DIGITAL ASSAYS WITH MULTIPLEXED ~~DETECTION~~
DETECTION OF TWO OR MORE TARGETS
IN THE SAME OPTICAL CHANNEL

~~Cross-References~~

CROSS-REFERENCES TO PRIORITY APPLICATIONS

[0001] This application is based upon and claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application Ser. No. 61/507,082, filed Jul. 12, 2011; and U.S. Provisional Patent Application Ser. No. 61/510,013, filed Jul. 20, 2011. Each of these priority applications is incorporated herein by reference in its entirety for all purposes.

CROSS-REFERENCES TO OTHER MATERIALS

[0002] This application incorporates by reference in their entireties for all purposes the following materials: U.S. ~~Patent~~Pat. No. 7,041,481, issued May 9, 2006; U.S. Patent Application Publication No. 2010/0173394 Al, published ~~July~~Jul. 8, 2010; U.S. ~~Provisional~~ patent application Ser. No. 13/251,016, filed Sep. 30, 2011; U.S. patent application Ser. No. 13/341,678, filed Dec. 30, 2011; U.S. patent application Ser. No. 13/424,304, filed Mar. 19, 2012; and Joseph R. Lakowicz, ~~Patent Application Serial No. 61/507,082, filed July 12, 2011; and Joseph R. Lakowicz,~~ PRINCIPLES OF FLUORESCENCE SPECTROSCOPY (~~2nd~~2"d Ed. 1999).

~~Introduction~~ INTRODUCTION

[0003] Digital assays generally rely on the ability to detect the presence or activity of individual copies of an analyte in a sample. In an exemplary digital assay, a sample is separated into a set of partitions, generally of equal volume, with each containing, on average, less than about one copy of the ~~analyte~~ana-lyte. If the copies of the analyte are distributed randomly among the partitions, some partitions should contain no copies, others only one copy, and, if the number of partitions is large enough, still others should contain two copies, three copies, and even higher numbers of copies. The probability of finding exactly 0, 1, 2, 3, or more copies in a partition, based on a given average concentration of analyte in the partitions, is described by a Poisson distribution. Conversely, the concentration of analyte in the partitions (and thus in the sample) may be estimated from the probability of finding a given number of copies in a partition.

[0004] Estimates of the probability of finding no copies and of finding one or more copies may be measured in the digital assay. Each partition can be tested to determine whether the partition is a positive partition that contains at least one copy of the analyte, or is a negative partition that contains no copies of the analyte. The probability of finding no copies in a partition can be approximated by the fraction of partitions tested that are negative (the "negative fraction"), and the probability of

finding at least one copy by the fraction of partitions tested that are positive (the "positive fraction"). The positive fraction or the negative fraction then may be utilized in a Poisson equation to determine the concentration of the analyte in the partitions.

[0005] Digital assays frequently rely on amplification of a nucleic acid target in partitions to enable detection of a single copy of an analyte. Amplification may be conducted via the polymerase chain reaction (PCR), to achieve a digital PCR assay. The target amplified may be the analyte itself or a surrogate for the analyte generated before or after formation of the partitions. Amplification of the target can be detected optically from a fluorescent probe included in the reaction. In particular, the probe can include a dye that provides a fluorescence signal indicating whether or not the target has been amplified.

[0006] A digital PCR assay can be multiplexed to permit detection of two or more different targets within each partition. Amplification of the targets can be distinguished by utilizing target-specific probes labeled with different dyes~~, which produce fluorescence detected in different detection channels, namely, at different wavelengths or wavelength regions ("colors")~~. If a detector for a digital PCR assay can distinguishably measure the fluorescence emitted by N different dyes, then the assay is effectively capable of measuring N different targets. However, instruments with more ~~detection~~optical channels, to detect emission from more ~~colors~~dyes, are more expensive than those with fewer ~~detection~~ channels. Also, increasing the number of distinguishable dyes is expensive and becomes impractical beyond a certain number. On the other hand, many applications, especially where sample is limited, could benefit greatly from higher degrees of multiplexing.

[0007] A new approach is needed to increase the multiplex levels of digital assays.

~~Summary~~SUMMARY

[0008] The present disclosure provides a system, including methods and apparatus, for performing a digital assay with multiplexed detection of two or more distinct targets in the same optical channel.

~~Brief Description of the Drawings~~

BRIEF DESCRIPTION OF THE DRAWINGS

~~Figure~~[0009] FIG. 1 is a flowchart of an exemplary method of performing a digital assay with multiplexed detection of two or more distinct targets in the same optical channel, in accordance with aspects of the present disclosure.

~~Figure~~[0010] FIG. 2 is a schematic view of an exemplary apparatus for performing the digital assay of ~~Figure~~FIG. 1, in accordance with aspects of the present disclosure.

~~Figure~~[0011] FIG. 3 is a schematic view of a pair of targets and corresponding probes capable of reporting the presence or absence of the targets via emitted light that may be detected together in the same channel for one or both targets in a digital PCR assay, in accordance with aspects of the present disclosure.

~~Figure~~[0012] FIG. 4 is an exemplary graph showing a signal that may be created by detecting light emitted from the probes of ~~Figure~~FIG. 3 in a digital PCR assay performed in droplets, with the signal created by detecting light from each target together in the same channel from a fluid stream containing the droplets, in accordance with aspects of the present disclosure.

~~Figure~~[0013] FIG. 5 is an exemplary graph showing droplet (or peak) intensities in arbitrary units ("arb.") for a C61/RPP30 droplet assay, where data from reporters for C61 and RPP30 are collected together in the same optical channel and plotted as a function of intensity in a FAM channel (vertical axis) versus intensity in a VIC channel (horizontal axis).

~~Figure~~[0014] FIG. 6 is a graph showing FAM intensities for the C61/RPP30 droplet assay of ~~Figure~~FIG. 5 for twelve different sets of assay conditions ~~(anneal~~, namely, amplification of the C61 and RPP30 targets with different annealing temperatures~~,~~ (given in Celsius and denoted, from left to right, 55.0, 55.2, .~~..~~, 64.6, and 64.9 ~~degrees Celsius~~).

