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(54) Title: METHODS AND DEVICES FOR IMAGING AND/OR OPTOGENETIC CONTROL OF LIGHT-RESPONSIVE NEURONS

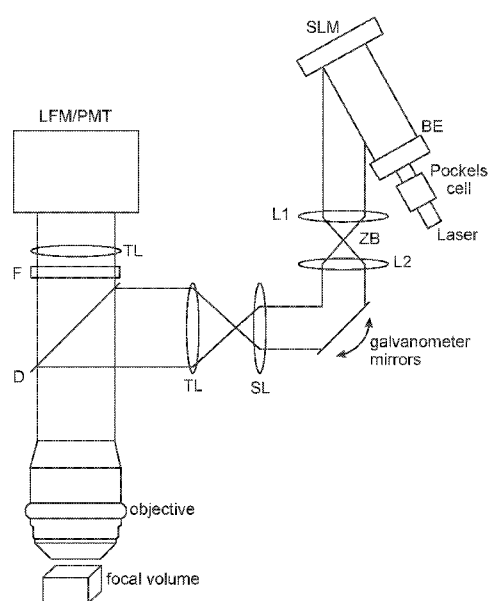


FIG. 8

(57) Abstract: A method for measuring the activity of one or more excitable cells, such as neurons, in a target tissue is provided. The present method may include measuring the activity of individual, selected excitable cells by projecting one or more three dimensional (3D) multi-focal laser light patterns into a target tissue containing excitable cells adapted to emit cellular electrical activity- sensitive fluorescence, to generate a multiplexed 2D diffraction pattern of fluorescence emitted by the neurons, and resolving the multiplexed 2D diffraction pattern. Also provided herein is a system configured to perform the present method, the system including a microscope configured to project one or more 3D multi-focal laser light patterns into a target tissue using a spatial light modulator and a mirror galvanometer, and a microlens array and an image detector to record individual and multiplexed 2D diffraction patterns of light emitted from the target tissue.



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METHODS AND DEVICES FOR IMAGING AND/OR OPTOGENETIC CONTROL OF LIGHT-RESPONSIVE NEURONS

CROSS-REFERENCE TO RELATED APPLICATIONS

- [0001]** Pursuant to 35 U.S.C. §119(e), this application claims the benefit of U.S. Provisional Patent Application Nos. 62/183,012, filed June 22, 2015, which application is incorporated herein by reference in its entirety.

INTRODUCTION

- [0002]** Fluorescent indicators of neuronal activity and optogenetic tools are increasingly used to optically measure and control neuronal activity, respectively, in neurons. Methods for optical recording of neural activity at single cell resolution in three dimensions include serial scanning techniques such as two photon microscopy and wide field detection techniques such as light sheet and light field microscopy.
- [0003]** Two-photon microscopy is a multiphoton fluorescence technique, in which red-shifted excitation light is used to excite fluorescent molecules in a sample. In two-photon microscopy, two photons of light are absorbed for each excitation.
- [0004]** In carrying out light sheet microscopy, a thin slice of the sample is illuminated perpendicularly to the direction of observation, where the illumination is provided by a laser beam that is focused only in one direction (i.e., a light sheet). In light field microscopy, a microlens array is inserted in to the optical path of a conventional microscope, which allows creation of focal stacks from a single image.

SUMMARY

- [0005]** A method for measuring the activity of one or more individual excitable cells, e.g., neurons, in a target tissue is provided. The present method may include measuring the activity of selected excitable cells by projecting one or more three dimensional (3D) multi-focal laser light patterns into a target tissue containing excitable cells adapted to emit cellular electrical activity-sensitive fluorescence, to generate a multiplexed 2D diffraction pattern of fluorescence emitted by the neurons, and resolving the multiplexed 2D diffraction pattern.
- [0006]** Another aspect of the present disclosure includes a method including the steps of obtaining individual two-dimensional (2D) diffraction patterns of multiple fluorescent excitable cells in a

target tissue, and measuring the activity of individual excitable cells by projecting one or more three dimensional (3D) multi-focal laser light pattern into the target tissue to generate a multiplexed 2D diffraction pattern of fluorescence emitted by the neurons, and resolving the multiplexed 2D diffraction pattern using the individual 2D diffraction patterns. Also provided herein is a system configured to perform the present methods, the system including a microscope configured to project a 3D multi-focal laser light pattern into a target tissue using a spatial light modulator and a mirror galvanometer, and a microlens array and an image detector to record individual and multiplexed 2D diffraction patterns.

[0007] An implementation of the present method may include the steps of i) selecting a first subset of excitable cells in a target tissue for analysis, wherein the target tissue is in a field of view of a microscope and wherein the target tissue comprises a plurality of excitable cells that are adapted to emit fluorescence sensitive to cellular electrical activity, ii) obtaining individual two-dimensional (2D) diffraction patterns for each excitable cell in the first subset of excitable cells by a) contacting an excitable cell in the first subset with a laser light, thereby causing the excitable cell to emit fluorescence, b) directing the emitted fluorescence from the contacted excitable cell through a microlens array to create a 2D diffraction pattern, and c) recording the 2D diffraction pattern, and iii) measuring the activity of individual excitable cells in the first subset by a) projecting one or more three-dimensional (3D) multi-focal laser light patterns into the target tissue, thereby causing at least a subset of excitable cells in the first subset of excitable cells in the target tissue to emit fluorescence, b) directing the fluorescence emitted from the target tissue in response to the one or more 3D multi-focal laser light patterns through the microlens array to generate a multiplexed 2D diffraction pattern, c) recording the multiplexed 2D diffraction pattern, and d) resolving the multiplexed 2D diffraction pattern using the individual 2D diffraction patterns of each individual excitable cell in the at least a subset of excitable cells in the first subset to determine the activity of each individual excitable cell illuminated by the one or more 3D multi-focal laser light patterns. The excitable cell may be a neuron and/or a muscle cell. In some embodiments, the target tissue is an in vivo neuronal tissue. In some cases, the target tissue is a light-scattering tissue. In some embodiments, the at least a subset of excitable cells in the first subset includes 20 or more excitable cells.

[0008] In any embodiment, the one or more 3D multi-focal laser light patterns may be configured to illuminate the at least a subset of excitable cells in the first subset with a laser light. In some embodiments, the one or more 3D multi-focal laser light patterns are configured to illuminate all individual excitable cells in the first subset with a laser light.

- [0009]** In any embodiment, the measuring the activity of an individual excitable cell may be performed at a frequency of from about 10 Hz to about 100 Hz.
- [0010]** In any embodiment, resolving the multiplexed 2D diffraction pattern may include deconvolving the multiplexed 2D diffraction pattern using the individual 2D diffraction patterns of each individual excitable cell in the at least a subset of excitable cells in the first subset, thereby resolving the contribution of fluorescence emitted from an individual excitable cell to the multiplexed 2D diffraction pattern. In some embodiments, deconvolving the multiplexed 2D diffraction pattern comprises determining individual point spread functions (PSFs) for each of the individual 2D diffraction patterns of each individual excitable cell in the at least a subset of excitable cells in the first subset and using the individual PSFs to deconvolve the multiplexed 2D diffraction pattern.
- [0011]** In any embodiment, projecting the one or more 3D multi-focal laser light patterns may include splitting a laser beam and projecting the one or more 3D multi-focal laser light patterns using a spatial light modulator. In some embodiments, the measuring step includes sequentially projecting each set of a plurality of sets of one or more 3D multi-focal laser light patterns into the target tissue. In some embodiments, each set of the plurality of sets of one or more 3D multi-focal laser light patterns is sequentially projected into the target tissue at a rate of from about 0.3 to about 0.8 times the maximum switching rate of the spatial light modulator. In some embodiments, each set of the plurality of sets of one or more 3D multi-focal laser light patterns is configured to contact a subset of excitable cells in the first subset of excitable cells, and wherein the method further comprises selecting members of each subset of excitable cells based on the individual 2D diffraction patterns. In some embodiments, the selecting step includes comparing the individual 2D diffraction patterns among each other, and selecting members of each subset such that the difference in the individual 2D diffraction pattern between any two excitable cells within the subset of excitable cells is above a threshold. In some embodiments, sequentially projecting each set of the plurality of sets of one or more 3D multi-focal laser light patterns into the target tissue includes horizontally translating the field of view of the spatial light modulator using a mirror galvanometer to project each set of the plurality of sets of one or more 3D multi-focal laser light patterns into at least two distinct sites in the target tissue. In some embodiments, the plurality of sets of one or more 3D multi-focal laser light patterns collectively illuminates excitable cells in a volume of the target tissue at least 1.5 times larger than the maximum volume of the target tissue that can be illuminated without horizontally translating the field of view of the spatial light modulator.

- [0012]** In any embodiment, the laser beam may be pulsed at a frequency of from about 100 kHz to about 500 kHz. In any embodiment, the laser beam may have an average power of from about 1.0 watts (W) to about 3.0 W. In any embodiment, the laser beam may have a pulse duration of from about 100 femtoseconds (fs) to about 500 fs. In any embodiment, the laser light may have a wavelength in the near-infrared range.
- [0013]** In any embodiment, recording the 2D diffraction pattern may include recording an image of the 2D diffraction pattern using an image detector. In some embodiments, the image detector may be a Complementary Metal Oxide Semiconductor (CMOS) detector, e.g., a CMOS camera.
- [0014]** In any embodiment, the method may include, before step i), placing the target tissue in the field of view of the microscope.
- [0015]** In any embodiment, each excitable cell of the first subset of the excitable cells may include an activity-regulated fluorescent dye or fluorescent protein. In some embodiments, each excitable cell of the first subset of the excitable cells expresses a genetically encoded fluorescent protein. In some embodiments, the activity-sensitive fluorescent dye or fluorescent protein is calcium-sensitive or voltage-sensitive.
- [0016]** In any embodiment, the method may include the steps of stimulating the target tissue, and measuring the activity of individual excitable cells in the first subset in response to the stimulation. In some embodiments, the target tissue includes a plurality of excitable cells in the first subset of excitable cells that are adapted to hyperpolarize and/or depolarize in response to a laser light stimulus, and the stimulating includes contacting the plurality of excitable cells with a laser light stimulus. In some embodiments, an excitable cell that is adapted to hyperpolarize and/or depolarize in response to a laser light stimulus is genetically modified to express one or more light-responsive polypeptides that hyperpolarize or depolarize the excitable cell when contacted by a laser light.
- [0017]** Also provided herein is a method of regulating the activity of a plurality of excitable cells in a target tissue, including: i) measuring the activity of a plurality of activity-sensitive fluorescence-emitting excitable cells in a target tissue containing a plurality of excitable cells, according to any method described above, and ii) modulating the activity of a plurality of excitable cells in the target tissue in response to the measured activity of the plurality of activity-sensitive fluorescence-emitting excitable cells. In some embodiments, the plurality of excitable cells are genetically modified to hyperpolarize and/or depolarize in response to a laser light stimulus, and the modulating includes contacting at least a subset of the plurality of genetically modified excitable cells with a laser light stimulus using one or more 3D multi-focal laser light pattern stimuli, wherein the one or more 3D multi-focal laser light pattern stimuli are controlled in

response to the measured activity of the at least a subset of the plurality of activity-sensitive fluorescence-emitting excitable cells.

- [0018]** Also provided herein is a system for measuring the activity of one or more excitable cells in a target tissue, the system, including a light microscope, one or more laser light sources, one or more spatial light modulators, one or more mirror galvanometers, a microlens array, an image detector, a controller, a processor, and a computer-readable medium comprising instructions that, when executed by the processor, cause the controller to perform any method described above. In certain embodiments, the microscope is configured to project one or more 3D multi-focal laser light patterns generated by a spatial light modulator and reflected off a mirror galvanometer into a target tissue that is in the field of view of the microscope, and is configured to direct light emitted from the target tissue through a microlens array onto an image detector.
- [0019]** In any embodiment, the one or more laser light sources may include a regenerative amplifier.
- [0020]** In any embodiment, the one or more laser light sources may be configured to produce a laser beam pulsed at a frequency of from about 100 kHz to about 100 MHz. In any embodiment, the one or more laser light sources may be configured to produce pulses of a laser beam having a duration of from about 100 fs to about 500 fs. In any embodiment, the one or more laser light sources may be configured to produce pulses of a laser beam having a wavelength in the near-infrared range.
- [0021]** In any embodiment, the spatial light modulator may be configured to split a laser beam generated by the one or more laser light sources into a plurality of laser lights and to generate the one or more 3D multi-focal laser light patterns projected into the target tissue.
- [0022]** In any embodiment, the one or more mirror galvanometers may be configured to project the one or more 3D multi-focal laser light patterns into the sample across a plurality of spatially distinct fields of view of the spatial light modulator.
- [0023]** In any embodiment, the image detector may be a Complementary Metal Oxide Semiconductor (CMOS) detector, e.g., a CMOS camera.
- [0024]** In any embodiment, the system further includes a zero order beam block configured to block zero order undiffracted laser light projected from the one or more spatial light modulators.
- [0025]** In any embodiment, the controller may measure the activity of each individual excitable cell among a plurality of excitable cells measured collectively at a frequency of from about 10 Hz to about 100 Hz.
- [0026]** Also provided herein is a method for collectively and specifically illuminating a plurality of individual excitable cells in a target tissue, the method including the steps of i) selecting a first subset of excitable cells in a target tissue for analysis, wherein the target tissue is in a field of

view of a microscope and wherein the target tissue includes a plurality of excitable cells that are adapted to emit fluorescence, and ii) sequentially projecting each 3D multi-focal laser light pattern of a plurality of 3D multi-focal laser light patterns generated by a spatial light modulator into the target tissue, wherein the plurality of 3D multi-focal laser light patterns is projected into at least two distinct sites in the target tissue, thereby collectively and specifically illuminating the plurality of excitable cells in the target tissue. In some embodiments, the method includes horizontally translating the field of view of the spatial light modulator using a mirror galvanometer to project the 3D multi-focal laser light patterns into the at least two distinct sites in the target tissue. In some embodiments, the target tissue includes a plurality of excitable cells that are adapted to hyperpolarize and/or depolarize in response to illumination by the laser light. In some embodiments, an excitable cell that is adapted to hyperpolarize and/or depolarize in response to a laser light stimulus is genetically modified to express one or more light-responsive polypeptides that hyperpolarize or depolarize the excitable cell when illuminated by the laser light.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0027] The present disclosure may be best understood from the following detailed description when read in conjunction with the accompanying drawings. Included in the drawings are the following figures:
- [0028] **FIGs. 1A-1D** are a collection of images depicting a scanless three-dimensional (3D) two-photon imaging system and the steps of using the system according to embodiments of the present disclosure.
- [0029] **FIGs. 2A-2B** are collection of images depicting the optimum laser beam frequency for use in the method according to embodiments of the present disclosure.
- [0030] **FIG. 3** is a collection of images showing the difference in the point spread functions obtained from empirical measurements in a target tissue and in a control sample.
- [0031] **FIGs. 4A-4E** are collection of images and graphs showing simultaneous 3D recording of activity in multiple neurons in an in vivo, neuronal target tissue, according to embodiments of the present disclosure.
- [0032] **FIGs. 5A-5B** are collection of images showing the increased field of view achieved by spatial-temporal multiplexing, according to embodiments of the present disclosure.

- [0033] **FIGs. 6A-6B** are a collection of schematic diagrams showing a time-division multiplexing strategy for laterally extending the SLM field of view (FOV), according to embodiments of the present disclosure.
- [0034] **FIGs. 7A-7B** are a collection of schematic diagrams and graphs showing timing considerations for SLM time-division multiplexing, according to embodiments of the present disclosure.
- [0035] **FIG. 8** is a schematic diagram showing an optical layout of a microscope setup for performing 3D Holographic Illumination with Time-Division Multiplexing, according to embodiments of the present disclosure.
- [0036] **FIGs. 9A-9B** are a collection of images showing experimentally measured increase in SLM field of view, according to embodiments of the present disclosure.
- [0037] **FIG. 10** is a graph showing theoretically computed and experimentally measured signal increase with SLM time-division multiplexing, according to embodiments of the present disclosure.
- [0038] **FIGs. 11A-11D** are a collection of schematic diagrams, graphs and images showing 3D two-photon *in vivo* calcium imaging using 3D Holographic Illumination with Time-Division Multiplexing, according to embodiments of the present disclosure.
- [0039] **FIG. 12** is a schematic diagram depicting a method of measuring the activity of excitable cells in a target tissue, according to embodiments of the present disclosure.
- [0040] **FIGs. 13A-13B** are a collection of schematic diagrams depicting a system for illuminating and/or measuring the activity of excitable cells in a target tissue, according to embodiments of the present disclosure.

DEFINITIONS

- [0041] The term “about” as used herein when referring to a measurable value such as a physical quantity, a temporal duration, a frequency and the like, is meant to encompass variations of $\pm 20\%$, such as $\pm 10\%$, such as $\pm 5\%$, $\pm 1\%$, including $\pm 0.1\%$ from the specified value, as such variations are typical of measurements characterizing the disclosed systems or appropriate to perform the disclosed methods.
- [0042] The terms “polynucleotide”, “nucleotide”, “nucleotide sequence”, “nucleic acid”, “nucleic acid molecule”, “nucleic acid sequence” and “oligonucleotide” are used interchangeably, and can also include plurals of each respectively depending on the context in which the terms are utilized. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides (DNA) or ribonucleotides (RNA), or analogs thereof. Polynucleotides may have any three-

dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA, ribozymes, small interfering RNA, (siRNA), microRNA (miRNA), small nuclear RNA (snRNA), cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA (A, B and Z structures) of any sequence, PNA, locked nucleic acid (LNA), TNA (treose nucleic acid), isolated RNA of any sequence, nucleic acid probes, and primers. LNA, often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' and 4' carbons. The bridge "locks" the ribose in the 3'-endo structural conformation, which is often found in the A-form of DNA or RNA, which can significantly improve thermal stability.

[0043] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[0044] As used herein, "expression" refers to the process by which a polynucleotide is transcribed into mRNA and/or the process by which the transcribed mRNA (also referred to as "transcript") is subsequently being translated into peptides, polypeptides, or proteins. The transcripts and the encoded polypeptides are collectively referred to as "gene product," depending on the context.

[0045] The term "genetic modification" refers to a permanent or transient genetic change induced in a cell following introduction into the cell of a heterologous nucleic acid (i.e., nucleic acid exogenous to the cell). Genetic change ("modification") can be accomplished by incorporation of the heterologous nucleic acid into the genome of the host cell, or by transient or stable maintenance of the heterologous nucleic acid as an extrachromosomal element. Where the cell is a eukaryotic cell, a permanent genetic change can be achieved by introduction of the nucleic acid into the genome of the cell. Suitable methods of genetic modification include viral infection, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate precipitation, direct microinjection, and the like.

[0046] The terms "light-activated," "light-responsive" in reference to a polypeptide or protein that is light-responsive, are used interchangeably and include light-responsive ion channels or opsins,

and ion pumps as described herein. Such light-responsive proteins may have a depolarizing or hyperpolarizing effect on the cell on whose plasma membrane the protein is expressed depending on the ion permeability of the activated protein, and the electrochemical gradients present across the plasma membrane.

[0047] A “plurality” contains at least 2 members. In certain cases, a plurality may have at least 10, at least 100, at least 1000, at least 10,000, at least 100,000, at least 10^6 , at least 10^7 , at least 10^8 or at least 10^9 or more members.

[0048] As used herein, the terms “treat,” “treatment,” “treating,” and the like, refer to 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, particularly in a human, 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; and (c) relieving the disease, e.g., causing regression of the disease, e.g., to completely or partially remove symptoms of the disease.

[0049] “Excitable cell,” as used herein, refers to electrically excitable cells, such as neurons and muscle cells. Excitable cells typically use changes in their membrane potential to transmit signals within the cell. Thus, an excitable cell may be characterized in having a resting state, where the membrane potential is at the resting membrane potential, and an excited state, where rapid depolarization of the membrane potential is transmitted across the cell as an action potential. The “cellular electrical activity” of an excitable cell may refer to the changes in the membrane potential or may refer to any indirect measure of the changes in membrane potential, such as the changes in intracellular calcium concentration or any other biochemical changes that is a functional measure of the change in the membrane potential.

[0050] “Target tissue,” as used herein, includes any tissue or portion thereof that is of interest and suitable for performing the present method. The target tissue may be an in vivo tissue, e.g., a part of the brain of a moving or immobilized animal, or may be an in vitro tissue sample, e.g., a brain slice tissue. The target tissue may be from any suitable subject, including fish, amphibian, reptile, bird, and mammal (including human, non-human primate, mouse, rat, etc.), etc.

[0051] “Field of view,” as used herein, refers to the maximum area over the surface of a target tissue that can be accessed optically by a component of the present microscope system when the relative position of the target tissue and the component is fixed. In some cases, the field of view of a

spatial light modulator is within the field of view of the image detector, which is in turn in the field of view of the microscope.

[0052] “Adapted,” as used in reference to a neuron adapted to perform a particular function, refers to the neuron being modified, e.g., genetically modified and/or otherwise being made to associate with a functional moiety, such as a fluorescent dye, to carry out the function.

[0053] “Fluorescence sensitive to cellular electrical activity,” as used herein, refers to one or more properties of the fluorescence emitted by an excitable cell, where changes in the membrane potential of the excitable cell can be measured, directly or indirectly, by a change in the fluorescence emitted. Indirect measures of cellular electrical activity may include changes in the intracellular calcium or other ion concentration, changes in biochemical activity caused by changes in the membrane potential, etc. The change in fluorescence may be a change in the intensity, wavelength, etc., of the fluorescence. (e.g., changes in).

[0054] “Distinct,” as used herein, includes a difference in whole or in part. As such, “distinct” includes “overlapping but different”. In some cases, something that is distinct from another may be measurably distinct in that particular system.

[0055] “Image detector,” as used herein, refers to any device that includes an optical sensor for detecting a two dimensional image formed by the incident electromagnetic radiation. Thus the image detector may detect in two dimensions the electromagnetic radiation impinging upon a focal plane positioned on the optical sensor. In certain cases, an image detector excludes devices, e.g., photomultiplier tubes (PMTs), that are configured to detect electromagnetic radiation without regard to the location of the source of radiation within the imaged target.

[0056] “Collectively,” as used herein, modifies an action taken or property to refer to the action taken by, or the property obtained by an ensemble of multiple individual parts. In some cases, multiple actions are taken at least substantially simultaneously with respect to the time scale of a functional output of the system, e.g., relative to the time scale of change in the measured fluorescence that is sufficient to characterize the cellular electrical activity, e.g., neuronal activity, or relative to the time scale of the cellular electrical activity that leads to a behavioral or therapeutic output. In some cases, the actions may be simultaneous or quasi-simultaneous, and may be effectively simultaneous within a given time point of measurement (e.g., as represented by the exposure time of an image detector). Thus in some cases, a collectively measured activity of multiple neurons includes the neuronal activity-sensitive fluorescence of each neuron measured simultaneously for all the neurons, quasi-simultaneously for all the neurons, or sequentially over different subsets of the neurons, at each time point, e.g., as defined by a single exposure of an image detector.

[0057] “Pattern,” as used herein, refers to a distribution of a signal, e.g., laser light, fluorescence, etc., projected on a two-dimensional (2D) area or into a three-dimensional (3D) space. The signal may be modulated in one or more properties (e.g., amplitude, frequency, polarity, etc.) across the 2D area or 3D space. In some instances, a pattern may exclude a distribution that is substantially uniform throughout the space or area.

DETAILED DESCRIPTION

[0058] A method for measuring the activity of one or more individual excitable cells, e.g., neurons, in a target tissue is provided. The present methods and systems employ spatio-temporal multiplexing of 3D holographic illumination, and 3D imaging using a microlens array to extend the field of view and maintain a desirable time-averaged signal for imaging. The present method may include measuring the activity of individual, selected excitable cells by projecting one or more three dimensional (3D) multi-focal laser light patterns into a target tissue containing excitable cells adapted to emit cellular electrical activity-sensitive fluorescence, to generate a multiplexed 2D diffraction pattern of fluorescence emitted by the neurons, and resolving the multiplexed 2D diffraction pattern.

[0059] Another aspect of the present disclosure includes a method including obtaining individual two-dimensional (2D) diffraction patterns of multiple fluorescent excitable cells, e.g., neurons, in a target tissue, and measuring the activity of individual excitable cells by projecting one or more three dimensional (3D) multi-focal laser light patterns into the target tissue to generate a multiplexed 2D diffraction pattern of fluorescence emitted by the excitable cells, and resolving the multiplexed 2D diffraction pattern using the individual 2D diffraction patterns.

[0060] Also provided herein is a system configured to perform methods of the present disclosure, the system including a microscope configured to project a 3D multi-focal laser light pattern into a target tissue using a spatial light modulator and a mirror galvanometer, and a microlens array and an image detector to record individual and multiplexed 2D diffraction patterns of light emitted from the target tissue.

[0061] The present methods and systems may achieve the extended field of view and/or desirable time-averaged signal with a suitable illumination power at the target tissue. In some cases, the power at the target tissue of the 3D multi-focal laser light patterns (e.g., the power of 5 3D multi-focal laser light patterns) used to illuminate all of the selected excitable cells (e.g., total of about 100 cells) is about 500 milliwatts (mW) or less, e.g., about 250 mW or less, about 200 mW or less, about 150 mW or less, about 100 mW or less, including about 80 mW or less. In some embodiments, the power at the target tissue of the 3D multi-focal laser light patterns used to

illuminate all of the selected excitable cells is from about 50 mW to about 500 mW, e.g., from about 55 mW to about 250 mW, from about 60 mW to about 200 mW, from about 65 mW to about 150 mW, including from about 70 mW to about 100 mW.

[0062] Before the present disclosure is described in greater detail, it is to be understood that embodiments of the present disclosure are not limited to particular examples described, 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 only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

[0063] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the present disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the present disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the present disclosure.

[0064] Certain ranges are presented herein with numerical values being preceded by the term "about." The term "about" is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes.

[0065] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the subject matter of the present disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, representative illustrative methods and materials are now described.

[0066] The practice of various embodiments of the present disclosure employs, unless otherwise indicated, conventional techniques of biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. *See* Green and Sambrook, MOLECULAR CLONING: A LABORATORY MANUAL, 4th edition (2012); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), and CELL AND TISSUE CULTURE: LABORATORY PROCEDURES IN BIOTECHNOLOGY (Doyle &

Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference.

[0067] All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the subject matter of the present disclosure is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0068] It is noted that, as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may 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.

[0069] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[0070] In further describing various aspects of embodiments of the present disclosure in greater detail, aspects of the systems and devices of various embodiments are reviewed first in greater detail, followed by a discussion of methods and kits according to certain embodiments of the present disclosure.

SYSTEMS

[0071] Aspects of the present disclosure include systems and devices thereof configured for imaging and/or optogenetic control of light-responsive excitable cells, e.g., neurons. With reference to FIG. 13A, the present system may include i) a light microscope **1310** having an excitation path and an emission path with respect to a target tissue, wherein the excitation path includes: a spatial light modulator **1312** for projecting a 3D multi-focal laser light pattern into the target tissue; and a pair of mirror galvanometers **1314**, **1315** configured to spatially translate a field of view of the spatial light modulator on the target tissue, and wherein the emission path includes a microlens

array **1316** configured to modify fluorescence emitted by the target tissue such that a 2D diffraction pattern is projected onto an image detector **1318**, ii) a controller **1320**; and iii) a processor **1330**. The processor may be configured to execute instructions **1352** that cause the controller to: illuminate the target tissue with different 3D multi-focal light patterns over different fields of view of the spatial light modulator, thereby causing the excitable cells to emit fluorescence, and record a multiplexed 2D diffraction pattern of the emitted fluorescence, and that cause the processor to resolve the recorded multiplexed 2D diffraction pattern.

