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(71) Applicants: **THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL** [US/US]; 100 Europa Drive, Suite 430, Chapel Hill, North Carolina 27517 (US). **NORTH CAROLINA STATE UNIVERSITY** [US/US]; Office of Technology Transfer, 1021 Main Campus Drive, 2nd Floor, Raleigh, North Carolina 27606 (US).

(72) Inventors: **SMITH, Spencer L.**; 470 Windy Ridge Road, Chapel Hill, North Carolina 27517 (US). **STIRMAN, Jeffrey**; 3B Valentine Lane, Chapel Hill, North Carolina 27516 (US). **KUDENOV, Michael**; 4604 Irene Way, Raleigh, North Carolina 27603 (US).

(74) Agent: **WILSON, Jeffrey L.**; Jenkins, Wilson, Taylor & Hunt, P.A., Suite 1200, University Tower, 3100 Tower Boulevard, Durham, North Carolina 27707 (US).

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(54) Title: STEERING DEVICES FOR TWO-PHOTON EXCITATION IMAGING SYSTEMS

(57) Abstract: Two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods are disclosed herein. In some aspects, the steering devices can include a first movable steering mirror, a second movable steering mirror, and at least one component in communication with the first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes. The steering devices can be implemented in a two-photon excitation imaging system. In some aspects, the imaging system can include a source for producing light pulses, a steering device, and a recombination relay. The steering device can be configured to direct first and second pulses of light, respectively, to the recombination relay, and an objective through which the first and second pulses of light are directed through to a specimen, after the first and second pulses of light are recombined.



STEERING DEVICES FOR TWO-PHOTON EXCITATION IMAGING SYSTEMS

DESCRIPTION

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CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application Serial No. 62/023,564 filed July 11, 2014, which is herein incorporated by reference in its entirety.

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STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under grant number A12-1051-001 awarded by the Human Frontier Science Program. The government has certain rights to this invention.

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TECHNICAL FIELD

The subject matter disclosed herein relates to multiphoton excitation laser scanning microscopy. More particularly, the subject matter disclosed herein relates to two-photon excited imaging systems, steering devices for two-photon excitation imaging systems, and related methods having an increased field of view (FOV) capable of high speed imaging in at least two regions of interest (ROIs) within the FOV.

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BACKGROUND

Ensemble neuronal activity is of key interest in system neuroscience, among other disciplines, to understand sensory coding, motor output, and cognitive function. Measuring neuronal activity in populations of neurons *in vivo* is technically challenging, due to the densely packed neuropil and sensitive

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neuroanatomy that is best probed with minimally invasive approaches. However, multiphoton imaging can overcome these technical challenges.

For example, two-photon population calcium imaging *in vivo* offers many distinct advantages over alternative approaches, such as metal electrodes.

5 Two-photon calcium imaging provides unambiguous identification of recorded neurons, particularly when genetically encoded indicators are employed. Two-photon calcium imaging can be used to record activity in tens to hundreds of neurons by imaging somata, dendrites, and/or axons in local populations. Furthermore, two-photon imaging can penetrate hundreds of microns into the
10 brain through an intact dura mater. Not only has this approach revealed cellular-level stimulus selectivity, but it can be used to measure neural activity in awake rodents (e.g., mice) performing a psychophysics task or navigating in a virtual reality environment.

Although multiphoton imaging has proven to be a powerful tool to study
15 ensemble activity in local populations of neurons in neocortex, conventional approaches are limited to a full field of view (FOV) of approximately 500 μm – 700 μm . This limitation precludes the simultaneous examination over a wide FOV or areas large enough to encompass multiple regions of interest (ROIs) within a FOV. While discrete functional areas of mammalian neocortex work in
20 concert to process sensory input and guide adaptive behavior, little is known about how cortical areas interact. Thus, in order to progress further in neuroscience a cellular-level view into ongoing neural activity across extended cortical networks is needed.

As a result, there is a need for two-photon excitation imaging systems,
25 steering devices for two-photon excitation imaging systems, and related methods that feature a wide FOV to enable high-speed imaging in spatially separate ROIs.

SUMMARY

In accordance with this disclosure, two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods are provided. In particular, the two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods can comprise an increased field of view (FOV) capable of high speed imaging in two discrete regions of interest (ROI).

In some aspects, a steering device for a two-photon excitation imaging system is provided. For example, the device can comprise a first movable steering mirror, a second movable steering mirror, and at least one component in communication with the first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes.

In other aspects, a two-photon excitation imaging system is provided. For example, the system can comprise a source for producing light pulses, a steering device comprising: a first movable steering mirror disposed in a first pathway along which a first pulse of light is directed, without delay, to the first movable steering mirror; a second movable steering mirror disposed in a second pathway along which a second pulse of light is directed to the second movable steering mirror; and at least one component in communication with the first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes. The system can also comprise a recombination relay configured to recombine the first and second pulses of light, wherein the first movable steering mirror and the second movable steering mirror are configured to direct the first and second pulses of light, respectively, to the recombination relay, and an objective through which the first and second pulses of light are directed through to a specimen, after the first and second pulses of light are recombined.

In other aspects, a two-photon excitation imaging method is provided. For example, the method can comprise at a two-photon excitation imaging system including a source for producing light pulses, a steering device comprising first movable steering mirror, a second movable steering mirror, and
5 at least one component in communication with the first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes, providing a first pulse of light and a second pulse of light, directing, without delay, the first pulse of light along a first pathway to the first movable steering mirror and the second pulse
10 of light along a second pathway to a second movable steering mirror, recombining the first and second pulses of light, and directing the recombined first and second pulses of light through an objective to a specimen.

Although some of the aspects of the subject matter disclosed herein have been stated hereinabove, and which are achieved in whole or in part by
15 the presently disclosed subject matter, other aspects will become evident as the description proceeds when taken in connection with the accompanying drawings as best described hereinbelow.

BRIEF DESCRIPTION OF THE DRAWINGS

20 The features and advantages of the present subject matter will be more readily understood from the following detailed description which should be read in conjunction with the accompanying drawings that are given merely by way of explanatory and non-limiting example, and in which:

Figure 1A illustrates a schematic of an exemplary two-photon excitation
25 imaging system according to some embodiments of the subject matter described herein;

Figure 1B illustrates a schematic of an exemplary afocal relay of the two-photon excitation imaging system according to Figure 1A;

Figure 1C illustrates a schematic of two exemplary and separate regions of interest (ROIs) within a field of view (FOV) generated by the two-photon excitation imaging system according to Figure 1A;

5 Figure 1D illustrates a schematic of two exemplary ROIs generated within a neural area FOV by an exemplary two-photon excitation imaging system according to some embodiments of the subject matter described herein;

Figure 2A illustrates a schematic of an exemplary objective, scanning lens, and tube lens of a two-photon excitation imaging system according to some embodiments of the subject matter described herein;

10 Figure 2B illustrates a schematic of the exemplary objective of the two-photon excitation imaging system according to Figure 2A;

Figure 3 illustrates a schematic of an exemplary steering device for use in a two-photon excitation imaging system according to some embodiments of the subject matter described herein; and

15 Figure 4 illustrates a flow diagram of an exemplary method for two-photon excitation imaging according to some embodiments of the subject matter described herein.

DETAILED DESCRIPTION

20 The present subject matter provides two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods. In one aspect, the present subject matter provides two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods that can comprise an increased field of view
25 (FOV) capable of high speed imaging in two discrete regions of interest (ROIs).

In one aspect, the present subject matter provides a novel two-photon excitation imaging system with a field of view greater than 3000 μm , resulting in an imaging area over 18-fold greater than that of conventional multiphoton imaging (> 7 square mm), while still achieving cellular resolution. To support

high speed imaging in two discrete ROIs within this FOV simultaneously, multiplexed excitation and high-speed photon counting can be used. As used herein, "multiplex" can refer to splitting a single pulse, delaying one pathway of the split pulse, and then recombining the two pulses.