~~Figure~~[0015] FIG. 7 is an exemplary graph showing droplet (or peak) intensities for a C63/RPP30 droplet assay, where data from reporters for C63 and RPP30 are collected ~~together in the same channel~~ and plotted as a function of intensity in a FAM channel (vertical axis) versus intensity in a VIC channel (horizontal axis).

~~Figure~~[0016] FIG. 8 shows FAM intensities (top panel; FIG. 8A) and VIC intensities (bottom panel; FIG. 8B) for the C63/ RPP30 droplet assay of ~~Figure~~FIG. 7 for twelve different sets of assay conditions ~~(anneal~~, namely, amplification of the C61 and RPP30 targets with different annealing temperatures~~,~~ (given in degrees Celsius and denoted, from left to right, 55.0, 55.2, . . ., 64.6, and 64.9 ~~degrees Celsius~~).

[0017] FIG. 9 is a schematic representation of an exemplary bulk phase mixture being separated into droplets during performance of an exemplary digital assay with multiplexed detection of three or more distinct targets (T1, T2, and T3) using a reporter for each target (a T1 probe, a T2 probe, and a T3 probe) each including a different luminophore (L1, L2, and L3, respectively), in accordance with aspects of the present disclosure.

[0018] FIG. 10 is a schematic representation of an exemplary graph showing droplet (or peak) intensities for a Tl/T2/ T3 droplet assay that may be performed with the droplets of FIG. 9, where data from the T1, T2, and T3 probes are collected in a first optical channel and a second optical channel and plotted as a function of intensity in the first channel (vertical axis) versus intensity in the second channel (horizontal axis), with intensities produced by exemplary populations or clusters of negative (NEG), Ti -positive (T1+), T2-positive (T2+), and T3-positive (T3+) droplets represented by filled ellipses, in accordance with aspects of the present disclosure.

~~Detailed Description~~ DETAILED DESCRIPTION

[0019] The present disclosure provides a system, including methods and apparatus, for performing a digital assay on a potentially greater number of targets

through multiplexed detection of signals from reporters for two or more distinct targets in a common or shared ~~detection~~ channel ("the same optical channel"). The reporters may include the same fluorophore, such as FAM or VIC, or different fluorophores with similar spectral characteristics, so that light from ~~each reporter~~two or more reporters may be collected simultaneously in the same optical channel. The assays may be constructed so that data for each target are distinguishable, for example, by choosing assays for each target that have sufficiently distinct endpoints (or time courses). ~~In some cases, preliminary assays may be run, using aliquots of the actual sample and/or materials representative of the sample, to determine suitable or optimal assay conditions, such as anneal temperatures capable of allowing reactions to run substantially toward or to completion in thermocycling assays.~~ The contents of each sample or sample partition may then be determined: those with no targets, those with a first assay (~~assay1~~assay1) target, those with a second assay (assay2) target, and those with both, in a two-target assay. The total number of droplets positive for each target (e.g., target 1 and target 2) can be estimated by taking into account the total number of droplets in each population. Concentrations for each target may be estimated based on the number of droplets positive for each target and the total number of droplets, for example, using Poisson statistics. Moreover, the relative numbers of different targets (including reference targets) may be estimated, allowing determination of copy number (CN)~~ and~~, copy number variation (CNV), and the presence/abundance of single nucleotide polymorphisms (SNPs), among other quantities. Copy number represents the number of copies of a given target present in a genome (e.g., humans have a diploid genome with a copy number of two for most autosomal genes). Copy number variation is a structural variation in the genome, such as deletions, duplications, translocations, and/or inversions, ~~that~~which may be a major source of heritable genetic variation, including susceptibility to disease (or disease itself) and responsiveness to disease treatment. [0020] The assays may be extended in various ways. In some embodiments, the assays may involve analysis of more than two targets in the same channel. For example, an assay for three targets ~~would~~may generate eight clusters or populations of data, separated by intensity. In the same or other embodiments, some targets may be analyzed in one channel (e.g., a FAM channel), and one or more other targets may be analyzed in one or more other channels (e.g., a VIC channel). Three targets, two in a first channel and one in a second channel, would again generate eight clusters or populations of data, but they would be separated in a two-dimensional intensity space and so in principle more easily resolvable.

[0021] Further aspects of the present disclosure are presented in the following sections: (I) system overview, and (II) examples.

I. ~~System Overview~~ SYSTEM OVERVIEW

[0022] This section provides an overview of exemplary methods and apparatus for performing digital assays, in accordance with aspects of the present disclosure.

~~Figure~~[0023] FIG. 1 shows a flowchart of an exemplary method 40 of performing a digital assay. The steps presented for method 40 may be performed in any suitable order and in any suitable combination. Furthermore, the steps may be combined with and/or modified by any other suitable steps, aspects, and/features of the present disclosure.

[0024] Sample Preparation.

~~Sample preparation.~~[0025] A sample may be prepared for the assay, indicated at 42. Preparation of the sample may include any suitable manipulation of the sample, such as collection, dilution, concentration, purification, lyophilization, freezing, extraction, combination with one or more assay reagents, performance of at least one preliminary reaction to prepare the sample for one or more reactions in the assay, or any combination thereof, among others. Preparation of the sample may include rendering the sample competent for subsequent performance of one or more reactions, such as one or more enzyme catalyzed reactions and/or binding reactions.

[0026] In some embodiments, preparation of the sample may include combining the sample with reagents for amplification and for reporting whether or not amplification occurred. Reagents for amplification may include any combination of primers for the targets, dNTPs and/or NTPs, at least one enzyme (e.g., a polymerase, a ligase, a reverse transcriptase, or a combination thereof, each of which may or may not be heat-stable), and/or the like. Accordingly, preparation of the sample may render the sample (or partitions thereof) capable of amplification of each of one or more targets, if present, in the sample (or a partition thereof). Reagents for reporting may include reporters for each target of interest. Accordingly, preparation of the sample for reporting may render the sample capable of reporting, or being analyzed for, whether or not amplification has occurred, on a target-by-target basis, and optionally the extent of any such amplification. The reporters each may be a labeled probe that includes a nucleic acid (e.g., an oligonucleotide) ~~and~~labeled with a luminophore, such as a fluorophore.

[0027] Sample Partitioning.