[0072] The spatial light modulator **1312** may be configured to modify light generated from a light source **1313**, to generate the 3D multi-focal laser light pattern projected into the target tissue.

[0073] In some embodiments, the system further includes input/output interfaces **1340** for a user of the system to enter input parameters and for the system to present outputs (e.g., recorded images, resolved images, measured activity of excitable cells, etc.) to the user. Input interfaces **1342** include, without limitation, a keyboard, mouse, touch pad, touch screen etc. Output interfaces **1344** include, without limitation, a screen, touch screen, speaker, indicator lights, etc. The system may further include a suitable computer-readable medium **1350** (e.g., a non-transient memory) storing the instructions **1352** as well as any input parameters **1354** that may be entered into the system by a user.

[0074] The input parameters may be any parameter that may be desirable to specify for illuminating and/or measuring activity of excitable cells in a particular target tissue, as described herein. Input parameters may include, without limitation, the coordinates of selected excitable cells in the target tissue for each set representing a field of view of the spatial light modulator; the optical model that can be used for resolving the multiplexed 2D diffraction patterns (such as an empirically determined 2D diffraction patterns for each individual cell that is selected for illumination; parameters for recording the multiplexed 2D diffraction patterns (e.g., exposure time of the image detector), etc. (see also, FIG. 13B).

[0075] In some embodiments, the subject systems include a light microscope, one or more laser light sources, one or more spatial light modulators, one or more mirror galvanometers, a microlens array, an image detector, a controller, a processor, and a computer-readable medium comprising instructions that, when executed by the processor, cause the controller to carry out one or more of the subject methods, described further herein. Each of these components is now described in greater detail, with further reference made to FIG. 1A or FIG. 8.

[0076] As summarized above, aspects of the present disclosure include a system that includes a light microscope. The light microscope may be configured to optically probe in three dimensions, using one or more three-dimensional (3D) multi-focal laser light patterns, a target tissue that is in

the field of view of the microscope. The target tissue may contain excitable cells, e.g., neurons, that are adapted to emit fluorescence, e.g., a two-photon excited fluorescence emission, when illuminated by an appropriate laser light. Thus, the light microscope may include a multi-focal near-infrared (NIR) excitation path and a visible detection path.

[0077] The multi-focal near-infrared (NIR) excitation path includes one or more laser light sources, one or more spatial light modulators (FIG. 1A and FIG. 8, “**SLM**”) and one or more mirror galvanometers (FIG. 1A, “**GM**”). The laser light source produces a laser beam with wavelength in the NIR range (from about 700 nm to about 2500 nm) and the laser beam is directed to a spatial light modulator (**SLM**), e.g., a phase spatial light modulator. The system may include a beam expander (as shown in FIG. 8, “**BE**”), e.g., an achromatic doublet, to expand the laser beam to cover the spatial light modulator. The spatial light modulator may be programmed with a phase mask that causes the laser beam to split into multiple beamlets (i.e., causes the laser beam to be spatially modulated in phase and/or amplitude) and thereby project any arbitrary 3D multi-focal laser light pattern into the target tissue placed in the focal volume. The beamlets are reflected off of a pair of mirror galvanometers (**GM**) positioned between the spatial light modulator and the target tissue. The mirror galvanometers are configured such that the 3D multi-focal laser light pattern may be projected into multiple distinct locations within the target tissue. In some cases, the spatially modulated laser beam is projected and focused onto a midpoint between the two mirror galvanometers which are spaced about 1 cm apart. The mirror galvanometers may have a mirror of any suitable size to hold the laser beam diameter over the angular range used (e.g., the mirror aperture may be in the range of about 3 mm to about 50 mm, e.g., about 3 mm to about 20 mm, about 3 mm to about 10 mm, including about 3 mm to about 7 mm).

[0078] The light microscope is configured to direct fluorescence emitted from the excitable cells in the target tissue upon illumination of the target tissue by the 3D multi-focal laser light pattern through the visible detection path. The visible detection path may include a diffractive optical element, e.g., a microlens array (FIG. 1A, “**MLA**”), that forms a two-dimensional (2D) diffraction pattern of the fluorescence emitted from the target tissue onto the optical sensor of an image detector, e.g., a scientific Complementary Metal Oxide Semiconductor (sCMOS) camera (FIG. 1A, “**sCMOS**”).

[0079] The laser light source may be any convenient laser light source for use in the present system and method. In certain embodiments, the laser light source generates a laser beam having a wavelength in the near-infrared (NIR) range (e.g., about 700 nm to about 2500 nm, 800 nm to 1500 nm, including 850 nm to 1000 nm). In some embodiments, the wavelength of the laser beam produced by the laser light source is tunable within its operating range. In certain embodiments,

the laser light source generates a laser beam having a pulse duration in the range of about 10 fs to about 1000 fs, e.g., about 50 fs to about 800 fs, about 100 fs to about 500 fs, about 100 fs to about 350 fs, including about 150 fs to about 250 fs.

[0080] The laser light source may be any suitable laser light source for use in the present system. In certain embodiments, the laser light source is a solid state laser, e.g., a mode-locked Titanium (Ti):Sapphire laser, such as those manufactured by Spectra-Physics of Mountain View California or by Coherent, Inc. of Santa Clara, California. Other suitable laser sources may include a dye laser, semiconductor laser (e.g., a diode laser), or a fiber laser, as described in, e.g., PCT Pub. No. WO2014/20513, which is incorporated herein by reference.

[0081] In certain embodiments, the laser light source includes a regenerative amplifier. The regenerative amplifier may be configured to receive a first seed laser beam and generate a second laser beam that has a higher pulse energy but lower average power than the first seed laser beam. In some cases, the laser light source includes a multipass amplifier as an alternative to the regenerative amplifier, or in conjunction with the regenerative amplifier. Any convenient regenerative amplifier and/or multipass amplifier may be used in the present system, as described in, e.g., U.S. Patent No. US RE042499; and U.S. App. Pub. No. 20070053401, which are incorporated herein by reference, and provided by Coherent (Santa Clara, CA).

[0082] In certain embodiments, the laser light source generates a laser beam pulsed at a frequency of about 100 MHz or less, including about 1 MHz or less, e.g., about 500 kHz or less, about 400 kHz or less, including about 300 kHz or less. In certain embodiments, the laser light source generates a laser beam pulsed at a frequency in the range of about 100 kHz to about 100 MHz, e.g., about 50 MHz to about 90 MHz, about 100 kHz to about 500 kHz, about 150 kHz to about 350 kHz, including about 200 kHz to about 300 kHz.

[0083] In some cases, the light source, e.g., laser light source, is configured to generate light, e.g., laser beam, having different properties, such as different pulse frequencies. A removable mirror (FIG. 1A, “RM1”) may be used to select between a first light and a second light having different properties, based on the position of the removable mirror.

[0084] In certain embodiments, the laser light source generates a laser beam with an average power in the range of about 1.0 W to 6.0 W, e.g., about 2.0 W to 5.5 W, about 1.4 W to 2.5 W, including about 1.6 to 2.0 W.

[0085] In certain embodiments, the system includes at least two laser light sources, at least one for use in measuring the activity of a plurality of activity-sensitive fluorescence-emitting excitable cells, e.g., neurons, in a target tissue, and at least one for use in performing standard two-photon scanning microscopy and/or photo-stimulation of excitable cells, as described below.

- [0086] The system may include a Pockels cell (FIG. 1A, “PC”) configured to receive the laser beam that leaves the laser light source and stabilize the intensity of the laser beam. The Pockels cell may be configured to modify the power of the light, e.g., laser beam, from the light source, e.g., laser light source, such that the 3D multi-focal light patterns collectively have a power at the target tissue of about 500 milliwatts (mW) or less, e.g., about 250 mW or less, about 200 mW or less, about 150 mW or less, about 100 mW or less, including about 80 mW or less, and in some cases, collectively have a power at the target tissue of about 10 mW or more, e.g., about 20 mW or more, about 30 mW or more, about 40 mW or more, about 50 mW or more, including about 70 mW or more. In some cases, the 3D multi-focal light patterns collectively have a power at the target tissue of from about 10 mW to about 500 mW, e.g., about 20 mW to about 250 mW, from about 20 mW to about 200 mW, from about 30 mW to about 200 mW, from about 30 mW to about 150 mW, from about 40 mW to about 100 mW, including from about 50 mW to about 80 mW.
- [0087] The system may further include half wave plates (FIG. 1A, “HWP”) that matches the polarization of the laser beam with that required by the spatial light modulator (FIG. 1A, “SLM”).
- [0088] The spatial light modulator (SLM) may be any suitable spatial light modulator for use in the present system and method. In certain embodiments, the spatial light modulator is a phase spatial light modulator. The phase spatial light modulator may be capable of $0-2\pi$ phase modulation of the incident laser light. In certain embodiments, the spatial light modulator may have a resolution of 2000 pixels or less x 2000 pixels or less, e.g., 1500 pixels or less x 1500 pixels or less, 1000 pixels or less x 1000 pixels or less, including 800 pixels or less x 800 pixels or less, and may have a resolution in the ranges of 200 pixels or more x 200 pixels or more, e.g., 256 pixels or more x 256 pixels or more, 350 pixels or more x 350 pixels or more, including 500 pixels or more x 500 pixels or more. In certain embodiments, the spatial light modulator has a resolution in the ranges of 200 pixels -2000 pixels x 200 pixels -2000 pixels, e.g., 256 pixels -1000 pixels x 256 pixels -1000 pixels, 400 pixels -1000 pixels x 400 pixels -1000 pixels, including 500 pixels -800 pixels x 500 pixels -800 pixels.
- [0089] The volume of the tissue in which excitable cells are collectively illuminated by a series of 3D multi-focal laser light patterns (i.e., 3D holographic illumination patterns) according to methods of the present disclosure may depend on the transition time of the spatial light modulator. Thus, the faster the transition time of the spatial light modulator, the more distinct 3D multi-focal laser light patterns may be projected into the tissue over a larger volume, within the exposure time of the image detector. In some cases, the spatial light modulator has a maximum

switching rate of at least about 40 Hz, e.g., at least about 50 Hz, at least about 75 Hz, at least about 100 Hz, at least about 120 Hz, at least about 150 Hz, including at least about 200 Hz, In certain embodiments, the spatial light modulator has a maximum switching rate in the range of about 10 Hz to about 200 Hz, e.g., about 15 Hz to about 150 Hz, about 20 Hz to about 120 Hz, including about 20 Hz to about 100 Hz. In certain embodiments, the spatial light modulator has a maximum switching rate of from about 40 Hz about 200 Hz, e.g., about 50 Hz to about 150 Hz, about 100 Hz to about 150 Hz, including about 120 Hz to about 150 Hz. The “maximum switching rate” as used herein, refers to the fastest rate at which the spatial light modulator can transition between spatially modulating a laser light beam to project two or more distinct, predetermined 3D patterns. The rate may be determined based on the time (t_{SLM}) taken by the spatial light modulator to achieve about 97% power when transitioning from one illumination pattern to another. Suitable spatial light modulators are provided by, e.g., Meadowlark Optics (Frederick, CO) and Hamamatsu (Bridgewater, NJ).

[0090] The present system is configured to image the spatial light modulator using a $2f_1 + 2f_2$ configuration relay lens pair (FIG. 1A, “L3”, “L4”; FIG. 8, “L1”, “L2”), onto a first member of the pair of mirror galvanometers and then onto a second member of the pair of mirror galvanometers, where the first and second mirror galvanometers are spaced apart equidistant from the conjugate spatial light modulator plane. Each member of the pair of galvanometers controls the horizontal translation in either the x or y direction of the 3D multi-focal laser light pattern within the field of view of the microscope and the image detector.

[0091] The mirror galvanometer may be any suitable mirror galvanometer for use in the present system. Suitable mirror galvanometers include those used in scanning laser microscopes described in, e.g., U.S. Patent Nos. 4,734,578; U.S. App. Pub. Nos. 20070171502, 20110279893, which are incorporated herein by reference.

[0092] A beam block (FIG. 1A and FIG. 8, “ZB”) is positioned between lens f_1 and f_2 (FIG. 1A, L3, L4; FIG. 8, L1, L2) to block the undiffracted, zero order light from the spatial light modulator.

[0093] The image of the spatial light modulator relayed from the mirror galvanometers is directed onto the back aperture of the objective placed over the target tissue via a pair of relay lenses and a short pass dichroic mirror.

[0094] Fluorescent light emitted from the target tissue upon illumination with a laser light is directed through the emission path. The emission path may be configured in any suitable manner, such as that described in Broxton et al., 2013, Optics Express 21(21), 25418, which is incorporated herein by reference. The emitted light is directed through the microlens array (MLA) that is placed at the image plane. The emission path may further include a tube lens (FIG. 1A and FIG. 8, “TL”)

positioned between the objective and the microlens array. The image detector is placed to capture the image at a plane one microlens-focal length behind the microlens array.

[0095] The microlens array may be any suitable microlens array for use in the present system. The size of individual lenses in the microlens array may vary from array to array, and within an array may be 50 μm or more x 50 μm or more, e.g., 70 μm or more x 70 μm or more, 100 μm or more x 100 μm or more, and may be 1000 μm or less x 1000 μm or less, e.g., 500 μm or less x 500 μm or less, including 200 μm or less x 200 μm or less. In some embodiments, the size of individual lenses in the microlens array may be in the ranges of 50-1000 μm x 50-1000 μm , e.g., 80-500 μm x 80-500 μm , including 100-200 μm x 100-200 μm . The f/number ($f/\#$) of the microlens array, defined as the ratio of the focal length to the distance between two parallel light beams incident on an individual lens of the microlens array at an angle perpendicular to the plane of the lens, may vary and may be 4 or more, e.g., 5 or more, including 8 or more, and may be 100 or less, e.g., 50 or less, including 20 or less. In some embodiments, the f/number of the microlens array may be in the range of 4 to 100, e.g., 5 to 50, including 8 to 15.

[0096] The image detector may be any suitable image detector for use in the present system. In some instances, the image detector is a digital camera, such as a CMOS detector, e.g., a scientific CMOS camera, or a charge-coupled device (CCD) detector, e.g., an electron-multiplying CCD (EMCCD) camera.

[0097] The emission path may further include a photodetector, e.g., a photomultiplier tube (FIG. 1A and FIG. 8, “PMT”), configured to receive light emitted from the target tissue upon illumination with a laser light. A second removal mirror (Fig. 1A, “RM2”) may be used to select whether the emitted light is directed onto the microlens array and thereby to the image detector, or to the photomultiplier tube.

[0098] The present system may include additional components extend the axial field of view. Thus, in some embodiments, the present system includes one or more tunable lenses, in the illumination path, the objective and/or the detection path of the microscope, where the tunable lenses are configured to extend the axial field of view. In some embodiments, the system includes one or more deformable mirrors, where the deformable mirrors are configured to extend the axial field of view.

[0099] As summarized above, aspects of the present disclosure include a controller, processor and computer readable medium that are configured or adapted to control or operate one or more components of the subject systems. In some embodiments, a system includes a controller that is in communication with one or more components of the systems, as described herein, and is configured to control aspects of the systems and/or execute one or more operations or functions

of the subject systems. In some embodiments, a system includes a processor and a computer-readable medium, which may include memory media and/or storage media. Applications and/or operating systems embodied as computer-readable instructions on computer-readable memory can be executed by the processor to provide some or all of the functionalities described herein (e.g., cause the controller to control the spatial light modulator and thereby generate a 3D multi-focal light pattern to illuminate selected excitable cells, control the mirror galvanometers to spatially translate the field of view of the spatial light modulator, record the multiplexed 2D diffraction pattern, and/or cause the processor to resolve a recorded multiplexed 2D diffraction pattern, etc.).

[00100] In some embodiments, a system includes a user interface, such as a graphical user interface (GUI), that is adapted or configured to receive input from a user, and to execute one or more of the methods as described herein. In some embodiments, a GUI is configured to display data or information to a user.

[00101] In some embodiment, the present system is configured to enable a user to provide one or more inputs to the system to control the various components (e.g., the spatial light modulator, laser light source, galvanometer mirrors, etc.) and/or to set parameters that the processor may use to execute an algorithm for performing a method as described herein.

[00102] In certain embodiments, the present system is a closed-loop system for measuring and controlling the activity of one or more excitable cells, e.g., neurons in a target tissue. In such instances, the controller may be programmed to illuminate a first set of excitable cells in the target tissue in response to the measured activity of a second set of excitable cells, according to a method as described herein. The first set and second set of excitable cells may be the same, or different (including different but overlapping) set of cells. The first set of excitable cells (e.g., neurons) may be configured to modulate activity (e.g., neural activity) upon being illuminated by an appropriate light stimulus, by, e.g., expressing a light-responsive polypeptide, as described further herein. Where a laser light illumination (non-spatially modulated, or spatially modulated) is used to modulate activity of the first set of excitable cells and to measure activity of a second set of excitable cells, the system may be configured so that spectral overlap between the modulating and measuring laser light illuminations is minimized.

METHODS

[00103] Aspects of the present disclosure include methods that may be used for imaging and/or optogenetic control of excitable cells, e.g., neurons. With reference to FIG. 12, in general terms, a method of the present disclosure may include selecting **1210** a set of excitable cells, e.g., neurons,

that are labeled with a cellular electrical activity-sensitive marker (e.g., a calcium indicator dye or protein) in a target tissue (e.g., a brain), and measuring the activity of each of the selected cells using a spatio-temporally multiplexed 3D holographic illumination **1220** and recording fluorescence signal from the illuminated cells that is further modified by a microlens array, as a multiplexed, 2D diffraction pattern **1230**, and resolving the recorded 2D diffraction pattern **1240**.

[00104] The 3D holographic illumination may be a 3D multi-focal light pattern generated by modifying light projected onto a target tissue using a spatial light modulator, where light is focused on a sub-volume of the total volume accessible within a field of the view of the spatial light modulator. The pattern of the 3D multi-focal light may be predetermined to match the location of excitable cells of interest preselected in the target tissue. Each excitable cell illuminated by the 3D multi-focal light pattern may be illuminated at any suitable portion of the excitable cell for measuring the electrical activity of the cell. Thus, in some cases, an excitable cell, such as a neuron, may be illuminated by the 3D multi-focal light pattern at the cell body (i.e., the region of the cell at and surrounding the location of the nucleus), and/or along an extension of the cell body, such as a neurite (e.g., axon, dendrite, etc.).

[00105] Spatio-temporally multiplexed 3D multi-focal light patterns are a collection of individual 3D multi-focal light patterns, each individual 3D multi-focal light pattern being generated by modulation of light projected onto the tissue using the spatial light modulator. The individual 3D multi-focal light patterns may generally have different patterns from each other. The individual 3D multi-focal light patterns may be sequentially (i.e., one after another) projected into the target tissue, and the field of view of the spatial light modulator may be spatially translated between any two or more of the individual 3D multi-focal light patterns, e.g., using a mirror galvanometer. As the field of view of the spatial light modulator is shifted between individual 3D multi-focal light patterns, a different subset of excitable cells, e.g., neurons, may become accessible for illumination by an individual 3D multi-focal light pattern. Thus, the spatio-temporally multiplexed 3D multi-focal light patterns can collectively illuminate excitable cells in a volume of the target tissue that is not accessible with an individual 3D multi-focal light pattern (i.e., without translating the field of view of the spatial light modulator).

[00106] The sets of excitable cells that are illuminated by individual 3D multi-focal light patterns at each field of view of the spatial light modulator may be different from each other. In some cases, overlapping but different sets of excitable cells are illuminated by individual 3D multi-focal light patterns as the spatial light modulator is translated across the target tissue and an individual 3D multi-focal light pattern is projected into the tissue at each position. The degree of overlap may be any suitable degree of overlap, and in some cases, may be 90% or less, e.g., 80% or less, 70% or

less, 60% or less, 50% or less, 40% or less, 30% or less, 20% or less, 10% or less, 5% or less, including 1 % or less, between any two sets of excitable cells illuminated by two different individual 3D multi-focal light patterns. In some cases, the sets of excitable cells illuminated by individual 3D multi-focal light patterns as the spatial light modulator is translated across the target tissue are non-overlapping sets.

[00107] The number of cells in each set of excitable cells that are illuminated by individual 3D multi-focal light patterns at each field of view of the spatial light modulator may vary depending on individual 3D multi-focal light pattern used to illuminate each set of cells. In general, each set of excitable cells illuminated by individual 3D multi-focal light patterns is smaller than the total number of excitable cells selected for illumination and/or measurement of activity. Thus, in some cases, a set of excitable cells illuminated by an individual 3D multi-focal light pattern has on average 90% or fewer, e.g., 80% or fewer, 70% or fewer, 60% or fewer, 50% or fewer, 40% or fewer, 30% or fewer, 20% or fewer, including 10% or fewer number of cells than the total number of excitable cells selected for illumination and/or measurement of activity. In some embodiments, the set of excitable cells illuminated by an individual 3D multi-focal light pattern has on average from 5% to 10%, from 10% to 20%, from 30% to 40 %, from 40% to 50%, from 50% to 60%, from 60% to 70%, from 70% to 80%, or from 80% to 90% fewer number of cells than the total number of excitable cells selected for illumination and/or measurement of activity.

[00108] In some embodiments, the subject method involves selecting a first subset of excitable cells, e.g., a first subset of neurons, in a target tissue for analysis, wherein the target tissue is in a field of view of a microscope and wherein the target tissue includes a plurality of excitable cells that are adapted to emit fluorescence sensitive to cellular electrical activity, e.g., neuronal activity, obtaining individual two-dimensional (2D) diffraction patterns for each excitable cell in the first subset of excitable cells by a) contacting an excitable cell in the first subset with a laser light, thereby causing the excitable cell to emit fluorescence, b) directing the emitted fluorescence from the contacted excitable cell through a microlens array to create a 2D diffraction pattern, and c) recording the 2D diffraction pattern, and iii) measuring the activity of individual excitable cells in the first subset by a) projecting one or more three-dimensional (3D) multi-focal laser light patterns into the target tissue, thereby causing at least a subset of excitable cells in the first subset of excitable cells in the target tissue to emit fluorescence, b) directing the fluorescence emitted from the target tissue in response to the one or more 3D multi-focal laser light patterns through the microlens array to generate a multiplexed 2D diffraction pattern, c) recording the multiplexed 2D diffraction pattern, and d) resolving the multiplexed 2D diffraction pattern using the individual 2D diffraction patterns of each individual excitable cell in the at least a subset of

excitable cells in the first subset to determine the activity of each individual excitable cell illuminated by the one or more 3D multi-focal laser light patterns. Aspects of the methods are now further described in greater detail below.

[00109] In general terms, excitable cells, e.g., neurons, whose activity patterns are of interest are first selected within a target tissue. To allow selection of the neurons, the target tissue may be in the field of view of a microscope, such as the microscope of the system described above, and the target tissue may contain neurons that are adapted to emit fluorescence so that they may be identified, e.g., by scanning the tissue using conventional scanning two-photon fluorescence microscopy and recording the emitted fluorescence with a photodetector (e.g., a photomultiplier tube), to generate a stack of full-frame 2D images. The fluorescence emitted by the neuron includes fluorescence sensitive to neuronal activity, e.g., intensity, wavelength, etc., of fluorescence changes in response to changes in membrane potential or intracellular calcium concentration. Fluorescent neurons suitable for use in the present method are described in further detail below.

[00110] For each excitable cell, e.g., each neuron, of interest, the cell may be illuminated with a laser light appropriate to cause the excitable cell to emit fluorescent light and the fluorescent light originating from the excitable cell and diffracted by the microlens array is captured on an image detector as an individual 2D diffraction pattern, which can be used to obtain an empirical measure of the point spread function of the excitable cell. In certain embodiments, the laser light is one or more 3D single-focal laser light patterns generated by the spatial light modulator. In some embodiments, the laser light is a plurality of 3D single-focal laser light patterns, temporally scanned across the excitable cell to generate an individual 2D diffraction pattern.

[00111] The activity of individual excitable cells, e.g., individual neurons, is then measured collectively for multiple excitable cells of interest in the field of view of the image detector by illuminating the excitable cells, or different subsets of excitable cells, using one or more 3D multi-focal laser light patterns. Measuring the activity of an excitable cell may include measuring the change in the activity level of the excitable cell over a time period. Thus, in such instances, the time period may be divided into multiple time points (e.g., exposure times of the image detector) at which the activity of the excitable cell is measured according to the method described herein. Thus, the activity of individual excitable cells may be measured collectively at each time point (e.g., single exposure of the image detector) for multiple excitable cells of interest in the target tissue by illuminating all the excitable cells, or excitable cells in one or more different subsets thereof, using one or more 3D multi-focal laser light patterns.

[00112] The 3D multi-focal laser light patterns may be generated, e.g., by splitting a seed laser beam into multiple beamlets using a spatial light modulator (in other words, spatially modulating the amplitude and/or phase of the seed laser beam using the spatial light modulator), and projecting the beamlets (i.e., the spatially modulated laser beam) into the target tissue as 3D multi-focal laser light patterns, where the 3D multi-focal laser light patterns may be configured such that excitable cells, e.g., neurons, of interest are specifically targeted for illumination by the beamlets of the 3D multi-focal laser light patterns (i.e., by the spatially modulated laser beam). The illumination may be spatio-temporally multiplexed within each time point (e.g., single exposure of an image detector) of the measurement such that each pattern of a plurality of 3D multi-focal laser light patterns are projected sequentially into the sample at one or more fields of view of the spatial light modulator. Thus in some cases, multiple 3D multi-focal laser light patterns may be projected into the sample at the same field of view of the spatial light modulator by switching the phase mask of the spatial light modulator. In some cases, a single 3D multi-focal laser light pattern may be projected into the sample at multiple distinct fields of view of the spatial light modulator by horizontally translating the spatial light modulator field of view using the mirror galvanometers. In some cases, multiple 3D multi-focal laser light patterns may be projected into the sample in at least two distinct fields of view of the spatial light modulator by switching the phase mask of the spatial light modulator and horizontally translating the spatial light modulator field of view using the mirror galvanometers.

[00113] In certain embodiments, an individual excitable cell, e.g., an individual neuron whose activity is selected to be measured, is illuminated by the beamlets forming one or more 3D multi-focal laser light patterns at each time point (e.g., a single exposure of the image detector of, e.g., 100 milliseconds (ms)). In such cases, a spatio-temporally multiplexed 3D multi-focal laser light pattern is projected into the target tissue for a time point, and some of the beamlets of the spatio-temporally multiplexed 3D multi-focal laser light pattern collectively illuminates a volume corresponding to the position of the individual excitable cell with a scanned diffraction limited spot. Thus, in some embodiments, the method includes projecting a spatio-temporally multiplexed 3D multi-focal laser light pattern into the target tissue to illuminate a set of excitable cells in each time point. In some embodiments, the method includes projecting a plurality of spatio-temporally multiplexed 3D multi-focal laser light patterns into the target tissue to illuminate a plurality of sets of excitable cells in each time point.

[00114] The rate at which multiple distinct 3D multi-focal laser light patterns are sequentially projected into the target tissue may vary, depending on the desired level of multiplexing and/or signal strength. In some cases, the spatio-temporally multiplexed 3D multi-focal light patterns are

projected into the target tissue by projecting a plurality of individual 3D multi-focal light patterns sequentially into the target tissue at a rate of at most about 0.75 times, e.g., at most about 0.7 times, at most about 0.65 times, at most about 0.6 times, at most about 0.55 times, including at most about 0.5 times the maximum switching rate of the spatial light modulator. In some embodiments, the rate may be in the range of about 0.3 times to about 0.8 times, e.g., about 0.35 times to about 0.75 times, about 0.4 times to about 0.7 times, including about 0.4 times to about 0.6 times the maximum switching rate of the spatial light modulator. In some embodiments, the rate at which distinct spatio-temporally multiplexed 3D multi-focal laser light patterns are sequentially projected into the target tissue may vary, and the rate may be in the range of about 0.3 to about 0.8, e.g., about 0.4 to about 0.7, including about 0.4 to about 0.6 times the maximum switching rate of the spatial light modulator.

