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(54) Title: CONCURRENT MULTI-REGION OPTICAL IMAGING

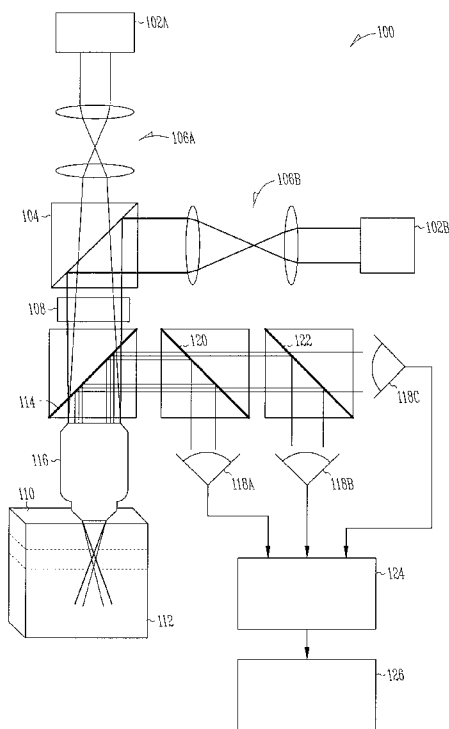


Fig. 1A

(57) Abstract: Concurrent multi-region static or dynamic imaging of different laterally or depth-displaced, same or different shaped regions of a tissue or other sample can be performed, such as by using spectral encoding and decoding, which can optionally be combined with temporal encoding and decoding. Detected response light emitted from the sample can include a fluorescence response, or a scattering response such as one including a higher order harmonic such as Second Harmonic Generation (SHG) or Third Harmonic Generation (THG), or an enhanced Raman signal such as one acquired using coherent anti-Stokes Raman Scattering (CARS) or stimulated Raman scattering (SRS) microscopy. The imaging technique can allow different concurrent responses to be concurrently observed, such as surface blood flow and deeper intracellular calcium changes in a brain tissue sample, blood flow at multiple layers of the brain, or calcium signals at multiple layers.



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CONCURRENT MULTI-REGION OPTICAL IMAGING

CLAIM OF PRIORITY

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This patent application claims the benefit of priority, under 35 U.S.C. Section 119(e), to Hillman et al. U.S. Provisional Patent Application Serial Number 61/424,465, entitled "SYSTEM AND METHOD FOR ACQUIRING MULTIPLE DEPTH PLANES SIMULTANEOUSLY IN MICROSCOPY," filed on December 10 17, 2010 (Attorney Docket No. 2413.126PRV), which is hereby incorporated by reference herein in its entirety.

This patent application claims the benefit of priority, under 35 U.S.C. Section 119(e), to Hillman et al. U.S. Provisional Patent Application Serial Number 61/488,518, entitled "SYSTEM AND METHOD FOR ACQUIRING MULTIPLE 15 DEPTH PLANES SIMULTANEOUSLY IN MICROSCOPY," filed on May 20, 2011 (Attorney Docket No. 2413.126PV2), which is hereby incorporated by reference herein in its entirety.

This patent application claims the benefit of priority, under 35 U.S.C. Section 119(e), to Hillman et al. U.S. Provisional Patent Application Serial Number 20 61/555,381, entitled "SPECTRALLY ENCODED DUAL-BEAM TWO-PHOTON MICROSCOPY FOR SIMULTANEOUS MULTI-REGION IMAGING," filed on November 3, 2011 (Attorney Docket No. 2413.126PV3), which is hereby incorporated by reference herein in its entirety.

25 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under: (1) CAREER 0954796 awarded by National Science Foundation; (2) 2009090524 graduate 30 fellowship awarded by National Science Foundation; (3) IDBR 1063315 awarded by National Science Foundation; (4) 1R01NS063226 awarded by National Institute of Health; (5) IR01NS076628 awarded by National Institute of Health; (6) R21NS053684 awarded by National Institute of Health (National Institute of

Neurological Disorders and Stroke); (7) R01NS063226 +/02S12 awarded by National Institute of Health (National Institute of Neurological Disorders and Stroke); (8) 1U54CA126513 awarded by National Institute of Health (National Cancer Institute); and (9) RGY0070/2007-C-HILLMAN from the Human Frontier
5 Science Program. The government has certain rights in this invention.

BACKGROUND

Optically imaging a target region, such as tissue, can include various techniques. Such techniques can include observing light scattering or fluorescence
10 from a target region in response to an optical excitation of the target region. For example, in a wide-field fluorescence microscope, the target region can be flooded evenly with light from a light source, and the resulting fluorescence response from the target region can be detected by a photodetector or photodetector array such as a camera associated with the fluorescence microscope apparatus. Confocal
15 microscopy sequentially illuminates discrete locations within the sample, and serially detects fluorescence resulting from this serial illumination. An aperture can be used to improve confocal imaging by attenuating out-of-focus light. Three dimensional (3D) image data can be reconstructed by translating the location of the illumination beam both laterally and to different depths.

20 Multi-photon (e.g., two-photon or more than two-photon) microscopy can allow imaging of living tissue at increased depths within the tissue. For example, in two-photon microscopy, the optical excitation can be provided at a wavelength at which two photons combine their energies to excite the fluorophores. Since this combination of photon energies is non-linearly related to incident power, it only
25 occurs at the very focus of the excitation beam, reducing the amount of light generated in out-of-focus regions as well as reducing photodamage in these regions. This also means that all fluorescence emission light coming from the tissue can be assumed to be coming from the excited focal spot, and an aperture is not required as in confocal microscopy. Longer wavelengths also generally pass through tissues
30 with less scattering and attenuation than shorter wavelengths used for one-photon

excitation in confocal microscopy. Another technique is second (or higher order) generation (SHG) microscopy, which exploits the non-linear phenomenon that two excitation photons can combine with a material with key physical properties to generate a single photon having an energy that is the sum of the two incident photons. As is the case with two-photon microscopy, SHG microscopy exploits the non-linear nature of its phenomenon to reject out of focus light.

Other nonlinear imaging techniques, such as coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) use two beams focused into a sample simultaneously in order to exploit intrinsic molecular vibrations. The signal detected is either a new electromagnetic field at the anti-Stokes frequency (CARS) or the intensity modulations in one of the input beams that result from occurrences of stimulated emission (SRS). Both signals are very wavelength-specific since they will match a vibrational frequency of the sample molecule. Like two-photon and SHG, these nonlinear phenomena can enable optical sectioning in highly scattering biological samples but involve point-by-point scanning

Thus, two-photon, SHG, and other types of nonlinear microscopy can be powerful methods for *in-vivo* and live tissue imaging, because of their unparalleled tissue penetration and depth sectioning capability. However, conventional serial point-by-point imaging schemes limit acquisition speed, particularly when imaging a 3D volume. Sheetz et al., "Advancing Multifocal Nonlinear Microscopy: Development and Application of a Novel Multibeam Yb:Kgd(WO₄)₂ Oscillator," Optics Express, 27 October 2008, Vol. 16, No. 22, p. 17574, discusses a technique of generating six beams of temporally delayed pulses that allows multifocal nonlinear microscopy.

Vizi et al. U.S. Patent No. 7,872,748 discusses a real-time, 3D, non-linear microscope measuring system and method for examining a set of microscopic image points in different image planes.

Tearney et al. U.S. Patent No. 7,847,949 discusses scanning a spectrally dispersed line.

Galimberti et al. U.S. Patent Publication No. 2010/0053743 discusses real-time 3D laser scanning microscopy, where single-photon fluorescence light, multiphoton fluorescence light, and higher order harmonics generated in the sample are detected.

5 So et al. U.S. Patent No. 7,372,985 discusses 3D imaging using two-photon scanning microscopy.

Carver U.S. Patent No. 7,817,267 discusses confocal multispectral scanning, including providing and receiving a temporal sequence of wavelength selective reflections.

10 Harter et al. U.S. Patent No. 6,020,591 discusses two-photon fluorescence microscopy using two different wavelength beams that are combined at a crossing angle within a specimen, including lateral scanning by adjusting the relative temporal delay between pulses of the respective beams.

15 imaging using simultaneous detection of two-photon fluorescence responses at N
spatially separated focal points, in respective response to an excitation beam
comprising a corresponding plurality of N sub-beams that are amplitude modulated
to create N respective temporal modulation patterns at the respective focal points.

20 OVERVIEW

The present inventors have recognized, among other things, that if more than one point in the sample is illuminated with more than one identical light beams, it is no longer possible to assume that all light coming from the tissue originated from a particular location. The present inventors have also recognized, among other things, that other approaches to multi-point measurement can rely on an imaging photodetector array such as a camera to spatially resolve the excited regions in lateral directions. However, signals from deeper regions will become scattered as they leave the tissue, losing their spatial accuracy, and meaning that such other approaches to multi-point scanning can only image to limited depths compared to single-beam configurations. The present inventors have also recognized, among

other things, that conventional serial point-by-point imaging schemes for nonlinear microscopy can limit acquisition speed, particularly when imaging a 3D volume. The present inventors have also recognized, among other things, that multi-point or random-access scanning approaches may trade off depth sensitivity or resolution for speed increases.

This document describes an approach that, among other things, can achieve parallelization without such trade-offs such as by using spectral encoding, among other things. This can be achieved by using multiple beams focused at different positions within the target tissue region, each beam at a different excitation wavelength, such as to allow their respective spectral emissions to be capable of being uniquely separated from one another. In an example of application of this approach, concurrent *in-vivo* imaging of two planes, for example, containing a mixture of fluorescent dyes within the rodent cortex can be performed, such as to explore the relative timing characteristics of spontaneous vascular fluctuations.

In an example, data can be concurrently acquired from two or more individually distinct sample regions (e.g., laterally or vertically displaced from each other, or both, with same or different shaped or sized sample regions) using spectral encoding. A linear or other spectral unmixing technique can be used to separate the fluorescence or other response signal that originated from a particular distinct sample region from a response signal that originated from another particular distinct sample region. This approach can offer similar advantages as temporal encoding, for example, it can provide truly parallel, concurrent measurements without requiring trading off between resolution and speed. Spectral encoding and decoding can also offer an advantage over temporal encoding in that spectral encoding and decoding need not include special detection electronics for performing the temporal decoding or delay lines for performing the temporal encoding or decoding. Phase-delay based temporal encoding can also be limited by the available temporal window for a particular laser repetition rate and fluorescence lifetime of a fluorophore dye, which, in turn, can limit the number of regions that can be concurrently imaged in parallel. Frequency modulation-based temporal encoding

involves expansion of system sampling bandwidth at least proportional to the number of encoding frequencies, or the application of specific very high bandwidth lock-in amplifiers for specific detection.