~~Sample partitioning.~~[0028] The sample may be separated into partitions, indicated at 44. Separation of the sample may involve distributing any suitable portion including up to all of the sample to the partitions. Each partition may be and/or include a fluid volume that is isolated from the fluid volumes of other partitions. The partitions may be isolated from one another by a fluid phase, such as a continuous phase of an emulsion, by a solid phase, such as at least one wall of a container, or a combination thereof, among others. In some embodiments, the partitions may be droplets disposed in a continuous phase, such that the droplets and the continuous phase collectively form an emulsion.

[0029] The partitions may be formed by any suitable procedure, in any suitable manner, and with any suitable properties. For example, the partitions may be

formed with a fluid dispenser, such as a pipette, with a droplet generator, by agitation of the sample (e.g., shaking, stirring, sonication, etc.), and/or the like. Accordingly, the partitions may be formed serially, in parallel, or in batch. The partitions may have any suitable volume or
volumes. The partitions may be of substantially uniform volume or may have different volumes. Exemplary partitions having substantially the same volume are monodisperse droplets. Exemplary volumes for the partitions include an average volume of less than about 100, 10 or 1 μL, less than about 100, 10, or 1 nL, or less than about 100, 10, or 1 pL, among others.

[0030] The partitions, when formed, may be competent for performance of one or more reactions in the partitions. Alternatively, one or more reagents may be added to the partitions after they are formed to render them competent for reaction. The reagents may be added by any suitable mechanism, such as a fluid dispenser, fusion of droplets, or the like.

[0031] Sample Reactions.

~~Sample reactions.~~[0032] One or more reactions may be performed in the partitions, indicated at 46. Each reaction performed may occur selectively (and/or substantially) in only a subset of the partitions, such as less than about one-half, one-fourth, or one-tenth of the partitions, among others. The reaction may involve a target, which may, for example, be a template and/or a reactant (e.g., a substrate), and/or a binding partner, in the reaction. The reaction may occur selectively (or selectively may not occur) in partitions containing at least one copy of the target.

[0033] The reaction may or may not be an enzyme-catalyzed reaction. In some examples, the reaction may be an amplification reaction, such as a polymerase chain reaction and/or ligase chain reaction. Accordingly, a plurality of amplification reactions for a plurality of targets may be performed simultaneously in the partitions.

[0034] Performing a reaction may include subjecting the partitions to one or more conditions that promote occurrence of the reaction. The conditions may include heating
the partitions and/or incubating the partitions at a temperature above room temperature, such as at a denaturation temperature, an annealing temperature, and/or an extension temperature. In some examples, the conditions may include thermally cycling the partitions to promote a polymerase chain reaction and/or ligase chain reaction.

[0035] Signal Creation.

~~Signal creation. Signals~~[0036] One or more signals may be created ~~for each of the partitions~~ that are representative of light detected from the partitions, indicated at 48. The ~~signals~~signal may represent ~~aspects~~an aspect of light, such as the intensity of the light, detected in the same optical channel ~~(i.e., in the same wavelength range (color regime) such as for FAM or VIC)~~ from reporters for two or more distinct targets. The signals optionally may include data collected in one or more different channels (~~i.~~e.g.,

in different wavelength ranges (color regimes) from reporters for the same and/or different targets). The light detected from each reporter may be light emitted from a ~~fluorophore~~luminophore. The light detected in a given channel may be detected with the same sensor at the same time such that light detected from different reporters is summed or accumulated without attribution to a particular reporter. Thus, the signal may be a composite signal that represents two, three, four, or more reactions and thus two, three, four, or more targets of the reactions.

[0037] An optical channel represents a particular detection regime with which emitted light is generated and detected. The detection regime may be characterized by a wavelength or waveband (i.e., a wavelength regime) for detection of emitted light. If pulsed excitation light is used in the detection regime to induce light emission, the detection regime may be characterized by a wavelength or waveband for illumination with excitation light and/or a time interval during which light emission is detected with respect to each light pulse. Accordingly, optical channels that are different from each other may differ with respect to the wavelength/waveband of excitation light, with respect to the wavelength/waveband of emitted light that is detected, and/or with respect to the time interval during which emitted light is detected relative to each pulse of excitation light, among others.

[0038] The signal may be created based on detected light emitted from one or more reporters in the partitions. The one or more reporters may report whether at least one of two or more particular reactions represented by the signal has occurred in a partition and thus whether at least one copy of at least one of two or more particular targets corresponding to the two or more particular reactions is present in the partition. The strength of the signal corresponding to the reporters may be analyzed to determine whether or not at least one of the particular reactions has occurred and at least one copy of one of the particular targets is present. The strength may vary among the partitions according to whether at least one of the particular reactions occurred or did not occur (e.g., above a threshold ~~extent~~and/or within a range) and at least one of the particular targets is present or absent in each partition.

[0039] Partitions may be analyzed and signals created at any suitable time(s). Exemplary times include at the end of an assay (endpoint assay), when reactions have run to completion and the data no longer are changing, or at some earlier time, as long as the data are sufficiently and reliably separated.

[0040] Number of Positives.

~~Number of positives.~~[0041] A number of partitions that are positive (or negative) for each target may be determined for the signal, indicated at 50. The signal detected from each partition, and the partition itself, may be classified as being positive or negative for each of the reactions/targets contributing to the signal. Classification may be based on the strength (and/or other suitable aspect) of the signal. If the signal/partition is classified as positive (+), for a given target, the reaction

corresponding to that target is deemed to have occurred and at least one copy of the target is deemed to be present in the partition. In contrast, if the signal/partition is classified as negative (-—), for a given target, the reaction corresponding to that target is deemed not to have occurred and no copy of the target is deemed to be present in the partition (i.e., the target is deemed to be absent from the partition). The data including all permutations of positives will generally fall into 2N2' populations or clusters, where N is the number of targets, assuming that each population is distinguishable. Exemplary results for one, two, and three target systems in which data are collected in a single channel are shown in the following tables:

	Target A	Intensityassemblies may be unconnected to the other assemblies, such that, for example, material/data is transferred manually. [0046] Apparatus 60 may operate as follows. Droplet generator 62 may form droplets disposed in a continuous phase. The droplets may be cycled thermally with thermocycler 64 to promote amplification of targets in the droplets. Signals may be detected from the droplets with detector 66. The signals may be processed by processor 68 to determine numbers of droplets and/or target levels, among others
Population 2	Target A Intensity	Highest
Population 2 Population 1	Highest Lowest	Lowest
	Target A Target B Intensity	

