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(71) Applicant: **TRUSTEES OF BOSTON UNIVERSITY** [US/US]; One Silber Way, Boston, MA 02215 (US).

(72) Inventors: **MERTZ, Jerome, Charles**; 16 Maplewood Aveenue, Newton, MA 02459 (US). **FORD, Timothy, Nehiley**; 15 Glenville Avenue, #15, Allston, MA 02134 (US). **CHU, Kengyeh, Ken**; 105 Walk Hill Street, #2, Jamaica Plain, MA 02130 (US).

(74) Agent: **LEE, Scott**; Law Office of Salvatore Arrigo and Scott Lee, LLP, 1050 Connecticut Ave. NW, 10th Floor, Washington, DC 20036 (US).

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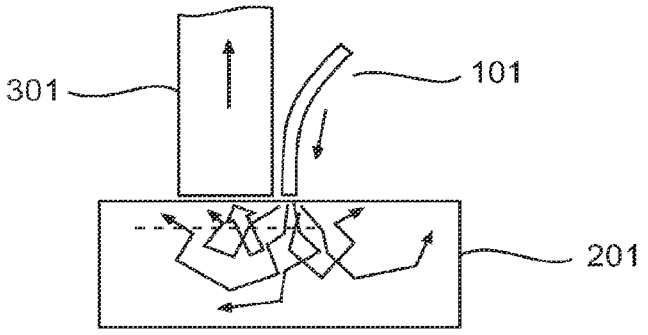

FIG. 1A

(57) Abstract: A method of creating a phase contrast image is provided. In some embodiments the method comprises illuminating the target region of a sample with a first light source to provide a first oblique back illumination of the target region of the sample, and detecting a first phase contrast image from light originating from the first light source and back illuminating the target region of the sample. In some embodiments the method further comprises illuminating the sample with a second light source to provide a second oblique back illumination of the target region of the sample, and detecting a second phase contrast image from light originating from the second light source and back illuminating the target region of the sample. In some embodiments a difference image of the target region of the sample is created by subtracting the second phase contrast image of the target region of the sample from the first phase contrast image of the target region of the sample. Apparatus for carrying out the methods are also provided. The methods and apparatus find use, for example, in endoscopy.

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PHASE CONTRAST MICROSCOPY WITH OBLIQUE BACK-ILLUMINATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/617,707, filed March 30, 2012, which is hereby incorporated herein by reference.

GOVERNMENT FUNDING

[0002] This invention was made with Government Support under Contract No. EB010059 awarded by the National Institutes of Health. The Government has certain rights in the invention.

INTRODUCTION

[0003] The standard technique to assess tissue pathology in clinical applications is to perform a biopsy [1]. In general, assessment is made based on purely morphological considerations. The technique often involves use of a device to observe tissue with high resolution. As successful and prevalent as this biopsy procedure has become, it faces certain drawbacks. For example, the process is laborious and time consuming, requiring hours or days to provide results. For certain applications it would be useful to have an alternative procedure that, in some embodiments, requires less time and/or work. For another example, tissue biopsies only provide a sparse sampling that may not be fully representative of the region of interest. For certain applications it would be useful to have an alternative procedure that, in some embodiments, enables more comprehensive sampling of the region of interest. For another example, tissue biopsies pose a risk of infection and/or other complications to the patient and can cause discomfort. For certain applications it would be useful to have an alternative procedure that, in some embodiments, causes less discomfort and/or has a reduced risk of infection and/or other complications to the patient.

[0004] The concept of an "optical biopsy" has long been sought by the biomedical imaging community [2], [3]. Nonetheless, efforts to develop optical biopsy techniques and equipment have had a limited success. Many strategies for optical biopsies have been proposed. In general, these can be separated into two broad categories, those based on imaging and those based on spectroscopy [3].

[0005] Phase contrast imaging is one of the most prevalent applications of wide field microscopy, and there exists an abundance of literature describing different wide field phase

contrast techniques. The most common of these, found in virtually every cellular biology lab, are Zernike phase contrast [4] or Normarski differential interference contrast (DIC)[5], [6]. The latter is also widely used in neurophysiology labs, since it is highly effective at revealing neurons in brain tissue slices. Other wide field phase contrast techniques include Schlieren microscopy [7], Hoffman contrast [8], or other variants of oblique field microscopies such as field contrast [9], [10]. None of these techniques is particularly quantitative in the sense that the measured signal cannot be easily converted into a measured phase. Nevertheless, the signals are phase dependent, and thus reveal variations in optical path length. Only recently (relatively speaking) has there been a trend toward the development of phase contrast techniques that are genuinely quantitative ([11–16]). The application of these techniques is limited, however, because each one works only in the transmission direction. This feature limits the use of these techniques to use with a transmission light source. The techniques are therefore applied to thin samples, such as cell monolayers or thin tissue slices.

[0006] Other techniques also work in the reflection direction, such as reflection confocal microscopy [17]. In this technique, signal arises from local reflectivities in the sample, which, in turn arise from refractive index variations. A difficulty with reflection confocal is that scattering in most biological tissues is dominantly in the forward direction. Only sharp interfaces (i.e. refractive index variations with high enough axial spatial frequencies) produce scattering in the backward direction, meaning that signal is weak. Moreover, the signal can easily be overwhelmed by multiply scattered light containing no image information. Both of these problems are solved by optical coherence tomography (OCT), which provides noiseless amplification of the directly back-scattered signal while rejecting multiply scattered background [18]. Nevertheless, the fact remains that OCT, like reflectance confocal, reveals mostly sharp axial interfaces in samples.

[0007] In most of their incarnations, reflectance confocal and OCT are based on scanning geometries, and thus require scanning mechanisms, somewhat complicating their operation in endoscopy applications. Certain designs have been proposed to overcome this feature [19–21].

[0008] In addition to OCT, other techniques have been developed to provide high resolution imaging in thick tissue. Examples are photo-acoustic microscopy (PAM) [23] and nonlinear microscopy [24], e.g. based on two-photon excited fluorescence (TPEF) or second harmonic generation (SHG). PAM reveals absorbing structures, TPEF reveals fluorophores,

and SHG reveals non-centrosymmetric structures. None provides phase contrast in the usual sense of the term. Moreover, PAM, TPEF and SHG are all scanning techniques.

[0009] Another class of thick tissue imaging techniques uses the detection of multiply scattered light. Examples are diffuse optical tomography (DOT) [30], and a beam scanning variant, laminar optical tomography (LOT) [31]. Image reconstruction with these techniques is based on mathematical models, and the extraction of data usually requires intensive numerical processing. These techniques can provide very deep tissue penetration, but it occurs at the expense of resolution. They reveal tissue properties such as absorption and/or scattering coefficients. They provide neither high resolution nor phase contrast. Nor have they been applied in endoscopy configurations.

[0010] Another technique is Orthogonal Polarization Spectral (OPS) imaging [32], [33], now commercialized as Cytoscan™ microscopy. This strategy is similar to OBM in that it generates backlighting from multiply scattered light (launched on-axis and distinguished by the fact that it is depolarized). This technique uses shadow-casting to reveal absorption contrast only. This technique cannot reveal phase contrast. Moreover, it provides only low resolution images with a rigid, handheld probe, and it cannot be combined with standard endoscopes.

[0011] Accordingly, there is a need for new imaging methods and apparatus that have useful properties. This disclosure meets that need by describing new imaging methods and apparatus, among other things.

SUMMARY

[0012] This disclosure describes a new phase contrast technique, sometimes referred to herein as oblique back-illumination microscopy (OBM). OBM works in a reflected light geometry (sometimes called epi-detection geometry), and is thus amenable to *in-vivo* endomicroscopy applications, among many others. OBM requires no labeling and provides high resolution DIC-like images of sub-surface sample morphology. As will become apparent from this disclosure, the methods and apparatus disclosed herein apply the new OBM technology in ways that offer useful improvements in various ways to other technologies currently available.

[0013] In a first asepct this disclosure provides methods of creating a phase contrast image, comprising: illuminating the target region of a sample with a first light source to provide a first oblique back illumination of the target region of the sample, and detecting a first phase contrast image from light originating from the first light source and back illuminating the target region of the sample. In some embodiments light from the first light source is the only light illuminating the sample when the first phase contrast image is detected from light originating from the first light source and back illuminating the target region of the sample.

[0014] In some embodiments the methods further comprise illuminating the sample with a second light source to provide a second oblique back illumination of the target region of the sample, and detecting a second phase contrast image from light originating from the second light source and back illuminating the target region of the sample. In some embodiments the methods further comprise creating a difference image of the target region of the sample by subtracting the second phase contrast image of the target region of the sample from the first phase contrast image of the target region of the sample. In some embodiments the methods further comprise creating an absorption contrast image of the target region of the sample by adding the first phase contrast image of the target region of the sample to the second phase contrast image of the target region of the sample.

[0015] In some embodiments the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source and back illuminating the target region are different. In some embodiments the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source and back illuminating the target region are different. In some embodiments the axis of detection of light originating from the first light source and back illuminating the target region and the axis of detection of light originating from the second light source and back illuminating the target region are different. In some embodiments the axis of detection of light originating from the first light source and back illuminating the target region and the axis of detection of light originating from the second light source and back illuminating the target region are the same. In some embodiments the wavelength of the light from the first light source and the wavelength of light from the second light source are different. In some embodiments the wavelength of the light from the first and second light sources is from 0.2 to 300 μm. In some embodiments the light source(s) is selected from a

light-emitting diode (LED), a laser, a supercontinuum light source, or a superluminescent diode (SLED). In some embodiments the detecting is by a photo detector array. In some embodiments the photo detector array is a charge coupled device (CCD) or a CMOS (complementary metal oxide semiconductor) camera sensor.

[0016] In some embodiments the methods comprise using an optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample. In some embodiments the optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample is selected from a fiber, an arrangement of fibers, a fiber bundle, a rigid lens, an arrangement of rigid lenses, a gradient index (GRIN) lens, or an arrangement of GRIN lenses. In some embodiments the same optical conduit communicates light toward the sample and away from the sample. In some embodiments different components of the same optical conduit communicates light toward the sample and away from the sample.

[0017] In some embodiments the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaced by from about 0.2 mm to about 10 mm. In some embodiments the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaced by from about 0.2 mm to about 10 mm.

[0018] In some embodiments the object plane of the target region is from the surface to about 300 μm below the surface of the sample. In some embodiments the lateral resolution of the image is from about 0.1 μm to about 10 μm.

[0019] In some embodiments the methods comprise detecting the first and second images during first and second non-overlapping time intervals. In some embodiments the methods comprise detecting the first and second images during first and second overlapping time intervals.

[0020] In some embodiments the first and second light sources illuminate the sample with light of different distinguishable wavelengths. In some embodiments the images of different distinguishable wavelengths are separated by a wavelength separator and directed onto separate camera sensors. In some embodiments the images of different distinguishable wavelengths are separated by a wavelength separator and directed onto different portions of a same camera sensor.

[0021] In some embodiments the first and second light sources illuminate the sample with orthogonally polarized light. In some embodiments the images of orthogonal polarization are separated by a polarization separator and directed onto separate camera sensors. In some embodiments the images of orthogonal polarization are separated by a polarization separator and directed onto different portions of a same camera sensor.

[0022] In some embodiments the difference image is axially resolved.

[0023] In some embodiments the methods comprise obtaining a series of two or more images and combining the images to provide a composite image larger than the field of view a single image. In some embodiments the methods comprise creating a phase contrast image of gastrointestinal tissue and examining the tissue to assess at least one of the presence and the absence of indicators of a disease. In some embodiments the gastrointestinal tissue is colonic mucosa disease is at least one of hyperplasia and adenomatous changes. In some embodiments the methods comprise creating a phase contrast image of lung tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease. In some embodiments the methods comprise creating a phase contrast image of liver tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease. In some embodiments the methods comprise creating a phase contrast image of bladder tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease. In some embodiments the methods comprise creating a phase contrast image of skin tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease. In some embodiments the methods comprise creating a phase contrast image of brain tissue and examining the tissue to assess tissue morphology. In some embodiments the methods comprise creating a phase contrast video of blood flow to assess blood flow velocity. In some embodiments the methods comprise creating a phase contrast video of blood flow to assess the cell count of at least one blood cell type.

[0024] In another aspect this disclosure provides an apparatus for creating a phase contrast image of a sample, comprising: a probe comprising 1) an optical radiation source or a first light conduit, and 2) a photo detector array or image conduit, and 3) a distal end; wherein the light conduit, the photo detector array or image conduit, and the distal end of the probe are configured to back illuminate the target region of a sample in contact or near contact with the distal end of the probe with a light from the first light source to provide a

first oblique back illumination of the target region of the sample, and to detect a first phase contrast image from light originating from the first light source and back illuminating the target region of the sample.

[0025] In some embodiments the distal end of the optical radiation source or first light conduit extend to the distal end of the probe. In some embodiments the distal end of the optical radiation source or first light conduit is recessed from the distal end of the probe by up to 10 cm. In some embodiments distal end of the photo detector array or image conduit is recessed from the distal end of the probe.

[0026] In some embodiments the probe comprises a first light conduit and the apparatus further comprises a first optical radiation source connected to or projected to a proximal end of the first light conduit. In some embodiments the probe comprises a photo detector array. In some embodiments the probe comprises an image conduit and a proximal end of the image conduit is connected to or imaged to a photo detector array.

[0027] In some embodiments the probe further comprises a second optical radiation source or a second light conduit; wherein the second optical radiation source or second light conduit, the photo detector array or image conduit, and the distal end of the probe are configured to illuminate the target region of a sample in contact or near contact with the distal end of the probe with a light from the second light source to provide a second oblique back illumination of the target region of the sample, and to detect a second phase contrast image from light originating from the second light source and back illuminating the target region of the sample.

[0028] In some embodiments the distal end of the optical radiation source or first light conduit extends to the distal end of the probe. In some embodiments the distal end of the optical radiation source or first light conduit is recessed from the distal end of the probe by up to 10 cm. In some embodiments the distal end of the photo detector array or image conduit is recessed from the distal end of the probe.

[0029] In some embodiments the probe comprises a second light conduit and the apparatus further comprises a second optical radiation source connected to or imaged to a proximal end of the first light conduit.

[0030] In some embodiments the apparatus comprises at least three optical radiation sources or a light conduits, wherein the at least three optical illumination sources or light conduits are located at distinct locations around the probe such that each is capable of creating oblique back illumination enabling the measurement and display of phase gradients in different directions relative to the others.

[0031] In some embodiments the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source and back illuminating the target region of the sample are different. In some embodiments the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are different.

[0032] In some embodiments the axis of detection of light originating from the first light source and reflected from the sample and the axis of detection of light originating from the second light source and illuminating the target region of the sample are different. In some embodiments the axis of detection of light originating from the first light source and reflected from the sample and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are the same. In some embodiments the wavelength of the light from the first light source and the wavelength of light from the second light source are different.

[0033] In some embodiments the apparatus is configured to detect the first and second images during first and second non-overlapping time intervals. In some embodiments the apparatus is configured to detect the first and second images during first and second overlapping time intervals. In some embodiments the apparatus is configured for illumination of the sample by the first and second light sources with light of different distinguishable wavelengths. In some embodiments the apparatus is configured for illumination of the sample by the first and second light sources with orthogonally polarized light.

[0034] In some embodiments the first and second light sources are capable of providing illumination at a range of wavelengths comprising from 0.2 to 300 μm.

[0035] In some embodiments the light source is selected from a light-emitting diode (LED), a laser, a supercontinuum light source, or a superluminescent diode (SLED). In some

embodiments the apparatus comprises a photo detector array. In some embodiments the photo detector array is a charge coupled device (CCD) or a CMOS (complementary metal oxide semiconductor) camera sensor.

[0036] In some embodiments the apparatus comprises an optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample.

[0037] In some embodiments the apparatus is configured so that the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaceable by from about 0.2 mm to about 5 mm. In some embodiments the apparatus is configured so that the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaceable by from about 0.2 mm to about 5 mm.

[0038] In some embodiments the apparatus is configured to obtain images of object planes of the target region from the surface of the sample to about 300 μm below the surface of the sample. In some embodiments the apparatus creates images laterally resolved at from about 0.3 μm to about 10 μm.

[0039] In some embodiments at least one of 1) the distal end of the first optical radiation source or first light conduit, 2) the distal end of the second optical radiation source or second light conduit, and 3) the distal end of the photo detector array or image conduit, extend through and end at the distal end of the probe.

[0040] In some embodiments at least one of 1) the distal end of the first optical radiation source or first light conduit, and 2) the distal end of the second optical radiation source or second light conduit, and 3) the distal end of the photo detector array or image conduit, is recessed from the distal end of the probe by up to 5 cm.

[0041] In another aspect an endoscope that comprises an apparatus of this disclosure is provided. In some embodiments the endoscope is portable.

[0042] In another aspect this disclosure provides a system that comprises an apparatus of this disclosure and a processor for processing images obtained from the apparatus. In

some embodiments the system comprises an endoscope that comprises an apparatus of this disclosure.

[0043] In another aspect this disclosure provides methods of creating at least one of a phase contrast image of a target region of a sample and a difference image of two phase contrast images of a target region of a sample, comprising: providing a sample comprising a target region; using an apparatus of this disclosure to create at least one phase contrast image of the target region of the sample using a method of this disclosure, and optionally creating a difference image from the two or more contrast images of the target region of the sample.

[0044] In another aspect this disclosure provides a phase contrast image created by a method of this disclosure. Also provided is a data set representing the phase contrast image. In some embodiments the phase contrast image is stored on a tangible computer-readable medium or machine-readable medium. Such media include, for example, hard disks, removable magnetic disks, removable optical disks (e.g., compact disks and digital video disks), magnetic cassettes, memory cards or sticks, random access memories (RAMs), read only memories (ROMs), and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] **Figure 1a** shows the principle of OBM. Illumination is launched into tissue via an offset fiber. Multiply scattered light leads to oblique trans-illumination of object plane (dashed).

[0046] **Figure 1b** shows the principle of OBM. Alternating illumination through two fibers, and imaging through flexible endoscope. **201** is a sample, the hatched line is the target region, **301** is an image conduit, **321** and **322** are optical elements in the image conduit, **101** and **102** are light conduits, **111** and **112** are LEDs, **401** is a camera.

[0047] **Figure 1c** shows that oblique illumination leads to phase gradient contrast. With no phase gradient, oblique illumination is partially blocked by aperture in detection optics.

[0048] **Figure 1d** shows that oblique illumination leads to phase gradient contrast. Phase gradients due to slopes in refractive index variations can lead to more blockage.

[0049] **Figure 1e** shows that oblique illumination leads to phase gradient contrast. Phase gradients due to slopes in refractive index variations can lead to less blockage.

[0050] **Figure 2** shows the principle of OBM. Illumination is launched into tissue via an offset fiber. Multiply scattered light leads to oblique trans-illumination of object plane (dashed).

[0051] **Figure 3** shows OBM images of onion skin. **(a)** and **(b)** are raw images acquired with left and right illumination. **(c)** is absorption image (a+b). **(d)** is a phase gradient image (a-b). Note: panel (d) contains negative values, meaning its zero level is gray. Scale bar = 100μm. Depth = 70μm.

[0052] **Figure 4a** shows an OCT image.

[0053] **Figure 4a** shows a representative reflected light path that generates the OCT image.

[0054] **Figure 4c** shows a DIC image.

[0055] **Figure 4d** shows a representative transmitted light path that generates the DIC image.

[0056] **Figure 5** shows an illumination module. In this embodiment computer-controlled LEDs can span from UV to NIR.

[0057] **Figure 6a** shows a lateral view of an embodiment of distal illumination optics. **601** is a probe, **301** is an image conduit that extends to the distal end of the probe (hidden from view), **101** and **102** are first and second light conduits.

[0058] **Figure 6b** shows a cross sectional view of an embodiment of distal illumination optics comprising a molded fiber end.

[0059] **Figure 6c** shows a cross sectional view of an embodiment of distal illumination optics comprising multiple thin fibers. In this case the first light conduit comprises five light conduits **(101)** and the second light conduit comprises five light conduits **(102)**. Each set of light conduits is located on opposite sides of the distal probe and thus each set acts as a light conduit to provide oblique back illumination of the target region of a sample.

[0060] **Figure 7a** shows an example of single shot OBM using polarization discrimination.

[0061] **Figure 7b** shows an example of single shot OBM using spectral discrimination.

[0062] **Figure 7c** shows an example of single shot OBM using polarization discrimination with the addition of polarizers in the aperture plane.

[0063] **Figure 7d** shows an example of single shot OBM using spectral discrimination with the addition of spectral bandpass filters in the aperture plane.

[0064] **Figure 8a** shows a Monte Carlo simulation of the "photon banana".

[0065] **Figure 8b** shows a Monte Carlo simulation of photon exit angles.

[0066] **Figure 8c** shows additional Monte Carlo simulations of photon exit angles.

[0067] **Figure 8d** shows estimates of the dependence of mean and median exit angles and detected intensity as a function of fiber-probe separation.

[0068] **Figure 9** shows OBM images of ex-vivo rat colon, acquired with 530nm LEDs. **(a)** and **(b)** are images acquired with left and right illumination after core removal (zoomed insets are before core removal). Exposure time per image is 2ms. **(c)** is sum image. **(d)** is difference image. Note: panel (d) contains negative values, meaning its zero level is gray. Field of view = 100μm. Depth = 70μm. Resolution = 6μm.