In an example, to spectrally encode, two (or more) beams at different
5 excitation wavelengths can be used, such as which, in a sample with a particular fluorophore or fluorophore combination, can result in unique emission spectra of the fluorophores excited by each beam, or spectrally distinct emissions of second harmonic generation (SHG), anti-Stokes fields (CARS), or probe wavelengths (SRS). In an illustrative two beam example, by acquiring three spectrally-resolved
10 emission channels, a signal that came in response to one excitation beam can be separated from a signal that came in response to the other, as explained herein. In an example, it was possible to use spectral unmixing to cleanly delineate fluorescence coming from two laser beams of different excitation wavelengths and, therefore, from two distinct imaging regions, such as by using a linear unmixing
15 technique.

This overview is intended to provide an overview of subject matter of the present patent application. It is not intended to provide an exclusive or exhaustive explanation of the invention. The detailed description is included to provide further information about the present patent application.

20

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings, which are not necessarily drawn to scale, like numerals may describe similar components in different views. Like numerals having different
25 letter suffixes may represent different instances of similar components. The drawings illustrate generally, by way of example, but not by way of limitation, various embodiments discussed in the present document.

FIG. 1A shows an example of a system that can be configured for concurrently imaging two or more distinct regions of a tissue or other sample, such
30 as using respective different optical excitation wavelengths respectively

corresponding to the distinct regions.

FIG. 1B shows an example of a system similar to that shown in FIG. 1A, but in which distinct X-Y scanners can be used, such as before combination of the beams.

5 FIG. 2 shows an example of a spectral encoding portion of an optical imaging system such as can use one laser source.

FIG. 3 shows an example of a temporal encoding portion of an optical imaging system, in which temporal encoding can be used, such as for example in addition to spectral encoding.

10 FIG. 4 shows an example of a spectral encoding portion of an optical imaging system, similar to the spectral encoding portion of the optical imaging system shown in FIG. 2, but which can omit the second prism, and which can image along an oblique axis to the surface of the sample.

FIG. 5 shows an example of a spectral encoding portion of an optical
15 imaging system, similar to the spectral encoding portion of the optical imaging system shown in FIG. 4, but which can use a static lenslet array.

DETAILED DESCRIPTION

FIG. 1A shows an example of a system 100 that can be configured for
20 concurrently imaging two or more distinct regions of a tissue or other sample, such as by using different optical excitation wavelength sets corresponding to the distinct regions. In an illustrative two beam example (which can be extended to a greater number of beams), laser or other light sources 102A-B can generate light at respective wavelength sets (e.g., a set of first wavelengths at about 780 nanometers
25 and at set of second wavelengths at about 840 nanometers, respectively) that are different from each other. The light sources 102A-B can be arranged (e.g., orthogonally or otherwise) to respectively deliver light to light combining optics, such as a Polarizing Beam Splitter (PBS) 104. This can be done via respective focusing or other lens optics, such as respective telescopes 106A-B, which can
30 respectively adjust divergence of the respective beams, such as for delivery to the

PBS 104. The resulting combined light beams provided by the PBS 104 can be provided to a scanner, such as the X-Y scanner 108 (e.g., galvo scanning mirrors, acousto-optic scanner, spatial light modulator (SLM) or other optical scanning device), such as for scanning in one or two dimensions of a plane at or parallel to a proximal surface 110 of a sample 112. Excitation light from the scanner 108 can be delivered to the sample 112 such as via a dichroic 114. The dichroic 114 can be configured to pass excitation light at the excitation wavelength sets to the sample 112, such as through focusing or other lens optics, such as a microscope 116.

The dichroic 114 can be configured such that, in an opposite direction, the dichroic 114 can reflect light at the response wavelengths, such as toward one or more photomultiplier tubes (PMTs) or other light detectors. For example, light detectors 118A-C can be configured for detecting light emitted from the sample 112 in response to the excitation light, such as at respective different wavelengths. In an example, the light detector 118A can be configured to detect blue light (e.g., at one or more wavelengths that are between 350 nanometers and 505 nanometers, inclusive), the light detector 118B can be configured to detect green light (e.g., at one or more wavelengths that are between 505 nanometers and 560 nanometers, inclusive), and the light detector 118C can be configured to detect red light (e.g., at one or more wavelengths that are between 560 nanometers and 650 nanometers, inclusive). Light from the sample 112 that is reflected by the dichroic 114 toward one or more light detectors can be spectrally separated for respective delivery to such light detectors 118. In an example, blue light from the sample 112 that is reflected by the dichroic 114 can be reflected by the dichroic 120 toward the blue light detector 118A, while green and red light can be passed on through the dichroic 120, such as for delivery to the dichroic 122. The dichroic 122 can reflect a green portion of such incident light toward the green light detector 118B, while red light can be passed on through the dichroic 122 for delivery to the red light detector 118C. In an example, the light detectors 118A-C can provide three spectrally-resolved detection channels. The light detectors 118A-C can transduce their respective input light signals into respective electrical signals having corresponding

fluctuations. The transduced electrical signals can be delivered to an imaging signal processor circuit 124, such as a microprocessor circuit or other combination of hardware, software, or firmware that can be configured to perform instructions to carry out acts for storing or signal-processing the transduced optical signals into one or more control signals, such as for driving a graphics circuit or an imaging display 126.

The signal processor circuit 124 can be configured to perform spectral unmixing, such as linear spectral unmixing. In an example of such spectral unmixing, the measured and transduced signal in the blue, green, and red emission channels (which can be designated as M_B , M_G , and M_R , respectively) is equal to the linear combination of the emission signals of the fluorescent dye or other fluorescent species excited by beams with different excitation wavelengths (which can be designated as λ_{ex1} and λ_{ex2} , respectively) at different positions (which can be designated as $r_{\lambda_{ex1}}$ and $r_{\lambda_{ex2}}$, respectively). An example of this relationship is shown in Equation 1:

$$\begin{pmatrix} M_B (r_{\lambda_{ex1}} + r_{\lambda_{ex2}}) \\ M_G (r_{\lambda_{ex1}} + r_{\lambda_{ex2}}) \\ M_R (r_{\lambda_{ex1}} + r_{\lambda_{ex2}}) \end{pmatrix} = \begin{pmatrix} F_{\lambda_{ex1}B} & F_{\lambda_{ex2}B} \\ F_{\lambda_{ex1}G} & F_{\lambda_{ex2}G} \\ F_{\lambda_{ex1}R} & F_{\lambda_{ex2}R} \end{pmatrix} \begin{pmatrix} k(r_{\lambda_{ex1}}) \\ k(r_{\lambda_{ex2}}) \end{pmatrix} \quad (1)$$

where $F_{\lambda_{ex1}B,G,R}$ are the characteristic emission signals of the dye mixture or other intrinsic (e.g., Nicotinamide Adenine Dinucleotide (NADH), Flavin Adenine Dinucleotide (FAD), etc.) or extrinsic fluorophore species measured using the light at excitation wavelength λ_{ex1} and $F_{\lambda_{ex2}B,G,R}$ are the characteristic emission signals of the dye mixture or other intrinsic or extrinsic fluorescent species measured using the light at the excitation wavelength λ_{ex2} . The coefficients $k(r_{\lambda_{ex1}})$ and $k(r_{\lambda_{ex2}})$ represent the relative components of the detected signals originating from each excitation light beam, for each scanned location in the sample, such as can be scanned using the scanner 108. In an example, values for F can be extracted such as from initial scans acquired with only one or other of the excitation light beams illuminating the sample. Images of k can be extracted such as using a non-negative

least squares fit for every data point (such as can be performed using instructions performed by the signal processor circuit 124 or using Matlab™).

Example A

5 In an example, a 1:2.5 dye combination of dextranconjugated fluorescein (e.g., FD2000S, Sigma-Aldrich, 5 milligram in 0.1 milliLiter saline) and dextran Texas Red (e.g., 01830, Invitrogen, 25 milligram in 2 milliLiter saline) was injected via a femoral cannula in the intact brain of an anesthetized rat to provide vascular contrast.

10 Images were acquired using a two-photon microscope. Two tunable Ti:Sapphire lasers (e.g., MaiTai XF, and MaiTaiHP, both Spectra Physics) were used for dual-beam excitation. The two excitation beams were configured to have perpendicular polarizations, and were combined using a PBS cube before being delivered to galvanometric scanning mirrors (e.g., Cambridge Technologies), which

15 were used to steer the beams in an x-y raster scan. Excitation light was focused into the sample with an Olympus XLUMPlanFI 206/0.95 W objective mounted on a z-translation stage (e.g., M-112.1DG, PI). Fluorescence was simultaneously collected by three spectrally resolved photomultiplier detectors (e.g., blue: 350-505 nanometer, green: 505-560 nanometer, and red: 560-650 nanometer emission

20 ranges, R3896 Hamamatsu). For SHG imaging, 390/10 nm and 430/10 nm filters were used with two spectrally resolved photomultiplier detectors. Software (e.g., written as a Matlab™ graphical user interface) controlled the microscope. Average power at the sample was 1-3 milliWatts and varied with wavelength. Single frame images were obtained at 400 x 400 pixels, and were acquired at 2.5 frames per

25 second. For fast acquisitions, 300 x 200 pixel images were acquired at 16 frames per second, or 250 x 250 pixel images at 6.4 frames per second, both of which correspond to a 500 x 500 micrometer field of view. For basic visualization of acquired images, red-green-blue (RGB) merges were created using data acquired with the three emission channels, with each channel scaled to its maximum after

thresholding out the highest 0.1% of pixels. Volume renderings were made using ImageJ.