II. EXAMPLES

	Target A	Target B	Intensity
Population 4 Population 3 Population 2 Population 1	+	• Highest Intermediate • Intermediate +Lowest	Highest[0047] This section presents selected aspects and embodiments of the present disclosure related to methods of performing digital assays with multiplexed detection of two or more targets in the same channel.
Population 3	+	-	Intermediate
Population 2	-	+	Intermediate

Population 1	-	-	Lowest

	Target A	Target B	Target C	Intensity
Population 8	+	+	+	Highest
Population 7	+	+	-	Intermediate
Population 6	+	-	+	Intermediate
Population 5	-	+	+	Intermediate
Population 4	+	-	-	Intermediate
Population 3	-	+	-	Intermediate
Population 2	-	-	+	Intermediate
Population 1	-	-	-	Lowest

Target A Target B Target C Intensity
Population 8 • Highest
Population 7 Intermediate
Population 6 • Intermediate
Population 5 • Intermediate
Population 4 Intermediate
Population 3 Intermediate
Population 2 • Intermediate
Population 1 Lowest
[0042] Target Level Determination.
Concentration estimate.[0043] A concentrationlevel of each target may be estimateddetermined, indicated at 52. The concentrationlevel of each target may be estimateddetermined based on the respective numbers of partitions positive for the target alone and for the target in combination with any other target(s). The calculation may be based on each target having a Poisson distribution among the droplets. The concentrationslevel may be a concentration. The levels may, for example, be estimated bybased on finding solutions to a series of linear equations. The total number of partitions may be counted or, in some cases, estimated. The dropletpartition data further may be used (e.g., directly and/or as concentration data) to estimate copy number (ONCN) and copy number variation (CNV), using any suitable algorithms such as those described elsewhere in the present disclosure.
[0044] Further aspects of sample preparation, droplet generation, signal detection, counting droplets, and target level determination, among others, that may be suitable for the system of the present disclosure are identified above in the Cross-References, which are incorporated herein by reference.

Figure[0045] FIG. 2 shows an exemplary apparatus 60 for performing the digital assay of FigureFIG. 1. Apparatus 60 may include a partitioning assembly, such as a droplet

generator 62 ("DG"), a thermal incubation assembly, such as a ~~thermocycler~~thermocy-cler 64 ("TC"), a detection assembly (a detector) 66 ("DET"), and a data processing assembly (a processor) 68 ("PROC"), or any combination thereof, among others. The data processing assembly may be, or may be included in, a controller that communicates with and controls operation of any suitable combination of the assemblies. The arrows between the assemblies indicate movement or transfer of material, such as fluid (e.g., a continuous phase of an emulsion) and/or partitions (e.g., droplets) or signals/data, between the assemblies. Any suitable combination of the assemblies may be operatively connected to one another, and/or one or more of the ~~assemblies may be unconnected to the other assemblies, such that, for example, material/data is transferred manually.~~

~~Apparatus 60 may operate as follows. Droplet generator 62 may form droplets disposed in a continuous phase. The droplets may be cycled thermally with thermocycler 64 to promote amplification of targets in the droplets. Signals may be detected from the droplets with detector 66. The signals may be processed by processor 68 to estimate concentrations of the targets.~~

~~II. Examples~~

~~This section presents selected aspects and embodiments of the present disclosure related to methods of performing digital assays with multiplexed detection of~~

~~two or more targets in the same channel.~~

Example 1~~. Exemplary Assay Formats~~

~~The example describes exemplary formats and conditions for performing digital assays with multiplexed detection.~~

~~The assays presented herein may be performed using any suitable techniques. Exemplary techniques, including assay formats and conditions, are described without limitation in Appendix A and U.S. Patent Application Serial No. 12/976,827, filed December 22, 2010, among others. These techniques may be performed using any suitable apparatus, including apparatus described without limitation in U.S. Patent No. 7,041,481, issued May 9, 2006; and U.S. Patent Application Publication No. 2010/0173394 Al, published July 8, 2010. All of the patent documents cross-referenced in this section are hereby incorporated by reference in their entireties.~~

~~Example 2.~~ Digital PCR Assays with Multiplexed Detection in the Same Channel

[0048] This example describes an exemplary digital PCR assay with multiplexed detection of two targets, using two probes, analyzed in the same channel. Other assays may involve three or more targets and three or more probes, where at least two targets are analyzed in the same channel.

~~Figure~~[0049] FIG. 3 shows a pair of targets 80, 82 ("Target 1" and "Target 2") and corresponding probes 84, 86 ("Probe 1" and "Probe 2") that may be used to create a dedicated signal for each target in a digital PCR assay. Each probe may include an

oligonucleotide 88, 90, a fluorophore 92, 94, and a quencher 96. Each of the fluorophore and the quencher may (or may not) be conjugated to the oligonucleotide by a covalent bond. The probe also or alternatively may include a binding moiety (a minor groove binder) for the minor groove of a DNA duplex, which may be conjugated to the oligonucleotide and which may function to permit a shorter oligonucleotide to be used in the probe.

[0050] Each oligonucleotide may provide target specificity by hybridization predominantly or at least substantially exclusively to only one of the two targets. Hybridization of the oligonucleotide to its corresponding target is illustrated schematically at 98.