[0069] **Figure 10** shows OBM images of various regions of ex-vivo rat intestine, acquired with 530nm LEDs. Top row is sum images; bottom row is difference (phase gradient) images. Panels **(a)-(d)** show small intestine (note high resolution content in panel (b); panel (d) highlights epithelial villi). Panels **(e)-(h)** show large intestine (panel (f) highlights a crease in tissue; panel (h) presumably highlights a blood vessel). Note much higher contrast of phase gradient images compared to relative featurelessness of absorption images. Field of view = 600μm. Depth = 70μm.

[0070] **Figure 11a and Figure 11b** are OBM phase-gradient images of mouse small-intestine villi (same sample as in Figure 10) acquired with Hopkins rod-lens. FIELD OF VIEW is 500μm. Working distance is 50μm. A and B are two frames from a movie taken

while focus depth was being adjusted. Green LED illumination is delivered through two diametrically opposed 1mm fibers.

[0071] **Figure 12** shows (a) a phase-gradient image of a 45μm bead embedded in agarose made scattering by 2μm beads (also apparent, demonstrating resolution), (b) phase gradient appears linear. Simultaneous (c) amplitude and (d) phase-gradient images of blood flow in chick embryo (movement in (c) is highlighted in (d)); scale bar = 75μm. Simultaneous (e) amplitude and (f) phase-gradient images acquired with higher magnification fiber bundle; scale bar = 30μm. (g) and (h) are high magnification phase-gradient images when blood flow is slowed (Note different shapes of blood cells); scale bar = 30μm.

[0072] **Figure 13** shows amplitude (top) and phase-gradient (bottom) mosaic of 300 frames acquired at 17.3 frames per second while manually scanning across a chick embryo. Scale bar = 75 μm. Box indicates single frame size (186 x 139 μm).

[0073] **Figure 14** shows a labeled version of Figure 12d. Visible morphological structures include (l) crypt lumens, (c) epithelial cells, (ap) apical border of epithelial cells, (bl) basolateral border of epithelial cells, and (lp) lamina propia.

[0074] **Figure 15** shows optical biopsy results. Panels **(a-c)** are images of **(a)** large, **(b)** mid and **(c)** small intestine regions. Note villi in panel (c) (FIELD OF VIEW = 600 μm). Panels **(d)** and **(e)** are images of **(d)** large, and **(e)** small intestine at higher magnification (Field of view = 240 μm). Note crypts in panel **(d)**.

[0075] **Figure 16** shows a comparison of added versus subtracted raw OBM images of a 45 μm polystyrene bead. Raw images under oblique back-illumination from two opposing directions are shown in Figures 16(a) and 16(b). Addition of (a) and (b) cancels phase gradient contrast and emphasizes absorption, as demonstrated in Figure 16(c). Subtraction of (a) and (b) cancels absorption contrast and emphasizes phase gradients, as shown in Figure 16(d).

[0076] **Figure 17** shows a demonstration of apparent axial resolution of OBM.

[0077] **Figure 18** shows images of mouse lung and liver. Fixed mouse liver imaged under phase gradient contrast OBM (a) and (b). Fixed mouse lung cross-section imaged under phase gradient contrast OBM (c) and (d).

[0078] **Figure 19** shows images of rat flank skin. (a) is an *en face* view 50 μm below the surface showing wavy collagen strands. (b) is an *en face* view 100 μm below the surface showing a cluster of adipocytes (stars).

[0079] **Figures 20** shows OBM penetration depth.

[0080] **Figure 21** shows shows OBM penetration depth.

[0081] **Figure 22** shows a comparison of OBM with epi-illumination reflection contrast.

[0082] **Figure 23 (a-c)** shows a dual-camera, multi-wavelength setup.

[0083] **Figure 24** shows OBM using a single illumination wavelength. (a) and (b) show individual phase gradient contrast OBM images under simultaneous red and NIR illumination. (c) shows a capillary visualized by a sliding 3-frame temporal variance filter. Multiplying frames in Figure 24(a, and b) by capillary mask Figure 24(c) yields the images in Figures 24(d, and e), where individual red blood cells are easily distinguished (white arrows). Figure 24(f) shows another capillary with a more tortuous path extracted from a separate segment from the same video as Figures 24(a-e).

[0084] **Figure 25** shows simultaneous co-registered multi-wavelength OBM. Phase gradient (a and c) images and corresponding amplitude images (b and d) were obtained under red and blue/green illumination, respectively.

[0085] **Figure 26** shows schematics of different OBM illumination configurations. (a) illustrates llumination delivered via fiber optic conduits in contact with the tissue surface. (b) illustrates illumination delivered by light sources not in contact with the tissue surface.

[0086] **Figure 27** shows a comparison of OBM with fiber-mediated illumination in contact with the tissue versus non-fiber-mediated illumination not in contact with the tissue. (a) and (b) are OBM images acquired with fiber-delivered LED light (~650nm). (c) and (d) are OBM images acquired with a 6-element LED flashlight held approximately 2 inches from the sample surface.

[0087] **Figure 28** shows a design of OBM based on illumination and detection through a common microscope objective.

[0088] **Figure 29** shows a design of miniature OBM endomicroscope probe.

DETAILED DESCRIPTION

[0089] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular. Generally, nomenclatures used in connection with microscopy, imaging and endoscopy, described herein are those well-known and commonly used in the art. Certain references and other documents cited herein are expressly incorporated herein by reference. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

[0090] The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., L. V. Wang and H.-i Wu, Biomedical Optics: Principles and Imaging, 1st ed. Wiley-Interscience, 2007; J. C. Mertz, Introduction to Optical Microscopy, Roberts and Company Publishers, 2009.

[0091] Before the present devices, systems, methods, and other embodiments are disclosed and described, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

[0092] The term "comprising" as used herein is synonymous with "including" or "containing", and is inclusive or open-ended and does not exclude additional, unrecited members, elements or method steps.

[0093] This disclosure provides a new phase contrast technique that works in a reflected light geometry and is thus amenable to use on tissues that for one or more reasons are not amenable to transmission lighting. One non-limiting example is endoscopy applications. This method is sometimes referred to herein as "oblique back-illumination microscopy, or "OBM". OBM requires no tissue labeling and provides high resolution

differential interference contrast (DIC)-like images of sub-surface sample morphology in an epi-detection configuration.

[0094] In some embodiments the methods, apparatus, and systems provided herein can be used for optical biopsies of tissue.

[0095] In certain embodiments OBM uses standard wide field detection optics. That is, light is projected from an object plane to an image plane with a series of lenses, and it is then detected with a camera (for example, a CCD or CMOS). In the case of an endoscope, some extra relay optics may be introduced, such as an imaging fiber bundle or a Hopkins rod-lens. This is not necessarily any different than standard wide field endoscopy. Where OBM differs is in the illumination path. A schematic is shown in Fig. 1a, depicting the distal end of an OBM endoscope. As illustrated, the illumination is launched into the tissue sample **(201)** via an off-axis optical fiber **(101)** (or a pair, as shown in Fig 1b **(101 and 102)**). Microscopic resolution endoscopes (or endomicroscopes) are invariably contact mode, or near contact mode. In such configurations, illumination light reflected directly from the sample surface is not detectable (this can be further ensured by, e.g., recessing the illumination fibers a bit). The only light that is detected is illumination light that has been multiply scattered to such a degree that it is re-directed toward the sample surface and incident upon a photo detector array or image conduit **(301)**. In this manner, the object plane (defined as the plane that is in focus with respect to the detection optics, and indicated by a hatched line in the Figure 1a), is back-illuminated. A critical point to note here is that, because the illumination source is off-axis, the back-illumination flux at the object plane is directed, on average, not quite vertically but with a slight tilt away from the illumination source. That is, the back-illumination is oblique, which results in OBM in this configuration.

[0096] Oblique illumination has been used in other contexts to obtain phase contrast, or, more precisely, phase gradient contrast. For example, the simple misalignment of the condenser in a standard transmission wide field microscope leads to phase gradient contrast. The reason for this can be understood intuitively from Figures 1c-1e. Oblique illumination locally originating from the object plane is partially blocked by the detection aperture **(351)**. The presence of a local phase slope (gradient) **(221)** in the object **(201)** refracts the illumination one way or the other, depending on the sign of the slope, thereby leading to a respective increase or decrease in the detected intensity. Thus, phase slopes are converted to intensity variations of related sign, yielding phase gradient contrast imaging. As shown

schematically in Figures 1c-1e, the aperture in the detection optics plays a role in creating phase gradient contrast images. Indeed, the inventors have shown in other contexts that phase gradient contrast can be further enhanced by combining oblique illumination with oblique detection [36]. Accordingly, in some embodiments of the methods and apparatus of this disclosure oblique illumination and oblique detection are combined.

[0097] While illumination with a single off-axis source is enough to achieve phase-gradient contrast in an oblique back illumination configuration, the inventors have also discovered that a second off-axis source approximately diametrically opposed to the first (Fig. 1b) allows for certain useful variations on the technique. The phase gradient contrast resulting from the second source has opposite sign to that resulting from the first. The acquisition of two raw images, one from each source, yields many useful features to certain embodiments of the methods and apparatus disclosed herein, such as without limitation: 1) in some embodiments the difference image enhances phase gradient contrast and cancels any absorption contrast; 2) in some embodiments the sum image reveals absorption contrast and cancels any phase gradient contrast; 3) in some embodiments the difference image rejects uniform background, providing axial resolution; and 4) the use of dual illumination provides, in certain embodiments, a more homogenous net distribution of light in the processed images.

[0098] Certain geometrical parameters of the OBM technique are shown in Figure 2. For simplicity, the figure only includes a single light source (101). This figure serves only as a schematic to depict how the illumination light can be roughly mimicked by a virtual light source a distance l_s^* below the physical light source. This approximation is reasonably valid for offset distances ρ on the order of l_s^* or greater.

[0099] OBM embodiments that utilize two light sources are based on the acquisition of two images, with illumination states of opposing obliquity, as obtained, for example, when the illumination sources have equal but opposing offsets from the optical axis. It is believed that the back-illumination is not only oblique but also non-uniform in intensity (see Fig 2). That is, regions of the image farther from the source will appear dimmer. To compensate for this intensity drop-off, as well as the possibility of non-equal source powers, the raw images are, in some embodiments, individually "flattened" and normalized (e.g. Figs 3a and 3b). This is done numerically prior to their subtraction or addition in embodiments in which images are combined. (Recall that image subtraction leads to phase gradient contrast, while image addition leads to absorption contrast.)

[00100] An useful property of the subtracted image is that it contains no uniform background. In fact, it contains no uniform signal at all, background or otherwise. Said differently, the difference image only contains non-zero spatial frequencies because it reveals phase derivatives instead of absolute phases. But by definition, non-zero lateral spatial frequencies in a standard microscope image are axially resolved [37]. That is, all the structure observed in Fig 3d. must arise from regions of the sample that are (more or less) close to the focal plane (in this case, 70μm deep). Because phase derivative images are intrinsically axially resolved, in some embodiments of the methods and apparatus of this disclosure the axial resolution is controlled. In some embodiments a particular axial resolution is specified by a user. In some embodiments high-pass spatial filters are used to provide tighter axial resolution (at the expense of low frequency image content). In some embodiments the types of filters, such as wavelets, are used.

[00101] OBM works in the reflection direction, like OCT. And yet, OBM images are not at all similar to OCT images. Instead, they are similar to DIC images, which are obtained in the transmission direction. Part of the reason for that is that OBM reveals phase gradients as opposed to absolute phase. But another more fundamental reason is that OBM is actually a transmission (i.e. trans-illumination) microscope in disguise.

[00102] OCT is based on the detection of reflected light (shown schematically in Figure 4b) whereas DIC is based on the detection of transmitted light (shown schematically in Figure 4d). For a tissue to cause reflection, it must exhibit index of refraction variations with very high axial spatial frequency (at least as high as 2k, where $k=2\pi/\lambda$ is the light wavenumber and λ the light wavelength in the medium). In contrast, for a tissue to deflect a beam by a small angle θ (less than 1), it need only exhibit lateral index of refraction spatial frequencies on the order $k\theta$. Thus, OCT reveals large axial spatial frequencies whereas DIC reveals much smaller lateral spatial frequencies. These differences are demonstrated by the images in Figure 4a (OCT) and 4c (a DIC image that is similar in image quality to OBM).

[00103] There are several useful consequences to working in the trans-illumination direction (which explains the popularity of transmission-based optical microscopes). The main advantage is that light scattering in tissue is dominantly forward directed, because scattering structures in tissue are typically micron scale or larger [40]. As noted, to obtain direct backward scattering from tissue requires structures that exhibit very high axial spatial frequencies, such as abrupt interfaces or tiny punctate scatterers. OCT beautifully reveals

such high axial-frequency structures, but it cannot reveal more subtle, lower frequency features that are prevalent in tissue. In contrast, trans-illumination microscopes, since they are sensitive to even minute deflections of light, do reveal low frequency features (in addition to high frequency features). This makes OBM very useful and enables its use, in some embodiments, as an effective tool for assessing tissue pathology. Indeed, the differences between healthy and diseased tissue are often very subtle.

[00104] Several parameters can affect signal to noise ratio (SNR), including shot noise. For example, OBM embodiments that combine images involve subtracting or adding two images of roughly equal intensity. In both cases, the shot noise in the final image is increased by a factor of the square root of 2. To maximize the SNR associated with shot noise one need simply maximize the amount of detected light. OBM is not a fluorescence technique, so scattered light is plentiful. Even for very short exposure times (~1 millisecond), a camera used to detect images generated with OBM can be operated close to saturation. Camera readout and dark noise play essentially no role in this regime. In some embodiments, camera pixel well capacity is as large as feasible. In other words, high-end scientific cameras designed for fluorescence imaging are not ideal for some embodiments. Instead, what is most useful for such embodiments are simple machine-vision cameras. In addition to featuring large well capacities ($>10^5$ e-), these offer additional benefits of high speed (>100fps), and low cost. An example currently on the market is cameras manufactured by Photonfocus AG.

[00105] In some embodiments of the methods and apparatus disclosed herein, real-time image information is captured at a near video rate. As described above, OBM is a two-shot system, meaning the maximum OBM frame rate is half the camera frame rate. Machine vision speeds easily satisfy real-time criteria; however, if there is a time delay between the two shots, and if the tissue (or probe) is rapidly moving or changing somehow, then motion artifacts could occur. Thus, in some embodiments a double-shutter camera (e.g. Pixelfy, Cooke Corp.) is used, which acquires images pairwise, with essentially zero inter-pair frame delay (<5μs). To reduce motion during the frame exposures fast exposures (and a lot of light) are used in some embodiments. In some embodiments the two exposures are merged into a single exposure.

[00106] In some embodiments of the two-shot technique, the shots are discriminated by time. In alternative embodiments other parameters are used to discriminate the shots, such as wavelength or polarization. In the former case, the left and right illumination sources

provide light of different colors, allowing a spectral separation within a same camera frame, for example. In the latter case, the left and right illumination sources are orthogonally polarized. The inventors have experimentally verified that polarization is partially preserved even after multiple scattering in a retro-reflection geometry (provided length scales are not too long), in agreement with previous studies [42–45]. This demonstrates the feasibility of polarization-based separation of both shots within the same camera frame.

[00107] It is believed that OBM will be particularly useful in endoscope configurations. However, in some embodiments OBM is deployed in a freestanding microscope configuration, for example. In some embodiments the OBM endoscope is a portable, standalone device. In some embodiments a fiber bundle is used to collect and relay the image to a photo detector array (or camera sensor).

[00108] In some embodiments, uneven spacing of the fiber cores of the image conduit causes raw images to appear corrupted by irregular sampling. This can be addressed with a fast image processing algorithm to very effectively remove these core-spacing related artifacts. The algorithm is based on a nonlinear, iterative segmentation-interpolation strategy that maintains high spatial resolution (described in detail in [35]). This algorithm, along with the two-shot triggering and data-transfer protocols necessary to operate HiLo microscopy are already coded in CUDA to run on a graphical processing unit (GPU). Reference 35 is hereby incorporated herein by reference. In some embodiments these are incorporated into an OBM system, apparatus, or method of this disclosure.

[00109] In some embodiments OBM is operated with LED illumination. This is useful for several embodiments. LEDs are inexpensive, robust, available in a variety of wavelengths, and can be rapidly turned on and off (30kHz measured). Moreover, they are spatiotemporally incoherent, meaning they do not produce speckle. However, incoherence also has a drawback. Incoherent light, which occupies a large "phase space", cannot be compressed (focused) into the small phase-space of a fiber without incurring significant power loss. Based on simple étendue arguments [37], the maximum coupling efficiency of LED light into a fiber is given by roughly $\mathrm{NA}_{fiber}^2(A_{fiber}/A_{LED})$, where NA_{fiber} and A_{fiber} are the fiber NA and area, and A_{LED} is the LED area. To accommodate this, in some embodiments a large area, multimode fiber is used. For example, in some embodiments a 400mW Luxeon LED coupled into a 1000μm fiber core delivers almost 30mW.

[00110] In some embodiments the LED(s) are housed in a module. For enhanced versatility with respect to wavelength in some embodiments the our module will houses several different color LEDs (see Fig 5), allowing the user to select different colors or perform multicolor imaging. The design of this module is similar in concept to the Zeiss Colibri™, with the difference that the LEDs are coupled into an optical fiber rather than a light pipe. In some embodiments two of these modules are used for a single OBM apparatus of this disclosure (left/right illumination). In some embodiments the control of the LEDs can be both analog (power) and digital (on/off). In some embodiments the housing can accept different size fibers via a standard interconnect.

[00111] In some embodiments the imaging fiber bundle, with its miniaturized distal imaging optics, is threaded through the accessory port of a probe, such as a standard flexible colonoscope. In some embodiments the diameter of this port is about 3.2mm. In some embodiments, the diameter of the distal optics is 2.8mm.

[00112] Some representative non-limiting examples of the distal end of an endoscope configuration of OBM are shown in Figure 6. Figure 6a is a lateral view, showing the distal end of the probe **(601)**, two illumination optical fibers **(101 and 102)**, and a light conduit **(301)**. In one embodiment, graded-index plastic optical fibers (Thorlabs or other), are heat-molded at their distal end into a more space conserving geometry (sufficient A_{fiber} is maintained for adequate throughput) (Figure 6b). As alternative plan, multiple thinner fibers arranged in arcs at the distal end and circular bundles at the proximal end are used (Figure 6c). Finally, an intriguing possibility is to incorporate mini LEDs directly into the endoscope housing itself. This would require connecting with electrical wires, but, in the end, may deliver the most optical power per utilized space. No heat sinks are necessary since the power requirements are very modest.

[00113] In some embodiments, lasers are used as the illumination source. These can deliver more power into thin optical fibers than LEDs, however they have the disadvantage of producing speckle, thereby possibly leading to image granularity. In other embodiments, superluminescent diodes (SLED) are used as the illumination source These are similar to lasers in that they can deliver more power into thin optical fibers than LEDs. Because they produce no speckle they can be preferable to lasers in certain embodiments.

[00114] A characteristic of OBM is that the illumination can be decoupled from the detection optics, such that the illumination does not go through the detection optics, as it does in many epi-imaging devices. This is useful because it avoids spurious back-reflections from glass interfaces, etc. It also makes extended image relay optics unnecessary in certain embodiments. Thus, in some embodiments a proximal camera is used in the OBM apparatus. In other embodiments the proximal camera is replaced by a miniaturized distal camera, such as by way of example one mounted directly at the end of the endoscope. Thus, in some embodiments the apparatus comprises an all-electric coupled distal end (illumination and detection).

[00115] Depending on their application, endoscopes can be flexible or rigid. Rigid endoscopes can be larger than flexible endoscopes—up to several millimeters in diameter. In some embodiments of a rigid endoscope the length scales involved are larger and longer illumination wavelengths are used. Fortunately, near infra-red LEDs are readily available.

[00116] A key source of usefulness of a rigid Hopkins-type endoscope is that, because it is based on simple lenses and free-space propagation, optical phase is preserved from the object plane to the detector plane. Moreover, the aperture plane can be accessed and in some embodiments oblique illumination is combined with complementary oblique detection [36]. In some embodiments oblique detection is achieved by introducing beam half-blocks in the detection aperture plane, and switching sides depending on which LED is illuminated. In some embodiments this is done in a single shot and with no moving parts. Two exemplary strategies for this are illustrated in Fig. 7. In both strategies, a Wollaston prism (or other beam separating mechanism) is placed at the aperture plane **(391)**, which splits the beam in two and projects the two images simultaneously onto the camera. In the case of polarization discrimination (Fig. 7a), simultaneous cross-polarized illumination is used (as noted above, polarization is partially maintained in tissue). In the case of spectral discrimination (Fig. 7b), simultaneous two-color illumination, which is assumed to be randomly polarized is used. Half-aperture blocks can then be inserted, either in the form of polarizers (Fig. 7c) or spectral bandpass filters (Fig. 7d), to confer some degree of obliqueness to the detection. It should be noted that, while oblique detection enhances phase gradient contrast, it degrades absorption contrast. Depending on how important the latter feature is in any particular embodiment, full oblique detection can or cannot be used as the user chooses.

[00117] This disclosure also provides methods of creating a phase contrast image. In some embodiments the method comprises illuminating the target region of a sample with a first light source to provide a first oblique back illumination of the target region of the sample, and detecting a first phase contrast image from light originating from the first light source and back illuminating the target region of the sample.

[00118] As used herein a "target region" is the portion of a sample that from which an image is desired. Alternatively, the "target region" is the portion of the sample from which an image is created and/or captured.

[00119] As used herein, "oblique back illumination" means illumination that results from the re-direction of light into the backward direction by a multiple scattering process within a tissue. Oblique back illumination is created by an off-axis illumination source. As a result, the back-illumination flux at the object plane is directed, on average, not quite perpendicular to the plane but with a slight tilt away from the illumination source. Oblique back illumination may be created with light sources that are in contact with a sample. Oblique back illumination may also be created by light sources that are not in contact with the sample. In some embodiments one or more of each type of light source are combined in an apparatus or used in a method.