5 Two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods can be advantageous for several reasons. Firstly, the wide FOV can offer extended overview imaging (e.g. of extended cortical networks). Since an excitation volume can be preserved across the FOV, the two-photon excitation imaging systems, steering
10 devices for two-photon excited imaging systems, and related methods can provide access to over seven-fold more spatial information than conventional multiphoton imaging systems. Secondly, the two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods can provide for two imaging pathways which can allow for
15 simultaneous imaging of spatially distinct ROIs within a FOV. Thirdly, the two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods can be flexible since imaging pulses can be freely repositioned during a single imaging session, e.g., two ROIs can be positioned in XYZ directions within the increased FOV simply by manipulating
20 motorized steering mirrors disposed within the system, which can allow a user, within a single specimen and imaging session, to image multiple combinations of extended cortical networks without having to move the specimen. Furthermore, the two ROIs can have independent control over a plane of focus. Each ROI can be as large or small as needed for the imaging session
25 depending on the scan speed required. For example, for large overview imaging with two depths, both imaging pathways can be set to a maximum FOV, and set to different image depths (e.g., range is approximately 100 μm). Alternatively, for example, to image a full field faster, the two ROIs can be smaller and spatially separated. .

Moreover, two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods can be used in several ways. Firstly, a number of light pulses can be extended, as long as an interval between any light pulse pulses (across all multiplexed pulses) is longer than a fluorescence lifetime of a fluorophore imaged. Secondly, spatially patterned optogenetic stimulation can be applied through a wide FOV objective, using a dichroic mirror disposed before any filters and a photomultiplier tube (PMT) on a detection arm of a scope. Optogenetic stimulation can be applied in any pattern across an entire FOV, even if calcium imaging is only targeted to two specific ROIs. To simultaneously image multiple cortical regions that are beyond the FOV, or to access subcortical brain regions, for example, the two-photon excitation imaging system can be used in conjunction with microendoscopes. Lateral views of a full cortical depth can be obtained using implanted microprisms with the system.

Notably, two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods as discussed herein can provide neuroscience with a flexible way to image cellular-level neuronal activity not only across multiple cortical areas, but also any model system that extends beyond spatial limits of conventional multiphoton or two-photon microscopy. Two-photon excitation imaging systems, steering devices for two-photon excitation imaging systems, and related methods can enable measurements of inter-area correlations at a single neuron level and monitoring of activity across extended neuronal circuits. However, one of skill in the art can readily understand that two-photon excitation imaging systems, steering devices, and related methods of the present subject matter are not limited to the uses, configurations, advantages, etc., discussed herein.

Now referring to Figure 1A, a schematic of an exemplary two-photon excitation imaging system, generally designated **100**, is illustrated. In some aspects, two-photon excitation imaging system **100** can comprise a two-photon

microscope, such as, for example, a Twin Region, Panoramic two-photon (Trepan2p) microscope, configured to provide a wide FOV and two imaging pulses that can be synchronously scanned, enabling simultaneous two-photon calcium imaging in spatially separate cortical areas of a specimen; thereby
5 doubling temporal resolution. In some aspects, where two-photon excitation imaging system **100** comprises a Trepan2p microscope, only a single, conventional microscope objective is needed, which can allow for flexible placement of sensory stimulation and behavior apparatus around a subject or specimen **104**. Alternately, custom objectives, such as a 0.8 numerical aperture
10 (NA) objective **102** (see Figs. 2A-2B) can be utilized in system **100**. Additionally, a working distance (e.g., approximately eight mm) can ensure that the Trepan2p microscope can be compatible with additional devices and/or systems, such as, for example, a simultaneous electrophysiology device and head-fixed behavior experimental systems.

15 Two-photon excitation imaging system **100** can comprise, in some aspects, a light source, generally designated **110**. Light source **110** can produce a pulse incident on a desired specimen **104**. Light source **110** can be a laser, such as, for example, a Ti: Sapphire laser, which can produce laser pulses following a directed pathway **112**. The laser pulses can be delivered at
20 approximately 12.5 ns intervals (e.g., 80 MHz) before being multiplexed. For example, the laser pulses can be attenuated and split into two pulses, each following its own directed pathway **114**, **116**, using two sets of polarization optics. However, the present subject matter can be readily extended into 2^n pulses, where $n = 1, 2, 3 \dots$. Whether or not a number of pulses can be extended
25 depends on whether an interval between any laser pulses (across all multiplexed pulses) is longer than a fluorescence lifetime of a fluorophore imaged.

The laser pulses produced by light source **110** can be directly detected by a photodiode (not shown) using a pick-off mirror (not shown). The

photodiode can also be used to synchronize photon-counting electronics, such as, for example a photon counting unit (e.g., **198**). Alternatively, synchronization output from light source **110** can also be used to synchronize photon-counting electronics.

5 Additionally, in some aspects, overall power of system **100** can be attenuated via a power attenuation mechanism, generally designated **120**. Power attenuation mechanism **120** can comprise at least one pulse block **130**, a first half wave plate **132A**, a second half wave plate **132B**, and a first polarizing pulse splitting (PBS) cube **134A**. Pulse block **130** can absorb excess
10 laser power. First PBS cube **134A** can be disposed in between first half wave plate **132A** and second half wave plate **132B** relative to the pathway **112** of laser pulses, where first half wave plate **132A** can be positioned before first PBS cube **134A**, while second half wave plate **132B** can be positioned after first PBS cube **134A**.

15 In some aspects, second half-wave plate **132B** can be used to determine a power ratio sent to each pathway **114**, **116** once light pulses have been split into two separate imaging pulses or pulses. Two imaging pulses can be, for example, directed to two different ROIs (e.g., two different cortical networks of a brain). In this example, one brain area might be deeper than the other, and thus
20 require more power for imaging than the other brain area. Thus, the power ratio between two pathways **114**, **116** can be adjustable.

 A mirror **136** can be provided after power attenuation mechanism **120** in order to direct pulses to a pulse expander, generally designated **140**. For example, pulse expander **140** can be disposed after second half-wave plate
25 **132B** in order to expand a size of the light pulses. In some aspects, a second pulse expander (not shown) can be utilized in system **100** after pulse recombination to overfill a back aperture of objective **102**.

 In some aspects, a PBS cube can be implemented in order to split the light pulses traveling along pathway **112** into two separate pulses or pulses

following two separate pathways **114**, **116**. For example, as illustrated in Figure 1A, second PBS cube **134B** is positioned after pulse expander **140** relative to pathway **112** of pulses and is configured to split pulses into a first pulse and a second pulse, each pulse following its own individual pathway **114**, **116**,
5 respectively. The first and second pulses can be configured with similar or different polarization. For example, the first pulse can be configured with p-polarization, while the second pulse can be configured with s-polarization. The first pulse can follow a first pathway **114** and can be configured to pass to a first steering mirror **160** without delay, while the second pulse can follow a second
10 pathway **116** and can be configured to pass to a second steering mirror **162** with a predetermined delay. First pathway **114** can be a similar or different length than second pathway **116**. For example, where it is desired to delay second pulse compared to first pulse, second pathway **116** can be longer in distance than first pathway **114**, such that it takes a light pulse of similar
15 properties a longer period of time to travel second pathway **116** compared to first pathway **114**. Alternatively, second pathway **116** can be shorter in distance than first pathway **114**.