[0051] Fluorophores 92, 94, which may be the same or different, create detectable but distinguishable signals in the same channel, allowing multiplexing in that channel. The signals may be distinguishable because an aspect of the fluorescence is different for one fluorophore than for the other fluorophore(s). For example, the intensity associated with one fluorophore, following reaction, may be lower or higher than the intensity(ies) associated with the other fluorophore (s). In some embodiments, one probe may be labeled with a different number of fluorophores than the other probe, and/or the probes may be located in slightly different local environments, creating a different level of fluorescence for each probe following reaction. Alternatively, or in addition, both probes may be labeled with the same number of fluorophores (e.g., one fluorophore), but there may be more or less of one probe than the other in the sample, so that a greater or smaller signal is created when the reactions have occurred. In some cases, the fluorophores themselves might be different, with one more or less intrinsically fluorescent than the other (e.g., due to differences in extinction coefficient, quantum yield, etc.), so long as each fluorophore can be detected in the same
channel. Exemplary fluorophores that may be suitable include FAM, VIC, ROX, TAMRA, JOE, etc., among others. [0052] Quencher 96 is configured to quench the signal produced by fluorophore 92 or 94 in a proximity-dependent fashion. Accordingly, light detected from the fluorophore may increase when the associated oligonucleotide 88 or 90 binds to the amplified target, to increase the separation between the fluorophore and the quencher, or when the probe is cleaved and the fluorophore and quencher become uncoupled during target amplification, among others. The quencher may be the same or different for each type of ~~fluorophore~~fluo-rophore. Here, the assay is designed so that the presence of a target gene leads to an increase in corresponding intensity, because amplification reduces quenching. In other assays, the reverse could be true, such that the presence of a target caused a decrease in corresponding intensity (although it typically is easier to detect a signal against a dark background than the opposite). Moreover, some embodiments may be constructed without a quencher, so long as the fluorescence and so the signal changes upon amplification.

~~Figure~~[0053] FIG. 4 shows an exemplary graph 102 of data corresponding to an exemplary digital PCR assay for Target 1 and Target 2 performed in droplets. The graph plots a signal 104 that represents light detected from probes 84, 86 (and/or one or more modified (e.g., cleavage) products thereof) (see ~~Figure~~FIG. 3). The signal is created from light detected over time in a single channel from a fluid stream containing the droplets and flowing through an examination region of the channel. The signal may be analyzed to determine whether neither Target, Target 1 alone, Target 2 alone, or both Targets 1 and 2 are present in each droplet. In particular, the strength or intensity of the signal in a system with two targets may be divided or thresholded into four intervals corresponding to no Target (Interval 1), Target 1 alone (Interval 2), Target 2 alone (Interval 3), or both Targets 1 and 2 (Interval 4):

- [0054] Peaks 106 with maxima in Interval 1 correspond to droplets containing no Target (T1-/T2-). The measured signal corresponds to background (e.g., background fluorescence, scattering, etc.) and does not reflect the presence or amplification of either Target.
- [0055] Peaks 108 with maxima in Interval 2 correspond to droplets containing Target 1 but not containing Target 2 (T1+/T2-). The measured signal corresponds to signal from Target 1 plus background and reflects amplification of Target 1 implying the presence of Target 1.
- [0056] Peaks 110 with maxima in Interval 3 correspond to droplets containing Target 2 but not containing Target 1 (T1-/T2+). The measured signal corresponds to signal from Target 2 plus background and reflects amplification of Target 2 implying the presence of Target 2.
- [0057] Peaks 112 with maxima in Interval 4 correspond to droplets containing both Targets 1 and 2 (T1+/T2+). The measured signal corresponds to signal from both Targets 1 and 2 plus background and reflects amplification of Targets 1 and 2 implying the presence of Targets 1 and 2.

[0058] In the present example, each droplet, whether positive or negative for each target, produces an increase in signal strength above the baseline signal that forms an identifiable peak 106, 108, 110, 112. Accordingly, the signal may vary in strength with the presence or absence of a droplet and with the presence or absence of a corresponding target.

[0059] The assignment of a droplet to a particular outcome (i.e., to one of T1 -/T2-, T1 +/T2-, T1-/T2+, and T1 +/T2+) may be performed using any suitable algorithm. In the example above, peak heights (i.e., intensity values) associated with each outcome are sufficiently different that each can be unambiguously identified and assigned. Specifically, the peaks are assigned based on intervals delineated by values lying between (e.g., half way between) the peak heights for one outcome and the peak heights for adjacent outcomes. In other cases, the peak heights for each outcome may overlap at their extremes, so that thresholding may be neither simple nor linear. In

such cases, statistical methods such as expectation maximization algorithms may be used to estimate the number of droplets or peaks associated with each outcome and the associated concentrations.

Example ~~3.~~ 2

Digital PCR Assay to Assess Copy Number of C61

[0060] This example describes a first exemplary digital PCR assay, in which multiplexing in a single channel is used to assess copy number of the C61 gene; see ~~Figures~~FIGS. 5 and 6. Specifically, signals from two probes, one for the gene of interest, C61, and one for a reference gene, RPP30, are collected together, as a single signal, in a single channel and used to assess the number of copies of the gene of interest relative to the number of copies of the reference gene.

[0061] The principles described here may be used with any suitable gene(s). In this example, C61 is a gene of interest, for which information on copy number is sought, and RPP30 is a reference gene, which codes for ribonuclease P protein subunit p30, that is known to have two copies per genome.

[0062] The principles described here also may be extended to additional genes of interest, for example, two or three or more genes of interest, and may or may not involve reference genes such as RPP30. The number of copies may be determined absolutely, if the copy number of at least one of the genes (e.g., the reference gene) is known, or relatively, if the copy number of none of the genes is known.

~~Figure~~[0063] FIG. 5 is a graph showing data for the exemplary C61/RPP30 system. Specifically, ~~Figure~~FIG. 5 shows intensity in the FAM channel plotted as a function of intensity in the VIC channel for each droplet in a digital PCR assay. Visually, the data comprise four distinct populations, corresponding to four distinct ranges of FAM intensity (the intensities in the VIC channel are all low and overlapping). The assay is constructed so that amplification of C61 leads to a lower FAM intensity than amplification of RPP30 (although it would work as well if the reverse were true). The four populations may be summarized as follows:

- [0064] Population 1, with the lowest FAM intensity, corresponds to droplets that are negative for RPP30 and C61 (i.e., droplets that did not include either gene).
- [0065] Population 2, with the lower of two intermediate FAM intensities, corresponds to droplets that are positive for C61 and negative for RPP30 (i.e., droplets that included the C61 gene but did not include the RPP30 gene).
- [0066] Population 3, with the higher of two intermediate FAM intensities, corresponds to droplets that are positive to RPP30 and negative for C61 (i.e., droplets that included the RPP30 gene but did not include the C61 gene).
- [0067] Population 4, with the highest FAM intensity, corresponds to droplets that are positive for RPP30 and C61 (i.e., droplets that included both genes).