[00115] The wavelength of the laser light used to contact or illuminate the excitable cells may be any suitable wavelength, depending on the fluorescence excitation wavelength of the cells. In some cases the present method employs two-photon excitation. Thus, in some cases, the wavelength of the laser light is in the near-infrared range (e.g., about 700 nm to about 2500 nm, 800 nm to 1500 nm, including 850 nm to 1000 nm).

[00116] Fluorescence emitted by the excitable cells illuminated by the one or more 3D multi-focal laser light patterns is directed through the microlens array, thereby creating a diffracted fluorescence emission and the diffracted fluorescence emission is imaged by an image detector as a 2D diffraction pattern. The 2D diffraction pattern may be a multiplexed 2D diffraction pattern, containing an incoherent linear sum of the fluorescence emitted by multiple individual excitable cells in the target tissue upon illumination by the 3D multi-focal laser light pattern. The relationship between the observed multiplexed 2D diffraction pattern and the contribution of fluorescence from each individual excitable cell can be represented by the equation: $\mathbf{f} = \mathbf{H}\mathbf{g}$, where $\mathbf{f}$ represents the observed fluorescence intensity at each pixel, $\mathbf{H}$ is a measurement matrix constructed from the empirically-determined individual 2D diffraction patterns, and $\mathbf{g}$ represents the contribution of fluorescence intensity from discrete volumes in the target tissue (Fig. 1D). Thus, the contribution of fluorescence emitted by each illuminated excitable cell to the multiplexed 2D diffraction pattern may be resolved by deconvolving the multiplexed 2D diffraction pattern using an optical model for light propagation through the emission path of the microscope, which, in some cases, may be the empirically-determined individual 2D diffraction patterns for each of the excitable cells. A suitable method is described in, e.g., Broxton et al., 2013, Optics Express 21(21), 25418, which is incorporated herein by reference. The optical model may be any suitable model for deconvolving the multiplexed 2D diffraction pattern, and in

some cases, may be an analytical model, or an empirical model, such as that described above. In some cases, the analytical model is a wave optics model.

- [00117]** The target tissue may be any suitable excitable tissue for measuring the activity of one or more individual excitable cells therein. In some embodiments, the target tissue is a light-scattering target tissue. In some cases, light passing through the light-scattering target tissue may be diffracted differently depending on the path of passage. Thus, the light-scattering tissue may induce variable, non-homogeneous diffraction patterns depending on the location within the tissue volume from which light, e.g., fluorescence, originates. In some cases, the light-scattering target tissue has variable, non-homogeneous indices of refraction throughout the tissue volume. The target tissue may also absorb light passing through. Thus, the intensity of light may decrease as light passes through a scattering target tissue.
- [00118]** In some embodiments, the decrease in light intensity per unit thickness of tissue may be defined as a scatter coefficient, S , as described in Aravanis et al., J Neural Eng 2007 Sep; 4(3) S143-56, which is incorporated by reference. S may be estimated by the Kubelka-Munk model for diffuse scattering media: $T = 1/(Sz+1)$, where T is the transmission fraction, and z is the thickness of the sample, assuming a planar, homogeneous, ideal diffuser illuminated from one side with diffuse monochromatic light, constant reflection and no absorption through the sample. The scatter coefficient, S , of the target tissue may vary, and may be in the range of about 5 mm^{-1} to about 20 mm^{-1} , e.g., about 8 mm^{-1} to about 15 mm^{-1} , including about 10 mm^{-1} to 12 mm^{-1} .
- [00119]** In some embodiments, the target tissue, e.g., the light-scattering target tissue, can be imaged using the present system and method at a depth of up to about $500 \text{ }\mu\text{m}$, e.g., up to about $400 \text{ }\mu\text{m}$, up to about $350 \text{ }\mu\text{m}$, up to about $300 \text{ }\mu\text{m}$, including up to about $250 \text{ }\mu\text{m}$ from the surface of the target tissue proximal to the objective.
- [00120]** The target tissue may be an in vivo neuronal tissue, a tissue slice preparation, a nerve fiber bundle, a neuromuscular junction, etc. The in vivo neuronal tissue may be neuronal tissue of an animal that is anesthetized or non-anesthetized, and is restrained or non-restrained, where the present system is configured so as to place the target tissue of interest in the field of view of the microscope. The target tissue of interest includes, but is not limited to, the neocortex, the hypothalamus, entorhinal and hippocampal formation cortex, mammillary bodies, septum, bed nucleus of stria terminalis, dorsal and ventral striatum, thalamus, amygdala, accumbens, brainstem, subcortical structures in general, muscle, spinal cord, cardiac tissue, etc.
- [00121]** In certain embodiments, the present method includes placing the target tissue in the field of view of the microscope of the present system, as described above. Placing the target tissue in the field of view of the microscope of the present system may be achieved by any suitable method

known in the art. In some instances, when the target tissue is an in vivo neuronal tissue, the system may include an interface for implanting or fixing a device to the animal such that the target tissue is placed in the field of view of the microscope.

[00122] Any suitable part of an excitable cell may be used to illuminate and/or measure activity therein. Thus, a reference to a “cell” is intended to include a portion of the cell, such as the cell body, which may in general refer to the area of the cell that is at and surrounds the cell nucleus, or any cytoplasmic extension of the cell that is amenable to illumination and/or optical measurement by methods of the present disclosure. In some cases, two spatially distinct portions of a single cell (e.g., distinct neurites or distinct synaptic termini of a neuron) may be considered to be different “cells”.

[00123] The number of excitable cells, e.g., neurons, illuminated by a single 3D multi-focal laser light pattern projected into the target tissue may be 10 or more excitable cells, e.g., 15 or more excitable cells, 20 or more excitable cells, 50 or more excitable cells, 80 or more excitable cells, including 100 or more excitable cells, and in some cases may be 1,000 or fewer excitable cells, e.g., 500 or fewer excitable cells, including 200 or fewer excitable cells. In some embodiments, the number of excitable cells, e.g., neurons, illuminated by a single 3D multi-focal laser light pattern projected into the target tissue may be in the range of 10 cells to 1,000 cells, e.g., 10 cells to 500 cells, 15 cells to about 500 cells, including 15 cells to 200 cells.

[00124] The number of excitable cells, e.g., neurons, whose activity-sensitive fluorescence is measured at each time point (e.g., collectively measured over each exposure of the image detector) may be 10 or more excitable cells, e.g., 15 or more excitable cells, 20 or more excitable cells, 50 or more excitable cells, 80 or more excitable cells, 100 or more excitable cells, 200 or more excitable cells and may be 5000 or fewer excitable cells, 1000 or fewer excitable cells, 500 or fewer excitable cells, including 200 or fewer excitable cells. In some embodiments, the number of excitable cells, e.g., neurons, whose activity-sensitive fluorescence is measured at each time point (e.g., collectively measured over each exposure of the image detector) may be in the range of 15 to 3000 excitable cells, e.g., 20 to 1000 excitable cells, 20 to 500 excitable cells, 50 to 300 excitable cells, including 100 to 250 excitable cells. In certain embodiments, the present method include selecting a subset of excitable cells in the target tissue and measuring the activity of all excitable cells in the subset at each time point by collectively illuminating each of the excitable cells with one or more 3D multi-focal laser light patterns.

[00125] In some embodiments, where multiple distinct 3D multi-focal laser light patterns are projected into the target tissue at each time point (e.g., each exposure of the image detector) of the measurement, the spatial light modulator phase mask for each 3D multi-focal laser light pattern is

defined so that the one or more 3D multi-focal laser light patterns illuminate a subset of excitable cells whose individual 2D diffraction patterns are sufficiently different from one another so as to improve the precision and/or accuracy of resolving the multiplexed 2D diffraction pattern generated by the excitable cells. The difference between individual 2D diffraction patterns may be determined by any convenient method, e.g., Euclidian distance, percent similarity in pixels, etc. Thus, the excitable cells assigned to a subset of excitable cells that are illuminated by a spatio-temporally multiplexed 3D multi-focal laser light pattern may be chosen by setting a minimum threshold for differences between any two individual 2D diffraction patterns within the subset. In some instances, the excitable cells assigned to a subset of excitable cells that are illuminated by a spatio-temporally multiplexed 3D multi-focal laser light pattern may be chosen by maximizing the minimum difference between any two individual 2D diffraction patterns within the subset.

[00126] In certain embodiments, the present method includes calibrating the spatial light modulator coordinate system and the 3D two-photon imaging coordinate system using the 2D diffraction pattern of the fluorescence generated by the microlens array as the reference coordinate system. In certain embodiments, the calibrating includes placing a fluorescent sample volume in the field of view of the microscope, illuminating discrete spots within the volume to cause emission of fluorescence from each spot, directing the fluorescence through the microarray lens to generate a reference 2D diffraction pattern for each location, recording the reference 2D diffraction pattern on the image detector and reconstructing the location of the spots.

[00127] In certain embodiments, the laser beam that is used to seed the multiplexed 3D multi-focal laser light pattern is pulsed at a frequency of about 100 kHz or more, e.g., about 150 kHz or more, about 200 kHz or more, and in some cases, may be pulsed at a frequency of about 1 MHz or less, e.g., about 800 kHz or less, about 600 kHz, or less, about 500 kHz or less, including about 400 kHz or less. In certain embodiments, the laser beam that is used to seed the multiplexed 3D multi-focal laser light pattern is pulsed at a frequency in the range of about 100 kHz to about 1 MHz, e.g., about 100 kHz to about 700 kHz, about 100 kHz to about 500 kHz, about 150 kHz to about 350 kHz, including about 200 kHz to about 300 kHz.

[00128] In certain embodiments, the laser beam that is used to seed the multiplexed 3D multi-focal laser light pattern has an average power in the range of about 1.0 W to about 3.0 W, e.g., about 1.5 W to about 2.5 W, about 1.6 W to about 2.0 W, including about 1.7 W to about 1.9 W.

[00129] In certain embodiments, the laser beam that is used to seed the multiplexed 3D multi-focal laser light pattern has a pulse duration in the range of about 10 fs to about 1000 fs, e.g., about 50

fs to about 800 fs, about 80 fs to about 500 fs, about 100 fs to about 300 fs, including about 150 fs to about 250 fs.

[00130] In some embodiments, the spatio-temporal multiplexing of the illumination patterns on the spatial light modulator increases the two photon signal generation efficiency. In some embodiments, the spatio-temporal multiplexing of the illumination patterns on the spatial light modulator provides for a more uniform illumination of the field of view of the image detector within each time point (e.g., each exposure of the image detector) during measurement of neural activity.

[00131] The present methods and systems may provide for illuminating and/or measuring cellular electrical activity, e.g., neural activity, from cells in a volume that is larger than the maximum volume that can be illuminated or imaged using a stationary spatial light modulator at the same temporal resolution. In some cases, the volume of the target tissue that is illuminated and/or imaged by the present methods and systems has a horizontal dimension (i.e., width or length along a plane perpendicular to the axis of view of the microscope) of about 300 μm or more, e.g., about 350 μm or more, about 400 μm or more, about 450 μm or more, including about 500 μm or more. In some embodiments, the volume of the target tissue that is illuminated and/or imaged by the present methods and systems has a horizontal dimension in the range of about 300 μm to about 1,000 μm , e.g., about 350 μm to about 900 μm , about 400 μm to about 800 μm , including about 500 μm to about 700 μm . In some cases, the volume of the target tissue that is illuminated and/or imaged by the present methods and systems has a depth (i.e., along the axis of view of the microscope) of about 50 μm or more, e.g., about 75 μm or more, about 100 μm or more, about 150 μm or more, including about 200 μm or more. In some embodiments, the volume of the target tissue that is illuminated and/or imaged by the present methods and systems has a horizontal dimension in the range of about 50 μm to about 500 μm , e.g., about 75 μm to about 400 μm , about 100 μm to about 350 μm , including about 150 μm to about 300 μm .

[00132] In certain embodiments, horizontally translating the field of view of the spatial light modulator using the mirror galvanometers increases the volume in the target tissue that can be illuminated by the spatial light modulator and therefore increases the number of excitable cells, e.g., neurons, that can be collectively illuminated, e.g., illuminated within each time point (e.g., each exposure of the image detector) during measurement of neural activity, by the 3D multi-focal laser light patterns. In other words, the excitable cells which are illuminated by the spatio-temporally multiplexed 3D multi-focal laser light patterns collectively occupy a volume of the target tissue that is larger than the volume that can be illuminated with an individual 3D multi-focal light pattern (i.e., without spatially translating the field of view of the spatial light

modulator). “Occupy” as used herein, may refer to at least the detectable presence of an excitable cell in a monolithic, contiguous volume (e.g., rectangular volume) of the target tissue. For measuring the activity of the excitable cell, the presence may be based on the portion of the cell body of the excitable cell in which the activity of the cell is to be measured (e.g., a rectangular volume of the target tissue in which the cell body or a neurite of a neuron is present). Thus, a volume collectively occupied by a group of excitable cells (e.g., neurons) may not extend beyond a monolithic, contiguous volume (e.g., a rectangular volume) that includes the cell bodies or the neurites at which the excitable cells are illuminated, even if other portions of the excitable cells (e.g., neurites) may exist outside of the collectively occupied volume.

[00133] In some cases, the excitable cells illuminated by the spatio-temporally multiplexed 3D multi-focal laser light patterns occupy a volume of the target tissue that is at least about 1.5 times, e.g., at least about 2 times, at least about 3 times, at least about 5 times, at least about 8 times, at least about 9 times, and up to about 10 times larger than the volume that can be illuminated without horizontally translating the field of view of the spatial light modulator. In some embodiments, the excitable cells illuminated by the spatio-temporally multiplexed 3D multi-focal laser light patterns occupy a volume of the target tissue that is from about 1.5 times to about 20 times, e.g., from about 2 times to about 15 times, from about 5 times to about 12 times, including from about 8 times to about 10 times larger than the volume that can be illuminated without horizontally translating the field of view of the spatial light modulator.

[00134] In some embodiments, the 3D multi-focal laser light patterns illuminated into the sample by horizontally translating the field of view of the spatial light modulator collectively illuminate excitable cells in a volume of the target tissue that is at least about 1.5 times, e.g., at least about 2 times, at least about 3 times, at least about 5 times, at least about 8 times, at least about 9 times, and up to about 10 times larger than the volume that can be illuminated without horizontally translating the field of view of the spatial light modulator. In some embodiments, the 3D multi-focal laser light patterns illuminated into the sample by horizontally translating the field of view of the spatial light modulator collectively illuminate excitable cells in a volume of the target tissue that is in the range of about 1.5 times to about 20 times, e.g., about 2 times to about 15 times, about 5 times to about 12 times, including about 8 times to about 10 times larger than the volume that can be illuminated without horizontally translating the field of view of the spatial light modulator.

[00135] In certain embodiments, the present method may include measuring the activity of one or more individual excitable cells, e.g., one or more individual neurons, in a target tissue, as described above, and manipulating the target tissue or subject to which the target tissue belongs in

response to the measured activity. The manipulation may include optical stimulation of light-responsive excitable cells, as described further below, or any other appropriate stimulation that produces a desired effect in response to the measured activity. In some embodiments, the method includes stimulating the target tissue and measuring the response, if any, of individual excitable cells to the stimulation. The stimulation may include, e.g., sensory stimulation of an animal when recording in vivo, electrical stimulation of different parts of the target tissue that may have direct or indirect functional connections to excitable cells in the field of view of the image detector or microscope, or optical stimulation of excitable cells in the field of view of the image detector or microscope where the excitable cells are adapted to hyperpolarize and/or depolarize in response to a laser light stimulus.

- [00136]** In any embodiment, the target tissue may include excitable cells, e.g., neurons, that are genetically modified to express a light-responsive polypeptide that hyperpolarizes or depolarizes the excitable cell in response to a laser light stimulus. The light-responsive polypeptide may include one or more various opsins that are known in the field, and are further described below.
- [00137]** Also provided herein is a method of illuminating a plurality of individual excitable cells, e.g., a plurality of individual neurons, in a target tissue, the method including i) selecting a first subset of excitable cells in a target tissue for analysis, wherein the target tissue is in a field of view of a microscope and wherein the target tissue contains a plurality of excitable cells that are adapted to emit fluorescence; and ii) sequentially projecting each 3D multi-focal laser light pattern of a plurality of 3D multi-focal laser light patterns generated by a spatial light modulator into the target tissue, wherein the plurality of 3D multi-focal laser light patterns is projected into at least two distinct sites in the target tissue, thereby collectively and specifically illuminating the plurality of excitable cells in the target tissue.
- [00138]** As described above, the plurality of 3D multi-focal laser light patterns may be one or more spatio-temporally multiplexed 3D multi-focal laser light patterns generated by switching the phase mask of the spatial light modulator and/or horizontally translating the field of view of the spatial light modulator using mirror galvanometers.
- [00139]** In certain embodiments, the excitable cells of the target tissue may contain a fluorescent dye or express a fluorescent protein. In certain embodiments, the fluorescent dye or fluorescent protein emit fluorescence in a cellular electrical activity-dependent manner, e.g., a neuronal activity-dependent manner, to enable measurement of cellular electrical activity, as described above.
- [00140]** The present disclosure also includes a method of measuring the activity of one or more individual excitable cells, e.g., one or more individual neurons, in a target tissue, as described

above, and modulating the activity of a plurality of excitable cells in the target tissue in response to the measured activity of the plurality of activity-sensitive fluorescence-emitting excitable cells. The modulating may be done by any convenient method of activating or inhibiting the plurality of excitable cells in the target tissue, including, but not limited to, sensory stimulation of the host organism to which the target tissue belongs, electric stimulation of excitable cells that are functionally (directly or indirectly) connected to the measured excitable cells, or photostimulation of excitable cells in the field of view of the microscope and that are functionally (directly or indirectly) connected to the measured excitable cells where the photostimulated excitable cells are adapted to hyperpolarizes or depolarizes in response to a laser light stimulus. In some embodiments, optically stimulating the genetically modified excitable cells may include contacting the excitable cells adapted to respond to light and functionally connected to the measured excitable cells using one or more 3D multi-focal laser light pattern stimuli, wherein the one or more 3D multi-focal laser light pattern stimuli are controlled in response to the measured activity of the measured excitable cells. The one or more 3D multi-focal laser light pattern stimuli may be projected into the target tissue using the spatial light modulator and/or mirror galvanometers, as described above.

[00141] The one or more 3D multi-focal laser light pattern stimuli may be selected to specifically hyperpolarize or depolarize a set of excitable cells, e.g., a set of neurons, depending on the desired outcome. For example, if the measured activity of one or more excitable cells is higher than desired, the one or more 3D multi-focal laser light pattern stimuli may be configured to stimulate excitable cells in the field of view of the microscope to reduce activity of the one or more excitable cells by, e.g., stimulating a hyperpolarizing light-responsive polypeptide expressed in the one or more excitable cells, by stimulating a hyperpolarizing light-responsive polypeptide expressed in excitable cells that (directly or indirectly) functionally activate the one or more excitable cells, or by stimulating a depolarizing light-responsive polypeptide expressed in excitable cells that (directly or indirectly) functionally inhibit the one or more excitable cells. In some embodiments, the method includes computationally compensating for the effect of any optical cross-talk between the 3D multi-focal laser light pattern stimuli and the fluorescence measurement for neural activity.

[00142] In certain embodiments, the present system and method achieves high-speed, high-signal measurement and/or control of neuronal activity in a target tissue. Thus, in some embodiments, the method includes controlling the mirror galvanometers to project multiple 3D multi-focal laser light patterns generated by the spatial light modulator into the sample across multiple spatially distinct fields of view of the spatial light modulator. The maximum rate at which a sequence of

3D multi-focal laser light patterns is projected into the sample across multiple spatially distinct fields of view of the spatial light modulator may vary depending on the maximum switching speed of the spatial light modulator and the desired signal rate, as described above.

[00143] The rate at which neuronal activity is collectively measured by the present systems and methods may be in the range of about 1 Hz to about 200 Hz, e.g., about 5 Hz to about 150 Hz, including about 10 Hz to about 100 Hz. The rate at which neuronal activity is collectively measured by the present systems and methods may be 1 Hz or more, e.g., about 5 Hz or more, about 10 Hz or more, about 20 Hz or more, about 30 Hz or more, including about 40 Hz or more, and in some cases, may be about 200 Hz or less, about 150 Hz or less, including about 100 Hz or less. The rate at which neuronal activity is collectively measured by the present systems and methods, when using a spatio-temporally multiplexed 3D multi-focal light pattern, may be in the range of about 1 Hz to about 80 Hz, e.g., about 5 Hz to about 70 Hz, including about 10 Hz to about 50 Hz. In some embodiments, the rate at which neuronal activity is collectively measured by the present systems and methods, when using a spatio-temporally multiplexed 3D multi-focal light pattern, may be about 1 Hz or more, e.g., about 5 Hz or more, about 10 Hz or more, about 20 Hz or more, about 30 Hz or more, including about 40 Hz or more, and in some cases, may be about 80 Hz or less, about 70 Hz or less, including about 60 Hz or less. The rate at which neuronal activity is collectively measured by the present systems and methods, when using a 3D multi-focal light pattern that is not spatio-temporally multiplexed, may be in the range of about 50 Hz to about 150 Hz, e.g., about 70 Hz to about 130 Hz, including about 80 Hz to about 120 Hz. In some embodiments, the rate at which neuronal activity is collectively measured by the present systems and methods, when using a 3D multi-focal light pattern that is not spatio-temporally multiplexed, may be about 50 Hz or more, e.g., about 70 Hz or more, about 80 Hz or more, 90 Hz or more, about 100 Hz or more, and in some cases, may be about 200 Hz or less, about 150 Hz or less, including about 120 Hz or less.

[00144] The rate at which neuronal activity is collectively measured and stimulated by the present systems and methods may be in the range of about 1 Hz to about 200 Hz, e.g., about 5 Hz to about 150 Hz, including about 10 Hz to about 100 Hz. The rate at which neuronal activity is collectively measured and stimulated by the present systems and methods may be about 1 Hz or more, e.g., about 5 Hz or more, about 10 Hz or more, about 20 Hz or more, about 30 Hz or more, including about 40 Hz or more, and in some cases, may be about 200 Hz or less, about 150 Hz or less, including about 100 Hz or less. The rate at which neuronal activity is collectively measured and stimulated by the present systems and methods, when using a spatio-temporally multiplexed 3D multi-focal light pattern, may be in the range of about 1 Hz to about 80 Hz, e.g., about 5 Hz to

about 70 Hz, including about 10 Hz to about 50 Hz. In some embodiments, the rate at which neuronal activity is collectively measured and stimulated by the present systems and methods, when using a spatio-temporally multiplexed 3D multi-focal light pattern, may be about 1 Hz or more, e.g., about 5 Hz or more, about 10 Hz or more, about 20 Hz or more, about 30 Hz or more, including about 40 Hz or more, and in some cases, may be about 80 Hz or less, about 70 Hz or less, including about 60 Hz or less. The rate at which neuronal activity is collectively measured and stimulated by the present systems and methods, when using a 3D multi-focal light pattern that is not spatio-temporally multiplexed may be in the range of about 50 Hz to about 150 Hz, e.g., about 70 Hz to about 130 Hz, including about 80 Hz to about 120 Hz. In some embodiments, the rate at which neuronal activity is collectively measured and stimulated by the present systems and methods when using a 3D multi-focal light pattern that is not spatio-temporally multiplexed may be about 50 Hz or more, e.g., about 70 Hz or more, about 80 Hz or more, about 90 Hz or more, about 100 Hz or more, and may be about 200 Hz or less, about 150 Hz or less, including about 120 Hz or less.

[00145] Also provided herein is a computer-implemented method of analyzing individual 2D diffraction patterns of plurality of excitable cells, e.g., plurality of neurons, and multiplexed 2D diffraction patterns, recorded according to the method described herein, to measure the activity of individual excitable cells, e.g. individual neurons. The computer-implemented method may include resolving the multiplexed 2D diffraction pattern based on the individual 2D diffraction patterns, as described herein. In some embodiments, the present computer-implemented method includes calculating the statistical significance of various parameters, e.g., statistical significance of the measured change in activity level of an excitable cell, statistical significance of the difference in the ensemble of excitable cells activated or inhibited by a manipulation, e.g., therapeutic manipulation, compared to an appropriate control condition, etc. In some embodiments, the present computer-implemented method includes compressing data and/or reducing the dimensionality of the data to facilitate analysis.

ADDITIONAL EMBODIMENTS

[00146] Additional embodiments of systems and methods of the present disclosure are now described.

Extending the SLM field of view with conjugated scanning mirrors

[00147] In a coherent illumination system, a desired object space intensity distribution, $I(x,y) = |\mathcal{F}\{H(u,v)\}|^2$ can be attained by controlling the complex electric field at the pupil plane, where

$$H(u, v) = A(u, v)e^{i\phi(u, v)}. \quad (1)$$

[00148] In holographic illumination systems with phase-only modulation, $A(u, v)$ is the fixed laser amplitude, typically uniform or Gaussian.

[00149] For a phase-only SLM with $0-2\pi$ modulation at wavelength λ at each of the $N \times N$ pixels, the $\phi(u, v) = \phi_{\text{SLM}}(u, v)$ for a given desired 2D or 3D intensity pattern can be computed. However, the area over which light can be steered, termed *field of view* (FOV) by, may be constrained by the pixel count of the SLM to $2x_{\text{max}} \times 2y_{\text{max}}$ where

$$x_{\text{max}} = y_{\text{max}} = \frac{\lambda}{4NA} N, \quad (2)$$

and N is the numerical aperture of the objective lens. The image of the SLM is assumed to be optically magnified such that the width of the SLM matches the diameter of the objective pupil (to jointly maximize spatial resolution and light transmission).

[00150] Often, the objective field of view is larger than the SLM-accessible field of view, as illustrated in Figs. 5A and 6A. To address this, a pair of galvanometer scanning mirrors, conjugated to the SLM plane, may be used to apply a lateral shift of $(\Delta x, \Delta y)$ by superimposing an additional linear phase term,

$$\phi_{\text{galvos}}(u, v; \Delta x, \Delta y) = c(u\Delta x + v\Delta y), \quad (3)$$

where c depends on the wavelength and lens parameters. The total phase modulation is the sum of the two,

$$\phi(u, v) = \phi_{\text{SLM}}(u, v) + \phi_{\text{galvos}}(u, v; \Delta x, \Delta y). \quad (4)$$

Rather than use the mirrors to enable smoother, continuous translation of a single hologram defining multiple optical traps, the time-sequential repositioning of the galvanometer mirrors and multiple different holograms may be used to achieve a larger effective FOV as shown in Figs. 5B and 6B.

[00151] In a typical multiphoton microscope, the galvo positioning time is less than ~ 1 ms, so the area within the objective field of view tiled by the present approach in a given exposure time is determined by the SLM transition time, illustrated in Figs. 7A and 7B.

[00152] **FIGS. 7A and 7B:** Timing considerations for SLM time-division multiplexing. (FIG. 7A) This particular SLM had an empirically measured 0 to $\sim 97\%$ transition time of 7.5 ms. (FIG. 7B) Timing diagram indicating SLM state throughout a given exposure time, T , for M sequentially illuminated subsets of focal spots. Each subset is laterally repositioned by galvanometer mirrors (y-galvo not shown).