As illustrated in Figure 1A, first pathway **114** can be configured without a predetermined delay portion, such that the first pulse travels without delay
20 through first pathway **114** before reaching first steering mirror **160**. By comparison, at least a portion of second pathway **116** can be configured as a delay path, such that the second pulse travels through the delay path of second pathway **116** prior to reaching second steering mirror **162**. In the delay path of second pathway **116**, the second pulse can pass between a plurality of mirrors
25 **150**. Plurality of mirrors **150** can for example include five (5) mirrors **152A-152E** spaced apart in such a manner that the second pulse travels from first mirror **152A** to second mirror **152B** to third mirror **152C** to fourth mirror **152D** to fifth mirror **152E**. In some aspects, there can be more or less mirrors depending on a length of the predetermined delay required. As illustrated in Figure 1A, mirrors

152A-152E are spaced apart such that the second pulse is delayed by approximately 6.25 ns due to traveling approximately 1.87 meters farther than the first pulse in the first pathway **114** ($6.25 \text{ ns} * c = 1.87 \text{ m}$). After traveling 1.87 m, the second pathway **116** can pass to second steering mirror **162**.

5 Steering mirrors **160**, **162** can each be separately and independently repositionable and/or movable relative to one another in an XY axis. As used herein, 'repositioning' of steering mirrors **160**, **162** can comprise independently pivoting each steering mirror **160**, **162** such that a center of a mirror surface of each of mirrors **160**, **162** does not translate. As a result, steering mirrors **160**,
10 **162** can be configured to direct respective light pulses to different regions of a FOV (e.g., FOV **106**, Figs. 1C) without 'clipping' of these light pulses. For example, by adjusting a deflection angle of one or more of the first and second pulses, steering mirrors **160**, **162** can define smaller ROIs within a larger FOV. In some aspects, steering mirrors **160**, **162** can comprise a large semi-diameter
15 steering mirror, including a semi-diameter of approximately 3.0 mm, although larger and smaller diameters are also contemplated. In some aspects, steering mirrors **160**, **162** can be motorized and can be remotely movable by a user for independently repositioning first and/or second movable steering mirrors **160**,
20 **162**. For example, steering mirrors **160**, **162** can comprise a servomotor configured to communicate with remote controls (see, Fig. 3). In other examples, a voice coil, piezo, galvanometer, etc., may be used to move steering mirrors **160**, **162**. Conversely, in other aspects, steering mirrors **160**,
25 **162** can be manually movable via at least one component in communication with first and second steering mirrors **160**, **162**, such as, for example, a joystick and/or other manual control mechanism. Where steering mirrors **160**, **162** are independently controllable by a motor, the mirrors can be moved along in XY axes at least, approximately 20 degrees or more, to reposition a deflection angle of the first and second pulses. For example, repositioning (e.g., tilt-tip) first steering mirror **160** can result in changing a deflection angle Ω_1 of the first

pulse along the XY axes, while independent repositioning (e.g., tilt-tip) of second steering mirror **162** can result in independently changing a deflection angle Ω_2 of the second pulse along the XY axes prior to recombination. Deflection angles Ω_1 , Ω_2 can determine the central locations (e.g., X_1 , Y_1 ; X_2 , Y_2) of individual ROIs within the larger FOV (see, Figs. 1C-1D). Consequently, it will be apparent to one of skill in the art that moving steering mirrors **160**, **162** can cause each corresponding ROI to similarly move along the XY axes within the FOV.

In some aspects, adjusting a position (e.g., focal plane) of pathways **114**, **116** along the Z-axis may be accomplished without repositioning either first or second steering mirrors **160**, **162**. Accordingly, electro-optical lenses, such as, for example, electrically tunable lenses (ETL) can be used to adjust a position (e.g., focal plane) of pathways **114**, **116** along the Z-axis relative to an imaging plane. For example, a first ETL **164A** can be disposed in first pathway **114** prior to first steering mirror **160** for adjusting a focal plane of first pathway **114**, while a second ETL **164B** can be disposed in second pathway **116** prior to second steering mirror **162** for adjusting a focal plane of second pathway **116**. First ETL **164A** and second ETL **164B** can each be independently controllable such that a focal plane of each pathway in the Z-axis is independently adjustable. First and/or second ETLs **164A**, **164B** can be disposed after first and/or second steering mirror **160**, **162**, respectively, to adjust convergence and/or divergence of the first and/or second pulses along the Z-axis. As an alternative to an electro-optical lens, a single angle translator, for example, may be used to adjust a focal plane of first and/or second pathways **114**, **116** of the first and/or second pulses along the Z-axis.

In some aspects, a pulse or pulse recombination relay **170** can be implemented to recombine the first and second pulses after a delay has been imposed upon one or more of the pulses. Recombination relay **170** can comprise relay units **172A-C** and a third PBS cube **134C**. Third PBS cube **134C**

can be positioned centrally in relation to relay units **172A-C** of recombination relay **170**. Each of the three relay units **172A-C** can be configured with a plurality of lenses to greatly reduce aberrations (e.g., astigmatism), which can allow for large angles (e.g., $\Omega_1, \Omega_2 \neq 0$) to be imparted on the individual imaging pathways **114, 116**. The plurality of lenses can comprise an achromatic lens and a positive meniscus lens configured such that a convex surface of the meniscus lens faces crown glass of the achromatic lens. For example, each relay unit **172A-C** can comprise a 50 mm achromatic lens and a 100 mm positive meniscus lens.

First steering mirror **160** can be configured to direct the first pulse from first pathway **114** to first relay unit **172A**, which can then direct the first pulse to third PBS cube **134C**. Second steering mirror **162** can be configured to direct the second pulse from second pathway **116** to second relay unit **172B**, which can then also direct the second pulse to third PBS cube **134C** for recombination.

In the above described multiplexing scheme, the light pulses are delivered from light source **110** at an 80 MHz pulse rate (12.5 ns intervals). Splitting the light pulses at second PBS cube **134B** results in a first pulse traveling along first pathway **114** without delay to first steering mirror **160**, while a second pulse experiences a 6.25 ns delay along second pathway **114** before reaching second steering mirror **162**. Thus, when each of the first and second pulses are delivered to PBS cube **134C**, the 6.25 ns delay applied to the second pulse results in perfectly interleaved pulses upon recombination, i.e., first and second pulses can be evenly spaced in time at 160 MHz after recombination.

After recombination at PBS cube **134C**, the first and second pulses can be directed to a third relay unit **172C** of recombination relay **170**. The first and the second pulses can then be relayed to X and Y galvanometer scanner, generally designated **180**. In some aspects, as illustrated in Figure 1B, X and Y

galvanometer scanner **180** can comprise X and Y scan mirrors **182A** and **182B**, respectively, that are connected via a first relay unit **184A** and a second relay unit **184B**. Scan mirrors **182A** and **182B** can be separated by a displacement distance, such as, for example, approximately 6 mm. In this manner, the first
5 and second pulses can be simultaneously raster scanned, where a field size is determined by scan amplitude, and with independently controlled spatial positions in the X and Y axes, as determined by Ω_1 , Ω_2 .

In some aspects and as illustrated in Figure 1B, first relay unit **184A** and second relay unit **184B** can be configured with a plurality of lenses. The plurality
10 of lenses can comprise an achromatic lens and a positive meniscus lens configured such that a convex surface of the meniscus lens faces crown glass of the achromatic lens. For example, each relay unit **184A-B** can comprise a 50 mm achromatic lens and a 100 mm positive meniscus lens. Notably, however, X and Y galvanometer scanner **180** can comprise alternative embodiments
15 having different types, sizes, etc., of lens in first relay unit **184A** and second relay unit **184B**.

In some aspects, axial movement of the meniscus lens of first relay unit **184A** and/or second relay unit **184B** can cause a small change in pulse convergence that can alter a focal plane for a particular path up to
20 approximately 100 μm . Therefore, each path can be fully independently positionable in the XY axes (e.g., range of approximately 3500 μm) and Z-axis (e.g., range of approximately 500 μm). This allows simultaneously scanning two different ROIs within a full FOV.