The number of droplets in each population may be counted using any suitable

mechanism(s), during or following data acquisition. Here, because the intensities are widely separated, the number may be counted by assigning suitable intensity ranges or intervals to each population, as in Example ~~2~~1, so that droplets falling within a selected intensity range are designated as falling within the population corresponding to that range. The results of such counting are summarized in the following table:

RPP30 C61	~~RPP30~~	~~C61~~	# Droplets
Population 4	~~+~~	~~+~~	1168
Population 3	~~+~~	~~-~~	2865
Population 2	~~-~~	~~+~~	2854
Population 1	~~-~~	~~-~~	6782

Here, + means that the assay is positive for the indicated gene (i.e., that the indicated gene is present), and — means that the assay is negative for the indicated gene (i.e., that the indicated gene was absent). There are 4033 droplets containing RPP30 (i.e., that are positive for RPP30, irrespective of whether they are positive or negative for C61), as determined by adding the number of droplets in Populations 3 and 4 (i.e., by adding 2865 and 1168, respectively). There are 4022 droplets containing C61 (i.e., that are positive for C61, irrespective of whether they are positive or negative for RPP30), as determined by adding the number of droplets in Populations 2 and 4 (i.e., by adding 2854 and 1168, respectively). Thus, the ratio of C61 to RPP30 is 4022/4033=0.997=1:1 within experimental error. Thus, because RPP30 is known to have two copies per genome, C61 must also have two copies (i.e., the copy number of C61 is two).

~~Figure~~[0068] FIG. 6 shows FAM intensities for the C61/RPP30 system of ~~Figure~~FIG. 5 for twelve different sets of ~~experimental conditions~~droplets exposed to twelve different annealing temperatures (denoted, from left to right, 55.0, 55.2, ..., 64.6, and 64.9)~~. These different conditions may correspond to anneal temperatures, in degrees Celsius, preceding the extend step(s) in a thermocycling assay~~ during thermal cycling to promote target amplification. The data show that there is sufficient resolution between the four populations to perform the assay in a single channel under a variety of experimental conditions. ~~This variety of conditions may correspond to relatively lower anneal temperatures (e.g., below about 60 degrees Celsius), in a thermocycling assay, sufficient to allow enough binding between template and primers for the thermocycling assay to run substantially toward or to completion.~~ The data shown in ~~Figure~~FIG. 5 correspond to one of the conditions in this plot~~. Less desirable conditions, such as relatively higher anneal temperatures (e.g., above about 60 degrees Celsius), may not allow sufficient resolution between populations, for example, because the signal from different partitions smears out as the extent of reaction varies from partition to partition.~~, namely, amplification with an annealing temperature of 59.1° C. (marked in FIG. 6 with an arrowhead).

[0069] The annealing temperature may be selected from among the various temperatures tested, based on comparison of collected intensity data. For example, the resolution or separation of each population of droplets from one another in the plot for the various annealing temperatures may be compared to permit selection of a suitable annealing temperature for further data collection and/or analysis. For example, here, the annealing temperature of 59.1° C. offers the best separation between each different population of droplets within the set. In particular, at this annealing temperature, data from double-positive droplets (RPP30+/C61+; the population of highest intensity) are well resolved from data for single-positive droplets (RPP30+ or C61+; the two populations of intermediate intensity), which in turn are well resolved from each other and from data for double-negative droplets (RPP30-/C61-; the population of lowest intensity).

[0070] The resolution provided by selection of an optimal annealing temperature may permit determination, for each target, a respective number of droplets that are positive for the target alone at the selected annealing temperature. Also, at least one number of droplets positive for more than one target may be determined. The total number of droplets positive for each target then may be determined based on the respective numbers and the at least one number.

[0071] In some cases, signal detection may be performed first on only a fraction of each set of droplets. Additional droplets from the particular set corresponding to the selected annealing temperature then may be run through the detector to provide additional data for analysis. In other cases, only a fraction of the data collected for each set of droplets may be plotted and/or compared, and then additional data collected for the particular set corresponding to the selected annealing temperature may be plotted and/or analyzed.

Example ~~4.~~ 3

Digital PCR Assay to Assess Copy Number of C63

[0072] This example describes a second exemplary digital PCR assay, in which multiplexing in a single channel is used to assess copy number of the C63 gene; see ~~Figures~~FIGS. 7 and 8. Specifically, signals from two probes, one for the gene of interest, C63, and one for a reference gene, again RPP30, are collected in a single channel and used to assess the number of copies of the gene of interest relative to the number of copies of the reference gene.

~~Figure~~[0073] FIG. 7 is a graph showing data for the exemplary C63/RPP30 system. Like ~~Figure~~FIG. 5, ~~Figure~~FIG. 7 shows intensity in the FAM channel plotted as a function of intensity in the VIC channel for each droplet in a digital PCR assay. However, unlike ~~Figure~~FIG. 5, this system includes probes for RPP30 and C63 instead of RPP30 and C61. The data again show four distinct populations, corresponding in order of decreasing intensity in the FAM channel to RPP30+/C63+, RPP30+/C63-, RPP30-/ C63+, and RPP30-/C63-.

[0074] The number of droplets counted in each of these bins corresponds to 3552 RPP30 positive droplets, 3441 C63 positive droplets, and 6224 negative droplets. Thus, the ratio of C63 to RPP30 is 3441/3552=0.969=1:1 within experimental error. Thus, like C61, C63 must have two copies (i.e., the copy number of C63 is two).
Figure 8[0075] FIG. 8A shows FAM intensities (top panel) and FIG. 8B shows VIC intensities (bottom panel) for the C63/RPP30 system of FigureFIG. 7 for twelve different sets of experimental conditions (i.e., different annealing temperatures for amplification). The data show that there is sufficient resolution between the four populations to perform the assay in a single channel under a variety of experimental conditions. The data shown in FigureFIG. 7 correspond to one of the conditions in these plots.

Example 5. Exemplary Assay Results4

Digital Assay with More Luminophores than Optical Channels

The[0076] This example describes an exemplary digital assay results, for a variety of targets, under a variety of conditions. The results are described without limitation in Appendix B. Some of the results correspond to and/or elaborate upon results presented in Examples 3 and 4.with multiplexed analysis of targets using more luminophores than the number of optical channels used for detecting signals from the luminophores; see FIGS. 9 and 10.