Increasing two-photon signal with sequential illumination

- [00153]** In two-photon excitation, signal rate depends quadratically on the average laser power P so the time-averaged signal integrated over a total period of exposure T is

$$S \propto P^2 T. \quad (5)$$

- [00154]** For an illumination pattern that divides the average laser power equally to n excitation sites with equal focal volumes, the total time-averaged signal from the n volumes is

$$S_{M=1} \propto n \left(\frac{P}{n} \right)^2 T, \quad (6)$$

which is a factor n times smaller than that in Eq. (5).

- [00155]** However, if the requirement for simultaneity of the illumination is relaxed, and M sets of n/M sites are allowed to be sequentially excited over the duration of the exposure time T , the total time-averaged signal may be

$$S(M) \propto n \left(\frac{PM}{n} \right)^2 \left(\frac{T}{M} - t_{SLM} \right), \quad (7)$$

where t_{SLM} is the time it takes the SLM to change the illumination pattern between each set of spots. This is greater than that in Eq. (6) by a factor of

$$Z(M; t_{SLM}) = M \left(1 - M \frac{t_{SLM}}{T} \right). \quad (8)$$

- [00156]** For a given t_{SLM} , Eq. (8) takes a maximum value of $M/2$ when $M = T/(2t_{SLM})$. Hence if the laser power is held fixed and the SLM transition time t_{SLM} is known, the optimum number of subsets to scan may be determined in order to maximize the total two-photon excited signal for a given total experimental exposure time.

EXCITABLE CELLS

- [00157]** The present disclosure also provides excitable cells, e.g., neurons, that are adapted to emit fluorescence and excitable cells, e.g., neurons, that are adapted to hyperpolarize and/or depolarize in response to a laser light stimulus, suitable for use in the present method and system.
- [00158]** In some embodiments, the excitable cells, e.g., neurons, are associated with a fluorescent moiety, e.g., a fluorescent dye or a fluorescent protein, that enables the excitable cells to emit fluorescence in response to illumination with an appropriate stimulus (e.g., appropriate wavelength and intensity of illumination). In some embodiments, the excitable cells, e.g.,

neurons, comprise a fluorescent moiety, e.g., a fluorescent dye or a fluorescent protein, that enables the excitable cells to emit fluorescence in response to illumination with an appropriate stimulus (e.g., appropriate wavelength and intensity of illumination). An excitable cell may be associated with the fluorescent moiety by any suitable method, including being labeled directly or indirectly with a fluorescent dye or fluorescent protein, or expressing a genetically encoded fluorescent protein. Any suitable method may be used to indirectly label an excitable cell with a fluorescent moiety. In some cases, a binding member, e.g., an antibody, that specifically binds to a cell surface marker on the excitable cell may be conjugated with a fluorescent dye or protein, and the fluorescently conjugated binding member may be used to label the excitable cell indirectly.

[00159] The fluorescence emitted from excitable cells, e.g., neurons, that are adapted to emit fluorescence may be generally insensitive to cellular electrical activity, e.g., neuronal activity, of the excitable cell that emits the fluorescence. In such cases, fluorescent dyes of interest include fluorescein, fluorescein isothiocyanate (FITC), rhodamine, tetramethylrhodamine isothiocyanate (TRITC), sulforhodamine 101 acid chloride (Texas Red®), phycoerythrin (PE), allophycocyanin, phycoerythrin-Texas Red® (PETR), 4-methylumbelliferone, etc. Fluorescent proteins of interest include green fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, etc., and variants thereof.

[00160] The fluorescence emitted from excitable cells, e.g., neurons, that are adapted to emit fluorescence may be sensitive to cellular electrical activity, e.g., neuronal activity, of the excitable cell that emits the fluorescence. Fluorescent moieties whose fluorescence properties are sensitive to cellular electrical activity include ratiometric/non-ratiometric dyes and fluorescent proteins. Fluorescent moieties whose fluorescence properties are sensitive to cellular electrical activity may be a fluorescence resonance energy transfer (FRET)-based reporter. Fluorescent moieties whose fluorescence properties are sensitive to cellular electrical activity may be sensitive to changes in intracellular concentration of ions such as calcium, sodium and protons or to changes in membrane potential. In such cases, fluorescent dyes of interest include, but are not limited to, calcium indicator dyes (Indo-1, Fura-2, and Fluo-3, Calcium Green®, Fluo-4, etc.); sodium indicator dyes (sodium-binding benzofuran isophthalate (SBFI), Sodium Green™, CoroNa™ Green, CoroNa™ Red, etc.); and proton indicator dyes (2',7' -bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), etc.). Cellular electrical activity-sensitive fluorescent proteins of interest include, but are not limited to, calcium indicators (Cameleon, GCaMP1, GCaMP2, GCaMP3, GCaMP6 and derivatives thereof, as well as those cited in US Patent No. 8,629,256, and Tian et al. 2012 Prog Brain Res, 196:79, which are incorporated herein by reference); and

voltage indicators (QuasAr1, QuasAr2, VSFP, and derivatives thereof, as well as those cited in US App. Pub. No. 20130224756, Hochbaum et al., Nat Methods 2014 11:825, Baker et al. Brain Cell Biol 2008 36:53; and Mutoh et al., Exp Physiol 2011 96:13, which are incorporated herein by reference). In some cases, the fluorescent moiety may be sensitive to biochemical changes in the excitable cell, such as changes in enzymatic activity (e.g., activation of kinases); changes in binding interactions (e.g., binding of transcription factors to DNA); changes in subcellular localization of proteins; etc. Exemplary fluorescent moieties are further described in, e.g., Mehta et al., Annu Rev Biochem. 2011; 80: 375, which is incorporated herein by reference.

[00161] In some embodiments, the excitable cells, e.g., neurons, are adapted to hyperpolarize and/or depolarize in response to a laser light stimulus. In some embodiments, the tissue surrounding the excitable cells may contain a photo-sensitive caged compound, e.g., a caged neurotransmitter, that, when uncaged by a light stimulus, binds to a receptor on a excitable cell nearby and contributes to hyperpolarizing or depolarizing the excitable cell, depending on the neurotransmitter and the receptor. In some cases, the caged neurotransmitter may be glutamate, dopamine, serotonin, GABA, etc., available from, e.g., Tocris, as well as those caged neurotransmitters described in, e.g., U.S. Pat. No. 8,178,496, which is incorporated herein by reference. Suitable methods of using caged neurotransmitters to stimulate neurons is described in, e.g., Noguchi et al., J Physiol. 2011, 589:2447, which is incorporated herein by reference.

[00162] In some embodiments, the excitable cells (e.g., neurons) are genetically modified to express a light-responsive polypeptide that, when stimulated by an appropriate light stimulus, hyperpolarizes or depolarizes the stimulated excitable cell. In some instances, the light-responsive polypeptide is a light-activated ion channel polypeptide. The light-activated ion channel polypeptides are adapted to allow one or more ions to pass through the plasma membrane of a target cell when the polypeptide is illuminated with light of an activating wavelength. Light-activated proteins may be characterized as ion pump proteins, which facilitate the passage of a small number of ions through the plasma membrane per photon of light, or as ion channel proteins, which allow a stream of ions to freely flow through the plasma membrane when the channel is open. In some embodiments, the light-responsive polypeptide depolarizes the excitable cell when activated by light of an activating wavelength. In some embodiments, the light-responsive polypeptide hyperpolarizes the excitable cell when activated by light of an activating wavelength.

[00163] In some embodiments, the light-responsive polypeptides are activated by blue light. In some embodiments, the light-responsive polypeptides are activated by green light. In some embodiments, the light-responsive polypeptides are activated by yellow light. In some

embodiments, the light-responsive polypeptides are activated by orange light. In some embodiments, the light-responsive polypeptides are activated by red light.

[00164] In some embodiments, the light-responsive polypeptide expressed in a cell can be fused to one or more amino acid sequence motifs selected from the group consisting of a signal peptide, an endoplasmic reticulum (ER) export signal, a membrane trafficking signal, and/or an N-terminal golgi export signal. The one or more amino acid sequence motifs which enhance light-responsive protein transport to the plasma membranes of mammalian cells can be fused to the N-terminus, the C-terminus, or to both the N- and C-terminal ends of the light-responsive polypeptide. In some cases, the one or more amino acid sequence motifs which enhance light-responsive polypeptide transport to the plasma membranes of mammalian cells is fused internally within a light-responsive polypeptide. Optionally, the light-responsive polypeptide and the one or more amino acid sequence motifs may be separated by a linker. In some embodiments, the light-responsive polypeptide can be modified by the addition of a trafficking signal (ts) which enhances transport of the protein to the cell plasma membrane. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the signal peptide sequence in the protein can be deleted or substituted with a signal peptide sequence from a different protein.

[00165] Exemplary light-responsive polypeptides and amino acid sequence motifs that find use in the present system and method are disclosed in, e.g., PCT App. Nos. PCT/US2011/028893 and PCT/US2015/23087. Representative light-responsive polypeptides that find use in the present disclosure are further described below.

[00166] In some embodiments, a depolarizing light-responsive polypeptide is a channelrhodopsin (ChR1 – NCBI Gene ID: 5724518, ChR2 - NCBI Gene ID: 5727376) derived from *Chlamydomonas reinhardtii*, wherein the polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. The light used to activate the light-responsive cation channel protein derived from *Chlamydomonas reinhardtii* can have a wavelength between about 460 and about 495 nm or can have a wavelength of about 480 nm. Additionally, light pulses having a temporal frequency of about 100 Hz can be used to activate the light-responsive protein. In some embodiments, activation of the light-responsive cation channel derived from *Chlamydomonas reinhardtii* with light pulses having a temporal frequency of about 100 Hz can cause depolarization of the excitable cells, e.g., neurons, expressing the light-responsive cation channel. The light-responsive cation channel protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular

wavelengths of light, and/or increase or decrease the ability of the light-responsive cation channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the light-responsive cation channel protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The light-responsive proton pump protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00167] In other embodiments, the light-responsive polypeptide is a step function opsin (SFO) protein or a stabilized step function opsin (SSFO) protein that can have specific amino acid substitutions at key positions in the retinal binding pocket of the amino acid sequence of ChR2. Further disclosure related to SFO or SSFO proteins can be found in International Patent Application Publication No. WO 2010/056970, the disclosure of which is hereby incorporated by reference in its entirety.

[00168] In some embodiments, a suitable light-responsive polypeptide is a cation channel derived from *Volvox carteri* (VChR1 - NCBI Gene ID: 9619570) and is activated by illumination with light of a wavelength of from about 500 nm to about 600 nm, e.g., from about 525 nm to about 550 nm, e.g., 545 nm. The light-responsive ion channel protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the light-responsive ion channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the light-responsive ion channel protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The light-responsive ion channel protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of an excitable cell in response to light.

[00169] In other embodiments, the light-responsive polypeptide is a SFO or an SSFO based on VChR1. In some embodiments an SFO or SSFO protein is capable of mediating a depolarizing current in the cell when the cell is illuminated with blue light. In some embodiments, the light has a wavelength of about 560 nm. Additionally, in some embodiments the light is delivered as a single pulse of light or as spaced pulses of light due to the prolonged stability of SFO and SSFO photocurrents. In some embodiments, activation of the SFO or SSFO protein with single pulses or spaced pulses of light can cause depolarization of an excitable cell, e.g., neuron, expressing the SFO or SSFO protein. In some embodiments, each of the disclosed step function opsin and

stabilized step function opsin proteins can have specific properties and characteristics for use in depolarizing the membrane of an excitable cell in response to light.

[00170] In other embodiments, the light-responsive cation channel protein is a C1V1 chimeric protein derived from the VChR1 protein of *Volvox carteri* and the ChR1 protein from *Chlamydomonas reinhardtii*, wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1; is responsive to light; and is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In some embodiments, the C1V1 protein further comprises a replacement within the intracellular loop domain located between the second and third transmembrane helices of the chimeric light responsive protein, wherein at least a portion of the intracellular loop domain is replaced by the corresponding portion from ChR1. In another embodiment, the portion of the intracellular loop domain of the C1V1 chimeric protein can be replaced with the corresponding portion from ChR1 extending to amino acid residue A145 of the ChR1. In other embodiments, the C1V1 chimeric protein further comprises a replacement within the third transmembrane helix of the chimeric light responsive protein, wherein at least a portion of the third transmembrane helix is replaced by the corresponding sequence of ChR1. In yet another embodiment, the portion of the intracellular loop domain of the C1V1 chimeric protein can be replaced with the corresponding portion from ChR1 extending to amino acid residue W163 of the ChR1.

[00171] In some embodiments, the C1V1 protein mediates a depolarizing current in the cell when the cell is illuminated with green light. In some embodiments, the light has a wavelength of between about 540 nm to about 560 nm. In some embodiments, the light can have a wavelength of about 542 nm. In some embodiments, the C1V1 chimeric protein is not capable of mediating a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein is not capable of mediating a depolarizing current in the cell when the cell is illuminated with light having a wavelength of about 405 nm. Additionally, in some embodiments, light pulses having a temporal frequency of about 100 Hz can be used to activate the C1V1 protein.

[00172] In some aspects, a suitable light-responsive polypeptide comprises substituted or mutated amino acid sequences, wherein the mutant polypeptide retains the characteristic light-activatable nature of the precursor C1V1 chimeric polypeptide but may also possess altered properties in some specific aspects. For example, the mutant light-responsive C1V1 chimeric proteins described herein can exhibit an increased level of expression both within an animal cell or on the animal cell plasma membrane; an altered responsiveness when exposed to different wavelengths

of light, particularly red light; and/or a combination of traits whereby the chimeric C1V1 polypeptide possess the properties of low desensitization, fast deactivation, low violet-light activation for minimal cross-activation with other light-responsive cation channels, and/or strong expression in animal cells.

[00173] Accordingly, suitable light-responsive proteins include C1V1 chimeric light-responsive proteins that can have specific amino acid substitutions at key positions throughout the retinal binding pocket of the VChR1 portion of the chimeric polypeptide.

[00174] In other embodiments, the light-responsive cation channel protein is a C1C2 chimeric protein derived from the ChR1 and the ChR2 proteins from *Chlamydomonas reinhardtii*, wherein the protein is responsive to light and is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. The light-responsive cation channel protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the light-responsive cation channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the light-responsive cation channel protein comprises one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The light-responsive proton pump protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00175] In some aspects, a depolarizing light-responsive polypeptide is a red shifted variant of a depolarizing light-responsive polypeptide derived from *Chlamydomonas reinhardtii*; such light-responsive polypeptides are referred to herein as a “ReaChR polypeptide” or “ReaChR protein” or “ReaChR.” The light used to activate the ReaChR polypeptide can have a wavelength between about 590 and about 630 nm or can have a wavelength of about 610 nm. The ReaChR protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the light-responsive cation channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the ReaChR protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The ReaChR containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00176] In some aspects, a depolarizing light-responsive polypeptide is a SdChR polypeptide (Genbank Accession No.: AHH02138) derived from *Scherffelia dubia*, wherein the SdChR polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. The light used to activate the SdChR polypeptide can have a wavelength between about 440 and about 490 nm or can have a wavelength of about 460 nm. The SdChR protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the SdChR protein to regulate the polarization state of the plasma membrane of the cell. In some instances, the SdChR protein comprises one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The SdChR protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00177] In some aspects, a depolarizing light-responsive polypeptide can be, e.g. CnChR2 (Genbank Accession No.: AHH02139), derived from *Chlamydomonas noctigama*, wherein the CnChR1 polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. The light used to activate the CnChR1 polypeptide can have a wavelength between about 560 and about 630 nm or can have a wavelength of about 600 nm. The CnChR1 protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the CnChR1 protein to regulate the polarization state of the plasma membrane of the cell. In some cases, the CnChR1 protein comprises one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The CnChR1 protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00178] In other embodiments, the light-responsive cation channel protein is a CsChrimson chimeric protein derived from a CsChR (Genbank Accession No.: AHH02144) protein of *Chloromonas subdivisa* and CnChR1 protein from *Chlamydomonas noctigama*, wherein the N terminus of the protein comprises the amino acid sequence of residues 1-73 of CsChR followed by residues 79-350 of the amino acid sequence of CnChR1; is responsive to light; and is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. The CsChrimson protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to

particular wavelengths of light, and/or increase or decrease the ability of the CsChrimson protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the CsChrimson protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A CsChrimson protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00179] In some aspects, a depolarizing light-responsive polypeptide can be, e.g. ShChR1 (Genbank Accession No.: AHH02106), derived from *Stigeoclonium helveticum*, wherein the ShChR1 polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. The light used to activate the ShChR1 protein derived from *Stigeoclonium helveticum* can have a wavelength between about 480 and about 510 nm or can have a wavelength of about 500 nm. The ShChR1 protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the ShChR1 protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the ShChR1 protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A ShChR1 protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00180] In some embodiments, a suitable hyperpolarizing light-responsive polypeptide is an Archaeorhodopsin (Arch - Genbank Accession No.: ADB03111) proton pump (e.g., a proton pump derived from *Halorubrum sodomense*) that can transport one or more protons across the plasma membrane of a cell when the cell is illuminated with light. The Arch protein can additionally have substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the Arch protein to transport ions across the plasma membrane of a target cell. Additionally, the Arch protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. An Arch protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a target cell in response to light.

[00181] In some embodiments, a suitable light-activated protein is an Archaeorhodopsin (ArchT - Genbank Accession No.: ABT17417) proton pump (e.g., a proton pump derived from *Halorubrum* sp. TP009) that can transport one or more protons across the plasma membrane of a

cell when the cell is illuminated with light. The light can have a wavelength between about 530 and about 595 nm or can have a wavelength of about 560 nm. The ArchT protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the ArchT protein to transport ions across the plasma membrane of a target cell. Additionally, the ArchT protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The ArchT protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a target cell in response to light.

[00182] In some embodiments, the light-responsive polypeptide is responsive to blue light and is a proton pump protein derived from *Guillardia theta*, wherein the proton pump protein is capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with blue light; such a protein is referred to herein as a “GtR3 protein” or a “GtR3 polypeptide”. The GtR3 (NCBI Gene ID: 17301498) protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the GtR3 protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the GtR3 protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The GtR3 protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to hyperpolarize the plasma membrane of an excitable cell, e.g., neuron, in response to light.

[00183] In some embodiments, a light-activated protein is an *Oxyrrhis marina* (Oxy - Genbank Accession No.: ADY17806) proton pump that can transport one or more protons across the plasma membrane of a cell when the cell is illuminated with light. The light can have a wavelength between about 500 and about 560 nm or can have a wavelength of about 530 nm. The Oxy protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the Oxy protein to transport ions across the plasma membrane of a target cell. Additionally, the Oxy protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The Oxy protein containing substitutions, deletions, and/or

insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a target cell in response to light.

[00184] In some embodiments, the light-responsive proton pump protein (referred to herein as “Mac protein” - NCBI Gene ID: 13287905) is responsive to light and is derived from *Leptosphaeria maculans*, wherein the Mac proton pump protein is capable of pumping protons across the membrane of a cell when the cell is illuminated with 520 nm to 560 nm light. The Mac protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the Mac protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the Mac protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A Mac protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to pump protons across the plasma membrane of an excitable cell, e.g., neuron, in response to light.

[00185] In some cases, a suitable light-responsive chloride pump protein is derived from *Natronomonas pharaonis*; such a protein is referred to herein as an “NpHR protein” or an “NpHR polypeptide.” In some embodiments, the NpHR (NCBI Gene ID: 3702828) protein can be responsive to amber light as well as red light and can mediate a hyperpolarizing current in the excitable cell, e.g., the neuron, when the NpHR protein is illuminated with amber or red light. The wavelength of light that can activate the NpHR protein can be between about 580 and 630 nm. In some embodiments, the light can be at a wavelength of about 589 nm or the light can have a wavelength greater than about 630 nm (e.g. less than about 740 nm). In another embodiment, the light has a wavelength of around 630 nm. In some embodiments, the NpHR protein can hyperpolarize a neural membrane for at least about 90 minutes when exposed to a continuous pulse of light. Additionally, the NpHR protein can comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the NpHR protein to regulate the polarization state of the plasma membrane of the cell. In some embodiments, the NpHR protein comprises one or more conservative amino acid substitutions. In some embodiments, the NpHR protein comprises one or more non-conservative amino acid substitutions. An NpHR protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to hyperpolarize the plasma membrane of an excitable cell in response to light.

- [00186] Further disclosure related to light-responsive chloride pump proteins can be found in U.S. Patent Application Publication Nos: 2009/0093403 and 2010/0145418 as well as in International Patent Application NO: PCT/US2011/028893, the disclosures of each of which are hereby incorporated by reference in their entireties.
- [00187] In some embodiments, a suitable light-responsive ion channel protein is, e.g., a DsChR protein (Genbank Accession No.: AEY68833) derived from *Dunaliella salina*, wherein the ion channel protein is capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with light. The light can have a wavelength between about 470 nm and about 510 nm or can have a wavelength of about 490 nm. The DsChR protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the DsChR protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the DsChR protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A DsChR protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of an excitable cell, e.g., a neuron, in response to light.
- [00188] In some embodiments, a hyperpolarizing light-responsive ion channel is based on a depolarizing light-responsive ion channel, as described in, e.g., PCT App. No. PCT/US2015/23087, which is incorporated herein by reference. In some embodiments, a light-responsive anion channel polypeptide is based on a C1C2 protein (Genbank Accession No.: AHA49646). In some embodiments, a suitable hyperpolarizing light-responsive polypeptide is based on the amino acid sequence of the protein ChR2 (Genbank Accession No.: AER29835). In some embodiments, a suitable hyperpolarizing light-responsive polypeptide is based on the amino acid sequence of the protein C1V1 (Genbank Accession No.: AEL28924). In some embodiments, a subject hyperpolarizing light-responsive polypeptide is based on the amino acid sequence of the protein ReaChR (Genbank Accession No.: AGT48260).
- [00189] Also provided herein is a fluorescent moiety or light-responsive polypeptide encoded in a nucleic acid, e.g., encoded as part of an expression vector. In such instances, the excitable cells, e.g., neurons, may be genetically modified with the nucleic acid to adapt the excitable cells to emit fluorescence and/or to hyperpolarize and/or depolarize in response to a laser light stimulus. Any suitable nucleic acid and expression vector may be used to encode the fluorescent moiety.
- [00190] In some embodiments, a portion of a nucleic acid encoding a fluorescent protein or light-responsive polypeptide is operably linked to a promoter sequence. Any suitable promoter that

functions in the excitable cell of interest in the target tissue can be used for expression of the subject nucleic acids. In certain embodiments, a promoter sequence can be a promoter that is specific to a particular target cell type or to a particular tissue type, such as a particular excitable cell, a particular muscle cell, a particular neuron or a pan-neuronal promoter. Initiation control regions of promoters, which are useful to drive expression of nucleic acids in a specific animal cell, are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving expression of the subject nucleic acids can be used. In some embodiments, the promoter used to drive expression of a subject protein can be the Thy1 promoter (See, e.g., Llewellyn, et al., 2010, Nat. Med., 16(10):1161-1166). In some embodiments, the promoter used to drive expression of a subject protein can be a human synapsin (hSyn) promoter, a human elongation factor 1- α (EF1 α) promoter, a cytomegalovirus (CMV) promoter, a CMV early enhancer/chicken β actin (CAG) promoter, a synapsin-I promoter (e.g., a human synapsin-I promoter), a human synuclein 1 promoter, a human Thy1 promoter, a calcium/calmodulin-dependent kinase II alpha (CAMKII α) promoter, or any other promoter capable of driving expression of the a subject nucleic acid sequence in a target cell.

[00191] In some embodiments, a promoter may be an inducible promoter. For example, the promoter may be induced by a trans-acting factor that responds to an exogenously administered drug. Examples of inducible promoters include, but are not limited to, tetracycline-on or tetracycline-off promoters, or tamoxifen-inducible CreER.

[00192] In some embodiments, a light-activated polypeptide-encoding nucleic acid may include a ribosomal skip sequence that can be used to generate two separate proteins from the same transcript. In such embodiments, a light-activated polypeptide-encoding nucleic acid will typically include a coding sequence that encodes a light-responsive protein as well as an activity-sensitive fluorescent protein, e.g., a neuronal activity-sensitive fluorescent protein. In these embodiments, a ribosomal skip sequence may be placed between the two coding sequences to produce two distinct proteins (namely, the light-responsive protein and the cellular electrical activity-sensitive fluorescent protein) from the same transcript.

[00193] Also provided herein are recombinant expression vectors containing a light-activated polypeptide-encoding nucleic acid or any variant thereof as described herein. Vectors according to the present disclosure also include vectors containing a nucleotide sequence that encodes an RNA (e.g., an mRNA) that when transcribed from the vector will result in the accumulation of a subject protein in the on excitable cells, e.g., neurons, in the target tissue, including accumulation of light-responsive ion channels on the plasma membrane. Vectors which may be used include, without limitation, lentiviral, HSV, adenoviral, and adeno-associated viral (AAV) vectors.

Lentiviruses include, but are not limited to HIV-1, HIV-2, SIV, FIV and EIAV. Lentiviruses may be pseudotyped with the envelope proteins of other viruses, including, but not limited to VSV, rabies, Mo-MLV, baculovirus and Ebola. Such vectors may be prepared using standard methods in the art.

[00194] In some embodiments, a vector may be a recombinant AAV vector. AAV vectors are DNA viruses of relatively small size that can integrate, in a stable and site-specific manner, into the genome of the cells that they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions that carry the encapsidation functions: the left-hand part of the genome that contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome that contains the cap gene encoding the capsid proteins of the virus.

[00195] AAV vectors may be prepared using standard methods in the art. Adeno-associated viruses of any serotype are suitable (see, e.g., Blacklow, pp. 165-174 of "Parvoviruses and Human Disease" J. R. Pattison, ed. (1988); Rose, Comprehensive Virology 3:1, 1974; P. Tattersall "The Evolution of Parvovirus Taxonomy" In Parvoviruses (JR Kerr, SF Cotmore. ME Bloom, RM Linden, CR Parrish, Eds.) p5-14, Huddersfield, London, UK (2006); and DE Bowles, JE Rabinowitz, RJ Samulski "The Genus Dependovirus" (JR Kerr, SF Cotmore. ME Bloom, RM Linden, CR Parrish, Eds.) p15-23, Huddersfield, London, UK (2006), the disclosures of each of which are hereby incorporated by reference herein in their entireties). Methods for purifying for vectors may be found in, for example, U.S. Pat. Nos. 6,566,118, 6,989,264, and 6,995,006 and WO/1999/011764 titled "Methods for Generating High Titer Helper-free Preparation of Recombinant AAV Vectors", the disclosures of which are herein incorporated by reference in their entirety. Methods of preparing AAV vectors in a baculovirus system are described in, e.g., WO 2008/024998. AAV vectors can be self-complementary or single-stranded. Preparation of hybrid vectors is described in, for example, PCT Application No. PCT/US2005/027091, the disclosure of which is herein incorporated by reference in its entirety. The use of vectors derived from the AAVs for transferring genes in vitro and in vivo has been described (See e.g., International Patent Application Publication Nos.: 91/18088 and WO 93/09239; U.S. Patent Nos.: 4,797,368, 6,596,535, and 5,139,941; and European Patent No.: 0488528, all of which are hereby incorporated by reference herein in their entireties). These publications describe various AAV-

derived constructs in which the rep and/or cap genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the gene of interest in vitro (into cultured cells) or in vivo (directly into an organism). The replication-defective recombinant AAVs according to the present disclosure can be prepared by co-transfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (rep and cap genes), into a cell line that is infected with a human helper virus (for example an adenovirus). The AAV recombinants that are produced are then purified by standard techniques.