Once both lasers were aligned through the objective, we adjusted the collimation of each beam's telescope so that each laser would focus at a different point along the axial dimension. An infinity-corrected microscope objective was optimized for creating a focal spot with collimated light entering at the back aperture of the objective, but if the light entering the objective is slightly converging or diverging, the beam will focus at a closer or further distance from the sample surface, respectively. In this configuration, the maximum achievable axial separation of the two imaging planes can be limited by deterioration in axial resolution as the light entering the back aperture of the objective becomes less collimated. However, beam integrity can be better preserved using remote focusing via a second objective or by using adaptive optics.

In an example, to demonstrate this technique, we used two beams at excitation wavelengths of 780 nm and 840 nm, focused at independent depth planes in the intact brain of an anesthetized rat. The beams were positioned at different depths by making one slightly divergent, such as by using different lens combinations in the light path of each beam before they are combined. The wavelengths were chosen based on the excitation-dependent emission spectra of a combination of intravascular dyes; Texas red dextran and dextran-conjugated fluorescein, such that shorter wavelength fluorescein fluorescence dominated at 780 nm excitation, while longer wavelength Texas red fluorescence dominated at 840 nm excitation. By combining beams and using one set of detectors, such as the detectors 118A-C, we were able to acquire raster-scan images that contain concurrent information from both planes of interest. Dynamic movies were acquired, in this example, in the form of 300 x 200 pixel images at 16 frames per second, or 250 x 250 pixel images at 6.4 frames per second, both corresponding to a 500 x 500 micrometer x-y field of view. The intravascular dyes allowed us to simultaneously monitor spontaneous vascular fluctuations within two planes separated by up to about 75 micrometers.

Blood flow changes in the brain underlie the signals detected by functional magnetic resonance imaging (fMRI). Propagation of vasodilation in the cortical vasculature during somatosensory stimulation can be used to determine the likely neurovascular mechanisms controlling evoked changes in blood flow. One approach can be to use two-photon microscopy to sequentially scan different vessel segments during repeated stimuli, from which it can be concluded that dilation initiates in deeper layers and propagates retrograde towards the cortical surface. Another approach to fMRI can use functional connectivity mapping (FCM) to exploit correlations in spontaneously occurring modulations in blood flow, to infer the connectivity of different brain regions. Here, we explored whether these spontaneous fluctuations are governed by similar neurovascular mechanisms to stimulus-evoked blood flow changes, such as by exploring whether they were governed by retrograde or anterograde propagation of vasodilation, which might imply local neuronal or systemic origins respectively. One difficulty with studying spontaneous dynamics can be the need for single trial recordings from multiple regions of the brain simultaneously, since spontaneous events cannot be reproduced for sequential measurements. Our approach can be ideal for studying this problem because two layers of the brain can be monitored in parallel to study the propagation of these fluctuations in vessel diameter.

In an example, our approach indicated that vascular modulations in deeper layers of the cortex preceded those in shallower layers in all cases ($n=3$ rats, 38 runs, $p < 7 \times 10^{-7}$). Since we can study dynamics anywhere within the two planes imaged, we further explored the timing of signals in many locations in the planes. We found that vasodilatory signals are propagating along the vessels themselves, but we found that temporal delay was not linear with respect to the 3D distance traveled along the vessel between sampled regions (for example, along the highlighted vessel in Figure 2A). This intriguing result indicates that signal propagation can also be related to the diameter of vessels, or organization of the vascular tree between sampled regions. These results demonstrate the ability of our

approach to two region (e.g., two-plane) spectrally encoded two-photon microscopy to simultaneously acquire dual-plane dynamic *in-vivo* data.

FIG. 1B shows an example of a system similar to that shown in FIG. 1A, but in which distinct X-Y scanners 108A-B can be used, such as before combination of the beams at PBS 104 or other light combining element. Use of separate X-Y scanners for the respective beams from the respective light sources 102A-B can allow concurrent or simultaneous imaging of different regions at different imaging speeds or at different imaging resolution scales, in addition or alternative to concurrent imaging of multiple different lateral regions or different-depth regions.

Spectrally encoded fluorescence imaging can be limited in the number of regions it is able to image simultaneously by the ability of dyes to produce unique emission patterns at different excitation wavelengths. One way to increase parallelization can be to increase the number of spectrally resolved detection channels, although dye combinations should be carefully considered. It can also be possible to combine spectral and temporal multiplexing, such as to increase the number of regions that can be sampled simultaneously.

In an example, the approach to spectrally encoded imaging described herein can also be applied to higher order harmonic generation, such as for example to second harmonic generation (SHG) or third harmonic generation (THG). SHG emission from a sample will be generated at half of the excitation wavelength. THG emission from a sample will be generated at a third of the excitation wavelength. Therefore, if enough spectrally distinct excitation beams are provided, almost unlimited spectrally narrow harmonics can be generated and separated, such as using a spectrally resolved detector or spectrometer.

In an example, spectrometer-based detection can be used together with spatio-spectrally patterned broadband light, such as from a super-continuum laser, such as to allow very high-speed volumetric imaging of SHG or THG contrast. In an example, we performed dual-beam SHG imaging of tissue, e.g., a fish tail. By using light detection filters centered at half the respective excitation wavelengths,

we could easily distinguish the SHG signal from two different planes without requiring any spectral unmixing, however, if the light detection filters are omitted, spectral unmixing can be used.

A similar approach can be used for CARS or SRS microscopy. By
5 providing multiple pump/probe beams to the sample, multiple spectrally distinct signals originating from different regions of the sample can be detected using a spectrally resolved light detector.

Thus, the present approach described herein to spectrally encoded imaging has demonstrated a method to parallelize laser scanning microscopy without cost to
10 resolution or penetration depth. Moreover, spectrally encoding in this way is compatible with dual beam two-photon and with SHG or THG microscopes as well as with confocal setups, broadband illumination, or stimulated Raman scattering (SRS) and CARS microscopy, and can be combined with temporal encoding. The approach need not be limited to concurrent imaging of dual or other multiple depth
15 planes, but can additionally or alternatively be applied to concurrent imaging of two or more laterally displaced regions, to multiple regions with differently sized or shaped respective fields of view (e.g., such as to a single cell, and to its network), or to multiple regions imaged at different speeds.

There are a number of ways to encode spatio-spectral or spatio-temporal
20 patterns into beams that can be used for scanning non-linear microscopy. For example, for temporal encoding, a purpose can be to generate multiple focused beams, each of which can be temporally encoded, for example, either by relative delay of a pulse (e.g., using lasers for non-linear microscopy that can be pulsed at between 20-80 MHz), or by amplitude modulating the beam, having a particular
25 excitation frequency, at a particular modulation frequency (this may be slower, and therefore may reduce achievable scan rates). Such modulation can be temporally encoded such as via a phase shift, rather than via specific frequency bands.

Spectral encoding can include generating multiple focused beams composed of different wavelengths of light. For exciting a fluorescence response, the number
30 of beams encoded may be limited by the properties of the dyes or fluorophores

being imaged, but for exciting a SHG response, because the emitted wavelengths are exactly half of the excitation wavelength (and half as spectrally wide as the excitation wavelength band), this allows for possibility for an almost continuum of sample points and wavelengths, which can allow very rapid volumetric imaging.

- 5 Several technologies can be used to provide a practical implementation of these encodings, such as one or more of spatial light modulators (SLMs), digital mirror devices (DMDs), or supercontinuum pulsed laser sources such as can provide broadband light. Some example configurations are described below.

FIG. 2 shows an example of a spectral encoding portion of an optical
10 imaging system 200 that can use a single pulsed or other supercontinuum light source 202, a spatial light modulator (SLM) or other diffractive optical element (DOE) capable of producing diffractive lenses or patterns 204, a first diffractive or refractive element such as a first prism 206 therebetween, a second diffractive or refractive element such as a second prism 208 between the DOE 204 and a target
15 sample 210 to be imaged, and a refractive lens 210 between the second prism 208 and the sample 210. The supercontinuum light source 202 can provide light including multiple wavelengths to the first prism 206. The first prism 206 can provide spectrally-separated light distributed in a specified manner to specific light
20 modulating components in the DOE 204. The SLM or custom lenslet array or other DOE 204 can be configured such that it imparts varying degrees of divergence on incoming light depending on the spatial location of the light on the DOE 204. .
Because the input light is spectrally separated, different color light can acquire different divergences or phase delays or one or more other characteristics after
25 interacting with the DOE 204, before being directed toward the second prism 208. In an example, the SLM or other DOE 204 can include an associated diffraction grating located at the DOE 204, such as to help direct the DOE-adjusted light to the second prism 208. The second prism 208 can recombine incident light from the DOE 204 such as to be co-axially directed toward the lens 212 and the sample 210.
30 The different colors of incident light from the DOE 204 can have different

divergences acquired at the DOE 204, such that the lens 212 can focus the different colors at different depths in the sample 210, such as coaxially at different depths along the same axial dimension. An X-Y scanner can be included, such as between the lens 212 and the sample 210, such as to move the illumination pattern delivered to the sample 210 to different X or Y positions within a plane at or parallel to a proximal surface of the sample 210. Z-direction scanning optics can also be included such as by an electric lens, a piezoelectric scanner, a motorized stage or encoded on the SLM or other DOE 204 itself.

At the different incident excitation focus locations at different depths in the sample 210, a response to the excitation light can be emitted from the sample 210, such as a fluorescence response, or a scattering response such as CARS, SRS, SHG, THG, or the like. The emitted light can be detected using a high speed spectrometer or other spectrally-resolved detector, such as the detector arrangement illustrated in FIGS. 1A-1B. The approach shown in and described with respect to FIGS. 1 and 2 can provide a 3D imaging improvement, relative to single-point serial scanning, that can be at least proportional to the number of defined wavelength bands provided by the light modulating components in the array of light modulating components in the SLM or other DOE 204.