In some aspects, a scan lens **190** in combination with a tube lens
25 **192** can direct the temporally recombined pulses towards a back aperture of objective **102** in order to expand the beam and provide low aberration beam scanning at the back aperture of objective **102**. For example, scan lens **190** and tube lens **192** can form a 4x telescope for this purpose (See, Fig. 2A), which in turn can determine a size of the FOV. In some aspects, a scan angle realized at

the back aperture of objective **102** can be made smaller by an inverse factor of beam expansion. Thus, a 4x telescope composed of, for example, scan lens **190** and tube lens **192**, can result in a scan angle being reduced to one-quarter of the scan angle of scan mirrors **182A**, **182B** of galvanometer **180**. Beam magnification, scan angles, and diameters of clear apertures of scan lens **190** and tube lens **192** can be optimized, along with aberration correction of scan lens **190** and tube lens **192**, in order to ensure a wide FOV. In some aspects and as illustrated in Figure 1C, a FOV, generally designated **106**, can be formed having a diameter based on a magnification of objective **102**. The magnification is a function of the focal length of objective **102**, smaller focal length corresponding to a greater magnification (see, Fig. 2). By decreasing a magnification of objective **102**, a diameter of FOV **106** can be increased. Conversely, by increasing a magnification of objective **102**, a diameter of FOV **106** can be decreased. Within FOV **106**, two separate ROIs, first ROI **108A** formed by first pathway **114** and second ROI **108B** formed by second pathway **116** can be scanned by system **100**. In some aspects, ROIs **108A** and **108B** can be repositioned within FOV **106** during a scanning session by repositioning steering mirrors **160**, **162**. For example, repositioning at least one of steering mirrors **160**, **162** can result in changing a deflection angle Ω_1 of the first pulse along the XY axis, while independent movement (e.g., tilt-tip) of second steering mirror **162** can result in independently changing a deflection angle Ω_2 of the second pulse along the XY axis prior to recombination. Angles Ω_1 , Ω_2 can determine the central locations (e.g., X_1 , Y_1 ; X_2 , Y_2) of individual ROIs **108A**, **108B** within FOV **106**. Additionally, as illustrated in Figure 1C, a focal plane parallel to an imaging plane is represented. In some aspects, ETLs **164A**, **164B** can be used to individually adjust or reposition the focal plane of pathways **114**, **116** along the Z-axis.

Referring now to Figure 1D, an exemplary embodiment of FOV **106** is illustrated. In Figure 1D, FOV **106** encompasses a neural area of specimen

104. Here a primary visual cortex **V1** surrounded by higher visual areas (e.g., posteromedial, anteromedial, anterior, rostrolateral, anterolateral, lateromedial, laterointermediate) is completely encompassed within FOV **106**. Portions of primary and secondary somatosensory cortex **S1**, **S2** and auditory cortex **AU** are also encompassed by FOV **106**. Changing a magnification and/or other components of system **100** may result in a diameter of FOV **106** increasing and/or decreasing. Within FOV **106**, first ROI **108A** can be positioned in one portion of primary visual cortex **V1**, while second ROI **108B** can be positioned in one portion of the anteromedial area. Notably, repositioning of steering mirrors **160** and/or **162** can result in a central location of ROIs **108A** and/or **108B** changing. Thus, within a same imaging session, multiple ROIs within one or more FOVs can be scanned, without moving system **100** or specimen **104**.

Referring back to Figure 1A, during operation of the two-photon excitation imaging system, such as system **100**, pulses from both imaging pathways **114**, **116** can overfill a back aperture of objective **102**. Overall, in such a configuration, system **100** can be configured to transmit approximately 13% of entering power. However, power does not have to be limited in this configuration, such that decreasing overfilling of objective **102** and scan mirrors **182A-B** of galvanometer **180** can increase power transmission, though with a possible decrease in excitation efficiency.

In some aspects, fluorescence can be collected using, for example, a dichroic mirror **194**, a plurality of lenses **196A** and **196B**, and a PMT **198** of system **100**. PMT **198** can comprise, for example, a GaAsP PMT having a high bandwidth amplifier (not shown) for measuring fluorescence signals from both pathways **114**, **116**. A photodiode (not shown) can be used to directly detect light pulses and synchronize photon counting electronics, e.g., PMT **198**. By synchronizing PMT **198** with the laser pulses, fluorescence events can be reliably assigned to an appropriate pathway (e.g., first pathway **114** or second pathway **116**, with minimal channel crosstalk (e.g., 1.5 – 6.6%).

Photon counting can provide an increase in signal to noise over analog integration for dim signals, which can be common with *in vivo* two-photon calcium imaging data. A digital acquisition board (not shown) can generate mirror command signals, and frame synchronization signals for PMT **198**. In some aspects, custom software (see, **304**, Fig. 3) can control steering mirrors **160**, **162**, communicate with PMT **198**, and can organize photon counting data into images. In some aspects, the pulsing of light source **110** can be measured using a photodiode (not shown) to determine which fluorescence signals came from which pathway.

Referring now to Figure 2A, a schematic, generally designated **200**, a scan engine composed of a scan lens **202**, a tube lens **204**, and an objective **206** is illustrated. A central optical axis is illustrated, in Figure 2A, by the broken line, while a maximum scan angle is illustrated by the solid line. Scan lens **202**, tube lens **204**, and objective **206** may each be utilized in a two-photon excitation imaging system, such as system **100**.

As illustrated in Figure 2A, a ratio TL_{FL}/SL_{FL} results in a factor by which an excitation beam is expanded. An inverse of this factor is the factor by which a scan angle Θ_1 is decreased to result in Θ_2 . To determine a width of the FOV an algorithm, such as, $2 * OBJ_{FL} * \tan(\Theta_2)$ can be used. A magnification of objective **206** can be a function of its focal length. Thus, both the focal length and/or magnification of objective **206**, as well as the scan angle can influence the FOV.

A schematic of one embodiment of objective **206** is illustrated in Figure 2B. In some aspects, a standard commercial objective can be utilized in system **200**, while in other aspects a custom objective can be used. For example, a custom objective, e.g., objective **206** can comprise an effective focal length (EFL) of 27.55 mm and an NA of 0.4. Objective **206** can be used in conjunction with scan lens **202**, tube lens **204**, and any associated relays (e.g., recombination relay **170**, afocal relay in galvanometer **180**, Figure 1) to

minimize aberrations to result in single neuron resolution with two-photon excitation across a wider FOV. In some aspects, a quality of a point spread function (PSF) of two-photon excitation imaging system is crucial to its efficiency, rejection of out of plane fluorescence, and image quality. For example, where a 0.80 NA objective (e.g., **102**) is utilized in system **100** a small PSF can result. This can provide a small excitation volume, good optical sectioning, and reduced contamination from out-of-plane fluorescent neuropil. Such a performance can be excellent on axis, but can degrade at large scan angles. Thus, performance of two-photon excitation imaging system **100** can be evaluated by measuring the off-axis excitation volume as a full-width at half-maximum (FWHM) of an intensity profile. Furthermore, a high performance objective and optimized relay lens systems can also lead to a flat FOV. Accordingly, configuring scan lens **202**, tube lens **204**, and objective **206** in a manner similar to that of Figure 2A enables a wide FOV, with only marginally decreased magnification and/or NA.

Referring now to Figure 3, a detailed schematic of an exemplary steering device, generally designated **300**, is illustrated. Steering device **300** can be used in a two-photon excitation imaging system, generally designated **316**. System **316** may comprise, in some aspects, components similar to that of system **100** (see, e.g., Fig. 1A). Steering device **300** can comprise a plurality of steering mirrors **312-314** controllable by at least one motorized component **310** interfacing with a computing platform **302** in order to impart a solid angle deflection (e.g., Ω_1 , Ω_2) to separate light pulses prior to recombination. Other entities that may be associated with the above-described steering device **300** may include a separate motorized component (not shown) for controlling an electro-optical lens (e.g., ETLs **164A-B**), which may also interface with computing platform **302**.