[00196] In some embodiments, the vector(s) for use in the system and method of the present disclosure are encapsidated into a virus particle (e.g. AAV virus particle including, but not limited to, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13, AAV14, AAV15, and AAV16). Accordingly, the present disclosure includes a recombinant virus particle (recombinant because it contains a recombinant polynucleotide) comprising any of the vectors described herein. Methods of producing such particles are known in the art and are described in U.S. Patent No. 6,596,535, the disclosure of which is hereby incorporated by reference in its entirety.

[00197] In some embodiments, the subject light-responsive proteins can be combined with various promoters and/or fluorescent proteins (XFP) for targeting specific neuronal populations in mammalian brains. For example, the following adeno associated vectors (AAVs) and components thereof may be used without limitation: AAV-CamKII-X-XFP, AAV-hSyn-X-XFP, AAV-mThy1-X-XFP, AAVmThy1-X-XFP, AAV-GFAP-X-XFP, AAV-VGAT-X-XFP, AAV-PET1-X-XFP, AAV-NPY-X-XFP, AAV-SST-X-XFP, AAV-AVP5.5-X-XFP, AAV-Ef1a-X-XFP, AAV-FLEX-rev-X-XFP, AAV-CAG-X-XFP, AAV-CAG-FLEX-X-XFP, where X is a light-responsive protein. Other AAV vectors that may be used in association with the polynucleotides include those with double floxed inverted reading frames (DIO) which allow expression of proteins under the control of recombinases such as Cre and Flp: AAV-Ef1a-DIO(Cre)-X-XFP (Cre-dependent expression), AAV-Ef1a-DIO(Flp)-X-XFP (Flp-dependent expression), AAV-Ef1a-DIO(Cre)-DIO(Flp)-X-XFP (Cre and Flp dependent expression), where X is a light-responsive protein.

[00198] Another major viral transduction system utilizes lentivirus including the following potential expression vectors: pLenti-CamKII-X-XFP, pLenti-Ef1a-X-XFP, pLenti-mThy1-X-XFP, pLenti-hThy1-X-XFP, pLenti-hSyn-X-XFP, pLenti-VGAT-X-XFP, pLenti-Hcrt-X-XFP, where X is a light-responsive protein. Herpes simplex virus (HSV) can be utilized to transport proteins of interest over synapses (anterograde) which includes the following expression vectors: HSV-EF1a-

X-XFP and HSVEF1a-DIO-X-XFP, where X is a light-responsive protein. Rabies and pseudorabies virus can be utilized for retrograde transports over synapses using the following expression vector: SAD(delta)G-X-XFP and SAD(delta)G-DIO-X-XFP. Other mammalian expression vectors include: pcDNA3.1-CMV-X-XFP and pCAGGS-X-XFP, where X is a light-responsive protein.

[00199] Neuron-specific promoters and other control elements (e.g., enhancers) are known in the art. Suitable neuron-specific control sequences include, but are not limited to, a neuron-specific enolase (NSE) promoter (see, e.g., EMBL HSENO2, X51956; see also, e.g., U.S. Pat. No. 6,649,811, U.S. Pat. No. 5,387,742); an aromatic amino acid decarboxylase (AADC) promoter; a neurofilament promoter (see, e.g., GenBank HUMNFL, L04147); a synapsin promoter (see, e.g., GenBank HUMSYNIB, M55301); a thy-1 promoter (see, e.g., Chen et al. (1987) Cell 51:7-19; and Llewellyn et al. (2010) Nat. Med. 16:1161); a serotonin receptor promoter (see, e.g., GenBank S62283); a tyrosine hydroxylase promoter (TH) (see, e.g., Nucl. Acids. Res. 15:2363-2384 (1987) and Neuron 6:583-594 (1991)); a GnRH promoter (see, e.g., Radovick et al., Proc. Natl. Acad. Sci. USA 88:3402-3406 (1991)); an L7 promoter (see, e.g., Oberdick et al., Science 248:223-226 (1990)); a DNMT promoter (see, e.g., Bartge et al., Proc. Natl. Acad. Sci. USA 85:3648-3652 (1988)); an enkephalin promoter (see, e.g., Comb et al., EMBO J. 17:3793-3805 (1988)); a myelin basic protein (MBP) promoter; a CMV enhancer/platelet-derived growth factor- β promoter (see, e.g., Liu et al. (2004) Gene Therapy 11:52-60); a motor neuron-specific gene Hb9 promoter (see, e.g., U.S. Pat. No. 7,632,679; and Lee et al. (2004) Development 131:3295-3306); and an alpha subunit of Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII α) promoter (see, e.g., Mayford et al. (1996) Proc. Natl. Acad. Sci. USA 93:13250). Other suitable promoters include EF1 α and DAT.

[00200] In some embodiments, a non-human animal in which the target tissue is present is genetically engineered to express a fluorescent protein or a light-responsive polypeptide, as described herein, by using any suitable method of genetically engineering the animal, e.g., via genetic manipulation of embryonic stem cells. In some cases, a cell in a target tissue is genetically modified to express a fluorescent protein or a light-responsive polypeptide, as described herein. A genetically modified cell present in a target tissue can be present in a mammal, e.g., a human, a non-human primate, a rodent, a lagomorph, etc.

[00201] Any suitable method may be used to adapt excitable cells, e.g., neurons, to emit fluorescence and/or to hyperpolarize and/or depolarize in response to a laser light stimulus. In some embodiments, excitable cells may be contacted with a fluorescent moiety (e.g., a fluorescent dye or a fluorescent dye conjugated to a specific binding member), or a photo-sensitive caged

compound, or a viral vector containing a nucleic acid encoding a light-responsive polypeptide and/or fluorescent protein, by delivering the fluorescent moiety, photo-sensitive caged compound, or the viral vector into the target tissue, locally or systemically. Any suitable composition for delivering the fluorescent moiety to a target tissue of interest may be used. Where compositions are to be delivered to a site in the brain, stereotactic injection can be used; see, e.g., Stein et al., *J. Virol.* 73:3424-3429, 1999; Davidson et al., *PNAS*, 97:3428-3432, 2000; Davidson et al., *Nat. Genet.* 3:219-223, 1993; and Alisky & Davidson, *Hum. Gene Ther.* 11:2315-2329, 2000, the contents of each of which are hereby incorporated by reference herein in their entireties.

APPLICATIONS

- [00202]** Using the subject method and system, the ordinarily skilled artisan will be able to perform in vivo recording of the activity of excitable cells, such as neurons, in scattering tissue volumes using multifocal two photon illumination from a phase spatial light modulator. In some embodiments, the microlens array and sCMOS detector on the emission path of the microscope allows for high speed encoding of the detected emission from multiple sites, and the use of a computational deconvolution process allows reconstruction of the activity traces at each focal point. In some embodiments, in vivo calcium imaging may be performed substantially simultaneously on 100 or more neurons at 30 Hz or more for each neuron.
- [00203]** The present system and method also find use in multifocal stimulation of neurons in a large volume of the target tissue by spatio-temporal multiplexing the field of view of the spatial light modulator using the mirror galvanometers.
- [00204]** The present system and method also find use in analyzing or mapping the connectivity of neurons in target tissues, such as the brain. For example, the present system and method may be used to measure the individual activity of a plurality of neurons in a target tissue volume in response to a stimulus, either to one or more neurons in the local area of the target tissue that is being analyzed, or to sites distal to the measurement site. The stimulus may be a sensory stimulation, an electrical stimulation through an electrode, or an optical stimulation. By observing the pattern of activity of the measured neurons in response to various stimuli and other manipulations, one may deduce the connectivity of the neurons in the observed area of the target tissue.
- [00205]** In some embodiments, the neuronal region targeted for measurement or stimulation by the present system and method include any neocortical region. With proper tissue exposure, the target neural region includes: the hypothalamus, entorhinal and hippocampal formation cortex, mammillary bodies, septum, bed nucleus of stria terminalis, dorsal and ventral striatum, thalamus,

amygdala, accumbens, brainstem, and subcortical structures in general. The target location may include: a cell, a portion of a cell, a plurality of cells, a bundle of nerve fibers, a neuromuscular junction, a central nervous system (CNS) tissue, a peripheral nervous system (PNS) tissue, muscle or cardiac tissue, or an anatomical region.

[00206] In some embodiments, the present system and method find use in screening in vitro/in vivo animal models of disease for neuronal circuit elements diagnostic of or causative for neuropsychiatric disease. Neuropsychiatric disease of interest may include disorders of mood and affect, anxiety, psychosis, personality, etc. The animal model may be any suitable model, including, but not limited to, rodents, cats, dogs, monkeys, and non-human primates. Perturbations used to model a neuropsychiatric disease include genetic models of neurological or psychiatric disease, such as autism; chronically induced models as with kainate or pilocarpine-induced epilepsy or chronic stress-induced depression; and acutely induced models as with hallucinogens or psychotogenic agents such as ketamine or phencyclidine (PCP). By comparing the difference in activity pattern between neurons in normal target tissue and neurons in abnormal target tissue, neural correlates of the neuropsychiatric disorder may be identified. Optical control of neurons in the target tissue may then allow identification of causative neuronal activity patterns for a particular neuropsychiatric disorder. These manipulations may potentially provide novel treatment targets.

[00207] In some embodiments, the present system and method find use in methods for identifying a treatment, e.g., a therapeutic treatment, with a desired activity on a group of neurons. If the desired outcome is known, then the present system and method may be used to screen for treatments, including, but not limited to, pharmacological agents, nonchemical based therapeutic treatment; behavioral treatment; electrical, magnetic, or optical based neural-modulation treatment; etc., that will bring about the desired neuronal activity pattern. The screening may be performed in any suitable animal model, either normal, or a model for a neurological disorder, such as Alzheimer's and Parkinson's disease, mild cognitive impairment, other dementias, and Down's Syndrome, as well as schizophrenia, autism, mood, affective, anxiety, and personality/developmental disorders.

[00208] In some embodiments, the present system and method find use in the treatment of a condition or disorder, such as a neurological or psychiatric condition using optogenetic control (closed loop control). As real time activity of neurons is monitored using the present system and method, a controller may be configured to modulate the activity of neurons in response to the imaged activity signals in such a way as to treat or reduce symptoms of the condition or disorder, at the behavioral and/or physiological levels.

[00209] Notwithstanding the appended claims, the present disclosure is also defined by the following clauses:

[00210] 1. A method for measuring the activity of excitable cells in a target tissue, the method comprising:

i) selecting a first plurality of excitable cells in a target tissue for measuring activity, wherein the excitable cells are adapted to emit fluorescence in a cellular electrical activity-sensitive manner; and

ii) measuring the activity of each of the excitable cells by:

a) illuminating the first plurality of excitable cells with spatio-temporally multiplexed three-dimensional (3D) multi-focal light patterns, thereby causing the excitable cells to emit fluorescence, wherein the 3D multi-focal light patterns are generated using a spatial light modulator and a pair of mirror galvanometers to spatially translate a field of view of the spatial light modulator on the target tissue, and wherein different subsets of excitable cells from the first plurality are illuminated among at least some of the 3D multi-focal light patterns;

b) recording, using an image detector, a multiplexed 2D diffraction pattern of the emitted fluorescence diffracted by a microlens array; and

c) resolving the multiplexed 2D diffraction pattern to determine the activity of each excitable cell illuminated by the plurality of 3D multi-focal light patterns.

[00211] 2. The method according to clause 1, wherein the illuminating comprises projecting a plurality of 3D multi-focal light patterns sequentially into the target tissue at a rate of at most about 0.75 times a maximum switching rate of the spatial light modulator.

[00212] 3. The method according to clause 1 or 2, wherein the illuminating comprises using a mirror galvanometer.

[00213] 4. The method according to any one of clauses 1 to 3, wherein the first plurality of excitable cells comprises at least 40 cells.

[00214] 5. The method according to any one of clauses 1 to 4, wherein the different subsets of excitable cells comprise on average 90% or fewer cells than the first plurality of excitable cells.

[00215] 6. The method according to any one of clauses 1 to 5, wherein the spatio-temporally multiplexed 3D multi-focal light patterns collectively have a power at the target tissue of about 500 mW or less.

[00216] 7. The method according to any one of clauses 1 to 6, wherein measuring the activity of an individual excitable cell is performed at a frequency of from about 5 Hz to about 100 Hz.

- [00217] 8. The method according to any one of clauses 1 to 7, wherein resolving the multiplexed 2D diffraction pattern comprises deconvolving the multiplexed 2D diffraction pattern using an optical model for light propagation through an emission path from the target tissue to the image detector.
- [00218] 9. The method according to clause 8, wherein the optical model comprises an analytical model.
- [00219] 10. The method according to clause 9, wherein the optical model comprises a wave optics model.
- [00220] 11. The method according to clause 8, wherein the optical model comprises an empirical model.
- [00221] 12. The method according to clause 11, wherein the optical model comprises individual 2D diffraction patterns for fluorescence emitted by the first plurality of excitable cells and diffracted by the microlens array.
- [00222] 13. The method according to clause 12, further comprising obtaining individual 2D diffraction patterns for each of the first plurality of excitable cells in the target tissue, by
- a) contacting an excitable cell of the first plurality with a laser light, thereby causing the contacted excitable cell to emit fluorescence; and
 - b) recording, using the image detector, a 2D diffraction pattern of the emitted fluorescence diffracted by the microlens array.
- [00223] 14. The method according to clause 13, wherein the contacting comprises two-photon 2D laser scanning.
- [00224] 15. The method according to any one of clauses 12 to 14, wherein the deconvolving comprises determining individual point spread functions (PSFs) for each of the individual 2D diffraction patterns and using the individual PSFs to deconvolve the multiplexed 2D diffraction pattern.
- [00225] 16. The method according to any one of clauses 12 to 15, further comprising selecting members of the different subsets of the first plurality of excitable cells based on the individual 2D diffraction patterns.
- [00226] 17. The method according to clause 16, wherein the selecting members of the different subsets comprises:
- comparing the individual 2D diffraction patterns among each other; and
 - selecting members of the different subset such that a minimum difference in the individual 2D diffraction patterns between excitable cells in any pair of the different subsets is at or above a threshold difference.

- [00227] 18. The method according to clause 17, wherein the threshold difference maximizes the minimum difference.
- [00228] 19. The method according to any one of clauses 1 to 18, wherein the 3D multi-focal laser light patterns are generated by spatially modulating a laser beam using the spatial light modulator.
- [00229] 20. The method according to clause 19, wherein the laser beam is pulsed at a frequency of from about 100 to about 500 kHz.
- [00230] 21. The method according to clause 19 or 20, wherein the laser beam has an average power of about 1.0 to about 3.0 W.
- [00231] 22. The method according to any one of clauses 19 to 21, wherein the laser beam has a pulse duration of about 100 to about 500 fs.
- [00232] 23. The method according to any one of clauses 1 to 22, wherein the selecting comprises: scanning the target tissue using a focused laser light, to generate an image of the target tissue; and identifying excitable cells in the image.
- [00233] 24. The method according to clause 23, wherein the focused laser light is pulsed at a frequency of about 10 to about 200 MHz.
- [00234] 25. The method according to clause 23 or 24, wherein the focused laser light is generated using a spatial light modulator.
- [00235] 26. The method according to any one of clauses 1 to 25, the image detector is a Complementary Metal Oxide Semiconductor (CMOS) detector.
- [00236] 27. The method according to any one of clauses 1 to 26, wherein the excitable cells comprise neurons.
- [00237] 28. The method according to any one of clauses 1 to 27, wherein the target tissue is an *in vivo* neuronal tissue.
- [00238] 29. The method according to any one of clauses 1 to 28, wherein the target tissue is a light-scattering tissue.
- [00239] 30. The method according to any one of clauses 1 to 29, wherein the excitable cells are adapted to emit fluorescence in a calcium- or voltage-sensitive manner.
- [00240] 31. The method according to clause 30, wherein the excitable cells comprise an activity-sensitive fluorescent dye.
- [00241] 32. The method according to clause 30, wherein the excitable cells comprise a genetically encoded, activity-sensitive fluorescent protein.

[00242] 33. The method according to any one of clauses 1 to 32, wherein the 3D multi-focal light patterns comprise light having a wavelength in the near-infrared range.

[00243] 34. The method according to any one of clauses 1 to 33, wherein the method further comprises:

stimulating the target tissue; and

measuring the activity of the first plurality of excitable cells in response to the stimulation.

[00244] 35. The method according to clause 34, wherein the target tissue comprises a second plurality of excitable cells that are adapted to hyperpolarize and/or depolarize in response to a laser light stimulus, and the stimulating comprises contacting the second plurality of excitable cells with a laser light stimulus.

[00245] 36. The method according to clause 35, wherein an excitable cell that is adapted to hyperpolarize and/or depolarize in response to a laser light stimulus is genetically modified to express one or more light-responsive polypeptides that hyperpolarize or depolarize the excitable cell when contacted by a laser light.

[00246] 37. A method of regulating the activity of a plurality of excitable cells in a target tissue, comprising:

i) measuring the activity of a first plurality of activity-sensitive fluorescence-emitting excitable cells in a target tissue comprising a second plurality of excitable cells, according to the method of any one of clauses 1 to 33; and

ii) modulating the activity of the second plurality of excitable cells in the target tissue in response to the measured activity of the first plurality of activity-sensitive fluorescence-emitting excitable cells.

[00247] 38. The method of clause 37, wherein excitable cells of the second plurality are genetically modified to hyperpolarize and/or depolarize in response to a laser light stimulus, and the modulating comprises contacting the genetically modified excitable cells of the second plurality with a light stimulus using one or more 3D multi-focal light pattern stimuli, wherein the one or more 3D multi-focal light pattern stimuli are controlled in response to the measured activity of one or more of the plurality of activity-sensitive fluorescence-emitting excitable cells.

[00248] 39. A system comprising:

i) a light microscope defining an excitation path and an emission path with respect to a target tissue, wherein the excitation path comprises:

a spatial light modulator configured to modify a first light generated by a light source, and to thereby project a 3D multi-focal laser light pattern into the target tissue; and

a pair of mirror galvanometers configured to spatially translate a field of view of the spatial light modulator on the target tissue,

and wherein the emission path comprises a microlens array configured to modify fluorescence emitted by the target tissue such that a 2D diffraction pattern is projected onto an image detector;

ii) a controller; and

iii) a processor configured to execute instructions that cause:

the controller to:

illuminate the target tissue with spatio-temporally multiplexed 3D multi-focal light patterns, thereby causing the excitable cells to emit fluorescence, and

record a multiplexed 2D diffraction pattern of the emitted fluorescence; and

the processor to resolve the recorded multiplexed 2D diffraction pattern.

[00249] 40. The system according to clause 39, wherein the light source is configured to generate a second light, and wherein the excitation path comprises a first movable mirror configured to direct

the first light onto the spatial light modulator when the first movable mirror is in a first position, and

the second light onto the spatial light modulator when the first movable mirror is in a second position.

[00250] 41. The system according to clause 39 or 40, wherein the light source is a laser light source and the light is a laser beam.

[00251] 42. The system according to clause 41, wherein the laser light source comprises a regenerative amplifier configured to modify the first laser beam.

[00252] 43. The system according to any one of clauses 39 to 42, wherein the emission path comprises a photodetector, and wherein the emission path comprises a second movable mirror configured to direct the fluorescence emitted by the target tissue to:

the microlens array when the second movable mirror is in a first position, and

the photodetector when the second movable mirror is in a second position.

[00253] 44. The system according to any one of clauses 39 to 43, wherein the emission path comprises an achromatic doublet configured to expand the first laser beam or the second laser beam directed onto the spatial light modulator.

[00254] 45. The system according to any one of clauses 39 to 44, wherein the spatial light modulator has a maximum switching rate of at least about 40 Hz.

- [00255] 46. The system according to any one of clauses 39 to 45, wherein the spatial light modulator has a resolution of at least 256 pixels x at least 256 pixels.
- [00256] 47. The system according to any one of clauses 39 to 46, wherein the first light has a wavelength in the near-infrared range.
- [00257] 48. The system according to any one of clauses 39 to 47, wherein the excitation path comprises a Pockels cell configured to reduce power of the first light such that the spatio-temporally multiplexed 3D multi-focal light patterns has an average power at the target tissue of about 500 mW or less.
- [00258] 49. The system according to any one of clauses 39 to 48, wherein the image detector is a CMOS detector.
- [00259] 50. A method for collectively and specifically illuminating a plurality of excitable cells in a target tissue, the method comprising:
- i) selecting a plurality of excitable cells in a target tissue, wherein excitable cells of the plurality are adapted to emit fluorescence; and
 - ii) sequentially projecting into the target tissue each 3D multi-focal light pattern of spatio-temporally multiplexed 3D multi-focal light patterns generated using a spatial light modulator and a pair of mirror galvanometers to spatially translate a field of view of the spatial light modulator on the target tissue, wherein the 3D multi-focal light patterns illuminate different subsets of the plurality of excitable cells,
- thereby collectively and specifically illuminating the plurality of excitable cells in the target tissue.
- [00260] 51. The method according to clause 50, wherein the 3D multi-focal light patterns are sequentially projected into the target tissue at a rate of at most about 0.75 times a maximum switching rate of the spatial light modulator.
- [00261] 52. The method according to clause 50 or 51, wherein the different subsets of excitable cells comprise on average 90% or fewer cells than the plurality of excitable cells.
- [00262] 53. The method according to any one of clauses 50 to 52, wherein the excitable cells are neurons.
- [00263] 54. The method according to clause 53, wherein the target tissue is an in vivo neuronal tissue.
- [00264] 55. The method according to any one of clauses 50 to 54, wherein the target tissue comprises a population of excitable cells that are adapted to hyperpolarize and/or depolarize in response to a light stimulus.

- [00265] 56. The method according to clause 55, wherein excitable cells of the population are genetically modified to express one or more light-responsive polypeptides that hyperpolarize or depolarize the excitable cell when illuminated by the light stimulus.
- [00266] 57. The method according to any one of clauses 50 to 56, wherein the 3D multi-focal light patterns collectively have a power at the target tissue of about 500 mW or less.
- [00267] 58. The method according to any one of clauses 50 to 57, wherein excitable cells of the plurality are adapted to emit fluorescence in a cellular electrical activity-sensitive manner, and wherein the sequentially projecting each of the 3D multi-focal light patterns is sufficient to cause the excitable cells of the first plurality in the target tissue to emit fluorescence when illuminated by a 3D multi-focal laser light pattern.
- [00268] 59. The method according to clause 58, further comprising iii) measuring the activity of excitable cells in the plurality by:
- recording, using an image detector, a multiplexed 2D diffraction pattern of the emitted fluorescence diffracted by a microlens array; and
 - resolving the multiplexed 2D diffraction pattern to determine the activity of each excitable cell illuminated by the plurality of 3D multi-focal light patterns.
- [00269] 60. The method according to clause 59, wherein the resolving comprises deconvolving the multiplexed 2D diffraction pattern using an optical model for light propagation through an emission path from the target tissue to the image detector.
- [00270] 61. The method according to clause 60, wherein the optical model comprises an analytical model.
- [00271] 62. The method according to clause 61, wherein the optical model comprises a wave optics model.
- [00272] 63. The method according to clause 60, wherein the optical model comprises an empirical model.
- [00273] 64. The method according to clause 63, wherein the optical model comprises individual 2D diffraction patterns for fluorescence emitted by the plurality of excitable cells and diffracted by the microlens array.
- [00274] 65. The method according to clause 63 or 64, wherein the individual 2D diffraction patterns are obtained by
- a) contacting an excitable cell in the plurality of excitable cells with a laser light, thereby causing the excitable cell to emit fluorescence;
 - b) directing the emitted fluorescence from the contacted excitable cell through a microlens array to create a 2D diffraction pattern; and

c) recording the 2D diffraction pattern.

[00275] 66. The method according to clause 65, wherein the contacting comprises two-photon 2D laser scanning.

[00276] 67. The method according to any one of clauses 63 to 66, further comprising selecting members of the different subsets of the plurality of excitable cells based on the individual 2D diffraction patterns.

[00277] 68. The method according to clause 67, wherein the selecting members of the different subsets comprises:
comparing the individual 2D diffraction patterns among each other; and
selecting members of the different subset such that a minimum difference in the individual 2D diffraction patterns between excitable cells in any pair of the different subsets is at or above a threshold difference.

[00278] 69. The method according to clause 68, wherein the threshold difference maximizes the minimum difference.

[00279] 70. The method according to any one of clauses 59 to 69, wherein measuring the activity of an excitable cell is performed at a frequency of from about 5 Hz to about 100 Hz.

[00280] 71. The method according to any one of clauses 59 to 70, wherein the image detector is a CMOS detector.

[00281] 72. The method according to any one of clauses 59 to 71, wherein the excitable cells are adapted to emit fluorescence in a calcium- or voltage-sensitive manner.

[00282] 73. The method according to any one of clauses 59 to 72, wherein the excitable cells comprise an activity-sensitive fluorescent dye.

[00283] 74. The method according to any one of clauses 59 to 72, wherein the excitable cells comprise a genetically encoded, activity-sensitive fluorescent protein.

[00284] 75. The method according to any one of clauses 59 to 74, wherein the method further comprises:

stimulating the target tissue; and

measuring the activity of individual excitable cells in the plurality of excitable cells in response to the stimulation.

[00285] 76. The method according to any one of clauses 50 to 75, wherein the target tissue is a light-scattering tissue.

[00286] 77. The method according to any one of clauses 50 to 76, wherein the first subset comprises 10 or more excitable cells.

- [00287] 78. The method according to any one of clauses 50 to 77, wherein the 3D multi-focal light pattern is generated by spatially modulating an amplitude and/or phase of a laser beam using the spatial light modulator.
- [00288] 79. The method according to clause 78, wherein the laser beam is pulsed at a frequency of from about 100 to about 500 kHz.
- [00289] 80. The method according to clauses 78 or 79, wherein the laser beam has an average power of from about 1.0 to about 3.0 W.
- [00290] 81. The method according to any one of clauses 78 to 80, wherein the laser beam has a pulse duration of from about 100 to about 500 fs.
- [00291] 82. The method according to any one of clauses 78 to 81, wherein the laser beam has a wavelength in the near-infrared range.
- [00292] 83. The method according to any one of clauses 50 to 82, wherein the selecting comprises: scanning the target tissue using a focused laser light, to generate an image of the target tissue; and identifying excitable cells in the image.
- [00293] 84. The method according to clause 83, wherein the focused laser light is pulsed at a frequency of from about 10 to about 200 MHz.
- [00294] 85. The method according to clause 83 or 84, wherein the focused laser light is generated using a spatial light modulator.

EXAMPLES

- [00295] As can be appreciated from the disclosure provided above, the present disclosure has a wide variety of applications. Accordingly, the following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use embodiments of the present disclosure, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results. Thus, the following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use embodiments of the present disclosure, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, dimensions, etc.) but some experimental errors and deviations should be accounted for.