FIG. 3 shows an example of a temporal encoding portion of an optical imaging system 300, in which temporal encoding can be used such as, for example, in combination with spectral encoding such as described herein. Incident light from one or more lasers, a supercontinuum light source, or output from the prism 206 can be received at a temporal modulation device, such as a frequency chopper 302. In an example, the incident light received by the frequency chopper 302 can be spectrally-encoded, such as described herein. The frequency chopper 302 can include a digital micromirror device (DMD) or can include a rotating disk with concentric rings of apertures 304 or other light transmissive elements that can be spaced apart along the path of a particular ring in the otherwise light-blocking frequency chopper 302 in a specified manner. Rotating the disk of the frequency chopper 302 can provide a temporal modulation that can include, for an individual

ring, a desired frequency modulation. The arrangement of apertures in the various rings can also be configured such that, between rings, a desired temporal delay or other temporal modulation feature can be specified. The frequency chopper 302 can provide temporally modulated (e.g., at a desired frequency modulation) collimated, and spectrally encoded light to an SLM or other DOE 204. Light from an individual ring of apertures in the frequency chopper 302 can be delivered to an individual light modulation element in an array of such light modulation elements or otherwise encoded on the SLM or physical lens or mirror array or other DOE 204. The temporally (e.g., frequency) modulated collimated light incident on the individual elements of SLM or DOE 204 can have its divergence or other optical characteristic adjusted, such that light provided by the respective elements of the SLM or DOE 204 for can be delivered to different regions (e.g., different depths or laterally shifted locations) of the sample 310. A lens arrangement or other optics, such as a static lenslet array 312, can be provided between the DOE 204 and the sample 310, such as to help focus the light at the different regions (e.g., different depths) of the sample 310. An X-Y scanner can be included, such as for example between the static lenslet array 312 and the sample 310, such as to move the illumination pattern delivered to the sample 310 to different X or Y positions within a plane at or parallel to a proximal surface of the sample 310. Z-direction scanning optics can also be included.

At the different incident excitation focus locations at different depths in the sample 310, a response to the excitation light can be emitted from the sample 310, such as a fluorescence response, or a scattering response such as SHG, THG, CARS, SRS, or other scattering response. The emitted light can be detected using a high speed spectrometer or other spectrally-resolved detector, such as the detector arrangement illustrated in FIGS. 1A-1B, which can be synchronized with the frequency chopper 302 to provide temporal decoding. The approach shown in and described with respect to FIG. 3 can provide a 3D imaging improvement, relative to single-point serial scanning, that can be at least proportional to the number of defined wavelength bands provided by the lens or mirror array of the SLM or DOE

204. In an example, the rotating disk frequency chopper 302 can be omitted, and the temporal (e.g., frequency) modulation can be provided by a digital micromirror device (DMD) or by controlling operation of the SLM or other DOE 204, however, the speed of such temporal modulation may be more limited than what can be
5 obtained using the rotating disk frequency chopper 302.

FIG. 4 shows an example of a spectral encoding portion of an optical imaging system 400, similar to the spectral encoding portion of the optical imaging system 200 shown in FIG. 2, but which can omit the second prism 208. In this example, spectrally encoded illumination localization at distinct lateral or depth
10 regions of the sample 410 can be provided in a different pattern than the coaxially aligned regional localization shown in FIG. 2. In an example, such an arrangement as shown in the example of FIG. 4 can be used such as for concurrently providing laterally and depth displaced spectrally-encoded illumination of distinct regions of the sample 410.

15 FIG. 5 shows an example of a spectral encoding portion of an optical imaging system 500, similar to the spectral encoding portion of the optical imaging system 200 shown in FIG. 2, but which can omit the second prism 208, and similar to the spectral encoding portion of the optical imaging system 400 shown in FIG. 4, but which can use a static lenslet array 312, such as in place of (or in addition to) the
20 lens 212 of FIG. 4. The lenslet array 312 can be configured to provide respective individual lenslets, in the lenslet array 312, corresponding to individual light modulating elements in the array of the SLM or other DOE 204. This can allow individualized focusing of light from the respective light modulating elements in the SLM or other DOE 204 for delivery to the laterally or depth-displaced individual
25 regions of the sample 410.

The examples described herein can be used to simultaneously acquire data from two different regions, either laterally or in different depth-planes. This can be valuable over a laser scanning microscopy approach that acquires data from a single point in space at any one time, making acquisition within 3D volumes slow. It is
30 valuable in many cases, but especially in *in-vivo* imaging, to be able to acquire

images at two different depths (or two different regions) at the same time, for example, to observe changes in blood flow at the surface of the brain while concurrently observing changes in intracellular calcium in neurons in a deeper layer, or for observing changes in blood flow in two layers simultaneously.

5 Spectral encoding and decoding can be used, by itself or in combination with temporal encoding or decoding, to concurrently detect light from distinct laterally or depth-displaced regions within a sample to be imaged. Image acquisition speed can be high enough to allow a time-series of images to be acquired as a movie, which can allow dynamics at the different laterally or depth-displaced regions to be
10 detected, resolved, or compared. The spectral encoding and decoding can be used together with temporal encoding, such as can provide intensity modulation of the beam, focused at different laterally or depth-displaced regions of the sample. Detection of such temporally intensity-modulated beams can include unmixing using frequency demodulation to extract components coming from different lateral
15 or depth-displaced regions. This approach can include generating an illumination beam with spatially-encoded frequency gradients, which can be compared to magnetic resonance imaging (MRI).

For temporal encoding and decoding, such as can optionally be combined with the spectral encoding and decoding described herein, phase delay can be used.
20 In an example, a two-photon laser can be pulsed at around 80 MegaHertz, with a pulsewidth of less than 100 femtoseconds. This means that, in this example, there can be a 12 nanosecond window between each 100 femtosecond pulse. Although fluorescence lifetime can be as long as 10 nanoseconds (longer for quantum dots), many have lifetimes of a few nanoseconds or under a nanosecond. This means that a
25 source of light can be configured that can phase shift pulses of light that can be focused at different depths within the 12 nanosecond window (or longer if, for example, a 40 MegaHertz repetition rate is used). Detected light can then be time-resolved such as to pick out signals emerging from the tissue sample at different
30 times in the temporal window, each of which would have come from a different depth.

Such spatiotemporal shaping of light in microscopy can include using a remote focusing approach, an SLM or other DOE, a DMD, a MEMS device, a lenslet array, a Pockels cell, other device to manipulate the focal plane, wavelength, or modulation of light. Temporal shaping of light beams can be used to improve axial resolution or for spatial encoding and can be used to augment the spectral coding and decoding techniques described herein.

In an example of spectral encoding, more than two spectral bands can be used, such as to illuminate more than two depth-displaced or laterally-displaced regions. The more than two spectral bands can be split from a single laser or otherwise provided, with an appropriate number of light detectors provided for spectral unmixing the response signal emitted from the sample. In an example, the light source can include an optical parametric oscillator, or a narrowband laser transmitted through a supercontinuum generating medium.

In an example, there can be two different dyes are of interest in two different planes, for example, a dye emitting in the red, and exciting at higher wavelengths is of interest in the superficial vasculature, and a dye with green emission, exciting at a lower wavelength is of interest at deeper depths. In this example, spectral unmixing may not necessarily be required to delineate signals coming from the two planes. However, spectral unmixing can still be used to distinguish between different laterally-displaced regions within one or both of these two planes. useful for fluorescence imaging.

Various Notes & Examples

Example 1 can include subject matter (such as an apparatus, a method, a means for performing acts, or a device readable medium including instructions that, when performed by the device, can cause the device to perform acts), such as can include delivering a first light excitation localized at a specified first region of a sample at a first excitation wavelength set of one or more wavelengths to excite a scattering or a multi-photon fluorescence first response light emitted from the specified first region of the sample. A second light excitation can be delivered such that the second light excitation can be localized at a different specified second

region of the sample at a different second excitation wavelength set of one or more wavelengths to excite a scattering or multi-photon fluorescence second response light emitted from the specified second region of the sample. The first and second responses can be concurrently detected from the different respective first and second regions of the sample, in respective response to the first and second light excitations, wherein the first and second responses are distinguishable by having different wavelengths from each other. The detected first and second responses can be spectrally resolved to provide location-resolved image data corresponding to the specified first and second regions of the sample.

Example 2 can include, or can optionally be combined with the subject matter of Example 1, to optionally include delivering the first light excitation localized at a specified first region of a sample at a first excitation wavelength set of one or more wavelengths comprises exciting a multi-photon fluorescence first response light emitted from the specified first region of the sample. The second light excitation can be delivered such that the second light excitation can be localized at a different specified second region of the sample at a different second excitation wavelength set of one or more wavelengths comprises exciting a multi-photon fluorescence second response light emitted from the specified second region of the sample. The first and second responses can comprise multi-photon fluorescence responses that are distinguishable by having different wavelengths from each other.

Example 3 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 or 2 to optionally include the delivering a first light excitation localized at a specified first region of a sample, such that such delivering includes delivering the light excitation localized at a specified first depth of the sample. The delivering a second light excitation localized at a different specified second region of the sample can include delivering the light excitation localized at a specified second depth of the sample that is different from the specified first depth of the sample. The concurrently detecting the first and second responses from the different respective first and second regions

of the sample, in respective response to the first and second light excitations, can include concurrently detecting the first and second responses from the different respective first and second depths of the sample. The spectrally resolving the detected first and second responses to provide location-resolved image data

5 corresponding to the specified first and second regions of the sample, can include spectrally resolving the detected first and second responses to provide depth-resolved image data corresponding to the specified first and second depths of the sample.

Example 4 can include, or can optionally be combined with the subject
10 matter of one or any combination of Examples 1 through 3 to optionally include the delivering a third light excitation localized at a specified third region of a sample at a third excitation wavelength set of one or more wavelengths, different from the first and second excitation wavelength sets, to excite a scattering or multi-photon fluorescence third response light emitted from the specified third region of the
15 sample. The detecting the first, second, and third responses from the different respective first, second, and third regions of the sample, in respective response to the first, second, and third light excitations, can be such that the first, second, and third responses are distinguishable by having different wavelengths from each other. The example can include spectrally resolving the detected first, second, and third
20 responses to provide location-resolved image data corresponding to the specified first, second, and second regions of the sample. In an example, at least two of the first, second, and third regions of the sample are laterally displaced from each other in the sample. In an example, at least two of the first, second, and third regions of the sample are depth-displaced from each other in the sample.

25 Example 5 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 4 to optionally include the delivering at least one of the first or second light excitation is temporally encoded using a phase delay or a frequency modulation at different frequencies. The detecting the at least one of the first or second response can be temporally decoded
30 using the phase delay or the frequency modulation.

Example 6 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 5 to optionally include the first and second regions of the sample being laterally displaced from each other in the sample.

