Docket No.: H0498.70509US05
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Xiaowei Zhuang
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For:	SYSTEMS AND METHODS FOR DETERMINING NUCLEIC ACIDS
Examiner:	J. T. Woitach
Art Unit:	1672

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Dated: November 22, 2023 Electronic Signature for Eileen M. MacKenzie: / Eileen M. MacKenzie /

RESPONSE

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

In response to the Office Action dated August 22, 2023, reconsideration is respectfully requested in view of the following:

The Claims are reflected in the listing of claims which begins on page 2 of this response.

Remarks begin on page 6 of this response.

10x Genomics EX2055
NanoString v. Harvard
IPR2024-00526

IN THE CLAIMS

Submitted below is a complete listing of the current claims, including marked-up claim(s) with insertions indicated by underlining and deletions indicated by strikeouts and/or double bracketing. This listing of claims replaces all prior versions, and listings, of claims in the application:

1-229. (Canceled)

230. (Currently amended) A method for imaging RNA spatial organization in a sample comprising:

a. contacting the sample comprising a plurality of distinct RNA species *in situ* with a plurality of primary nucleic acid probe pools each of the nucleic acid probes comprising a target sequence and one or more read sequences, wherein each pool of nucleic acid probes hybridize to a distinct RNA species and each pool of probes encode a N-bit ~~binary~~ codeword with a Hamming weight of at least 2 that was assigned to each distinct RNA species, wherein each assigned N-bit codeword is a valid codeword with a Hamming distance equal to or greater than 2 between valid codewords and wherein each of the read sequences correspond to a bit value of 1 [[of]] for the ~~binary~~ codewords assigned to the distinct RNA species;

b. contacting the bound primary nucleic acid probes with a plurality of readout probes comprising a fluorescent label, wherein the readout probes hybridize to the read sequences of the primary nucleic probes;

c. imaging the readout probes bound to the primary nucleic acid probes; and,

d. repeating steps b) and c) in one or more sequential hybridization and imaging rounds until all N positions in the ~~binary~~ N-bit codeword have been imaged providing an imaged ~~binary~~ codeword corresponding to each distinct RNA species in a spatial organization.

231. (Cancelled)

232. (Currently amended) The method of claim 230, wherein the assigned ~~binary codes~~ N-bit codewords have a Hamming distance equal to or greater than 4 between each of the ~~assigned binary codes~~ valid codewords.

233. (Currently amended) The method of claim 230, wherein the ~~binary~~ N-bit codeword has a Hamming weight of 4.

234. (Currently amended) The method of claim ~~231~~ 230, wherein the imaged ~~binary~~ codeword is matched to ~~the assigned binary code~~ a valid codeword assigned to a distinct RNA species ~~and hybridization errors, if present, are identified~~.

235. (Currently amended) The method of claim ~~234~~ 230 wherein the Hamming distance is 4 or greater ~~and the hybridization errors are identified and corrected~~ and the imaged codewords are matched to valid codewords or discarded.

236. (Currently amended) The method of claim ~~234~~ 230, wherein the ~~binary~~ N-bit codeword has a Hamming weight of 4 and the Hamming distance is 4 or greater and ~~hybridization errors, if present, are identified and the imaged binary code correctly matched to the assigned binary code, or discarded~~ the imaged codewords are matched to valid codewords or discarded.

237. (Previously Presented) The method of claim 230, wherein the RNA species is a RNA transcript.

238. (Previously Presented) The method of claim 230, comprising determining the spatial organization of the transcriptome from a single cell.

239. (Previously Presented) The method of claim 230, wherein each primary nucleic acid probe pool comprises at least 10 different primary nucleic acid probes.

240. (Previously Presented) The method of claim 230, wherein each primary nucleic acid probe pool comprises at least four distinct read sequences.

241. (Previously Presented) The method of claim 230, wherein each primary nucleic acid probe pool comprises four distinct read sequences.

242. (Previously Presented) The method of claim 230, wherein each primary nucleic acid probe pool comprises at least eight distinct read sequences.