EXAMPLE 1: Multisite Random Access Two-Photon Three-Dimensional In Vivo Optical
Recording of Neural Activity

Materials and Methods

Scanless three-dimensional two-photon imaging

- [00296] The microscope included a multifocal near-infra-red (NIR) excitation path and a visible detection path (Fig. 1A). In the two-photon excitation path, pulses from a 2Watt, 80 MHz, 150 fs, Ti:Sapphire laser (Coherent, Chameleon®) tuned to 920nm were either sent directly to the rest of the microscope optics through a Pockels cell (Conoptics) for intensity control, or used as a seed laser for a regenerative amplifier (Coherent RegA® 9000) customized to produce 1.8 W at 250 kHz, 150 fs pulses which are sent to a separate Pockels cell (Conoptics). Both beams were passed through half wave plates (Thorlabs) used to match the beam polarizations to that required by the spatial light modulator (SLM). One of the two lasers were selected using a motorized mirror (Newport) and were expanded using NIR achromatic doublets (Thorlabs) to a $1/e^2$ diameter of 10 mm before reflecting off a 19.2 mm x 19.2 mm, 512x512, $0-2\pi$ phase spatial light modulator (Meadowlark) at a 15 degree angle from the normal. The SLM was imaged using a $2f_1 + 2f_2$ configuration relay lens pair on to a 6-mm galvanometer mirror (Cambridge Technology) and a 6-mm y-galvo spaced 1 cm apart, equidistant from the conjugate SLM plane. In between lens f_1 and lens f_2 , a beam block (2mm steel pin inserted at center of glass window, Thorlabs) was used to block the zero order undiffracted light from the SLM. Finally, another pair of relay lenses (Leica scan lens, Nikon tube lens) relayed the image of the SLM through a short pass dichroic (Chroma) and onto the back aperture of the objective.
- [00297] One arm of the emission path followed the light field microscope configuration. On the emission path, light went through the short pass dichroic, through a tube lens (Nikon) and onto a microlens array (RPC Photonics, 125 μ m pitch, f/10) placed at the image plane. A pair of relay lenses (Nikon 35mm, Nikon 50mm) set to infinity focus and with aperture wide open were used to image the camera (Hamamatsu OrcaTM Flash4.0 V2) to a plane one microlens focal length behind the microlens array (1.25 mm). This path was used to simultaneously record fluorescence emission from multiple focal points, and for three dimensional (3D) calibration of the microscope.
- [00298] The second arm of the emission path, realized by a removable mirror before the tube lens in the previous illumination path, sent the emission through another separate tube lens (Nikon) through a focusing lens and onto a photo multiplier tube (Hamamatsu).

Calibration

[00299] A calibration procedure was used to ensure the cell locations defined by coordinates in the reference 3D stack of dense image frames can be accurately illuminated by the SLM excitation path. In order to achieve this, spots in a fluorescent volume (Sharpie® highlighter dye in a cuvette (Thorlabs) with coverslip) were illuminated and the 3D location of those spots was reconstructed using the light field microscope as a reference coordinate system. This calibration was done for the Chameleon® SLM path, the RegA® SLM path, and the Chameleon® SLM galvo paths. For each calibration, a 2 dimensional (2D) affine transform was fitted at one of two z-depths, and linear interpolation was used to determine the affine transform for other z-depths. With the same fluorescent volume, the position of the wave plates and the grating in the dispersion compensation unit could be optimized to maximize signal. This procedure was repeated once at the beginning of each experiment day.

Data acquisition

[00300] First, a 3D stack of dense image frames was acquired by scanning with the galvanometer mirrors a single laser point focused to a given z-depth by the SLM and recording the emission onto the PMT. ScanImage software was used to analyze the emissions. Cells were then hand selected for imaging.

[00301] Next, each cell was illuminated one at a time, and the emission was recorded through the light field microscope emission path onto the camera. These empirical measurements of the optical model were later used for deconvolution.

[00302] All the cells to non-overlapping subsets can be assigned by maximizing the minimum similarity between two cells in any given subset.

[00303] Finally, for actual recording, a set of phase masks were pre-computed, where each mask illuminates one subset of cells.

Control electronics and timing

[00304] A computer running Windows® 7, 64-bit and custom MATLAB® software was used to control all hardware. Analog and digital control signals were generated using DAQ cards (NI DAQ). The camera was operated with external triggering. ScanImage software was used to control the galvanometer mirrors for acquiring the 3D stack of dense image frames.

Results

[00305] A combination of simultaneous multi-focal two-photon excitation, simultaneous 3D imaging and the use of regenerative amplifier, in conjunction with an empirically measured optical model and computational deconvolution, were utilized to enable fast recording of neuronal activity with genetically-encoded calcium indicators with single cell resolution deep in tissue (FIGs. 1A-1D).

A regenerative amplifier was used to generate over an order of magnitude more two photon fluorescence signal compared with a conventional Ti:Saph laser, both operating at 920nm for GCaMP6.

[00306] FIGs. 1A-1D: Design of a multisite random access targeting microscope. (FIG. 1A)

Schematics of microscope setup. PC, Pockels cell; S, shutter; HWP, half-wave plate; RM1-2, removable mirrors; L1-6, lenses; SLM, spatial light modulator; ZB, zero order beam block; GM, galvanometer mirrors; SL, scan lens; TL, tube lens; F, filter; PMT, photomultiplier tube; MLA, microlens array. (FIG. 1B) A phase spatial light modulator generates simultaneous 3D multifocal illumination patterns which are positioned and dithered by galvanometer mirrors on sites of interest pre-specified from a 3D two-photon image stack. (FIG. 1C) The resultant two-photon excited fluorescence emission is imaged through a microlens array onto an image sensor, where the emission from each target has a unique diffraction pattern (FIG. 1D) A measurement matrix constructed from individually illuminated targets is used to solve for the fluorescence at each target for each sensor image.

[00307] Cycling through illuminating subsets of targets at exactly half the maximum SLM switching speed was the optimal speed for maximizing signal rate (Fig. 2).

[00308] The output of this laser system was focused into a multifocal 3D excitation pattern using a phase spatial light modulator at an optical plane conjugate to the back aperture of the microscope objective with a phase pattern computed using the Gerchberg Saxton algorithm. The resulting time varying fluorescence from multiple locations in the sample in 3D was imaged through the detection configuration used in a microlens array positioned at a conjugate image plane, onto a sCMOS image sensor placed via relay optics at exactly one microlens focal length behind the microlens array. Each recorded camera frame was an incoherent linear sum of the unique but potentially overlapping diffraction patterns from the various 3D locations, and the individual time traces for each point in 3D could be recovered by solving a least squares deconvolution problem. In order to accurately account for tissue aberrations of the visible wavelength emission though, rather than use an analytically calculated diffraction pattern for each point from a wave optics model of light propagation in an aberration free medium, the diffraction pattern from each point in a sample was explicitly measured during a calibration procedure before each recording experiment (FIGs. 1B and 1C; FIG. 3). This empirically measured optical model enabled the faithful reconstruction of sources even in the presence of refractive index heterogeneities (FIG. 1D).

[00309] FIGs. 2A-2B: Laser repetition rate and signal rate. For the same average power, the 250 kHz repetition rate (FIG. 2B) yields approximately 230 times higher signal rate than the standard laser

at 80 MHz repetition rate (FIG. 2A). This agrees well with the theoretical 240 times higher signal, where the difference may be attributable to differences in pulse duration.

[00310] FIG. 3: Necessity for using empirically measured point spread functions for deconvolution. Actual point spread functions measured in mouse cortex (magenta) are broader and often shifted from those measured in a scattering free (green) control sample (fluorescent dye in water).

[00311] The capabilities of this approach were demonstrated by *in vivo* imaging of mouse cortex neurons using a genetically encoded calcium indicator (FIGs. 4A-4E). After first acquiring full-frame 2D images across 51 z-depths spanning a volume of 700 μm by 700 μm by 200 μm using standard laser scanning two photon microscopy (with standard Ti:Saph laser) in a process of a few minutes, 106 cells from which to record were hand selected (FIG. 4B). In another process requiring several minutes, each cell was illuminated for a single focal point, and its unique diffraction pattern recorded on the image sensor (FIG. 1B). All of the cells were then illuminated simultaneously and images recorded at 10 Hz for spontaneous activity and stimulus-evoked activity (FIGs. 4C-4E).

[00312] FIGs. 4A-4E: 3D *in vivo* calcium imaging in an awake mouse responding to a whisker deflection stimulus (FIG. 4A) *In vivo* experiment setup. (FIG. 4B) Target recording sites are selected from a 3D two-photon image stack. Scale bar, 50 μm . (FIG. 4C) Spontaneous activity measured in 106 neurons. (FIG. 4D) Whisker deflection evoked responses measured in 106 neurons. (FIG. 4E) Representative traces from adjacent cells demonstrating single cell recording resolution.

[00313] Next, the requirement for simultaneity was relaxed, and a subset of 21 cells were illuminated each for 10 ms, to record from 106 cells at 10 Hz. This recording strategy, although not truly simultaneous, allows one to record from both more cells and to more easily separate the various sources, and may be considered as simultaneous within the limits of the particular calcium sensor in the present experiments. Reducing the number of cells illuminated to a subset that is smaller by a factor M allows one to record from the same number of cells but with $\sqrt{M}$ power, or to use the same total laser power to record from $\sqrt{M}$ more cells.

[00314] The SLM field of view could be increased by temporally multiplexing using galvanometer mirrors (FIGs. 5A-5B). Using temporal multiplexing of illumination patterns on the SLM can also increase two photon signal generation efficiency (FIGs. 5A-5B).

[00315] FIGs. 5A-5B: Improving signal rate and extending the field of view using spatio-temporal multiplexing. Without spatio-temporal multiplexing (FIG. 5A), the field of view is limited by the SLM resolution and diffraction efficiency. With spatio-temporal multiplexing (FIG. 5B),

galvanometer mirrors are used to time division multiplex the illumination to various spatial regions, allowing for both more uniform illumination and higher signal rate.

[00316] The current limitations on number of cells that can be recorded from with reasonable SNR could be improved through a combination of using better lasers, faster spatial light modulators and faster cameras. In addition, real time motion correction methods could be used compensate for residual brain motion even in head fixed imaging settings.

EXAMPLE 2: Extended Field-of-View and Increased-Signal 3D Holographic Illumination with Time-Division Multiplexing

Experimental Setup

[00317] The time-division multiplexing illumination approach was implemented on a microscope equipped with a SLM-based illumination system and galvanometer scanning mirrors, as illustrated in FIG. 8. A single-snapshot 3D imaging system (light field microscope) and a single-beam laser scanning two-photon imaging system were additionally incorporated to enable quantification of the two-photon excitation efficiency and 3D *in vivo* calcium imaging.

[00318] **FIG. 8:** Optical layout. The SLM, illuminated by a 920 nm femtosecond laser through a beam expander (BE), is imaged through lenses (L1, L2) and zero-order beam block (ZB) to the midpoint between two galvanometer scanning mirrors, and then to the objective pupil plane through a scan lens (SL), tube lens (TL) and short-pass dichroic mirror (D). Excited fluorescence filtered by an emission filter (F) is captured by either a photomultiplier tube (PMT) or light field microscope (LFM) with sCMOS camera, enabling single-snapshot 3D visualization of the excited fluorescence.

Optical setup

[00319] In the excitation path, 920 nm pulses from a 80 MHz Ti:Sapphire laser (Coherent Chameleon® Ultra II) seeded a regenerative amplifier (Coherent RegA® 9000) producing 1.8 W at 250 kHz with 170 fs pulses. A Pockels cell (Conoptics 350-80-LA-02-RP KD*P) both reduced power and served as a high-speed shutter. The beam passed through a half wave plate (Thorlabs AHWP10M-980) and was expanded using achromatic doublets (Thorlabs) to slightly overfill a 6.14 mm × 6.14 mm, 256 × 256 resolution, 0-2 π phase SLM (Meadowlark HSP256-1064-P8) at a ~15 degree angle from the normal. The SLM was imaged ($f_1 = 300$ mm, $f_2 = 200$ mm) onto the midpoint between two 5-mm galvanometer mirrors (Cambridge Technology 6215H), spaced ~1 cm apart. A beam block (2 mm ϕ steel pin in a glass window, Thorlabs WG12012-B) blocked zero order undiffracted light. Finally, two lenses (Leica scan lens VIS-IR-TCS-SP2, $f = 39$ mm and Thorlabs tube lens ITL200, $f = 200$ mm) brought the conjugated SLM and scanning mirrors

through a dichroic (Semrock FF670-SDi01-25x36) and onto the objective pupil (Nikon 16x 0.8NA), after which ~20% of the total laser power remained.

[00320] The fluorescence emission path followed the light field microscope configuration in Broxton et al., *supra*. Emission passed through a filter (Semrock FF01-535/50), an identical tube lens, microlens array (RPC Photonics, 125 μm pitch, f/10), two relay lenses (Nikon 35 mm, Nikon 50 mm), and an sCMOS camera (Hamamatsu OrcaTM Flash4.0 V2). A MATLAB® program (MathWorks) controlled all hardware with a data acquisition card (National Instruments PCIe-6343).

[00321] 3D holograms computed with the multi-plane Gerchberg-Saxton method were used. For calibration and characterization experiments, a green fluorescent dye (Sharpie® highlighter) in a cuvette (Thorlabs CV10Q3500) capped with a 0.17 mm coverslip was used. The amplifier compressor stage was adjusted to compensate for pulse dispersion from the optics.

Calibration

[00322] The SLM transition time was determined to be $t_{SLM} = 7.5$ ms by steering a single focal spot between two photodiodes placed just after ZB in Fig. 8 and measuring power as a function of time, plotted as the solid line in Fig. 7A, where the surrounding shaded region illustrates one standard deviation from three measurements.

[00323] With the geometric calibration procedure in Quirin et al. (Optics express **21**(13), 16007-16021 (2013)), the 2D affine transforms between each z-depth of the light field microscope and SLM field of view was determined for each of 9 galvanometer mirror positions. For each mirror position at two z-depths, the 2D transform was measured by illuminating multiple focal spots in fluorescent dye and recording the resulting 3D light field coordinates; the transforms for the other z-depths were linearly interpolated.

Modifications for 3D two-photon in vivo calcium imaging with time-division multiplexing

[00324] To enable 3D *in vivo* calcium imaging, two flip mirrors (Newport 8892-K) were used to switch between the 80 MHz and 250 kHz lasers, and between a photomultiplier tube (Hamamatsu H10770P A-40) and the sCMOS camera/microlens array, enabling single-beam 2D laser-scanning two-photon microscopy. The geometric calibration procedure described above (“Calibration”) was extended to include this third coordinate system by registering the corners of the 2D scanned field of view in dye with the light field microscope coordinates at each z-depth.

[00325] A 512×512 resolution, but slower ($t_{SLM} = 10$ ms), SLM (Meadowlark HVHSPDM512-532) was used, and to match the larger 19.2 mm SLM size to the objective pupil, the beam expansion (BE) was adjusted accordingly using $f_1 = 750$ mm and $f_2 = 150$ mm.

[00326] Imaging was performed 6 months after a chronic cranial window was implanted. Briefly, mice were sedated with isoflurane and a 5 mm diameter, #1 thickness cover slip (Warner) was implanted over barrel cortex after injection of 1000 μL of AAVdj- Camk2a-GCaMP6m virus. In addition, a plate for head-fixation during imaging was affixed to the skull using dental cement (Parkell). The Stanford University Institutional Animal Care and Use Committee approved all experimental protocols.

Results

Extending field of view

[00327] 3D-reconstructed volumes of a fluorescent dye solution illuminated with and without an extended field of view were analyzed. In each case, the same 324 focal points in a $420\text{ }\mu\text{m} \times 420\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ volume were illuminated within a single camera exposure of $T = 100\text{ ms}$ using 36 mW (at the sample) to avoid saturation. In Fig. 9A, all focal points were illuminated simultaneously with the galvanometer mirrors in a fixed position. The lateral extent of the xy maximum projection after applying a 5% intensity threshold was $140\text{ }\mu\text{m}$, matching the theoretical value of $140\text{ }\mu\text{m}$ as described by Eq. (2) above.

[00328] **FIGs. 9A and 9B:** Experimentally measured increase in SLM field of view during $T = 100\text{ ms}$ exposure. Measured 3D illumination patterns in a fluorescent dye solution (maximum intensity projections shown) illustrate the field of view increase, denoted by dashed lines, from (FIG. 9A) $140\text{ }\mu\text{m} \times 140\text{ }\mu\text{m}$ without, to (FIG. 9B) $380\text{ }\mu\text{m} \times 380\text{ }\mu\text{m}$ with, time-sequential galvanometer scanning.

[00329] In Fig. 9B time-division multiplexing of 9 SLM fields of view with 9 galvanometer positions was used to extend the field of view to $380\text{ }\mu\text{m}$, an increase in area of 7.4 times. In addition, it should be possible to address the spatially varying diffraction efficiency visible in Fig. 9A by choosing the time-sequentially scanned fields of view to overlap further.

Increasing two-photon signal

[00330] To quantify the two-photon signal gain from time-division multiplexing empirically, the illumination in a fluorescent dye solution was restricted to the same 33 focal points within the same single SLM field of view (as in Fig. 6A), to control for excitation and emission path vignetting effects, and measured the total detected counts on the image sensor as a function of the multiplexing factor, M , again with fixed laser power and camera exposure time of $T = 100\text{ ms}$. A pockels cell shuttered the laser during the SLM transition periods in Fig. 7B. Measurements for each M were normalized with $M = 1$, and compared with Eq. (8) in Fig. 10.

[00331] **FIGs. 6A and 6B:** Illustration of time-division multiplexing strategy for laterally extending the SLM field of view (FOV) of a holographic illumination system (FIG. 6A), where a pair of

galvanometer mirrors at a conjugate pupil plane enable lateral time-sequential scanning of one of 9 different holograms to each of 9 regions (FIG. 6B). Drawings are to scale for the Nikon 16x 0.8NA objective lens (assuming 20mm field number) and 256×256 SLM used in the experiments.

[00332] **FIG. 10:** Theoretically computed and experimentally measured signal increase with SLM time-division multiplexing, for fixed average laser power, total number of illuminated sites and exposure time ($T = 100$ ms was used). SLMs with faster transition times may enable larger increases in total time-averaged two-photon signal with a greater number of subsets of focal sites illuminated sequentially. Error bars indicate one standard deviation.

[00333] For $t_{SLM} = 7.5$ ms and $t_{SLM} = 10$ ms, the maximum signal occurs at $M = 7$ and $M = 5$, respectively, compared with $M = 6.7$ and $M = 5$ predicted by Eq. (8). The slower $t_{SLM} = 10$ ms SLM was approximated by keeping the pockels cell shuttered for this longer duration. The fact that the experimentally measured traces are slightly lower than the theoretically predicted curves might be explained by the limited precision with which the hardware can be synchronized, including the SLM, pockels cell and camera.

[00334] Finally, Eq. (8) plotted in Fig. 10 suggested that recent SLMs with even shorter transition times may approach the limit where t_{SLM} tends toward 0 ms, where the increase in signal should scale exactly linearly with the number of multiplexed subsets.

Two-photon 3D in vivo calcium imaging with time-division multiplexing

[00335] The 3D *in vivo* neuronal calcium imaging was applied using the microlens array in the light field microscope.

[00336] Briefly, fluorescence representing calcium activity of the neurons in an awake, head-fixed mouse, shown in Fig. 11A, was imaged through the cranial window using the following approach. In the first step, lasting several minutes, single-beam 2D laser scanning two-photon imaging yielded high-spatial resolution images identifying the locations of neurons at each of 51 z-depths defined by an SLM-implementation of a quadratic phase. 104 neurons of interest spanning $600 \mu\text{m} \times 600 \mu\text{m} \times 200 \mu\text{m}$ from this 3D image stack were then select for high-speed recording and were grouped into $M = 5$ subsets. Next, the time-division multiplexing approach was used with 250 kHz laser with 71 mW (at the sample) to excite fluorescence at all sites, which was recorded through the microlens array and in a single $T = 100$ ms camera exposure. From each camera image the fluorescence contribution from each of the illuminated sites were extracted (more below), and by recording an image sequence, the underlying calcium activity was sampled at each site at a rate of 10 Hz, for a total duration of 50 seconds, as shown in Fig. 11C.

[00337] FIGs. 11A-D: 3D two-photon *in vivo* calcium imaging. (FIG. 11A) Neuronal activity in barrel cortex of an awake, head-fixed mouse is recorded. (FIG. 11B) 104 recording sites, centered on neurons, divided into $M = 5$ subsets (annotated by number and subset) and spanning $600\ \mu\text{m} \times 600\ \mu\text{m} \times 200\ \mu\text{m}$, are selected from a single-beam raster-scanned two-photon image stack, and then illuminated using the present time-division multiplexing approach, enabling the sampling of (FIG. 11C) calcium signals across all sites at a rate of 10 Hz ($T = 100\ \text{ms}$). (FIG. 11D) Top 10 largest magnitude responses from (FIG. 11C).

[00338] In total, the $M = 5$ illuminated sub-regions spanned an area about 4 times larger than the original SLM field of view of $300\ \mu\text{m} \times 300\ \mu\text{m}$, and Fig. 11C demonstrated that spontaneous neural activity was observed in sites within every region. Additionally, the signal increase from multiplexing, in combination with other efficiency improvements, enabled *in vivo* mouse cortex recording using only 71 mW. Importantly, the limited kinetics of the GCaMP6m calcium sensor enabled time-division multiplex sampling of the sites with $T = 100\ \text{ms}$ without sampling any less information than if all sites were had been sampled simultaneously.

[00339] For completeness, the following describes how the calcium measurements shown in Fig. 11C were extracted from raw camera images of the illuminated sites. The light field microscope projected fluorescence from each site in 3D to a unique image sensor diffraction pattern, which was calibrated empirically by recording an image of each site illuminated in isolation. (The unique image sensor diffraction pattern may also be determined analytically.) Because each raw camera image represents a linear superposition of the known, possibly overlapping, diffraction patterns of each of the illuminated sites, Richardson-Lucy deconvolution was used to solve for the fluorescence at each site. The $\Delta F/F$, a normalized measure of activity, of each site plotted in Fig. 11C was this computed fluorescence across all camera frames divided by the baseline fluorescence, estimated using the 25th percentile.

EXAMPLE 3: Method of Implementing Closed-Loop Control of Neural Activity in a Target Tissue

[00340] The present system and method to spatially resolve and detect fluorescence in conjunction with temporal multiplexing is used for implementing closed-loop 2D and 3D control. The system is adapted with an interface that allows implantation or fixation to a living and/or moving subject at a target location. The subject is a patient diagnosed with a neurological disorder, and the closed-loop control is configured to deliver feedback to the target tissue in response to an action taken either by the device, the software, the patient or by a caregiver. Real-time activity in defined excitable-tissue cells and circuit elements is imaged using chemical or genetically-encoded reporters of activity (such as Ca^{2+} , voltage, or biochemical signals) in conjunction with a

real-time system for modulating the membrane potential or activity level of cells in response to imaged activity signals. The system also includes circuit elements containing optical control tools, such as microbial opsins, and the present system configured to illuminate and observe responses in target locations with light, and to modulate the wave length and/or the intensity of the light, frequency and/or the duration of the pulses of light. The system is configured to illuminate the target spot location in response to a user input, and to minimize spectral overlap between light used for control and light used for imaging.

[00341] The subject methods and systems may be used to treat a patient for a condition or disorder, such as a neurological or psychiatric condition or disorder, wherein closed-loop principles involve action taken either by the device, the software, the patient or by a caregiver) to deliver feedback to the target tissue.

EXAMPLE 4: Method of Determining the Anatomical and Neural Activity Correlates of Neuropsychiatric Diseases

[00342] The present system and method to spatially resolve and detect fluorescence in conjunction with temporal multiplexing is used for circuit screening in animal models for endophenotypes of neuropsychiatric disease. The system is adapted with an interface that allows implantation or fixation to a living and/or moving subject, e.g., a model organism, at a target location. Neural activity is assessed in a neural region having multiple subfields by observing a cellular response in at least one subfield or circuit element or ensemble thereof, in conjunction with or due to neural activity in another region or set of regions, in response to a behavioral event, a sensory stimulus, or a circuit intervention (e.g. electrical or optical). For optical control of the circuit, the cells in the target region express a light-activated channel/pump, and the cellular electrical response is evoked by optical stimulation of the light-activated channel/pump protein. Captured image data of the ensemble response is at a level sufficiently detailed in space and time to differentiate between portions of the subfield.

[00343] Identification of abnormalities or dysfunction is assessed in normal tissue and animals as well as in genetically, pharmacologically or physiologically perturbed tissues and animals. Novel classes of events, motif, or ensemble activity are detected and observed with joint statistics across the 3D volume representing a crucial novel feature, allowing detection of volumetric motifs representing or encoding dysfunction. Highly novel treatment targets may be identified at the circuit or tissue ensemble level.

EXAMPLE 5: Method of Screening for or Monitoring the Effects of Therapeutic Treatments for Neurological Disease

[00344] The present system and method to spatially resolve and detect fluorescence in conjunction with temporal multiplexing is used for assessing effects of a treatment on endophenotypes or other readouts of physiological activity of the neural region or different subfields within the region. Readouts of physiological activity are compared with and without a treatment for the neural region. Endophenotypes and their response to intervention are assessed with respect their predictive value relative to a disorder's occurrence/magnitude and the extent of the treatment response.

[00345] Although the foregoing disclosure has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of the present disclosure that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

[00346] Accordingly, the preceding merely illustrates the principles of the present disclosure. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the present disclosure and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the present disclosure and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the present disclosure as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present disclosure, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present disclosure is embodied by the appended claims.

CLAIMS

What is claimed is:

1. A method for measuring the activity of excitable cells in a target tissue, the method comprising:
 - i) selecting a first plurality of excitable cells in a target tissue for measuring activity, wherein the excitable cells are adapted to emit fluorescence in a cellular electrical activity-sensitive manner; and
 - ii) measuring the activity of each of the excitable cells by:
 - a) illuminating the first plurality of excitable cells with spatio-temporally multiplexed three-dimensional (3D) multi-focal light patterns, thereby causing the excitable cells to emit fluorescence, wherein the 3D multi-focal light patterns are generated using a spatial light modulator and a pair of mirror galvanometers to spatially translate a field of view of the spatial light modulator on the target tissue, and wherein different subsets of excitable cells from the first plurality are illuminated among at least some of the 3D multi-focal light patterns;
 - b) recording, using an image detector, a multiplexed 2D diffraction pattern of the emitted fluorescence diffracted by a microlens array; and
 - c) resolving the multiplexed 2D diffraction pattern to determine the activity of each excitable cell illuminated by the plurality of 3D multi-focal light patterns.
2. The method according to claim 1, wherein the illuminating comprises projecting a plurality of 3D multi-focal light patterns sequentially into the target tissue at a rate of at most about 0.75 times a maximum switching rate of the spatial light modulator.
3. The method according to claim 1 or 2, wherein the illuminating comprises using a mirror galvanometer.
4. The method according to any one of claims 1 to 3, wherein the first plurality of excitable cells comprises at least 40 cells.
5. The method according to any one of claims 1 to 4, wherein the different subsets of excitable cells comprise on average 90% or fewer cells than the first plurality of excitable cells.
6. The method according to any one of claims 1 to 5, wherein the spatio-temporally multiplexed 3D multi-focal light patterns collectively have a power at the target tissue of about 500 mW or less.

7. The method according to any one of claims 1 to 6, wherein measuring the activity of an individual excitable cell is performed at a frequency of from about 5 Hz to about 100 Hz.
8. The method according to any one of claims 1 to 7, wherein resolving the multiplexed 2D diffraction pattern comprises deconvolving the multiplexed 2D diffraction pattern using an optical model for light propagation through an emission path from the target tissue to the image detector.
9. The method according to claim 8, wherein the optical model comprises an analytical model.
10. The method according to claim 9, wherein the optical model comprises a wave optics model.
11. The method according to claim 8, wherein the optical model comprises an empirical model.
12. The method according to claim 11, wherein the optical model comprises individual 2D diffraction patterns for fluorescence emitted by the first plurality of excitable cells and diffracted by the microlens array.
13. The method according to claim 12, further comprising obtaining individual 2D diffraction patterns for each of the first plurality of excitable cells in the target tissue, by
 - a) contacting an excitable cell of the first plurality with a laser light, thereby causing the contacted excitable cell to emit fluorescence; and
 - b) recording, using the image detector, a 2D diffraction pattern of the emitted fluorescence diffracted by the microlens array.
14. The method according to claim 13, wherein the contacting comprises two-photon 2D laser scanning.
15. The method according to any one of claims 12 to 14, wherein the deconvolving comprises determining individual point spread functions (PSFs) for each of the individual 2D diffraction patterns and using the individual PSFs to deconvolve the multiplexed 2D diffraction pattern.
16. The method according to any one of claims 12 to 15, further comprising selecting members of the different subsets of the first plurality of excitable cells based on the individual 2D diffraction patterns.