5 Example 7 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 6 to optionally include the first and second regions of the sample being depth-displaced from each other in the sample.

10 Example 8 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 7 to optionally include the first and second regions of the sample both being laterally displaced and depth-displaced from each other in the sample.

15 Example 9 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 8 to optionally include the first and second regions of the sample, at which the respective first and second light excitations are localized, being different in at least one of size or shape.

20 Example 10 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 9 to optionally include the delivering a first light excitation localized at a specified first region of a sample at a first excitation wavelength set of one or more wavelengths comprising exciting a scattering first response light, comprising a second or higher order harmonic, emitted from the specified first region of the sample. The example can include delivering a second light excitation localized at a different specified second region of the sample at a different second excitation wavelength set of one or more
25 wavelengths comprises exciting a scattering second response light, can comprise a second or higher order harmonic, emitted from the specified second region of the sample. The example can include concurrently detecting the first and second responses comprises concurrently detecting a second or higher order harmonic response from the different respective first and second regions of the sample, in
30 respective response to the first and second light excitations, wherein the first and

second responses are distinguishable by having different wavelengths from each other. The example can include spectrally resolving the detected first and second responses comprises spectrally resolving a second or higher order harmonic of the first light excitation from a second or higher order harmonic of the second light
5 excitation to provide location-resolved image data corresponding to the specified first and second regions of the sample.

Example 11 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 10 to optionally include the spectrally resolving comprising spectral unmixing.

10 Example 12 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 11 to optionally include the spectrally resolving comprising using different fluorophore enhancing agents introduced at the different regions.

Example 13 can include, or can optionally be combined with the subject
15 matter of one or any combination of Examples 1 through 12 to optionally include perturbing the sample in conjunction with at least one of: (A) the delivering the first light excitation localized at a specified first region of a sample; or (B) the delivering the second light excitation localized at a different specified second region of the sample. The perturbing can include at least one of: (1) using multi-photon
20 uncaging of a photoactivatable caged substance; (2) using a light activatable glutamate receptor; (3) genetically encoding a cell in the sample to express a light-sensitive membrane channel; (4) genetically encoding a photosensitizer; (5) topically applying a photosensitizer; (6) photoablating a cell in the sample; (7) photocoagulating cells in the sample; (8) optically generating a physical force
25 applied to a cell in the sample; or (9) magnetically generating a physical force applied to a cell in the sample.

Example 14 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 13 such that the delivering at least one of the first light excitation or the second light excitation includes

adjusting a divergence of the first light excitation to be different than the divergence of the second light excitation.

Example 15 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 14 to optionally include
5 the adjusting the divergence including diffracting.

Example 16 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 15 to optionally include the delivering at least one of the first light excitation or the second light excitation including: obtaining light from a light source; diffractively or refractively spectrally
10 separating the light for delivery to a diffractive optical element; adjusting the divergence of the spectrally-separated light using diffraction by the diffractive optical element; diffractively or refractively axially aligning the divergence-adjusted spectrally-separated light; and refracting the axially-aligned divergence-adjusted spectrally-separated light for delivery to different depths of the sample.

Example 17 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 16 to optionally include obtaining light from a light source, adjusting at least the divergence of the light using a spatial light modulator (SLM), and refracting divergence-adjusted light from
15 different optical elements of the SLM for delivery to different depths of the sample.

Example 18 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 17 to optionally include at least one of frequency modulating or phase multiplexing the light for delivery to the SLM.
20

Example 19 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 18 to optionally include
25 the refracting divergence-adjusted light from different optical elements of the SLM for delivery to different depths of the sample including using respective refractive elements of a static refractive element array.

Example 20 can include, or can optionally be combined with the subject
30 matter of one or any combination of Examples 1 through 19 to optionally include

the delivering the first light excitation localized at a specified first region of a sample at a first excitation wavelength set to excite a first response light emitted from the specified first region of the sample. The example can include the delivering a second light excitation localized at a different specified second region
5 of the sample at a different second excitation wavelength set to excite a second response light emitted from the specified second region of the sample, comprising scanning across a plane to movably image the specified first and second regions of the sample.

Example 21 can include, or can optionally be combined with the subject
10 matter of one or any combination of Examples 1 through 20 to optionally include the scanning across the plane comprising scanning across an X-Y plane that is parallel to a proximal surface of the sample.

Example 22 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 21 to optionally include
15 subject matter (such as an apparatus, a method, a means for performing acts, or a device readable medium including instructions that, when performed by the device, can cause the device to perform acts) that can include a first light source, which can be configured for delivering a first light excitation localized at a specified first region of a sample at a first excitation wavelength set of one or more wavelengths to
20 excite a scattering or a multi-photon fluorescence first response light emitted from the specified first region of the sample. A second light source can be configured for concurrently delivering a second light excitation localized at a different specified second region of the sample at a different second excitation wavelength set of one or more wavelengths to excite a scattering or multi-photon fluorescence second
25 response light emitted from the specified second region of the sample. A light detector can be configured for concurrently detecting the first and second responses from the different respective first and second regions of the sample, in respective response to the first and second light excitations, wherein the first and second responses are distinguishable by having different wavelengths from each other. The
30 example can also include a signal processor circuit, coupled to the light detector, the

signal processor circuit configured for spectrally resolving the detected first and second responses to provide location-resolved image data corresponding to the specified first and second regions of the sample.

Example 23 can include, or can optionally be combined with the subject
5 matter of one or any combination of Examples 1 through 22 to optionally include or use a Polarizing Beam Splitter, configured to combine light from the first light source and light from the second light source. An X-Y scanner can be configured to create a scanning pattern upon the sample. A microscope can be configured to receive light from the Polarizing Beam Splitter for delivery to the sample. A
10 dichroic can be configured to pass the first and second responses to the light detector. The light detector can include first, second, and third wavelength selective light detection channels.

Example 24 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 23 to optionally include or use the first and second light sources comprising separate laser light sources.

Example 25 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 24 to optionally include or use a supercontinuum light source. A first diffractive or refractive element can be coupled to receive light from the supercontinuum light source for separation into the first and second light sources respectively providing the first and second light excitations respectively including the respective first and second excitation wavelength sets.

Example 26 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 25 to optionally include or use a diffractive optical element that can be configured to receive and diffractively adjust at least one optical characteristic of the first and second light excitations.

Example 27 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 26 to optionally include or use the diffractive optical element including a Spatial Light Modulator.

Example 28 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 27 to optionally include or use a refractive optical element, configured to receive light from the Spatial Light Modulator and to provide the first and second light excitations to the different first and second target regions of the sample.

Example 29 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 28 to optionally include or use a temporal modulator configured to provide a temporal modulation of the first and second light excitations.

Example 30 can include, or can optionally be combined with the subject matter of one or any combination of Examples 1 through 29 to optionally include or use the temporal modulator comprising a frequency chopper.

Each of these non-limiting examples can stand on its own, or can be combined in any permutation or combination with any one or more of the other examples.

The above detailed description includes references to the accompanying
5 drawings, which form a part of the detailed description. The drawings show, by way of illustration, specific embodiments in which the invention can be practiced. These embodiments are also referred to herein as “examples.” Such examples can include elements in addition to those shown or described. However, the present inventors also contemplate examples in which only those elements shown or
10 described are provided. Moreover, the present inventors also contemplate examples using any combination or permutation of those elements shown or described (or one or more aspects thereof), either with respect to a particular example (or one or more aspects thereof), or with respect to other examples (or one or more aspects thereof) shown or described herein.

15 In the event of inconsistent usages between this document and any documents so incorporated by reference, the usage in this document controls.

In this document, the terms “a” or “an” are used, as is common in patent documents, to include one or more than one, independent of any other instances or

usages of “at least one” or “one or more.” In this document, the term “or” is used to refer to a nonexclusive or, such that “A or B” includes “A but not B,” “B but not A,” and “A and B,” unless otherwise indicated. In this document, the terms “including” and “in which” are used as the plain-English equivalents of the
5 respective terms “comprising” and “wherein.” Also, in the following claims, the terms “including” and “comprising” are open-ended, that is, a system, device, article, composition, formulation, or process that includes elements in addition to those listed after such a term in a claim are still deemed to fall within the scope of that claim. Moreover, in the following claims, the terms “first,” “second,” and
10 “third,” etc. are used merely as labels, and are not intended to impose numerical requirements on their objects.

Method examples described herein can be machine or computer-implemented at least in part. Some examples can include a computer-readable medium or machine-readable medium encoded with instructions operable to
15 configure an electronic device to perform methods as described in the above examples. An implementation of such methods can include code, such as microcode, assembly language code, a higher-level language code, or the like. Such code can include computer readable instructions for performing various methods. The code may form portions of computer program products. Further, in an example,
20 the code can be tangibly stored on one or more volatile, non-transitory, or non-volatile tangible computer-readable media, such as during execution or at other times. Examples of these tangible computer-readable media can include, but are not limited to, hard disks, removable magnetic disks, removable optical disks (e.g., compact disks and digital video disks), magnetic cassettes, memory cards or sticks,
25 random access memories (RAMs), read only memories (ROMs), and the like.

The above description is intended to be illustrative, and not restrictive. For example, the above-described examples (or one or more aspects thereof) may be used in combination with each other. Other embodiments can be used, such as by one of ordinary skill in the art upon reviewing the above description. The Abstract
30 is provided to comply with 37 C.F.R. §1.72(b), to allow the reader to quickly

ascertain the nature of the technical disclosure. It is submitted with the understanding that it will not be used to interpret or limit the scope or meaning of the claims. Also, in the above Detailed Description, various features may be grouped together to streamline the disclosure. This should not be interpreted as
5 intending that an unclaimed disclosed feature is essential to any claim. Rather, inventive subject matter may lie in less than all features of a particular disclosed embodiment. Thus, the following claims are hereby incorporated into the Detailed Description as examples or embodiments, with each claim standing on its own as a separate embodiment, and it is contemplated that such embodiments can be
10 combined with each other in various combinations or permutations. The scope of the invention should be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

THE CLAIMED INVENTION IS:

1. A method comprising:
delivering a first light excitation localized at a specified first region of a sample at a first excitation wavelength set of one or more wavelengths to excite a scattering or a multi-photon fluorescence first response light emitted from the specified first region of the sample;
delivering a second light excitation localized at a different specified second region of the sample at a different second excitation wavelength set of one or more wavelengths to excite a scattering or multi-photon fluorescence second response light emitted from the specified second region of the sample;
concurrently detecting the first and second responses from the different respective first and second regions of the sample, in respective response to the first and second light excitations, wherein the first and second responses are distinguishable by having different wavelengths from each other; and
spectrally resolving the detected first and second responses to provide location-resolved image data corresponding to the specified first and second regions of the sample.
2. The method of claim 1, wherein:
delivering the first light excitation localized at a specified first region of a sample at a first excitation wavelength set of one or more wavelengths comprises exciting a multi-photon fluorescence first response light emitted from the specified first region of the sample;
delivering the second light excitation localized at a different specified second region of the sample at a different second excitation wavelength set of one or more wavelengths comprises exciting a multi-photon fluorescence second response light emitted from the specified second region of the sample; and
the first and second responses comprise multi-photon fluorescence responses that are distinguishable by having different wavelengths from each other.