In some aspects, a computing platform **302** can be configured to control at least one motorized component **310** of steering device **300** and/or system

316 through an interface **308**. Computing platform **302** may represent any suitable entity or entities (e.g., a control platform or a server farm) for controlling at least one motorized component **310**. For example, computing platform **302** may control at least one motorized component **310**, which in turn, may adjust steering mirrors **312-314** of system **316**. In some aspects, computing platform **302** may be a stand-alone tool, device, or software executing on a processor, and may not be integrated into steering device **300** and/or system **316**. In some aspects, computing platform **302** may be a single node or may be distributed across multiple computing platforms or nodes. Thus, when configured as described herein, computing platform **302** becomes a special purpose computing platform that can improve the technological field of two-photon excitation imaging by independently repositioning steering mirrors to thereby independently reposition temporally multiplexed excitation pathways to simultaneously image separate ROIs within an expanded FOV.

Computing platform **302** may include control module (CM) **304**. CM **304** may be any suitable entity (e.g., software executing on a processor) for performing one or more aspects associated with two-photon excitation imaging. CM **304** may include functionality for controlling at least one motorized component **310**, controlling at least one electro-optical lens (e.g., **164A-B**, Fig. 1A), communicating with photon counting electronics (e.g., **198**, Fig. 1A), and organizing photon counting data into images. For example, CM **304** may be configured to automatically control at least one motorized component **310** to thereby adjust a position of one or more steering mirrors **312-314**. Where each steering mirror **312-314** is controlled by an individual motorized component, CM **304** may be configured to independently, automatically control individual motorized components.

In some aspects, CM **304** may include or access data storage **306** containing data and/or images related to controlling at least one motorized component **310**. For example, CM **304** may access data storage **306** containing

mapped coordinate systems, profiles, settings, or configurations that may enable CM **304** to automatically control at least one motorized component **308**. Where CM **304** is configured for additional functionality, data storage **306** may comprise previously stored photon counting data. Data storage **306** may include
5 non-transitory computer readable media, such as flash memory, random access memory, or other storage devices. In some aspects, data storage **306** may be external to and/or or integrated with computing platform **302** and/or CM **304**.

In some aspects, computing platform **302** and/or CM **304** may include one or more interfaces **308**. For example, one or more interfaces **308** may
10 include remote controls used to operate steering mirrors **312-314** either wirelessly or with wired contact. A CM user (not shown) may be able to interact with one or more interfaces **308** to remotely control movement of steering mirrors **312-314** via at least one motorized component **310**. In some aspects, a CM user may be an automated system or may be controlled or controllable by a
15 human user to operate steering mirrors **312-314**. For example, interface **308** may include a joystick or other control implement for a CM user to manipulate. Through manipulation of interface(s) **308** steering mirrors **312-314** may be adjusted into the directed positions. In other aspects, the CM user may interact with one or more interfaces **308** in order to provide instructions to CM **304** to
20 remotely control movement of steering mirrors **312-314** via at least one motorized component **310**.

At least one motorized component **310** can comprise a servomotor, voice coil, piezo, galvanometer, etc., configured to manipulate, adjust, move and/or reposition components of steering device **300**. For example, at least one
25 motorized component **310** can comprise two separate servomotors that can be configured to independently adjust steering mirrors **312**, **314**. At least one motorized component **310** can be integrated with or configured separately from steering mirrors **312**, **314**.

In some aspects, there can be more than two steering mirrors of steering device **300**. However, regardless of a number of steering mirrors, steering device **300** can be configured such that each steering mirror is independently repositionable and adjustable. Thus, where a CM user manipulates interfaces
5 **308** of computing platform **302** and/or CM **304**, motorized component **310** can be configured to adjust steering mirrors **312**, **314** along XY axes at least, approximately 20 degrees or more, to move a deflection angle of the first and second pulses.

Referring now to Figure 4, a flow chart depicting an exemplary method,
10 generally designated **400**, for two-photon excitation imaging is illustrated. Method **400** can be accomplished at a two-photon excitation imaging system, such as system **100**, including a source (e.g., **110**) for producing light pulses, a steering device (e.g., **300**) comprising first movable steering mirror, a second movable steering mirror, and at least one component in communication with the
15 first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes. However, one having ordinary skill in the art will understand that method **400** is in no way limited to system **100**.

In block **402**, a first pulse of light and a second pulse of light are
20 provided. For example, a light source **110** can be configured to generate a light pulse that can be split by PBS cube **134B** into a first pulse of light and a second pulse of light.

In block **404**, the first pulse of light is directed, without delay, along a first pathway to a first movable steering mirror, while the second pulse of light is
25 directed along a second pathway to a second movable steering mirror. For example, first steering mirror **160** may be provided on a first pathway **114** so that the first pulse of light can travel without delay to first steering mirror **160**. By comparison, for example, second steering mirror **162** may be provided on a second pathway **116**, a portion of which is configured as a delay path, so that

the second pulse of light travels with a 6.25 ns delay to second steering mirror **162**.

In block **406**, the first and second pulses of light are recombined. For example, the first and second pulses of light can be recombined at a recombination relay **170** and a PBS cube **134C**.

In block **408**, the recombined first and second pulses of light are directed through an objective to a specimen. For example, the first and second pulses of light can be directed through a scan lens **190** and a tube lens **192** and be directed into a back aperture of an objective **102** to a specimen **104**.

In some aspects, method **400** can comprise remotely and independently repositioning, by at least one motorized component, the first movable steering mirror and the second movable steering mirror in the XY axes. For example, at least one motorized component **310** can be used to repositioning a first movable steering mirror **160** and a second movable steering mirror **162**.

In some aspects, method **400** can comprise individually repositioning, by at least one tunable lens, a focal plane for at least one of the first pathway and the second pathway. For example, at least one tunable lens can be an electrically tunable lenses (ETL) **164A, B** for a focal plane for at least one of a first pathway **114** and a second pathway **116**.

In some aspects, at least one of the first movable steering mirror and the second movable steering mirror are configured to impart solid angle deflections on at least one of the first pulse of light and the second pulse of light. For example, solid angle deflections Ω_1 , Ω_2 may be imparted on the first pulse of light and/or the second pulse of light. In some aspects, the solid angle deflections are configured to determine central locations of two individual regions of interest (ROIs) within a field of view (FOV). For example, solid angle deflections Ω_1 , Ω_2 may be configured to determine central locations of two individual ROIs **108A**, **108B** within a FOV **106**. In some aspects, the FOV is of a

neural area, and the two individual ROIs are generated within the neural area (see, Fig. 1D).

It will be appreciated that exemplary process **400** is for illustrative purposes and that different and/or additional actions may be used. It will also be appreciated that various actions described herein may occur in a different order or sequence.

The present subject matter can be embodied in other forms without departure from the spirit and essential characteristics thereof. The embodiments described therefore are to be considered in all respects as illustrative and not restrictive. Although the present subject matter has been described in terms of certain preferred embodiments, other embodiments that are apparent to those of ordinary skill in the art are also within the scope of the present subject matter.