[00120] In some embodiments of the method, the method further comprises illuminating the sample with a second light source to provide a second oblique back illumination of the target region of the sample, and detecting a second phase contrast image from light originating from the second light source and back illuminating the target region of the sample. In some embodiments the method further comprises creating a difference contrast image of the target region of the sample by subtracting the second phase contrast image of the target region of the sample from the first phase contrast image of the target region of the sample. In some embodiments the method further comprises creating an absorption contrast image of the target region of the sample by adding the first phase contrast image of the target region of the sample to the second phase contrast image of the target region of the sample. In some embodiments the method the difference contrast image and the absorption contrast image are analyzed together to infer at least one property of the sample. In some embodiments one of the difference contrast image and the absorption contrast image is analyzed in a way that the other is not in order to infer at least one property of the sample.

[00121] In some embodiments of the method, the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source and back illuminating the target region of the sample are different. That is, the light source is off axis, meaning among other things that it is delivered independently of the detection optics. In some embodiments of the method, the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are different. In some embodiments of the method, the axis of detection of light originating from the first light source and back illuminating the target region of the sample, the axis of detection of light originating from the second light source back illuminating the target region of the sample are different. Note that in such embodiments the illumination and the detection are both oblique. In some embodiments of the method, the axis of detection of light originating from the first light source and back illuminating the target region of the sample and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are the same.

[00122] In some embodiments of the method, the first and second light sources illuminate the sample with light of different distinguishable wavelengths. In some embodiments of the method, the first and second light sources illuminate the sample with distinguishable orthogonally polarized light. In both of these types of embodiments it is possible to detect light from the first and second light sources simultaneously, although the method need not be conducted that way.

[00123] In some embodiments the method comprises detecting the first and second images during first and second non-overlapping time intervals. In such embodiments the wavelength of light from the first and second light sources can be (but need not be) the same.

[00124] In some embodiments of the method, the wavelength of the light from at least one of the first and second light sources is from 0.2 to 300 μm, from 0.2 to 1 μm, from 0.4 to 0.7 μm, from 0.2 to 0.3 μm, from 0.3 to 0.4 μm, from 0.4 to 0.5 μm, from 0.5 to 0.6 μm, or from 0.6 to 0.7 μm. In some embodiments of the method, the light source is selected from a light-emitting diode (LED), a laser, or a superluminescent diode (SLED). In some embodiments of the method, the detecting is by a photo detector array. In some embodiments of the method, the photo detector array is a charge coupled device (CCD) or a CMOS (complementary metal oxide semiconductor) camera sensor. In some embodiments the

method comprises using an optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample.

[00125] In some embodiments of the method, the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaced by from about 0.2 mm to about 3 mm, from about 0.5 mm to about 2.5 mm, from about 1 mm to about 2 mm, from about 1.5 mm to about 2.5 mm, or from about 2 mm to about 3 mm. In some embodiments axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaced by about 0.2 mm, about 0.3 mm, about 0.4 mm, about 0.5 mm, about 1.0 mm, about 1.5 mm, about 1.75 mm, about 2.0 mm, about 2.25 mm, about 2.5 mm, about 3.0 mm, about 3.5 mm, about 4.0 mm, or about 5.0 mm. In some embodiments of the method, the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaced by from about 0.2 mm to about 3 mm, from about 0.5 mm to about 2.5 mm, from about 1 mm to about 2 mm, from about 1.5 mm to about 2.5 mm, or from about 2 mm to about 3 mm.. In some embodiments axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaced by about 0.2 mm, about 0.3 mm, about 0.4 mm, about 0.5 mm, about 1.0 mm, about 1.5 mm, about 1.75 mm, about 2.0 mm, about 2.25 mm, about 2.5 mm, about 3.0 mm, about 3.5 mm, about 4.0 mm, or about 5.0 mm. In some embodiments the displacement of the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source, and the displacement of the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are the same. In some embodiments the displacement of the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source, and the displacement of the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are different.

[00126] In some embodiments of the method, the object plane of the target region is from the sample surface to about 350 μm below the surface of the sample, from about 100 to about 300 μm below the surface of the sample, from about 150 to about 250 μm below the surface of the sample, from about 175 to about 225 μm below the surface of the sample. In some embodiments it is below the sample surface, greater than about 5 μm below the surface

of the sample, greater than about 10 μm below the surface of the sample, greater than about 15 μm below the surface of the sample, greater than about 20 μm below the surface of the sample, greater than about 25 μm below the surface of the sample, greater than about 30 μm below the surface of the sample, greater than about 35 μm below the surface of the sample, greater than about 40 μm below the surface of the sample, greater than about 45 μm below the surface of the sample, greater than about 50 μm below the surface of the sample, greater than about 75 μm below the surface of the sample, greater than about 100 μm below the surface of the sample, greater than about 150 μm below the surface of the sample, greater than about 200 μm below the surface of the sample, greater than about 250 μm below the surface of the sample, greater than about 300 μm below the surface of the sample, or greater than about 350 μm below the surface of the sample.

[00127] In some embodiments of the method, the lateral resolution of the image is from about 0.3 μm to about 2 μm, the lateral resolution of the image is from about 1 μm to about 3 μm, the lateral resolution of the image is from about 2 μm to about 3 μm, the lateral resolution of the image is from about 2 μm to about 5 μm, the lateral resolution of the image is from about 2 μm to about 10 μm. In some embodiments of the method, the lateral resolution of the image is at least about 9 μm, the lateral resolution of the image is at least about 8 μm, the lateral resolution of the image is at least about 7 μm, the lateral resolution of the image is at least about 6 μm, the lateral resolution of the image is at least about 5 μm, the lateral resolution of the image is at least about 4 μm, the lateral resolution of the image is at least about 3 μm, the lateral resolution of the image is at least about 2 μm, the lateral resolution of the image is at least about 1 μm, the lateral resolution of the image is at least about 0.9 μm, the lateral resolution of the image is at least about 0.8 μm, the lateral resolution of the image is at least about 0.7 μm, the lateral resolution of the image is at least about 0.6 μm, the lateral resolution of the image is at least about 0.5 μm, the lateral resolution of the image is at least about 0.4 μm, or the lateral resolution of the image is at least about 0.3 μm.

[00128] In some embodiments of the method, the difference image is axially resolved.

[00129] In some embodiments the method further comprises obtaining a series of two or more images and combining the images to provide a composite image larger than the field of view a single image.

[00130] In some embodiments the method comprises creating a phase contrast image of gastrointestinal tissue and examining the tissue to assess at least one of the presence and the absence of indicators of a disease. In some embodiments the gastrointestinal tissue is colonic mucosa disease is at least one of hyperplasia and adenomatous changes.

[00131] Also provided herein are apparatus for creating a phase contrast image of a sample. In some embodiments the apparatus comprises a first light conduit, a photo detector array or an image conduit, and a distal end, wherein a distal end of the light conduit and the distal end of the photo detector array or image conduit extend to the distal end of the probe. In some embodiments the distal end of the light conduit and the distal end of the photo detector array or image conduit extend through and end at the distal end of the probe. In some embodiments at least one of the distal end of the light conduit and the distal end of the photo detector or image conduit is recessed from the distal end of the probe.

[00132] Two examples of OBM designed that use recessed illumination are shown in Figures 28 and 29. Figure 28 shows a design of OBM based on illumination and detection through a common microscope objective. Collimated, oblique illumination beams are (roughly) focused to off-axis spots on the sample surface. Imaging of the sample is restricted by a field stop (somewhere in the detection optics) to a field of view that does not encompass the illumination spots. Figure 29 shows a design of miniature OBM endomicroscope probe. Proximal end is shown on left, where two collimated, off-axis illumination beams are (roughly) focused into an imaging fiber bundle with an objective (OBJ). Distal end is shown on right, where a GRIN lens is optically cemented to the fiber bundle, centered by a clear support ring. Imaging is performed in the reverse direction with the GRIN lens and central fiber-bundle cores. Total probe diameter can be as small as 1mm.

[00133] In some embodiments the apparatus further comprises a first optical radiation source connected to or imaged to a proximal end of the light conduit. In some embodiments the light conduit, the photo detector array or image conduit, and the distal end of the probe are configured to back illuminate the target region of a sample in contact or near contact with the distal end of the probe with a light from the first light source to provide a first oblique back illumination of the target region of the sample, and to detect a first phase contrast image from light originating from the first light source and back illuminating the target region of the sample. In some embodiments of the apparatus, the probe comprises a photo detector array.

In some embodiments of the apparatus, the probe comprises an image conduit and a proximal end of the image conduit is connected to or imaged to a photo detector array.

[00134] In some embodiments of the apparatus the probe further comprises a second light conduit, wherein a distal end of the second light conduit extends through and ends at the distal end of the probe. In some embodiments a second optical radiation source connected to or imaged to a proximal end of the second light conduit. In some embodiments the second light conduit, the photo detector or image conduit, and the distal end of the probe are configured to illuminate the target region of a sample in contact or ear contact with the distal end of the probe with a light from the second light source to provide a second oblique back illumination of the target region of the sample, and to detect a second phase contrast image from light originating from the second light source and back illuminating the target region of the sample.

[00135] In some embodiments of the apparatus, the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source and back illuminating the target region of the sample are different. In some embodiments of the apparatus, the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are different.

[00136] In some embodiments of the apparatus, the axis of detection of light originating from the first light source and back illuminating the target region of the sample and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are different. In some embodiments of the apparatus, the axis of detection of light originating from the first light source and back illuminating the target region of the sample and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are the same. In some embodiments of the apparatus, the wavelength of the light from the first light source and the wavelength of light from the second light source are different. In some embodiments the apparatus is configured to detect the first and second images during first and second non-overlapping time intervals. In some embodiments the apparatus is configured to detect the first and second images during first and second overlapping time intervals. In some embodiments the apparatus is configured for illumination of the sample by the first and second light sources with light of different distinguishable wavelengths. In some

embodiments the apparatus is configured for illumination of the sample by the first and second light sources with orthogonally polarized light.

[00137] In some embodiments of the apparatus the first and second light sources are capable of providing illumination at a wavelength of from 0.2 to 300 μm, from 0.2 to 1 μm, from 0.4 to 0.7 μm, from 0.2 to 0.3 μm, from 0.3 to 0.4 μm, from 0.4 to 0.5 μm, from 0.5 to 0.6 μm, or from 0.6 to 0.7 μm. In some embodiments of the apparatus the first and second light sources are capable of providing illumination at a wavelength that comprises a range selected from at least one of from 0.2 to 300 μm, from 0.2 to 1 μm, from 0.4 to 0.7 μm, from 0.2 to 0.3 μm, from 0.3 to 0.4 μm, from 0.4 to 0.5 μm, from 0.5 to 0.6 μm, or from 0.6 to 0.7 μm. In some embodiments of the apparatus, the light source is selected from a light-emitting diode (LED), a laser, or a superluminescent diode (SLED). In some embodiments of the method, the detecting is by a photo detector array. In some embodiments of the method, the photo detector array is a charge coupled device (CCD) or a CMOS (complementary metal oxide semiconductor) camera sensor. In some embodiments the method comprises using an optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample.

[00138] In some embodiments of the apparatus the light source is selected from a light-emitting diode (LED), a laser, or a superluminescent diode (SLED). In some embodiments the apparatus comprises a photo detector array. In some embodiments the photo detector array is a charge coupled device (CCD) or a CMOS (complementary metal oxide semiconductor) camera sensor.

[00139] In some embodiments of the apparatus, the apparatus comprises an optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample.

[00140] In some embodiments of the apparatus, the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaced by from about 0.2 mm to about 3 mm, from about 0.5 mm to about 2.5 mm, from about 1 mm to about 2 mm, from about 1.5 mm to about 2.5 mm, or from about 2 mm to about 3 mm. In some embodiments the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaced by about 0.2 mm, about 0.3 mm, about 0.4 mm, about 0.5 mm, about 1.0 mm,

about 1.5 mm, about 1.75 mm, about 2.0 mm, about 2.25 mm, about 2.5 mm, about 3.0 mm, about 3.5 mm, about 4.0 mm, or about 5.0 mm. In some embodiments of the apparatus, the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaced by from about 0.2 mm to about 3 mm, from about 0.5 mm to about 2.5 mm, from about 1 mm to about 2 mm, from about 1.5 mm to about 2.5 mm, or from about 2 mm to about 3 mm. In some embodiments the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaced by about 0.2 mm, about 0.3 mm, about 0.4 mm, about 0.5 mm, about 1.0 mm, about 1.5 mm, about 1.75 mm, about 2.0 mm, about 2.25 mm, about 2.5 mm, about 3.0 mm, about 3.5 mm, about 4.0 mm, or about 5.0 mm. In some embodiments the displacement of the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source, and the displacement of the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are the same. In some embodiments the displacement of the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source, and the displacement of the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are different.

[00141] In some embodiments of the apparatus, the apparatus is configured to allow for imaging a sample in which the object plane of the target region is from the sample surface to about 350 μm below the surface of the sample, from about 100 to about 300 μm below the surface of the sample, from about 150 to about 250 μm below the surface of the sample, from about 175 to about 225 μm below the surface of the sample. In some embodiments it is below the sample surface, greater than about 5 μm below the surface of the sample, greater than about 10 μm below the surface of the sample, greater than about 15 μm below the surface of the sample, greater than about 20 μm below the surface of the sample, greater than about 25 μm below the surface of the sample, greater than about 30 μm below the surface of the sample, greater than about 35 μm below the surface of the sample, greater than about 40 μm below the surface of the sample, greater than about 45 μm below the surface of the sample, greater than about 50 μm below the surface of the sample, greater than about 75 μm below the surface of the sample, greater than about 100 μm below the surface of the sample, greater than about 150 μm below the surface of the sample, greater than about 200 μm below the surface of the sample, greater than about 250 μm below the surface of the sample, greater

than about 300 μm below the surface of the sample, or greater than about 350 μm below the surface of the sample.

[00142] In some embodiments of the apparatus, the apparatus is configured to create images in which the lateral resolution of the image is from about 0.3 μm to about 2 μm, the lateral resolution of the image is from about 1 μm to about 3 μm, the lateral resolution of the image is from about 2 μm to about 3 μm, the lateral resolution of the image is from about 2 μm to about 5 μm, the lateral resolution of the image is from about 2 μm to about 10 μm. In some embodiments of the apparatus, the lateral resolution of the image is at least about 9 μm, the lateral resolution of the image is at least about 8 μm, the lateral resolution of the image is at least about 7 μm, the lateral resolution of the image is at least about 6 μm, the lateral resolution of the image is at least about 5 μm, the lateral resolution of the image is at least about 4 μm, the lateral resolution of the image is at least about 3 μm, the lateral resolution of the image is at least about 2 μm, the lateral resolution of the image is at least about 1 μm, the lateral resolution of the image is at least about 0.9 μm, the lateral resolution of the image is at least about 0.8 μm, the lateral resolution of the image is at least about 0.7 μm, the lateral resolution of the image is at least about 0.6 μm, the lateral resolution of the image is at least about 0.5 μm, the lateral resolution of the image is at least about 0.4 μm, or the lateral resolution of the image is at least about 0.3 μm.

[00143] Also provided is an endoscope, comprising an apparatus according to this disclosure. In some embodiments the endoscope is portable.

[00144] Also provided is a system, comprising an apparatus according to this disclosure and a processor for processing images obtained from the apparatus.

[00145] Method examples described herein can be machine-implemented or computer-implemented at least in part. Some examples can include a tangible computer-readable medium or machine-readable medium encoded with instructions operable to 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, the code may be tangibly stored on one or more volatile or non-volatile computer-readable media during execution or at other times. These computer-readable media may

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, random access memories (RAMs), read only memories (ROMs), and the like.

EXAMPLES

Example 1: Monte Carlo Analysis of OBM

[00146] As described herein, OBM uses an off axis light source. Illumination light that is multiply scattered in the object is re-directed toward the sample surface and is detected. In this manner, the object plane (defined as the plane that is in focus with respect to the detection optics), is back-illuminated. Because the illumination source is off-axis, the back-illumination flux at the object plane is directed, on average, not quite vertically but with a slight tilt away from the illumination source. That is, the back-illumination is oblique. The illumination source is mimicked by an effective virtual source a distance l^*_s directly below it (l^*_s being the transport scattering length of the medium) (Figure 2). The overlap of the illumination and detection spread functions of the illuminating and detected light is referred to as a "photon banana". Understanding the distribution of photons in this system will help characterize the geometrical constraints of OBM. In particular, it will help define how far off axis can and/or should the light source be located.

[00147] Standard Monte Carlo simulations of light propagation in scattering tissue were performed to validate our claim that light launched into scattering tissue via an off-axis light conduit (e.g., optical fiber) undergoes multiple scattering that leads to an average tilt in the back illumination of an on-axis target region. Only a single source is shown, for simplicity. For our simulations, we chose system parameters that mimicked our OBM endomicroscope device, such as the illumination fiber diameter and numerical aperture (NA_{ill}) the detection field of view (FOV) and numerical aperture (NA_{det}). Figures 8a and 8b depict the photon banana, corresponding to the density distribution of photons that are both launched into the tissue by the optical fiber and detected by the endomicroscope distal optics. We also chose scattering parameters l_s and l_s^* that mimicked biological tissue such as brain tissue. The number of photons in our simulation was chosen to obtain reasonable statistical accuracy in our plots. Figure 8b depicts the distribution of illumination tilt angles emanating from the target region in the sample. As expected, this distribution is skewed and tilted away from the illumination source. The median tilt angle for our parameters is about 11 degrees.

[00148] To further characterize the method, Monte Carlo simulations were used to estimate photon exit angle distributions at different fiber-detector separations. The exit angle corresponds to the tilt angle of the detected photon's path relative to the micro-objective optical axis (positive angles point away from the source). Five fiber-probe separations were considered: 1,830, 1,730 and 1,910 μm correspond to the middle, left, and right extremes of the 2.5× micro-objective FOV, respectively, while 910 and 3,970 μm correspond to roughly half and twice these distances, respectively. The results are shown in Figure 8(c). The median exit angle for each distribution is noted with a dashed line in Figure 8(c). A Lambertian exit angle distribution, as would be obtained from isotropic diffuse light in the sample, is also shown for comparison.

[00149] Monte Carlo simulations were also used to estimate the dependence of mean and median exit angles and detected intensity as a function of fiber-probe separation. The exit angle corresponds to the tilt angle of the detected photon's path relative to the micro-objective optical axis. The detected intensity is integrated over all exit angles. Three illumination conditions were simulated: a point-source with zero illumination NA, a point source with 0.48 NA, and a 1,000 μm diameter illumination fiber with 0.48 NA, as shown in Figure 8d. The shaded band represents the actual fiber-probe separation range used in our experiment with the 2.5× micro-objective (FOV = 240 μm).

[00150] The main conclusion from the Monte Carlo simulations is that, for the parameters used in our experiments, including the light source separation, the back-illumination at the target region of interest is indeed expected to be oblique.

Example 2: OBM Images of Onion Skin

[00151] To demonstrate the resolution and image quality of the OBM technique, images of onion skin were acquired with a flexible endomicroscope configuration. (Figure 3.) The endomicroscope setup is based on the use of a flexible fiber-bundle probe containing 30,000 fibers. One probe variant provides a 600μm field-of-view with 6.5μm resolution. Another probe variant provides a 240μm field of view with 2.5μm resolution. The outer diameter of both probes is 2.8mm. Imaging depth is 70μm. Illumination is provided by LEDs, and there are no moving parts. The frame rate of the system is 17Hz.

[00152] Panels (a) and (b) of Figure 3 are raw images acquired with left and right illumination. Panel (c) is an absorption image (a+b). Panel (d) is a phase gradient image (a-

b). Note that panel (d) contains negative values, meaning its zero level is gray. Note that the difference and sum images are very different despite having been obtained simultaneously with the same raw data, demonstrating that phase and absorption images can indeed highlight different sample structures. Note also the DIC-like appearance of the phase gradient image, and the absence of speckle compared to OCT images.

Example 3: Images of rodent Colon

[00153] To further demonstrate the resolution and image quality of the OBM technique, as well as to demonstrate its clinical relevance, images of the exposed surface of an excised and fixed rat colon were obtained using the endomicroscope setup described in Example 2. Figs 9a and 9b were obtained with left and right illumination, respectively, after core removal (raw core artifacts shown in insets). It is difficult to make out structure without core removal. Figs 9c and 9d are the resulting sum (absorption) and difference (phase gradient) images. Both images highlight manifestly different information. In particular, colonic crypts are clearly apparent.

Example 4: Images of rodent Intestine

[00154] To further demonstrate the resolution and image quality of the OBM technique, as well as to demonstrate its clinical relevance, images of the exposed surface of other regions of the excised and fixed rat intestine were obtained. (Figure 10.) Figure 10 shows OBM images of various regions of the rat intestine, acquired with 530nm LEDs. The top row presents sum images and the bottom row difference (phase gradient) images. Panels **(a)-(d)** show small intestine (note high resolution content in panel (b); panel (d) highlights epithelial villi). Panels **(e)-(h)** show large intestine (panel (f) highlights a crease in tissue; panel (h) presumably highlights a blood vessel). Note much higher contrast of phase gradient images compared to relative featurelessness of absorption images. The phase gradient images in these cases are much more revealing than the absorption images. These are high resolution images of thick, unlabeled tissue obtained through a flexible endomicroscope.