17. The method according to claim 16, wherein the selecting members of the different subsets comprises:
- comparing the individual 2D diffraction patterns among each other; and
 - selecting members of the different subset such that a minimum difference in the individual 2D diffraction patterns between excitable cells in any pair of the different subsets is at or above a threshold difference.
18. The method according to claim 17, wherein the threshold difference maximizes the minimum difference.
19. The method according to any one of claims 1 to 18, wherein the 3D multi-focal laser light patterns are generated by spatially modulating a laser beam using the spatial light modulator.
20. The method according to claim 19, wherein the laser beam is pulsed at a frequency of from about 100 to about 500 kHz.
21. The method according to claim 19 or 20, wherein the laser beam has an average power of about 1.0 to about 3.0 W.
22. The method according to any one of claims 19 to 21, wherein the laser beam has a pulse duration of about 100 to about 500 fs.
23. The method according to any one of claims 1 to 22, wherein the selecting comprises:
- scanning the target tissue using a focused laser light, to generate an image of the target tissue; and
 - identifying excitable cells in the image.
24. The method according to claim 23, wherein the focused laser light is pulsed at a frequency of about 10 to about 200 MHz.
25. The method according to claim 23 or 24, wherein the focused laser light is generated using a spatial light modulator.

26. The method according to any one of claims 1 to 25, the image detector is a Complementary Metal Oxide Semiconductor (CMOS) detector.
27. The method according to any one of claims 1 to 26, wherein the excitable cells comprise neurons.
28. The method according to any one of claims 1 to 27, wherein the target tissue is an *in vivo* neuronal tissue.
29. The method according to any one of claims 1 to 28, wherein the target tissue is a light-scattering tissue.
30. The method according to any one of claims 1 to 29, wherein the excitable cells are adapted to emit fluorescence in a calcium- or voltage-sensitive manner.
31. The method according to claim 30, wherein the excitable cells comprise an activity-sensitive fluorescent dye.
32. The method according to claim 30, wherein the excitable cells comprise a genetically encoded, activity-sensitive fluorescent protein.
33. The method according to any one of claims 1 to 32, wherein the 3D multi-focal light patterns comprise light having a wavelength in the near-infrared range.
34. The method according to any one of claims 1 to 33, wherein the method further comprises:
stimulating the target tissue; and
measuring the activity of the first plurality of excitable cells in response to the stimulation.
35. The method according to claim 34, wherein the target tissue comprises a second plurality of excitable cells that are adapted to hyperpolarize and/or depolarize in response to a laser light stimulus, and the stimulating comprises contacting the second plurality of excitable cells with a laser light stimulus.
36. The method according to claim 35, wherein an excitable cell that is adapted to hyperpolarize and/or depolarize in response to a laser light stimulus is genetically modified to express one or more light-

responsive polypeptides that hyperpolarize or depolarize the excitable cell when contacted by a laser light.

37. A method of regulating the activity of a plurality of excitable cells in a target tissue, comprising:

i) measuring the activity of a first plurality of activity-sensitive fluorescence-emitting excitable cells in a target tissue comprising a second plurality of excitable cells, according to the method of any one of claims 1 to 33; and

ii) modulating the activity of the second plurality of excitable cells in the target tissue in response to the measured activity of the first plurality of activity-sensitive fluorescence-emitting excitable cells.

38. The method of claim 37, wherein excitable cells of the second plurality are genetically modified to hyperpolarize and/or depolarize in response to a laser light stimulus, and the modulating comprises contacting the genetically modified excitable cells of the second plurality with a light stimulus using one or more 3D multi-focal light pattern stimuli, wherein the one or more 3D multi-focal light pattern stimuli are controlled in response to the measured activity of one or more of the plurality of activity-sensitive fluorescence-emitting excitable cells.

39. A system comprising:

i) a light microscope defining an excitation path and an emission path with respect to a target tissue, wherein the excitation path comprises:

a spatial light modulator configured to modify a first light generated by a light source, and to thereby project a 3D multi-focal laser light pattern into the target tissue; and

a pair of mirror galvanometers configured to spatially translate a field of view of the spatial light modulator on the target tissue,

and wherein the emission path comprises a microlens array configured to modify fluorescence emitted by the target tissue such that a 2D diffraction pattern is projected onto an image detector;

ii) a controller; and

iii) a processor configured to execute instructions that cause:

the controller to:

illuminate the target tissue with spatio-temporally multiplexed 3D multi-focal light patterns, thereby causing the excitable cells to emit fluorescence, and

record a multiplexed 2D diffraction pattern of the emitted fluorescence; and

the processor to resolve the recorded multiplexed 2D diffraction pattern.

40. The system according to claim 39, wherein the light source is configured to generate a second light, and wherein the excitation path comprises a first movable mirror configured to direct the first light onto the spatial light modulator when the first movable mirror is in a first position, and the second light onto the spatial light modulator when the first movable mirror is in a second position.
41. The system according to claim 39 or 40, wherein the light source is a laser light source and the light is a laser beam.
42. The system according to claim 41, wherein the laser light source comprises a regenerative amplifier configured to modify the first laser beam.
43. The system according to any one of claims 39 to 42, wherein the emission path comprises a photodetector, and wherein the emission path comprises a second movable mirror configured to direct the fluorescence emitted by the target tissue to: the microlens array when the second movable mirror is in a first position, and the photodetector when the second movable mirror is in a second position.
44. The system according to any one of claims 39 to 43, wherein the emission path comprises an achromatic doublet configured to expand the first laser beam or the second laser beam directed onto the spatial light modulator.
45. The system according to any one of claims 39 to 44, wherein the spatial light modulator has a maximum switching rate of at least about 40 Hz.
46. The system according to any one of claims 39 to 45, wherein the spatial light modulator has a resolution of at least 256 pixels x at least 256 pixels.
47. The system according to any one of claims 39 to 46, wherein the first light has a wavelength in the near-infrared range.

48. The system according to any one of claims 39 to 47, wherein the excitation path comprises a Pockels cell configured to reduce power of the first light such that the spatio-temporally multiplexed 3D multi-focal light patterns has an average power at the target tissue of about 500 mW or less.

49. The system according to any one of claims 39 to 48, wherein the image detector is a CMOS detector.

50. A method for collectively and specifically illuminating a plurality of excitable cells in a target tissue, the method comprising:

i) selecting a plurality of excitable cells in a target tissue, wherein excitable cells of the plurality are adapted to emit fluorescence; and

ii) sequentially projecting into the target tissue each 3D multi-focal light pattern of spatio-temporally multiplexed 3D multi-focal light patterns generated using a spatial light modulator and a pair of mirror galvanometers to spatially translate a field of view of the spatial light modulator on the target tissue, wherein the 3D multi-focal light patterns illuminate different subsets of the plurality of excitable cells,

thereby collectively and specifically illuminating the plurality of excitable cells in the target tissue.

51. The method according to claim 50, wherein the 3D multi-focal light patterns are sequentially projected into the target tissue at a rate of at most about 0.75 times a maximum switching rate of the spatial light modulator.

52. The method according to claim 50 or 51, wherein the different subsets of excitable cells comprise on average 90% or fewer cells than the plurality of excitable cells.

53. The method according to any one of claims 50 to 52, wherein the excitable cells are neurons.

54. The method according to claim 53, wherein the target tissue is an in vivo neuronal tissue.

55. The method according to any one of claims 50 to 54, wherein the target tissue comprises a population of excitable cells that are adapted to hyperpolarize and/or depolarize in response to a light stimulus.

56. The method according to claim 55, wherein excitable cells of the population are genetically modified to express one or more light-responsive polypeptides that hyperpolarize or depolarize the excitable cell when illuminated by the light stimulus.

57. The method according to any one of claims 50 to 56, wherein the 3D multi-focal light patterns collectively have a power at the target tissue of about 500 mW or less.

58. The method according to any one of claims 50 to 57, wherein excitable cells of the plurality are adapted to emit fluorescence in a cellular electrical activity-sensitive manner, and wherein the sequentially projecting each of the 3D multi-focal light patterns is sufficient to cause the excitable cells of the first plurality in the target tissue to emit fluorescence when illuminated by a 3D multi-focal laser light pattern.

59. The method according to claim 58, further comprising iii) measuring the activity of excitable cells in the plurality by:

recording, using an image detector, a multiplexed 2D diffraction pattern of the emitted fluorescence diffracted by a microlens array; and

resolving the multiplexed 2D diffraction pattern to determine the activity of each excitable cell illuminated by the plurality of 3D multi-focal light patterns.

60. The method according to claim 59, wherein the resolving comprises deconvolving the multiplexed 2D diffraction pattern using an optical model for light propagation through an emission path from the target tissue to the image detector.

61. The method according to claim 60, wherein the optical model comprises an analytical model.

62. The method according to claim 61, wherein the optical model comprises a wave optics model.

63. The method according to claim 60, wherein the optical model comprises an empirical model.

64. The method according to claim 63, wherein the optical model comprises individual 2D diffraction patterns for fluorescence emitted by the plurality of excitable cells and diffracted by the microlens array.

65. The method according to claim 63 or 64, wherein the individual 2D diffraction patterns are obtained by

- a) contacting an excitable cell in the plurality of excitable cells with a laser light, thereby causing the excitable cell to emit fluorescence;
- b) directing the emitted fluorescence from the contacted excitable cell through a microlens array to create a 2D diffraction pattern; and
- c) recording the 2D diffraction pattern.

66. The method according to claim 65, wherein the contacting comprises two-photon 2D laser scanning.

67. The method according to any one of claims 63 to 66, further comprising selecting members of the different subsets of the plurality of excitable cells based on the individual 2D diffraction patterns.

68. The method according to claim 67, wherein the selecting members of the different subsets comprises:

- comparing the individual 2D diffraction patterns among each other; and
- selecting members of the different subset such that a minimum difference in the individual 2D diffraction patterns between excitable cells in any pair of the different subsets is at or above a threshold difference.

69. The method according to claim 68, wherein the threshold difference maximizes the minimum difference.

70. The method according to any one of claims 59 to 69, wherein measuring the activity of an excitable cell is performed at a frequency of from about 5 Hz to about 100 Hz.

71. The method according to any one of claims 59 to 70, wherein the image detector is a CMOS detector.

72. The method according to any one of claims 59 to 71, wherein the excitable cells are adapted to emit fluorescence in a calcium- or voltage-sensitive manner.

73. The method according to any one of claims 59 to 72, wherein the excitable cells comprise an activity-sensitive fluorescent dye.
74. The method according to any one of claims 59 to 72, wherein the excitable cells comprise a genetically encoded, activity-sensitive fluorescent protein.
75. The method according to any one of claims 59 to 74, wherein the method further comprises:
stimulating the target tissue; and
measuring the activity of individual excitable cells in the plurality of excitable cells in response to the stimulation.
76. The method according to any one of claims 50 to 75, wherein the target tissue is a light-scattering tissue.
77. The method according to any one of claims 50 to 76, wherein the first subset comprises 10 or more excitable cells.
78. The method according to any one of claims 50 to 77, wherein the 3D multi-focal light pattern is generated by spatially modulating an amplitude and/or phase of a laser beam using the spatial light modulator.
79. The method according to claim 78, wherein the laser beam is pulsed at a frequency of from about 100 to about 500 kHz.
80. The method according to claims 78 or 79, wherein the laser beam has an average power of from about 1.0 to about 3.0 W.
81. The method according to any one of claims 78 to 80, wherein the laser beam has a pulse duration of from about 100 to about 500 fs.
82. The method according to any one of claims 78 to 81, wherein the laser beam has a wavelength in the near-infrared range.
83. The method according to any one of claims 50 to 82, wherein the selecting comprises:

scanning the target tissue using a focused laser light, to generate an image of the target tissue; and identifying excitable cells in the image.

84. The method according to claim 83, wherein the focused laser light is pulsed at a frequency of from about 10 to about 200 MHz.

85. The method according to claim 83 or 84, wherein the focused laser light is generated using a spatial light modulator.

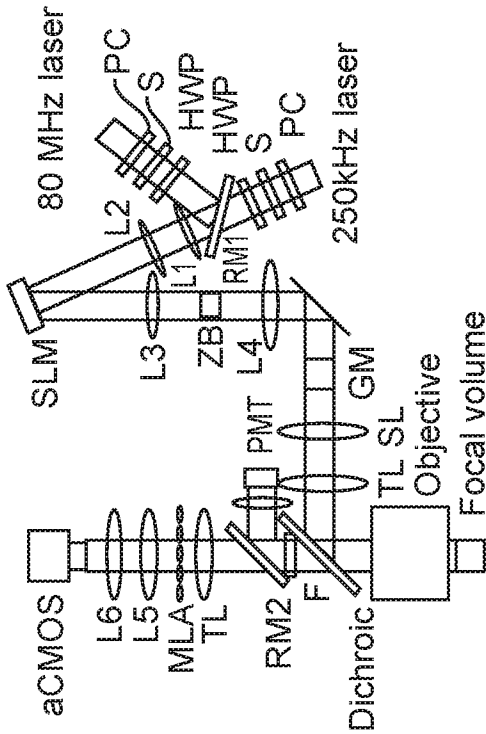


FIG. 1A

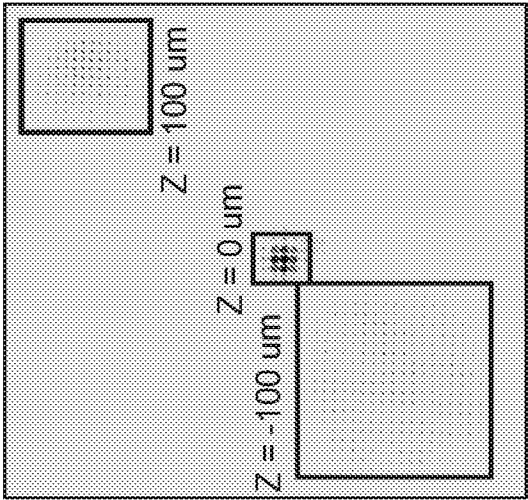


FIG. 1C

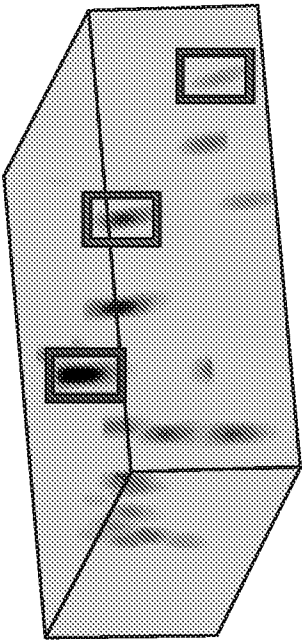


FIG. 1B

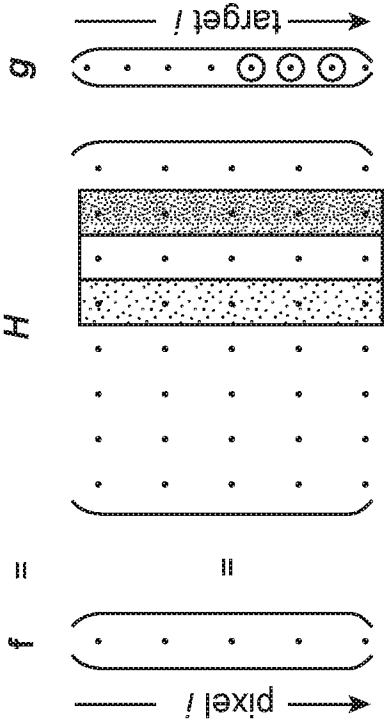


FIG. 1D

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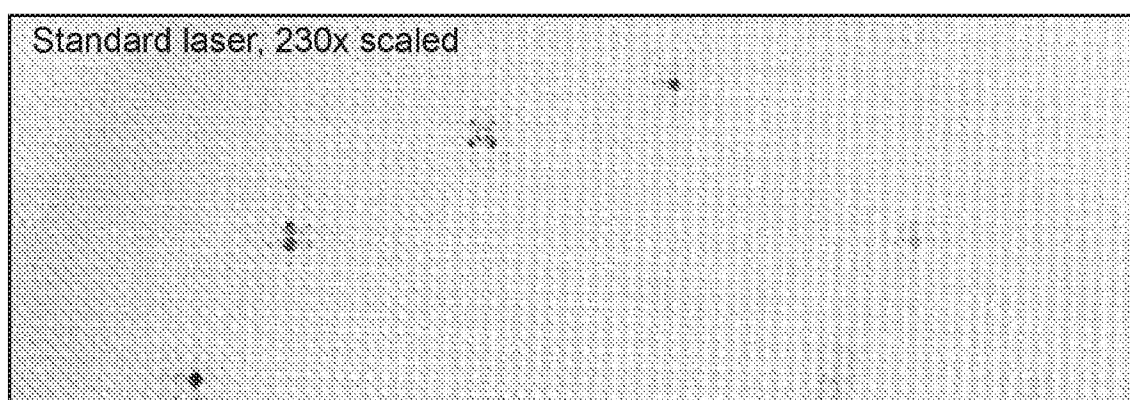


FIG. 2A

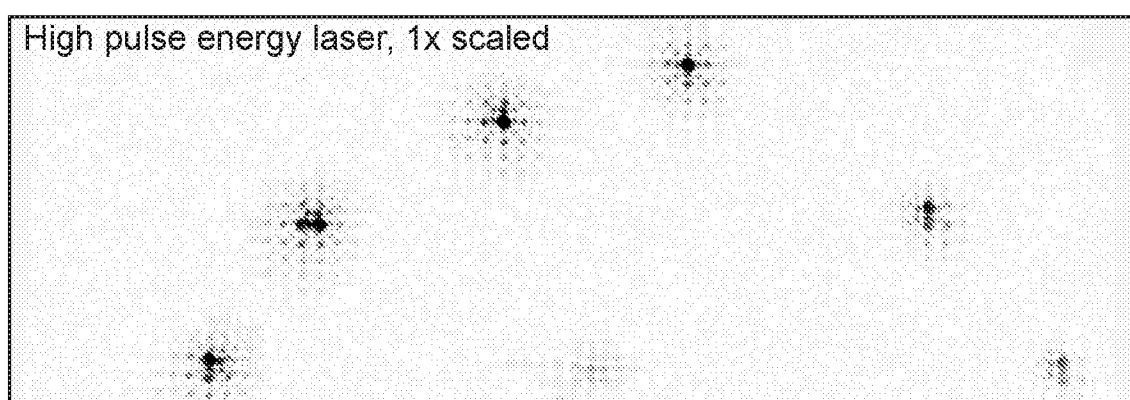


FIG. 2B

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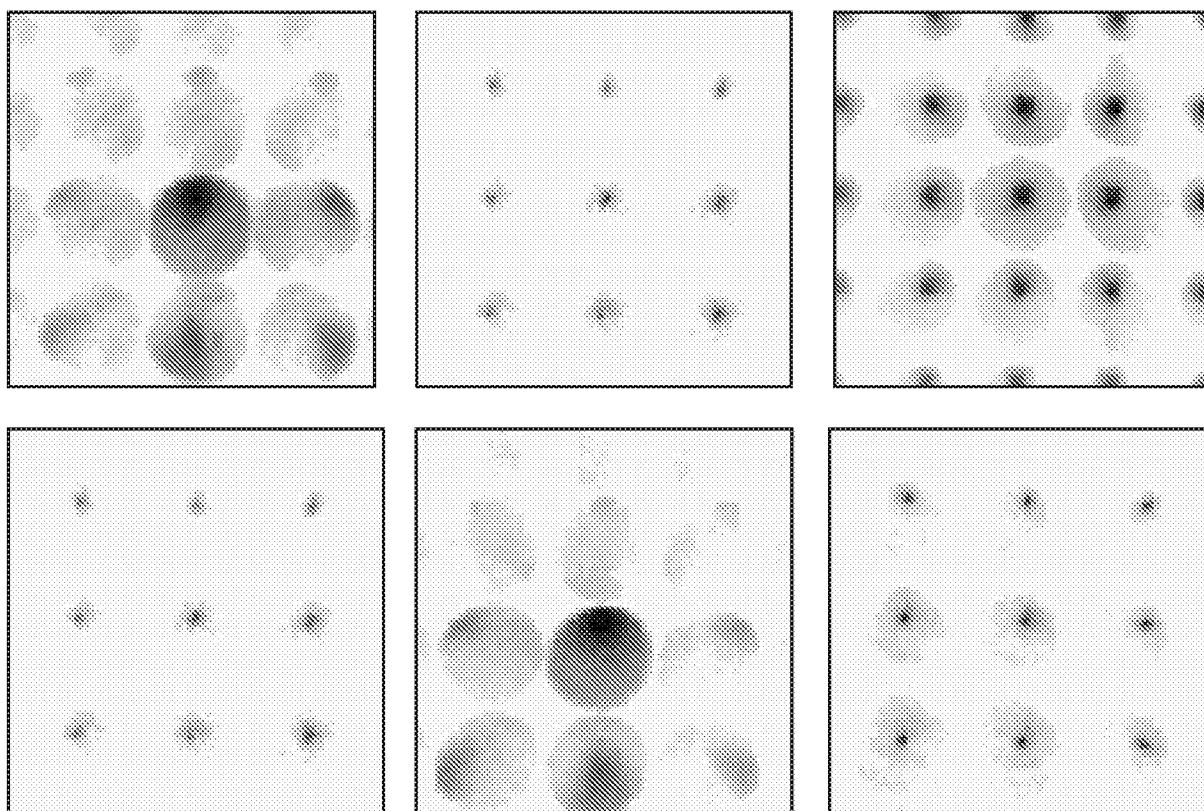


FIG. 3

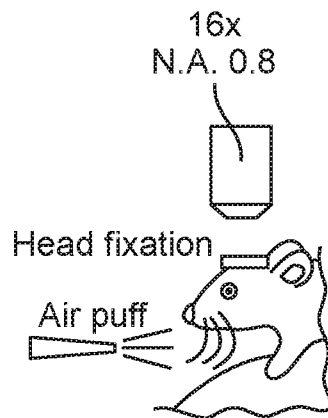


FIG. 4A

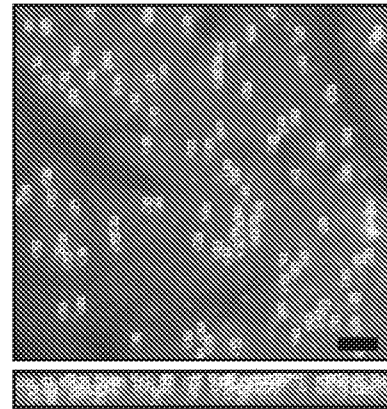


FIG. 4B

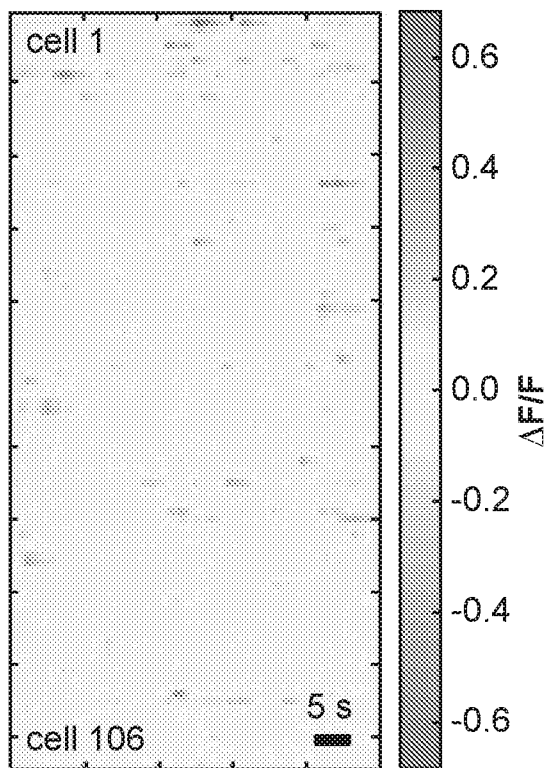


FIG. 4C

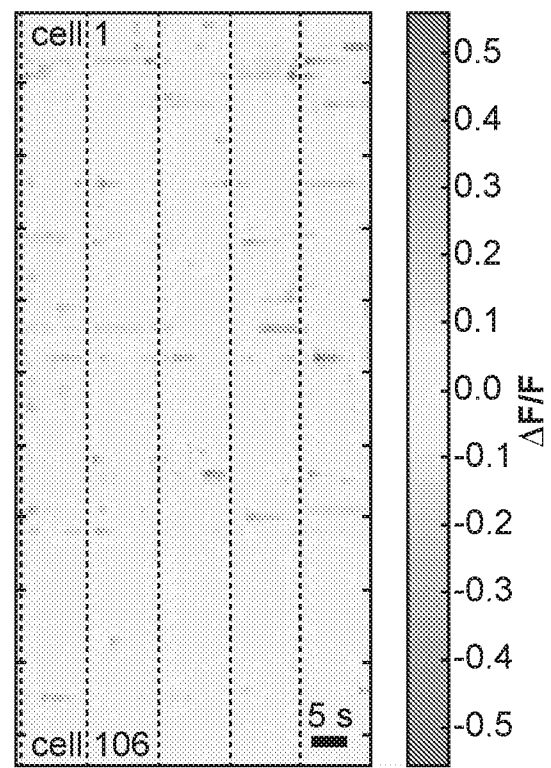


FIG. 4D

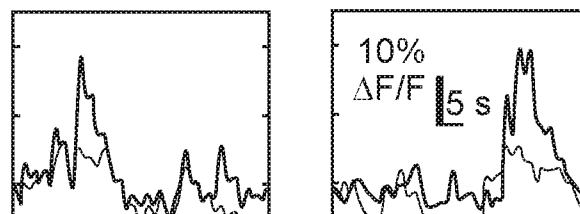


FIG. 4E

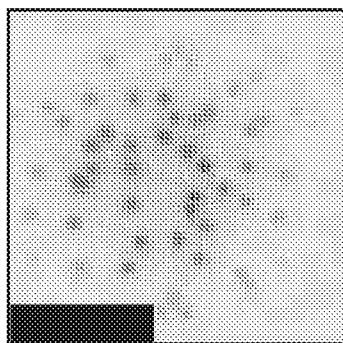
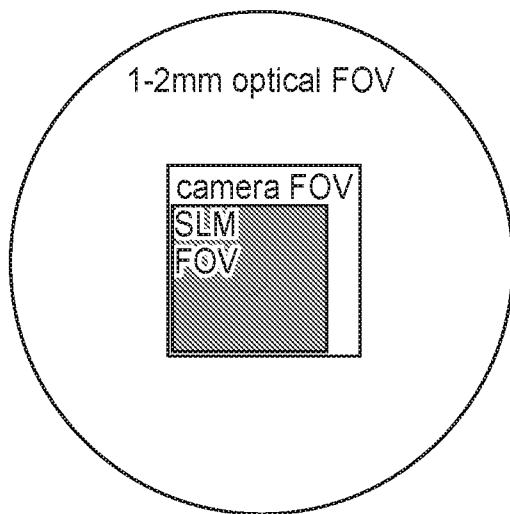


