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(54) Title: RNA FIXATION AND DETECTION IN CLARITY-BASED HYDROGEL TISSUE

(57) Abstract: Methods, kits, and systems for fixation of RNA permitting its detection in intact tissue, such as, large volume of mammalian tissue are disclosed. The methods, kits, and systems utilize carbodiimide-based chemistry to stably retain RNAs in tissue clarified using CLARITY. Also provided herein are methods, kits, and systems for detection of RNAs in clarified tissue.

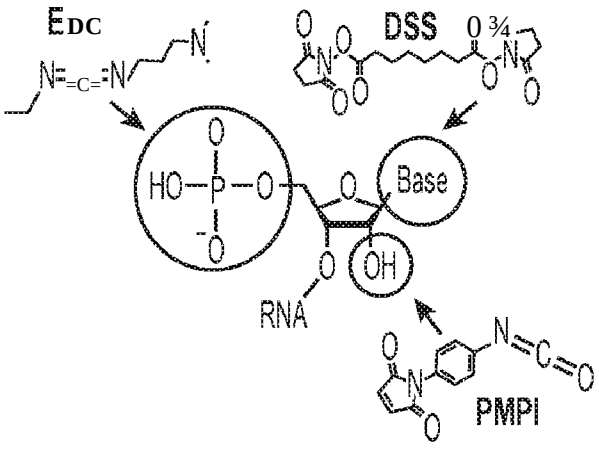

FIG. 1A

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RNA FIXATION AND DETECTION IN CLARITY-BASED HYDROGEL TISSUE

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/293,490, filed February 10, 2016, which application is incorporated herein by reference in its entirety.

INTRODUCTION

[0002] An exciting theme in modern biology is moving toward joint maximization of the content and context of molecular-level observations—that is, obtaining high-resolution and content-rich information about the biological system, while also maintaining this system largely or fully intact to preserve crucial contextual information. Historically these two goals of content and context have been in opposition, since higher-resolution analyses have tended to require disassembling the system or accepting a limited field of view. But the value of obtaining and integrating information about the identity, function and connectivity of cells in intact 3D volumes has been increasingly appreciated.

[0003] For example, one of the current challenges in neuroscience is to query molecular identity, activity level, and circuit wiring of individual cells within intact brain networks, which would require linkage of information spanning several orders of magnitude in spatial scale. Until recently, investigating the structure of neural networks in this way required sectioning for optical access and molecular labeling, followed by computer-assisted alignment and 3D reconstruction (Denk and Horstmann, 2004; Micheva and Smith, 2007; Oh et al., 2014). Such reconstructions have been valuable, but are often laborious, limited to small volumes, and susceptible to loss of information at section boundaries, making tract-tracing and circuit-mapping particularly difficult (Wanner et al., 2015). However, tissue-clearing techniques have emerged that, to various degrees, enable the visualization of cell morphology (and in some cases molecular phenotype, as well as local and long-range wiring) embedded withm intact neural circuits (Chung et al., 2013; Tomer et al., 2014; Yang et al, 2014; Dodt et al, 2007; Erturk et al., 2012; Hama et al., 201 l; Kuwajima et al, 2013; Renier et al., 2014; Richardson and Lichtman, 2015; Staudt et al., 2007; Susaki et al, 2014; Tainaka et al, 2014).

[0004] To date these technologies have chiefly focused on interrogating proteins, whether transgenically-expressed or immunohistochemically-detected (with the exception of single probes tested in CLARITY-based hydrogei experiments in sectioned

tissue; Chung et al., 2013; Yang et al., 2014), and many such approaches may not be compatible with accessing the wealth of biological information contained in the RNA of large intact volumes. This untapped opportunity spans untranslated species, including microRNAs (which, among other reasons for investigation, are particularly relevant to human genetically-determined diseases; Esteller, 2011), the majority of splice variants, many immediate early gene (IEG) RNAs used to infer activity of particular regions or cells during behavior (Guzowski et al, 1999; Loebrich and Nedivi, 2009), and even the vast majority of translated gene products, due to limited antibody specificity and availability.

[0005] Thus, there remains a need for the development of methods for visualizing RNA in intact tissue. The present disclosure fulfills this need and provides methodology, tools, and resources for cellular-resolution transcriptional profiling of large and intact transparent mammalian tissue volumes, with reliable detection of diverse markers for non-coding transcripts, cell identity, and activity history.

SUMMARY

[0006] Methods, kits, and systems for fixation of RNA permitting its detection in intact tissue, such as, large volume of mammalian tissue are disclosed. The methods, kits, and systems utilize carbodiimide-based chemistry to stably retain RNAs in tissue clarified using CLARITY. Also provided herein are methods, kits, and systems for detection of RNAs in clarified tissue.

[0007] An aspect of the present disclosure includes a method of preparing a biological specimen for microscopic analysis of a target RNA analyte, the method including fixing the specimen with a plurality of hydrogel subunits; polymerizing the hydrogel subunits to form a hydrogel-embedded specimen; fixing RNA in the specimen using carbodiimide mediated crosslinking; clearing the hydrogel-embedded specimen wherein the RNA is substantially retained in the specimen; and contacting the specimen with a nucleic acid probe for a target RNA analyte.

[0008] In some embodiments, the carbodiimide comprises l-Ethyl-3-3-dimethyl-aminopropyl carbodiimide (EDC). In some embodiments, the nucleic acid probe undergoes a Hybridization Chain Reaction (HCR). In certain embodiments, the nucleic acid probe is a DNA probe. In some embodiments, the nucleic acid probe is a RNA probe. In some embodiments, the specimen is stored for at least one week prior to the contacting. In some embodiments, the specimen is stored at 4°C for a period of one

week to a year prior to the contacting. In some embodiments, the specimen is stored at 4°C for a period of one week to six months prior to the contacting. In some embodiments, the contacting comprises contacting the specimen with a plurality of nucleic acid probes for a plurality of target RNA analytes. In some embodiments, the clearing comprises substantially removing a plurality of cellular components from the specimen. In some embodiments, the clearing comprises substantially removing lipids from the specimen. In some embodiments, the the clearing comprises electrophoresing the specimen. In some embodiments, the electrophoresing comprises using a buffer solution comprising an ionic surfactant. In some embodiments, the specimen is a biopsy specimen or autopsy specimen. In some embodiments, the specimen is from a human. In some embodiments, the method further comprises imaging the specimen using confocal microscopy, two-photon microscopy, light-field microscopy, tissue expansion microscopy, and/or CLARITYTM-optimized light sheet microscopy (COLM).

BRIEF DESCRIPTION OF THE FIGURES

[0009] FIG. 1A-1K depict fixation in EDC significantly improves RNA retention in **CLARITY** volumes.

[0010] FIG. 2A-2B show characterization of tissue formulation and storage time for *in situ* hybridization.

[0011] FIG. 3A-3G illustrate that **DNA** diffuses into CLARITY tissue more quickly than antibodies.

[0012] FIG. **4A-4F** show comparison of antibody-based and **DNA** -based amplification.

[0013] **FIG.5A-5C** show validation of amplification specificity.

[0014] FIG. 6A-60 depict cell-type phenotyping in CLARITY tissue using DNA probes and HCR amplification.

[0015] FIG. 7A-7B exemplify application of **EDC-CLARITY** to non-neural tissue.

[0016] FIG. **8A-8H** show characterization of **HCR** probe design and amplification sensitivity.

[0017] FIG. 9A-9B illustrate detecting activity-induced transcripts and non-coding RNAs in CLARITY volumes.

[0018] FIG. **1OA-10C** illustrate detection of microRNAs in CLARITY tissue.

[0019] FIG. **11A-11B** illustrate multiplexed detection of mRNAs in CLARITY.

[0020] FIG. **12A-12B** show characterization of orthogonal hairpins.

[0021] FIG. 13 depicts Table **1** that lists probes for detection of target RNA.

DETAILED DESCRIPTION

[0022] The present disclosure provides methods, systems, and kits for preparing a tissue specimen for microscope analysis of a target RNA analyte present or suspected of being present in the tissue. These methods, systems, and kits utilize hydrogel subunits to form hydrogel-embedded specimen and carbodiimide for fixing RNA present in tissue prior to clearing the hydrogel-embedded specimen. The tissue specimen prepared using the methods, systems, and kits disclosed herein provides superior retention and increased stability of RNA analyte in the tissue compared to the other methods that have been used for preparing tissue specimen for microscopic analysis of RNA.

[0023] All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entireties.

[0024] It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and 'the" include plural referents unless the content clearly dictates otherwise. It is further noted that the claims can be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0025] Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

[0026] Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

METHODS

[0027] The present disclosure provides methods for preparing a biological specimen for microscopic analysis of a target RNA analyte. In certain embodiments, the method comprising fixing the specimen with a plurality of hydrogel subunits; polymerizing the hydrogel subunits to form a hydrogel-embedded specimen; fixing RNA in the specimen using carbodiimide crosslinking; clearing the hydrogel-embedded specimen wherein the

RNA is substantially retained in the specimen; and contacting the specimen with a nucleic acid probe for a target RNA analyte.

[0028] Aspects of the present methods include fixing the specimen in the presence of hydrogel subunits. By "fixing" the specimen it is meant exposing the specimen, i.e., the components present throughout the specimen, such as within cells of the specimen, to a fixation agent such that the cellular components become cross-linked to one another. By "hydrogel" or "hydrogel network" is meant a network of polymer chains that are water-insoluble, sometimes found as a colloidal gel in which water is the dispersion medium. A detailed description of suitable hydrogels may be found in published U.S. patent application 20100055733, herein incorporated by reference. By "hydrogel subunits" or "hydrogel precursors" is meant hydrophiiic monomers, prepoiymers, or polymers that can be cross-linked, or "polymerized", to form a three-dimensional (3D) hydrogel network.

[0029] The specimen may be fixed in the presence of hydrogel subunits and a fixation agent or a fixative to fix the specimen in the presence of the hydrogel subunits. Suitable fixatives, without limitation, include an aldehyde containing fixative, such as, formaldehyde, paraformaldehyde, glutaraldehyde. Other fixatives such as, acetone, ethanol, methanol, and the like may also be used. The fixative used in the presence of the hydrogel subunits may be at a concentration of about 1-10%, e.g. 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, or 10%. In certain embodiments, the fixative may be formaldehyde or paraformaldehyde.

[0030] The hydrogel subunits may comprise any convenient hydrogel subunits, such as, but not limited to, acryiamide, bis-acrylamide, poly(ethylene glycol) and derivatives thereof (e.g. PEG-acrylate (PEG-DA), PEG-RGD), polyaliphatic polyurethanes, polyether polyurethanes, polyester polyurethanes, polyethylene copolymers, polyamides, polyvinyl alcohols, polypropylene glycol, polytetramethyiene oxide, polyvinyl pyrrolidone, polyacrylamide, polyihydroxy ethyl acryiate), and poly(hydroxy ethyl methacrylate), collagen, hyaluronic acid, chitosan, dextran, agarose, gelatin, alginate, protein polymers, methyiceliulose, and the like, and combinations thereof.

[0031] The type and concentration of fixative(s) and hydrogel subunits used in the presently disclosed methods can be selected based on a number of factors, such as, the type of tissue, volume of tissue, thickness of the tissue, duration of fixing and polymerization, and the like. Thus, for example, a fixative/hydrogel composition used for fixing and polymerization steps may comprise an acryiamide monomer at a

concentration of from about 1% w/v to about 20% w/v, e.g., about 2% to about 15%, about 3% to about 10%, about 4% to about 8%, and a concentration of bis-acrylamide cross linker in the range of about 0.01% to about 0.075%, e.g., 0.01%, 0.02%, 0.025%, 0.03%, 0.04%, 0.05%, 0.06%, or 0.075%; or, for example, the fixative/hydrogel composition may comprise PEG prepolymers having a molecular weight ranging from at least about 2.5Kto about 50K, e.g., 2.5K or more, 3.5K or more, 5K or more, 7.5K or more, 10K or more, 15K or more, 20K or more, but typically not more than about 50K, at a concentration in a range from about 1% w/w to about 50% w/w, e.g., 1% or more, 5% or more, 7.5% or more, 10% or more, 15% or more, 20% or more, 30% or more, 40% or more, and usually not more than about 50%s. Concentrations of hydrogei subunits and modifiers that provide desired hydrogei characteristics may be readily determined by methods in the art or by the methods described in the working examples below. In certain embodiments, the hydrogei subunits used in the present methods may be acryiamide, bis-acrylamide or a combination thereof. In certain embodiments, the tissue specimen may be fixed by contacting it with a solution that includes acryiamide, bis-acrylamide, and formaldehyde or paraformaldehy de. In certain embodiments, the tissue specimen may be fixed by contacting it with a solution that includes l%-4% acryiamide, 0.00125 %-0.05% bis-acrylamide, and 1%-10% formaldehyde or paraformaldehyde.

[0032] The fixative/hydrogel solution may be delivered to the specimen by any convenient method, e.g., perfusion, injection, instillation, absorption, application, immersion/submersion, etc. The specimen will typically be fixed in the presence of the hydrogei for 15 minutes or more, for example, for 30 minutes or more, 1 hour or more, 2 hours or more, 4 hours or more, 6 hours or more, 12 hours or more, in some instances, for 16 hours or more, 20 hours or more, or 24 hours or more.

[0033] Following fixation of the specimen, the hydrogei subunits are polymerized, i.e., covalently or physically cross-linked, to form a hydrogei network. Polymerization may be by any method including, but not limited to, thermal crosslinking, chemical crosslinking, physical crosslinking, ionic crosslinking, photo-crosslinking, irradiative crosslinking (e.g., x-ray, electron beam), and the like, and combinations thereof and may be selected based on the type of hydrogei used and knowledge in the art. The length of time for polymerization will depend on the type of hydrogei subunits used and the chosen polymerization method, but will typically be about 15 minutes to about 48 hours, 30 min to about 10 hours, lhour to about 8 hours, 2 hours to about 6 hours, for example,

15 minutes or more, 1 hour or more, 2 hours or more, 3 hours or more, 4 hours or more,5 hours or more, 6 hours or more, 12 hours or more, 16 hours or more, 24 hours or more, or in some instances, 48 hours. In certain cases, a thermal initiator may be included in the fixative/hydrogel composition used for fixing and polymerization of the tissue specimen. The optimal time and combination of reagents will be known to the ordinarily skilled artisan or may be determined empirically or from any number of publicly available resources (e.g., on the world wide web at piercenet.com; see also, Macroporous Polymers: Production Properties and Biotechnologieal/Biomedieai Applications. Edited by Bo Mattiasson, Ashok Kumar, and Igor Yu. Galeaev. CRC Press 2010; and Crosslinking Reagents Technical Handbook, Pierce Biotechnology, Inc., 2006). In certain cases, the polymerization of the hydrogel may be initiated by incubating the fixed tissue specimen at a high temperature of at least 35°C to about a 100°C. such as, 37°C or more, 40°C or more, or 50°C or more, 60°C or more, 70°C or more, 80°C or more, 90°C or more, or 100°C.

[0034] In certain embodiments, the fixing the specimen with a plurality of hydrogel subunits and polymerizing the hydrogel subunits to form a hydrogel -embedded specimen may be carried out using the methods disclosed in U.S. patent application publication 20150144490, which is herein incorporated by reference in its entirety.

[0035] Once polymerized, the hydrogel -embedded (i.e., hydrogel-hybridized) specimen is exposed to a reagent for fixing the RNA present in the specimen using carbodiimide for crosslinking the RN A to the components of the hydrogel-embedded specimen. In certain aspects, the carbodiimide may crosslink the RNA present in the specimen to amine containing cellular components, such as, proteins and peptides.

[0036] Any suitable carbodiimide may be utilized. In certain embodiments, the carbodiimide may be selected from the group consisting of 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HC1), l-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluenesulfonate (CMC), N,N'-dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide (DIC), and l-ethyI-3-(3-dimethylaminopropyl)-carbodumide methiodide (EDC-Mel). In another aspect, the carbodiimide may be 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), l-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCi) or ll-ethyl-3-(3-dimethylaminopropyO-carbodiimide methiodide (EDC-Mel). The concentration of the

carbodiimide may range from lmM to 500mM, or lOmM to 300mM, or 30mM to 200mM, or 50mM to lOOmM, e.g., 50mM, 100 mM, 2()()mM, or 3()0mM.

[0037] In one aspect of the disclosed methods, the hydrogel-embedded specimen is contacted with a solution comprising a carbodiimide at a temperature ranging from about 20°C to about 70°C. For example, the hydrogel-embedded specimen may be contacted with a solution comprising a carbodiimide at a temperature ranging from 30°C to 50°C, or 35°C to 45°C, or 35°C to 40°C, such as, 30°C, 35°C, 37°C, or 40°C.

[0038] In one aspect of the disclosed methods, the hydrogel-embedded specimen is contacted with a solution comprising a carbodiimide for a duration of at least 15 min to 10 days, or 1 hour to 5 days, or 3 hours to 3 days, or 5 hours to 1 day, or 5 hours to 16 hours, or 10 hours to 18 hours, for example, 5 hours, or 6 hours, or 7 hours, or 8 hours, or 10 hours, or 12 hours, or 14 hours, or 16 hours, or 18 hrs.

[0039] In another aspect of the disclosed methods, the solution comprising a carbodiimide has a pH of about 6.0 to about 10.0. In an embodiment, the solution comprising a carbodiimide has a pH of about 7.0 to about 9.0, such as, 7.5-9.0, 7.5-8.75, 7.75-8.5, 8.0-8.75, 8.0-8.5, e.g., 7.5, 7.75, 8.0, 8.5, or 8.75. The carbodiimide may be present in any suitable buffer, such as, 1-methylimidazole buffer or MES (4-morpholinoethanesulfonic acid) buffer.

[0040] In certain embodiments, the fixing the RNA in the specimen may include contacting the hydrogel-embedded specimen with a solution comprising a carbodiimide and a heterocyclic derivative selected from the group consisting of an imidazole, pyrazole, tnazole or tetrazole or a combination thereof. In another aspect, the heterocyclic derivative is selected from the group consisting of 1-methylimidazole, imidazole, 1-hydroxyl-benzotriazole, 5-ethylthiotetrazole, and 2- chloromidazole. In an embodiment, the heterocyclic derivative is 5-ethylthiotetrazole. In another embodiment, the heterocyclic derivative comprises 1-methylimidazole and 5-ethylthiotetrazole. In a particular embodiment, the solution for fixing the RNA may include EDC, EDC-HC1, or EDC-Mel and 1-methylimidazole and 5-ethylthiotetrazole.

[0041] In certain embodiments, the fixing the RNA in the specimen may be carried out using the methods and reagents disclosed in U.S. application publication no. 20140220574, which is herein incorporated by reference in its entirety.

[0042] The presently disclosed methods may be used for fixing any type of RNA in the tissue specimen. For example, the RNA may be messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), microRNA (miRNA), long non-coding RNA

(IncRNA), mitochondrial RNA (mtRNA), small nucleolar RNA (snoRNA) or other RNA that may be present in the tissue.

[0043] Once the RNA is fixed, the hydro-gel embedded specimen may be cleared. By "clearing" a specimen it is meant that the specimen is made substantially transparent, i.e., permeable to light. In other words, about 70% or more of the visual (i.e., white) light, ultraviolet light or infrared light that is used to illuminate the specimen will pass through the specimen and illuminate cellular components therein, e.g., 75% or more of the light, 80% or more of the light, 85% or more of the light, in some instances, 90% or more of the light, 95% or more of the light, 98% or more of the light, e.g. 100% of the light will pass through the specimen. This change in the optical properties of the specimen provides for the visualization of cellular and subcellular components internal to the tissue.

[1044] Any treatment that forces cellular components, e.g., lipids, from the specimen, that draws cellular components, e.g., lipids, from a specimen, or that causes cellular components, e.g., lipids, to break down, i.e., dissolve, within a specimen may be used to clear the specimen, including, without limitation, exposure to organic solvents such as xylenes, ethanol or methanol, exposure to detergents such as saponin, Triton X-100 and Tween-20, exposure to ionic surfactants, e.g., sodium dodecyl sulfate (SDS), electrophoresis, hydrodynamic pressure, ultrasonic vibration, solute contrasts, microwave radiation, vascular circulation, and the like.

[0045] In some embodiments, clearing may be conducted using an ionic surfactant, e.g., SDS, in order to expedite the clearing process by actively transporting charged ionic micelles out of the specimen that is being cleared. Clearing may be performed in any convenient buffer that is compatible with the selected clearance method, e.g., saline, phosphate buffer, phosphate buffered saline (PBS), sodium borate buffer, sodium tetraborate buffer, boric acid buffer, citric acid buffer, etc., as known in the art, and will typically take about 1-10 days per centimeter thickness of specimen, i.e., usually about 1 day, in some instances 2 days, sometimes 5 days, and typically no more than 10 days per cubic centimeter. Optimal time may be readily determined by visual inspection of the specimen for clarity. The clearing may be conducted at a temperature ranging from 20°C-100°C, or 20°C-50°C, or 25°C-45°C, or 30°C -45°C, or 35°C-40°C, such as 35°C, 37°C, or 40°C. In certain embodiments, the clearing time is shorter than the clearing time required when an agent other than a carbodiimide is used for the RNA fixation step. For example, the clearing time is half of that required when using p-maleimidophenyl isocyanate

(PMP1) for fixing the RNA in the hydro-gel embedded tissue specimen to obtain the same degree of clearing.

[0046] In some embodiments, clearing the hydrogel-embedded specimen comprises eiectrophoresing the specimen. In some embodiments, the specimen is eiectrophoresed using a buffer solution that comprises an ionic surfactant. In some embodiments, the ionic surfactant is sodium dodecyl sulfate (SDS). In some embodiments, the specimen is eiectrophoresed using a voltage ranging from about 10 to about 60 volts. In some embodiments, the specimen is eiectrophoresed for a period of time ranging from about 15 minutes up to about 10 days.

[0047] After clearing, a sample will generally be substantially free of lipids. By "substantially free of lipids" is meant that the original amount of lipids present in the sample before clearing has been reduced by approximately 70% or more, such as by 75% or more, such as by 80% or more, such as by 85% or more, such as by 90% or more, such as by 95% or more, such as by 99% or more, such as by 100%.

[0048] In certain embodiments, the fixing the specimen with a plurality of hydrogei subunits, polymerizing the hydrogei subunits to form a hydrogel-embedded specimen and clearing the hydrogel-embedded specimen may be carried out using the methods disclosed in U.S. patent application publication 20150144490, which is herein incorporated by reference in its entirely. This method is also referred to as the CLARITY method or process and the specimen prepared is referred to as CLARTTY-based hydrogei tissue.

[0049] After clearing the hydro-gel embedded tissue specimen may be contacted with a nucleic acid probe, such as, a DNA probe, RNA probe. Peptide nucleic acid (PNA) probe, locked nucleic acid (LNATM) probe, 2'-0-methyl (2'-OMe) oiigoribormcleotide probe, 2'-0-ethyl (2'-OEt) oligoribonucleotide probe, 2'-0-methoxyethyl (MOE) oligoribonucleotide probe or 2',4'-contrained MOE bicyclic nucleic acid (cMOE BNA) probe or 2',4'-contrained 2'-0-ethyl bicyclic (cEt BNA) probe or S-DNA probe, and the like. The sequence of the nucleic acid probe may be determined based on the sequence of the target RNA analyte. The target RNA analyte may be any RNA present in the tissue. In certain embodiments, a plurality of different nucleic acid probes specific for a plurality of target RNA analytes may be used in the disclosed methods. As used herein, the term "specific" in the context of a probe and the target RNA analyte refers to a probe that binds to a target RNA analyte that has a nucleotide sequence that is substantially complementary to the nucleotide sequence of the probe and does not bind to a RNA that

has a nucleotide sequence that is not substantially complementary to the nucleotide sequence of the probe. As used **herein,** substantially complementary refers to a complementarity of at least about 70% or more, such as, 80%, 85%, 90%, 95%, 99% or more. It is understood that the contacting of the specimen with the probe will be conducted under hybridization conditions that increase specific binding between a probe and its target RNA while decreasing non-specific binding. The specificity of binding between a probe and its target RNA anaiyte can be affected by the length of the probe and presence of repetitive sequences. Appropriate probes may be designed using available algorithms. The probe may be single stranded or double stranded. Probes may be 10 to 20, 11 to 30, 31 to 40, 41 **to** 50, 51-60, 61 to 70, 71 to 80, 80 to 100, 100 to 150, 150 to 200 or 200-250 nucleotides in length, for example 25,50, 100 or 150 nucleotides. In certain embodiments, the target RNA anaiyte may be one or more of the RNA listed in Table 1 in Fig. 13. In certain embodiments, a probe(s) for **a** target RNA anaiyte may be one **or** more probes listed **in** Table 1 in Fig. 13.

[0050] In certain embodiments, the probe may be a labeled probe. **The** phrase 'labeled probe" refers **to** a probe that contains a detectable moiety. The detectable moiety may produce a signal directly or indirectly. Examples of a detectable moiety that produces a signal directly include fluorescent molecules, radioactive isotopes, electron dense moieties, etc. Detectable moieties that produce a signal indirectly include moieties that produce a signal upon exposure to detection reagents such as substrates or antibodies, **etc. A** detectable moiety that produces a signal directly can optionally be detected by-indirect means such as by using a labeled antibody that binds to the moiety. In certain cases, a signal may be of a particular wavelength which is detectable by a **photodetector,** e.g., a **light** microscope, a spectrophotometer, a fluorescent microscope, a fluorescent sample reader, or a florescence activated cell sorter, etc. **A** probe may be labeled **with** more than one detectable moiety. In certain embodiments, a plurality (e.g., 2-20) of probes may be used for detecting a plurality **of RNA analytes** in the tissue specimen. In certain embodiments, a plurality of probes may be used for detecting a single RNA anaiyte. As used herein, RNA anaiyte refers to a molecule containing a continuous stretch of ribonucleotides.