Example 5: OBM Images Acquired With Hopkins Rod-Lens

[00155] To further demonstrate OBM, phase-gradient images of the same small intestine sample used in Example 3 (Figure 10), were acquired with a contact mode Hopkins rod-lens using a two-shot implementation. The field of view was 500μm and the working distance 50μm. Two frames from a movie taken while focus depth was being adjusted are

shown in Figure 11. For these images green LED illumination was delivered through two diametrically opposed 1mm fibers. Note the high resolution of the small intestinal villi. This indicates that OBM will be useful for optical biopsy applications.

Example 6: Bead and Resolution

[00156] To quantify the resolution of OBM, a 45 μm bead was embedded in agarose made scattering by 2 μm beads. As shown in Figure 12a, not only are the margins of the 45 μm bead crisp and clear but the 2 μm beads embedded in the agarose are themselves very clearly visible.

[00157] Figure 12b is the line profile of the intensity difference image of the bead shown in Figure 12a. As expected, this line profile reveals the phase gradient induced by the bead.

Example 7: Moving Tissues in Chick Embryo

[00158] To characterize the ability of the OBM technique to image moving tissues the inventors collected images of blood flow in chick embryos. Figure 12 shows simultaneous (c) amplitude and (d) phase-gradient images of blood flow in chick embryo (movement in (c) is highlighted in (d)); scale bar = 75μm. Simultaneous (e) amplitude and (f) phase-gradient images acquired with higher magnification fiber bundle (scale bar = 30μm) are also shown. When blood flow is slowed down, the OBM technique provides high resolution images of individual blood cells as shown in Figure 12 (g) and (h). Note the different types of blood cell morphologies apparent in the images. These images demonstrate the versatility and applicability of the technique.

Example 8: Mosaic Images and Movies

[00159] To demonstrate the ability of the OBM technique to acquire images across a large target area, Figure 13 shows an amplitude (top) and phase-gradient (bottom) mosaic of 300 frames acquired at 17.3 frames per second while manually scanning across a chick embryo. The scale bar is75 μm and the box indicates the size of a single frame (186 x 139 μm). This example further demonstrates the versatility of the system.

[00160] The inventors have also successfully used the OBM technique to create movies. In a first implementation, a phase-gradient movie of subsurface capillaries in a chick embryo (day 11 post-fertilization) was taken using an OBM endomicroscope based on

a flexible fiber bundle placed on the embryo surface. The focal depth was varied during acquisition of the movie to demonstrate the pseudo optical-sectioning capacity of instrument. The frame rate was 17.5Hz (actual frame rate of movie) and the fiber probe was manually scanned over the sample.

[00161] In a second implementation, a simultaneous amplitude and phase-gradient movie of vascular and extravascular structure obtained with an OBM endomicroscope probe placed along the yolk membrane in a chick embryo (day 11 post fertilization) was obtained. Again, the frame rate was 17.5Hz and the fiber probe was manually scanned over the sample.

[00162] In a third implementation, a phase-gradient movie of capillaries draining into a venule in a chick embryo (day 7 post fertilization) was obtained. The magnification was 2.5x lower than in videos 1 and 2. The frame rate was 17.5Hz. The movie was stabilized a posteriori to correct for heart-beat motion. Inter-frame variations (i.e. movement) in the amplitude movie were used to highlight venule and capillaries in the phase-gradient movie.

Example 9: Diagnostics

[00163] Hyperplasia is a non-neoplastic proliferation of colonic mucosa that results from reduced exfoliation of normal epithelium, and adenoma is a pre-malignant condition that arises from unregulated epithelial growth. These lesions are commonly found on routine screening colonoscopy. Accordingly, the ability to distinguish normal colonic mucosa from that exhibiting hyperplasia or adenomatous changes is useful. Visible morphological structures used to evaluate mucosa during colonoscopy screening include (l) crypt lumens, (c) epithelial cells, (ap) apical border of epithelial cells, (bl) basolateral border of epithelial cells, and (lp) lamina propia.

[00164] Figure 14 shows a labeled version of Figure 15d. Visible morphological structures include (l) crypt lumens, (c) epithelial cells, (ap) apical border of epithelial cells, (bl) basolateral border of epithelial cells, and (lp) lamina propia. (Wang TD et al. "Functional imaging of colonic mucosa with a fibered confocal microscope for real time in vivo pathology" Clin Gastroenterol Hepatol 5(11):1300-1305 (2007).) As shown in Figure 14, the high resolution phase contrast images generated by OBM allow identification of each of these morphological structures.

Example 10: Comparison of Added Versus Subtracted Raw OBM Images

[00165] In this example, a 45 μm polystyrene bead embedded in scattering tissue phantoms was analyzed to compare added versus subtracted raw OBM images. Raw images under oblique back-illumination from two opposing directions are shown in Figures 16(a) and 16(b). Addition of (a) and (b) cancels phase gradient contrast and emphasizes absorption, as demonstrated in Figure 16(c). Subtraction of (a) and (b) cancels absorption contrast and emphasizes phase gradients, as shown in Figure 16(d). The 2 μm beads used to render the tissue phantom scattering are readily visible only when in focus. Lateral resolution is ~2.6 μm, limited by the fiber core spacing of the imaging fiber bundle (here 3.3 μm, demagnified 2.5x due to the distal micro-objective). Scale bars 20 μm.

Example 11: Axial Response

[00166] In this example the apparent axial resolution of OBM was explored. A z-stack of OBM phase gradient images was acquired by axially translating a scattering tissue phantom with a step size ~100 nm. The contrast of five 5.5 × 5.5 μm (12 × 12 pixel) regions each bounding single 2 μm diameter beads was computed as (max – min) / (max + min) for every frame in the z-stack. The resulting contrast profiles were normalized and co-registered and are presented in Figure 17. Increasing z corresponds to deeper imaging.

Example 12: Imaging Mouse Lung and Liver Tissue

[00167] Mouse lung and liver images were acquired using the OBM setup based on a fiber bundle.Fixed mouse liver imaged under phase gradient contrast OBM reveals collagen strands (Figure 18(aa, arrows) and groups of hepatocytes (Figure 18(b), arrows). Fixed mouse lung cross-section imaged under phase gradient contrast OBM reveals fine microstructure (Figure 18(c)) and alveoli (Figure 18(d), stars). Scale bars are 30 μm. These images demonstrate the usefulness of the OBM technique for analyzing tissue morphology.

Example 13: Imaging Rat Flank Skin

[00168] OBM was implemented in a bench top microscope to access longer working distances. Figure 19 shows rat flank skin under phase gradient OBM 50 μm and 100 μm below the surface. Figure 19(a) is an *en face* view 50 μm below the surface showing wavy collagen strands. Figure 19(b) is an *en face* view 100 μm below the surface showing a cluster of adipocytes (stars). It is noteworthy that contrast remains high even when imaging through ~30 μm of collagen fibers. Scale bars are 50 μm. These images demonstrate the usefulness

of the OBM technique for analyzing tissue morphology at depths of 50 μm and 100 μm below the tissue surface and further demonstrate the general usefulness of the technique in such contexts.

Example 14: OBM Depth Penetration in Mouse Brain Slices

[00169] To illustrate OBM penetration depth, the micro-objective and flexible fiber bundle were replaced with a traditional microscope objective (Olympus 40× water immersion, 0.80 NA, working distance 3.3 mm). The two illuminating fibers were guided along the objective housing and placed in contact with the sample. The separation between fiber and objective axes was 4.3 mm. As shown in Figure 20, a z-stack of a fixed mouse brain slice (sagittal cut through the brain mid-line) illustrates imaging to a depth of 100 μm, beyond which OBM contrast became too weak to reveal structure. In addition to providing greater working distance, the microscope setup afforded improved spatial resolution (~700 nm, camera pixel limited) compared to the flexible fiber bundle system. Scale bar 30 μm.

Example 15: OBM Depth Penetration in Mouse Ventral Skin

[00170] To illustrate penetration depth, OBM was configured in a microscope setup as described in Example 14. As shown in Figure 21, a z-stack of fixed mouse ventral skin shows keratin filaments in the stratum corneum down to 80 μm, where the deeper layer of stratum granulosum becomes apparent. Scale bar 30 μm.

Example 16: Comparison of OBM With Epi-Illumination Reflection Contrast

[00171] To further characterize OBM, the technique was compared to traditional epi-illumination reflection contrast. To generate epi-illumination reflection contrast, illumination was delivered to the sample directly through the fiber bundle rather than through separate illumination fibers. To minimize extraneous back reflections from the fiber bundle surface, the illumination and detection paths were cross-polarized. The sample was fixed mouse cardiac muscle tissue. As shown in Figure 22, the phase gradient image (OBM –) exhibits significantly higher contrast than either the absorption image (OBM +) or reflection image (epi). The left panels are displayed with a linear grayscale mapping such that 0 is represented by the middle gray level and the image is scaled to fill the dynamic range of the display. The right panels have been autoscaled by subtracting the minimum values before scaling up to fill the dynamic range of the display, bringing the contrast of the images to 100%. Note that while the removal of the large biases in the absorption and reflection images improves

contrast, signal-to-noise ratio remains too low to reveal meaningful structure. Scale bar 20 μm.

Example 17: Dual-camera multi-wavelength OBM endomicroscopy

[00172] A dual-camera, multi-wavelength setup was used to simultaneously acquire data under different illumination wavelength ranges (see Fig. 23) Two multi-wavelength fiber-coupled light emitting diode (LED) modules (Mightex WFC-H4, SLC-AA04-US) coupled light into optical fibers (Thorlabs BFL48-1000; 0.48 NA; 1000 μm core; 2 m length) through a standard SMA connection. A summary of the available wavelengths and optical powers is shown in Table 1.

Table 1. Multi-wavelength illumination parameters

Channel	Peak Wavelength (nm)	Bandwidth ($1/e^2$, nm)	Optical Power* (mW)
blue	464	43	22
green	527	60	10
red	632	32	10
NIR	736	47	7.5

*after transmission through 2 m optical fiber

[00173] The optical fibers were placed in contact with the sample alongside a contact-mode micro-objective (Mauna-Kea Technologies; 2.6 mm diameter; 2.5× magnification; 60 μm working distance; water-immersion; 0.8 NA) coupled to an imaging fiber bundle (30,000 cores; 600 μm active area). The source-detector separation was approximately 1.8 mm. The image at the proximal face of the fiber bundle was relayed to matching monochrome complementary metal oxide semiconductor (CMOS) cameras (PhotonFocus MV1-D1312-160-CL-12, 12-bit mode) with an achromatic objective (Olympus UPLFLN10X2; U Plan Fluorite; 10×, 0.3 NA) and tube lens (Thorlabs AC254-200-A-ML). An image-splitting dichroic beamsplitter (Semrock FF560-FDi01-25×35) was used to send blue and green light to the first camera and red and near infrared (NIR) light to the second. Images were streamed from the camera along a camera-link interface and captured with a dual-base frame grabber (BitFlow KBN-PCE-CL2-D). Frame rate differed by experiment and was typically limited by available illumination power and acceptable signal to noise ratio (SNR).

[00174] The dual-camera multi-wavelength OBM endomicroscope setup is illustrated in Figure 23. As shown in Figure23(a) light from two fiber-coupled multi-wavelength LED modules is transmitted along multimode fibers alongside an imaging fiber bundle probe. The image on the proximal face of the fiber bundle is relayed to two high speed CMOS cameras

through an image-splitting dichroic beamsplitter such that each camera is sensitive to complementary portions of the visible and NIR spectrum. As shown in Figure 23(b) normalized emission spectra of four wavelengths was available in each LED module (solid blue, green, red and magenta lines) along with normalized absorption (blue dashed line) and emission (green dashed line) spectra of fluorescein, and transmission spectra of the dichroic mirror (solid black line) and fluorescein emission filter (solid orange line). Figure 23(c) shows a contact-mode 2.5× water-immersion micro-objective is fixed to the end of the imaging fiber bundle to give additional magnification and 60 μm working distance. Offset optical fibers deliver light which obliquely back-illuminates the sample at the focal plane. A pre-processing procedure was used to remove the appearance of the fiber bundle cores before OBM-specific processing was performed. This was done using a segmentation-interpolation algorithm wherein dark regions between the fiber cores were "filled in" with interpolated values based on closest neighbor fiber core signals.

[00175] Several modes of operation are available to the dual-camera multi-wavelength setup. Non-limiting examples follow.

Example 18: Single Wavelength OBM

[00176] One mode of operation is to perform OBM with a single illumination wavelength by synchronously toggling power between the left and right optical fibers. Raw camera frames can then be combined pair-wise to produce either a phase-gradient contrast or amplitude contrast composite image (by subtracting or adding normalized images, respectively). Multiple wavelengths are available to be used individually or in concert; the LED controller allows independent configuration and triggering of each wavelength. This mode utilizes only one of the two cameras, and was used to visualize capillary blood flow through the human eyelid epidermis *in vivo* (see Fig. 24). Specifically, capilary blood flow was measured *in vivo* through human eyelid epidermis. Figures 24(a) and (b) show individual phase gradient contrast OBM images under simultaneous red and NIR illumination. Figure 24(c) shows a capillary visualized by a sliding 3-frame temporal variance filter. Multiplying frames in Figure 24(a, and b) by capillary mask Figure 24(c) yields the images in Figures 24(d, and e), where individual red blood cells are easily distinguished (white arrows). Figure 24(f) shows another capillary with a more tortuous path extracted from a separate segment from the same video as Figures 24(a-e). Scale bars are 20 μm.

Example 19: Simultaneous Co-Registered Multi-Wavelength OBM

[00177] Another available mode of operation to perform OBM is a dual-camera configuration. Such a configuration can be used to simultaneously acquire co-registered OBM images using different wavelengths. In this case four raw images are acquired in the time span of two exposures. This mode was used to simultaneously visualize phase gradient contrast in the red/NIR spectrum and amplitude contrast in the blue/green spectrum. Stratified squamous epithelium of the buccal mucosa was imaged *in vivo* under the flexible OBM endomicroscope probe. Phase gradient (Figures 25(a and c)) images and corresponding amplitude images (Figures 25(b and d)) were obtained under red and blue/green illumination, respectively. Scale bars are 30 μm for Figures 25(a-b) and 20 μm for Figures 25(c-d). The phase gradient images highlight buccal cell borders (dashed lines in Figure 25(a)), cell nuclei as well as sub-cellular features. The amplitude images reveal cell nuclei in high contrast (arrows in Figures 25(b,d)). All images are still frames from a video acquired at 30 fpps (60 fps).

Example 20: Recessed Illumination

[00178] Figure 26 is a schematics of different OBM illumination configurations. Figure 26(a) illustrates llumination delivered via fiber optic conduits in contact with the tissue surface. Figure 26(b) illustrates illumination delivered by light sources not in contact with the tissue surface. In both cases, illumination is depicted only from one source (left); however, alternative configurations with two or more light sources may also be used. As shown in the figure, OBM can be achieved with light sources that are not necessarily in contact with the sample, but can be recessed by several centimeters. For example, the illumination could be delivered by light sources or fibers that are offset from the optical axis but are set back from the sample surface. In this case, the light sources can diverge over large angles. Illumination obliquity is then assured by the shadow cast by the probe housing itself (as illustrated in Fig 26(b)). Alternatively, if detection is performed through an objective, the illumination can be delivered through the same objective but producing offset illumination spots at the sample surface that are outside the field of view of the detection optics (as illustrated in Fig XXXa). Alternatively, if detection is performed through a fiber bundle, the illumination can be delivered through the peripheral fiber cores in the bundle and detection can be performed through the central cores of the bundle where imaging is performed via

micro-objective (such as a gradient index (GRIN) lens of diameter smaller than the fiber bundle (as illustrated in Fig XXXb).

[00179] A comparison of OBM with fiber-mediated illumination in contact with the tissue versus non-fiber-mediated illumination not in contact with the tissue is shown in Figure 27. For this example the sample was a 45um bead just under the surface of a tissue phantom (comprising 2um beads in 2% agarose gel, ls*~1 mm). Images were acquired with a rigid laparascope setup. All images have been phase-gradient OBM processed after averaging approximately 20 frames to reduce shot noise. The camera was a QImaging Retiga 2000R. Panels (a) and (b) are OBM images acquired with fiber-delivered LED light (~650nm). In (b) the subtraction of images has opposite sign as in (a), leading to a change in sign in the phase gradient. Panels (c) and (d) are OBM images acquired with a 6-element LED flashlight held approximately 2 inches from the sample surface. The 45 um bead is observed but with lower normalized contrast than the fiber illumination. Background 2um beads remain barely discernible. Scale bar is 20um. This example demonstrates the usefulness of OBM with a recessed light source and demonstrates that the light source need not be structurally associated with the probe or the detection optics.

[00180] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

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CLAIMS:

1. A method of creating a phase contrast image, comprising:

illuminating the target region of a sample with a first light source to provide a first oblique back illumination of the target region of the sample, and

detecting a first phase contrast image from light originating from the first light source and back illuminating the target region of the sample.

2. The method of claim 1, wherein light from the first light source is the only light illuminating the sample when the first phase contrast image is detected from light originating from the first light source and back illuminating the target region of the sample.

3. The method of claim 2, further comprising illuminating the sample with a second light source to provide a second oblique back illumination of the target region of the sample, and

detecting a second phase contrast image from light originating from the second light source and back illuminating the target region of the sample.

4. The method of claim 3, further comprising creating a difference image of the target region of the sample by subtracting the second phase contrast image of the target region of the sample from the first phase contrast image of the target region of the sample.

5. The method of claim 3 or claim 4, further comprising creating an absorption contrast image of the target region of the sample by adding the first phase contrast image of the target region of the sample to the second phase contrast image of the target region of the sample.

6. The method of any one of claims 1-5, wherein the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source and back illuminating the target region are different.

7. The method of any one of claims 1 and 3-6, wherein the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source and back illuminating the target region are different.

8. The method of any one of claims 3-7, wherein the axis of detection of light originating from the first light source and back illuminating the target region and the axis of detection of light originating from the second light source and back illuminating the target region are different.

9. The method of any one of claims 3-7, wherein the axis of detection of light originating from the first light source and back illuminating the target region and the axis of detection of light originating from the second light source and back illuminating the target region are the same.

10. The method of any one of claims 3-9, wherein the wavelength of the light from the first light source and the wavelength of light from the second light source are different.

11. The method of any one of claims 1-10, wherein the wavelength of the light from the first and second light sources is from 0.2 to 300 μm.

12. The method of any one of claims 1-11, wherein the light source(s) is selected from a light-emitting diode (LED), a laser, a supercontinuum light source, or a superluminescent diode (SLED).

13. The method of any one of claims 1-12, wherein the detecting is by a photo detector array.

14. The method of claim 13, wherein the photo detector array is a charge coupled device (CCD) or a CMOS (complementary metal oxide semiconductor) camera sensor.

15. The method of any one of claims 1-14, comprising using an optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample.

16. The method of any of claims 1-15, wherein the optical conduit to communicate light in at least one direction selected from toward the sample and away from

the sample is selected from a fiber, an arrangement of fibers, a fiber bundle, a rigid lens, an arrangement of rigid lenses, a gradient index (GRIN) lens, or an arrangement of GRIN lenses.

17. The method of any of claims 1-16, wherein the same optical conduit communicates light toward the sample and away from the sample.

18. The method of any of claims 1-17, wherein different components of the same optical conduit communicates light toward the sample and away from the sample.

19. The method of any one of claims 1-18, wherein the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaced by from about 0.2 mm to about 10 mm.

20. The method of any one of claims 3-19, wherein the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaced by from about 0.2 mm to about 10 mm.

21. The method of any one of claims 1-20, wherein the object plane of the target region is from the surface to about 300 μm below the surface of the sample.

22. The method of any one of claims 1-21, wherein the lateral resolution of the image is from about 0.1 μm to about 10 μm.

23. The method of any one of claims 3-22, comprising detecting the first and second images during first and second non-overlapping time intervals.

24. The method of any one of claims 3-22, comprising detecting the first and second images during first and second overlapping time intervals.

25. The method of claim 24, wherein the first and second light sources illuminate the sample with light of different distinguishable wavelengths.

26. The method of claim 25, wherein the images of different distinguishable wavelengths are separated by a wavelength separator and directed onto separate camera sensors.

27. The method of claim 25, wherein the images of different distinguishable wavelengths are separated by a wavelength separator and directed onto different portions of a same camera sensor.

28. The method of claim 24, wherein the first and second light sources illuminate the sample with orthogonally polarized light.

29. The method of claim 28, wherein the images of orthogonal polarization are separated by a polarization separator and directed onto separate camera sensors.

30. The method of claim 28, wherein the images of orthogonal polarization are separated by a polarization separator and directed onto different portions of a same camera sensor.

31. The method of any one of claims 4-30, wherein the difference image is axially resolved.

32. The method of any one of claims 1-31, further comprising obtaining a series of two or more images and combining the images to provide a composite image larger than the field of view a single image.

33. The method of any one of claims 1-32, comprising creating a phase contrast image of gastrointestinal tissue and examining the tissue to assess at least one of the presence and the absence of indicators of a disease.

34. The method of claim 33, wherein the gastrointestinal tissue is colonic mucosa disease is at least one of hyperplasia and adenomatous changes.

35. The method of any one of claims 1-32, comprising creating a phase contrast image of lung tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease.

36. The method of any one of claims 1-32, comprising creating a phase contrast image of liver tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease.

37. The method of any one of claims 1-32, comprising creating a phase contrast image of bladder tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease.

38. The method of any one of claims 1-32, comprising creating a phase contrast image of skin tissue and examining the tissue to assess at least one of the presence and the absence of at least one indicator of a disease.

39. The method of any one of claims 1-32, comprising creating a phase contrast image of brain tissue and examining the tissue to assess tissue morphology.

40. The method of any one of claims 1-32, comprising creating a phase contrast video of blood flow to assess blood flow velocity.