FIG. 5A

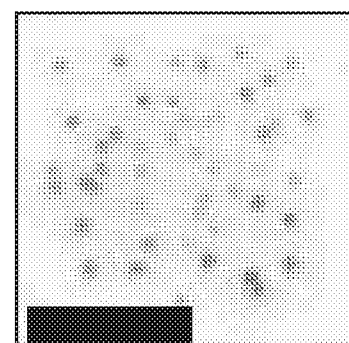
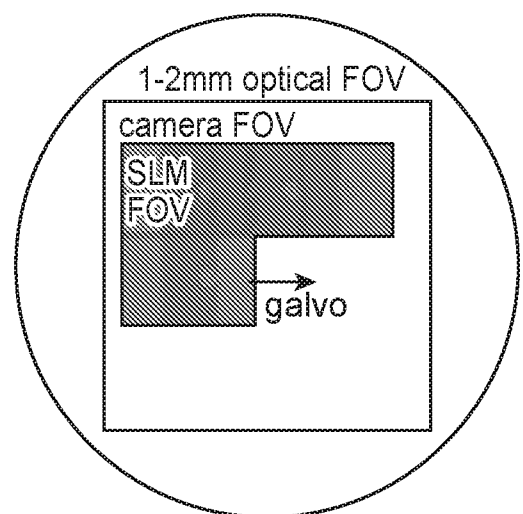


FIG. 5B

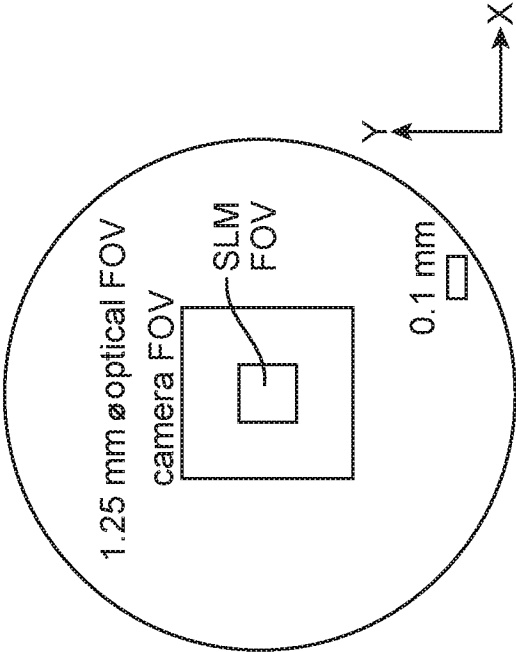


FIG. 6A

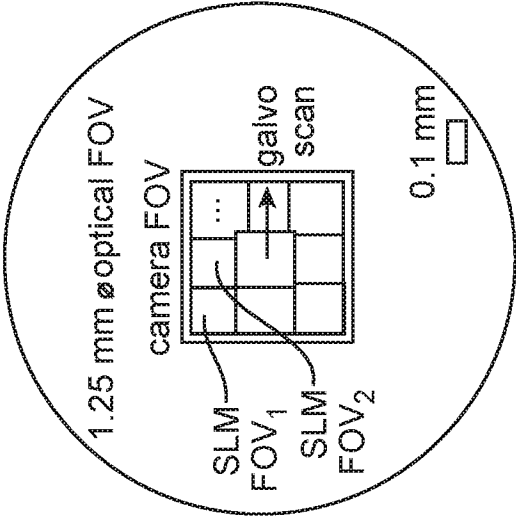


FIG. 6B

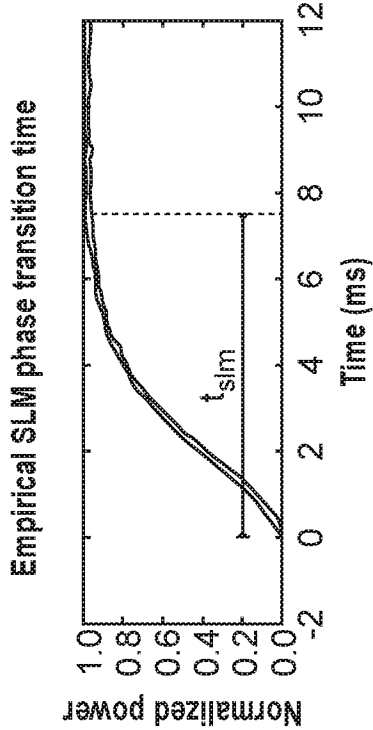


FIG. 7A

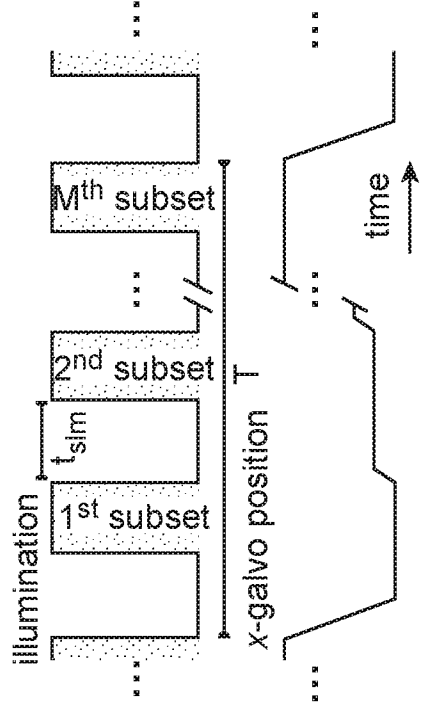


FIG. 7B

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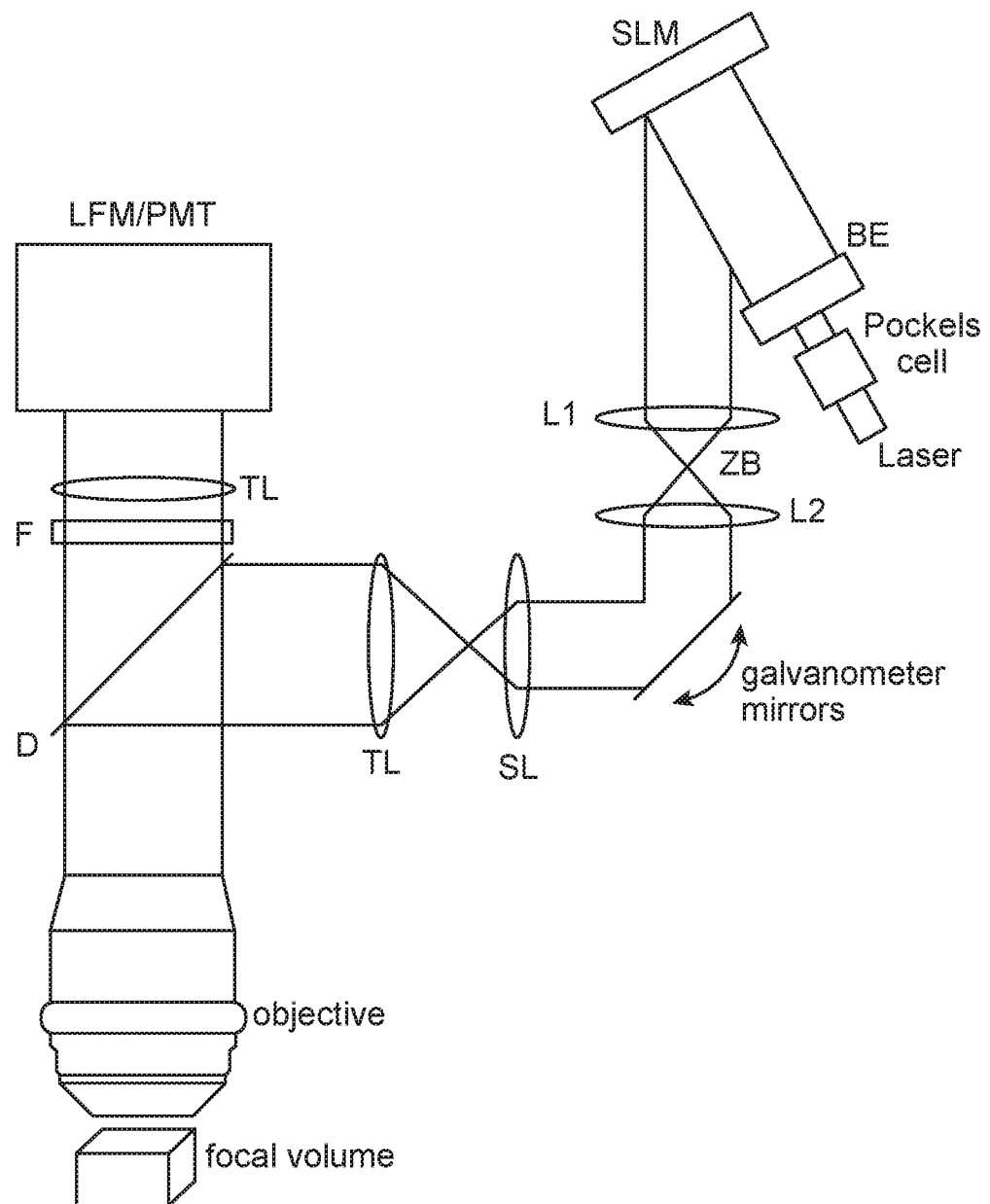


FIG. 8

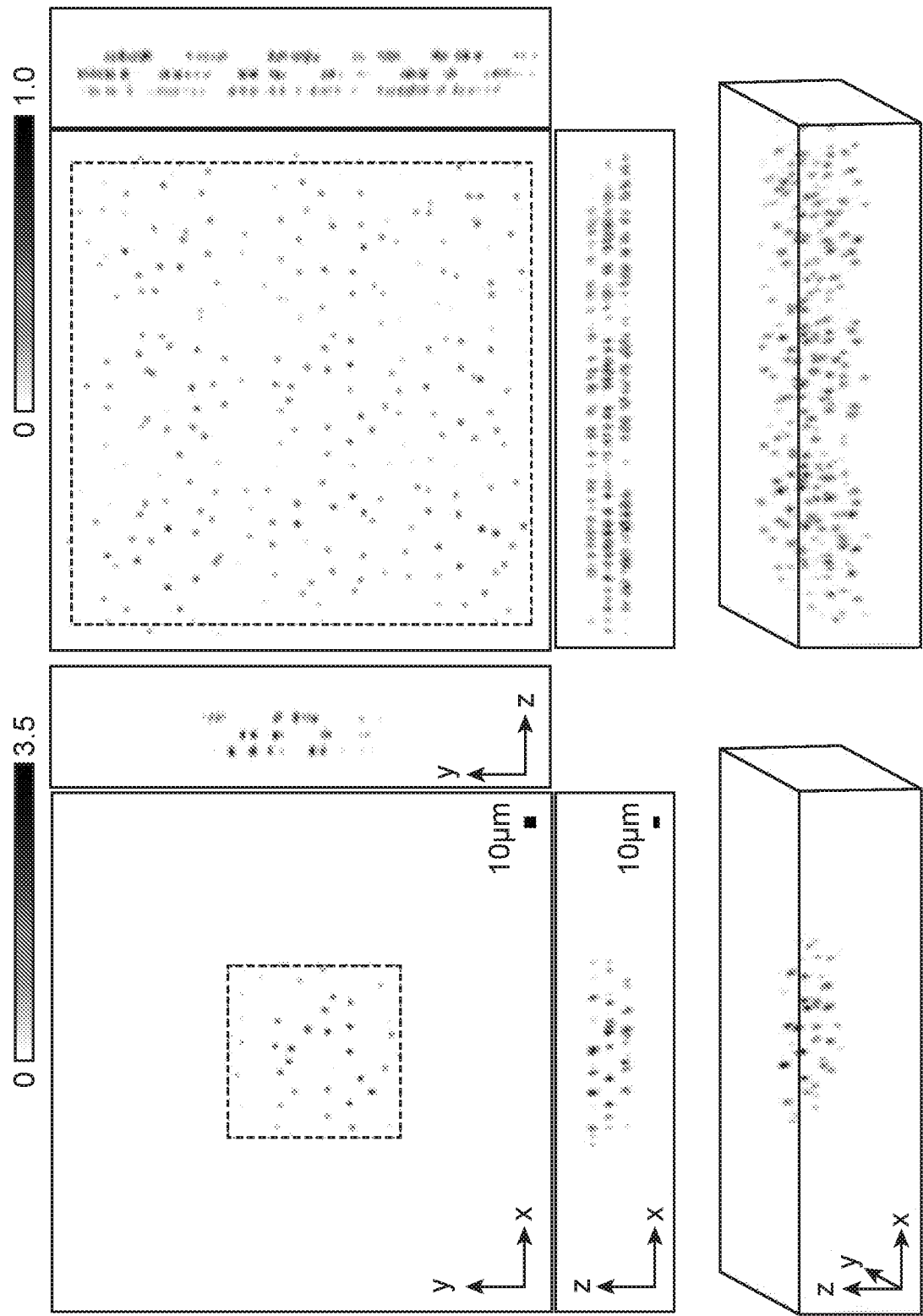


FIG. 9B

FIG. 9A

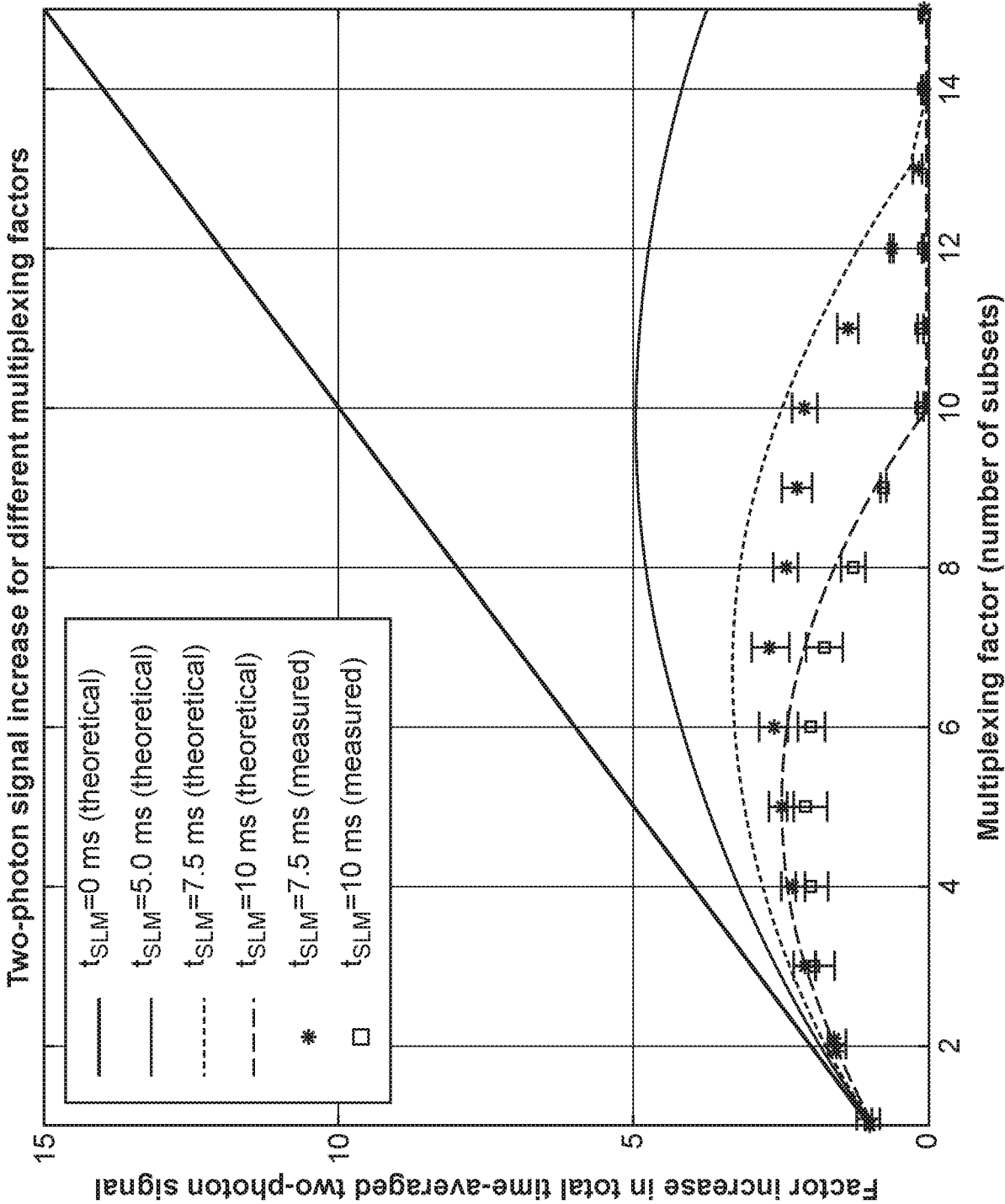


FIG. 10

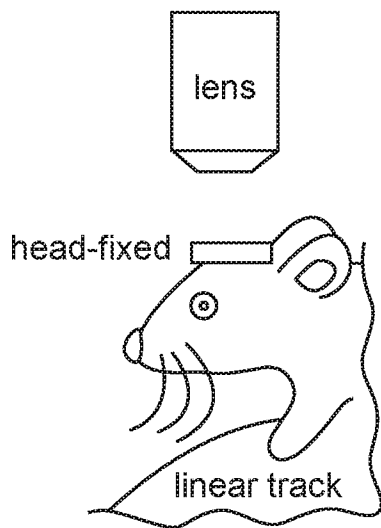


FIG. 11A

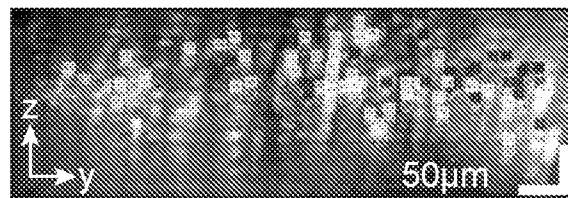
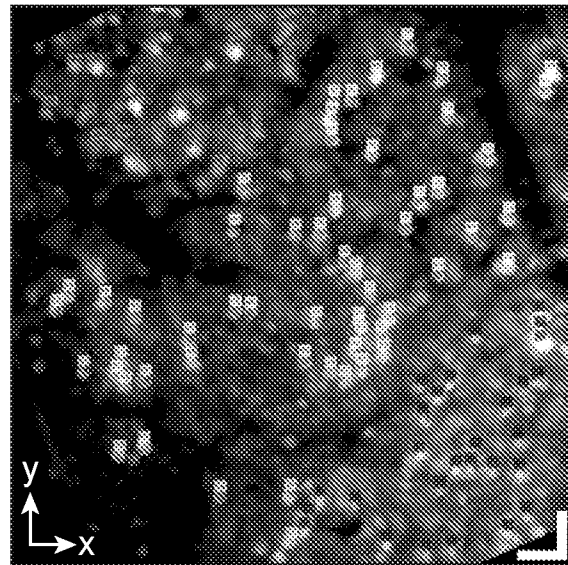


FIG. 11B

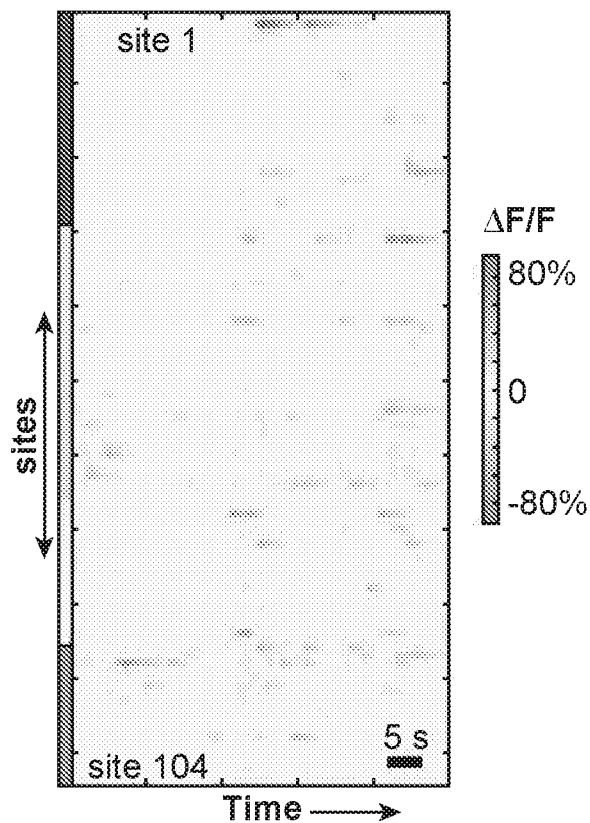


FIG. 11C

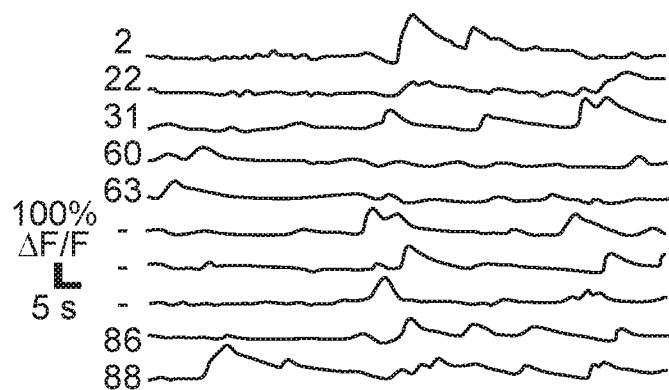


FIG. 11D

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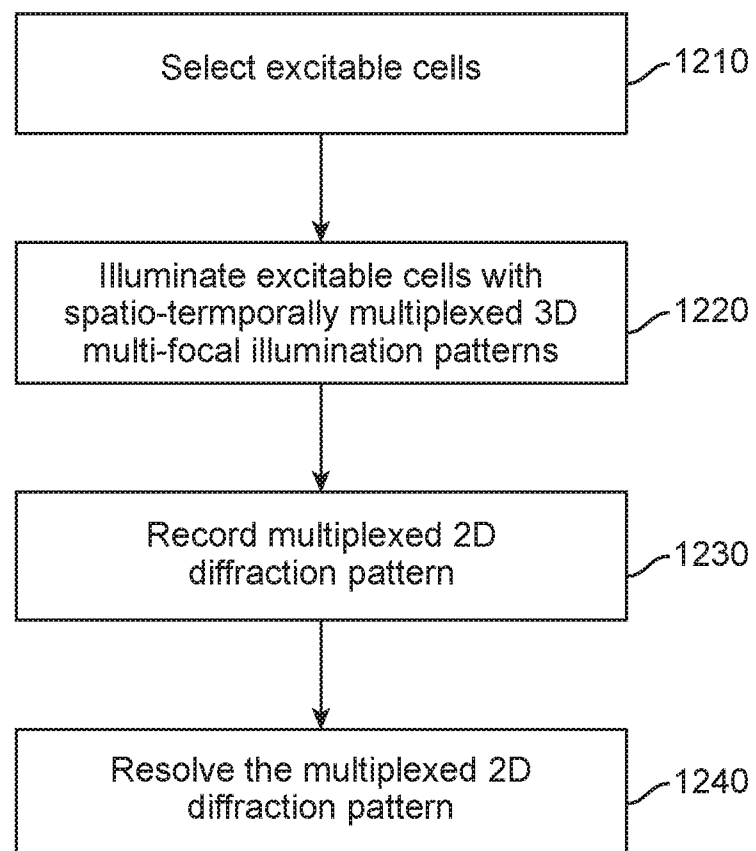


FIG. 12

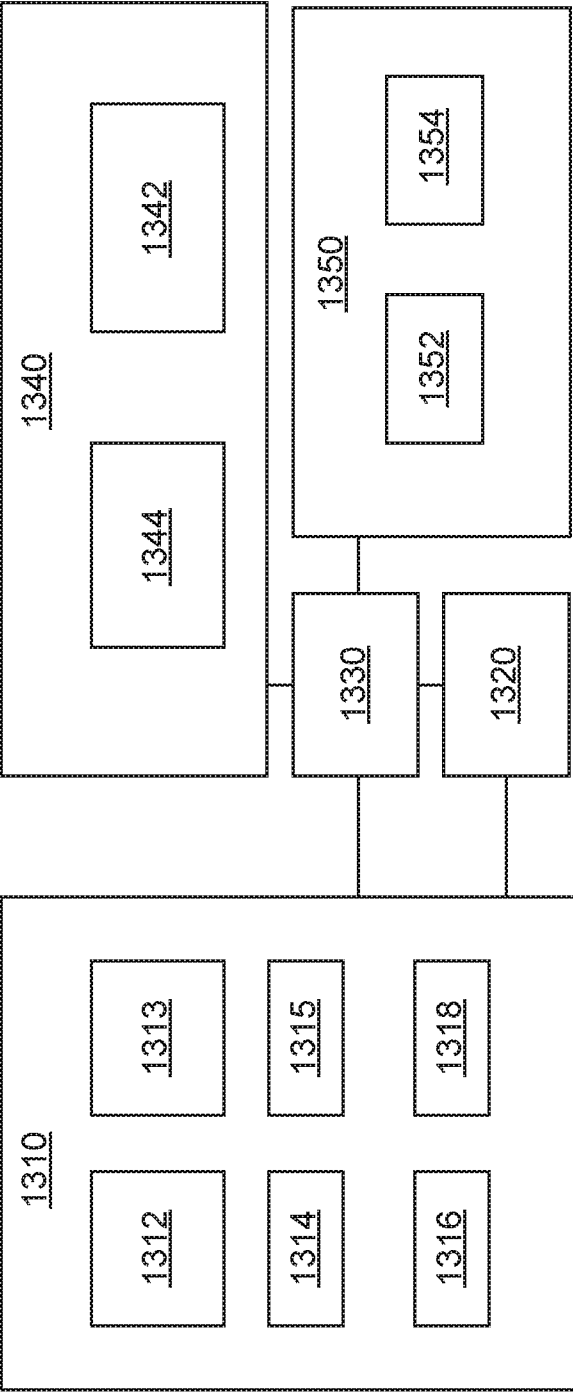


FIG. 13A



FIG. 13B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/37271

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - H04N 7/18 (2016.01)

CPC - H04N 7/18

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)

IPC (8) - H04N 7/18 (2016.01)

CPC - H04N 7/18

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

CPC - G02B21/002, G02B27/095, G02B21/06 (See Keywords below)

USPC - 348/79, 204/157.15, 422/82.05, 422/186

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

Thomsoninnovation.com; Patbase; Google Scholar; Google Patents; Gogole.com; Freepatentsonline; ProQuest Dialog

Search Terms: Measure, monitor, determine, activity, fluorescence, diffraction, cell, tissue, neuron, 3D, excitable cells, illuminate, light, laser, beam, amplify, multi focal, spatial light Modulator, galvanometer, microlens, patterns, i

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0286181 A1 (BETZIG et al.), 31 October 2013 (31.10.2013), entire document, especially Abstract; Para [0014], [0071], [0085], [0087], [0099], [0109], [0161]	1-3, 39-42 and 50-52
Y	WO 2013/126762 A1 (THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES OFFICE OF TECHNOLOGY TRANSFER, NATIONAL INSTITUTES OF HEALTH), 29 August 2013 (29.08.2013), entire document, especially Abstract; Para [0030], [0034], [0038]	1-3, 39-42 and 50-52
Y	US 2002/0190922 A1 (TSAO), 19 December 2002 (19.12.2002), entire document, especially Abstract; Para [0039]-[0040]	2-3 and 51-52
Y	US 6,108,081 A (HOLTOM et al.), 22 August 2000 (22.08.2000), entire document, especially Abstract; col 4, ln 1-20	42
A	WO 2014/117079 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK), 31 July 2014 (31.07.2014), entire document	1-3, 39-42 and 50-52
A	US 2011/0233046 A1 (NIKOLENKO et al.), 29 September 2011 (29.09.2011), entire document	1-3, 39-42 and 50-52

☐ 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" earlier application or patent but published on or after the 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

"T" 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

15 August 2016 (15.08.2016)

Date of mailing of the international search report

09 SEP 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/37271

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.: 4-38, 43-49 and 53-85
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III 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.