[0051] In certain embodiments, the nucleic acid probe may be fluorescent. In other embodiments, the nucleic acid probe hybridized to a RNA target anaiyte in the tissue may be detected by contacting the tissue with a first antibody that binds to a detectable moiety in the probe. The first antibody may be conjugated to a fluorescent moiety or to

an enzyme that produces a detectable reaction product. In other cases, a second antibody that binds to the first antibody may be used. The second antibody may be conjugated to a fluorescent moiety or to an enzyme that produces a detectable reaction product. In certain cases, the probe may labeled with biotin, digoxygenin, avidin, and the like.

[0052] In certain embodiments, the RNA target anaiyte may be amplified prior to contacting the tissue specimen with a probe. In other embodiments, the specimen may be contacted with a probe in absence of amplification of the target RNA anaiyte.

[0053] In certain cases, a target RNA anaiyte fixed in the hydro-gel embedded tissue specimen may be detected using hybridization chain reaction (HCR). HCR is a method for the triggered chain of hybridization of nucleic acid molecules starting from stable, monomer hairpins or other more complicated nucleic acid structures. HCR is described in U.S. patent nos. 8,124,751 and 8,105,778. In the simplest version of this process, stable monomer hairpins undergo a chain reaction of hybridization events to form a nicked helix when triggered by a nucleic acid initiator strand. The fundamental principle behind HCR is that short loops are resistant to invasion by complementary single-stranded nucleic acids. This stability allows for the storage of potential energy in the form of loops; potential energy is released when a triggered conformational change allows the single-stranded bases in the loops to hybridize with a complementary strand. In certain embodiments, the probe for contacting the hydrogel-embedded specimen may include a target region and an initiation region. The target region is able to specifically bind to the target RNA anaiyte, while the initiation region is able to initiate the polymerization of labeled nucleic acid monomers. Thus, the specimen is contacted with a first metastable monomer comprising an initiator region that is complementary to the initiation region of the probe and a second metastable monomer comprising a region complementary to a portion of the first monomer. One or both of the monomers may be labeled with a fluorescent dye. They may also be labeled with a fluorescence quencher such that prior to polymerization the fluorescence is quenched. A fluorescent signal is thus generated upon formation of a polymer and background is reduced.

[0054] In other embodiments utilizing HCR, a triggered probe may be used, such that the initiation region is only made available to interact with the monomers when the probe is bound to the target RNA anaiyte. For example, in some embodiments the probe undergoes a conformational change upon binding to the anaiyte such that the initiation region is available to stimulate polymerization. In this way, non-specific polymerization resulting from non-specific probe binding is reduced. In certain embodiments, the in situ

HCR reactions can be multiplexed to identify the presence of multiple RNA analytes of interest simultaneously.

[0055] In another aspect, methods of in situ imaging are provided in which a biological sample is contacted with a probe comprising a target region capable of specifically binding to an **analyte** of interest and an initiator region, such that the probe binds to the anaiyte of interest. The sample is then contacted with at least **two** fluorescently labeled monomers, whereby the initiator region of the bound probe hybridizes to at least one of the monomers. As a result, the monomers form a **fluorescently** labeled polymer tethered to the analyte via the probe. The fluorescently labeled polymer can then be visualized.

[0056] Tissue specimens suitable for use **with** the methods and systems described herein generally include any type of tissue specimen collected from living **or** dead subjects, such as, e.g., biopsy specimens and autopsy specimens. Tissue specimens may be collected and processed using **the** methods, kits and systems described herein and subjected to microscopic analysis immediately following processing, or may be preserved and subjected to microscopic analysis at a future time, e.g., after storage for an extended period of time (at least for 1 day or more, such as up to 5 years, for example, 1 **day-3** years, 3 days-! year, 10 **days-9** months, **or** 2 **weeks-6** months, such as, up **to** 5 years, 3 years, 1 years, or 6 months). In some embodiments, the methods described herein may be used to preserve tissue specimens in a stable, accessible and fully intact form for future analysis. For example, tissue specimens, such as, e.g., human brain tissue specimens, may be processed as described above and cleared to remove a **plurality** of cellular components, such as, e.g., lipids, and then stored for future analysis. In some embodiments, the methods and systems described herein may be used to analyze a previously-preserved or stored tissue specimen.

[0057] By "microscopic analysis" is meant the analysis of a specimen using techniques that provide for the visualization of aspects of a specimen that cannot be seen **with** the unaided eye, i.e., **that** are not within the resolution range of the normal human eye. Such techniques may include, without limitation, optical microscopy, e.g., bright field, oblique illumination, dark field, phase contrast, differential interference contrast, interference reflection, **epifluorescence, confocal** microscopy, CLARITY™**-optimized** light sheet microscopy (COLM), light field microscopy, tissue expansion microscopy, etc., laser microscopy, such as, two photon microscopy, electron microscopy, and scanning probe microscopy. By "preparing a biological specimen **for** microscopic analysis" is generally

meant rendering the specimen suitable for microscopic analysis at an unlimited depth within the specimen.

[0058] Tissue specimens suitable for use **with** the methods and systems described herein generally include any type of tissue specimens collected from living or dead subjects, such as, e.g., biopsy specimens and autopsy specimens. Tissue specimens may be collected and processed using the methods and systems described herein and subjected to microscopic analysis immediately following processing, **or** may be preserved and subjected to microscopic analysis at a future time, e.g., after storage for an extended period of time. In some embodiments, the methods described herein may be used **to preserve** tissue specimens in a stable, accessible and fully intact form for future analysis. For example, tissue specimens, such as, e.g., human brain tissue specimens, may be processed as described above and cleared to remove a plurality of cellular components, such as, e.g., lipids, and then stored for future analysis. In some embodiments, the methods and systems descnbed herein may be used to analyze a previously-preserved or stored tissue specimen. For example, in some embodiments a previously-preserved tissue specimen that has not been subjected to the CLARITY process may be processed and analyzed as described herein.

[0059] In some instances, the target RNA analyte may be endogenous to the cells in the tissue specimen. In other instances, the target RNA analyte may be ectopically provided. For example, stereotactic surgery is often used in **the** field of **neuroscience to** provide **biomolecules** such as proteins, viruses, chemicals to neural tissue that label, or "trace", the projections and/or the connectivity of subsets of neurons in vivo or ex vivo. In this technique, a needle comprising a labeling macromolecule is lowered into CNS tissue at a precise location and the labeling molecule is released into the tissue. The molecule will fill the neurons in the vicinity of the injection site and, depending on the type of macromolecule delivered, may be transported across synapses to label their efferent targets ("anterograde tracing") and/or across dendrites to label the afferent neurons from which they are receiving signals ("retrograde tracing"). Examples of agents **that** may be used to label neurons stereotacticaily are well known in the art, including, for example, viral tracers, e.g. Herpes simplex virus **typel** (HSV) and the **Rhabdoviruses.** In some instances, the nucleic acid probe may be passively transported into the specimen. **In** other words, the nucleic acid probe diffuses into the specimen. In other instances, the nucleic acid probe is actively transported into the specimen, e.g. by electroporation,

hydrodynamic pressure, ultrasonic vibration, solute contrasts, microwave radiation, vascular circulation, or the like.

[0060] To **microscopically** visualize specimens prepared by the subject methods, in some embodiments the specimen is embedded in a mounting medium. Mounting medium is typically selected based **on** its suitability for the reagents used to visualize the cellular **biomolecules,** the refractive index of the specimen, and the microscopic analysis to be performed. For example, for phase-contrast work, the refractive index of the mounting medium should be different from the refractive index of the specimen, whereas for bright-field work the refractive indexes should be similar. As another example, **for** epifluorescence work, **a** mounting medium should be selected **that** reduces fading, **photobleaching** or quenching during microscopy or storage. **In** certain embodiments, a mounting medium or mounting solution may be selected to enhance or increase the optical clarity of the cleared tissue specimen. NonliraUing examples of suitable mounting media that may be used include glycerol, CC/Mount™, **Fluoromount™** Fluoroshield™, **lmmunHistoMount 1™, Vectashield™, Permount™, Acrytol™,** CureMoimt™, FocusClear™, or equivalents thereof.

[0061] In some instances, the **hydrogel**-embedded specimen is permanently mounted. In other words, once mounted in mounting medium, the **hydrogel-embedded** specimen cannot be removed for further manipulation. **In** other instances, the specimen is temporarily, or reversibly, mounted. In other words, the hydrogel-embedded specimen may be removed from the mounting medium and re-stained after microscopy to visualize **alternative/additional** biomolecules or subcellular structures. In such instances, **macromolecules** that were previously added to the specimen, e.g. to visualize certain biomolecules, may be removed after microscopic analysis by, e.g., **exposure** to organic solvents such as xylenes, ethanol or methanol, exposure to detergents such as sodium **dodecyl** sulfate (SDS), saponin, Triton **X**-100 and Tween-20, electrophoresis, hydrodynamic pressure, ultrasonic vibration, solute contrasts, microwave radiation, vascular circulation, and the like. The hydrogel-embedded specimen is then contacted with different macromolecules specific for other biomolecules or subcellular structures. As such, iterative staining may be performed on the same specimen.

[0062] Specimens prepared using the subject methods may be analyzed by any of a number of different types of microscopy, for example, optical microscopy (e.g. bright field, oblique illumination, dark field, phase contrast, differential interference contrast,

interference reflection, epifluorescence, confocal, etc., microscopy), laser microscopy, electron microscopy, and scanning probe microscopy.

KITS

[0063] The present disclosure provides kits for carrying out the methods of the present **disclosure.** The kits may include one or more of the following: fixative; **hydrogel** subunits; clearing reagents; nucleic acid probes, in situ hybridization buffer, labeled and or un-iabeled antibodies, buffers, e.g. buffer for fixing, washing, clearing, and/or staining specimens; mounting medium; embedding molds; dissection tools; etc. The subject reagents and kits thereof may vary greatly and may include a sub-set of the foregoing reagents.

[0064] Also provided are specimens that have been prepared by the subject methods for use in, **for** example, studying tissue at the cellular and subcellular level. For example, fixed, polymerized specimens, with carbodiimide cross-linked RNA, or specimens that have been fixed, polymerized, cross-linked using a carbodiimide and cleared, are provided for use in studying the expression of genes of interest, for screens to **identify** candidate agents **that** target cells and/or subcellular structures **of interest,** etc. Such prepared specimens may also be provided as a positive control in one of the kits or systems as described herein.

[0065] In addition to the above components, the subject kits may further include instructions for practicing the subject methods. These **instructions** may be present **in the** subject kits **in** a variety of forms, one or more of which may be present **in** the kit. One form **in** which these instructions may be present is as printed information on a suitable medium or substrate, e.g., **a** piece or pieces of paper **on** which the information is printed, in the packaging of the kit, in a package insert, etc. Yet another means would be a computer readable medium, e.g., diskette, CD, digital storage medium, etc., on which the information has been recorded. Yet another means that may be present is a website address which may be used via the Internet to access the information at a removed site. Any convenient means may be present in the kits.

SYSTEMS

[0066] Also disclosed herein are systems that include devices for conducting the methods disclosed herein. The subject system may include devices, such as, electrophoresis apparatus, ultrasounds, microwaves, needles, tubing, perfusion pumps,

etc., for fixing, clearing, fixing RNA, contacting with nucleic acid probes, and labeling probes if needed.

[0067] Electrophoresis devices suitable for use in the subject methods will generally comprise an electrophoresis chamber into which a buffer solution and the hydrogel-embedded specimen may be placed. The electrophoresis chamber may generally be any suitable size to accommodate a hydrogel-embedded sample of interest, and may be constructed of any material that will retain solution within the chamber, for example glasses and plastics, such as, for example, acrylics, polycarbonates, polystyrenes, polymethyl methacrylates, polyethylene, polyfluoroethylene, polypropylene, polyurethane, polyethylene terephthalate, polytetrafluoroethylene and the like.

[0068] In certain cases, the tissue specimen may be at least about 0.1 mm thick, such as, about 10 mm-O.lmm, 8 mm-1 mm, 6 mm- lmm, 4 mm- lmm, 3 mm- lmm, 3 mm-0.5mm, 3 mm-0.3mm, 3 mm-O.lmm, 5 mm-0.5mm, 5 mm-0.3mm, 5 mm-O.lmm, 10 mm-0.3mm, or 10 mm-O. lmm thick. As used herein, the thickness of the tissue specimen is measured along the same plane as that traversed by the illumination beam used for detecting a signal from the tissue. In certain embodiments, the electrophoresis chamber may be sized to hold the tissue sample and to contain space for solutions needed to preparing the specimen for microscopic analysis.

[0069] The system for conducting all or some steps of the methods disclosed herein may be automated completely or partially.

APPLICATIONS

[0070] Using the subject methods, reagents, kits, systems and devices, the ordinarily skilled artisan will be able to prepare any biological tissue for microscopic analysis. Methods, reagents, kits, systems and devices may be used to prepare a specimen from any plant or animal, including but not limited to transgenic animals, e.g., vertebrate or invertebrate, e.g. insect, worm, xenopus, zebrafish, mammal, e.g. equine, bovine, ovine, canine, feline, murine, rodent, non-human primate or human. Tissue specimens may be collected from living subjects (e.g., biopsy samples) or may be collected from dead subjects (e.g., autopsy or necropsy samples). The specimens may be of any tissue type, e.g. hematopoietic, neural (central or peripheral), glial, mesenchymal, cutaneous, mucosal, stromal, muscle (skeletal, cardiac, or smooth), spleen, reticulo-endotheiial, epithelial, endothelial, hepatic, kidney, pancreatic, gastrointestinal, pulmonary, fibroblast, and other cell types. In some instances, the specimen is the entire organism,

e.g. a worm, an insect, a **zebrafish.** In other instances, the specimen is a whole organ, e.g., the whole brain of a rodent. In other instances, the specimen is a portion of an organ, i.e. a biopsy, **e.g.** a biopsy **of** a transplanted tissue. The specimen may be freshly isolated or preserved, e.g. snap frozen. **In** some embodiments, the specimen may be a previously preserved specimen, such as, e.g., a preserved specimen from a tissue bank, e.g., a **preserved** specimen of a human brain obtained from a tissue collection program. **In** some instances, a specimen may be from a subject known to suffer from a specified disease or condition, such as, e.g., a sample **of brain** tissue from an autistic human. **In** other instances, a sample may be from a "normal" subject that does not suffer from a specific disease or condition. **In** some instances, a sample may be from a transgenic subject, such as, e.g., a transgenic mouse.

[0071] The **carbodiimide** mediated crosslinking of the RNA in conjunction with the CLARITY based tissue preparation provides a robust method for detecting a target RNA **analyte in** an intact tissue volume. The carbodiimide mediated crosslinking **of** the RNA provides increased retention of RNA in the tissue as compared to other RNA fixation methods, such as, those utilizing disuccinimidyl suberate (DSS). In addition, the use of carbodiimide mediated crosslinking of the RNA does not substantially increase the time required to achieve clearing of the hydro-gel embedded specimen while other RNA fixation methods, such as the use of **PPMI** almost doubles the clearing time.

[0072] The subject methods find many uses. For example, the subject methods may be used for in situ hybridization for detection **of target** RNA analyt**es** present in low levels in the tissue (for example, 50 copies/cell or less, such as 50-5 copies/cell, 45-5 copies/cell, 40-5 copies/cell, 35-5 copies/cell, 20-5 copies/cell, or 15-5 copies/cell), **for** quantitation of amount of the target RNA analyte, **for** visualization of subcellular localization of the target RNA analyte, detection of transiently expressed RNA, and the like.

[0073] As another example, the subject methods may be employed to evaluate, diagnose or monitor a disease. "Diagnosis" as used herein generally includes a prediction of a subject's susceptibility to a disease or disorder, determination as to whether a subject is presently affected by a disease or disorder, prognosis of a subject affected by a disease or disorder (e.g., identification of cancerous states, stages of cancer, likelihood that a patient **will** die from the cancer), prediction of a subject's responsiveness to treatment for a disease or disorder (e.g., a positive response, a negative response, no response at all to, e.g., allogeneic hematopoietic stem cell transplantation, chemotherapy, radiation therapy.

antibody therapy, small molecule compound therapy) and use of therametrics (e.g., monitoring a subject's condition to provide information as to the effect or efficacy of therapy). For example, **a** biopsy may be prepared from a cancerous tissue and microscopically analyzed to determine the type of cancer, the extent **to** which the cancer has developed, whether the cancer **will** be responsive to therapeutic intervention, etc.

[0074] As **another** example, a biopsy may be prepared from a diseased tissue, e.g. kidney, pancreas, stomach, etc., to determine the condition of the tissue, the extent to which the disease has developed, the likelihood that a treatment will be successful, etc. The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable **to** the disease. "Treatment" **as** used herein covers any treatment of a disease in a mammal, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, **i.e.,** arresting its development; or **(c)** relieving the disease, i.e., causing regression of the disease. **The therapeutic** agent may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms **of the** patient, is of particular interest. Such treatment **is** desirably performed prior to complete loss of function in the affected tissues. The subject therapy **will** desirably **be** administered dunng the symptomatic stage of the disease, and **in** some cases after the symptomatic stage of the disease. The terms "individual," "subject," "host," and "patient," are used interchangeably herein and refer to any mammalian subject **for** whom diagnosis, treatment, or therapy is desired, particularly humans. Examples of diseases that are suitable to evaluation, analysis, diagnosis, prognosis, and/or treatment using the subject methods and systems include, but are not limited **to,** cancer, immune system disorders, **neuropsychiatric** disease, endocrine/reproductive disease, cardiovascular/pulmonary disease, musculoskeletal disease, gastrointestinal disease, and the like.

[0075] Similarly, the subject methods may be used to monitor tissue grafts to determine how well the subject has accepted a transplanted organ/tissue, e.g. heart, kidney, liver, or other organ. In such instances, a biopsy of the transplanted organ may be prepared by

the subject methods, and the specimen microscopically analyzed for, e.g., tissue integrity, tissue vascularization, the infiltration of immune cells, etc.

[0076] The subject methods may also be used to evaluate normal tissues, organs and cells, for example to evaluate the relationships between cells and tissues of a normal tissue specimen, e.g., a tissue specimen taken from a subject not known to suffer from a specific disease or condition. The subject methods may be used to investigate, e.g., relationships between cells and tissues during fetal development, such as, e.g., during development and maturation of the nervous system, as well as to investigate the relationships between cells and tissues after development has been completed, e.g., the relationships between cells and tissues of the nervous systems of a fully developed adult specimen. In some embodiments, the subject methods may be used on samples collected from transgenic animals to investigate the effects of genetic changes on the development and/or function of specific cells, tissues, and/or organs.

[0077] The subject methods also provide a useful system for screening candidate therapeutic agents for their effect on a tissue or a disease. For example, a subject, e.g. a mouse, rat, dog, primate, human, etc. may be contacted with a candidate agent, an organ or a biopsy thereof may be prepared by the subject methods, and the prepared specimen microscopically analyzed for one or more target RNA analytes.

EXAMPLES

[0078] Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

[0079] Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Materials **and Methods**

[0080] CLARITY Tissue Preparation. CLARITY tissue was prepared as described in Tomer et al. (2014). In brief, C57/B16 8-12 weeks of age were anaesthetized with beuthanasia (100 mg/kg) and transcardially perfused with cold PBS, followed by cold hydrogel solution (1% or 4% aerylamide, 0.0125% bisacrylamide (for 1% acrylamide) or 0.05% bisacrylamide (for 4%> acrylamide), 0.25% VA-044 initiator, 1x PBS, 4%> PFA in dH20). Tissues were removed and post-fixed overnight at 4°C. For induction of

immediate early genes, animals were injected with either saline or kainic acid (12mg/kg, i.p.) 2 hours prior to perfusion and monitored for seizure activity.

[0081] For A4P0 samples, tissues were prepared as described (Yang et al., 2014). For A4P0 samples, tissues were first perfused in 4% PFA, post-fixed in 4% PFA for 24h (4°C), then transferred to a PFA-free embedding solution (4% acrylamide, 0.25% VA-044 initiator, 1x PBS in dH20) for 48h. Conical tubes containing samples were degassed under vacuum for 10 minutes, chamber was flooded with nitrogen, oil was quickly added to the surface of the hydrogel solution and tubes were immediately capped. Gel was polymerized at 37°C for 5 hours, removed from hydrogel solution and sectioned where indicated using a vibratome (500 μm sections) or sectioning block (1, 2, or 3mm sections). Tissue was incubated with RNA fixatives after acrylamide polymerization (EDC, 0.1M; PMPL 0.1M; or DSS, 0.1M, overnight at 37°C). Tissue was cleared passively in a 4%SDS/ 0.2M Boric acid (pH=8.5) clearing solution at 37°C with gentle shaking (0.5mm, ~1 week; 1mm, 1-2 weeks; 2-3mm, ~3 weeks) until transparent. Clearing solution was changed every 1-2 days. Cleared tissue was washed three times (1 hour each), plus overnight, and stored in 1x PBS with 0.3% TX-100.

[0082] Total RNA Isolation and **Acridine** Orange **Staining.** Cleared tissue was homogenized in 20 μg/ml proteinase K, extracted with Trizol and then acidic phenol:chloroform:isoamyl alcohol before precipitation with ethanol. For acridine orange staining, sections were rinsed in sodium citrate (SC) buffer for 10 minutes, incubated in acridine orange solution (100 ug/mL) for 3h, then rinsed in SC buffer, then PBS, and transferred to refractive index matching in FocusClear.

[0083] Probe Design. Riboprobes were generated from cDNA templates, reverse transcribed with DIG-labeled dNTPs (Roche), and purified. smFISH probes were designed and synthesized by BioSearch (Petaluma, CA). DNA 50mer oligonucleotide probes were purchased from Molecular Instruments (Caltech) or designed using OligoWiz software (Wernersson et al., 2007) and synthesized by Integrated DNA Technologies. LNA probes were synthesized by Exiqon.

[0084] For riboprobes, (FIG, 3B,C), cDNA templates for somatostatin (Probe #1, Accession#BC010770, 280-429) or paralbumin (Probe #2, Accession#BC027424, 203-352) were generated by Genscript. Vectors were linearized and reverse transcribed using T7 RNA polymerase and DIG-labeled dNTPs (Roche), and purified by phenol chloroform extraction. smFISH probes (FIGs 6J-6L) were designed and synthesized by Biosearch Technologies (Petaluma, CA). DNA SOmer initiator-labeled oligonucleotide

probes (FIGs 4A-F, 6A-60, 9A-9B) were either purchased from Molecular Instruments (Pasadena, CA; Parvalbumin, Tacl, Th, 10 probes each), or designed using OiigoWiz software (Wernersson et al., 2007) and synthesized by Integrated DNA Technologies (FIG. 6A-60 and 9A-9B, somatostatin, NPY, VIP, Tac2, Malatl, Npas4, Arc, 4-6 probes each). LNA probes were synthesized by Exiqon (FIG 9A-9B).

[0085] Sequences and concentration of probes used are indicated in Table 1 shown in FIG. 13.

[0086] Probe and Antibody **Diffusion.** For RNA and DNA probe diffusion, cleared tissue (2mm) was incubated in hybridization solution for the time indicated, then cooled to 4°C, fixed with PFA, and re-sectioned (200um). Cross-sections of the center of tissue were selected for staining with anti-DIG antibody conjugated to HRP and detected with TSA.

[0087] For antibody diffusion, tissue was incubated in 50mer DIG-labeled oligonucleotides overnight in 40% formamide and 2xSSC, cooled to 4°C, fixed in 4% PFA for one hour at RT. The tissue was then incubated with anti-DIG Fab fragment antibody coupled to HRP (1:1000) in PBST for the corresponding time and further processed as above for re-sectioning and TSA amplification.

[0088] ***In Situ* Hybridization.** For all in situ hybridizations, cleared tissue was equilibrated in hybridization solution for lh, hybridized in the same solution overnight at 37°C unless otherwise noted, then stringency washes were performed at the hybridization temperature to remove excess or non-specifically bound probe. Solutions and temperatures varied for each probe type and are as follows. Qligo(dT): hybridization with 15% formamide, 2xSSC, 10% dextran sulfate, 50nM probe; stringency 3x1 hour in 15% formamide, 2 x SSC then 2x1 hour in 2xSSC. DIG-labeled 50mers: hybridization with 50% formamide, 5xSSC, 0.5mg/ml yeast tRNA; stringency 3xlhour in 50% formamide, 5xSSC plus 2x1 hour in 2xSSC and then transferred to PBST. Initiator-labeled 50mers: hybridization with 40% formamide, 2xSSC, 10% dextran sulfate, 0.5mg/ml yeast tRNA; stringency 3xlhour in 40% formamide, 2xSSC plus 2xlhour in 2xSSC. DNA 20mers (smFISH sets): hybridization with 10% formamide, 2xSSC, 10% dextran sulfate; stringency 3x1 hour in 10% formamide, 2xSSC plus 2x1 hour in 2xSSC. LNA probes: hybridization with 50% formamide, 5xSSC, 0.5mg/ml yeast tRNA, 12.5nM DIG labeled probe at 20°C below Tm; stringency 2x1 hour in 5xSSC plus 1 hour in 2xSSC at the same temperature.

[0089] For DIG labeled probes, tissue was washed in PBST after stringency. Tissue was incubated overnight in anti-DIG antibody conjugated to HRP (1:500) for 2 days per mm tissue thickness, washed overnight in PBST, developed with tyramide signal amplification (1:50 dilution, 30 minutes), washed 3x in PBST, and transferred to FocusClear for imaging. For initiator probes, tissue was equilibrated in amplification buffer (5xSSC, 0.1% Tween20, 10% dextran sulfate). DNA hairpins were separately-heated to 90°C, cooled to RT, and added to amplification buffer. Tissue was incubated in hairpins overnight at RT, then washed 5x1 hour with 5xSSC plus 0.1% Tween20, and transferred to FocusClear for imaging.

[0090] Propidium iodine staining, where applicable, was performed using a PropI/RNase solution after stringency washes. Sections were transferred to FocusClear for 4 hours prior to imaging. Tissue shrinks once equilibrated to FocusClear for imaging; ail scale bars represent the imaged volume, which is approximately 50% of original tissue volume.

[0091] **Human** Tissue. Human tissue is putative healthy tissue obtained from temporal lobe resections from two patients (46 y.o. female, 18 y.o. male). Tissue was equilibrated in 1% hydrogei solution for 2 days at 4°C, polymerized for 5 hours at 37°C, and cleared for 5 weeks in 4% SDS at 37°C.