41. The method of any one of claims 1-32, comprising creating a phase contrast video of blood flow to assess the cell count of at least one blood cell type.

42. An apparatus for creating a phase contrast image of a sample, comprising:

a probe comprising 1) an optical radiation source or a first light conduit, and 2) a photo detector array or image conduit, and 3) a distal end,;

wherein the light conduit, the photo detector array or image conduit, and the distal end of the probe are configured to back illuminate the target region of a sample in contact or near contact with the distal end of the probe with a light from the first light source to provide a first oblique back illumination of the target region of the sample, and to detect a first phase contrast image from light originating from the first light source and back illuminating the target region of the sample.

43. The apparatus of claim 42, wherein the distal end of the optical radiation source or first light conduit extend to the distal end of the probe.

44. The apparatus of claim 42, wherein the distal end of the optical radiation source or first light conduit is recessed from the distal end of the probe by up to 10 cm.

45. The apparatus of any one of claims 42-44, wherein the distal end of the photo detector array or image conduit is recessed from the distal end of the probe.

46. The apparatus of claim 42, wherein the probe comprises a first light conduit and the apparatus further comprises a first optical radiation source connected to or projected to a proximal end of the first light conduit.

47. The apparatus of claim 42, wherein the probe comprises a photo detector array.

48. The apparatus of claim 42, wherein the probe comprises an image conduit and a proximal end of the image conduit is connected to or imaged to a photo detector array.

49. The apparatus of any one of claims 42-48, wherein the probe further comprises:

a second optical radiation source or a second light conduit;

wherein the second optical radiation source or second light conduit, the photo detector array or image conduit, and the distal end of the probe are configured to illuminate the target region of a sample in contact or near contact with the distal end of the probe with a light from the second light source to provide a second oblique back illumination of the target region of the sample, and to detect a second phase contrast image from light originating from the second light source and back illuminating the target region of the sample.

50. The apparatus of claim 49, wherein the distal end of the optical radiation source or first light conduit extends to the distal end of the probe.

51. The apparatus of claim 49, wherein the distal end of the optical radiation source or first light conduit is recessed from the distal end of the probe by up to 10 cm.

52. The appratus of any one of claims 49-51, wherein the distal end of the photo detector array or image conduit is recessed from the distal end of the probe

53. The apparatus of claim 49, wherein the probe comprises a second light conduit and the apparatus further comprises a second optical radiation source connected to or imaged to a proximal end of the first light conduit.

54. The apparatus of any one of claims 42-53, comprising at least three optical radiation sources or a light conduits, wherein the at least three optical illumination sources or light conduits are located at distinct locations around the probe such that each is capable of creating oblique back illumination enabling the measurement and display of phase gradients in different directions relative to the others.

55. The apparatus of any one of claims 42-54, wherein the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source and back illuminating the target region of the sample are different.

56. The apparatus of any one of claims 49-55, wherein the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are different.

57. The apparatus of any one of claims 49-56, wherein the axis of detection of light originating from the first light source and reflected from the sample and the axis of detection of light originating from the second light source and illuminating the target region of the sample are different.

58. The apparatus of any one of claims 49-56, wherein the axis of detection of light originating from the first light source and reflected from the sample and the axis of detection of light originating from the second light source and back illuminating the target region of the sample are the same.

59. The apparatus of any one of claims 49-58, wherein the wavelength of the light from the first light source and the wavelength of light from the second light source are different.

60. The apparatus of any one of claims 49-59, configured to detect the first and second images during first and second non-overlapping time intervals.

61. The apparatus of any one of claims 49-60, configured to detect the first and second images during first and second overlapping time intervals.

62. The apparatus of claim 61, configured for illumination of the sample by the first and second light sources with light of different distinguishable wavelengths.

63. The apparatus of claim 61, configured for illumination of the sample by the first and second light sources with orthogonally polarized light.

64. The apparatus of any one of claims 42-63, wherein the first and second light sources are capable of providing illumination at a range of wavelengths comprising from 0.2 to 300 μm.

65. The apparatus of any one of claims 42-64, wherein the light source is selected from a light-emitting diode (LED), a laser, a supercontinuum light source, or a superluminescent diode (SLED).

66. The apparatus of any one of claims 42-65, comprising a photo detector array.

67. The apparatus of claim 66, wherein the photo detector array is a charge coupled device (CCD) or a CMOS (complementary metal oxide semiconductor) camera sensor.

68. The apparatus of any one of claims 1-67, comprising an optical conduit to communicate light in at least one direction selected from toward the sample and away from the sample.

69. The apparatus of any one of claims 42-68, configured so that the axis of illumination of the sample with the first light source and the axis of detection of light originating from the first light source are displaceable by from about 0.2 mm to about 5 mm.

70. The apparatus of any one of claims 49-69, configured so that the axis of illumination of the sample with the second light source and the axis of detection of light originating from the second light source are displaceable by from about 0.2 mm to about 5 mm.

71. The apparatus of any one of claims 42-70, configured to obtain images of object planes of the target region from the surface of the sample to about 300 μm below the surface of the sample.

72. The apparatus of any one of claims 42-71, that creates images laterally resolved at from about 0.3 μm to about 10 μm.

73. The apparatus of any one of claims 42-72, wherein at least one of 1) the distal end of the first optical radiation source or first light conduit, and 2) the distal end of the second optical radiation source or second light conduit, and 3) the distal end of the photo detector array or image conduit, extend through and end at the distal end of the probe.

74. The apparatus of any one of claims 42-72, wherein at least one of 1) the distal end of the first optical radiation source or first light conduit, and 2) the distal end of the second optical radiation source or second light conduit, and 3) the distal end of the photo detector array or image conduit, is recessed from the distal end of the probe by up to 5 cm.

75. An endoscope comprising an apparatus according to any one of claims 42-74.

76. The endoscope of claim 75, wherein the endoscope is portable.

77. A system comprising an apparatus according to any one of claims 42-74 or an endoscope according to claim 75 or claim 76, and a processor for processing images obtained from the apparatus.

78. A method of creating at least one of a phase contrast image of a target region of a sample and a difference image of two phase contrast images of a target region of a sample, comprising:

providing a sample comprising a target region; using an apparatus of any one of claims 42-74 to create at least one phase contrast image of the target region of the sample using a method according to any one of claims 1-41, and optionally creating a difference image from the two or more contrast images of the target region of the sample.