3. The method of claim 1,
wherein the delivering a first light excitation localized at a specified first
region of a sample includes delivering the light excitation localized at a specified
5 first depth of the sample;
wherein the delivering a second light excitation localized at a different
specified second region of the sample includes delivering the light excitation
localized at a specified second depth of the sample that is different from the
specified first depth of the sample;
10 wherein the concurrently detecting the first and second responses from the
different respective first and second regions of the sample, in respective response to
the first and second light excitations, comprises concurrently detecting the first and
second responses from the different respective first and second depths of the
sample; and
15 wherein the spectrally resolving the detected first and second responses to
provide location-resolved image data corresponding to the specified first and second
regions of the sample, comprises spectrally resolving the detected first and second
responses to provide depth-resolved image data corresponding to the specified first
and second depths of the sample.
20
4. The method of claim 1, comprising:
delivering a third light excitation localized at a specified third region of a
sample at a third excitation wavelength set of one or more wavelengths, different
from the first and second excitation wavelength sets, to excite a scattering or multi-
25 photon fluorescence third response light emitted from the specified third region of
the sample;
detecting the first, second, and third responses from the different respective
first, second, and third regions of the sample, in respective response to the first,
second, and third light excitations, wherein the first, second, and third responses are
30 distinguishable by having different wavelengths from each other;

spectrally resolving the detected first, second, and third responses to provide location-resolved image data corresponding to the specified first, second, and second regions of the sample;

wherein at least two of the first, second, and third regions of the sample are
5 laterally displaced from each other in the sample; and

wherein at least two of the first, second, and third regions of the sample are depth-displaced from each other in the sample.

5. The method of claim 1, wherein:
10 the delivering at least one of the first or second light excitation is temporally encoded using a phase delay or a frequency modulation at different frequencies; and
the detecting the at least one of the first or second response is temporally decoded using the phase delay or the frequency modulation.

15 6. The method of claim 1, wherein the first and second regions of the sample are laterally displaced from each other in the sample.

7. The method of claim 1, wherein the first and second regions of the sample are depth-displaced from each other in the sample.

20 8. The method of claim 1, wherein the first and second regions of the sample are both laterally displaced and depth-displaced from each other in the sample.

9. The method of claim 1, wherein the first and second regions of the sample,
25 at which the respective first and second light excitations are localized, are different in at least one of size or shape.

10. The method of claim 1, wherein:
the delivering a first light excitation localized at a specified first region of a
30 sample at a first excitation wavelength set of one or more wavelengths comprises

exciting a scattering first response light, comprising a second or higher order harmonic, emitted from the specified first region of the sample;

the delivering a second light excitation localized at a different specified second region of the sample at a different second excitation wavelength set of one or more wavelengths comprises exciting a scattering second response light, comprising a second or higher order harmonic, emitted from the specified second region of the sample;

concurrently detecting the first and second responses comprises concurrently detecting a second or higher order harmonic response from the different respective first and second regions of the sample, in respective response to the first and second light excitations, wherein the first and second responses are distinguishable by having different wavelengths from each other; and

spectrally resolving the detected first and second responses comprises spectrally resolving a second or higher order harmonic of the first light excitation from a second or higher order harmonic of the second light excitation to provide location-resolved image data corresponding to the specified first and second regions of the sample.

11. The method of claim 1, wherein the spectrally resolving comprises spectral unmixing.

12. The method of claim 1, wherein the spectrally resolving comprises using different fluorophore enhancing agents introduced at the different regions.

13. The method of claim 1, further comprising perturbing the sample in conjunction with at least one of: (A) the delivering the first light excitation localized at a specified first region of a sample; or (B) the delivering the second light excitation localized at a different specified second region of the sample; and wherein the perturbing comprises at least one of:

(1) using multi-photon uncaging of a photoactivatable caged substance;

- (2) using a light activatable glutamate receptor;
- (3) genetically encoding a cell in the sample to express a light-sensitive membrane channel;
- (4) genetically encoding a photosensitizer;
- 5 (5) topically applying a photosensitizer;
- (6) photoablating a cell in the sample;
- (7) photocoagulating cells in the sample;
- (8) optically generating a physical force applied to a cell in the sample; or
- (9) magnetically generating a physical force applied to a cell in the sample.
- 10
14. The method of claim 1, wherein the delivering at least one of the first light excitation or the second light excitation includes adjusting a divergence of the first light excitation to be different than the divergence of the second light excitation.
- 15 15. The method of claim 14, wherein adjusting the divergence includes diffracting.
16. The method of claim 14, wherein the delivering at least one of the first light excitation or the second light excitation includes:
- 20 obtaining light from a light source;
- diffractively or refractively spectrally separating the light for delivery to a diffractive optical element;
- adjusting the divergence of the spectrally-separated light using diffraction by the diffractive optical element;
- 25 diffractively or refractively axially aligning the divergence-adjusted spectrally-separated light; and
- refracting the axially-aligned divergence-adjusted spectrally-separated light for delivery to different depths of the sample.
- 30 17. The method of claim 1, comprising:

obtaining light from a light source;
adjusting at least the divergence of the light using a spatial light modulator (SLM);
refracting divergence-adjusted light from different optical elements of the
5 SLM for delivery to different depths of the sample.

18. The method of claim 17, comprising at least one of frequency modulating or phase multiplexing the light for delivery to the SLM.

10 19. The method of claim 17, wherein the refracting divergence-adjusted light from different optical elements of the SLM for delivery to different depths of the sample includes using respective refractive elements of a static refractive element array.

15 20. The method of claim 1, wherein the delivering the first light excitation localized at a specified first region of a sample at a first excitation wavelength set to excite a first response light emitted from the specified first region of the sample, and the delivering a second light excitation localized at a different specified second region of the sample at a different second excitation wavelength set to excite a
20 second response light emitted from the specified second region of the sample, comprises scanning across a plane to movably image the specified first and second regions of the sample.

21. The method of claim 20, wherein the scanning across the plane comprises
25 scanning across an X-Y plane that is parallel to a proximal surface of the sample.

22. An apparatus comprising:
a first light source, configured for delivering a first light excitation localized at a specified first region of a sample at a first excitation wavelength set of one or

more wavelengths to excite a scattering or a multi-photon fluorescence first response light emitted from the specified first region of the sample;

5 a second light source, configured for concurrently delivering a second light excitation localized at a different specified second region of the sample at a different second excitation wavelength set of one or more wavelengths to excite a scattering or multi-photon fluorescence second response light emitted from the specified second region of the sample;

10 a light detector, configured for concurrently detecting the first and second responses from the different respective first and second regions of the sample, in respective response to the first and second light excitations, wherein the first and second responses are distinguishable by having different wavelengths from each other; and

15 a signal processor circuit, coupled to the light detector, the signal processor circuit configured for spectrally resolving the detected first and second responses to provide location-resolved image data corresponding to the specified first and second regions of the sample.

23. The apparatus of claim 22, comprising:

20 a Polarizing Beam Splitter, configured to combine light from the first light source and light from the second light source;

an X-Y scanner, configured to create a scanning pattern upon the sample;

a microscope, configured to receive light from the Polarizing Beam Splitter for delivery to the sample;

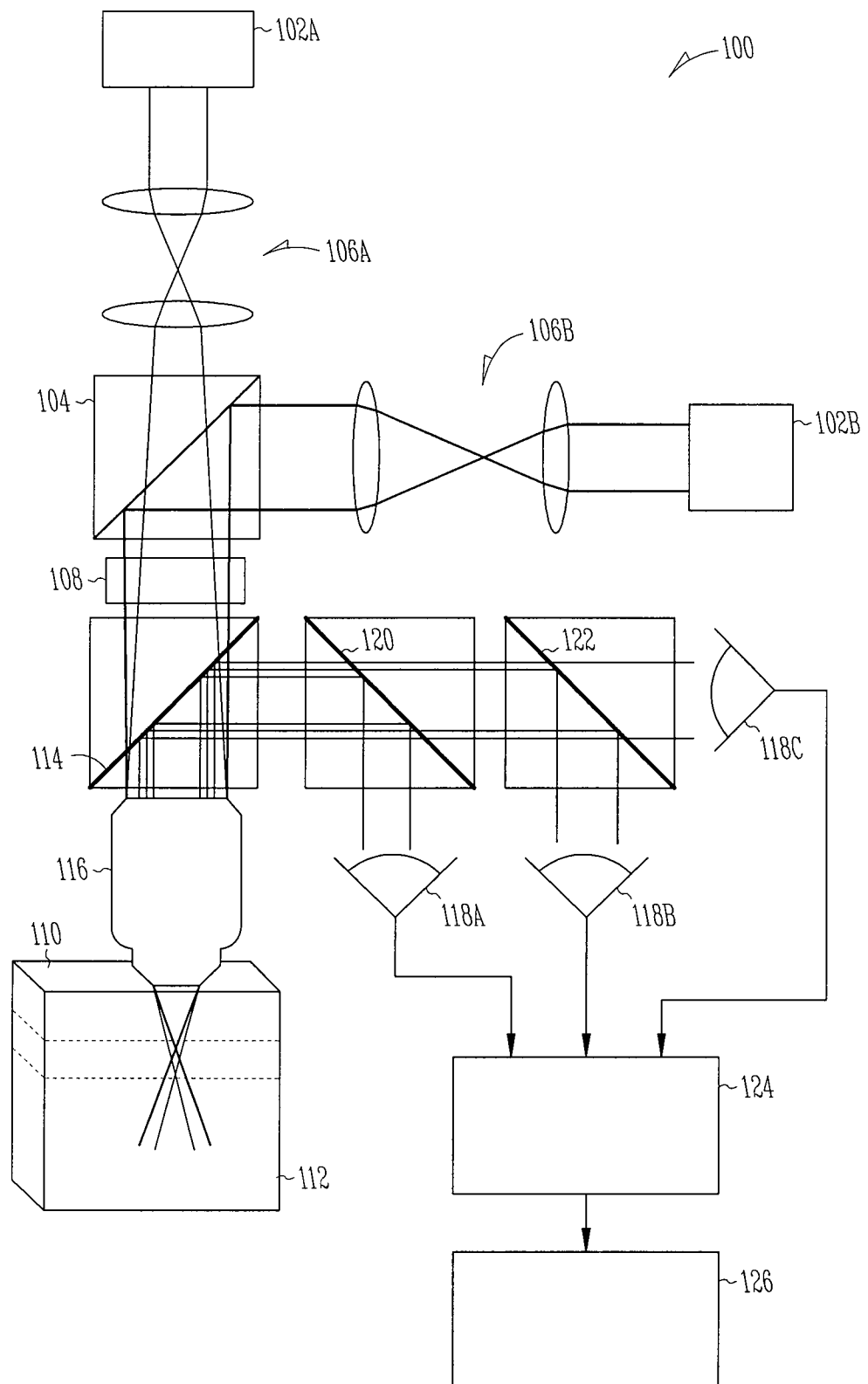
25 a dichroic, configured to pass the first and second responses to the light detector; and