[0092] **Coiifocal** Microscopy. All images were taken on a Leica SP5 confocal microscope with a 1Ox/0.4 objective (WD: 2.2 mm) or 20x/0.75 objective (WD: 0.66 mm) at 488 nm (FITC), 514 nm, 543 nm, or 647 nm excitation.

[0093] **Experimental** subjects. Animal husbandry and all aspects of animal care and euthanasia as described were in accordance with guidelines from the National Institutes of Health and have been approved by members of the Stanford institutional Animal Care and Use Committee. Use of surgical and post-mortem human tissue was in accordance with guidelines from the National Institutes of Health and approved by the Stanford Institutional Review Board.

[0094] **CLARITY Tissue** Preparation ***for In Situ*** Hybridization

[0095] Passive tissue clearing is performed as described in Tomer et al. Nature, 2014. In brief:

[0096] 1. Perfuse animal with cold PBS, then cold CLARITY hydrogei solution:

Hydrogel Solution

Chemical	Volume in 400ml	Final Concentration
Acrylamide (40%)	10 fflL	1% final cone
Bis-acrylamide (2%)	2.5 mL	0.00125 % final cone
VA-044 Initiator	i g	0.25% final cone

10X PBS	40 mL	IX
16% PFA	100 mL	4%
d H_20	247.5 mL	-

[0097] 2. Postfix brain in 20ml of hydrogel solution at 4°C overnight.

[0098] 3. Degas solution under vacuum to remove dissolved oxygen, which inhibits polymerization. This can be done by degassing, flooding the chamber with nitrogen, then quickly capping the tube.

[0099] 4. Incubate 5 hours at 37°C

[00100] 5. Section tissue, if applicable.

[00101] 6. Transfer tissue to methylimidizole buffer (80µl methylimidizole in 10ml water) for 15 minutes.

[00102] 7. Incubate tissue in EDC solution at 37°C o/n. This compound acts as a fixative for 5' terminal phosphates (Pena et al., 2009; Tymianski et al., 1997) . This fixative is particularly helpful in preserving and detecting small RNAs, but also increases retention of mRNAs. To note: EDC fixation will increase clearing time by few days.

EDC Fixative Solution

Chemical	Mass in 10ml	Final Concentration
EDC	0.19g	0.1M
ETT	0.13g	0.1M
Methylimidizole Buffer (80µl Methylimidizole in 10 ml in H_2O	80 µl	

pH to 8.5 with NaOH.

[00103] 8. Move post-fixed sections to clearing solution. Passive clearing in 4%SDS/ 0.2M Boric acid (pH=8.5) clearing solution at 37°C until clear. Switch out solutions every day for at least first few days, then every other day should suffice.

Clearing Solution

Chemical	Mass in 1L	Final Concentration
Sodium tetraborate	40.24	0.2M
SDS	40g	4%
H_2O	1L	-

pH to 8.5 with NaOH.

[00104] 9. After clearing, wash 3x in PBST (PBS + 0.3% Triton), 1 hour each, at RT and once overnight.

Example 1

Advancing clarified tissue chemistry **with** carfeodiimide-based UNA retention

[00105] Many existing clearing methods rely on incubation of tissue for prolonged periods of time at temperatures of 37°C or greater (Chung et al., 2013; Tomer et al., 2014; Yang et al, 2014; Renier et al., 2014; Susaki et al, 2014; Tainaka et al, 2014);

however, formalin is known to revert its crosslinks at elevated temperatures, and the bonds made to nucleic acids are particularly vulnerable (Masuda et al., 1999; Srinivasan et al., 2002). Therefore, to improve retention of RNA during high-temperature tissue clearing, we sought to introduce temperature-resistant covalent linkages to RNA molecules prior to clearing, by targeting functional groups on the RNA molecule for fixation to surrounding proteins or the hydrogel matrix.

[00106] We explored three tissue-chemistry strategies: EDC (1-Ethyi-3-3-dimethyl-aminopropyl carbodiimide) for linkage of the 5'-phosphate group to surrounding arnine-containing proteins (Pena et al., 2009; Tymianski et al, 1997); PMPI (p-maleimidophenyl isocyanate) for linkage of the 2' hydroxy! group to surrounding sulfydryl-containing proteins (Shen et al., 2004); and DSS (disuccinimidyl suberate) for linkage of amine-containing side chains in RNA to surrounding amine-containing proteins (Mattson et al., 1993) (FIG. 1A). These crosslinks were introduced after hydrogel embedding (Chung et al, 2013). After fixation, samples were fully cleared and RNA was extracted from each preparation. We observed that although DSS provided no significant increase in RNA yield (potentially due to overfixation of RNA through multiple amine groups on each RNA molecule), there was markedly improved retention of RNA in EDC and PMPI-fixed samples compared with control for both 1% and 4% acrylamide hydrogel compositions (FIG IB). However, since PMPI doubled tissue-clearing time, while EDC only marginally increased clearing time (1-2 extra days in lmm tissue blocks), we proceeded with EDC as an RNA-fixation agent for CLARITY.

[00107] To complement these quantitative total-RNA biochemical measures with direct visualization of retained RNA within tissue, we stained tissue samples of different hydrogel compositions with acridine orange, an intercalating RNA dye. We found significantly increased RNA staining in EDC fixed samples, with EDC-treated 1% CLARITY tissue showing the best RNA labeling (FIG. 1C, ID). While promising, these total RNA measures did not specifically address mRNA, the population most relevant to molecular phenotyping and activity-dependent gene expression (in contrast to the more abundant rRNA, which by virtue of tight association with proteins could contribute disproportionately to the improvement seen with EDC). To determine if EDC improved mRNA preservation, we performed in situ hybridization with a 50 base deoxy-thymine oligonucleotide (oligo(dT)) to target the polyA tail of mature mRNA. Again, we found that 1% CLARITY with EDC samples exhibited the highest RNA signal (FIGs 1E, IF). Surprisingly, the 4% acrylamide hydrogel composition with EDC exhibited significantly

reduced RNA detection with both acridine orange staining and oligo(dT) in situ hybridization (as well as weaker staining in target-specific in situ hybridization; FIG. 2A). This consistent picture may reveal that the dense hydrogel network in 4% CLARITY makes mRNA targets less accessible for probe hybridization. In support of this notion, we find high concentrations (10M) of EDC also reduced mRNA staining, whereas more modest fixation (0.1M-1.0M EDC) provided the most effective labeling of RNAs (FIG. 1G, 1H).

[00108] A major motivation for RNA detection includes broad application to clinical tissue, but human samples are particularly prone to RNA degradation, since pre-fixation post-mortem intervals vary, immersion-fixation crosslinks tissue more slowly than transcardial perfusion, and clinical samples are often banked for extended periods of time. We have also found that human tissue clears more slowly and, in some cases, demands higher clearing temperatures. To test if EDC could improve RNA retention in human tissue, we compared two human samples collected during temporal lobe resection, one treated with EDC and one an untreated control (1% CLARITY hydrogel). Although both samples showed comparably strong mRNA signal prior to clearing, we found that only the EDC-treated sample exhibited detectable mRNA after clearing (FIG. II, 1J). We reasoned that EDC might not only be critical for the immediate processing of CLARITY samples, but might enable long-term storage with little RNA loss. To test this idea, we extracted and measured total RNA from rodent tissue during each stage of the clearing process. After a small loss of RNA during clearing, there was no significant loss during subsequent storage at 4°C for up to 6 months (FIG. IK), demonstrating a surprising level of stability (also reflected in target-specific in situ hybridization; FIG. 2B). Together, these data identify and validate a nucleic acid-tuned CLARITY chemistry with EDC.

[00109] FIG 1A-1K Fixation in EDC significantly improves RNA retention in CLARITY volumes. (FIG. 1A) Chemical compounds targeting functional groups on RNA (red circles) were characterized and assessed for RNA fixation and retention. (FIG. 1B) lmm mouse brain blocks were embedded in CLARITY hydrogel containing either 1% or 4% acrylamide, then either immediately processed for RNA extraction (uncleared), or post-fixed overnight in PMPI, DSS, EDC, or no-fix, then cleared until visually transparent, and processed for RNA extraction. There was a significant increase in RNA yield in PMPI and EDC treated groups relative to cleared no-fix controls (*** $P < 0.001$, one-way ANOVA, with Sidak's post-hoc multiple comparisons test n = 6 slices per group).

(FIG. 1C) lmm blocks embedded in hydrogel (containing 1% or 4% acrylamide, or 4% acrylamide with no PFA) were post-fixed with EDC (+) or no fix (-), then cleared and stained with acridine orange to visualize total RNA levels (false colored; RNA signal in pink). Scale bar, 200 μη. Relative intensities are quantified in FIG. ID. (FIG. ID) 1% hydrogel embedded slices post-fixed in EDC showed significantly more RNA than ail other conditions tested. Fluorescence intensities are normalized to mean intensity for all conditions (P<0.01, one-way ANOVA, with Sidak's post-hoc multiple comparisons test. n = 5 slices per group). (FIG. 1E) 1mm blocks prepared as in (FIG. 1C), hybridized with an oligo(dT) probe to detect mRNA (false colored). Scale bar, 50 μη. Relative intensities are quantified in (FIG. 1F). (FIG. IF) 1% hydrogel embedded slices post-fixed in EDC showed more mRNA than all other conditions tested. Fluorescence intensities are normalized to mean intensity for all conditions for each experiment (PO.01 One way ANOVA, Tukey's post-hoc test for multiple comparisons. n = 4 slices per condition). (FIG. 1G) lmm blocks of tissues were embedded in a 1% CLARITY hydrogel and post fixed with 0, 0.1, *I*, or 10M EDC, either for 3 hours or 1 day at 37°C. Oiigo(dT) was performed as in (FIG. IE). Relative intensities are quantified in (FIG. 1H). (FIG. 1H) Fixation with 0.1M or 1M EDC for 1 day produced optimal RNA hybridization in 1% CLARITY tissue. Fluorescence intensities from oligo(dT') are normalized to mean intensity of the no EDC condition. Asterisks indicate statistical significance compared to 0M EDC, 3 hour condition (***P<0.001, *P<0.05, One way ANOVA, Tukey's post-hoc test for multiple comparisons). n=4 slices per condition. (FIG. II) In situ hybridization in human tissue from temporal lobe resection. Left, small samples of resection from each patient were PFA fixed and ohgo(dT) hybridization was performed to confirm that mRNA was intact before clearing. The remaining tissue was immersion fixed in 1% CLARITY hydrogel (2 days), embedded, then cleared immediately (-EDC), or fixed in EDC overnight at 37°C prior to clearing (+EDC). Right, mRNA was detected by oligo(dT) and DNA was stamed with propidiurn iodide (PropI). Scale bar, 100 μη. (FIG. 1J) 3D rendering of EDC fixed human temporal lobe volume (same patient as in (FIG. 1G)). Scale bar, 100 μη. (FIG. IK) 1 mm tissue blocks (1% hydrogel, EDC postfix) were processed for RNA extraction at various time points: uncleared (immediately after post-fixation); 1 day and 1 week (while in clearing solution); 1 month and 6 months (after clearing and stored in PBST). There is no significant loss of RNA during storage even up to 6 months at 4°C (n = 6 slices per group, n.s. paired t-test). All data are means ± S.D. See also FIG. 2A, 2B.

[00110] FIG. 2A-2B. Characterization of tissue formulation and storage time for in situ hybridization, related to FIG 1A-1K. (FIG. 2A) CLARITY sections (1mm) of mouse tissue from cortex embedded in 1% CLARITY hydrogel, 4% CLARITY hydrogel, 1% CLARITY hydrogel with EDC postfixation, or A4P0 (4% aciylamide, no bis-acrylamide, no PFA during acrylamide polymerization), were cleared in 4% SDS until transparent, and in situ hybridization for somatostatin was performed on the cleared tissue. Images are maximum z-projactions from 5 planes, z-interval = 20μm. Scale bar, 100 μm. (FIG. 2B) EDC-CLAR1TY sections (1mm, 1% hydrogel) were cleared until transparent and stored in PBST for the times indicated. In situ hybridization for somatostatin was performed and confocal images were acquired. Images are maximum z-projections from 5 planes, z-interval = 20μm. Scale bar, 100 μm.

Example 2

Quantifying diffusion of ***in situ*** hybridization components into clarified tissue

[00111] After ensuring stable retention of RNAs, we next focused on access to target RNAs for specific labeling in transparent tissue volumes. Traditional in situ hybridization (ISH) uses labeled DNA or RNA probes, which are detected by enzyme-conjugated antibodies that catalyze the deposition of chromophores or fiuorophores at the target location. Interrogation of RNA by these methods requires the penetration of each component to the target location. Since prior work had only shown detection of RNA in small volumes (100-500um thick; Chung et al, 2013; Yang et al., 2014), we sought to test the ability of ISH components to diffuse into intact EDC-CLARITY tissue.

[00112] We began by characterizing the diffusion of nucleic acid probes into EDC-CLARITY tissue. We incubated tissue blocks with 50-base DIG-labeled DNA or RNA probes, and visualized the diffusion profile of these probes by cutting cross-sections through the center of the tissue blocks and quantifying probe density on the newly exposed surface via antibody-based enzymatic amplification (tyramide signal amplification; TSA) (FIG. 3A). We found that DNA probes diffused significantly faster into EDC-CLARITY tissue than corresponding RNA probes (FIG. 3B-3D); this important effect may be due to greater nonspecific tissue binding of RNA at this temperature, hindering penetration. Strikingly (and with substantial implications for nucleic acid labeling as the potential approach of choice for transparent tissue molecular phenotyping), we consistently observed DNA probes reaching the center of 2 mm tissue blocks within 3 hours. It should be noted that this detection method (TSA) may saturate

at higher concentrations and obscure more subtle underlying concentration gradients expected to be present at 1-3 hour time points, but these diffusion rates are still considerably faster than observed for antibodies (Chung et al., 2013; Tomer et al, 2014).

[00113] At 37°C (optimized for DNA-RNA hybridization), DNA probes reached the center of a 2 mm-thick block in < 1 hour (FIG. 3E). In contrast, enzyme-linked Fab antibody fragments penetrated only ~500 μη into tissue even after 2 days (FIG. 3F). Importantly, the rate of diffusion for the Fab fragment was almost two orders of magnitude slower than that of the DNA oligonucleotide (FIG. 3G) under the EDC-CLARITY-ISH condition. Taken together, these experiments reveal that short DNA probes rapidly diffuse throughout large volumes of EDC-CLARJTY tissue and suggest that an optimal approach to labeling native RNA species in large intact volumes could leverage the speed and specificity of short DNA probes in addition to EDC tissue chemistry'.

[00114] FIG. 3A-3G. DNA diffuses into CLARITY tissue more quickly than antibodies. (FIG. 3A) Tissue configuration for B,C,E, and F. 2 mm EDC-CLARITY blocks are incubated in nucleic acid probes or antibody for time indicated and fixed in 4%» PFA. 200 μm cross-sections are cut, probe diffusion is detected by TSA on the newly exposed tissue surface, and ROIs are selected as indicated by the dotted box and quantified in B,C,E, and F. (FIG. 3B, 3C) 3h incubation with DIG-labeled nboprobes or DNA oligonucleotides (50 bases) targeting two different mRNAs in 50% formamide, 5x SSC at 55°C. Top, example ROIs of tissue as shown in (FIG. 3A), pseudocolored. Cross-section is incubated in anti-DIG Fab fragment antibody conjugated to HRP and detected with TSA using FITC. Bottom, quantification of signal intensity as a function of depth for 10-15 ROIs from 3 experiments. For each ROI, no probe control is subtracted, and signal is normalized to peak intensity. (FIG. 3D) Quantification of ratio of signal intensity at tissue edge to center, calculated as maximum intensity over first 100 μm to average intensity of last 100 μm. (****P<0.0001, One way ANOVA, Tukey's post hoc test for multiple comparisons). (FIG. 3E) Diffusion of 50 base DNA oligonucleotide at shorter incubation times with hybridization conditions optimized for in situ hybridization with DNA probes (30, 60 and 180 minutes; 2x SSC, 40% formamide, 37°C). *n*= 6-12 ROIs. (FIG. 3F) Antibody diffusion. CLARITY tissue is incubated in 50 base oligonucleotide probes overnight, washed, and transferred to anti-DIG antibody conjugated to HRP for time indicated. Tissue is sectioned as in (A), and antibody-diffusion is detected by TSA. For 4 hours, n=25 ROIs; 12 hours, n~8; 24 hours, n=17; 48

hours, n=24. (FIG. 3G) Diffusion constants and R2 values for nucleic acid and antibody diffusion in CLARITY tissue. Constants calculated by fitting average curves to Fick's Law: y=nO*erfc(x/(2 *V(D*t))) for one dimensional diffusion in a uniform medium with constant boundary condition. Diffusion rate is slower than reported previously in CLARITY tissue (Li et al., 2015), which may arise from additional crosslinking during EDC fixation, or changes in tissue properties during in situ hybridization. Curves used for fitting: RNA, 3 hours incubation; DNA, 30 mm incubation; Antibody, 4 hours incubation. All error bars indicate SEM. All scale bars = 100 μm.

Example 3

In **situ** hybridization **in EDC-CLARITY**

[00115] Based on these findings that demonstrate stable retention of RNA with EDC-CLARITY and rapid penetration with short DNA probes, we next sought to develop a panel of oligonucleotide-based ISH techniques for application to large transparent tissue volumes. We began with digoxigenin (DIG)-labeled DNA oligonucleotide probes targeting somatostatin mRNA (3 probes) and amplified with anti-DIG HRP-conjugated antibody and TSA (FIG. 4A). In initial tests, we were readily able to resolve individual cells expressing somatostatin mRNA, demonstrating that specific mRNA species within the EDC-CLARITY hydrogel can be retained and are accessible to ISH probes (FIG. 4C).

[00116] However, using this technique in larger volumes revealed two major limitations: (1) the surface of the tissue sections showed non-specific staining that could result in false positives during cell detection, and (2) the signal was visible only to a depth of <300 μm (FIG. 4C). A similar pattern was seen in parallel experiments with a probe set targeting YFP mRNA in a Thyl-YFP transgenic mouse, confirming that under these conditions TSA signal at the tissue surface lacks specificity (FIG. 5C). We hypothesized that the main sources of surface staining and signal heterogeneity resulted from a concentration gradient of antibody penetrating the EDC-CLARITY hydrogel and, consequently greater surface deposition of fluorophore during enzymatic amplification. We and others have found that probes can be labeled directly with fluorophore when RNA copy-number is high and little amplification needed (Yang et al., 2014), though with limitations on sensitivity and volume size (up to 1 mm blocks, still far greater than the 20-40μm queried with traditional techniques). Nevertheless, this restriction in volume, the need for exclusion of superficial tissue, and the severe limitation to highly-

expressed transcripts together pointed to the need for further innovation to exploit the speed of DNA penetration into EDC-CLARITY tissue (FIG. 3B, 3E).

[00117] FIG. 4A-4F. Comparison of antibody-based and DNA-based amplification. (FIG. 4A) Workflow for TSA reaction. DIG-labeled probes are hybridized to target mRNA. HRP conjugated anti-DIG antibodies bind to hybridized probes and are detected by TSA. (FIG. 4B) Workflow for HCR reaction. Initiator-labeled probes are hybridized to target mRNA. In a second step, initiator sequences hybridize to toehold of fluorophore bearing hairpins, starting a chain reaction of hairpin assembly. (FIG. 4C-4D) In situ hybridization for somatostatin mRNA in CLARITY tissue. Above, z = 100 μτη, 200 μτη, and 300 μηι into CLARITY tissue volume of mouse cortex using traditional ISH (FIG. 4C) or hybridization chain reaction (FIG. 4D). Scale bars, 100 μιη. Below, yz-subsections of CLARITY volume and 3D rendering of lmm sections. Arrowheads indicate the z-location of the sections above. Arrows indicate the tissue surface. Due to high surface background, the top 130 μιη of tissue are not shown for the 3D rendering in (FIG. 4C). Scale bars, 100 μm (left), 300 μm (right). (FIG. 4C) DIG-labeled oligonucleotide probes detected with anti-DIG antibody (2 days) and TSA. Somatostatin expressing cells can be detected, but surface has high background and signal diminishes deeper in the tissue. (FIG. 4D) Initiator-labeled oligonucleotide is detected with HCR reaction (1 day), resulting in more uniform staining. (FIG. 4E) Number of cells as a function of tissue depth after local thresholding and cell segmentation on each imaging plane, 10 μιη z-interval. High surface background in TSA reaction yields a large number of putative false positives 0-75 μm into the tissue section. Detection with HCR amplification shows a more uniform labeling of cells, comparable to the distribution of somatostatin cells in a genetically encoded reporter mouse (Sst-TFP). (FIG. 4F) Ratio of signal to background as a function of depth in tissue, calculated from ratio of mean signal intensities segmented in (F) to the mean background intensity. For (FIG. 4E-4F), No Probe, **n=3;** Scrambled Control, n=3; TSA, n=5; HCR, n=v; Sst-TFP, n=4. PO.05, Kruskal-Wallis test on mean ratio over entire depth. All error bars indicate SEM. See also FIG. 5A-C.

[00118] FIG. 5. Validation of amplification specificity, related to FIG. 4A-4F. (FIG. 5A) EDC-CLARITY sections (1 mm) of tissue from Thyl-YFP mouse cortex. YFP probes were either hybridized at 45°C (50% formamide, 5x SSC) or at 37°C (40% formamide, 2x SSC) overnight and amplified using HCR. (Top) Three dimensional rendering of YFP fluorescence after in situ hybridization. (Bottom) Three dimensional rendering of YFP mRNA by HCR-based in situ hybridization. Scale bar, 200 μηι (FIG. 5B) YFP

fluorescence from EDC-CLARITY tissue after in situ hybridization at 37°C or 45°C, calculated as the ratio of mean intensity of signal to the mean intensity of the background. Each data point represents one volume. Representative volumes in (FIG. 5A) are indicated in red. (FIG. 5C) EDC-CLARITY sections are hybridized with YFP probes labeled with either DIG or initiator sequences and amplified with TSA or HCR, respectively. Cells identified by YFP protein fluorescence (green) or YFP ISH (black) after local thresholding and cell segmentation are plotted against tissue depth; 10 μm z-interval. High background on tissue surface with TSA amplification produces many false positives 0-50 μτη from the tissue surface that are not seen in the YFP protein controls (n=3 for each condition, error bars represent SEM).

Example 4

DNA-based ISH signal **amplification**

[00119] We hypothesized that an all-DNA based amplification system rather than the traditional antibody approach might be an ideal solution. Recent work has capitalized upon the programmable base-pairing of DNA molecules to design DNA structures that amplify signal by several orders of magnitude (Battich et al., 2013; Choi et al., 2010). We explored integrating this approach with EDC-CLARITY tissue chemistry, selecting the hairpin chain reaction amplification system (HCR; Choi et al., 2010) for further development since HCR (a) involves only small DNA oligonucleotides (<150 bases) which self-assemble at the target mRNA, and (b) requires only two hybridization steps (FIG. 4B).

[00120] In the first hybridization step, an oligonucleotide probe containing a 36-base initiator sequence binds to target mRNA. In a second step, two fluorophore-tagged oligonucleotides are added, which are kineticaliy trapped in a hairpin conformation in the absence of the initiator sequence. As they diffuse into the tissue and encounter initiator sequences on hybridized probes, base pairing between the initiator sequences and the single-stranded toehold on Hairpm 1 open the hairpin, revealing a new initiator sequence capable of opening Hairpm 2. In turn, Hairpm 2 opens to reveal the original initiator sequence, starting the cycle anew. As the chain self-assembles, fluorophores accumulate at the target location. It is estimated that the hairpin chain reaction can amplify the signal approximately 200 fold (Choi et al., 2014), and we expected that this degree of amplification might be sufficient to detect RNA in EDC-CLARITY.

[00121] To test this approach, we appended initiator sequences to the 3' and 5' ends of the three somatostatin oligonucleotide probes used above, hybridized the probes to EDC-CLARITY tissue, and amplified with HCR hairpins. We found that the combination of EDC-CLARITY and HCR amplification exhibited excellent signal, low background, produced no non-specific surface staining and signiiicantly improved the depth at which we could identify individual ceils (FIG. 4D). The signal-to-background ratio was significantly higher than in TSA-based amplification (FIG. 4F) with the characteristic sparse pattern of somatostatin mRNA expression clearly distinguishable from background (FIG. 4D). Moreo ver, the distribution and cell density detected with HCR amplification mirrors somatostatin expression in transgenic repoiter mice, underscoring the specificity of this method (Sst-TFP, FIG. 4E).

Example 5

in situ **hybridization in** intact tissue

[00122] Linking information on cellular morphology, connectivity, and activity to information on RNA expression will be of substantial value; accordingly, we sought conditions for in situ hybridization in EDC-CLARITY that maintained fluorescence of transgenically expressed proteins. As a proof of concept, we performed in situ hybridization for YFP mRNA on Thy 1-YFP transgenic mouse tissue and formulated a hybridization buffer that allowed reduction of hybridization temperature from 45°C to 37°C, which improved fluorescence in dendrites and axons while maintaining ISH specificity (FIG. 6A, 5A-5C). To provide a generalizable framework for HCR-based RNA detection in EDC-CLARITY, we used these hybridization conditions to design, test, and refine sets of 50mer DNA probes for several representative and broadly-useful target RNAs for molecular phenotyping in nervous system tissue: somatostatin, parvalbumin, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), tachykininl, tachykinin2, tyrosine hydroxylase, and Malatl. These targets showed reliable signal in EDC-CLARITY tissue and corresponded to known anatomical distributions in both neural and non-neural tissue (FIG. 6B-6I, FIG. 7A,B).