Example 6. Selected Embodiments

This example describes selected aspects and embodiments related to digital assays with multiplexed detection of two or more targets in the same channel, in accordance with aspects of the present disclosure, presented without limitation as a series of numbered paragraphs.

[0077] FIG. 9 shows an exemplary bulk phase mixture 140 being separated into droplets during performance of an exemplary digital assay with multiplexed detection of three or more distinct targets (e.g., identified here as T1, T2, and T3). Mixture 140 may include a forward primer 142 and a reverse primer 144 for each target. The mixture also may include a reporter for amplification of each target (such as a T1 probe, a T2 probe, and a T3 probe). Each reporter may be a probe including an oligonucleotide labeled with a different lumino-phore (namely, L 1 , L2, and L3, respectively). Accordingly, luminescence from the T1 -T3 probes, and more particularly, the Ll, L2, and L3 luminophores, may report whether or not target T1, T2, and T3, respectively, are present in each individual droplet.

[0078] FIG. 10 schematically represents an exemplary graph showing droplet (or peak) intensities for a T 1/T2/T3 droplet assay that may be performed with the droplets of FIG. 9. Data from the T1, T2, and T3 probes are collected in a first optical channel and a second optical channel and plotted as a function of intensity in the first channel (vertical axis) versus intensity in second channel (horizontal axis). Intensities

produced by exemplary populations or clusters of negative, Ti -positive, T2-positive, and T3-positive droplets are identified by filled ellipses, with the size of each ellipse corresponding to the number of droplets in each population. To simplify the presentation, double-positive populations (T1+/T2+, T1+/T3+, and T2+/T3+) and the triple-positive population (T1+/T2+/T3+) are not shown.

[0079] Each luminophore may be at least predominantly or substantially exclusively detectable in only one optical channel, or may be substantially detectable in two or more optical channels. For example, L1 of the T1 probe is substantially detectable in the first channel but not the second channel, L2 of the T2 probe is substantially detectable in the second channel but not the first channel, and L3 of the T3 probe is substantially detectable in both channels. More generally, L3 may generate a distinguishable intensity and/or a different ratio of signal intensities for amplification of the T3 target in each channel relative to the intensity and/or ratio produced by Ll for amplification of the T1 target and produced by L2 for amplification of the T2 target. As a result, the population of T3-only positives is resolved from the T1 -only and T2-only populations. Also, the populations containing more than one target may be resolved and distinguishable from one another and from the single-target populations and the negative population.

[0080] The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of features, functions, elements, and/or properties may be claimed in applications claiming priority from this or a related application. Such claims, whether directed to a different invention or to the same invention, and whether broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the inventions of the present disclosure. Further, ordinal indicators, such as first, second, or third, for identified elements are used to distinguish between the elements, and do not indicate a particular position or order of such elements, unless otherwise specifically stated.

We claim:

1. A method of performing a digital assay, comprising ~~(A) separating a sample containing at least two types of~~: amplifying two or more targets ~~into partitions~~in droplets containing a reporter for each ~~type of~~ target; ~~(B)~~ detecting ~~from each partition~~ a signal from the droplets representing combined emission of light from the reporters for ~~each type of target~~the targets, wherein each reporter provides a portion of the signal having an intensity that varies according to

whether or not the corresponding ~~type of~~ target is present in a ~~partition; (C) counting a number of the partitions that are positive or that are negative for each type of target based on the signal; and (D)~~ droplet, and wherein a plurality of the droplets contain more than one target and have a distinguishable intensity of the signal relative to droplets containing only one target;

determining for each target a number of droplets that are positive for only one of the targets;

determining at least one number of droplets that are positive for more than one target; and

determining a quantity representative of a level of at least one ~~type of~~ target ~~in the sample~~.

2. The method of ~~paragraph~~claim 1, wherein the quantity representative of a level is a concentration of the at least one target ~~in the sample~~.

3. The method of ~~paragraph 1~~claim 2, wherein the step of determining a quantity representative of a level further comprises ~~(D1)~~a step of ascertaining a total number of ~~partitions~~ droplets based on the signal~~;~~, and ~~(D2)~~ obtaining a concentration of the at least one target based on the ~~counted number of partitions and the~~ total number of ~~partitions~~droplets.

4. The method of ~~paragraph~~claim 1, wherein the quantity representative of a level is a copy number of the at least one target in ~~the~~a sample disposed in the droplets.

5. The method of ~~paragraph~~claim 1, wherein the step of determining a quantity representative of a level further comprises a step of obtaining a copy number of a first target by comparing the number of ~~partitions~~droplets positive for the first target with the number of ~~partitions~~droplets positive for a second target, the first and second targets being among the ~~at least~~ two ~~types of~~or more targets ~~contained in the sample~~.

6. The method of ~~paragraph~~claim 5, wherein the copy number of the second target is known, and wherein the copy number of the first target is determined ~~as an absolute number (e.g., one, two, three, etc.)~~ by calculating the ratio of the number of ~~partitions~~droplets positive for the first target to the number of ~~partitions~~droplets positive for the second target and multiplying the ratio by the copy number of the second target.

7. The method of ~~paragraph~~claim 5, wherein the copy number of the second target is unknown, and wherein the copy number of the first target is obtained relative to the copy number of the second target as the ratio of the number of ~~partitions~~droplets positive for the first target to the number of ~~partitions~~droplets positive for the second target.

8. The method of ~~paragraph~~claim 1, wherein the ~~step of counting a number of the partitions that are positive or that are negative for each type of target includes treating a partition as positive if the partition produces a signal that is above a preselected~~

~~(e.g., threshold) level and treating a partition as negative if the partition produces a signal that is below the preselected level.~~reporters each are labeled with the same luminophore.

9. The method of ~~paragraph~~claim 8, wherein the ~~signal is a fluorescence signal and the preselected level is a preselected fluorescence intensity~~luminophore is selected from the group consisting of FAM, VIC, ROX, TAMRA, and JOE.