243. (Previously Presented) The method of claim 230, wherein the primary nucleic acid probes comprise a target sequence, with an average length of between 10 and 200 nucleotides, that hybridize the distinct RNA species.

244. (Previously Presented) The method of claim 230, wherein each primary nucleic acid probe pool comprises at least 10 different target sequences.

245. (Previously Presented) The method of claim 230, wherein after each hybridization and imaging round the fluorescent read out probe is quenched to inactivate.

246. (Previously Presented) The method of claim 230, wherein after each hybridization and imaging round the fluorescent read out probe is inactivated by chemically or enzymatically cleaving the fluorescent label from the read out probe.

247. (Previously Presented) The method of claim 230, wherein the N-bit binary code comprises at least a 16-bit code.

248. (Previously Presented) The method of claim 230, wherein the plurality of fluorescent readout probes comprise at least two distinct fluorescent labels.

249. (Previously Presented) The method of claim 230, wherein the plurality of fluorescent readout probes comprise at least three distinct fluorescent labels.

250. (Previously Presented) The method of claim 230, wherein the spatial organization of the distinct RNA species is imaged in 2 dimensions.

251. (Previously Presented) The method of claim 230, wherein the spatial organization of the distinct RNA species is imaged in 3 dimensions.

252. (Previously Presented) The method of claim 230, further comprising determining abundance for the distinct RNA species.

REMARKS

I. Status of the Claims

Prior to entry of the attached, amended claims 230-252 are pending in the present application. Claims 1-229 were previously cancelled.

II. The Amendments and Support

Claims 231 has been herein cancelled and the subject matter incorporated into claim 230. Claims 232-236 have been herein amended to clarify certain embodiments. Support for those amendments can be found in the claims as filed and throughout the specification. In addition, the specification has been amended to correct the spelling of "SECDED" on page 26. It is believed that no new matter has been added by any of these amendments and the Examiner is respectfully requested to enter them.

The amendments to the claims are made solely to obtain expeditious allowance of the instant application and not for reasons related to patentability. Amendment of the claims is made without prejudice, without intent to abandon any originally claimed subject matter, and without intent to acquiesce in any rejection of record. The right to file one or more continuing applications hereof containing the canceled or unamended claims is expressly reserved. The amendments to the claims are fully supported by the applications as filed.

III. Double Patenting Rejections

Claims 230-252 have been rejected under the judicially-created doctrine of obviousness-type double patenting as being unpatentable over the claims of U.S. Pat. No. 11,098,303.

While not acquiescing to the reasoning offered in the Office Action, but to expedite prosecution to allowance, submitted herewith is a Terminal Disclaimer in compliance with 37 C.F.R. § 1.321(c). In view of this Terminal Disclaimer, withdrawal of the rejection of these claims is therefore respectfully requested.

However, the filing of a terminal disclaimer is not an admission that the double patenting rejection was proper. Rather, "[i]n legal principle, the filing of a terminal disclaimer simply serves

the statutory function of removing the rejection of double patenting, and raises neither presumption nor estoppel on the merits of the rejection." *Quad Environ. Tech. Corp. v. Union Sanitary Dist.*, 946 F.2d 870 (Fed. Cir. 1991); see also M.P.E.P. § 804.02.

IV. Provisional Double Patenting Rejections

Claims 230-252 have been provisionally rejected under the judicially-created doctrine of obviousness-type double-patenting as being unpatentable over claims the claims of co-pending U.S. Pat. Apl. Ser. Nos. 18/046,409, 17,374,000, 18/185,048, and 18/297,378.

These rejections are asserted. It is believed that the present claims are patentably distinct and non-obvious over pending claims of U.S. Ser. No. 18/046,409; 17/374,000; 18/185,048 and 18/297,378.

However, should the Patent Office maintain the rejection, it is respectfully requested that the rejection be further held in abeyance until notification of allowable subject matter. The Applicant will then file a terminal disclaimer that complies with 37 C.F.R. §1.321(c) or 1.321(d), explain why the present application is patentably distinct from the asserted claims of the co-pending Applications, or request issuance of the claims without the need of a terminal disclaimer pursuant to § 804 of the M.P.E.P.