CLAIMS

1. A steering device for a two-photon excitation imaging system, the steering device comprising:
 - a first movable steering mirror;
 - a second movable steering mirror; and
 - at least one component in communication with the first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes.
2. The steering device of claim 1, further comprising a computing platform including at least one hardware processor and a memory, and an interface in communication with the computing platform.
3. The steering device of claim 2, wherein the interface comprises remote controls configured for remotely and independently repositioning the first and/or second movable steering mirrors via at least one motorized component.
4. The steering device of claim 1, wherein at least one of the first movable steering mirror and the second movable steering mirror are configured to impart solid angle deflections on at least one of a first pulse of light and a second pulse of light.
5. The steering device of claim 4, wherein the solid angle deflections are configured to determine central locations of two individual regions of interest (ROIs) within a field of view (FOV).
6. The steering device of claim 1, wherein the first movable steering mirror is disposed in a first pathway along which a first pulse of light is directed, without delay, to the first movable steering mirror, and wherein the second movable

steering mirror is disposed in a second pathway along which a second pulse of light is directed, with a delay, to the second movable steering mirror.

7. The steering device of claim 6, wherein the first movable steering mirror and the second movable steering mirror are configured to direct the first and second pulses of light, respectively, to a recombination relay.
8. The steering device of claim 7, wherein the recombination relay is configured to direct the first and second pulses of light to an afocal relay of a galvanometer scanner.
9. The steering device of claim 1, wherein the first movable steering mirror and the second movable steering mirror each comprise a semi-diameter of approximately 3.0 mm.
10. A two-photon excitation imaging system comprising:
 - a source for producing light pulses;
 - a steering device comprising:
 - a first movable steering mirror disposed in a first pathway along which a first pulse of light is directed, without delay, to the first movable steering mirror;
 - a second movable steering mirror disposed in a second pathway along which a second pulse of light is directed to the second movable steering mirror; and
 - at least one component in communication with the first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes;
 - a recombination relay configured to recombine the first and second pulses of light, wherein the first movable steering mirror and the second movable

steering mirror are configured to direct the first and second pulses of light, respectively, to the recombination relay; and

an objective through which the first and second pulses of light are directed through to a specimen, after the first and second pulses of light are recombined.

11. The system of claim 10, wherein the first movable steering mirror and the second movable steering mirror are configured to be remotely and independently repositioned in the XY axes via at least one motorized component.
12. The system of claim 10, wherein at least one of the first movable steering mirror and the second movable steering mirror are configured to impart solid angle deflections on at least one of the first pulse of light and the second pulse of light.
13. The system of claim 12, wherein the solid angle deflections are configured to determine central locations of two individual regions of interest (ROIs) within a field of view (FOV).
14. The system of claim 13, wherein the FOV is of a neural area, and the two individual ROIs are generated within the neural area.
15. The system of claim 10, further comprising at least one tunable lens configured to individually reposition a focal plane for at least one of the first pathway and the second pathway.
16. The system of claim 10, wherein at least a portion of the second pathway is configured as a delay path, so that the second pulse of light travels with a 6.25 ns delay to the second movable steering mirror.
17. A two-photon excitation imaging method comprising:

at a two-photon excitation imaging system including a source for producing light pulses, a steering device comprising first movable steering mirror, a second movable steering mirror, and at least one component in communication with the first movable steering mirror and the second movable steering mirror for independently repositioning the first and/or second movable steering mirror in XY axes:

providing a first pulse of light and a second pulse of light;

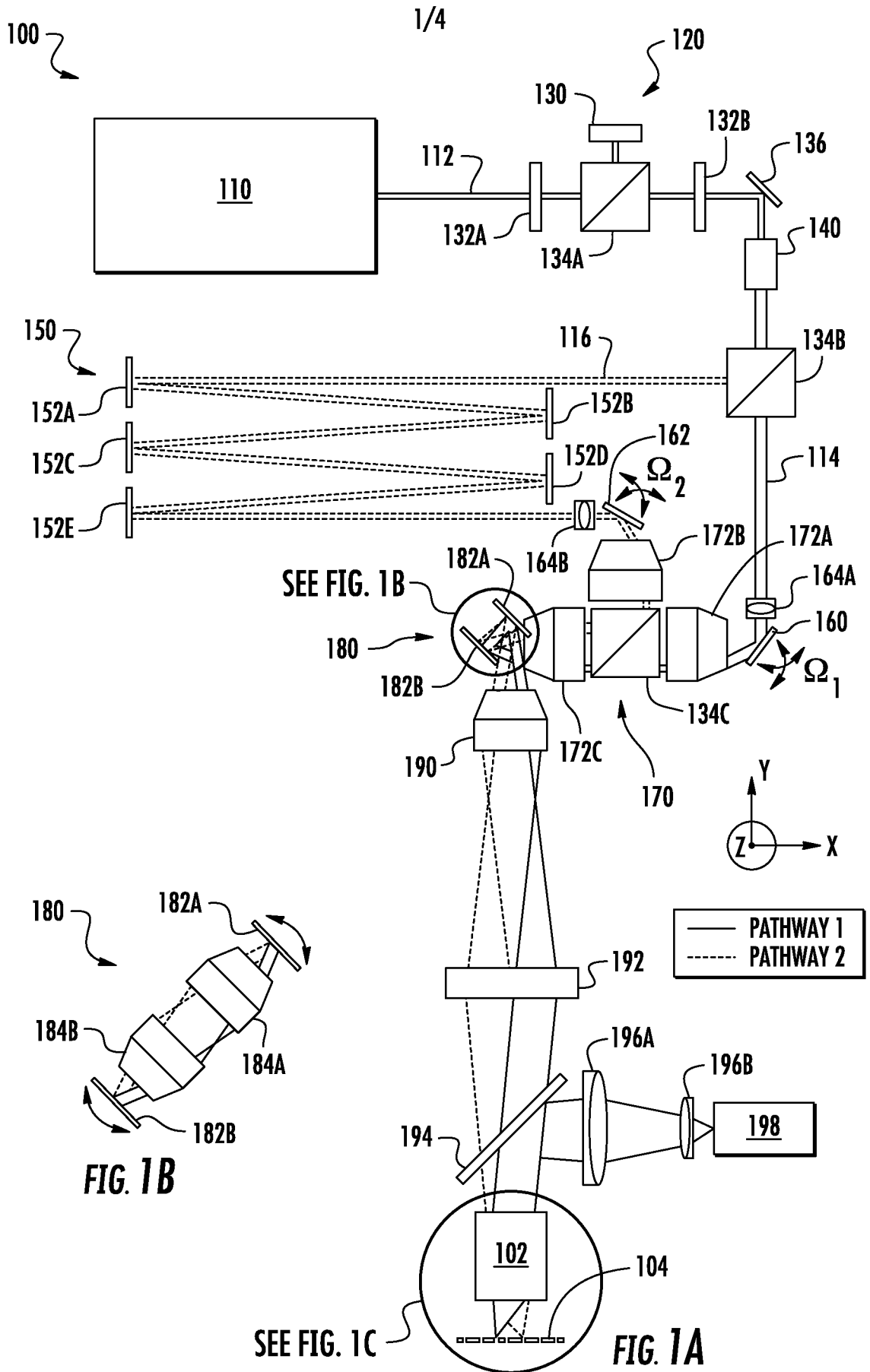
directing, without delay, the first pulse of light along a first pathway to the first movable steering mirror and the second pulse of light along a second pathway to a second movable steering mirror;