79. A phase contrast image created by the method of any one of claims 1-41 and 78.

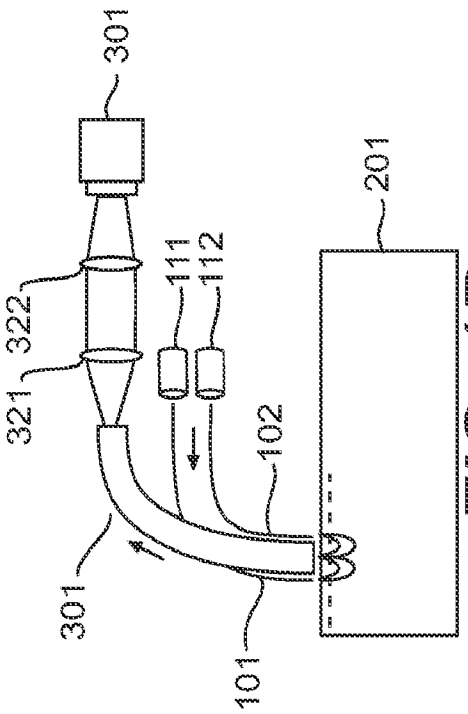


FIG. 1B

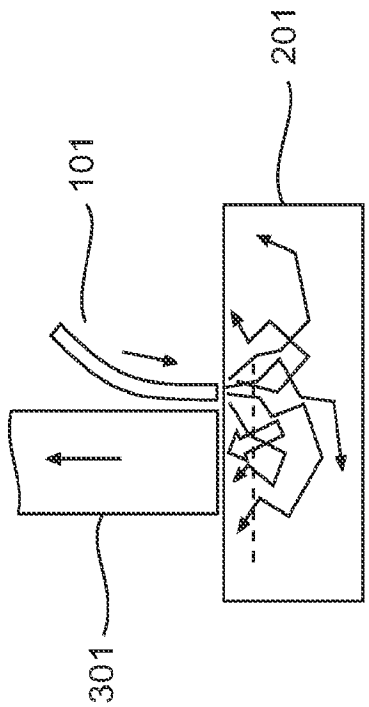


FIG. 1A

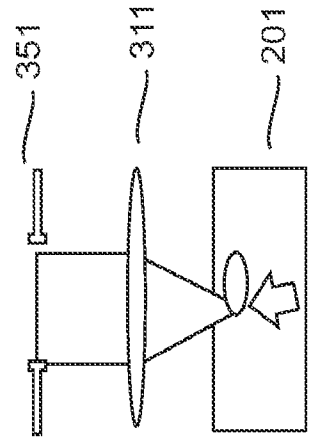


FIG. 1E

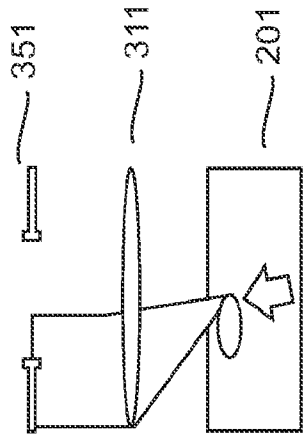


FIG. 1D

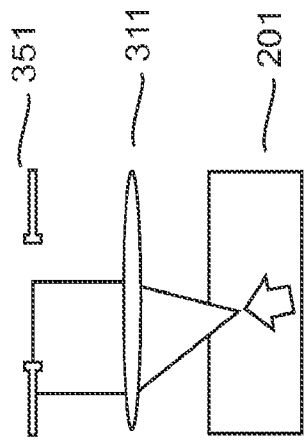


FIG. 1C

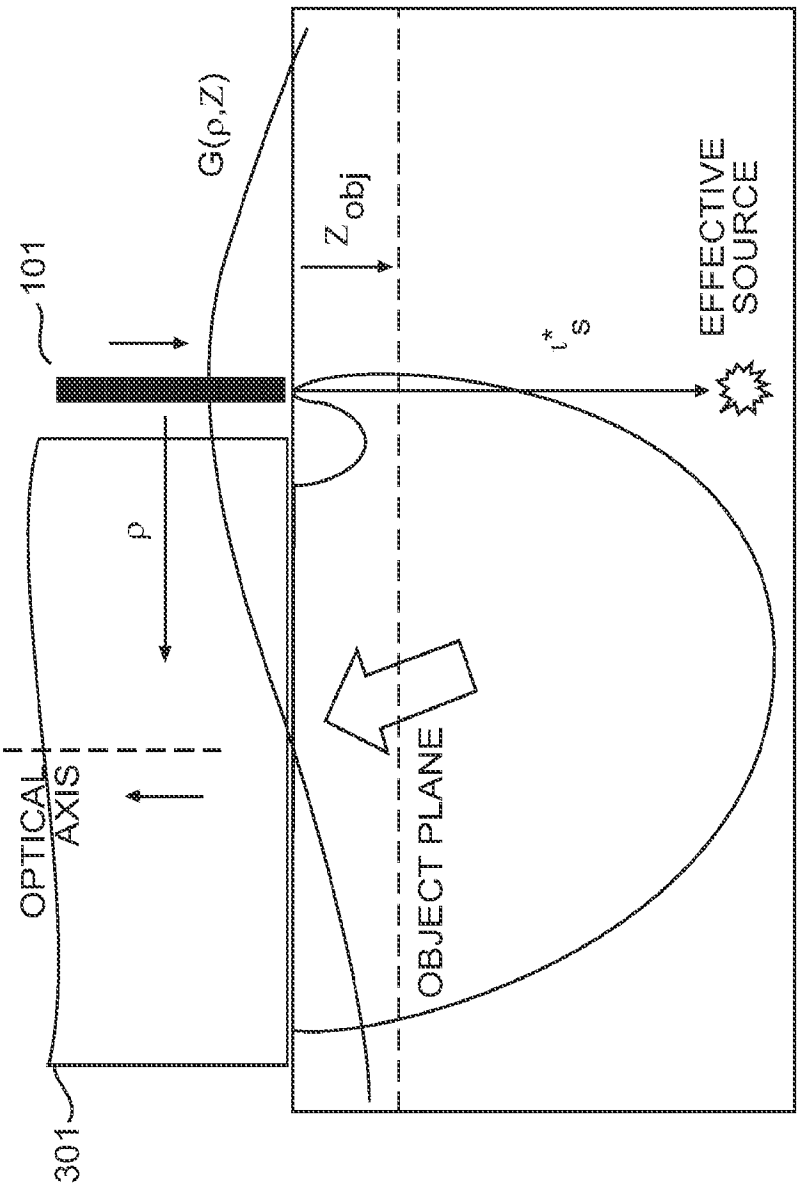

101
G(ρ,Z)
Z_{obj}
EFFECTIVE SOURCE
t^*_S
ρ
OPTICAL AXIS
OBJECT PLANE
301

FIG. 2

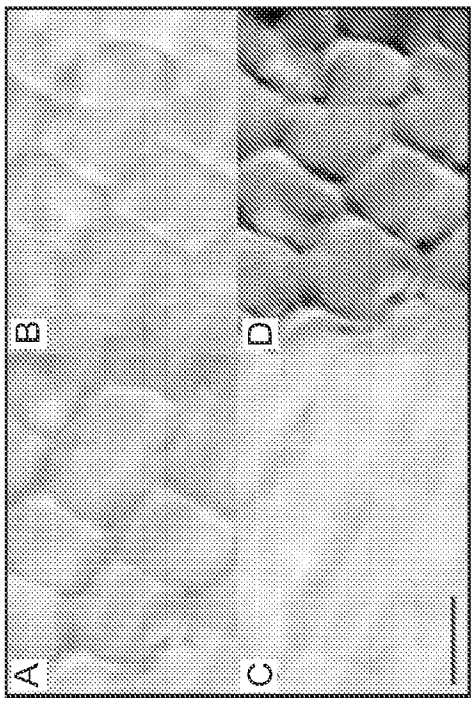


FIG. 3

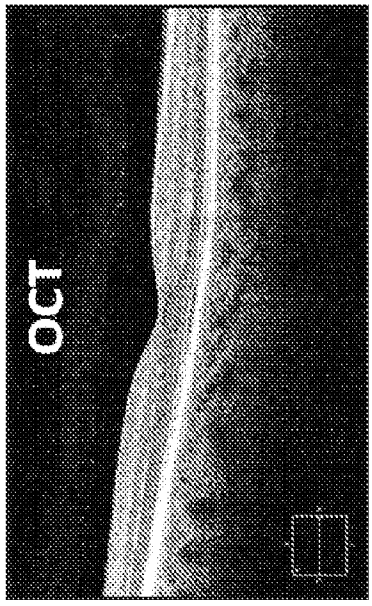


FIG. 4A

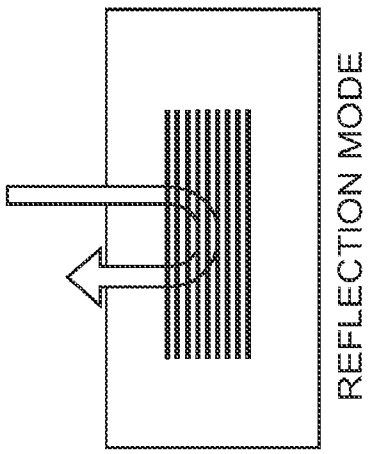


FIG. 4B

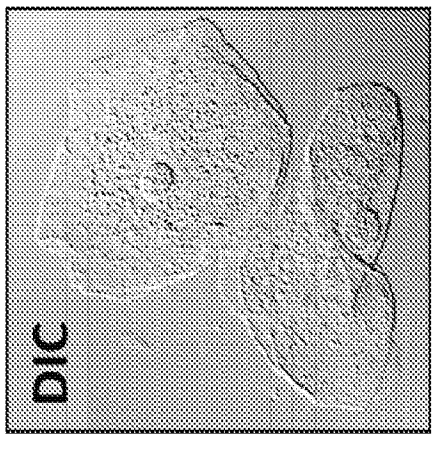


FIG. 4C

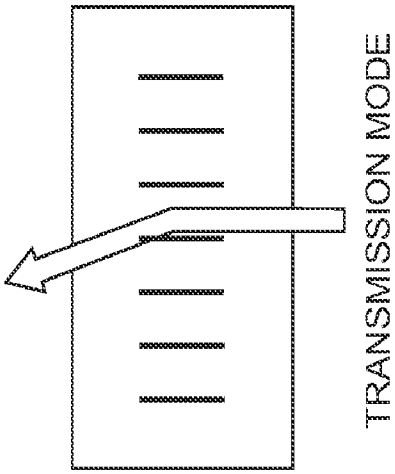


FIG. 4D

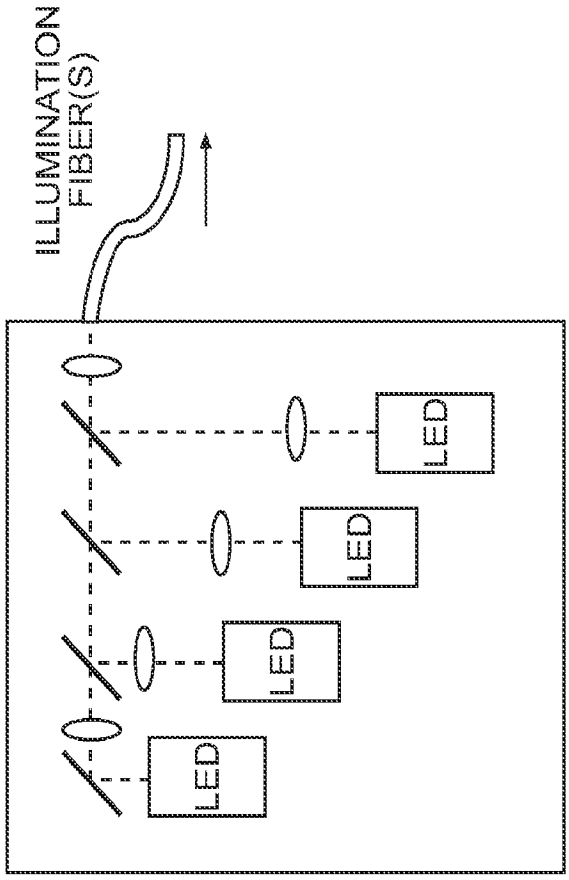


FIG. 5

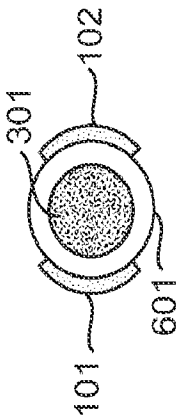


FIG. 6B

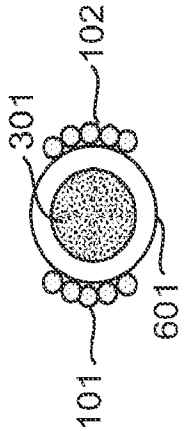


FIG. 6C

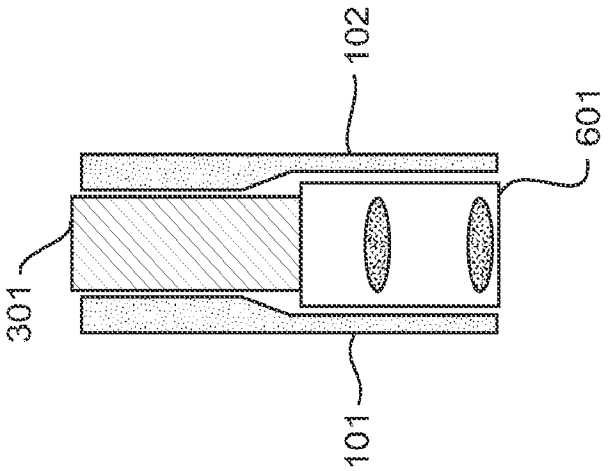


FIG. 6A

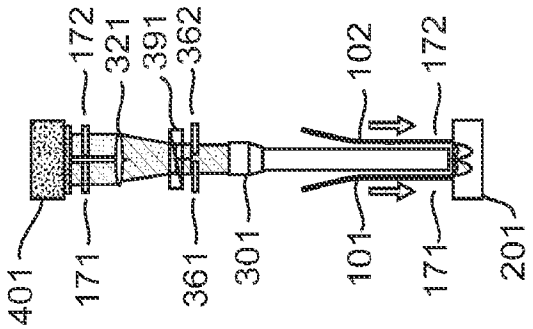


FIG. 7D

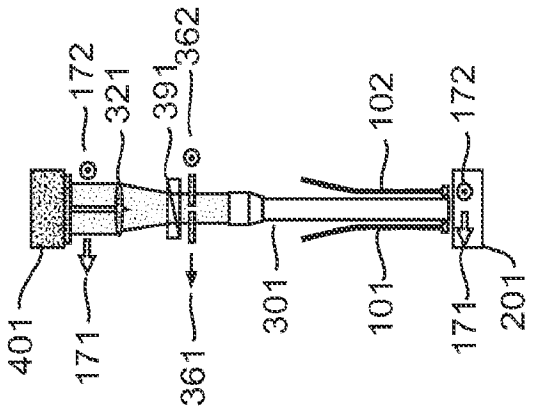


FIG. 7C

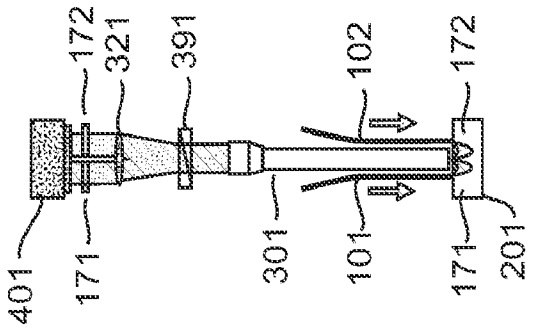


FIG. 7B

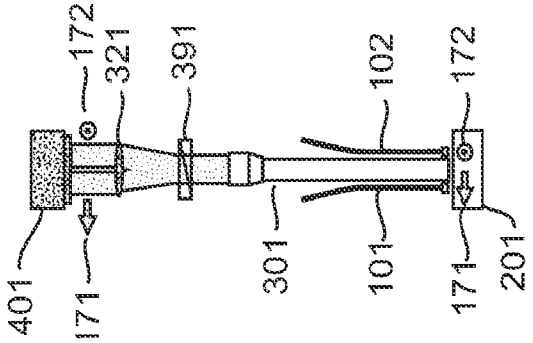


FIG. 7A

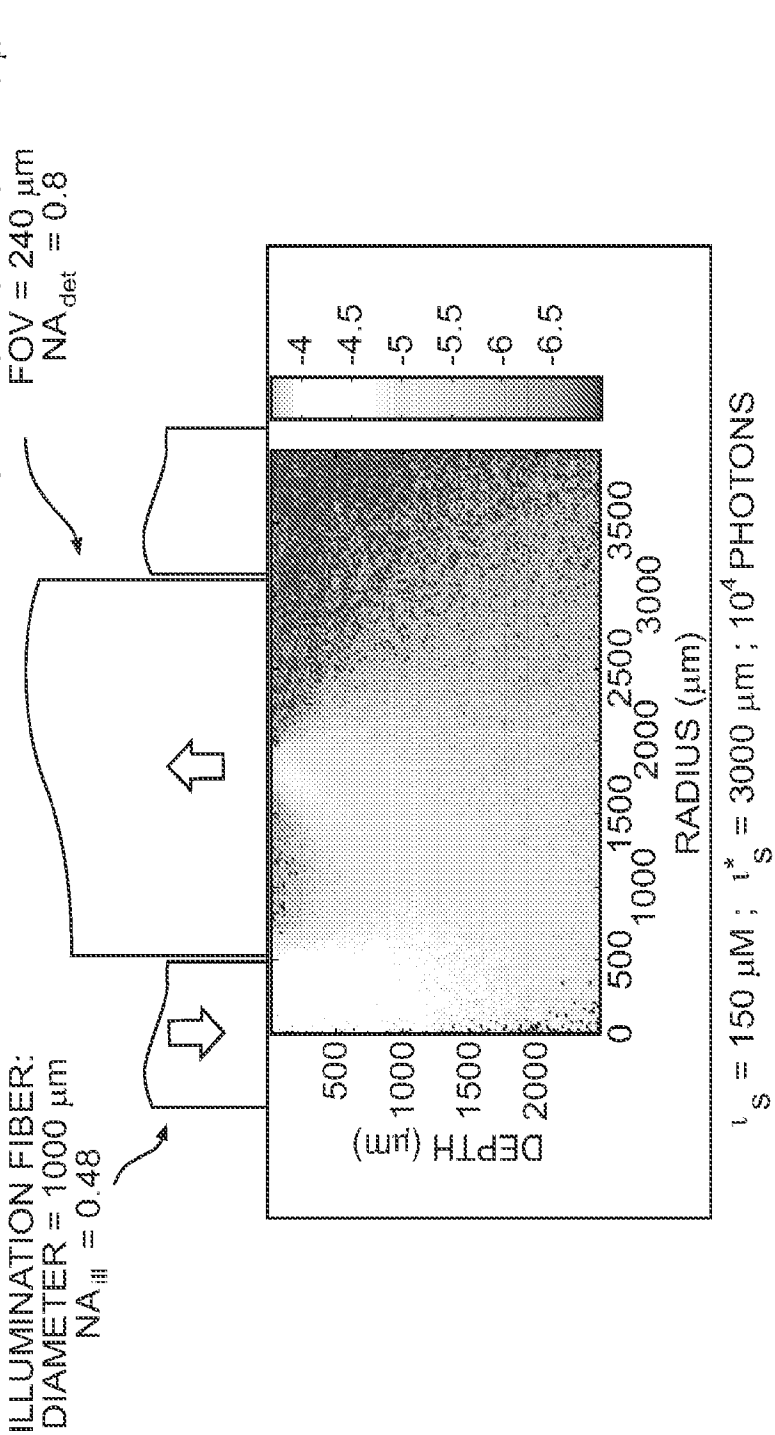

DETECTION OPTICS:
WORKING DISTANCE = 70 μm
FOV = 240 μm