V. Rejections under 35 U.S.C. § 101

Claims 230-252 were rejected under 35 U.S.C. 101 as allegedly being directed to non-statutory subject matter (an abstract idea) without significantly more.

According to the Patent Office, claim 230 is directed to "spatially imaging RNA in a sample" wherein "the judicial exception of the claims are the steps of assessing sequence data based on target probe hybridization and imaging the target probe with a second probe specific for the tag read sequence associated with the target portions" and "a set of instructions for analysis of sequence image data derived from probing RNA in a sample, and appears to fall into the category of a **Mental Process**, that is concepts performed in the human mind."

The rejection is traversed for at least the following reasons.

It is noted that the judicial exceptions have been categorized as "laws of nature," "natural phenomena," and "abstract ideas" and include further groupings such as "mental processes." *See* M.P.E.P. § 2106.04(I). Abstract ideas are enumerated in the following groupings as "mathematical concepts," "certain methods of organizing human activity," and "mental processes." *See* M.P.E.P. § 2106.04(a). Courts have acknowledged that while "all inventions at some level embody, use, reflect, rest upon, or apply laws of nature, natural phenomenon or abstract idea, not all claims recite an abstract idea" (*Alice Corp. Pty. Ltd. v. CLS Bank, Int'l.*, 573 U.S. 208 (2014)), but that to be accurately determined subject matter ineligible a claim must be "directed to" a judicial exception. Accordingly, USPTO guidance requires a claim to ***recite*** (i.e., set forth or describe) an abstract idea to be deemed ineligible in Step 2A Prong One of the subject matter eligibility analyses, thereby distinguishing claims reciting abstract ideas from those that are merely based on or involve an abstract idea. *See* M.P.E.P. § 2016.04(a)(1). The grouping of "metal processes" is defined ***as concepts performed in the human mind***, and examples of mental processes include observations, evaluations, judgements, and opinions. *See* MPEP § 2106.04(a)(2)(III). Moreover "a claim with limitations that cannot practically be performed in the human mind does not recite a mental process."

It is submitted the claimed subject matter: a) does not "recite," "set forth," or "describe" an abstract idea; and, b) is directed to a *process* reciting active steps using tangible products that *cannot practically be performed in the human mind.* It is believed that the claimed subject matter and remarks provided below, when viewed as a whole, illustrate the **Eligibility** of the claims as pending. Accordingly, it is submitted that the pending claims are subject matter **Eligible** under 35 U.S.C. § 101.

Claim 1 recites the following steps:

> a. contacting the sample comprising a plurality of distinct RNA species *in situ* with a plurality of primary nucleic acid probe pools each of the nucleic acid probes comprising a target sequence and one or more read sequences, wherein each pool of nucleic acid probes hybridize to a distinct RNA species and each pool of probes encode a N-bit codeword with a Hamming weight of at least 2 that was assigned to each distinct RNA species, wherein each assigned N-bit codeword is a valid codeword with a Hamming distance equal to or greater than 2 between valid codewords and wherein each of the read sequences correspond to a bit value of 1 for the codewords assigned to the distinct RNA species….

In this step, the sample is ***contacted*** with "a plurality of primary nucleic acid pools" wherein those probes are further defined in the recited step. This step cannot practically be performed in the human mind, as it is a physically active step performed with tangible reagents (e.g., primary nucleic acid probes and a biological sample). Accordingly, this step does not "recite," "set forth," or "describe" a mental process.

b. contacting the bound primary nucleic acid probes with a plurality of readout probes comprising a fluorescent label, wherein the readout probes hybridize to the read sequences of the primary nucleic probes;

In this step, the primary nucleic acid probes, hybridized to the RNA species of the sample from step a., are ***contacted*** with fluorescent labeled "readout probes" which hybridize to the readout sequences of the primary nucleic acid probes. This step cannot practically be performed in the human mind, as it is a physically active step performed with tangible reagents (e.g., readout nucleic acid probes). Accordingly, this step does not "recite," "set forth," or "describe" a mental process.