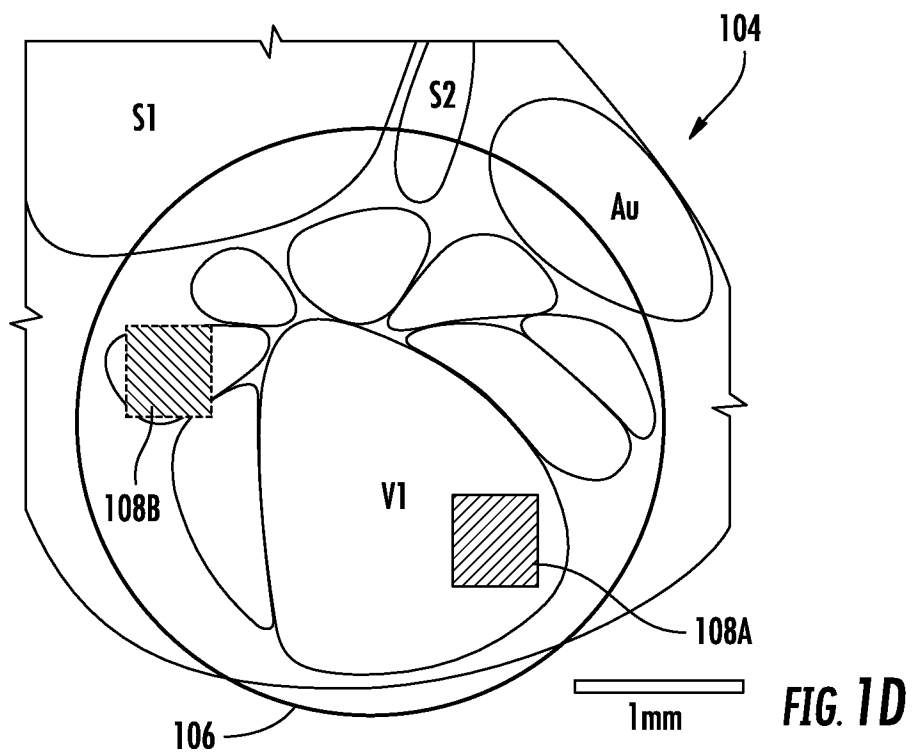
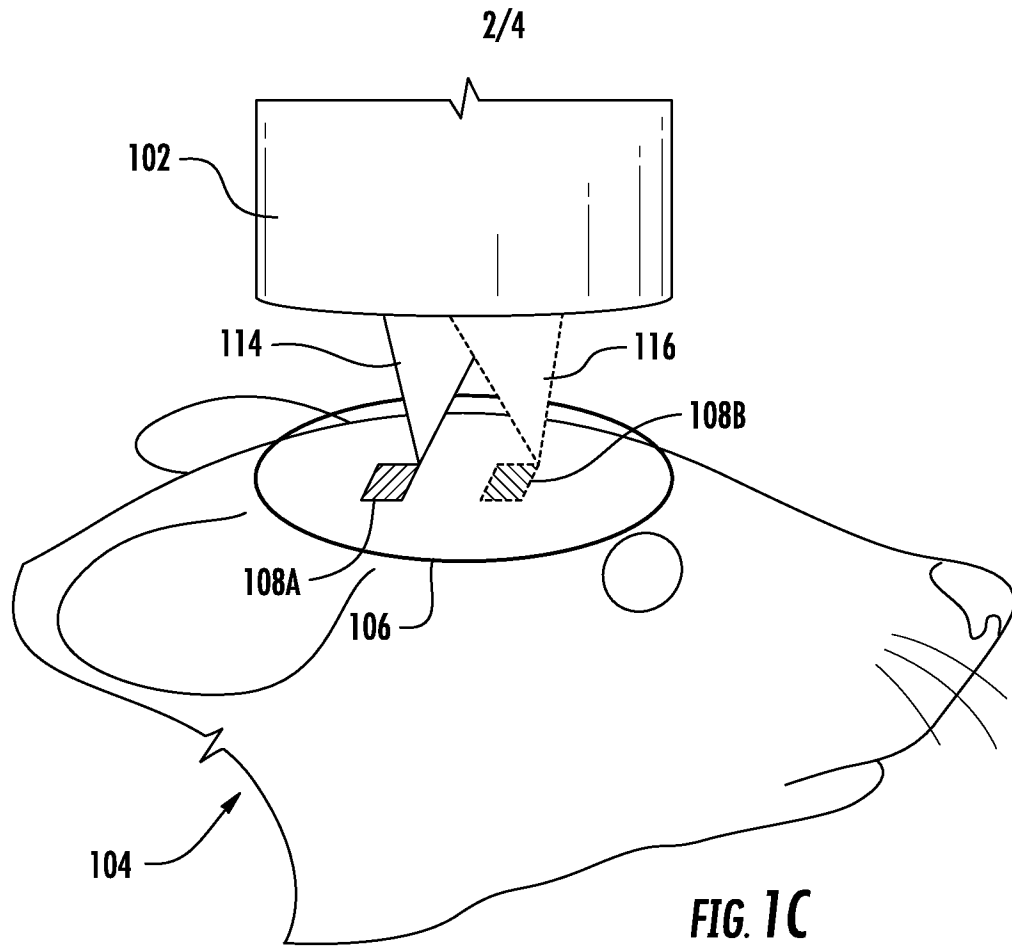
recombining the first and second pulses of light; and

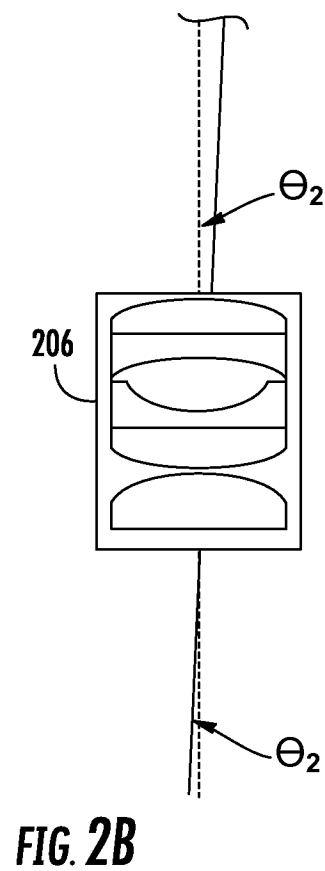
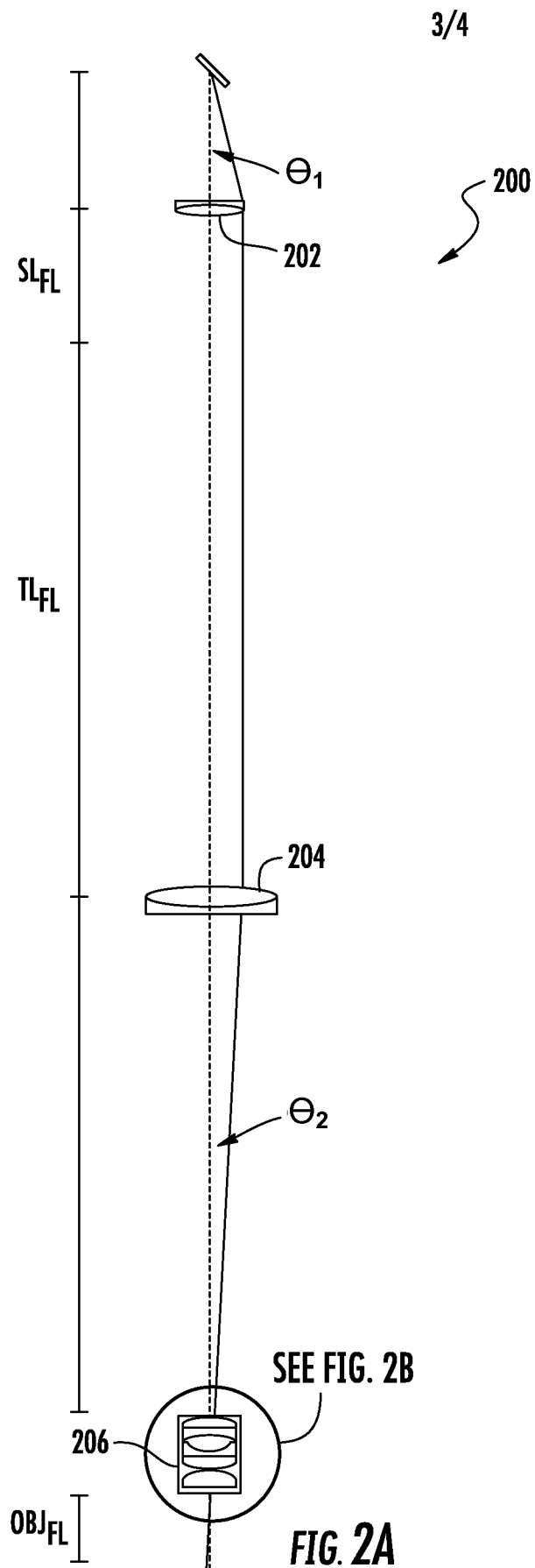
directing the recombined first and second pulses of light through an objective to a specimen.