wherein the light detector comprises first, second, and third wavelength selective light detection channels.

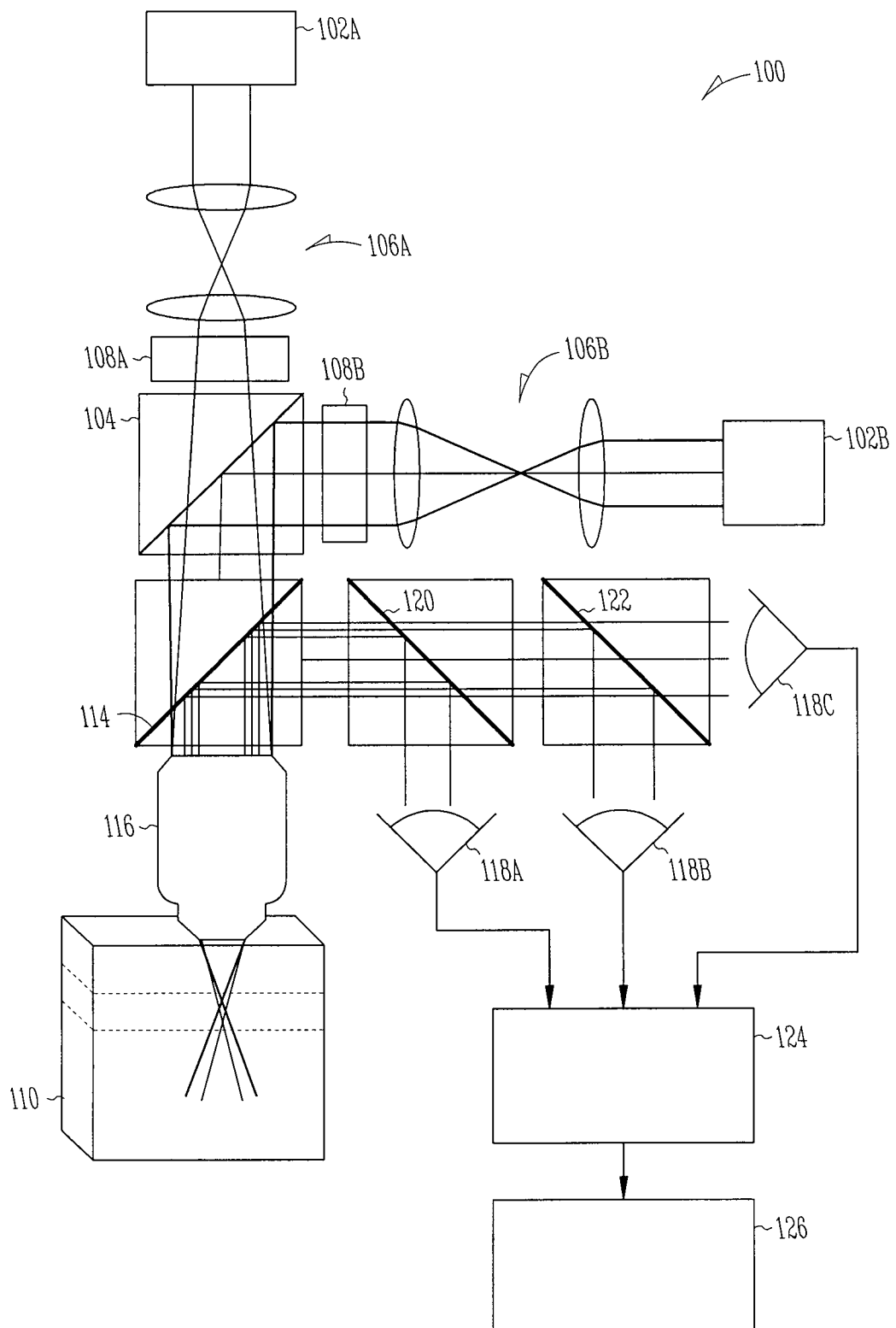
24. The apparatus of claim 22, wherein the first and second light sources comprise separate laser light sources.

25. The apparatus of claim 22, comprising:
a supercontinuum light source; and
a first diffractive or refractive element, coupled to receive light from the supercontinuum light source for separation into the first and second light sources respectively providing the first and second light excitations respectively including the respective first and second excitation wavelength sets.
26. The apparatus of claim 25, comprising a diffractive optical element, configured to receive and diffractively adjust at least one optical characteristic of the first and second light excitations.
27. The apparatus of claim 26, wherein the diffractive optical element comprises a Spatial Light Modulator.
28. The apparatus of claim 27, comprising a refractive optical element, configured to receive light from the Spatial Light Modulator and to provide the first and second light excitations to the different first and second target regions of the sample.
29. The apparatus of claim 22, comprising a temporal modulator configured to provide a temporal modulation of the first and second light excitations.
30. The apparatus of claim 29, wherein the temporal modulator comprises a frequency chopper.