c. imaging the readout probes bound to the primary nucleic acid probes; and,

In this step, as is well-understood in the art, ***imaging*** the readout probes involves the use of a fluorescent instrument with appropriate excitation and emission filters for a given fluorescent label providing a fluorescent signal and image. Fluorescent wavelengths are outside the visible range for humans, and it is impossible to perform this step without an appropriate instrument, which is a physically active step involving a tangible instrument. Accordingly, this step does not "recite," "set forth," or "describe" a mental process.

d. repeating steps b) and c) in one or more sequential hybridization and imaging rounds until all N positions in the N-bit codeword have been imaged providing an imaged codeword corresponding to each distinct RNA species in a spatial organization.

In this step, steps b and c are repeated (which have been discussed above, and are not mental processes) "until all N positions in the N-bit codeword have been imaged providing an imaged codeword corresponding to each distinct RNA species in a spatial organization." Thus, steps b and

c are simply repeated until all N positions (e.g., 16 positions in a 16-bit code) have been imaged, thereby providing an image of RNA species in a spatial organization.

While based on use of an algorithm or a "set of instructions" to match an imaged codeword with a valid codeword, not only can that not be performed in a human mind, but the unrecited underlying process does not meet the threshold for "reciting," "setting forth," or "describing" a mental process. To this point, further instructive are the Examples provided by the USPTO, e.g., Example 37, 38, 39 and 46 (claim 4). Each of these examples is directed to a process and some of which recite a judicial exception (e.g., mental process) but all of which were deemed to be Subject Matter **Eligible**. For example, claim 1 of Example 38 ("Stimulating an Analog Audio Mixer" was deemed to **Eligible** because "the claim does not recite a mental process," which is similar to the present claims that recite active steps even when some of the limitations may be based on an abstract idea. The same analysis applies to Example 39, "Method of Training a Neural Network for Facial Detection," wherein claim 1 was deemed Eligible because "the claim does not recite a mental process because the steps are not practically performed the human mind." *See* also MPEP § 2106.04(a)(1), "Examples of Claims that Do Not Recite Abstract Ideas."

Based on the analysis provided above, it is believed that claim 230, as well as any claims dependent thereupon, are **Eligible** because the claims do not recite a judicial exception:

Step	Analysis of claim 230
1: Statutory Category?	**Yes.** The claim recites a series of steps and, therefore, is a process. "A method for imaging RNA spatial organization in a sample…."
2A: Prong 1: Judicial Exception Recited?	**No.** The claim does not recite any of the judicial exceptions enumerated in MPEP 2106.04(a)(2). For instance, the claim does not recite any mathematical relationships, formulas, or calculations. While some of the limitations may be based on mathematical concepts, the mathematical concepts are not recited in the claims. ***Further, the claim does not recite a mental process because the steps are not practically performed in the human mind.*** Finally, the claim does not recite any method of organizing human activity such as a fundamental

	economic concept or managing interactions between people. Thus, the claim is **Eligible** because it does not recite a judicial exception.
2A – Prong 2: Integrated into a Practical Application	**N/A**
2B – Claim provides an Inventive Concept?	**N/A**

Based on the above analysis, and guidance provided by M.P.E.P. § 2106.04(a), it is believed that claims 230-252 qualify as **Eligible** subject matter under 35 U.S.C. § 101. Withdrawal of the rejection is accordingly respectfully requested.

VI. Rejections Under 35 U.S.C. § 103

Claims 122-124, 126-132, and 135 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over Femino, *et al.*, "Visualization of Single RNA Transcripts in Situ," *Science*, 280(5363):585-590, 1998 ("Femino et al."), Lubeck, *et al.*, "Single cell in situ RNA profiling by sequential hybridization," *Nat. Methods*, 11(4):360-361, 2014 ("Lubeck 2014"), Lubeck and Cai "Single cell systems biology by super-resolution imaging and combinatorial labelling," *Nat. Methods*, 9(7):743-748, 2012 ("Lubeck and Cai"), and Fortina, *et al.*, "Digital mRNA profiling," *Nat. Methods*, 26(3):293-294 ("Fortina").

