensing particle may be formed without a bottom layer. In this embodiment, the cavity may be tapered to inhibit the passage of the particle from the cavity, through the bottom of the supporting member. FIG. 27A-D, depicts the formation of a supporting member from a silicon substrate. In this embodiment, a photoresist layer 750 is formed upon an upper surface of a silicon substrate 752, as depicted in FIG. 27A. The photoresist layer 750 may be patterned and developed such that the regions of the silicon substrate in which the cavities will be formed are exposed.

Cavities 754 may now be formed, as depicted in FIG. 27B, by subjecting the silicon substrate to an anisotropic etch. In one embodiment, a potassium hydroxide etch is used to produced tapered cavities. The etching may be controlled such that the width of the bottom of the cavities 750 is less than a width of the particle. After the cavities have been etched, a particle 756 may be inserted into the cavities 754 as depicted in FIG. 27C. The particle 756 may be inhibited from passing out of the cavities 754 by the narrower bottom portion of the cavities. After the particle is positioned within the cavities 754, a cover 758 may be formed upon the silicon substrate 752, as depicted in FIG. 27D. The cover may be formed of any material substantially transparent to the light produced by the light source used for analysis. Openings 759 may be formed in the cover 758 to allow the fluid to pass into the cavity from the top face of the supporting member 752. The openings 759 in the cover and the opening at the bottom of the cavities 754 together may allow fluid to pass through the cavity during use.

In another embodiment, a supporting member for a sensing particle may be formed from a plurality of layers of a photoresist material. In this embodiment, the cavity may be tapered to inhibit the passage of the particle from the cavity, through the bottom of the supporting member. FIGS. 28A-E depict the formation of a supporting member from a plurality of photoresist layers. In an embodiment, a first photoresist layer 760 is developed and etched to form a series of openings 762 which are positioned at the bottom of subsequently formed cavities, as depicted in FIG. 28A. As depicted in FIG. 28B, a second layer of photoresist material 764 may be formed upon the first photoresist layer 760. The second photoresist layer may be developed and etched to form openings substantially aligned with the openings of the first photoresist layer 760. The openings formed in the second photoresist layer 764, in one embodiment, are substantially larger than the layers formed in the first photoresist layer 760. In this manner, a tapered cavity may be formed while using multiple photoresist layers.

As depicted in FIG. 28C, additional layers of photoresist material 766 and 768 may be formed upon the second photoresist layer 764. The openings of the additional photoresist layers 766 and 768 may be progressively larger as each layer is added to the stack. In this manner, a tapered cavity may be formed. Additional layers of photoresist material may be added until the desired thickness of the supporting member is obtained. The thickness

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of the supporting member, in one embodiment, is greater than a width of a particle. For example, if a layer of photoresist material has a thickness of about 25 μm and a particle has a width of about 100 μm, a supporting member may be formed from four or more layers of photoresist material. While depicted as pyramidal, the cavity may be formed in a number of different shapes, including but not limited to, rectangular, circular, oval, triangular, and trapezoidal. Any of these shapes may be obtained by appropriate patterning and etching of the photoresist layers as they are formed.

In some instances, the photoresist material may be substantially transparent to the light produced by the light source. As described above, the use of a transparent supporting member may lead to "cross-talk" between the cavities. To reduce the occurrence of this "cross-talk", a substantially reflective layer 770 may be formed along the inner surface of the cavities 762, as depicted in FIG. 28D. In one embodiment, the reflective layer is composed of a metal layer which is formed on the inner surface of the cavities 762. The metal layer may be deposited using chemical vapor deposition or other techniques for depositing thin metal layers. The presence of a reflective layer may inhibit "cross-talk" between the cavities.

After the cavities 762 are formed, particles 772 may be inserted into the cavities 762, as depicted in FIG. 28D. The narrow portions of the cavities 762 may serve as a support for the particles 772. The particles 772 may be inhibited from being displaced from the cavities 762 by the lower portion of the cavities. After the particles 772 are placed in the cavities 762, a cover 774 may be placed upon the upper surface of the top layer 776 of the supporting member, as depicted in FIG. 28E. In one embodiment, the cover 774 is also formed from a film of photoresist material. After the cover layer is formed, openings 778 may be formed in the cover 774 to allow the passage of the fluid into the cavities.