[00123] In refining these probe sets, we typically performed initial testing on pools of 5-10 probes; in cases where we observed non-specific staining, we then tested probes individually to identify and discard probes contributing significantly to background, which improved image quality (FIG. 8A). Under these conditions, we estimate that HCR in CLARITY tissue results in -50 fold amplification per double-initiator-labeled probe

(FIG. 8D-8F). In agreement with previous results, increasing the number of initiators, whether by adding initiators to both 5' and 3' ends or by adding more probes, enhances signal substantially. This effect may eventually saturate if limited by slightly sublinear amplification (FIG. 8C), but low copy number transcripts may still benefit from a larger set of probes. In comparing these results to published data from single-ceil transcriptomics (Zeisel et al., 2015), we find that our data capture relative differences among gene expression levels across 2 orders of magnitude (FIG. 8G-8H); indeed, with 4 probes per target, this approach allows detection of mRNAs present at as low as -50 copies/cell (FIG. 8H). Although not as sensitive as RNAseq, probe sets can be expanded as shown below, and the large volumes processed in a single CLARITY experiment enable inclusion of spatial information and sampling from many more cells than would be achieved with RNAseq (particularly important if genes are expressed in sparse subsets within a tissue).

[00124] Since low copy-number transcripts may benefit from additional probes, and since it was important to determine if our methodology could be readily adapted to diverse probe design strategies, we tested the feasibility of using a larger set of shorter probes by attaching initiators to the 5' end of probe sets originally designed for single-molecule fluorescent in situ hybridization (smFISH), which typically uses 20mer oligonucleotides (30-50 probes) that tile the mRNA target sequence. As with directly fluorophore-labeled 20mer probes, we expected that with many HCR-labeled 20raers, the on-target signal would accumulate in cells in which many probes bind and amplify (whereas off-target binding would be uniform across the sample); we did not, however, expect that HCR with these probes would provide single-molecule capability. Using this strategy, we were able to detect tyrosine hydroxylase, SERT, and Drd2 mRNA in EDC-CLARITY tissue, demonstrating that the HCR approach is adaptable to other probe types in CLARITY and compatible with larger pools of short probes (FIG. 6J-6L).

[00125] Because longer nucleotides are more expensive to synthesize and purify, the strategy of using short probes would reduce overall cost and may enable significantly greater signal amplification. Likely owing to the quick and uniform diffusion of DNA probes and hairpins, we find that tissue blocks up to at least 3 mm thick could be reliably-used for intact in situ hybridization (FIG. 6M). Another unique advantage of nucleic acid detection (relative to antibody-based detection) is that once the target sequence is known, it is possible to design probes for the target which are highly specific, permanently renewable, and cost-effective. We therefore anticipate that this methodology for RNA

detection in EDC-CLARITY may be versatile for probing a variety of transcriptional products across many tissue-types and species.

[00126] FIG. 6A-60. Cell-type phenotyping in CLARITY tissue using DNA probes and HCR amplification. (FIG. 6A) 3D rendering of 1-mm-thick coronal section from Thyl-YFP mouse, in situ hybridization for YFP mRNA in red, endogenous YFP fluorescence in green. Scale bar, 200 μιη. Inset, 3D rendering of boxed section in cortex. Scale bar, 50 μm. (FIG. 6B-6L) 3D rendering of in situ hybridization performed in 0.5mm coronal CLARITY sections using 50mer DNA oligonucleotide probes. Scale bars, 500 μτη; insets, 50 μη, unless otherwise noted. FIG. 6B) Somatostatin mRNA (4 probes). (FIG. 6C) Parvalbumin mRNA (4 probes). Inset scale bar, 70 μη. (FIG. 6D) Neuropeptide Y mRNA (5 Probes). (FIG. 6E) Tyrosine hydroxylase mRNA (10 probes). (FIG. 6F) Vasoactive Intestinal Peptide (VIP) mRNA (10 probes). (FIG. 6G) Tachykininl mRNA (5 probes). Scale bar, 1 mm; inset 100 μη. (FIG. 6H) Tachykinin2 mRNA (4 probes). Scale bar, 1000 μm; inset of BNST and cortex, 50 μτη. (FIG. 61) Malatl mRNA (4 probes). (FIG. 6J-6L) 3D rendering of in situ hybridization performed in 0.5mm CLARITY sections using 20mer DNA oligonucleotides. (FIG. 6J) SERT mRNA (47 probes). (FIG. 6K) Tyrosine hydroxylase mRNA (39 probes). (FIG. 6L) Drd2 mRNA (39 probes). Scale bar, 1500 μm, inset; 50 μτη. (FIG. 6M) Left, 3D rendering of 2mm block of mouse cortex, processed with EDC-CLARITY with in situ hybridization for somatostatin using HCR amplification. (Middle) orthogonal view of volume at left, showing signal throughout tissue depth. Scale bars, 200 μη. (Right) magnified view of somatostatin expressing cells in cortex from volume at left. Scale bar, 50 μηι. See also FIG. 7A-7, 8A-8H.

[00127] FIG. 7A-7B. Application of EDC-CLARITY to non-neural tissue, related to FIG 6A-60. (FIG. 7A) Left, Three-dimensional rendering of a lmm thick EDC-CLARITY section of mouse pancreas with in situ hybridization for somatostatin. Scale bar, 300μτη. Right, expanded view of box at right. Delta cells in pancreatic islets are prominently labeled. Scale bar, 50μm. (FIG. 7B) Left, Three dimensional rendering of an EDC-CLARITY section of mouse small intestine with in situ hybridization for VIP. Scale bar, lOOμm. Right, orthogonal views of tissue at left. Large cells positive for VIP (arrows) are putative peripheral neurons in the submucosal plexus. Smaller puncta in the circular folds correspond well with the distribution of VIP expressing enteroendocrine cells (arrowheads). Scale bar, 200μm.

[00128] FIG. 8A-8H. Characterization of HCR probe design and amplification sensitivity, related to FIG 6A-60. (FIG. 8A) In situ hybridization for parvalbumin in 500 μη EDC-CLARITY sections. Confocal images of parvalbumin ISH in cortex. Initial testing of 5 probe cocktail targeting parvalbumin (PV1-5) had high background. Probes were tested individually and probes 1-3 showed specific signal. Omitting probes 4 and 5 decreased background. A second set of 4 probes contains one specific probe (PV8). For parvalbumin, all working probes targeted the coding region of the mRNA. Blue arrows indicated probes tested, red outlines indicate successful probes. All scale bars, 100 μm. (FIG. 8B) Histogram of cell fluorescence intensities for individual somatostatin probes, or combinations of 2 or 4 probes. In situ hybridizations were performed on 500fim thick CLARJTY-EDC sections and amplified with HCR. Cells are segmented, mean fluorescence intensity is plotted, and normal distributions are fit to the data. (n=3 experiments). (FIG. 8C) Average of mean cell intensities per experiment, from data shown in (FIG. 8A), normalized to the average of all individual probes (columns 1-4). Dotted lines represent the linear sum of 2 or 4 probes. (n=3 experiments). Data are means ± S.D. (FIG. 8D) Estimation of fold amplification with HCR. 500μη EDC-CLARITY tissue was hybridized under two conditions. In the first condition, one set of somatostatin probes is labeled with B1 initiators and another set targeting difference sequences is labeled with B5 initiators. Both are amplified with HCR, but with different fluorophores: Bl-Alexa647 and B5-Alexa514. In a second condition, one group of probes is labeled with B5 initiators amplified with Alexa514, but the other probes are labeled directly with Alexa647. (FIG. 8E) Somatostatin containing cells were identified using the control Alexa514 channel and the mean fluorescence intensity for both channels was calculated for each cell (average background of each ROI was subtracted from mean intensity). Data for one representative experiment is plotted in (FIG. 8E) as the signal intensity in the Alexa647 channel (for either directly-labeled or HCR-amplified probes) as a function of the control HCR-amplified, Alexa514. Inset highlights low range of y-axis. There is good correlation between the two channels and the relationship is linear, suggesting amplification is proportional to transcript number. (FIG. 8F) Histogram of fluorescence intensities of the Aiexa 647 channel for directly labeled or HCR-amplified probes from 4 experiments as in (FIG. 8E). The ratio of the mean HCR value to the mean directly labeled value suggests that there is -40 fold amplification. (FIG. 8G) Genes with mRNA copy numbers ranging over several orders of magnitude were selected from a published dataset in which single cell RNA-seq data were collected from 1691 cells in mouse

cortex (Zeisel et al, 2015). To compare with our dataset, which predominantly uses interneuron cell markers that are highly expressed in one subpopulation but much lower elsewhere, we excluded cells in the RNA-seq data set corresponding to detection of fewer than 5 molecules. Red bars indicate the mean of all cells with >5 transcripts of the gene indicated. (FIG. 8H) In situ hybridizations for 7 different mRNAs were performed in parallel on 500 μη EDC-CLARITY sections of cortex with comparable ROIs taken with identical imaging parameters. To improve cell identification for more weakly expressing mRNA, images were first acquired at the same gain, and then weak signals were imaged again at increased gain. High gain was used to detect cells, but all measurements are from the low gain images, which were the same across all transcripts. Representative cells are shown in the inset and pseudocolored. Below, mean fluorescence intensities are plotted for all cells from three separate experiments, using the same imaging and cell detection parameters for each mRNA. For ease of visualization, a random subset of 1000 cells is shown for Malatl. Red bars indicated the mean intensity for segmented cells; blue bars indicate mean background fluorescence. We are able to detect Npas4 expressing cells, which RNA-seq data from (Zeisel et al., 2015) suggests contain -50 copies of Npas4 per cell (average molecules detected is -10, adjusted for a 22% capture rate).

Example 6

Detection of **activity-dependent** genes and non-coding **RNAs** in intact volumes

[00129] Many mRNAs are transiently up-regulated by activity, a fact that has been instrumental in identifying cells and circuits recruited during particular behaviors (e.g. Loebrich and Nedivi, 2009). Using such immediate early genes (lEGs), it has been possible to identify neurons involved in complex behaviors (even multiple behaviors separated in time; Guzowski et al., 1999; Reijmers et al, 2007), to visualize behavioraily relevant neurons in transgenic mice, in some cases long after the behavior itself (Barth et al, 2004; Guenthner et al., 2013; Smeyne et al, 1992), and to manipulate these IEG-expressmg neurons to modify or recapitulate the observed behavior (Gamer et al, 2012; Liu et al., 2012; Ramirez et al, 2013). Yet a major unmet goal is linking form and function: to align these transcriptional activity changes with molecular phenotype and connectivity information in large intact volumes. We therefore next designed HCR probe sets against several canonical activity-regulated transcripts: Arc (Lyford et al., 1995), c-fos (Sheng et al., 1990), and Npas4 (Bioodgood et al., 2014), and tested these probes in a

kainic acid seizure model (known to induce robust hippocampal transcription of many activity-regulated genes; Nedivi et al., 1993)). We found that we were able to reliably track changes in expression of all of these activity-regulated genes in EDC-CLARITY. For example, Npas4 is normally expressed in scattered cells in cortex but robustly transcribed in both hippocampal pyramidal cells and interneurons after seizure activity (FIG. 9A). In parallel experiments, increases in c-Fos transcription in hilar neurons and Arc transcription in dentate granule cells were readily detectable (FIG. 9B-9C), as described previously in hippocampal seizure models (Lyford et al., 1995).

[00130] Lastly, we assessed detection of small-noncoding RNAs- a major motivation for this entire approach since these are undetectable by antibodies yet also are 1) critical for the modulation of post-transcriptional gene expression; 2) play key roles in human genetic diseases (Esteller, 2011); and 3) represent a wealth of biological information not yet approached by any tissue clearing technique. Indeed, due to small size, microRNAs have fewer amines to react with paraformaldehyde or acrylamide and are easily lost from fixed tissues (Pena et al, 2009; Renwick et al, 2013).

[00131] Consistent with this expected challenge, we found that post-treatment with EDC was critical for the retention of miRNAs in EDC-CLARITY (FIG 10A). Using DTG-labeled locked nucleic acid probes, we targeted several miRNAs with known function in the mammalian brain and in neuropsychiatric disease (miR-10, miR-124, miR-128), as well as a miRNA known to exist only outside the mammalian brain (miR-21) to serve as a negative control (Landgraf et al, 2007). We detected robust expression of these miRNAs in volumes of mouse brain, in a pattern largely limited to areas with anticipated expression (as inferred from miRNA deep sequencing experiments). miR-10 signal (for instance) was almost exclusively recovered in the thalamus, miR-124 was observed to be more ubiquitously present throughout the brain, and miR-128 showed characteristic forebrain and cerebellar enrichment (FIG 9D, 10B-IOC). We observed minimal signal for miR-21 under the same detection and amplification conditions, as expected, highlighting the specificity of the miRNA signals observed.

[00132] miR-128 is particularly well-studied in the context of its known disease-relevance for oncogenic suppression (Pang et al., 2009) and predisposition to mood disorders (Zhou et al, 2009) but until now, miR-128 expression has not been visualized volumetrically in the mammalian brain at single cell resolution, which we were readily able to achieve here (FIG. 9D). To further test potential utility of this approach in the study of brain disease, we endeavored to detect miR-128 in human clinical samples to

determine if differences in expression might be associated with human glioblastomas (suggested but not directly observed; Ciafre et al., 2005). We indeed were able to detect miR-128 in human GBM samples processed in EDC-CLARITY hydrogel; moreover, by integrating antibody staining (in this case, GFAP to mark tumor location) with ISH in EDC-CLARITY, we could track the crucial relative relationships of GFAP and miR-128 expression across the tissue volume at cellular resolution (FIG. 9E). Such an approach designed to provide 3D volumetric access to miRN As in biopsied or post-mortem human brain samples may be valuable in the search for tissue-level disease insights, biomarkers, and therapeutic targets for neurological and psychiatric disease.

[00133] FIG. 9A-9B. Detecting activity-induced transcripts and non-coding RNAs in CLARITY volumes. (FIG. 9A-B) 3D rendering of 0.5mm CLARITY section, HCR in situ hybridization in control saline injected (left) and kainic acid injected (right) animals. Kainic acid, 12 mg/kg, i.p., 2 hours prior to perfusion. (FIG. 9A) Npas4 mRNA (4 probes). Scale bar, 200 μη1. Right, magnified view of indicated boxes. Scale bar, IOOμιη. (FIG. 9B) Arc mRNA (5 probes). Right, magnified view of indicated boxes. Scale bar, 50μm. (C) c-fos mRNA (45 probes). Scale bars 500um; Right, magnified view of dentate gyrus as indicated by dotted box. Scale bar, 100 fim. (D) Left, projection image of 1 mm mouse brain sagittal section, cleared, and hybridized with DIG-labeled LNA probes for mature miR-128 sequence. Scale bar, 500 μm. Middle, right 10x zoom of hippocampal and striatal volumes respectively. Scale bar, 150 μm. (E) Left, projection images of human brain control (left) and tumor (GBM) (middle) samples, cleared and in situ hybridized for miR-128 (green). Scale: 50 μm (Right). Volume reconstruction of human GBM tumor biopsy sample (200 μη1 thick; scale: 50 μτη) also stained with antibody to GFAP (red). miR-128 and GFAP have orthogonal signal gradients within the tumor preparation. See also FIG. 1OA-IOC.

[00134] FIG. lOA-lOC. Detection of microRNAs in CLARITY tissue, related to FIG. 9A-9B. (FIG. 10A) Projection images of 5x confocally acquired and tiled 1 mm mouse brain sagittal sections, cleared, and in situ hybridized with DIG-labeled LNA probes complementary to the mature miR-128 sequence without (left) and with (right) EDC fixation. Brain regions indicated as follows: forebrain (brackets), hippocampus (asterisk), thalamus (arrowhead). Scale: 700 μη1 (left) and 800 μm (right). (FIG. 10B) Projection images of 10* confocally acquired 1 mm mouse brain coronal sections, cleared and in situ hybridized with DIG-labeled LNA probes complementary to the mature miR-128, miR-lOb, miR-124, and miR-21 sequences. miR-128 is preferentially expressed in

hippocampus (asterisk), miR-10 in thalamus (arrowhead), and miR-124 in both. There is minimal expression of miR-21 in either structure (consistent with sequencing data suggesting lack of miR-21 expression in adult brain tissue). Scale: 100/100/100/50 μm. (FIG. 1OC) Projection images of 5x confocally acquired and tiled 1 mm mouse brain sagittal section, cleared and in situ hybridized with DIG-labeled LNA probes complementary to the mature miR-128, miR-10, and miR-21 sequences. Scale: 150 μm. Brain regions indicated as follows: hippocampus (asterisk), thalamus (arrowhead).

Example 7

Multiplexed molecular **phenotyping**

[00135] Finally, we sought to develop methods for multiplexed detection of RNA in EDC-CLARITY to address the critical and rapidly-growing need for multiple overlaid markers of cell identity or activity in the native anatomical context. Using multiplexed hybridization and amplification with orthogonal hairpin sets (Choi et al, 2014), we were able to simultaneously label multiple mRNAs in EDC-CLARITY. Of note, although orthogonal hairpins were equivalent in amplification, individual fiuorophores varied in fluorescence signal, as may be expected by differences in tissue autofluorescence, fluorophore efficiency, and light iransmittance at different wavelengths (FIG 12A, 12B). Nevertheless, somatostatin, parvalbumin, and tyrosine hydroxylase could be simultaneously hybridized and amplified with sets of orthogonal hairpins carrying Alexa514, Alexa647 and Alexa546 fiuorophores, respectively (FIG. 11A). We were also able to combine in situ hybridization for cell-type markers with in situ hybridization for activity markers (FIG. 1IB). Taken together, these data demonstrate key steps toward integrated investigation of cellular structure and typology, microRNA expression, and activity-regulated gene transcription within intact tissue volumes.

[00136] FIG. 11A-11B. Multiplexed detection of mRNAs in CLARITY. (FIG. 11A) Left, multiplexed in situ hybridization of 0.5mm coronal CLARITY section treated with kainic acid, using somatostatin (red), parvalbumin (blue) and tyrosine hydroxylase (green) probe sets. Scale bar, 500 μm. Middle, inset of caudal hippocampus showing parvalbumin and somatostatin interneurons in CA1 region. Scale bar, 50 μm. Right, parvalbumin and tyrosine hydroxylase positive cells in midbrain. Scale bar, 100 μm. (FIG. 1IB) 3D rendering of lmm CLARITY block, HCR in situ hybridization for somatostatin (red) and Arc mRNA (green) in control saline injected (left) and kainic acid

injected (right) animals. Right, magnified view of indicated boxes. Scale bar, 100 μm. See also FIG. 12A-12B.

[00137] FIG. 12A-12B. Characterization of orthogonal hairpins, related to FIG 11A-11B. (FIG. 12A) Four orthogonal hairpins sets have equivalent amplification in CLARITY-EDC tissue. In situ hybridizations for somatostatin were performed using the same probe sequences and fluorophore (Alexa647), with 4 different hairpin sets (B1, B2, B4, and B5, as described in (Choi et al., 2014)), and normalized to the mean intensity of all conditions (n=3) Data are means ± S.D. (FIG. 12B) Relative fluorescent intensities of different Alexa fluorophores. In situ hybridization for somatostatin was performed on 500 μm CLARITY tissue using B1 hairpins conjugated to the dyes indicated. Fluorescence intensity of somatostatin cells was calculated, background auto fluorescence was subtracted for each channel, and then normalized to the mean intensity of all conditions (n=3) Data are means ± S.D.

[00138] Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined herein.

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Claims

What is claimed is:

1. A method of preparing a biological specimen for microscopic analysis of a target RNA analyte, the method comprising:

fixing the specimen with a plurality of hydrogel subunits;

polymerizing the hydrogel subunits to form a hydrogel-embedded specimen;

fixing RNA in the specimen using carbodiimide mediated crosslinking:

clearing the hydrogel-embedded specimen wherein the RNA is substantially retained in the specimen; and

contacting the specimen with a nucleic acid probe for a target RNA analyte.

2. The method of claim 1, wherein the carbodiimide comprises l-Ethyl-3-3-dimethyl-aminopropyl carbodiimide (EDC).

3. The method of any one of claims 1-2, wherein the nucleic acid probe undergoes a Hybridization Chain Reaction (HCR).

4. The method of any one of claims 1-3, wherein the nucleic acid probe is a DNA probe.

5. The method of any one of claims 1-3, wherein the nucleic acid probe is a RNA probe.

6. The method of any one of claims 1-5, wherein the specimen is stored for at least one week prior to the contacting.

7. The method of any one of claims 1-5, wherein the specimen is stored at 4°C for a period of one week to a year prior to the contacting.

8. The method of any one of claims 1-5, wherein the specimen is stored at 4°C for a period of one week to six months prior to the contacting.

9. The method of any one of claims 1-8, wherein the contacting comprises contacting the specimen with a plurality of nucleic acid probes for a plurality of target RNA analytes.

10. The method of any one of claims 1-9, wherein the clearing comprises substantially removing a plurality of cellular components from the specimen.

11. The method of any one of claims 1-9, wherein the clearing comprises substantially removing lipids from the specimen.

12. The method of any one of claims 1-11, wherein the clearing comprises electrophoresing the specimen.

13. The method of claim 12, wherein the electrophoresing comprises using a buffer solution comprising an ionic surfactant.

14. The method of claim any one of claim 1-13, wherein the specimen is a biopsy specimen or autopsy specimen.

15. The method of claim any one of claim 1-14, wherein the specimen is from a human.

16. The method any one of claims 1-15, wherein the method further comprises imaging the specimen using confocal microscopy, two-photon microscopy, light-field microscopy, tissue expansion microscopy, and/or CLARITY™-optimized light sheet microscopy (COLM).