Briefly, the Patent Office has asserted that each of Femino et al., Lubeck 2014, Lubeck and Cai, and Fortina teach all the elements of the claims other than "the design of the probe consider a Hamming distance." According to the Patent Office , "providing sequential hybridization for specific but different barcode sequences appears to provide for Hamming distances greater than 2 or more." Thus, the Examiner asserted it would have been obvious to one of ordinary skill in the art to "perform in situ hybridization with a panel of target specific probes containing barcode tags capable of distinguishing between the specific targets for each of the probes."

This rejection is respectfully traversed.

When determining whether a claim is obvious, an examiner must make "a searching comparison of the claimed invention—*including all its limitations*—with the teaching of the prior art." *In re Ochiai*, 71 F.3d 1565, 1572 (Fed. Cir. 1995) (emphasis added). Thus, "obviousness

requires a suggestion of all limitations in a claim." *CFMT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2003) (*citing In re Royka*, 490 F.2d 981, 985 (CCPA 1974)). Moreover, as the Supreme Court stated, "*there must be some articulated reasoning* with some rational underpinning to support the legal conclusion of obviousness." *KSR Int'l v. Teleflex Inc.*, 127 S. Ct. 1727, 1741 (2007) (quoting *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006) (emphasis added)).

As described in more detail below, it is submitted that the pending claims are non-obvious and patentably distinct from the cited art because those references fail to disclose, teach or reasonably suggest all of the claim elements. In particular, the claimed subject matter is a patentably distinct advancement over the methods taught in the cited art for multiplexed *in situ* RNA spatial imaging, as described more fully below. Moreover, it is submitted that the Patent Office has provided no articulated reasoning as to how the teaching of the cited art renders the claims obvious.

Claim 230 is directed to a method for imaging RNA spatial organization in a sample, wherein the claims recite, in part, "… a. contacting the sample comprising a plurality of distinct RNA species *in situ* with a plurality of primary nucleic acid probe pools each of the nucleic acid probes comprising a target sequence and one or more read sequences, wherein each pool of nucleic acid probes hybridize to a distinct RNA species and each pool of probes encode a N-bit codeword with a Hamming weight of at least 2 that was assigned to each distinct RNA species, wherein each assigned N-bit codeword is a valid codeword with a Hamming distance equal to or greater than 2 between valid codewords and wherein each of the read sequences correspond to a bit value of 1 for the codewords assigned to the distinct RNA species; b. contacting the bound primary nucleic acid probes with a plurality of readout probes comprising a fluorescent label, wherein the readout probes hybridize to the read sequences of the primary nucleic probes; c. imaging the readout probes bound to the primary nucleic acid probes; and, d. repeating steps b) and c) in one or more sequential hybridization and imaging rounds until all N positions in the N-bit codeword have been imaged providing an imaged codeword corresponding to each distinct RNA species in a spatial organization." Rejected claims 232-252 depend from and incorporate the patentable subject matter of Independent claim 230.

It is believed that at least the underlined claim recitations above were not taught or suggested by Femino et al., Lubeck 2014, Lubeck and Cai, or Fortina, either alone, or in combination.

The present specification discloses and teaches the use of error-robust *in situ* hybridization nucleic acid probes ("encoding probes") that reduce misidentification of nucleic acid targets in a sample wherein probe pools for distinct targets encode a N-bit codeword. Those nucleic acid probes comprise a target sequence and one or more read sequences wherein the read sequence corresponds to a position within the N-bit codeword, which is imaged in sequential hybridization rounds using fluorescent readout probes that bind to the read sequences of the nucleic acid probes. *See* Example 1; Figure 5E, 11 and 12A-12C. In other words, the claimed subject matter utilizes two sets of probes which provide for the sequential imaging and generation of a codeword using a combinatorial strategy with readout probes binding to read sequences of the primary nucleic acid probes. For example, a 16-bit code (e.g., claim 247) with a Hamming weight of 2 or more (e.g., claim 230) would contain at least two "1" and the remaining N-bit positions would be "0" wherein the number of "1" positions corresponds to the number of unique read sequences in pool of primary nucleic acid probes. *See* Fig 6D.