In another embodiment, a supporting member for a sensing particle may be formed from photoresist material which includes a particle support layer. FIGS. 29A-E depict the formation of a supporting member from a series of photoresist layers. In an embodiment, a first photoresist layer 780 is developed and etched to form a series of openings 782 which may become part of subsequently formed cavities. In another embodiment, a cavity having the appropriate depth may be formed by forming multiple layers of a photoresist material, as described previously. As depicted in FIG. 29B, a second photoresist layer 784 may be formed upon the first photoresist layer 780. The second photoresist layer 784 may be patterned to form openings substantially aligned with the openings of the first photoresist layer 782. The openings formed in the second photoresist layer 784 may be substantially equal in size to the previously formed openings. Alternatively, the openings may be variable in size to form different shaped cavities.

For reasons described above, a substantially reflective layer 786 may be formed along the inner surface of the cavities 782 and the upper surface of the second photoresist layer 784, as depicted in FIG. 29C. In one embodiment, the reflective layer is composed of a metal layer. The metal layer may be deposited using chemical vapor deposition or other techniques for depositing thin metal layers. The presence of a reflective layer may inhibit "cross-talk" between the cavities.

After the metal layer is deposited, a particle support layer 788 may be formed on the bottom surface of the first photoresist layer 780, as depicted in FIG. 29D. The particle support layer 788 may be formed from photoresist material, silicon dioxide, silicon nitride, glass or a substantially transparent plastic material. The particle support

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layer 788 may serve as a support for the particles placed in the cavities 782. The particle support layer, in one embodiment, is formed from a material that is substantially transparent to the light produced by the light source.

After the particle supporting layer 788 is formed, particles 785 may be inserted into the cavities 782, as depicted in FIG. 29E. The particle support layer 788 may serve as a support for the particles. Thus the particles 785 may be inhibited from being displaced from the cavities by the particle support layer 788. After the particles 785 are placed in the cavities 782, a cover 787 may be placed upon the upper surface of the second photoresist layer 784, as depicted in FIG. 29E. In one embodiment, the cover is also formed from a film of photoresist material. After the cover is formed, openings 789 may be formed in the cover 787 to allow the passage of the fluid into the cavities. In this embodiment, the fluid is inhibited from flowing through the supporting member. Instead, the fluid may flow into and out of the cavities via the openings 789 formed in the cover 787.

A similar supporting member may be formed from a plastic material, as depicted in FIGS. 30A-D. The plastic material may be substantially resistant to the fluid which includes the analyte. The plastic material may be substantially transparent or substantially opaque to the light produced by the light source. After obtaining a suitable plastic substrate 790, a series of cavities 792 may be formed in the plastic substrate 790. The cavities may be formed by drilling (either mechanically or with a laser), transfer molding (e.g., forming the cavities when the plastic substrate is formed using appropriately shaped molds), or using a punching machine to form the cavities. In one embodiment, the cavities extend through a portion of the plastic substrate, terminating proximate the bottom of the plastic substrate, without passing through the plastic substrate. After the cavities 792 are formed, particles 795 may be inserted into the cavities 792, as depicted in FIG. 30B. The bottom of the cavity may serve as a support for the particles 795. After the particles are placed in the cavities, a cover 794 may be placed upon the upper surface of the plastic substrate 790, as depicted in FIG. 30C. In one embodiment, the cover may be formed from a film of photoresist material. After the cover 794 is formed, openings 796 may be formed in the cover to allow the passage of the fluid into the cavities. While depicted as rectangular, is should be understood that the cavities may be formed in a variety of different shapes, including triangular, pyramidal, pentagonal, polygonal, oval, or circular. It should also be understood that cavities having a variety of different shapes may be formed into the same plastic substrate, as depicted in FIG. 30D.