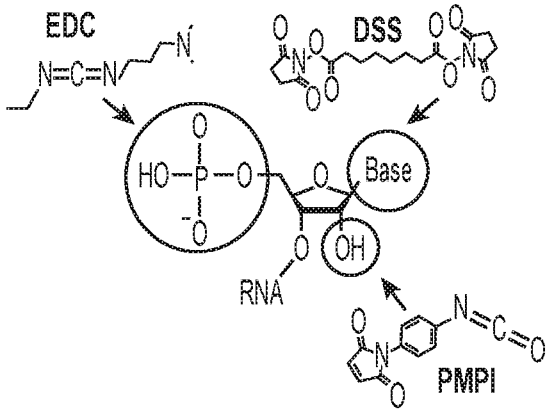


FIG. 1A

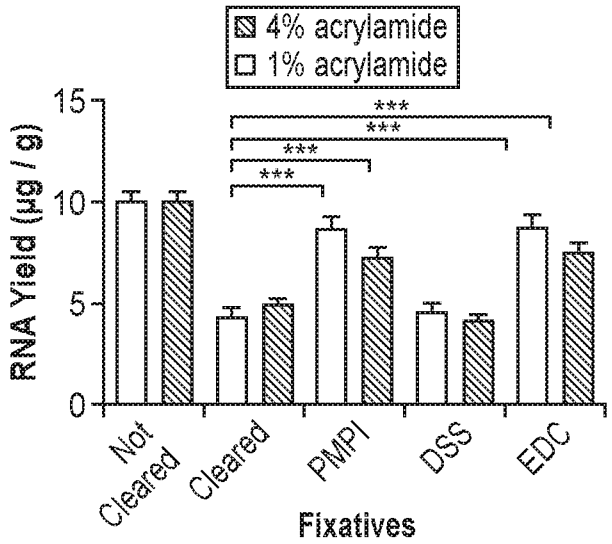


FIG. 1B

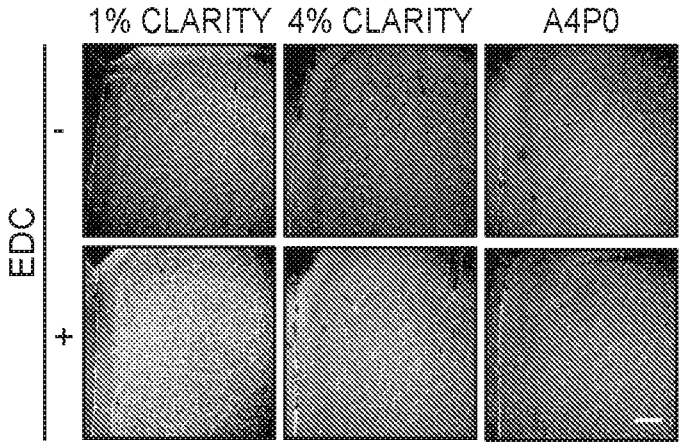


FIG. 1C

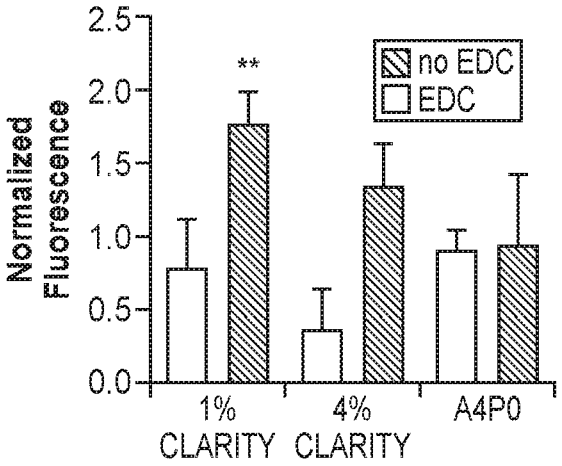


FIG. 1D

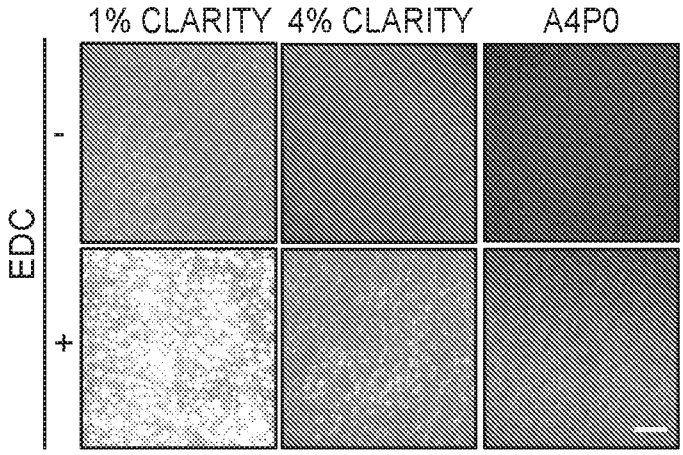


FIG. 1E

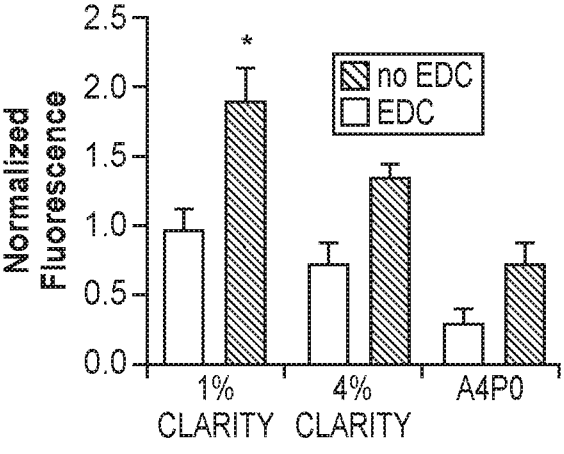


FIG. 1F

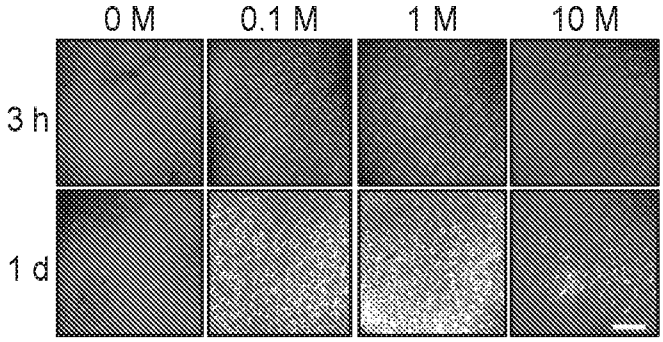


FIG. 1G

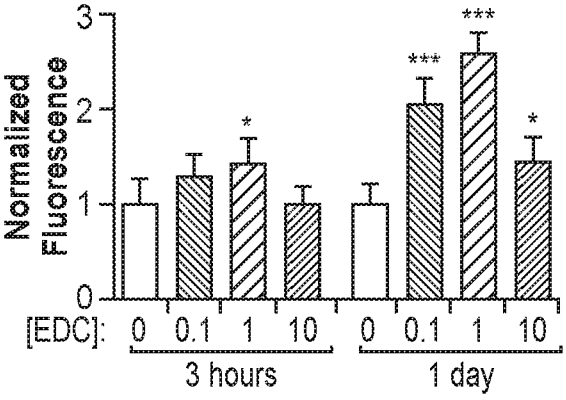


FIG. 1H

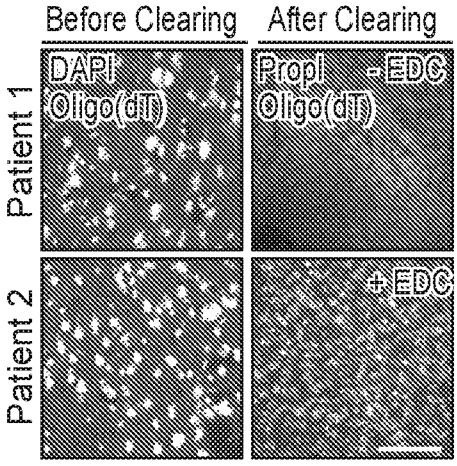


FIG. 1I

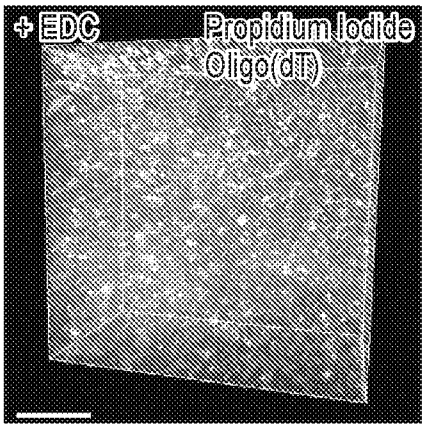


FIG. 1J

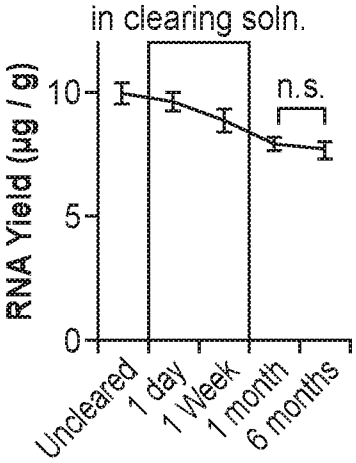


FIG. 1K

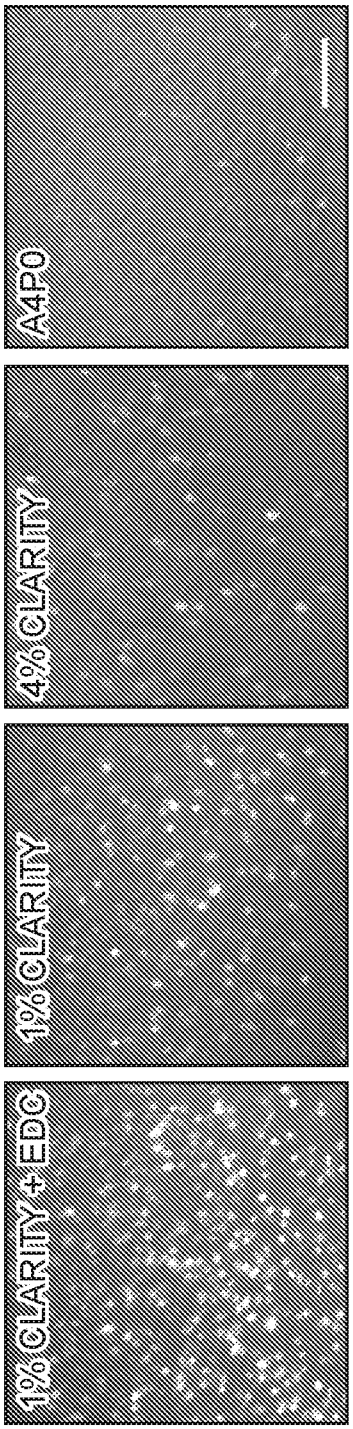


FIG. 2A

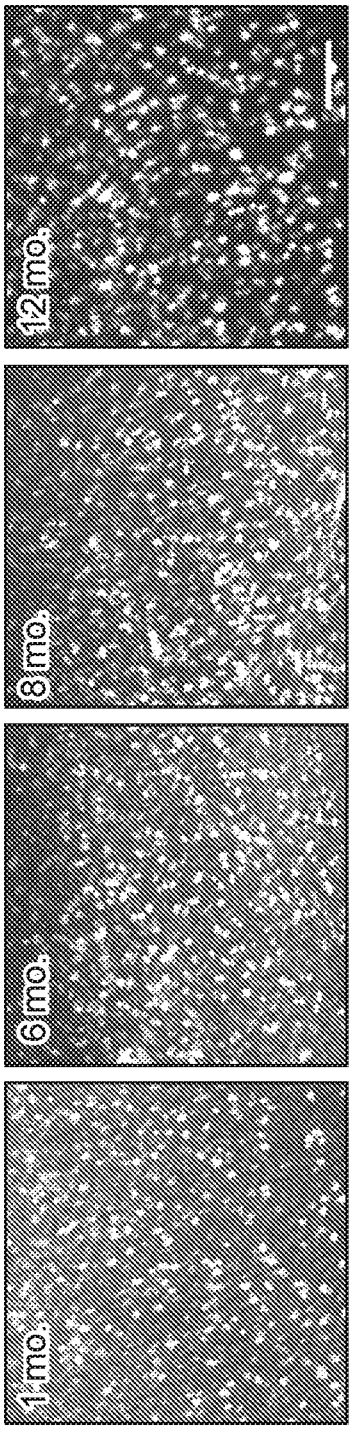


FIG. 2B

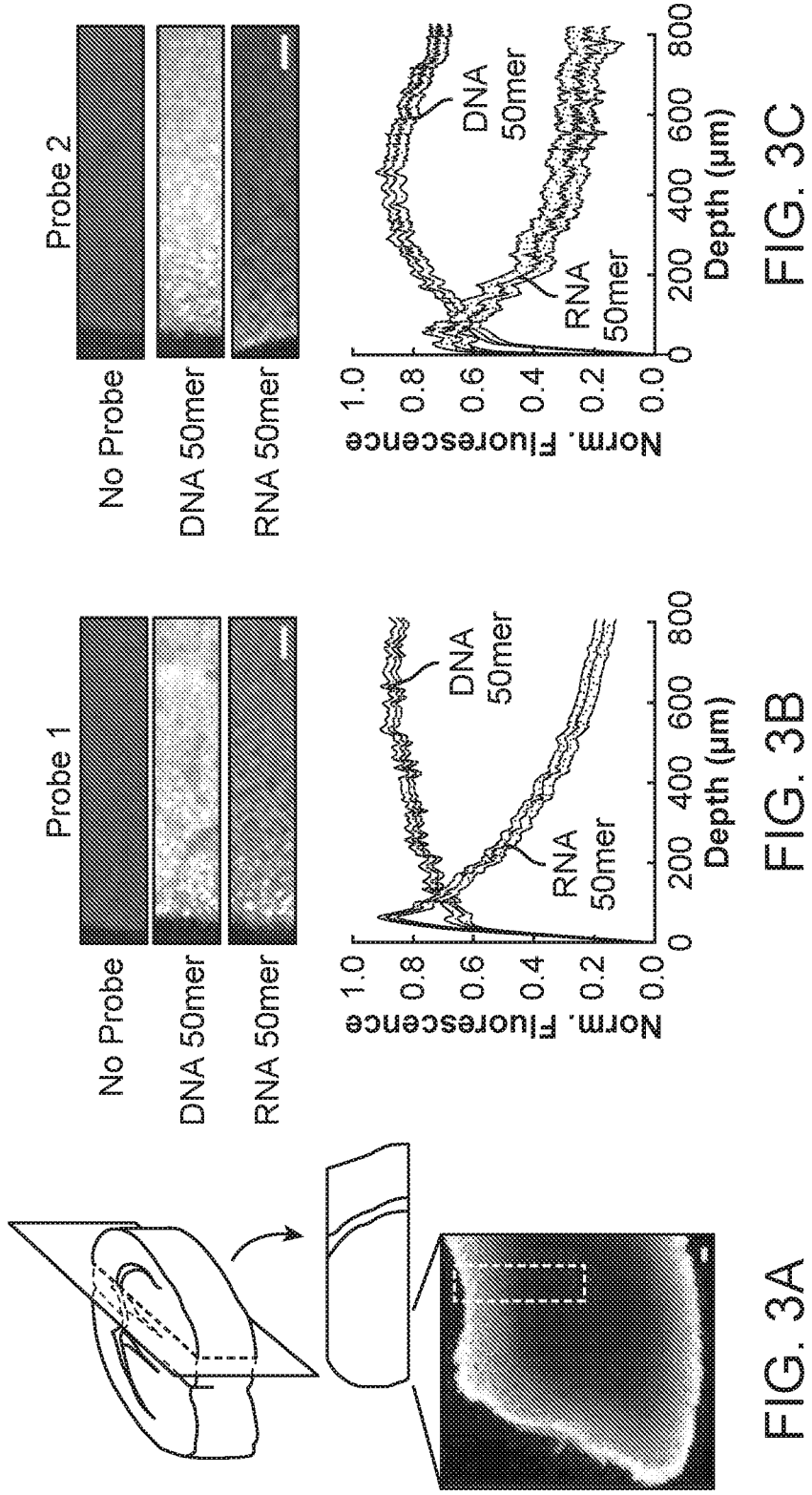


FIG. 3C

FIG. 3B

FIG. 3A

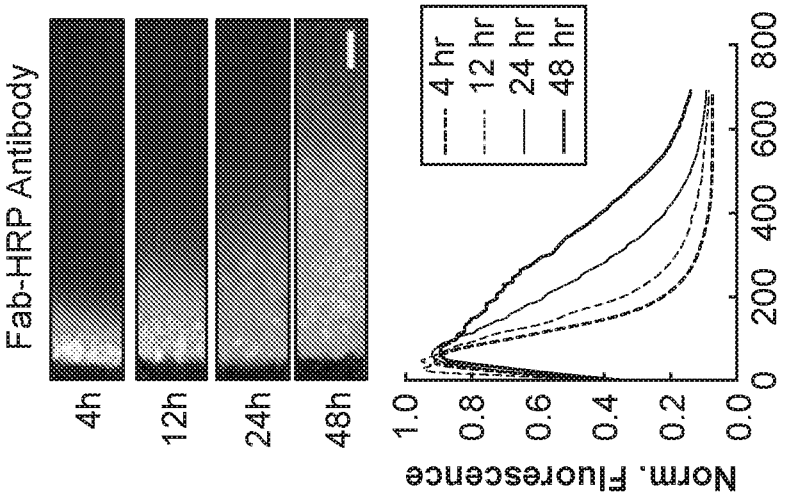


FIG. 3F

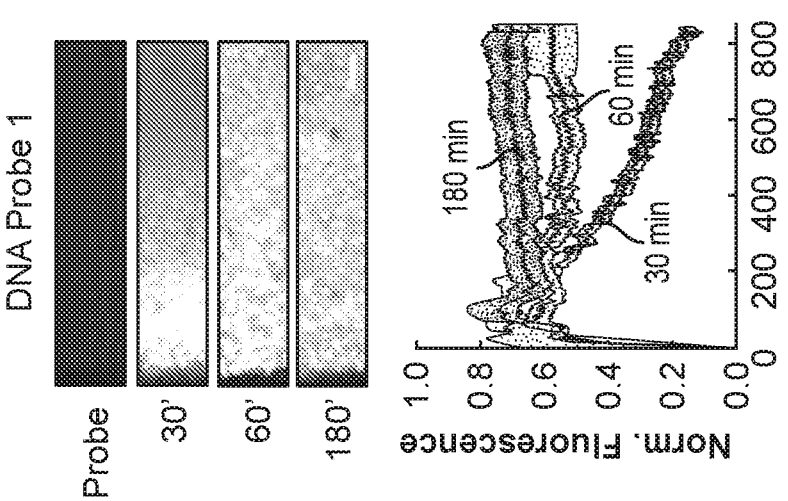


FIG. 3E

Diffusion Constant $\mu m^2/s$		
RNA 50mer #1 (55°C)	5.1	R^2 = .99
RNA 50mer #2 (55°C)	4.6	R^2 = .92
RNA 50mer #1 (37°C)	41	R^2 = .99
Fab Fragment (55°C)	0.60	R^2 = .99

FIG. 3G

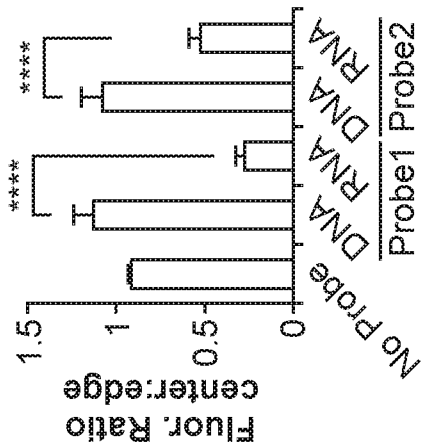
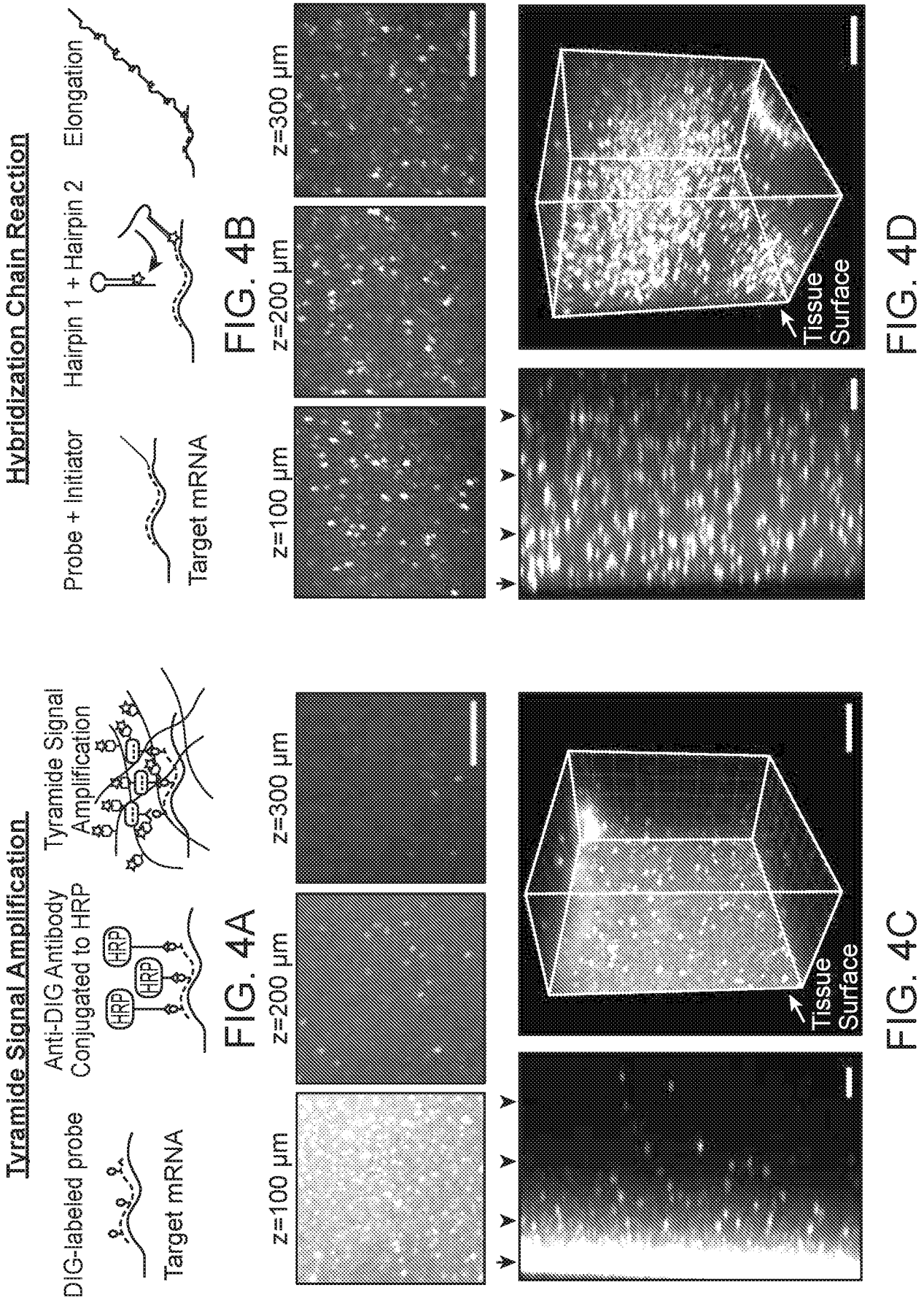


FIG. 3D


Tyramide Signal Amplification
DIG-labeled probe
Target mRNA
Anti-DIG Antibody Conjugated to HRP
HRP
Tyramide Signal Amplification
z=100 µm
z=200 µm
z=300 µm
FIG. 4A
Hybridization Chain Reaction
Probe + Initiator
Target mRNA
Hairpin 1 + Hairpin 2
Elongation
z=100 µm
z=200 µm
z=300 µm
FIG. 4B
Tissue Surface
FIG. 4C
Tissue Surface
FIG. 4D

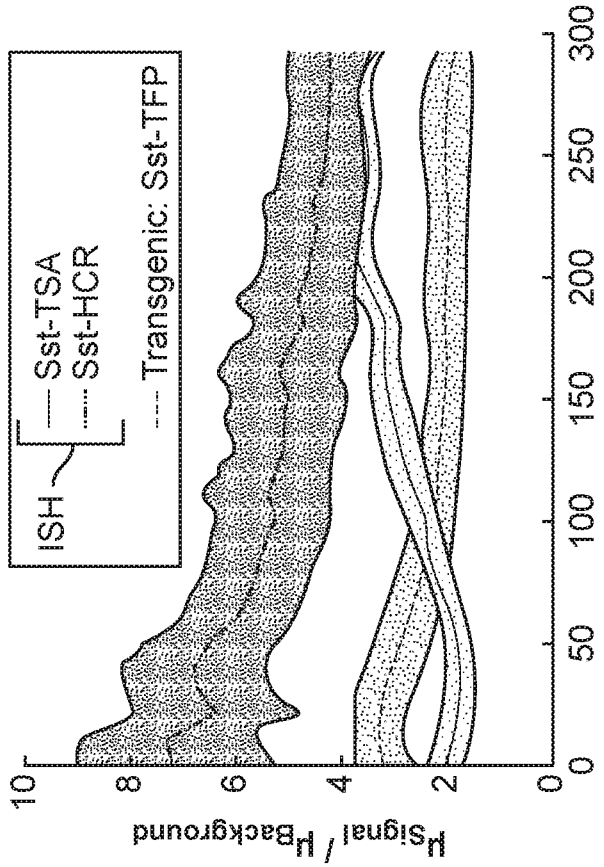

ISH
Sst-TSA
Sst-HCR
Transgenic: Sst-TFP
μSignal / μBackground
Depth (μm)
0
50
100
150
200
250
300
2
4
6
8
10

FIG. 4F

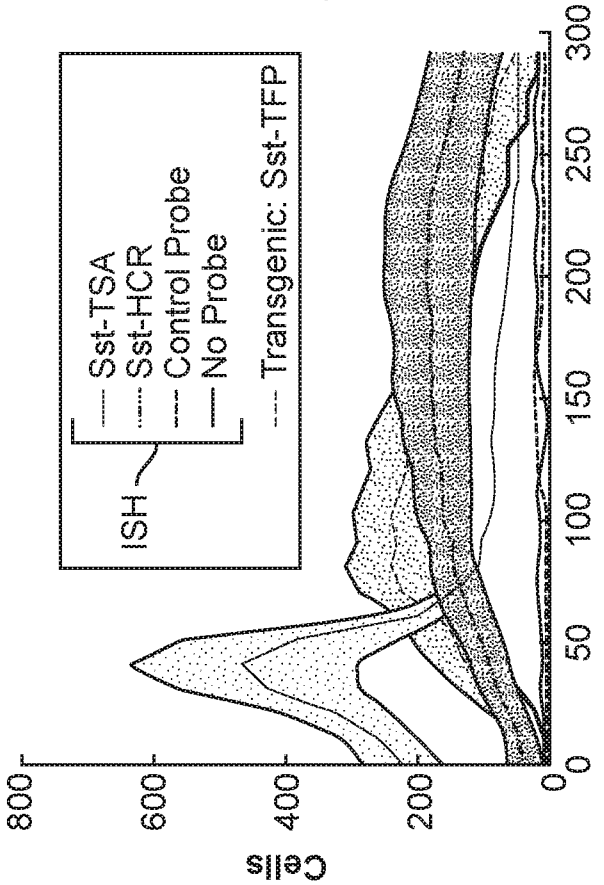

ISH
Sst-TSA
Sst-HCR
Control Probe
No Probe
Transgenic: Sst-TFP
Cells
Depth (μm)
0
50
100
150
200
250
300
200
400
600
800