Those figures (Figure 5E, 6D, 11 and 12A-12C) illustrate the "plurality of primary nucleic acid probe pools" wherein "each of the nucleic acid probes comprising a target sequence and one or more read sequences" that "hybridize to a distinct RNA species and each pool of probes encode a N-bit codeword with a Hamming weight of at least 2 that was assigned to each distinct RNA species." Moreover, "each assigned N-bit code is a valid codeword with a Hamming distance of 2 or more between valid codewords and wherein each of the read sequences correspond to a bit value of 1 for the codewords assigned to the distinct RNA species." A Hamming weight identifies the number of positive hybridization signals (e.g., "1") in each codeword wherein "each codeword may be unique and separated from the other codewords by a Hamming distance, h, which measures the number of letters or positions that must be incorrectly read for one codeword to be misinterpreted as a different one." *See* the present application as published at [0152]. Moreover, so that each distinct RNA species is imaged in a spatial organization *in situ*, the distinct RNA species are each assigned a valid codeword. None of those elements are disclosed, taught or reasonably suggested by any of the cited art.

Femino et al. teaches the use of FISH probes (e.g., primary nucleic acid probes) that hybridize directly to a ***single RNA species*** and which contain "five fluorochromes per molecule." *See* Abstract. The use of multiple primary probes with multiple fluorochromes that hybridize the same RNA transcript provides the amplified signal needed to visualize single RNA transcripts *in situ*. This method is known in the art as single molecule FISH ("smFISH") and correspondingly the probes as "smFISH probes," wherein the general strategy utilizes multiple FISH probes (e.g., primary nucleic acid probes) to the same transcript with the same fluorophore (one or more per probe) to visualize individual RNA transcripts. (*See*, for example, Raj, *et al.*, "Imaging individual mRNA molecules using multiple singly labeled probes," *Nat. Methods*, 5:877-879, 2008.)

Accordingly, while Femino et al. teach simultaneous imaging of β-actin gene and β-actin nascent transcripts (each with multiple probes to the gene or transcript and each with multiple fluorophores) do not teach any of the presently claimed steps (e.g., contacting a sample with a pool of primary nucleic acid probes comprising a target sequence and one or more read out sequence; contacting those hybridized primary nucleic acid probes with read out probes comprising a fluorescent label, wherein the readout probes hybridize to the read sequences of the primary nucleic probes; imaging and repeating until all positions in the N-bit codeword have been imaged). Femino et al. teach visualizing single RNA transcripts *in situ* but provide absolutely no teaching or motivation for how to multiplex imaging of a plurality of RNA transcripts *in situ*.

Lubeck and Cai also teach a method for visualizing ***single RNA transcripts***, with limited multiplexing, using optical super-resolution microscopy (SRM) and combinatorial labeling of primary nucleic acid probes. This multiplexed method using smFISH probes is known in the art as "seqFISH." The presently claimed subject matter is believed to be patentably distinct from the disclosure of Lubeck and Cai, as described below.

Lubeck and Cai utilize smFISH probes, wherein each primary probe is labeled with a fluorophore to generate color barcodes to identify up to 32 RNA transcripts in *S. cerevisiae* cells. *See* Figure 1. In particular, the labeled probes are tiled along the mRNA in a 5' to 3' spatial ordered fashion or the identify of mRNA is coded by the combination of fluorophores, ignoring spatial order. *See* "Spatial coding of single mRNAs" p. 2 and "Spectral Coding" p. 4. Both of those strategies are unrelated to the claimed methods using the present primary nucleic acid probes

"comprising a target sequence and one or more read sequences, wherein each pool of nucleic acid probes hybridize to a distinct RNA species and each pool of probes encode a N-bit codeword with a Hamming weight of at least 2 that was assigned to each distinct RNA species, wherein each assigned N-bit codeword is a valid codeword with a Hamming distance equal to or greater than 2 between valid codewords and wherein each of the read sequences correspond to a bit value of 1 for the codewords assigned to the distinct RNA species" and "readout probes comprising a fluorescent label, wherein the readout probes hybridize to the read sequences of the primary nucleic probes."