10. The method of ~~paragraph~~claim 1, wherein the two or more targets include a first target and a second target, wherein the step of determining for each target includes a step of counting a number of ~~the partitions that are positive or that are negative for each type of target includes using an expectation maximization algorithm to estimate the numbers of partitions that are positive and/or negative for at least one of the targets.~~droplets positive for the first target and not the second target and a number of the droplets positive for the second target and not the first target, and wherein the step of determining at least one number of droplets includes a step of counting a number of droplets positive for both the first target and the second target.

~~11. The method of paragraph 1, wherein the reporters each are labeled with the same fluorophore.~~

~~12. The method of paragraph 11, wherein the fluorophore is selected from the group consisting of FAM, VIC, ROX, TAMRA, and JOE.~~

~~13~~11. The method of ~~paragraph~~claim 1, the signal being a first signal, further comprising ~~(E)~~ :

detecting from ~~each partition~~the droplets a second signal representing emission of light from an additional reporter for an ~~additional type of~~ additional target, the additional reporter providing at least a portion of a second signal having an intensity that varies according to whether or not the corresponding additional ~~type of~~ target is present in a ~~partition~~droplet, wherein the first signal and second signal correspond to emission of light in different wavelength regimes; ~~(F) counting a number of the partitions that are positive or that are negative for the~~ counting a number of the droplets that are positive for the additional ~~type of~~ target based on the second signal; and ~~(G)~~ determining a quantity representative of a level of the ~~additional type of~~ additional target in the ~~sample~~droplets.

~~14~~12. The method of ~~paragraph 13~~claim 11, wherein the first signal is collected from a first ~~type of fluorophore~~luminophore and the second signal is collected from a second ~~type of fluorophore~~luminophore.

~~15.~~13. The method of ~~paragraph 14~~claim 12, wherein the first ~~type of fluorophore~~luminophore is one of FAM, VIC, ROX, TAMRA, and JOE, and wherein the second ~~type of fluorophore~~luminophore is another of FAM, VIC, ROX, TAMRA, and JOE.

~~16.~~14. The method of ~~paragraph~~claim 1, wherein the ~~partitions~~droplets include a first probe

reporting a presence or absence of a first target in individual ~~partitions~~droplets, a second probe reporting a presence or absence of a second target in individual ~~partitions~~droplets, and a third probe reporting a presence or absence of a third target in individual ~~partitions~~droplets, and wherein a signal from the first and second probes represents light emitted in a first wavelength regime and a signal from the third probe represents light emitted in a second wavelength regime distinct from ~~and nonoverlapping with~~ the first wavelength regime.

~~17.~~15. The method of ~~paragraph~~claim 1, wherein ~~the step of separating a sample forms the partitions with an average concentration per partition of less than about one copy of each of the types of targets.~~each reporter is a probe including an oligonucleotide, and wherein each target is a nucleic acid.

~~18. The method of paragraph 1, wherein the step of separating a sample forms one or more partitions containing no copies of a target for each of the types of targets.~~

~~19. The method of paragraph 1, wherein the partitions are droplets.~~

~~20. The method of paragraph 1, wherein the partitions have a substantially~~

16. A method of performing a digital assay, comprising:

amplifying two or more targets in droplets containing a reporter for each target, wherein amplifying is performed with a plurality of different annealing temperatures on respective sets of the droplets;

detecting a signal from each set of droplets representing combined emission of light from the reporters for the targets, wherein each reporter provides a portion of the signal having an intensity that varies according to whether or not the corresponding target is present in a droplet;

comparing the signals from the sets of droplets;

selecting an annealing temperature for which amplification of each target is distinguishable based on the signal detected from the corresponding set of droplets;

determining for each target a number of droplets that are positive for amplification at the selected annealing temperature; and

~~same volume relative to~~determining a quantity representative of a level of at least one ~~another~~target.

~~21.~~17. The method of ~~paragraph 1~~claim 16, wherein the step of detecting is performed on a fraction of each set of droplets, further comprising a step of ~~performing an amplification reaction in one or more of the partitions before~~selectively repeating the step of detecting a signal for the set of droplets corresponding to the selected annealing temperature after the step of selecting an annealing temperature.

~~22. The method of paragraph 21, wherein the step of performing an~~

~~amplification reaction includes a step of thermally cycling partitions to perform a polymerase chain reaction.~~

~~23. The method of paragraph 22, wherein the amplification reaction includes sequential melt, anneal, and extend steps, and wherein the anneal steps are performed at a sufficiently low temperature to allow sufficient annealing to occur so that the reaction can run to completion during subsequent extend step(s).~~

~~24. The method of paragraph 1, wherein each target is a nucleic acid.~~

~~25. The method of paragraph 24, further comprising a step of cleaving the targets into fragments prior to, or concomitant with, the step of separating a sample into partitions, so that the partitions are enriched in fragments of the targets rather than uncleaved (full-size) targets.~~

~~26. The method of paragraph 25, wherein the step of cleaving the targets includes a step of exposing the targets to one or more restriction enzymes.~~

~~27.~~18. The method of ~~paragraph 26, wherein the assay is for one or more genes of interest, and wherein the restriction enzymes are selected so as not to cleave the targets within the genes of interest.~~claim 16, wherein a plurality of the droplets contain more than one target and have a distinguishable intensity of the signal relative to droplets containing each target alone after amplification at the selected annealing temperature.

19. A method of performing a digital assay, comprising: amplifying at least three targets in droplets containing a respective reporter for each target, wherein each reporter includes a different luminophore;

detecting at least two signals from the droplets, each signal representing combined emission of light from at least two of the reporters detected in a different optical channel;

determining for each target a number of droplets that are positive based on the at least two signals; and determining a quantity representative of a level of at least one target.

~~28.~~20. The method of ~~paragraph 1, wherein the signal is representative of light detected from fluid carrying droplets through an examination region.~~claim 19, further comprising a step of determining at least one number of droplets that are positive for more than one of the targets.

* * *

~~29. An apparatus for performing the method of any of paragraphs 1-28 on a sample containing at least two targets, comprising (A) a partitioning assembly; (B) a thermal incubation assembly; (C) a detection assembly; and (D) a data processing assembly configured (i) to communicate with and control operation of at least one of the partitioning assembly, the thermal incubation assembly, and the detection assembly, and (ii) to determine a quantity representative of as level of at least one type of target in the sample.~~

~~The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein.~~