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*Fig. 1A*

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*Fig. 1B*

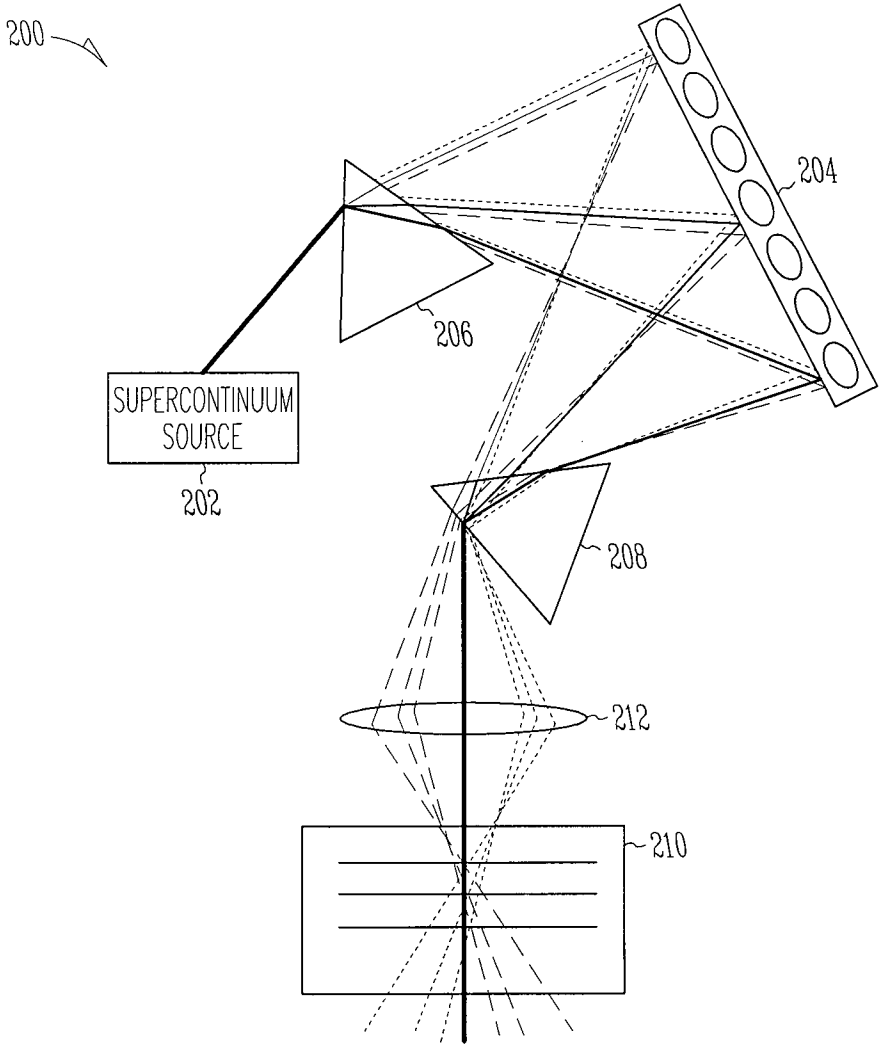


Fig. 2

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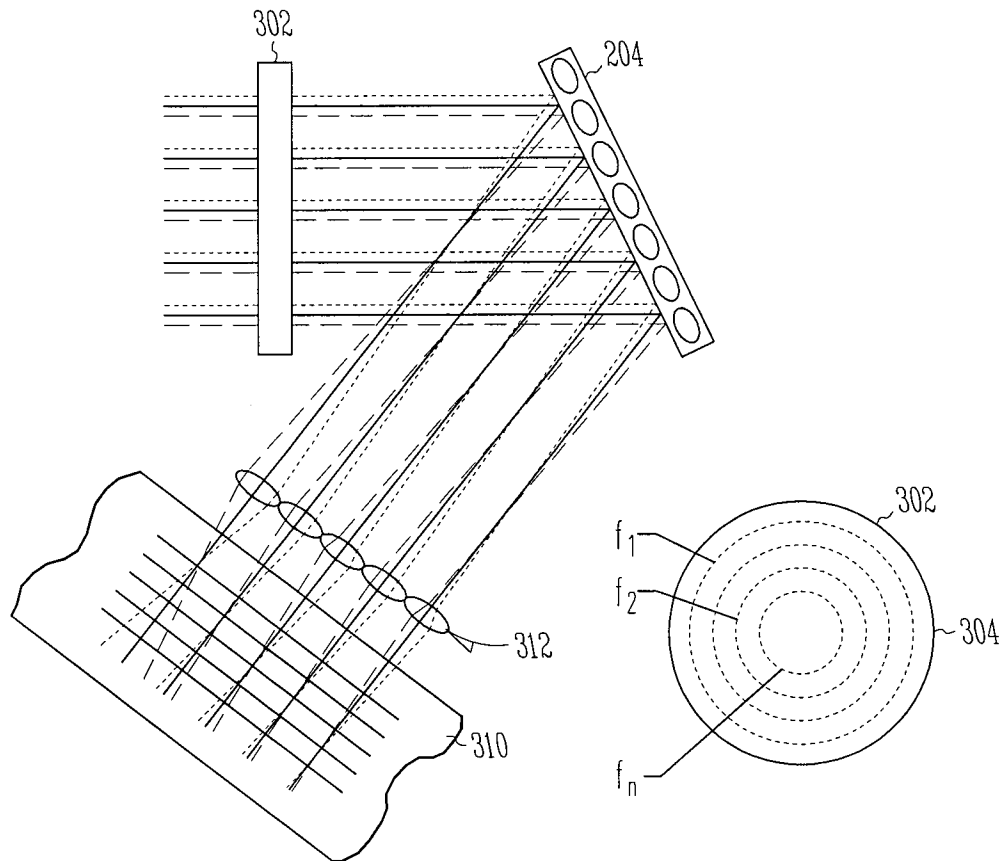
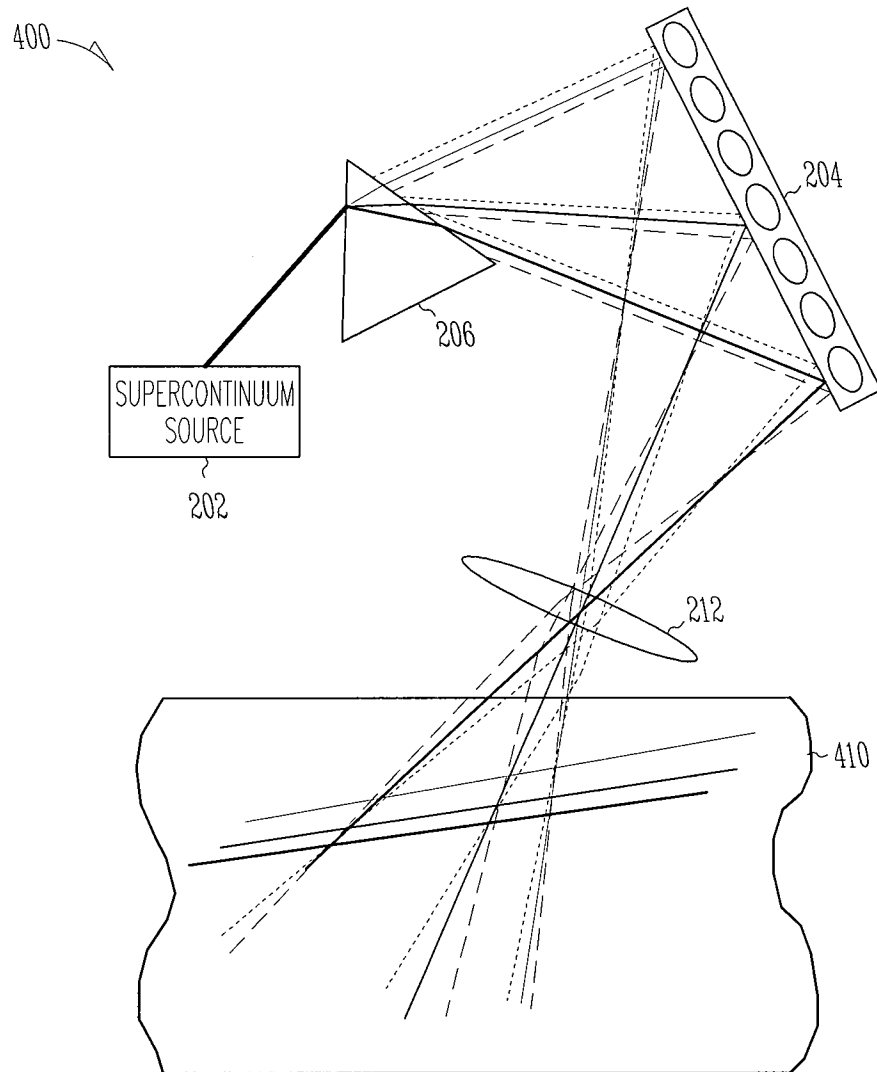


Fig. 3

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*Fig. 4*

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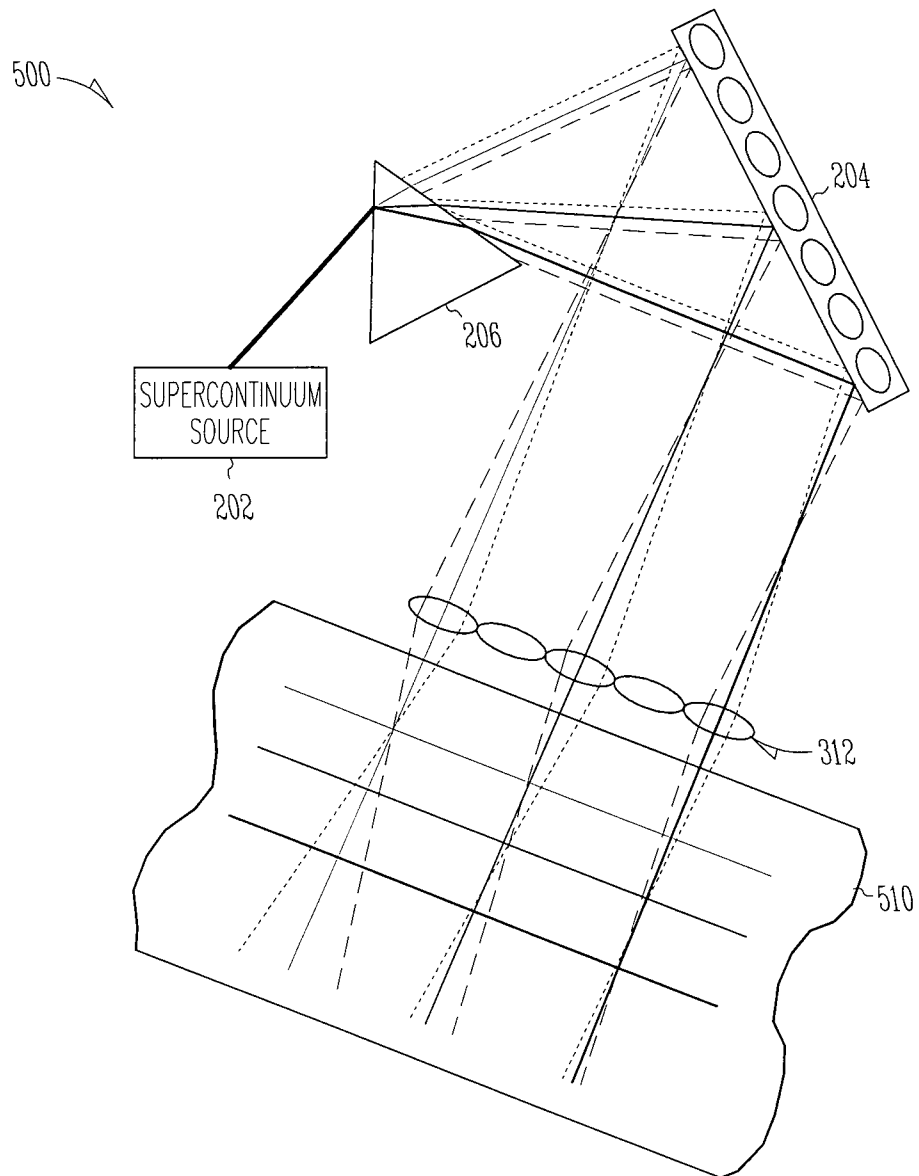


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/065562

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61B 5/00 (2012.01) USPC - 600//310 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) - A61B 5/00, 5/145, 5/1455 (2012.01) USPC - 356//300-02, 311, 318-24; 600/309-10, 321, 476 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) PatBase, MicroPatent, Google Patents		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0168586 A1 (HILLMAN et al) 01 July 2010 (01.07.2010) entire document	1-30
Y	US 6,917,421 B1 (WIHL et al) 12 July 2005 (12.07.2005) entire document	1-30
Y	US 2010/0053743 A1 (GALIMBERTI et al) 04 March 2010 (04.03.2010) entire document	2, 5, 10, 13, 29-30
Y	US 2010/0292931 A1 (WANG et al) 18 November 2010 (18.11.2010) entire document	17-19, 27-28
Y	US 2009/0177094 A1 (BROWN et al) 09 July 2009 (09.07.2009) entire document	25-28
A	US 2008/0058629 A1 (SEIBEL et al) 06 March 2008 (06.03.2008) entire document	1-30
A	US 2006/0269450 A1 (KIM et al) 30 November 2006 (30.11.2006) entire document	1-30
A	US 2005/0146784 A1 (VOGT) 07 July 2005 (07.07.2005) entire document	1-30
A	US 2007/0076200 A1 (MARTIN et al) 05 April 2007 (05.04.2007) entire document	1-30
A	US 5,111,821 A (POTTER) 12 May 1992 (12.05.1992) entire document	1-30
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Date of the actual completion of the international search 12 April 2012		Date of mailing of the international search report 25 APR 2012
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. 571-273-3201		Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774