Lubeck 2014 is a published letter to the editor of *Nature Methods*, further refining their smFISH and seqFISH methodology of Lubeck and Cai for single cell *in situ* RNA profiling by sequential hybridization. In this method the smFISH probes are individually labeled with a single fluorophore and each round of hybridization utilizes a pool of the smFISH probes with the same fluorophore providing a color barcode and amplification of signal ("increasing brightness of the FISH signal"). *See* Figure 1a. This methodology, however, is unrelated to the presently claimed methods which do not use smFISH probes. Hence, Lubeck 2014 fails to teach or reasonably suggest the present primary nucleic acid probes "comprising a target sequence and one or more read sequences, wherein each pool of nucleic acid probes hybridize to a distinct RNA species and each pool of probes encode a N-bit codeword with a Hamming weight of at least 2 that was assigned to each distinct RNA species, wherein each assigned N-bit codeword is a valid codeword with a Hamming distance equal to or greater than 2 between valid codewords and wherein each of the read sequences correspond to a bit value of 1 for the codewords assigned to the distinct RNA species" and "readout probes comprising a fluorescent label, wherein the readout probes hybridize to the read sequences of the primary nucleic probes."

Fortina teaches a solution-phase hybridization scheme for RNA expression profiling using Nanostring nCounter System and reagents (e.g., reporter and capture probes). The method of Fortina uses two probes which are both complementary to the target (RNA transcript): the capture probe linked to biotin for immobilization and purification and a reporter probe containing a unique fluorescent barcode. Once the RNA-probe complexes are hybridized, those complexes are captured on a streptavidin coated surface using the biotin tag of the capture probes and the fluorescent barcode tag imaged.

This method of Fortina (and Nanostring Technologies) is both unrelated to the claimed subject matter (e.g., different probes and coding scheme) and incompatible with visualizing RNA spatial organization *in situ* as presently claimed.

Each of Femino et al., Lubeck and Cai, and Lubeck 2014 discloses and teaches various methods using smFISH probes for single molecule detection *in situ*, including multiplexed imaging (e.g., seqFISH). The presently claimed subject matter does not relate to smFISH probes (e.g., primary probes directly labeled with a fluorophore and which directly hybridize to the RNA), but instead uses primary probes that tile across the RNA target and which comprise one or more readout sequences. These readout sequences correspond to a specific position in the N-bit codeword assigned to that RNA species, but which are randomly located across the length of the RNA via the primary nucleic acid probes. Those readout sequences are then imaged using readout probes which comprise a fluorophore and hybridize to the readout sequence only during the corresponding hybridization round for that position of the N-bit codeword. That combination of primary and secondary probes with readout sequences corresponding to a specific position in the N-bit codeword provide a unique coding scheme for multiplex imaging of single molecules *in situ*. The Patent Office has not provided any articulated reasoning as to why, or how, one of skill in the art would have modified any of Femino et al., Lubeck and Cai, and/or Lubeck 2014 to arrive at the claimed subject matter.

As to Fortina, given the proprietary nature of the reagents disclosed therein (i.e., reporter probes from Nanostring Technologies), it is unclear what the elements of those reporter probes were. However, what is clear from the teachings of Fortina is that the method uses a solution-phase hybridization of probes to the target, and therefore does not teach or reasonably suggest *in situ* hybridization.