NA$_{det}$ = 0.8
ILLUMINATION FIBER:
DIAMETER = 1000 μm
NA$_{ill}$ = 0.48
-4
-4.5
-5
-5.5
-6
-6.5
0
500
1000
1500
2000
2500
3000
3500
RADIUS (μm)
DEPTH (μm)
500
1000
1500
2000
μ_s = 150 μM ; μ_s^* = 3000 μm ; 10^4 PHOTONS

FIG. 8A

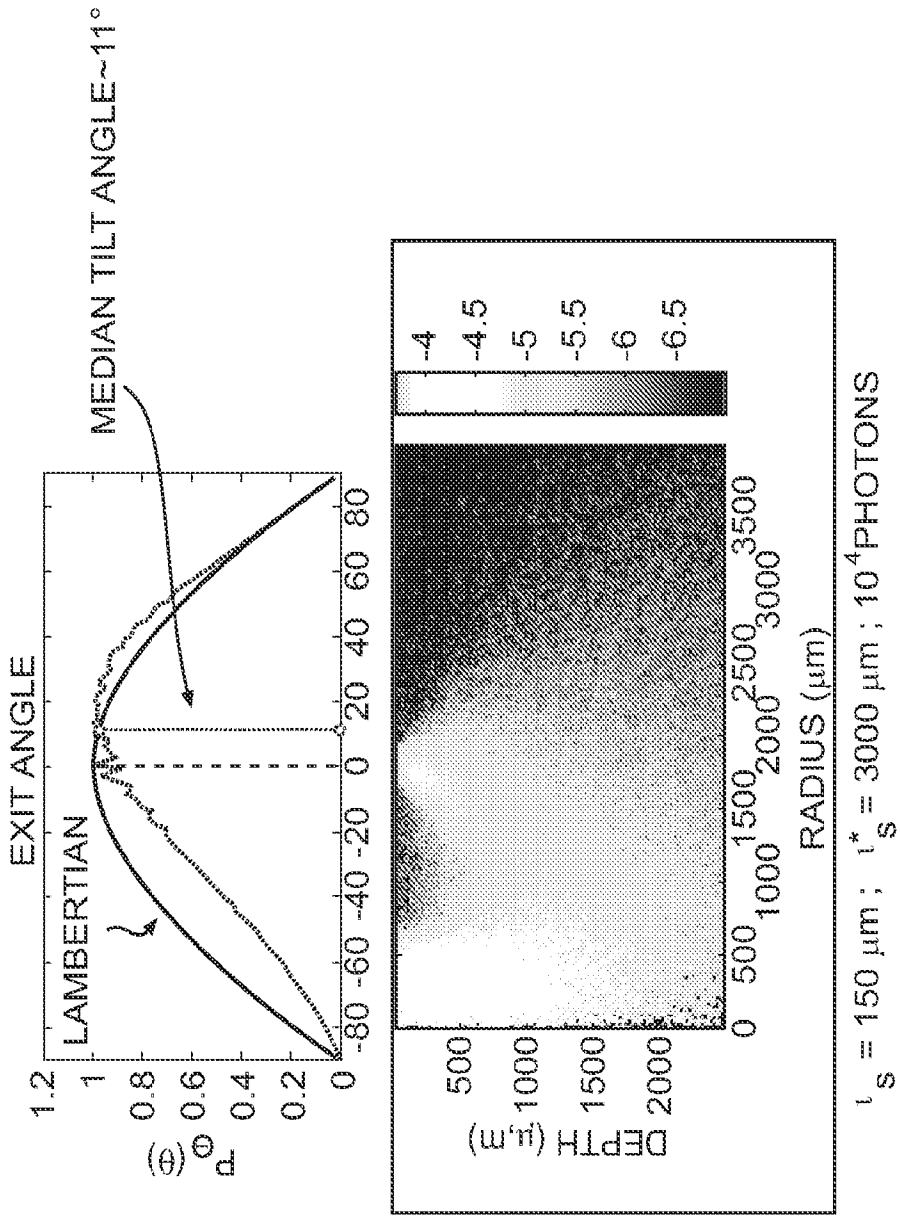

MEDIAN TILT ANGLE~11°
EXIT ANGLE
LAMBERTIAN
$P_Q(\theta)$
1.2
1
0.8
0.6
0.4
0.2
0
-80 -60 -40 -20 0 20 40 60 80
DEPTH (μm)
500
1000
1500
2000
RADIUS (μm)
0 500 1000 1500 2000 2500 3000 3500
-4
-4.5
-5
-5.5
-6
-6.5
ι_s = 150 μm ; ι^*_s = 3000 μm ; 10^4 PHOTONS

FIG. 8B

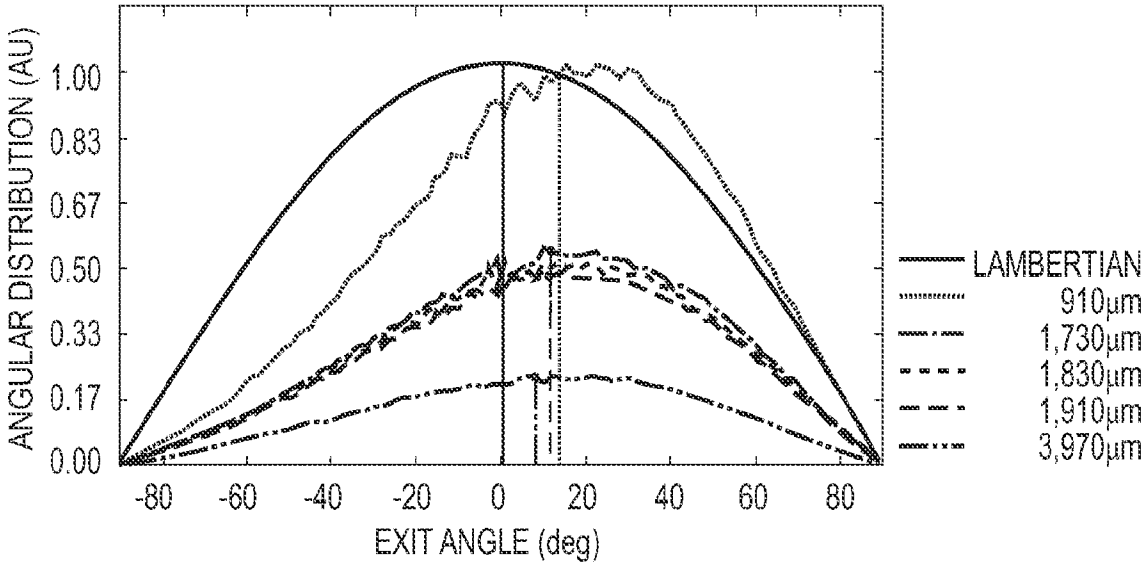

ANGULAR DISTRIBUTION (AU)
1.00
0.83
0.67
0.50
0.33
0.17
0.00
-80
-60
-40
-20
0
20
40
60
80
EXIT ANGLE (deg)
LAMBERTIAN
910µm
1,730µm
1,830µm
1,910µm
3,970µm

FIG. 8C

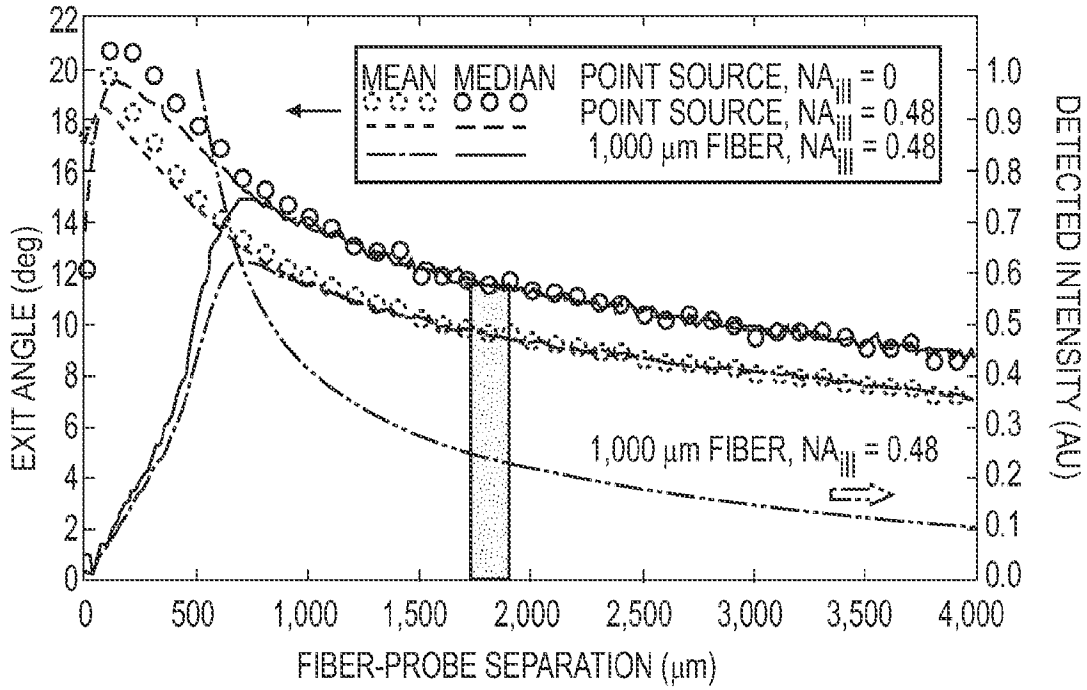

EXIT ANGLE (deg)
DETECTED INTENSITY (AU)
FIBER-PROBE SEPARATION (µm)
MEAN MEDIAN
POINT SOURCE, NA||| = 0
POINT SOURCE, NA||| = 0.48
1,000 µm FIBER, NA||| = 0.48
1,000 µm FIBER, NA||| = 0.48

FIG. 8D

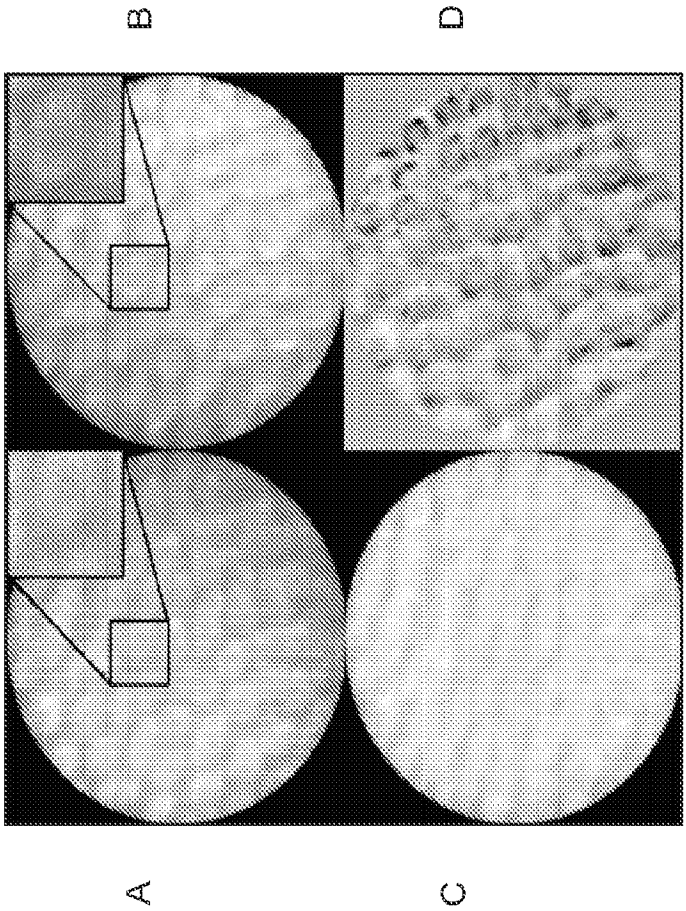


FIG. 9

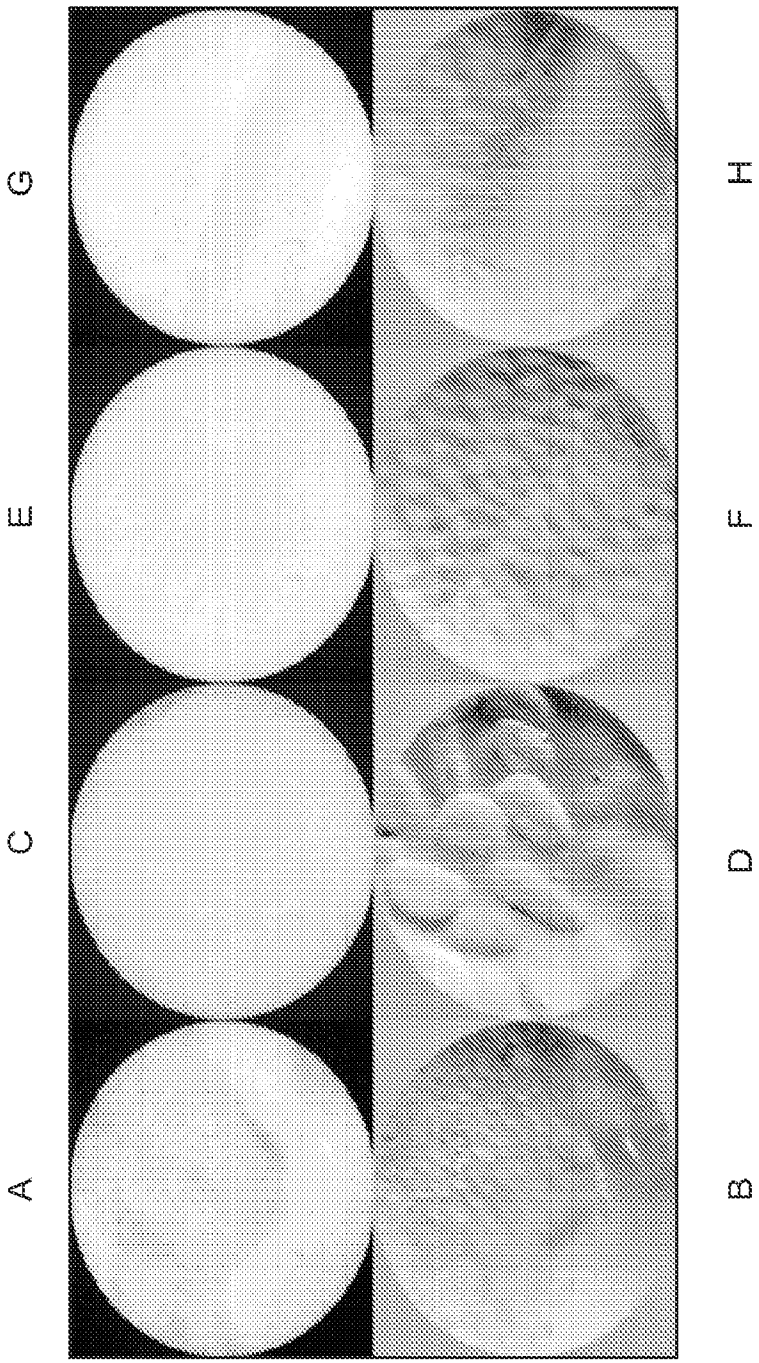


FIG. 10

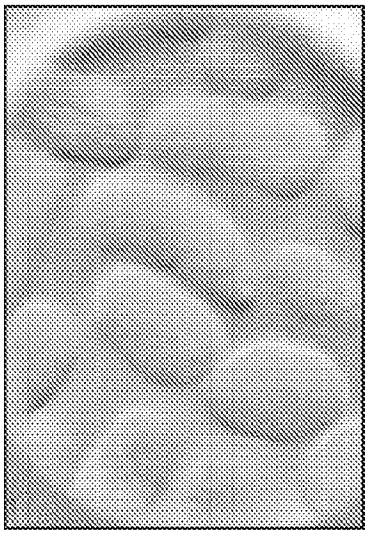


FIG. 11B

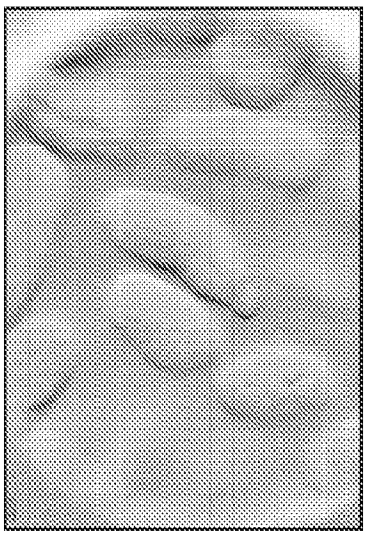
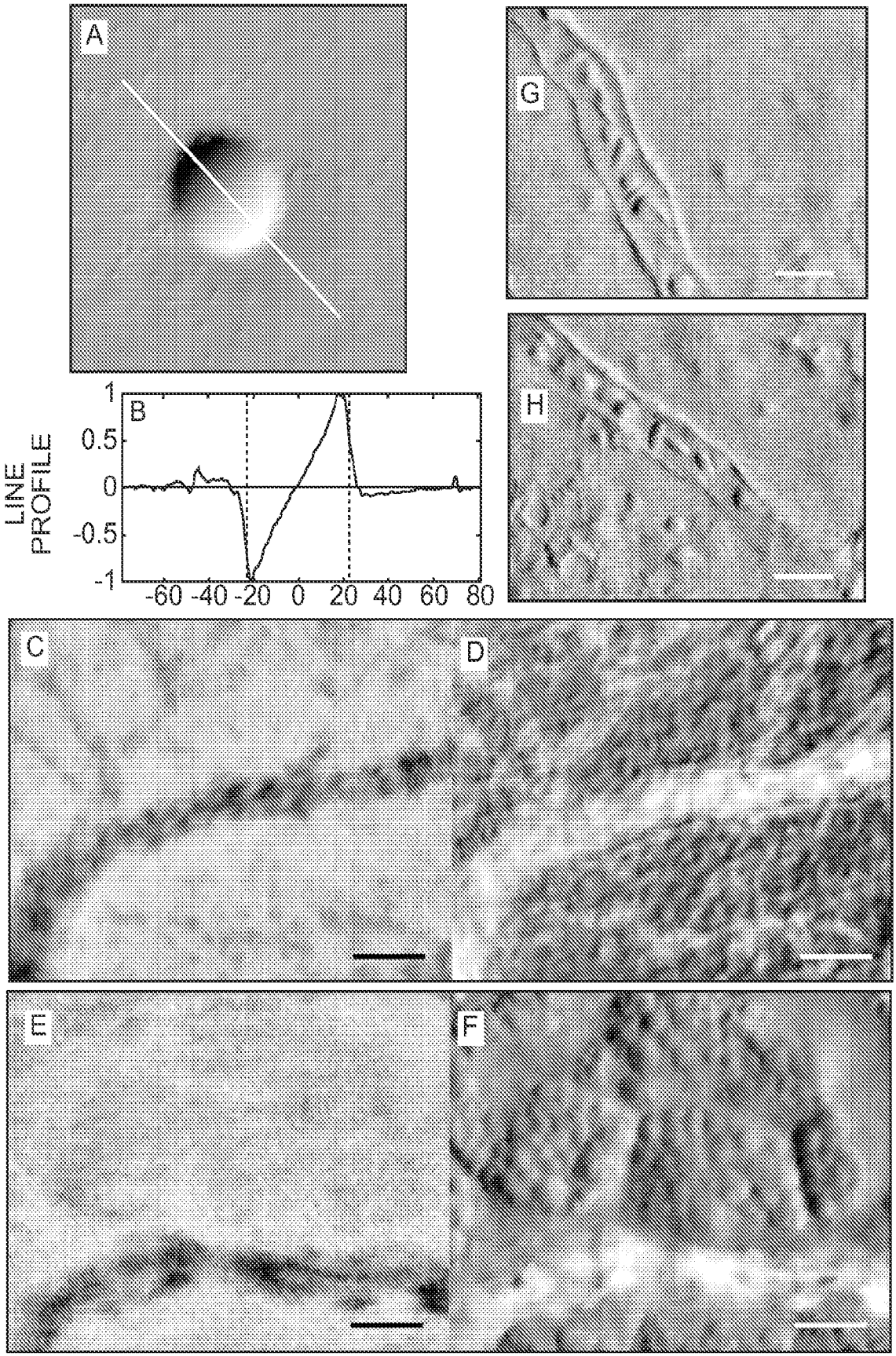


