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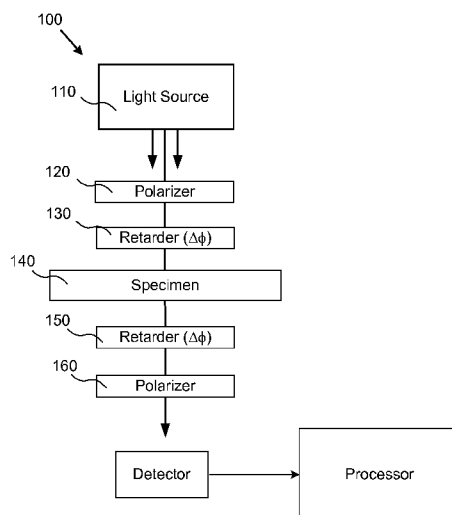


FIG. 1

(57) Abstract: Described herein is a polarization microscope that uses spectral-encoding of the polarization state for cellular imaging. The spectral-encoded polarization microscope is both sufficiently fast for cellular imaging and is compatible with high extinction optics required to image molecular structures and assemblies. The spectral-encoded microscope allows for the polarization state of light presented to the specimen to sample discrete states over the entirety of the Poincaré sphere while simultaneously giving a null measurement of the observed cellular birefringence. Sampling over the entire Poincaré sphere allows the microscope to determine of specimen phase retardation due to both linear and circular birefringence. The spectral-encoded polarization microscope can be operated in a slightly off-null state that will improve signal-to-noise.



**TITLE: SPECTRALLY-ENCODED HIGH-EXTINCTION POLARIZATION
MICROSCOPE**

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention generally relates to polarized light microscopy.

2. Description of the Relevant Art

Cell structures such as polymers, membranes or vesicles are birefringent. Birefringence can be detected and imaged based on how these structures interact with polarized light. However, to fully characterize these interactions, the polarized light must be modulated to allow different polarization states incident on the sample.

Modulated polarization microscopy (MPM) has the demonstrated ability to image cytoskeletal elements and other structures in living cells. To visualize these structures, images must be acquired while the polarization state of the illuminating light is modulated or varied over time. State of polarized light that interacts with the sample is modified. Image detail is encoded as small changes in intensity as the polarization state is modulated. Detecting changes in image intensity as one modulates the polarization state allows one to determine specific birefringence signals of the specimen from the detected signal. However, lateral movement of cellular objects over the time period of polarization modulation degrades the value of recorded data. Such movement artifacts are most readily observed with tiny structures that may move one or more pixels during the course of polarization modulation. Therefore, an important factor that limits the performance of MPM is the speed of modulation and the ability of the camera to record high resolution images (both in spatial resolution and bit depth) at rates sufficiently fast to mitigate movement artifacts.

Procedures to modulate the polarization state have, heretofore, involved devices such as mechanical rotation of polarizers or waveplates (e.g., $\frac{1}{2}$ wave); liquid crystal retarders, or Faraday rotators. Mechanical rotation of polarizers or $\frac{1}{2}$ -wave plates is limited by the mechanical inertia of the element and is a slow process and frequently introduces vibration and image blurring. Furthermore, mechanical rotation of a single element (e.g., a polarizer or $\frac{1}{2}$ -

waveplate) may not provide sufficient sampling of the Poincaré sphere and determination of both linear or circular birefringence may not be possible. Liquid crystal retarders can provide a complete sampling of the Poincaré sphere but they are slow and they provide poor polarization purity (contrast ratios). Poor polarization purity reduces the intensity changes due cellular birefringence. The slow speed of liquid crystal retarders prevents observation of many cellular processes in real time, while the poor polarization purity limits the types of cellular structures that can be observed. Faraday rotators are fast and compatible with high polarization purity. Faraday rotators, however, introduce technical challenges and do not provide a full sampling of the Poincaré sphere and thus are not able to isolate linear and circular birefringence.

None of these devices are capable of providing the necessary modulation of the polarization state of light at a speed sufficiently fast to visualize moving, living specimens.

SUMMARY OF THE INVENTION

In one embodiment, a polarization microscope includes a variable wavelength light source; a first polarizer that transmits light in a pure polarization state (linear, circular, elliptical, radial, or azimuthal) optically coupled to the variable wavelength light source. The first polarizer is optically coupled to a first retarder (linear, circular or elliptical). A specimen stage is optically coupled to the first retarder, wherein the specimen stage holds a specimen in the optical pathway of the light received from the first retarder. A second retarder, having the opposite-sign to the first retarder, is optically coupled to the specimen stage to receive light that passes through the specimen stage. A second polarizer is optically coupled to the second retarder, the second polarizer being arranged orthogonal to the first polarizer. An optical capture system is optically coupled to the second polarizer.

The variable wavelength light source, in one embodiment, is capable of producing light having a wavelength from between about 350nm to about 800nm.

In one embodiment, the first polarizer and/or the second polarizer is a polarizing prism.

In one embodiment, the first and second retarders are crystal polarized light rotators. The crystal polarized light rotators may be composed of tellurium dioxide. In one embodiment, the polarization microscope comprises a first crystal polarized light rotator and a second crystal polarized light rotator, wherein the first crystal polarized light rotator and the second crystal polarized light rotator are matched crystal rotators. The degree of rotation of the first polarized light rotator and the second polarized light rotator may be based, in part, on the wavelength of the incident light.

In one embodiment, the optical capture device is a charged coupled device or scientific CMOS imager with adequate sensitivity, resolution and speed of image capture.