In one embodiment, a series of channels may be formed in the supporting member interconnecting some of the cavities, as depicted in FIG. 3. Pumps and valves may also be incorporated into the supporting member to aid passage of the fluid through the cavities. A schematic figure of a diaphragm pump 800 is depicted in FIG. 31. Diaphragm pumps, in general, include a cavity 810, a flexible diaphragm 812, an inlet valve 814, and an outlet valve 816. The flexible diaphragm 812, during use, is deflected as shown by arrows 818 to create a pumping force. As the diaphragm is deflected toward the cavity 810 it may cause the inlet valve 814 to close, the outlet valve 816 to open and any liquid which is in the cavity 810 will be forced toward the outlet 816. As the diaphragm moves away from the cavity 810, the outlet valve 816 may be pulled to a closed position, and the inlet valve 814 may be opened, allowing additional fluid to enter the cavity 810. In this manner a pump may be used to pump fluid through the cavities. It should be understood that the pump depicted in FIG. 31 is a generalized version of a diaphragm based pump. Actual diaphragm pumps may have different shapes or may have inlet and outlet valves which are separate from the pumping device.

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In one embodiment, the diaphragm 810 may be made from a piezoelectric material. This material will contract or expand when an appropriate voltage is applied to the diaphragm. Pumps using a piezoelectric diaphragms are described in U.S. Patent Nos. 4,344,743, 4,938,742, 5,611,676, 5,705,018, and 5,759,015, all of which are incorporated by reference. In other embodiments, the diaphragm may be activated using a pneumatic system. In these systems, an air system may be coupled to the diaphragm such that changes in air density about the diaphragm, induced by the pneumatic system, may cause the diaphragm to move toward and away from the cavity. A pneumatically controlled pump is described in United States Patent No. 5,499,909 which is incorporated by reference. The diaphragm may also be controlled using a heat activated material. The diaphragm may be formed from a temperature sensitive material. In one embodiment, the diaphragm may be formed from a material which is configured to expand and contract in response to temperature changes. A pump system which relies on temperature activated diaphragm is described in United States Patent No. 5,288,214 which is incorporated by reference.

In another embodiment, an electrode pump system may be used. FIG. 32 depicts a typical electrode based system. A series of electrodes 820 may be arranged along a channel 822 which may lead to a cavity 824 which includes a particle 826. By varying the voltage in the electrodes 820 a current flow may be induced in the fluid within the channel 822. Examples of electrode based systems include, but are not limited to, electroosmosis systems, electrohydrodynamic systems, and combinations of electroosmosis and electrohydrodynamic systems.

Electrohydrodynamic pumping of fluids is known and may be applied to small capillary channels. In an electrohydrodynamic system electrodes are typically placed in contact with the fluid when a voltage is applied. The applied voltage may cause a transfer in charge either by transfer or removal of an electron to or from the fluid. This electron transfer typically induces liquid flow in the direction from the charging electrode to the oppositely charged electrode. Electrohydrodynamic pumps may be used for pumping fluids such as organic solvents.

Electroosmosis, is a process which involves applying a voltage to a fluid in a small space, such as a capillary channel, to cause the fluid to flow. The surfaces of many solids, including quartz, glass and the like, become variously charged, negatively or positively, in the presence of ionic materials, such as for example salts, acids or bases. The charged surfaces will attract oppositely charged (positive or negative) counterions in aqueous solutions. The application of a voltage to such a solution results in a migration of the counterions to the oppositely charged electrode, and moves the bulk of the fluid as well. The volume flow rate is proportional to the current, and the volume flow generated in the fluid is also proportional to the applied voltage. An electroosmosis pump system is described in United States Patent No. 4,908,112 which is incorporated by reference.

In another embodiment, a combination of electroosmosis pumps and electrohydrodynamic pumps may be used. Wire electrodes may be inserted into the walls of a channel at preselected intervals to form alternating electroosmosis and electrohydrodynamic devices. Because electroosmosis and electrohydrodynamic pumps are both present, a plurality of different solutions, both polar and non-polar, may be pump along a single channel. Alternatively, a plurality of different solutions may be passed along a plurality of different channels connected to a cavity. A system which includes a combination of electroosmosis pumps and electrohydrodynamic pumps is described in United States Patent No. 5,632,876 which is incorporated by reference.

In an embodiment, a pump may be incorporated into a sensor array system, as depicted in FIG. 32. A sensor array 830 includes at least one cavity 832 in which a particle 834 may be placed. The cavity 832 may be configured to allow fluid to pass through the cavity during use. A pump 836 may be incorporated onto a portion of

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