FIG. 4E

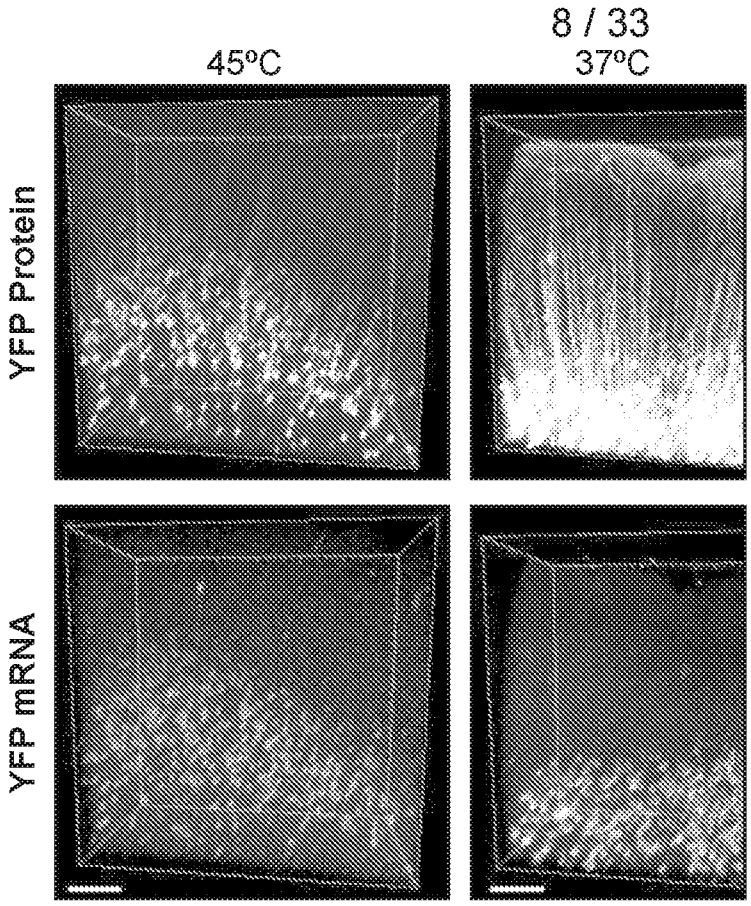


FIG. 5A

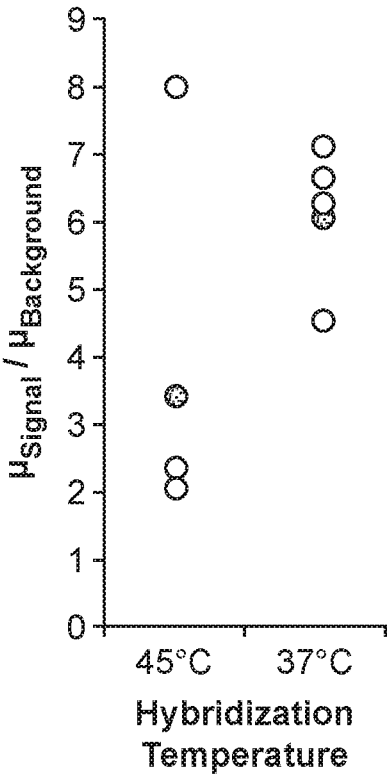


FIG. 5B

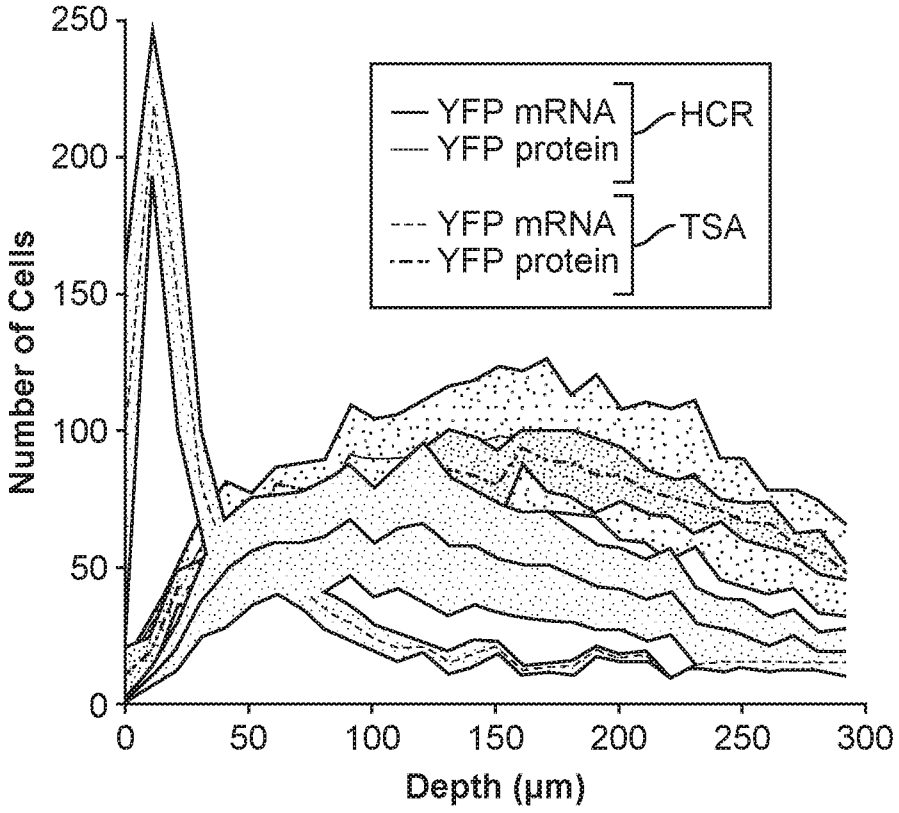


FIG. 5C

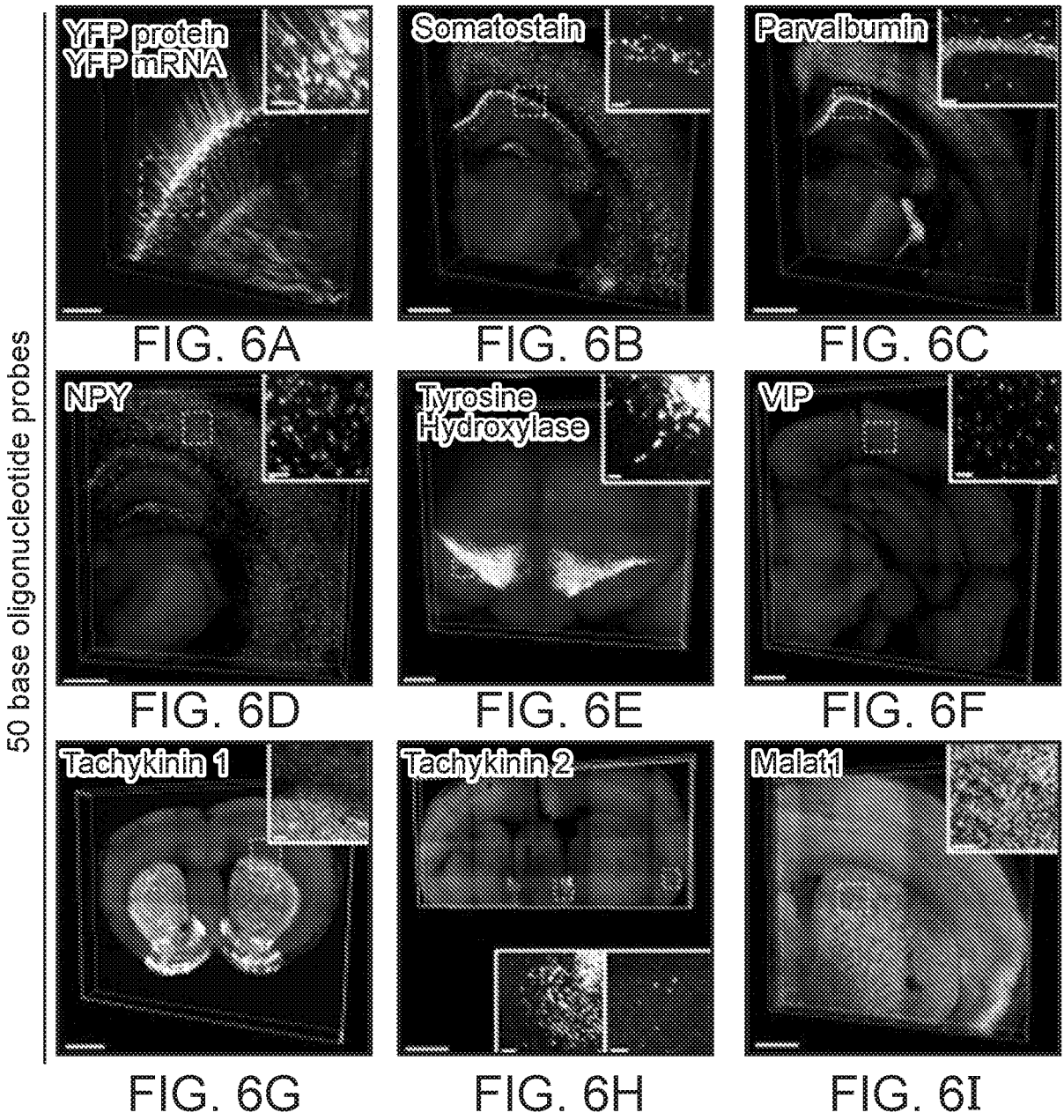


FIG. 6A FIG. 6B FIG. 6C

FIG. 6D FIG. 6E FIG. 6F

FIG. 6G FIG. 6H FIG. 6I

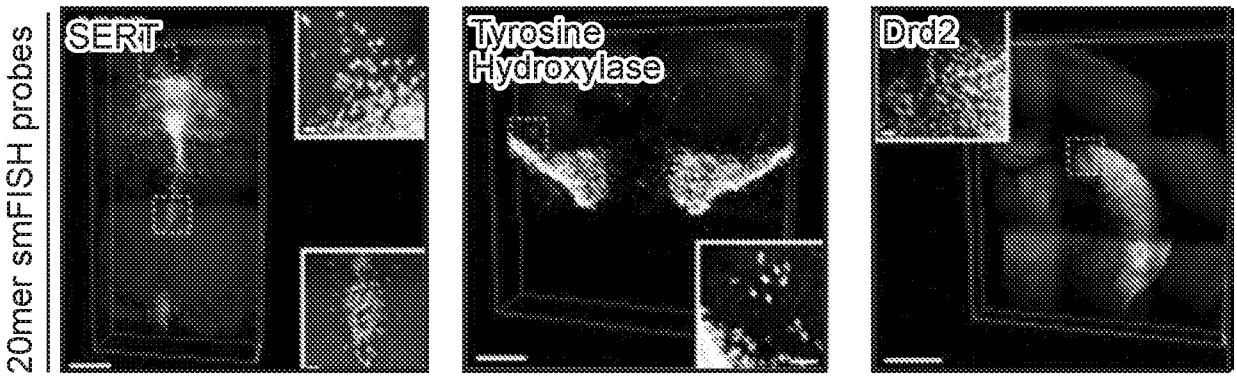


FIG. 6J FIG. 6K FIG. 6L

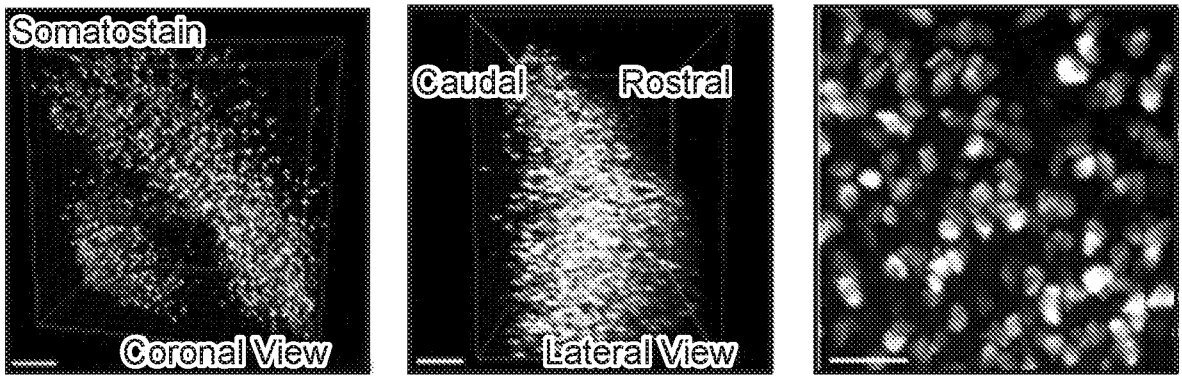


FIG. 6M FIG. 6N FIG. 6O

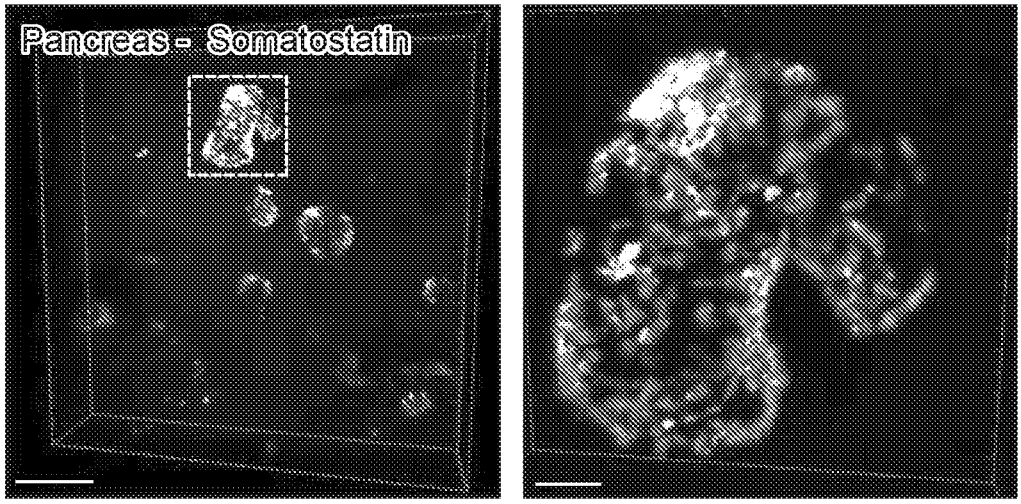

Pancreas - Somatostatin

FIG. 7A

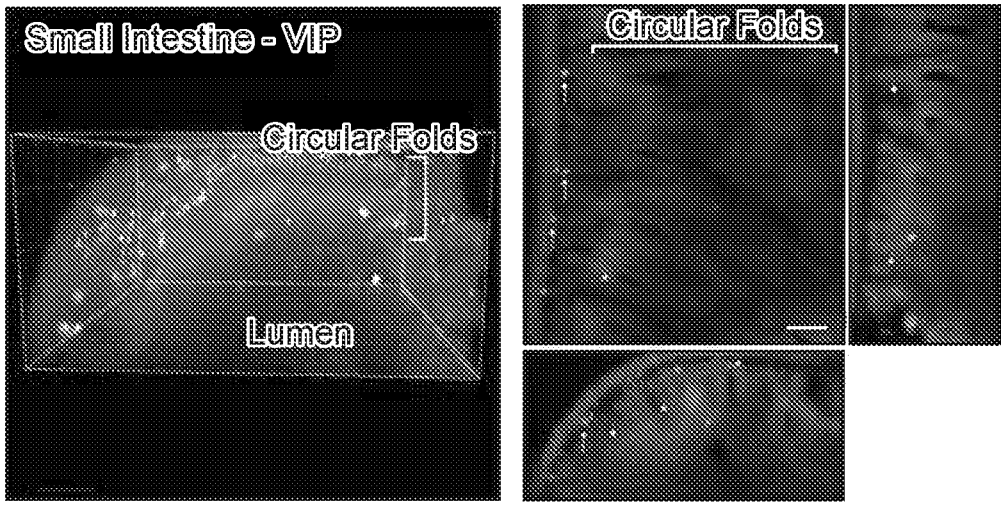

Small Intestine - VIP
Circular Folds
Lumen
Circular Folds

FIG. 7B

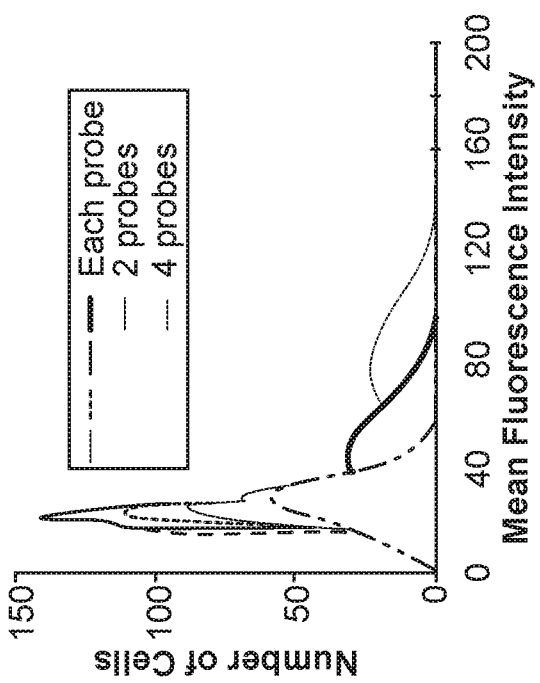


FIG. 8B

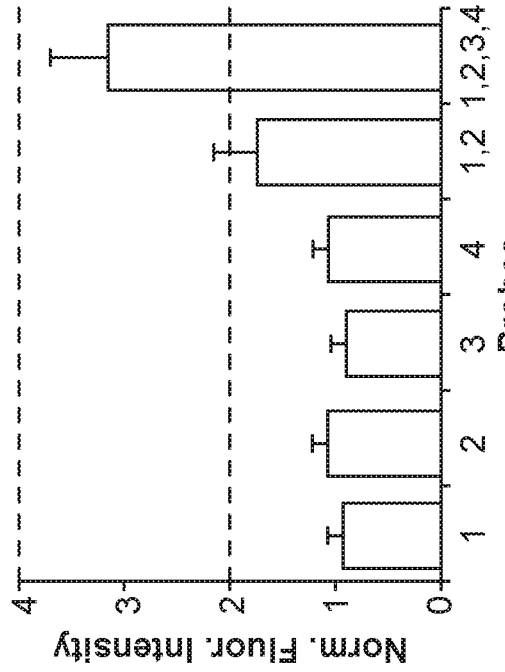


FIG. 8C

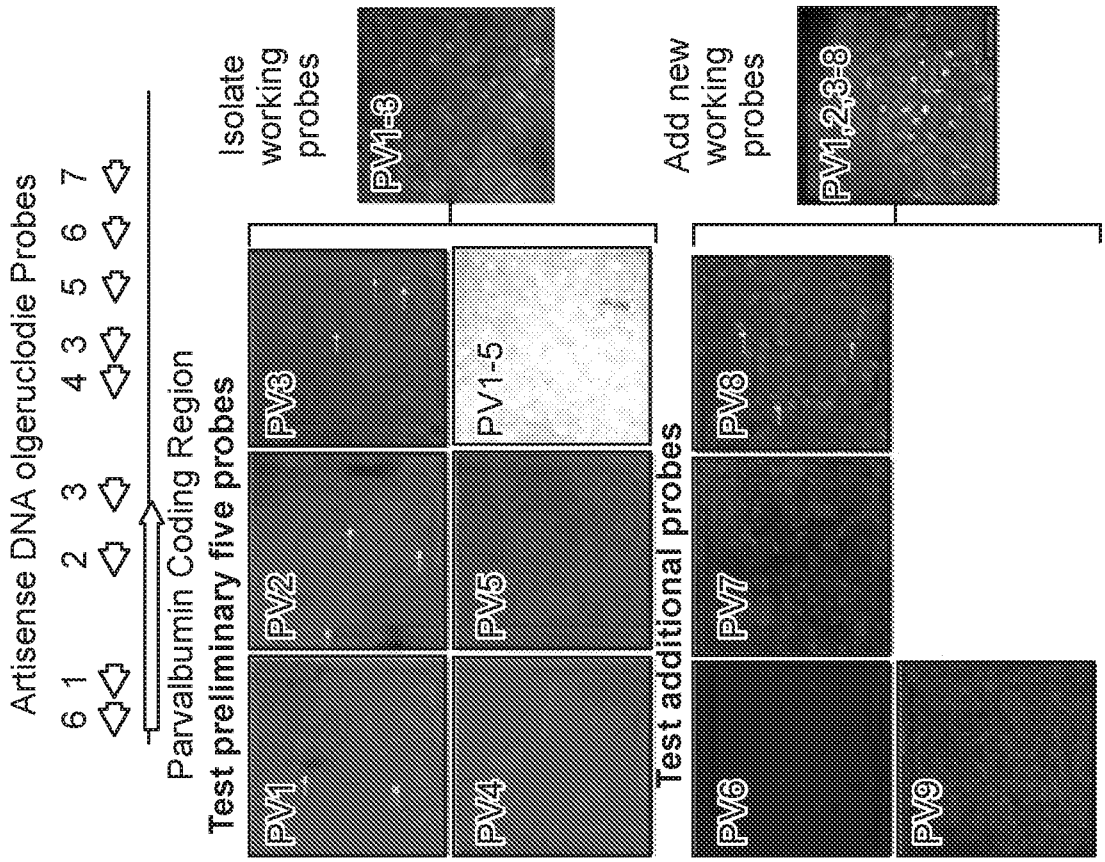


FIG. 8A

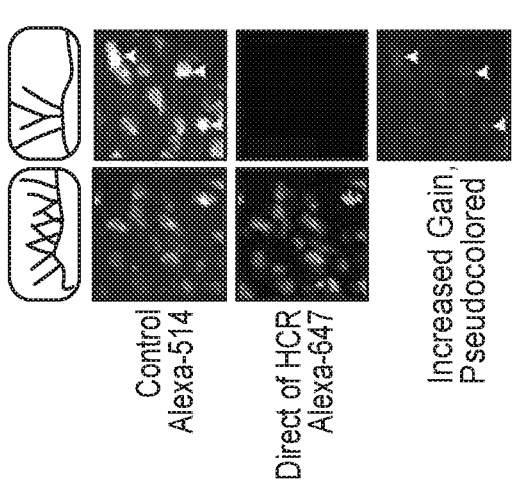


FIG. 8D

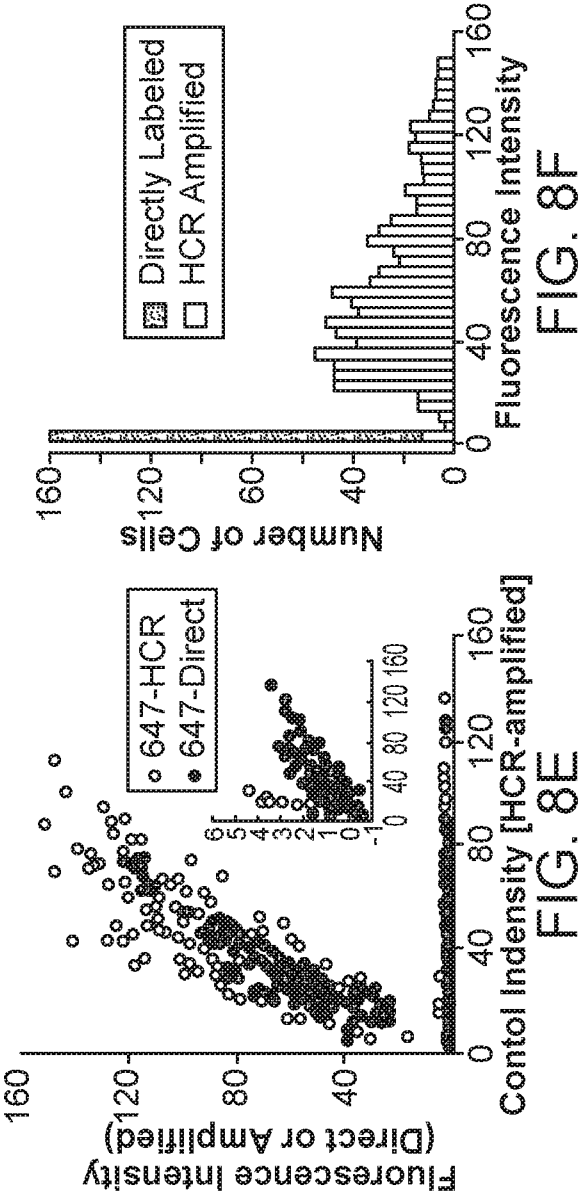


FIG. 8E

FIG. 8F

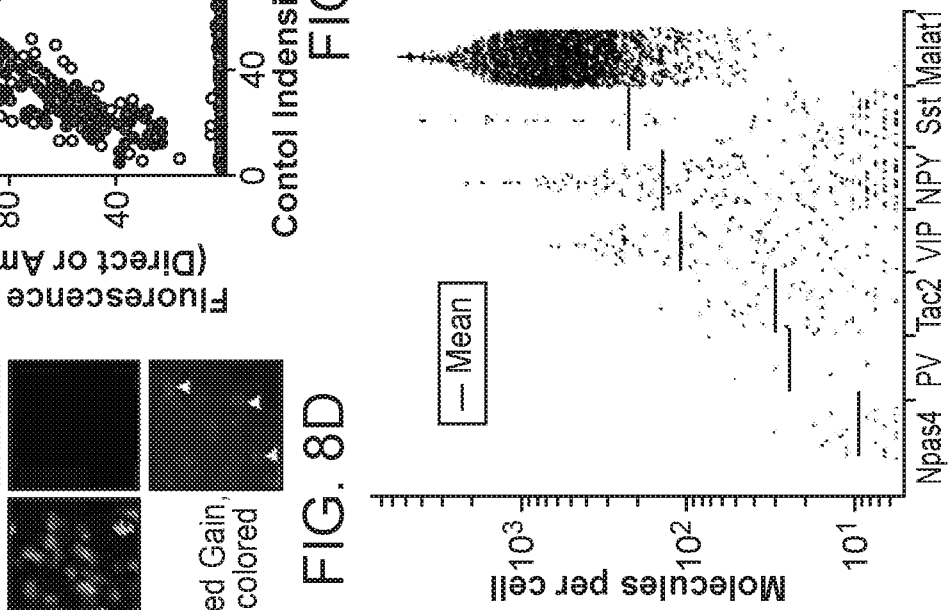


FIG. 8H

FIG. 8G

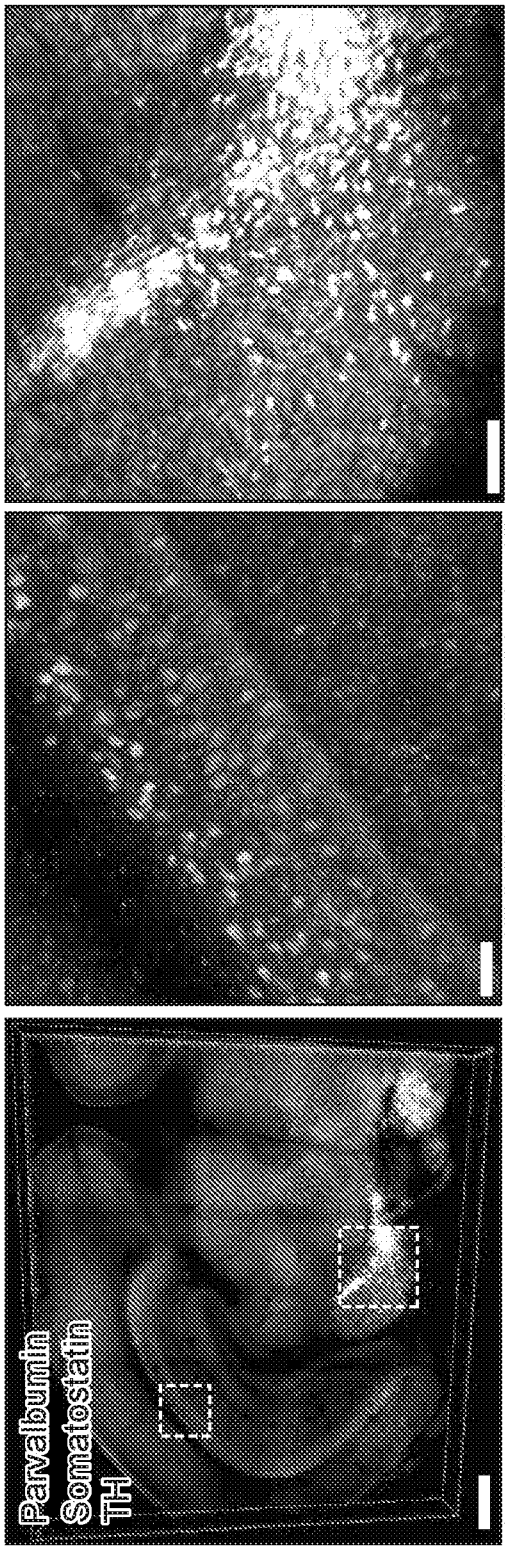

Parvalbumin
Somatostatin
TH

FIG. 9A

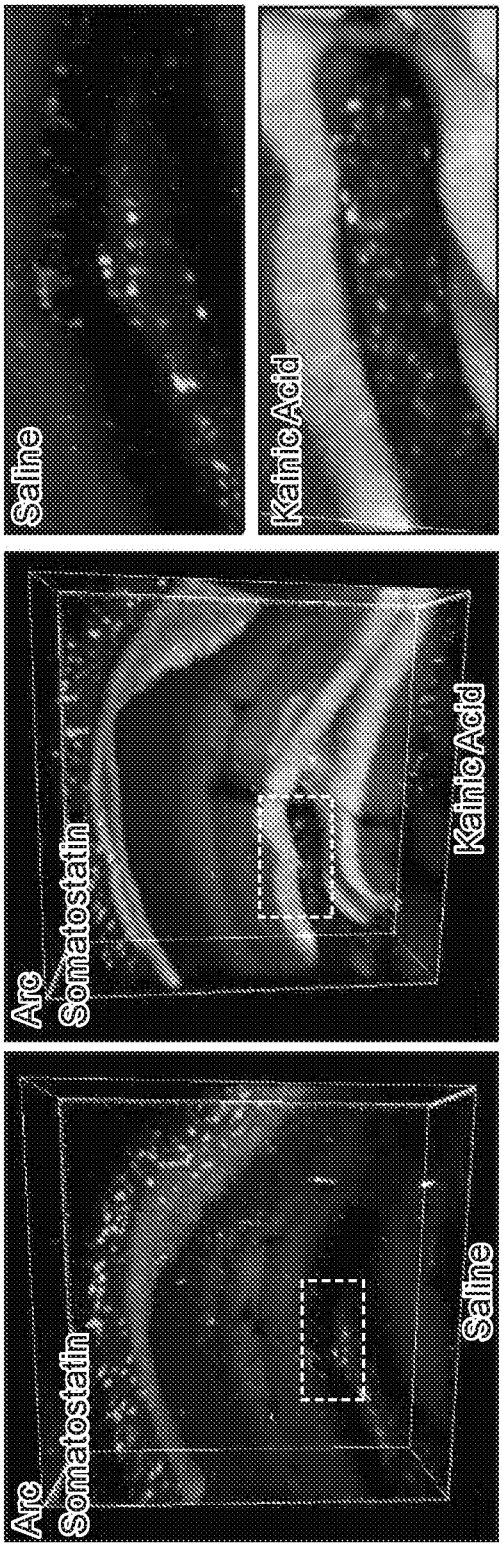

Saline
Kainic Acid
Arc
Somatostatin
Kainic Acid
Arc
Somatostatin
Saline

FIG. 9B

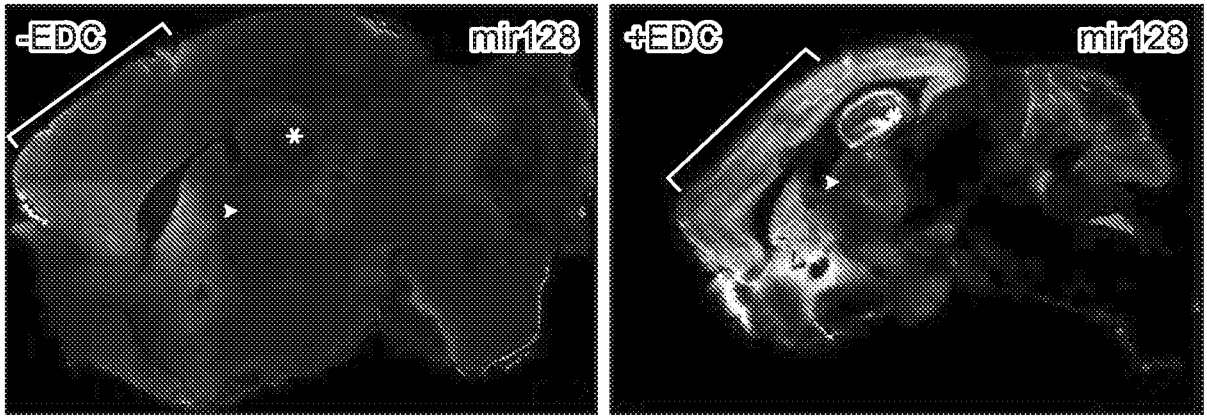


FIG. 10A

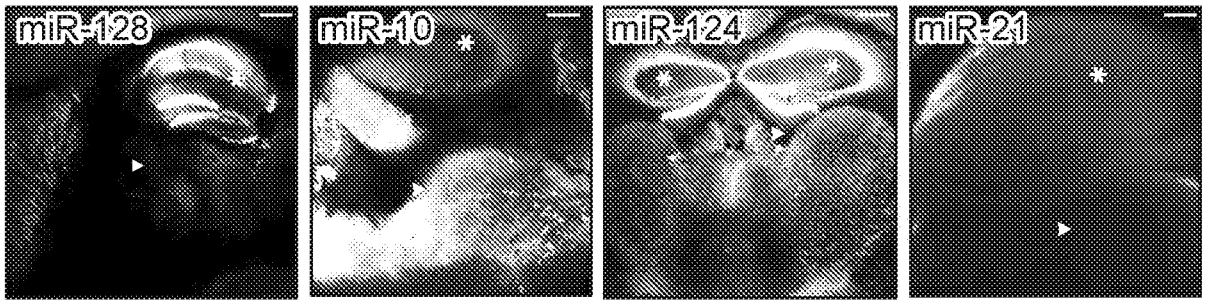


FIG. 10B

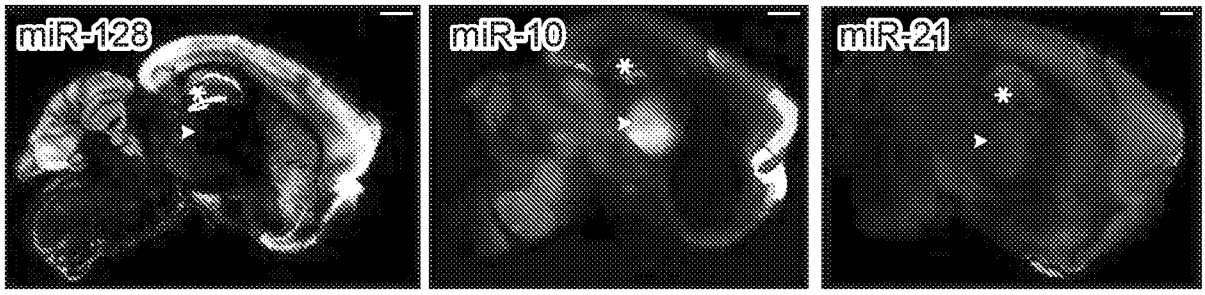


FIG. 10C

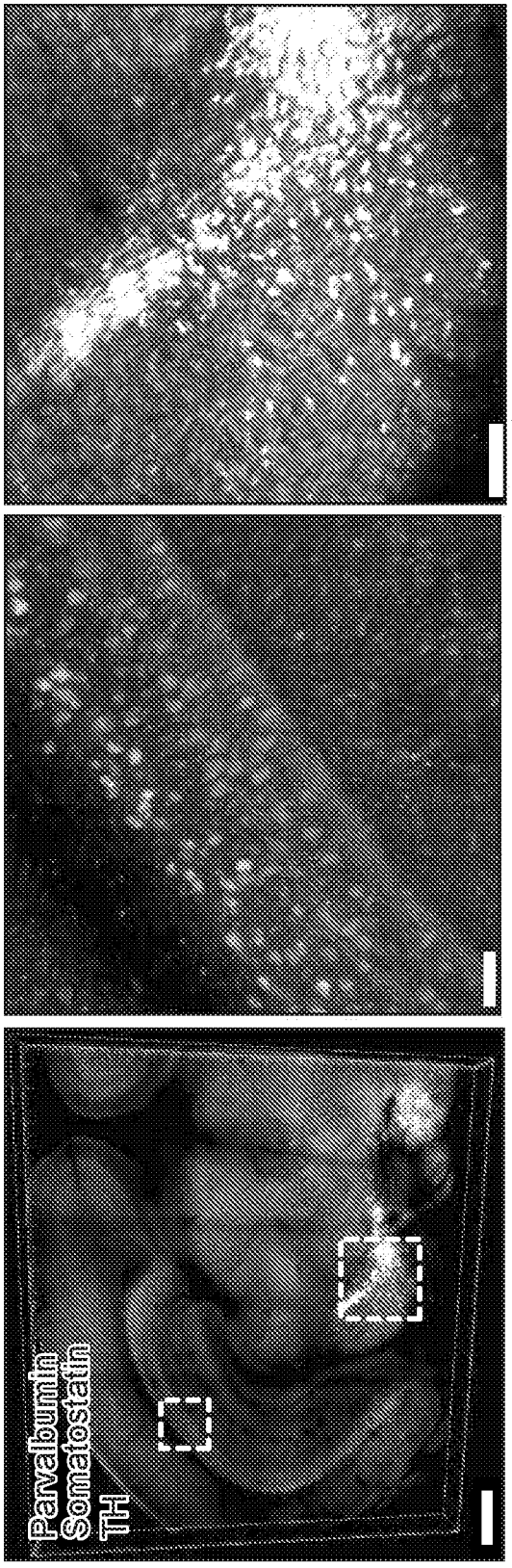


FIG. 11A

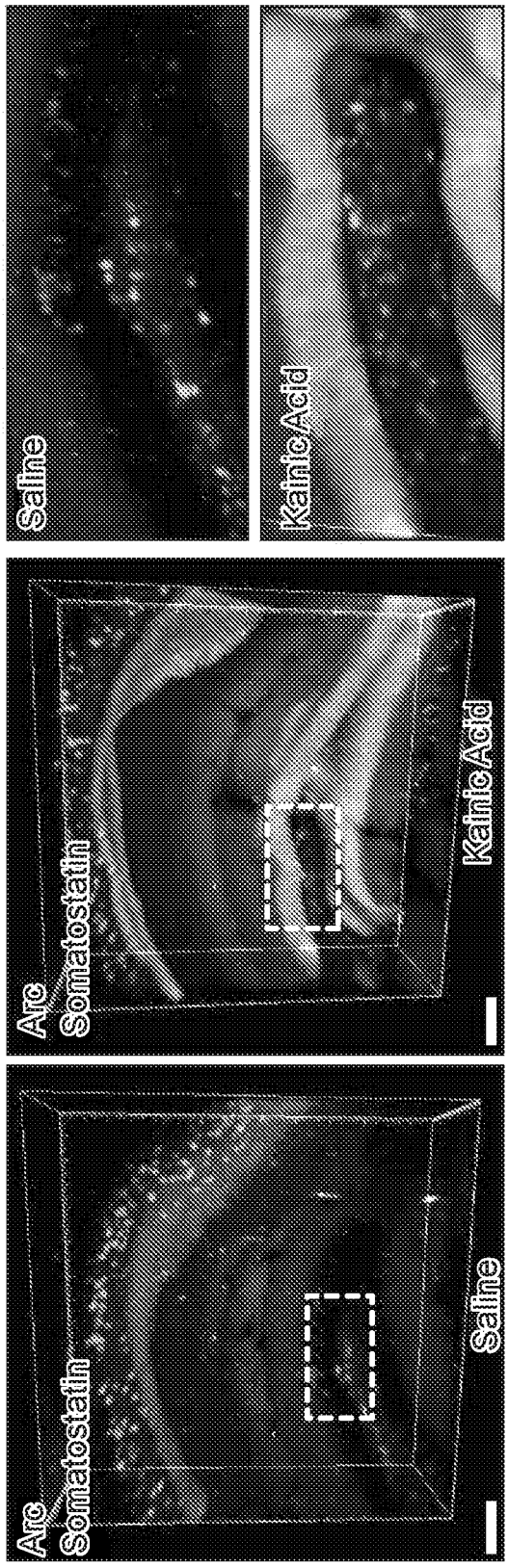


FIG. 11B

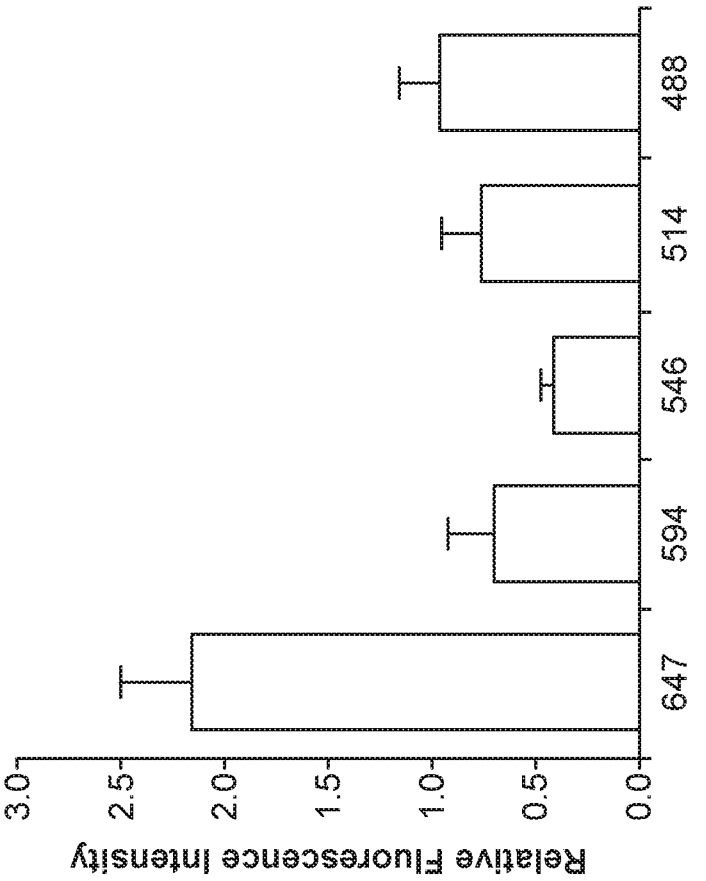


FIG. 12B

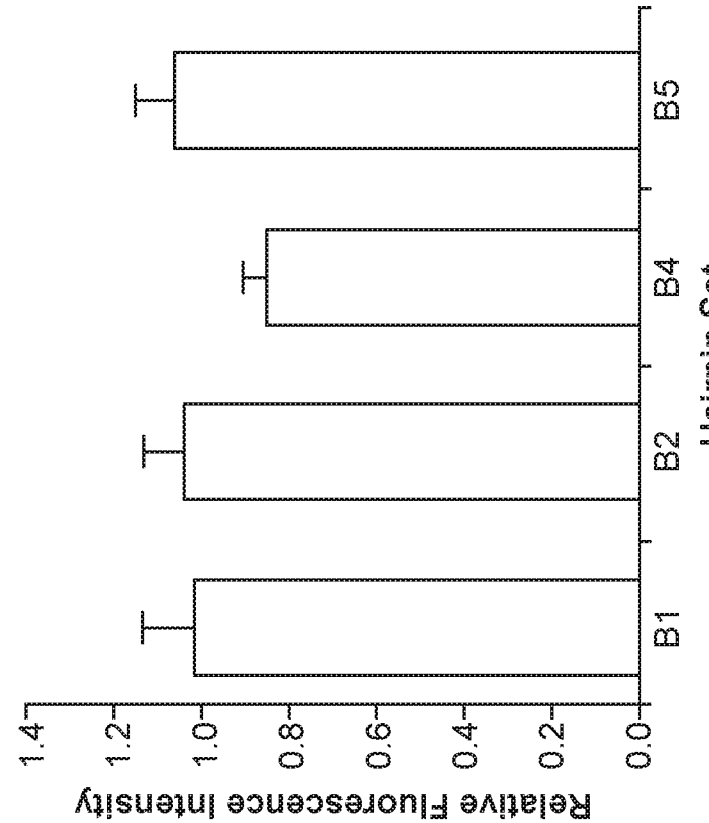
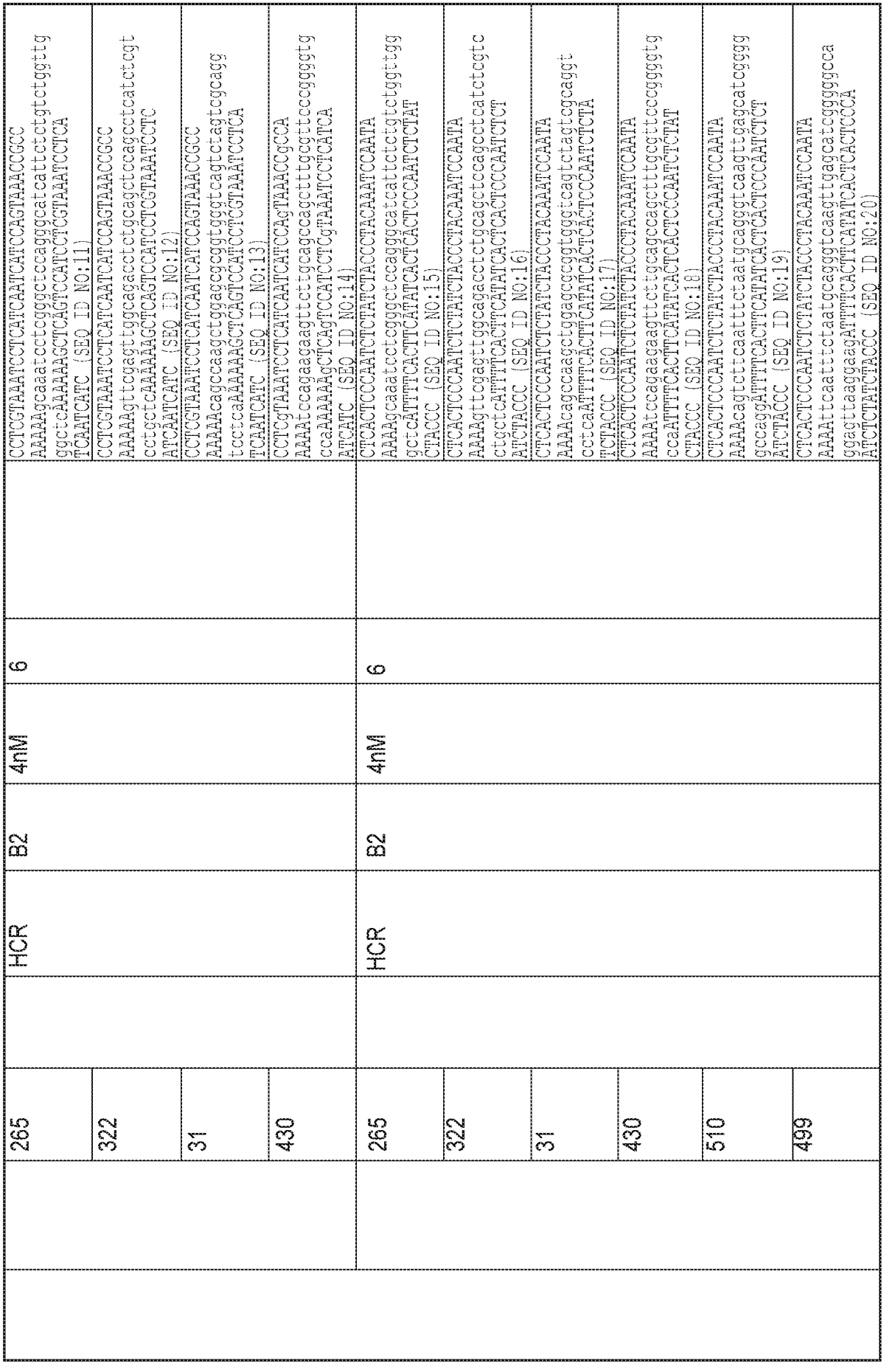


FIG. 12A

Target	Accession #	Probe Position	Probe type	Amplification Type	Hairpin Set	[Probe]	Figure	Region	Sequences
Somatostatin	BC010770	380-429	DIG-labled riboprobe	anti-DIG+ TSA	-	200ng/ul		Cortex, Hippocampus, Striatum, pancreas, weak in stomach	gaatgtcttccagaagaagttcttgcagccagctttgcgttcccggg gtg (SEQ ID NO:1)
		330-429	DIG-labled riboprobe	anti-DIG+ TSA	-	200ng/ul			gaatgtcttccagaagaagttcttgcagccagctttgcgttcccggg gtgccattgctgggttcgagttggcagacctctgcagctccagcctc atctcg (SEQ ID NO:2)
		280-429	DIG-labled riboprobe	anti-DIG+ TSA	-	200ng/ul			gaatgtcttccagaagaagttcttgcagccagctttgcgttcccggg gtgccattgctgggttcgagttggcagacctctgcagctccagcctc atctcgtcctgctcagcctgcctgggggcaaatcctcgggctccaggggc atcatctc (SEQ ID NO:3)
		265	DIG-labeled DNA 50mer	anti-DIG+ TSA	-	50nM	3		gcaaatcctcgggctccagggcatcattctctgtctggttgggctc (SEQ ID NO:4)
		322							gttcgagttggcagacctctgcagctccagcctcatctcgtcctgctcc (SEQ ID NO:5)
		31							cagccaagctgggagcgcggtgggtcagtctagtcgcaggtcctca (SEQ ID NO:6)
		265	~50mer DNA oligonucleotide	HCR	B1	4nM	11		GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcaaatcctcgggctcccagggcatcattctctgtctggtt gggctcATATAGCATTCTTTCTTGAGGAGGGCAGCAA ACGGGAAGAG (SEQ ID NO:7)
		322							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgttcgagttggcagacctctgcagctccagcctcatctcg tcctgctcATATAGCATTCTTTCTTGAGGAGGGCAGCA AACGGGAAGAG (SEQ ID NO:8)
		31							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcagccaagctgggagcgcggtgggtcagtctagtcgcag gtcctcaATATAGCATTCTTTCTTGAGGAGGGCAGCA AACGGGAAGAG (SEQ ID NO:9)
		430							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTccagaagaagttcttgcagccagctttgcgttcccggg gtgccaATATAGCATTCTTTCTTGAGGAGGGCAGCAA ACGGGAAGAG (SEQ ID NO:10)

FIG. 13