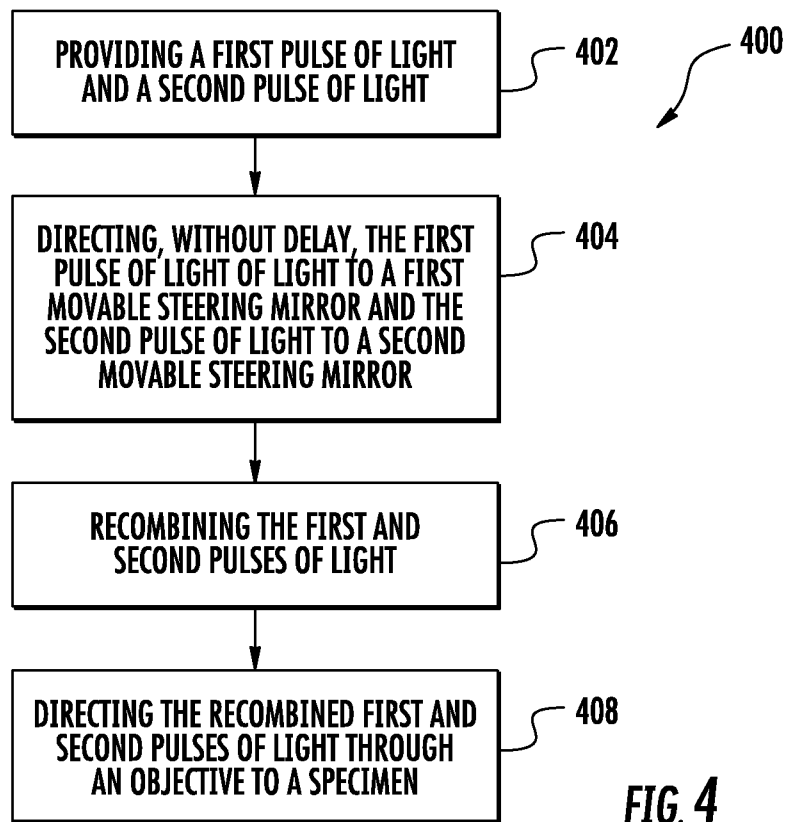
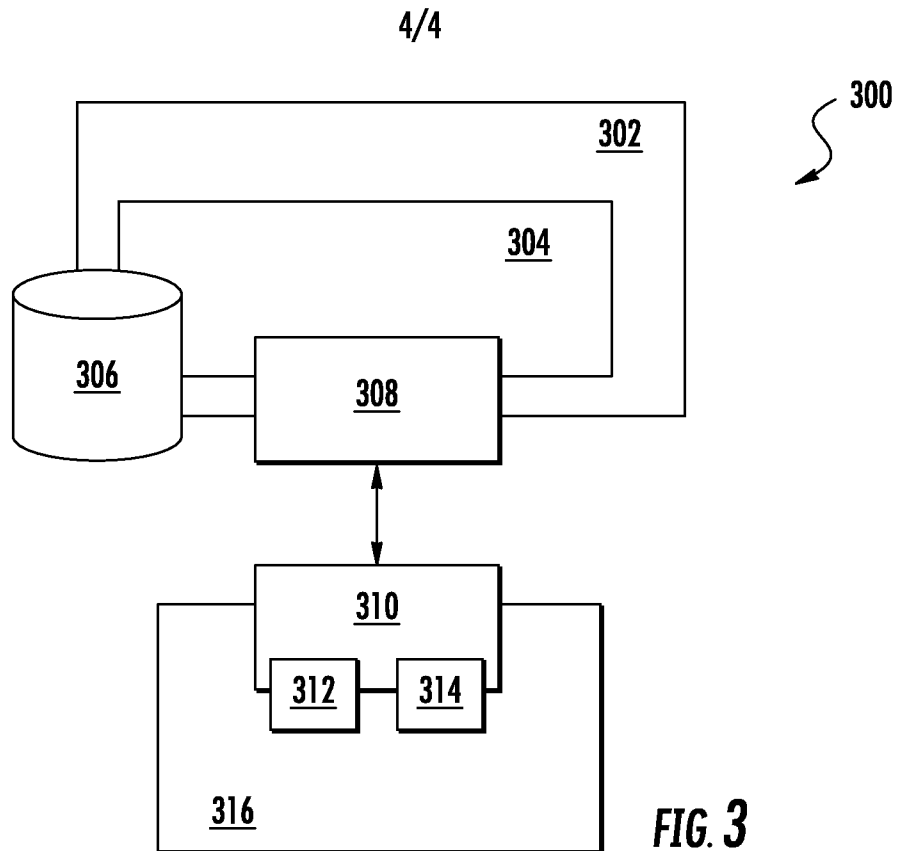
18. The method of claim 17, further comprising remotely and independently repositioning, by at least one motorized component, the first movable steering mirror and the second movable steering mirror in the XY axes.
19. The method of claim 17, wherein at least one of the first movable steering mirror and the second movable steering mirror are configured to impart solid angle deflections on at least one of the first pulse of light and the second pulse of light.
20. The method of claim 19, wherein the solid angle deflections are configured to determine central locations of two individual regions of interest (ROIs) within a field of view (FOV).
21. The method of claim 20, wherein the FOV is of a neural area, and the two individual ROIs are generated within the neural area.

22. The method of claim 17, further comprising individually repositioning, by at least one tunable lens, a focal plane for at least one of the first pathway and the second pathway.
23. The method of claim 17, wherein directing the second pulse of light to the second movable steering mirror comprises directing the second pulse of light to at least a portion of the second pathway configured as a delay path, so that the second pulse of light travels with a 6.25 ns delay to the second movable steering mirror.









INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/040173

A. CLASSIFICATION OF SUBJECT MATTER		
G02B 21/18 (2006.01)		
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)		
G02B 21/00, 21/18		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
PatSearch (RUPTO internal), USPTO, PAJ, Esp@cenet, DWPI, EAPATIS, PATENTSCOPE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5034613 A (CORNELL RESEARCH FOUNDATION, INC.) 23.07.1991, abstract, col. 4, line 61-col. 5, line 9, fig. 1	1-6, 9
A		7, 8, 10-23
Y	JP 2005301065 A (OLYMPUS CORP) 27.10.2005, abstract, claim 19, [0005], [0007], [0015], [0017], [0040], fig. 1	1-6, 9
A		7, 8, 10-23
A	JP 2008096778 A (NATIONAL INSTITUTE OF ADVANCED INDUSTRIAL & TECHNOLOGY) 24.04.2008, abstract	1-23
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents:	“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
“A” document defining the general state of the art which is not considered to be of particular relevance	“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
“E” earlier document but published on or after the international filing date	“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
“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)	“&”	document member of the same patent family
“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		
Date of the actual completion of the international search	Date of mailing of the international search report	
20 October 2015 (20.10.2015)	12 November 2015 (12.11.2015)	
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer R. Artemiev Telephone No. 499-240-25-91	