Moreover, the inventors of the present specification published a paper exemplifying certain embodiments within the scope of the claimed subject matter. *See* Chen, *et al.*, "Spatially resolved, highly multiplexed RNA profiling in single cells," *Science.* 348(6233):412, 2015 ("Chen"). Chen demonstrates that multiplexing requires an error robust coding scheme to accurately image spatial organization of RNA transcripts *in situ* and that the methods of seqFISH (Lubeck and Cai, and Lubeck 2014) are inadequate for those purposes ("scaling up the throughput of smFISH to the

systems scale faces significant challenge because not only does the addressable RNA species increases exponentially with *N* [each RNA species by multiple (*N*) distinct signals], but the detection error rates also grow exponentially with *N*"). *See* Chen at p. 2 "Combinatorial labeling with error-robust encoding schemes". Moreover, "as *N* increase, the fraction of RNAs properly detected (the calling rate) would rapidly decrease and, more troubling, the fraction of RNAs that are identified as incorrect species (the misidentification rate) would rapidly increase", wherein "the majority of RNA molecules would be misidentified after 16 rounds of hybridization!" To address that problem of using smFISH probes in multiplex assays, the inventors developed error-robust encoding schemes, in which only a subset of 2^N-1 words separated by a certain Hamming distance are used to encode RNAs. This was exemplified with a coding scheme "in which the number of "1" bits [Hamming weight] were kept both constant and relatively low—only four per word—to reduce error and avoid biased detection". *See* Chen at p. 3, first full paragraph. In other words, the present inventors devised a unique two-step labeling scheme to encode and readout cellular RNA to perform multiplexed error-robust fluorescence *in situ* hybridization (MERFISH). *See* Chen at Figure 1. This error-robust multiplexed method using encoding and readout probes is now known in the art as "MERFISH." The claimed subject matter is distinct from those methods using smFISH probes and provide an advancement over seqFISH (multiplexed methods using smFISH probes).

The use of smFISH probes, as taught by each of Femino et al., Lubeck and Cai, and Lubeck 2014, requires long incubation periods (e.g., overnight) for the smFISH probes to hybridize to the RNA target *in situ*. *See* Lubeck at "Probe design, Purification and Hybridization" in the Methods section. The primary binding step with the RNA requires overnight to a few days, as the kinetics of binding are slow. To perform multiplexing from this scheme, the first signal (e.g., a smFISH probe) is removed, a second signal from a second set of smFISH probes are added to the sample and imaged. This is not a practical approach to multiplexing if each round of hybridization requires at least an overnight incubation with a new set of smFISH probes. The present inventors solved that problem by developing a two-step probe binding method, wherein the primary probe is only added in one initial step (which is the longest step and takes more than a day) and then repeated hybridization rounds are performed with secondary probes that bind to the primary probes. This is not a trivial step and greatly enhances the multiplexed FISH methods. Accordingly, the claimed

subject matter is a patentably distinct advancement over the cited art and solved two problems—providing an error-robust coding scheme that enables accurate FISH multiplexing and a shorter method using a two-step probe binding process wherein multiple rounds of hybridization can be performed in a practical time frame.

For those reasons, it is submitted that the teachings of the references are insufficient to make a *prima facie* case of obviousness. Even if combined, the aggregate teachings, legally, do not relate to the subject matter of interest. Therefore, it is respectfully requested that the rejections of claims 230-252 be withdrawn.

VII. General Comments on Dependent Claims

Since each of the dependent claims depends from a base claim that is believed to be in condition for allowance, for the sake of brevity, it is believed that it is unnecessary at this time to argue the further distinguishing features of the dependent claims. However, the interpretation of the previously presented dependent claims as set forth in the Office Action, nor the basis for rejection of any of the previously presented dependent claims is proper, is conceded. Therefore, the right to specifically address the further patentability of the dependent claims in the future is hereby reserved.

CONCLUSION

In view of the foregoing remarks, this application should now be in condition for allowance. A notice to this effect is respectfully requested. If the Examiner believes, after the foregoing remarks, that the application is not in condition for allowance, the Examiner is requested to call the undersigned at 617-646-8000.

If this response is not considered timely filed and if a suitable request for an extension of time is otherwise absent, any necessary extension of time is hereby requested. If there is a fee occasioned by this response, including an extension fee, the Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have

been filed herewith to Deposit Account No. 23/2825, under Docket No. H0498.70509US05, from which the undersigned is authorized to draw.

Dated: November 22, 2023

Respectfully submitted,

Electronic signature: /Tani Chen/
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