	265		HCR	B2	4nM	6	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCC AAAAAgcaaatcctcggctccagggcatcattcctgtctggttg ggctccAAAAAAGCTCAGTCCATCCTCGTAAATCCTCA TCAATCATC (SEQ ID NO:11)
	322						CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCC AAAAAgttcgagttggcagacctctgcagctccagcctcatctcgt cctgctcAAAAAAGCTCAGTCCATCCTCGTAAATCCTC ATCAATCATC (SEQ ID NO:12)
	31						CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCC AAAAAcagccaagctggaggcggtggctcagtcctcagtcgcagg tcctcaAAAAAAAGCTCAGTCCATCCTCGTAAATCCTCA TCAATCATC (SEQ ID NO:13)
	430						CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCCA AAAAtccagaaggaagttcttgcagccagcctttgcgttcccgggggtg ccaAAAAAAgCTCAgTCCATCCTCgTAAATCCTCATCA ATCATC (SEQ ID NO:14)
	265		HCR	B2	4nM	6	CTCACTCCCAATCTCTATCTACCCTACAAATCCAATA AAAAgcaaatcctcggctccagggcatcattcctgtctggttgg gctcATTTTCACTTTCATATCACTCATCTCCAATCTCTAT CTACCC (SEQ ID NO:15)
	322						CTCACTCCCAATCTCTATCTACCCTACAAATCCAATA AAAAgttcgagttggcagacctctgcagctccagcctcatctcgtc ctgctcATTTTCACTTTCATATCACTCATCTCCCAATCTCT ATCTACCC (SEQ ID NO:16)
	31						CTCACTCCCAATCTCTATCTACCCTACAAATCCAATA AAAAcagccaagctggaggcggtggctcagtcctcagtcgcaggt cctcaATTTTCACTTTCATATCACTCATCTCCAATCTCTA TCTACCC (SEQ ID NO:17)
	430						CTCACTCCCAATCTCTATCTACCCTACAAATCCAATA AAAAtccagaaggaagttcttgcagccagcctttgcgttcccgggggtg ccaATTTTCACTTTCATATCACTCATCTCCAATCTCTAT CTACCC (SEQ ID NO:18)
	510						CTCACTCCCAATCTCTATCTACCCTACAAATCCAATA AAAAcagtctttcaatttctaatgcagggtcaagttgagcatcgggg gcccaggATTTTCACTTTCATATCACTCATCTCCCAATCTCT ATCTACCC (SEQ ID NO:19)
	499						CTCACTCCCAATCTCTATCTACCCTACAAATCCAATA AAAAttcaatttctaatgcagggtcaagttgagcatcgggggcca ggagttaaggaagATTTTCACTTTCATATCACTCATCTCCCA ATCTCTATCTACCC (SEQ ID NO:20)

FIG. 13 (Cont. 1)

Parvalbumin	BC027424	14	~50mer DNA oligonucleotide	HCR	B1	0.5nM	6,11	Cortex,TRN, Cerebellum, weaker in hippocampus
		72						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTttcttgatgtcctcagcgctgagcacgtctgtcatcgaca tcctgcaactATAATAGCATTCTTTCTTCTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:21)
		210						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgtctcccagcgcccagaagcgtctttgttcctttagcaga caagtcctgATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:22)
		303						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgtcctccagcgcccagaagcgtctttgttcctttagcaga caagtcctgATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:23)
								GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcggagaggtgggggacccaagcagtcagcgcccacttag ctttcagccacCATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG(SEQ ID NO:24)
		Tested and removed	400				7	tacaggtggtgtccgattggtacagcctttattgttcticcagcattic c (SEQ ID NO:25)
			473					agtaccaagcaggcaggagatatcgggggcgttgtccttgactaatc tca (SEQ ID NO:26)
			683					aagatggacgatccatcacccccatctccttgtgggaaaggtgcag agat (SEQ ID NO:27)
			762					ggagtccttgatctagctagtcctgaaggactcaacccctttcccttic cc (SEQ ID NO28)
			550					aagaaagaaaaaaaaactgcccaaaccaacaccctccaggcctg ggtcct (SEQ ID NO:29)
Tyrosine Hydroxylase	M69200	303	~50mer DNA oligonucleotide	HCR	B1	2nM	6	Midbrain	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTactctgcccgccgtccaatgaacttgggacgtgacagc ctcggcctgctATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:30)
		817						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctggaagcccagtccgttccttcaagaagtgagacacatc ctccagctgtgATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:31)