FIG. 11A


A
G
H
B
LINE PROFILE
1
0.5
0
-0.5
-1
-60 -40 -20 0 20 40 60 80
C
D
E
F

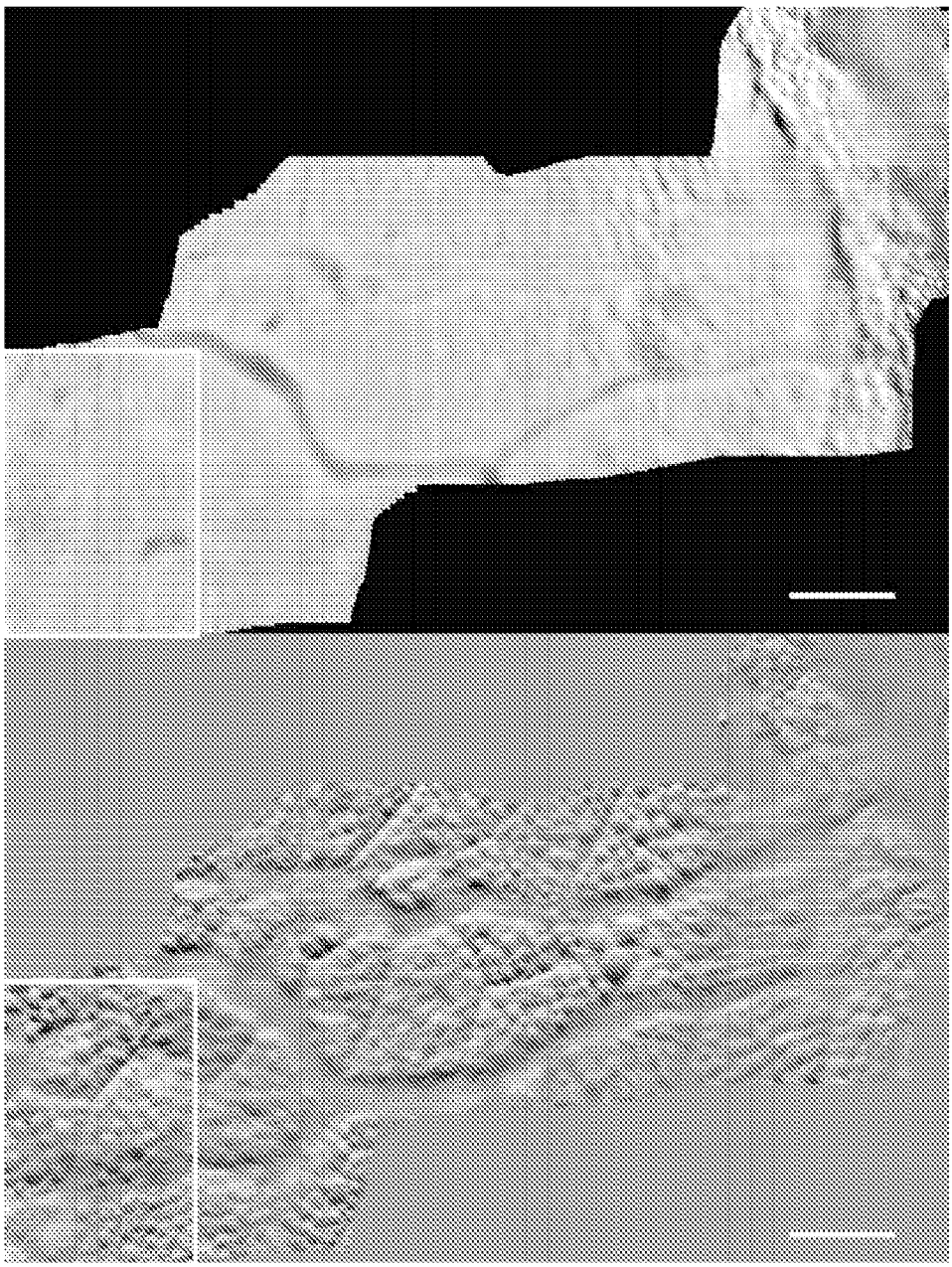


FIG. 13

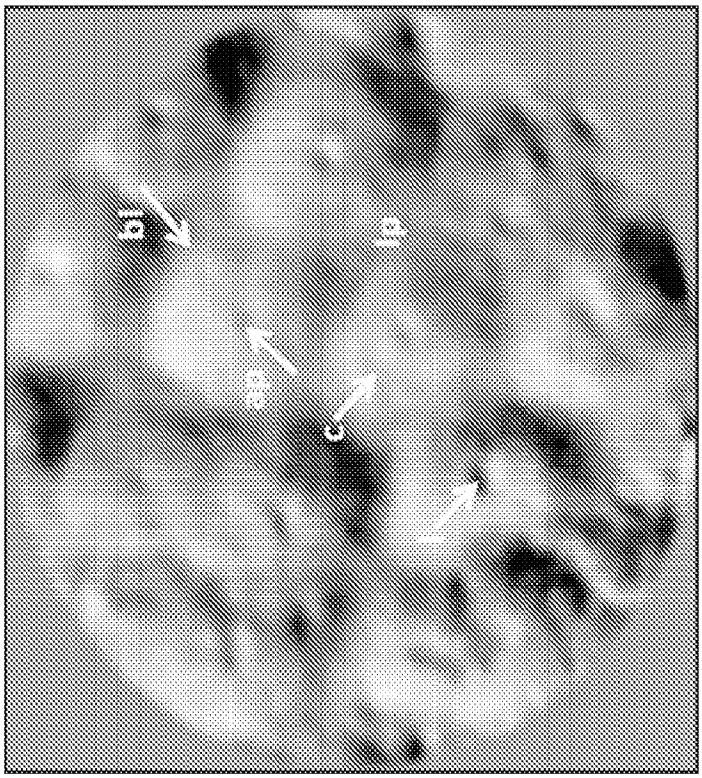


FIG. 14

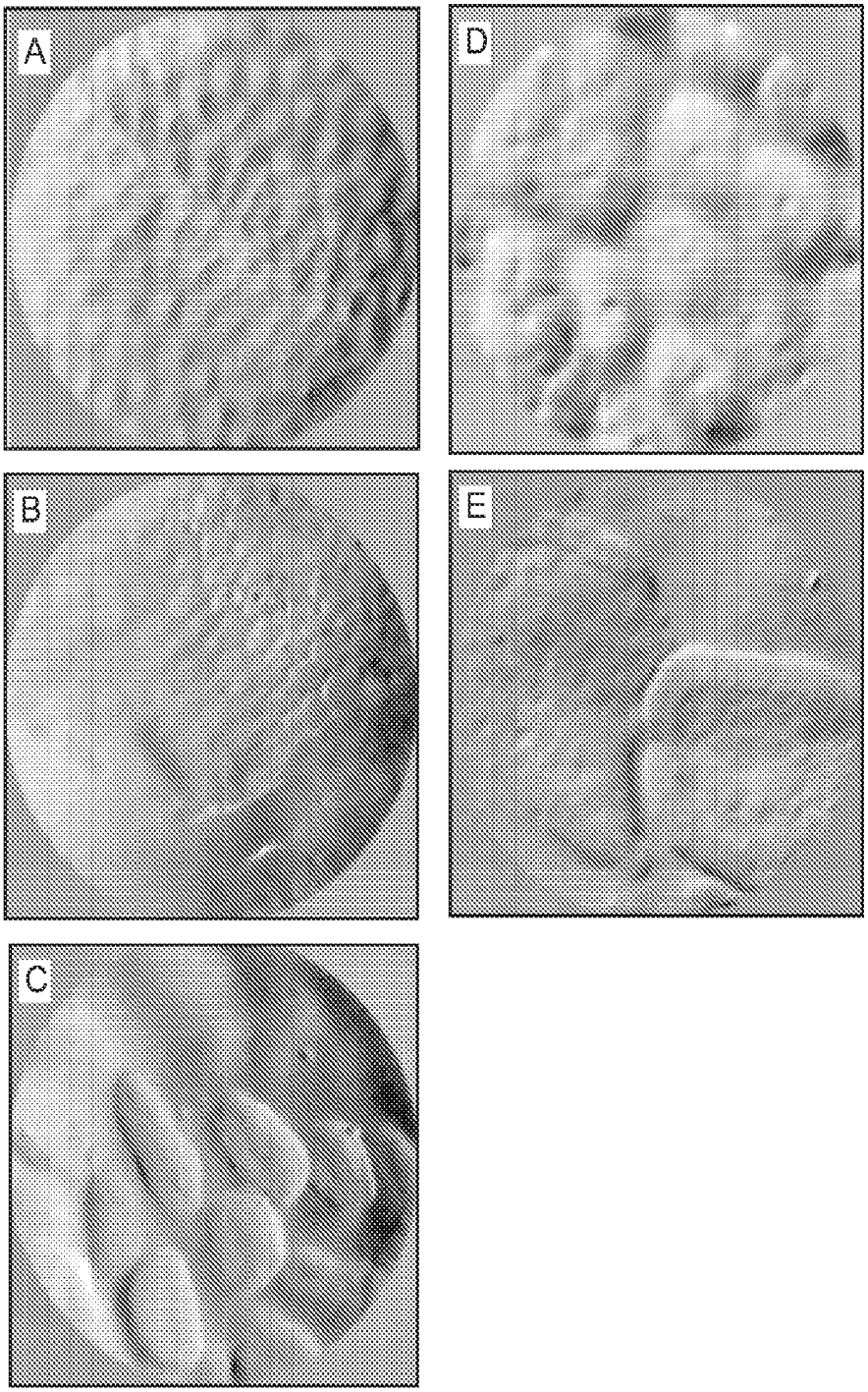


FIG. 15

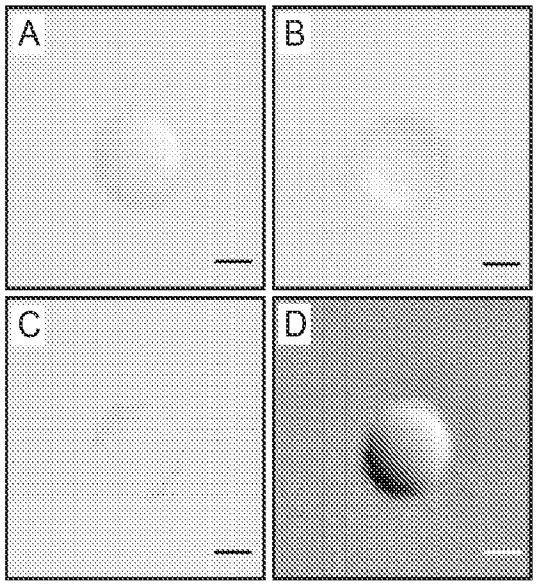

A
B
C
D

FIG. 16

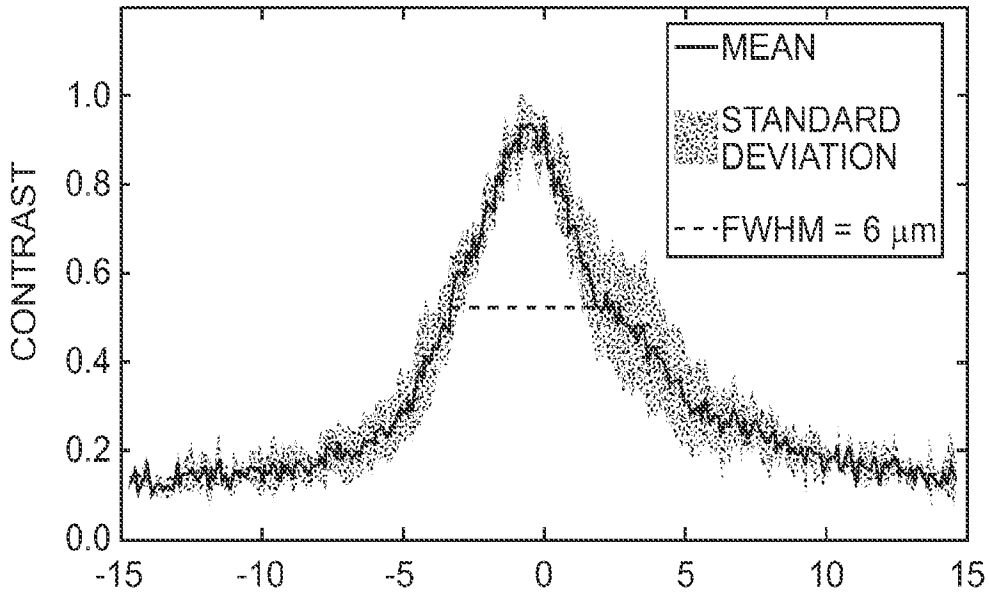

MEAN
STANDARD DEVIATION
FWHM = 6 μm
CONTRAST
1.0
0.8
0.6
0.4
0.2
0.0
-15
-10
-5
0
5
10
15

FIG. 17

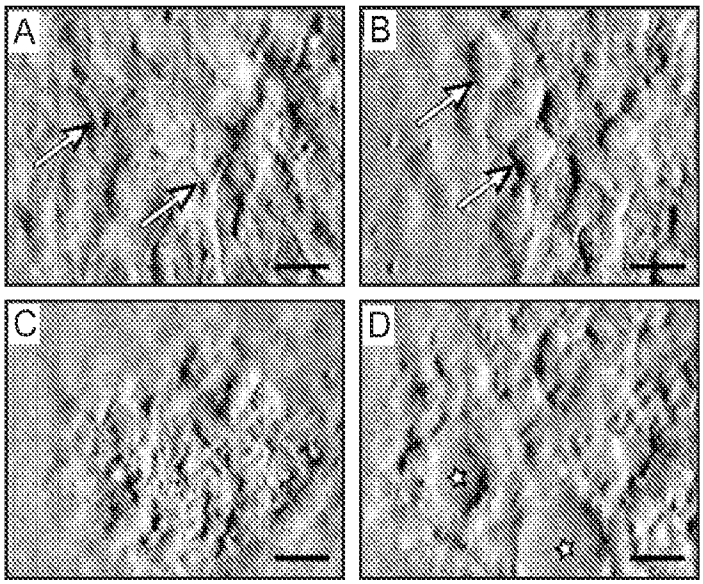


FIG. 18

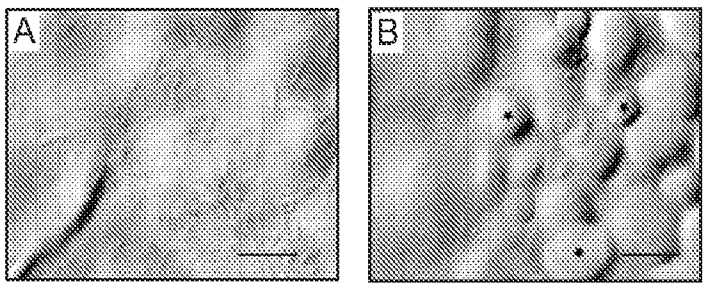


FIG. 19

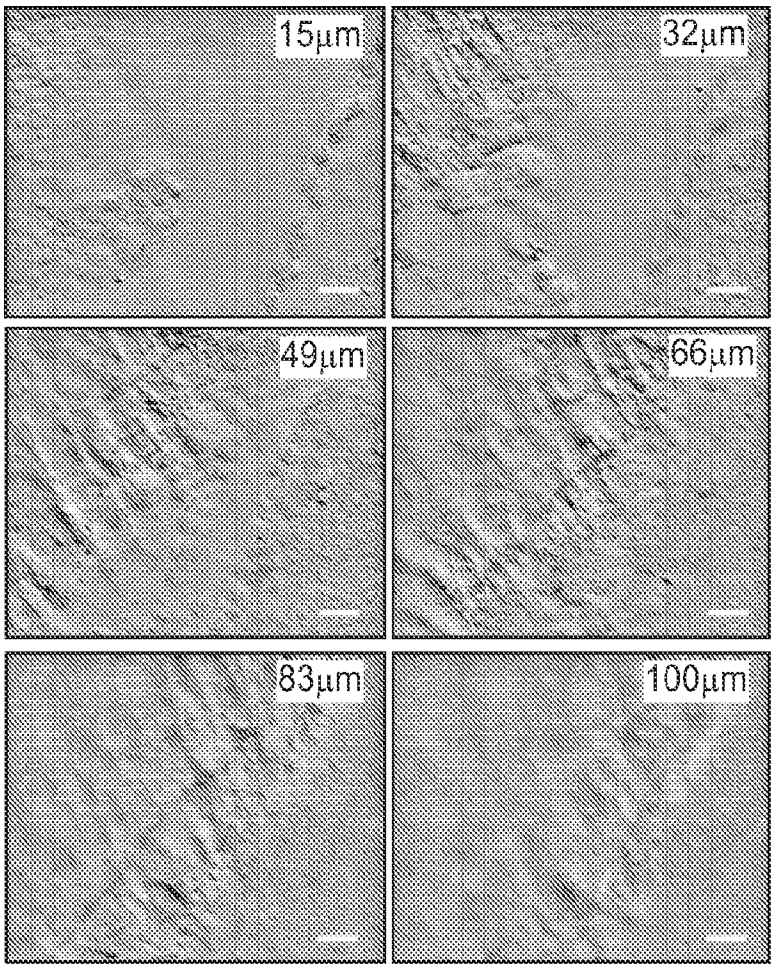


FIG. 20

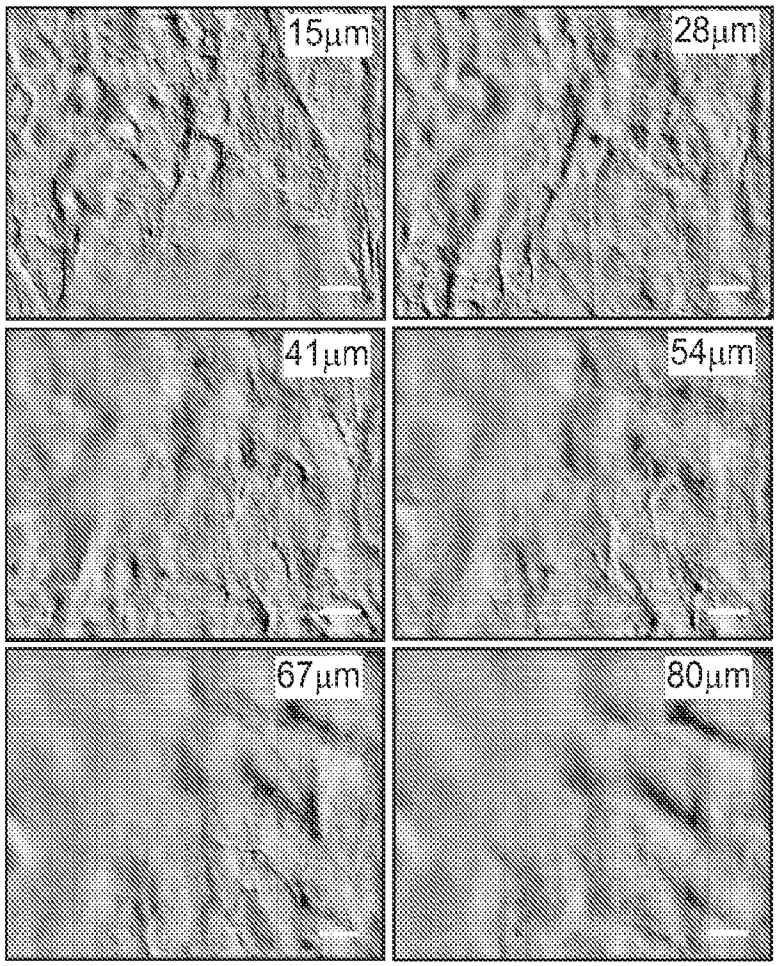


FIG. 21

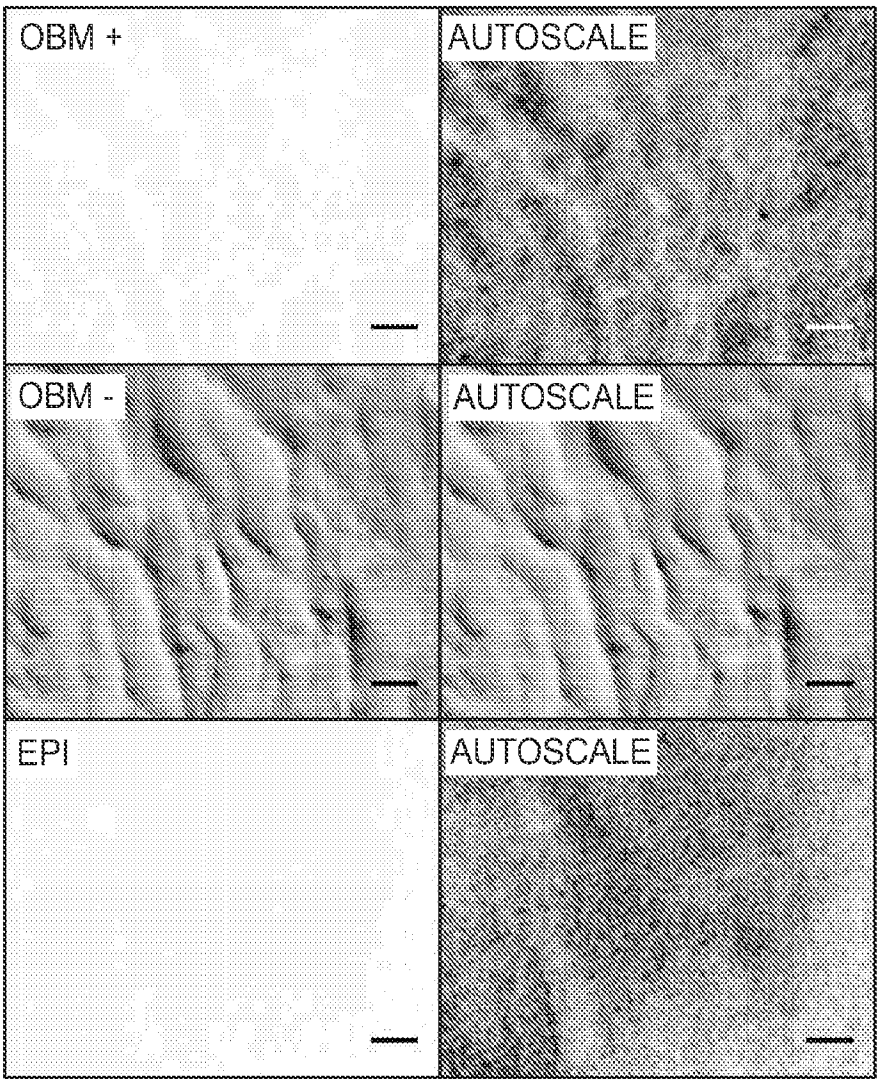


FIG. 22

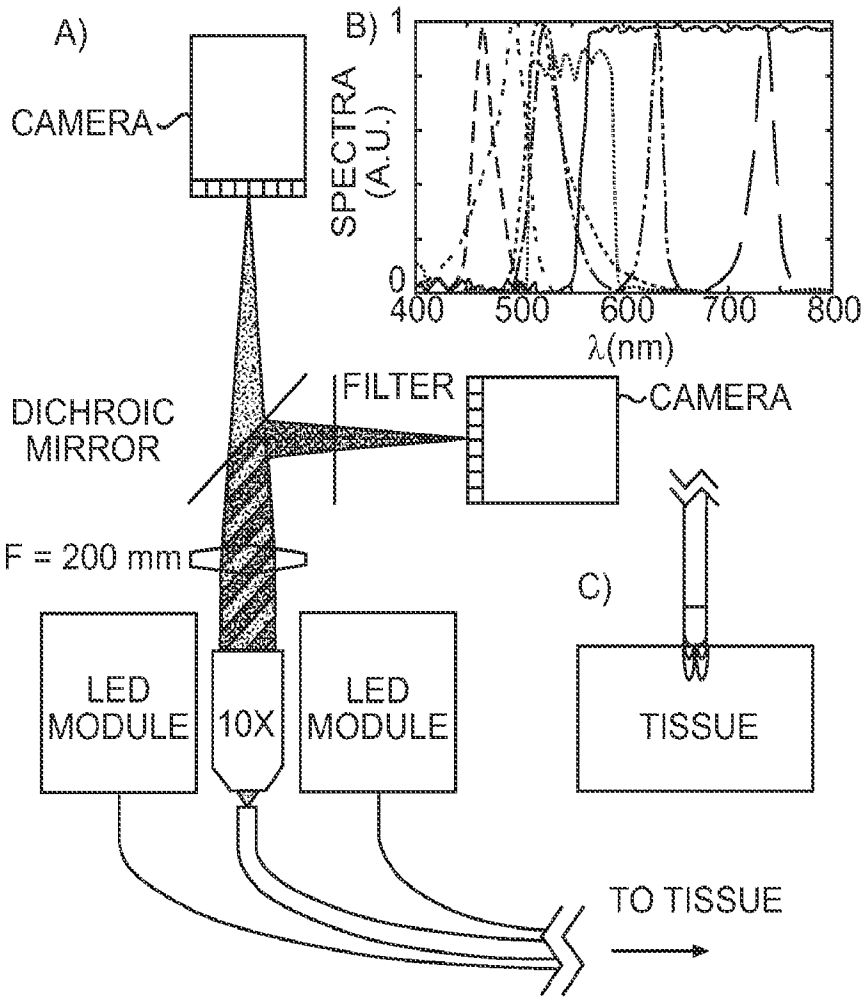

A)
CAMERA
B) 1
SPECTRA (A.U.)
0
400
500
600
700
800
λ(nm)
DICHROIC MIRROR
FILTER
CAMERA
F = 200 mm
C)
LED MODULE
10X
LED MODULE
TISSUE
TO TISSUE

FIG. 23

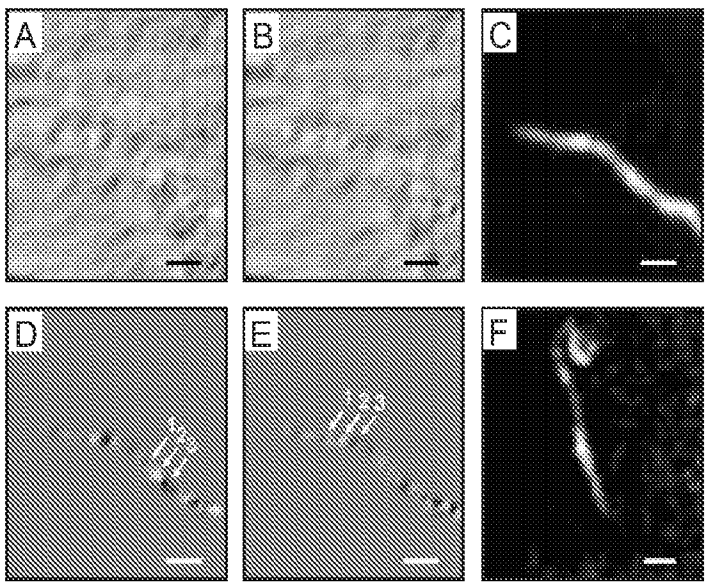

A
B
C
D
E
F

FIG. 24

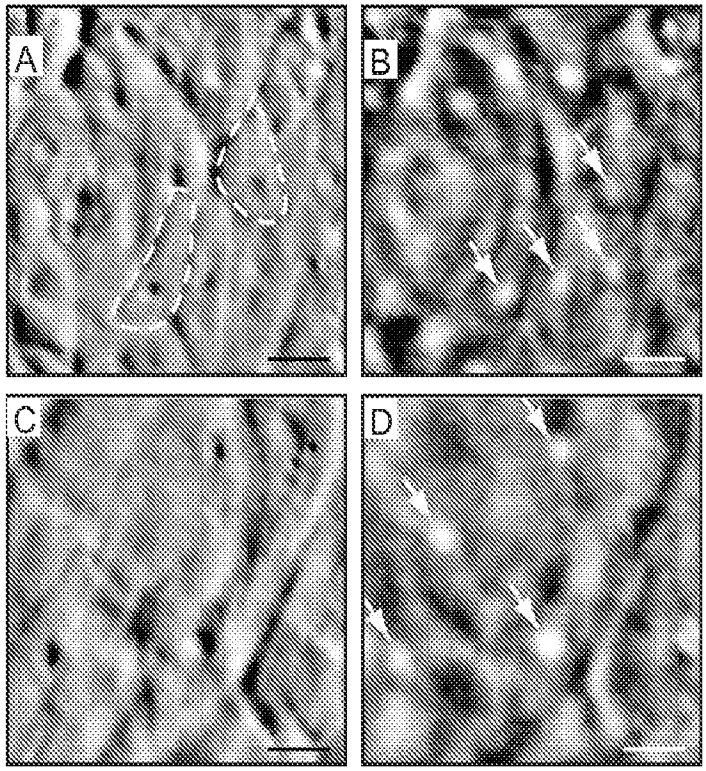


FIG. 25

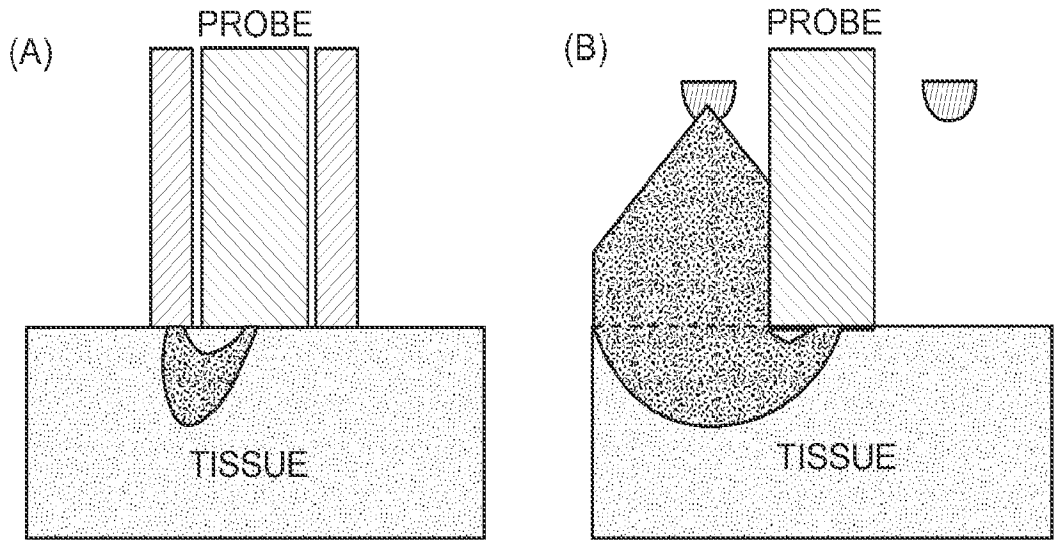


FIG. 26

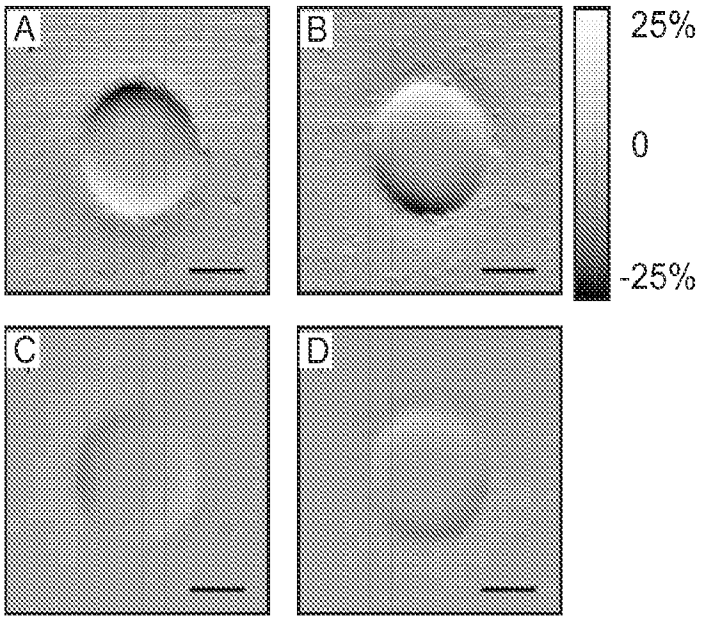


FIG. 27

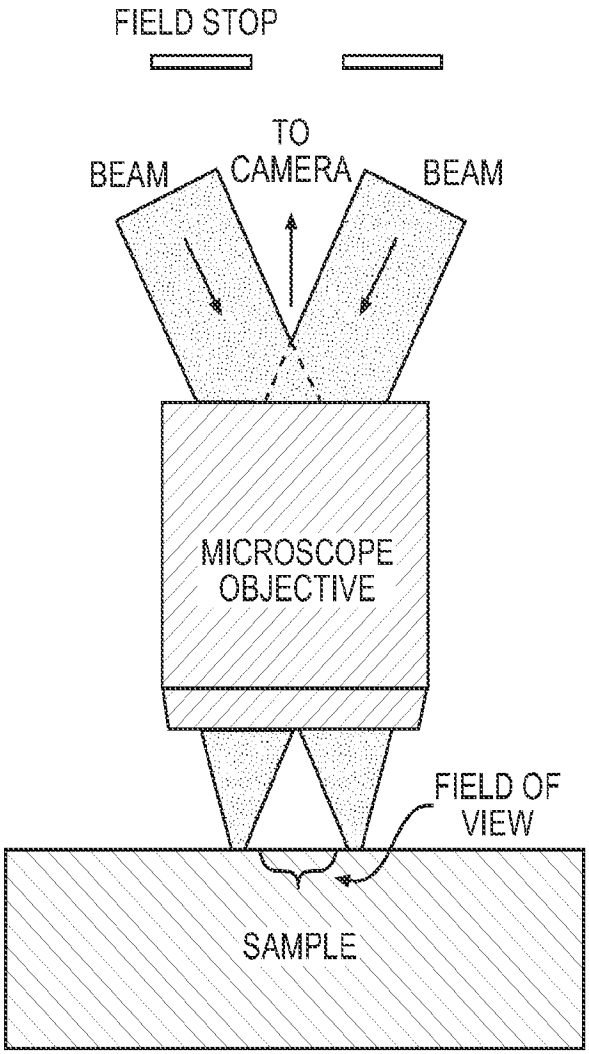

FIELD STOP
TO CAMERA
BEAM
BEAM
MICROSCOPE OBJECTIVE
FIELD OF VIEW
SAMPLE

FIG. 28

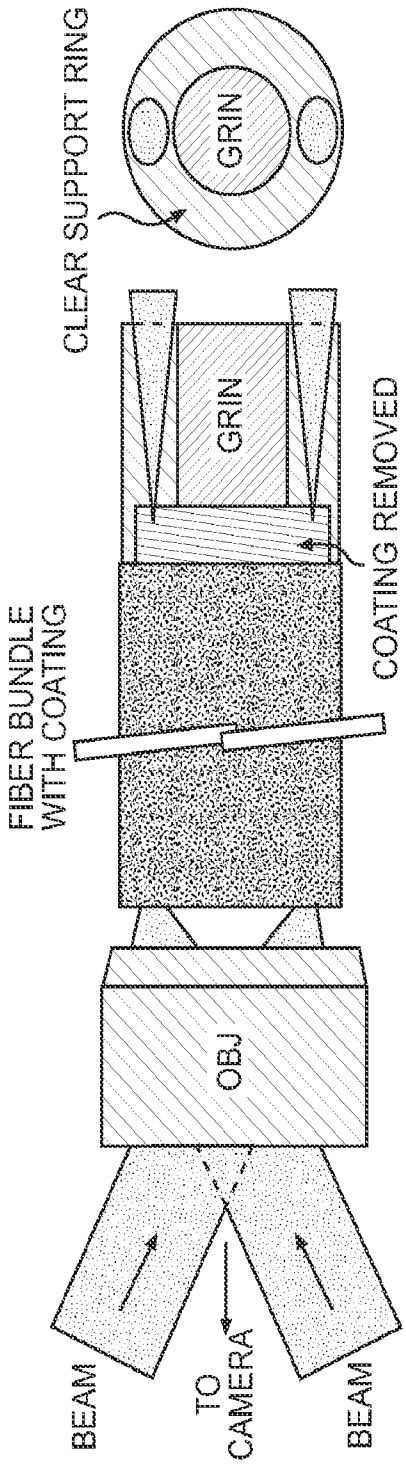

CLEAR SUPPORT RING
GRIN
GRIN
COATING REMOVED
FIBER BUNDLE
WITH COATING
OBJ
BEAM
TO
CAMERA
BEAM

FIG. 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032517

A. CLASSIFICATION OF SUBJECT MATTER

G02B 21/00(2006.01)i, G01N 21/00(2006.01)i, G01N 33/483(2006.01)i

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; G02B 21/14; H61B 6/00; C12Q 1/02; G01B 9/04; G01N 21/49; G01N 21/64; C12N 1/00; A61B 5/1455; A61B 1/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: phase contrast microcopy, oblique back illumination, back scattered, and optical fiber

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 08-194160 A (MITSUBISHI HEAVY IND., LTD.) 30 July 1996 See abstract; paragraphs 12-14 and figure 2.	1-2,42-48
A		3-5
A	US 2011-0263955 A1 (NARITA et al.) 27 October 2011 See abstract; paragraphs 63,64,82-88 and figures 2,3,8.	1-5,42-48
A	US 5973779 A (ANSARI et al.) 26 October 1999 See abstract; column 6, line 30-column 7, line 28 and figures 1,3.	1-5,42-48
A	US 5827190 A (PALCIC et al.) 27 October 1998 See abstract; column 8, line 38-column 9, line 52 and figures 2,5.	1-5,42-48
A	US 6905838 B1 (BITTNER, CHRISTOPH) 14 June 2005 See column 5, line 21-column 6, line 3 and figure 5.	1-5,42-48

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

☒ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
20 June 2013 (20.06.2013)	**21 June 2013 (21.06.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
AHN, Jae Yul
Telephone No. 82-42-481-8525

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/032517

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 08-194160A	30.07.1996	JP 3434064 B2	04.08.2003
US 2011-0263955 A1	27.10.2011	JP 2010-008286 A	14.01.2010
		JP 2010-008287 A	14.01.2010
		JP 2010-057566 A	18.03.2010
		JP 2010-060330 A	18.03.2010
		JP 2010-060331 A	18.03.2010
		JP 2010-060332 A	18.03.2010
		WO 2011-081141 A1	07.07.2011
US 05973779 A	26.10.1999	None	
US 05827190 A	27.10.1998	EP 0752825 A1	21.04.1999
		EP 0752825 B1	20.09.2000
		EP 0920831 A1	09.06.1999
		EP 0920831 B1	17.08.2005
		EP 1472972 A1	03.11.2004
		EP 1472972 B1	11.10.2006
		JP 10-500588 A	20.01.1998
		JP 2002-301009 A	15.10.2002
		JP 2004-154592 A	03.06.2004
		JP 3683271 B2	17.08.2005
		JP 3694667 B2	14.09.2005
		US 5590660 A	07.01.1997
		US 5827190 A	27.10.1998
		WO 95-26673 A3	12.10.1995
US 6905838 B1	14.06.2005	AT 293787 T	15.05.2005
		AU 2000-79187 A1	23.04.2001
		AU 7918700 A	23.04.2001
		CN 1181334 C0	22.12.2004
		CN 1399717 A0	26.02.2003
		DE 19949029 A1	17.05.2001
		DE 19949029 C2	21.11.2002
		EP 1248947 A2	16.10.2002
		EP 1248947 B1	20.04.2005
		JP 2003-529747 A	07.10.2003
		WO 01-27591 A2	19.04.2001

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032517

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

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

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

2. ☒ Claims Nos.: 14,25-30,34,50,51,53,62,63,67,76
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 14,25-30,34,50,51,53,62,63,67,76 are unclear, because they refer to multiple dependent claims which does not comply with PCT Rule 6.4(a). Therefore, they do not meet the requirement of PCT Article 6.

3. ☒ Claims Nos.: 6-13,15-24,31-33,35-41,49,52,54-61,64-66,68-75,77-79
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.