FIG. 13 (Cont. 2)

1277						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcatcactgaagctctctgacacgaagtacaccggctggt aggtttgatctATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:32)
1398						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcttaaggagccccggatggtgtgaggactgtccagtac atcaatggccagATATAGCATTCTTTCTTGAGGAGGGC AGCAAACGGGAAGAG (SEQ ID NO:33)
307						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTccgggtctctaagtggtgaattttggcttcaaatgtctca aacactttcaATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:34)
956						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcagctcgtgccagccagtctggctcgggtgagtgcatag gtgaggaggcaATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:35)
1332						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtcacagagaatgggcgctggatacgaggcatagtt cctgagcttgtcATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:36)
387						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcagcccaggtcgccactgggcacctcgaagccgcacaaa gtactccaggtgATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:37)
1158						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaggagctctccataggaagacagcagccctgcaccgta agccttcagctcATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:38)
514						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTagggtcaaacttggtgaccaggtggtgacacttatccaa ctctgacactcATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:39)
76	~50mer DNA oligonucleotide	HCR	B2	2nM	11	Midbrain	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatctgcccgccgtccaatgaaccttgggacgtgacagc ctcggcctgctATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:40)

FIG. 13 (Cont. 3)

817							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctggaagccagtccgttcctcaagaagtgagacacatc ctccagctgtgATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:41)
1277							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcatcactgaagcctcctgacacgaagtacaccggctggt aggtttgatcATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:42)
1398							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctaaggagccccgatggtgtgaggactgtccagtac atcaatggccagATATAGCATTCTTTCTTGAGGAGGGC AGCAAACGGGAAGAG (SEQ ID NO:43)
307							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcgggtctctaagtggtggattttggcttcaaatgtctca aacactttcaATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:44)
956							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgagctcgtggcagccagtctcggctcgggtgagtgcatag gtgaggaggcaATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:45)
1332							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtcacagagaatgggcgcctggatacgaggcatagtt cctgagcttgtcATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:46)
387							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcagcccaggtcgccactgggcacctcgaagcggcacaaa gtactccaggtgATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:47)

FIG. 13 (Cont. 4)

									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaggagctcttccatcaaggaagacagcagccctgcaccgta agccttcagccCCATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:48)
	1158								
	514								GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTagggtccaaacttggtgaccaggtggtgacacttatccaa ctctgacacttATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:49)
Tachykinin 1	NM_009311	250	~50mer DNA oligonucleotide	HCR	B1	2nM	6	Striatum, Cortex	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatccaaagaactcctgaggcctgggtcttcgggcgattc tctgcagaagaATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:50)
	441								GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTttacgtctcttcttcgtagttctgcatcggcgcttctttcata agccacagATATAGCATTCTTTCTTGAGGAGGGCAGC AAACGGGAAGAG (SEQ ID NO:51)
	528								GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatcatcctcataggcgcacatttatttaccgttcactgc tcactgacaATATAGCATTCTTTCTTGAGGAGGGCAGC AAACGGGAAGAG (SEQ ID NO:52)
	799								GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTccagggtagccgtgagagagacgcacaggagtctctgc ttccagcagccaATATAGCATTCTTTCTTGAGGAGGGC AGCAAACGGGAAGAG (SEQ ID NO:53)
	874								GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTacaggaaacatgctgctaggatacaaatcagtcaaat accgaagtctcaATATAGCATTCTTTCTTGAGGAGGGC AGCAAACGGGAAGAG (SEQ ID NO:54)

FIG. 13 (Cont. 5)

Tachykinin 2	BC051476.1	253	~50mer DNA oligonucleotide	HCR	B1	1nM	6	BNST, Cortex	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgctcagcacttccagcaatcctccagcagagacagggc ggctgtATATAGCATTCTTTCTTGAGGAGGGCAGCAA ACGGGAAGAG (SEQ ID NO:55)
		324							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcataagtcccacaaagaagtcgtgcatctcacgtttct ctggaatgaATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:56)
		379							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTggtgttcttcaaccacgtcggtgggagtgtctggttg gctgttccATATAGCATTCTTTCTTGAGGAGGGCAGCA AACGGGAAGAG (SEQ ID NO:57)
		489							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgggagagagaggaagcagggagcactgctttta tgatgcaatATATAGCATTCTTTCTTGAGGAGGGCAGC AAACGGGAAGAG (SEQ ID NO:58)
		Tested and removed	184						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaggcagctgatagagatcagagtcctactgagcctcc ctccctATATAGCATTCTTTCTTGAGGAGGGCAGCAA ACGGGAAGAG (SEQ ID NO:59)
			549						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgttcatatgcagcttctatgcagagtagggaagggagc caacaggagATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:60)
	Arc	BC023127.1	281	~50mer DNA oligonucleotide	anti-DIG + TSA	-	50nM		gccggtgggtcctccgtacgtgttccagcatctcagctcggcactta cc (SEQ ID NO:61)
			942						Tcctcttgaacticccacccagttctctcaccgagccctgcttgaactcc caccact (SEQ ID NO:62)
			1061						ccccgttgggccagaggtaactggtcgagtggttcaccctgcttctg (SEQ ID NO:63)
			1284						gctggtaagagcaggtgtgagtgcctccgtctcatcctctgtgggca gtgggggtgccag (SEQ ID NO:64)

FIG. 13 (Cont. 6)

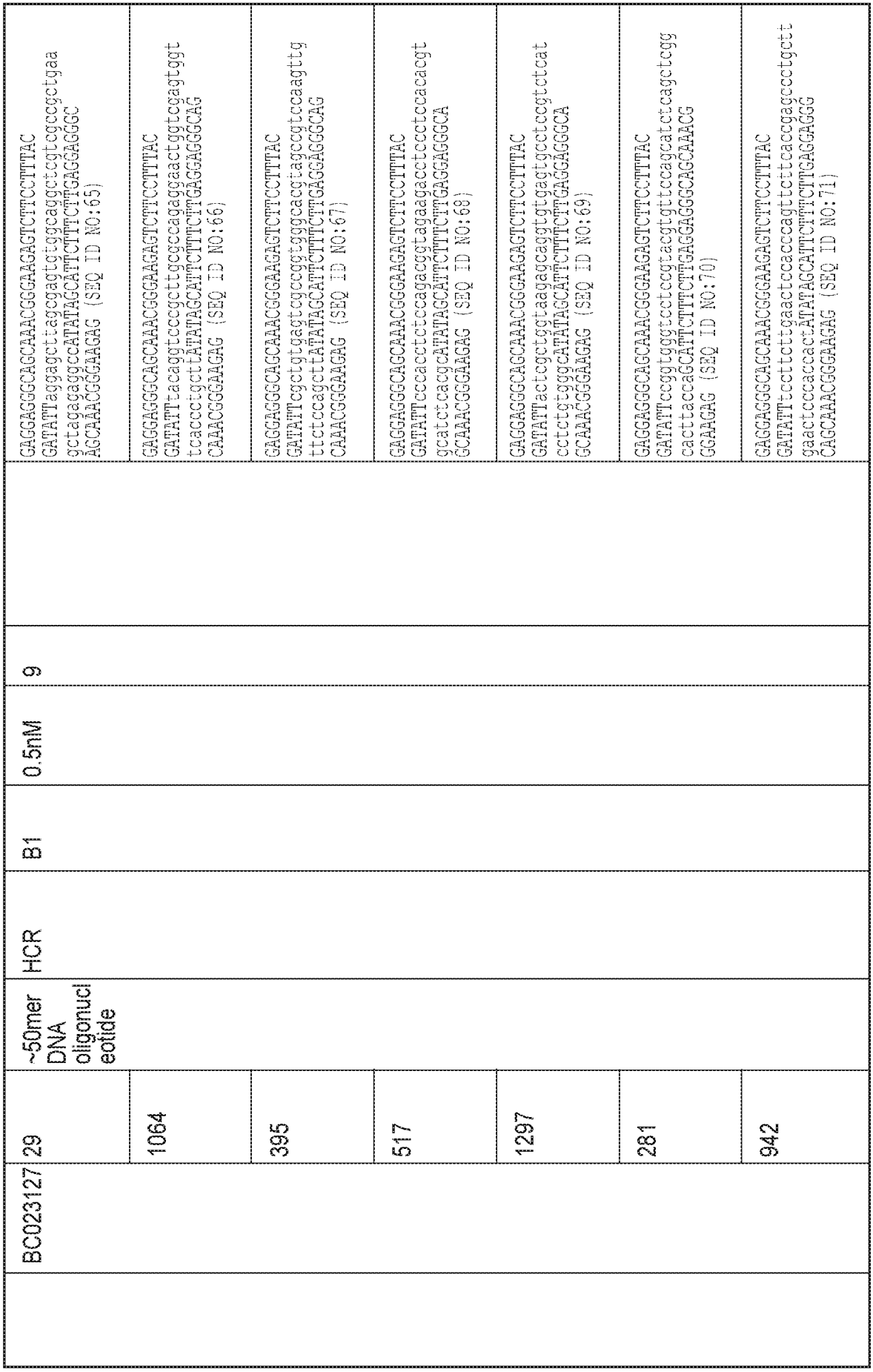

BC023127 29
~50mer DNA oligonucleotide
HCR
B1
0.5nM
9
1064
GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC
GATATTaggagctagcgagtgtggcaggctcgtcgccgctgaa
gctagagaggccATATAGCATTCTTTCTTGAGGAGGGC
AGCAAACGGGAAGAG (SEQ ID NO:65)
395
GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC
GATATTcgctgtgagtcgccggtgggcaggtagccgttcaagttg
ttcttccagcttATATAGCATTCTTTCTTGAGGAGGGCAG
CAAACGGGAAGAG (SEQ ID NO:67)
517
GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC
GATATTcccacctctccagacggtagaagacctccctccacacgt
gcatcttcacgcATATAGCATTCTTTCTTGAGGAGGGCA
GCAAACGGGAAGAG (SEQ ID NO:68)
1297
GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC
GATATTactcgctggtaagagcaggtgtgagtgcctccgtctctcat
cctctgtgggcATATAGCATTCTTTCTTGAGGAGGGCA
GCAAACGGGAAGAG (SEQ ID NO:69)
281
GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC
GATATTccgtgtgggtcctccgtacgtgttccagcatctcagctcgg
cacttaccaGCATTCTTTCTTGAGGAGGGCAGCAAACG
GGAAGAG (SEQ ID NO:70)
942
GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC
GATATTcctttcttgaactccacccagttctttcaccgagccctgctt
gaactcccaccactATATAGCATTCTTTCTTGAGGAGGG
CAGCAAACGGGAAGAG (SEQ ID NO:71)

FIG. 13 (Cont. 7)

								GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTccagcttgccgccacaggaaactggtcgagtggttcaccct gcttctgATATAGCATTCTTTCTTGAGGAGGGCAGCAA ACGGGAAGAG (SEQ ID NO:72)
	1061	1284						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgctggtaagagcaggtgtgagtgccttcggtctcatcctct gtgggcagtgggtgcccagATATAGCATTCTTTCTTGAGG AGGGCAGCAAACGGGAAGAG (SEQ ID NO:73)
Npas4	NM_153553	1154	~50mer DNA oligonucleotide	HCR	B1	0.5nM	9	Activity-induced in hippocampus superficial cortex GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgaatgagggtagcacaggctgggttcctaggacatagg ctgcctggATATAGCATTCTTTCTTGAGGAGGGCAGCA AACGGGAAGAG (SEQ ID NO:74)
		1889						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgctgagctagggccaggagtgtacagtccccattacca gggctgctaATATAGCATTCTTCTTCTTGAGGAGGGCAGC AAACGGGAAGAG (SEQ ID NO:75)
		1288						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTggagggttgggaagcctttctggtgttgagatccacacc tagttcaggagATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:76)
		1569						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcaactgtccttgtagtggaactggttagtgggtctgggaa ggtagcactgctgggATATAGCATTCTTTCTTGAGGAGG GCAGCAAACGGGAAGAG (SEQ ID NO:77)
		1032						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcagtaaaagggccttctggaccttctggagtatagcatgc agtaaatccatgtcccaATATAGCATTCTTTCTTGAGGAGG GCAGCAAACGGGAAGAG (SEQ ID NO:78)

FIG. 13 (Cont. 8)

		1798							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcctcgggcgaagtaagtcttggtaggattgggctcag ttgctctgggaagcATATAGCATTCTTTCTTGAGGAGGG CAGCAAACGGGAAGAG (SEQ ID NO:79)
VIP	NM_011702.3	5	~50mer DNA oligonucleotide	HCR	B1	1nM	9	Cortical and hippocampal interneurons, intestinal enteroendocrine cells	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTacactgaagagtatcaggaatgccaggaactgaggctt gcttctggcttATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:80)
		158							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatttctgctaagggattctgcaagatgtcaagtctgct tttaaagagATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:81)
		243							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTagaaatctgacccagaagtctcctgtaatcgctggtgaa aactccatcagcatgcATATAGCATTCTTTCTTGAGGAGG GCAGCAAACGGGAAGAG (SEQ ID NO:82)
		328							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctcactgctcctcttccattcaggatggagttcaggtatt tcttATATAGCATTCTTTCTTGAGGAGGGCAGCAAAC GGGAAGAG (SEQ ID NO:83)
		433							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgtgtcgttgattggcacaggatcttccgagatgctgctg ctgattcgtttATATAGCATTCTTTCTTGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:84)
NPY	NM_023456.3	21	~50mer DNA oligonucleotide	HCR	B1	1nM	5	Cortical and hippocampal interneurons	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcgtcgctgagcggcggctgctcctcctgggtgcctctgt gagagaaagagatccaATATAGCATTCTTTCTTGAGGAGG GCAGCAAACGGGAAGAG (SEQ ID NO:85)
		134							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcggagggggtacccctcagcccaagaatgcccaaacacacg agcagagatagagccATATAGCATTCTTTCTTGAGGAGG GCAGCAAACGGGAAGAG (SEQ ID NO:86)
		218							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatgagattcgatgtagtgtcgcagagcggagtagtatctg gccatgtcctctATATAGCATTCTTTCTTGAGGAGGGCA GCAAACGGGAAGAG (SEQ ID NO:87)
		398							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgggatgagatgagatgagggtggaaacttggaaaag tcgggagaacaagtATATAGCATTCTTTCTTGAGGAGGG CAGCAAACGGGAAGAG (SEQ ID NO:88)

FIG. 13 (Cont. 9)

		451							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcggagtccagcctagtggtgcatgcattgggac aggcagactggtttATATAGCATTCTTTCTTGAGAGGG CAGCAAACGGGAAGAG (SEQ ID NO:89)
Malat1	FJ209304.1	1461	~50mer DNA oligonucleotide	HCR	B1	2nM	9	Nuclear localization throughout the brain	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaacgtacatgccgccagtcgaaggattttataacggccg tcaacttaacctaATATAGCATTCTTTCTTGAGAGGC AGCAAACGGGAAGAG (SEQ ID NO:90)
		1898							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTggctctaccattcatccccttcgagcggtttaaagacaa cttgccatctaccattATATAGCATTCTTTCTTGAGAGG GCAGCAAACGGGAAGAG (SEQ ID NO:91)
		2407							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTacacactgggtttagagaaggcgtgtactgctatgctgtt ggcacgacacttATATAGCATTCTTTCTTGAGAGGGC AGCAAACGGGAAGAG (SEQ ID NO:92)
		4598							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaagtgtcttacctagatgtttagcccatggtcaaattaga ccccctgacttATATAGCATTCTTTCTTGAGAGGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:93)
		6231							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaaccgatatgcaacgtgacctcaaggatccagctactg gctgcatcaaATATAGCATTCTTTCTTGAGAGGGCAG CAAACGGGAAGAG (SEQ ID NO:94)
Sert		117	20mer smFISH DNA probe	HCR		2nM per probe	6	dorsal raphae	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcattggataatttgtcccaggc (SEQ ID NO:95)
		141							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTttgtactccgaactgccgcag (SEQ ID NO:96)
		169							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgtagagtgtggcgctccatc (SEQ ID NO:97)
		268							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgccatagccaatgcagacacacag (SEQ ID NO:98)
		299							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcggaaaaacgcccagatgttgc (SEQ ID NO:99)
		321							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcaccattcttggtagccatatg (SEQ ID NO:100)
		362							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaaagatggcccatggtcgtgt (SEQ ID NO:101)
		392							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcgcttccatgtagaagagtg (SEQ ID NO:102)

FIG. 13 (Cont. 10)

		429						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtagaaatgcacccatttcgg (SEQ ID NO:103)
		453						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaaatcgggcagatcttcttc (SEQ ID NO:104)
		514						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgtgttatagtaggaggcgat (SEQ ID NO:105)
		557						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcgtgaaggaggagatgaggt (SEQ ID NO:106)
		597						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtgttccaagagttcttgcag (SEQ ID NO:107)
		621						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcgaagtagttggtgcagttg (SEQ ID NO:108)
		662						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaggtgacgtggaatggagtg (SEQ ID NO:109)
		686						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatggcgcaagtaaaactcct (SEQ ID NO:110)
		710						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctttgactgatggatctgca (SEQ ID NO:111)
		766						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatgagcatgatgcagagagc (SEQ ID NO:112)
		796						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTttccagatgctgaagtagat (SEQ ID NO:113)
		818						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcttgccagacgttttgactc (SEQ ID NO:114
		933						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgccagttgggtttcaagtaa (SEQ ID NO:115)
		1027						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctagcaaacgccaggagaac (SEQ ID NO:116)
		1063						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcatcttggtaacagttgtt (SEQ ID NO:117)
		1092						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtcatgcagttcaccacactg (SEQ ID NO:118)
		1114						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTacaaagccagagacgaagct (SEQ ID NO:119)
		1138						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatgtagccaagcaccgtgaa (SEQ ID NO:120)
		1164						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTacacgtcttcgttcctcatc (SEQ ID NO:121)
		1218						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcctccgcatatgtgatgaaa (SEQ ID NO:122)

FIG. 13 (Cont. 11)

							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTgcgaacgtactatcccaaacc (SEQ ID NO:123)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTcaacacaacgctgtgatcacac (SEQ ID NO:124)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTcccagatgtgaggaaaactca (SEQ ID NO:125)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTcgatagaccacaaaccattcc (SEQ ID NO:126)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTcccaagatgccaagtgatgac (SEQ ID NO:127)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTctgatgtcagtgtgaccagg (SEQ ID NO:128)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTccgatgagaccacggtgag (SEQ ID NO:129)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTgaaccaagacacgacgacgg (SEQ ID NO:130)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTtgcagaactgagtgattcca (SEQ ID NO:131)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTaaaaccatccgggctgaag (SEQ ID NO:132)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTcaggagaaacagagggctga (SEQ ID NO:133)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTgttgggtcgactcatcaaaa (SEQ ID NO:134)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTctccagtcgggataattgta (SEQ ID NO:135)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTatgcagtagcccaacatga (SEQ ID NO:136)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTagggatgcagatgacacacg (SEQ ID NO:137)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTgtgctgatcagccgataaat (SEQ ID NO:138)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTataatgcgcctccttaagtgt (SEQ ID NO:139)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTggtgtttcaggatgatact (SEQ ID NO:140)	
							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC	
							GATATTatgtccccacacgggaattttc (SEQ ID NO:141)	
Drd2	19	20mer smFISH	HCR		2nM per probe	6	striatum	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC
								GATATTagatcatcatcgtaccaggga (SEQ ID NO:142)
	1303							
	1334							
	1356							
	1386							
	1408							
	1431							
	1504							
	1526							
	1548							
	1590							
	1631							
	1668							
	1702							
	1724							
	1751							
	1780							
	1807							
	1831							
	1855							

FIG. 13 (Cont. 12)

		96	DNA probe						GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtggcatagtagttgtagtgg (SEQ ID NO:143)
		118							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaagatgaggagggtgagcag (SEQ ID NO:144)
		141							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcacattgccaaagacgatg (SEQ ID NO:145)
		168							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtctctctggatacagccatg (SEQ ID NO:146)
		191							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgttggtggtggtctgcaaag (SEQ ID NO:147)
		214							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTacagcgaggctgactatcag (SEQ ID NO:148)
		238							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTagtgtggccaccagaagatc (SEQ ID NO:149)
		260							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatagacgacccagggcataa (SEQ ID NO:150)
		292							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctgctgaatttccactcacc (SEQ ID NO:151)
		316							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgtgacaaagatgtcacagtg (SEQ ID NO:152)
		338							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtgtgcacatcatgacatcca (SEQ ID NO:153)
		404							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcaacataggcatggccacag (SEQ ID NO:154)
		426							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtggagctgtagcgtgtgtta (SEQ ID NO:155)
		448							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatcatgacagtaactcggcg (SEQ ID NO:156)
		472							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaaggacaggacccagacaat (SEQ ID NO:157)
		496							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaagagcagtgggcaagagat (SEQ ID NO:158)
		520							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTttctggtctgtgttgttgag (SEQ ID NO:159)
		542							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaggggttggcaatgatacact (SEQ ID NO:160)
		564							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtggaggagtagaccacgaag (SEQ ID NO:161)
		587							GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgaagggcacgtagaacgaga (SEQ ID NO:162)

FIG. 13 (Cont. 13)

614									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTttgataataggaccagcaggg (SEQ ID NO:163)
636									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgcttgcggagaacgatgtag (SEQ ID NO:164)
680									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTctgaaagctcggctgcctac (SEQ ID NO:165)
702									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtgagtggtgtcttcaggttg (SEQ ID NO:166)
736									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTagtttcatgtcctcagggtg (SEQ ID NO:167)
758									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTagacttcatgataacggtgc (SEQ ID NO:168)
780									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgttcactgggaaactcccca (SEQ ID NO:169)
882									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtgggaggatgggctatac (SEQ ID NO:170)
913									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgatggatccgggagagtgag (SEQ ID NO:171)
985									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTttgacaatcttggcatgcccc (SEQ ID NO:172)
1007									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaaagaacttggcaatcctgg (SEQ ID NO:173)
1029									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcattgggcatggtctggatc (SEQ ID NO:174)
1059									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTtcatcgtcttaagggaggtc (SEQ ID NO:175)
1115									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTaatggcaagccatctgagtgg (SEQ ID NO:176)
1144									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTagccagcagatgatgaacac (SEQ ID NO:177)
1169									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTcaggatgcgcgtgatgaaga (SEQ ID NO:178)
drd2.a.11 92									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTatgttgcagtcacagtgtat (SEQ ID NO:179)
drd2.a.13 10									GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTAC GATATTgtgcaggatcttcatgaagg (SEQ ID NO:180)

FIG. 13 (Cont. 14)

Th	proprietary	20mer smFISH DNA probe	HCR	B1	2nM per probe	6	midbrain	Proprietary, Biosearch (Petaluma, CA)
cfos	proprietary	20mer smFISH DNA probe	HCR	B1	2nM per probe	9	hippocampus	Proprietary, Biosearch (Petaluma, CA)
miR-10		LNA probe	anti-DIG + TSA	-	12.5nM	8		cacaaattcggttctacagggta (SEQ ID NO:181)
miR-21		LNA probe	anti-DIG + TSA	-	12.5nM	8		agtctgataagctta (SEQ ID NO:182)
miR-124		LNA probe	anti-DIG + TSA	-	12.5nM	8		ggcattcaccgcgtgccttta (SEQ ID NO:183)
miR-128		LNA probe	anti-DIG + TSA	-	12.5nM	8	forebrain	aaagagaccggttcactgtga (SEQ ID NO:184)

FIG. 13 (Cont. 15)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/17251

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01 N 1/28; G02B 21/06; G01N 33/559; C07H 21/04 (2017.01)
CPC - G01 N 1/30; G01 N 1/28; G02B 21/34; G01 B 9/04; C12N 15/11; C12Q 1/6876

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0144490 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 28 May 2015 (28.05.2015), Abstract, para [0003], [0005], [0007], [0008], [0009], [0045], [0048], [0051], [0052], [0058], [0061], [0064], [0081], [0118], [0141], and [0161]	1-3
Y	CHOI et al., Next-Generation in Situ Hybridization Chain Reaction: Higher Gain, Lower Cost, Greater Durability. ACS Nano. 2014, Vol. 8(5), p. 4284-94. Abstract; pg 4284, col 1, lower para; pg 4285, Fig 1; pg 4287, col 2; pg 4288, Fig 4; and pg 4289, Fig 7	1-3
Y	LAI et al., Characterization of Cross-Linked Porous Gelatin Carriers and Their Interaction with Corneal Endothelium: Biopolymer Concentration Effect. PLoS One. 2013, Vol. 8(1):e54058. PDF File: pg 1-12. Abstract; and pg 2, col 1, middle para and last para	1-3
A	CHUNG et al., Structural and molecular interrogation of intact biological systems. Nature. 2013, Vol. 497(7449), p. 332-7. Entire documentation, especially Abstract; pg 333, Fig 1 and Fig 2; and pg 336, col 2, para 1	1-3
A	CHEN et al., RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. Science. 2015 Apr, Vol. 348(6233), p. 412 and aaa6090-1-14. Entire documentation, especially pg 412, col 1, and col 2, lower para; pg aaa6090-1, Abstract, and col 2, middle para; and pg aaa6090-10, col 2, para 1, and col 3, top para	1-3
A	US 2015/0299784 A1 (CELLULAR RESEARCH, INC.) 22 October 2015 (22.10.2015), para [0267], [0269], [0328], [0347], [0366], and [0385]	1-3

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlierapplication or patent but published on orafierthe international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"p." later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 March 2017	28 APR 2017

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/17251

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	SHAH et al., Single-molecule RNA detection at depth by hybridization chain reaction and tissue hydrogel embedding and clearing. Development. 2016 Aug, Vol. 143(15), p. 2862-7. Epub 2016 Jun 24. Entire documentation, especially Abstract; pg 2862, col 2, middle para; pg 2863, Fig 1; and pg 2865, col 1, lower para	1-3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/17251

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.