During use, light from the first polarized light rotator passes through the specimen held on the specimen stage. Alternatively, light from the first crystal polarized light rotator is reflected from the specimen held on the specimen stage.

In an embodiment, the retarders are a pair of matched rotators (circular retarders) that cause a phase delay between left and right circular polarized light. In an embodiment, the retarders are matched elliptical retarders formed by a rotator (circular retarder) and a waveplate (linear retarder) optically coupled to each other. In another embodiment, the retarders are elliptical retarders formed by two waveplates oriented with respect to each other.

In an embodiment, a method of visualizing a specimen using a polarization microscope includes: placing the specimen on a specimen stage of a polarization microscope as described above and obtaining images of the specimen at one or more wavelengths. Obtaining images of the specimen may be performed by periodically changing the wavelength of light impinging on the specimen and capturing images of the specimen after each change of wavelength of light. Each change of wavelength may be accomplished in between 1 nanosecond and 500 microseconds whereas acquisition of one image can be accomplished in as little as 10 milliseconds. Imaging of the biological specimen may be performed continuously or intermittently as long as a set of images required for calculating polarization state are acquired time. In one embodiment, a set of images (e.g., 2 to 25 images) is produced by capturing images in succession (e.g. 10-20 milliseconds apart).

In an embodiment, the method further comprises modulating the polarization state of the light on the Poincaré sphere to produce both azimuthal (about the poles) and longitudinal (about an equatorial axis) movement on the Poincaré sphere by altering the wavelength of the light produced by the variable wavelength light source.

In an embodiment, the method further comprises: adjusting the orientation axis of the first polarizer with respect to the second polarizer so that the first polarizer and the second polarizer are not fully orthogonal; and obtaining images of the specimen while the first polarizer and second polarizer are not fully orthogonal. The method may also include calibrating the polarization microscope by determining the polarization state of the light when the second polarized light rotator and the second waveplate are removed from the optical path. Determining the polarization state of the light, in one embodiment, may be performed by methods known in the art of light polarization analysis such as rotating the second polarizer through discrete angles and analyzing the data collected by the optical capture device.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects and advantages of the invention will become apparent upon reading the following detailed description and upon reference to the accompanying drawings in which:

FIG. 1 depicts a schematic diagram of a polarization microscope;

FIG. 2 depicts a schematic diagram of a Poincaré sphere; and

FIG. 3 depicts a schematic diagram of an alternate embodiment of a polarization microscope.

While the invention is susceptible to various modifications and alternative forms, specific embodiments thereof are shown by way of example in the drawing and will herein be described in detail. It should be understood, however, that the drawings and detailed description thereto are not intended to limit the invention to the particular form disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present invention as defined by the appended claims.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is to be understood the present invention is not limited to particular devices or methods, which may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include singular and plural referents unless the content clearly dictates otherwise. Furthermore, the word “may” is used throughout this application in a permissive sense (i.e., having the potential to, being able to), not in a mandatory sense (i.e., must). The term “include,” and derivations thereof, mean “including, but not limited to.” The term “coupled” means directly or indirectly connected.

As used herein the term “retarder” refers to an optical element (composed of one or more optical components) is an optical element that provides an optical phase delay ($\Delta\phi$) between a pair of orthogonal states. The orthogonal states may be linear states oriented at ninety degrees, left- and right-circular polarization states, or orthogonal elliptical states. A retarder is specified by either of the two polarization states (sometimes called eigenstates) that propagate through the retarder element without a phase delay and the phase delay ($\Delta\phi$) between the two eigenstates. For example, a waveplate is linear retarder and is specified by two linear orthogonal states that when propagating through the retarder experience a phase delay. The retarder pair in the microscope are configured so that if these two elements were positioned sequentially light would experience no change in the polarization state. An elliptical retarder can be formed, for example, by a sequential combination of rotator (circular retarder) and a waveplate (linear retarder). Other combinations are known in the art, for example, an elliptical retarder may be formed from two waveplates that are oriented at 45 degrees.

As used herein the term “polarizer” refers to optical elements that transmit light in a pure polarization state (linear, circular or elliptical) with high extinction for each light wavelength emitted from the light source. One embodiment of a polarizer is a linear retarder the transmits a pure linear polarization state.

Described herein is a new approach to rapidly modulate the polarization state of light on the Poincaré sphere to produce both azimuthal (about the poles) and longitudinal (about an equatorial axis) movement on the Poincaré sphere. High speed modulation of the polarization state is combined with a relatively high speed imager (100 Hz or faster frame rates) that can

capture images corresponding to discrete polarization states of light interacting with the specimen. To the extent that movement of sample constituents can be frozen during the period of modulation, much better birefringence signal-to-noise ratio for individual objects is obtained. The design of the microscope is simple, and utilizes components that maintain high polarization contrast ratios. Furthermore, as will be explained, the design provides, for obtaining null or near null measurements of polarization states. The microscope design can be easily implemented by other laboratories making it useful for a wide range of studies.

Microscopy has been advancing on several fronts to improve resolution in both space and time. Better Z-axis resolution has been achieved using confocal or two-photon microscopy whereas a variety of approaches have now broken through the diffraction barrier to obtain superresolution. However, these techniques are largely based on fluorescence which can require labeling specific proteins or structures. As powerful and important as fluorescence microscopy is, fluorescent probes bleach thereby limiting the time of observation.

Polarized light microscopy provides a different mode of imaging with contrast based on molecular structure and orientation. Polarized light microscopy has long been used for imaging spindle microtubules based on their birefringence. However, there are many cellular structures that can be detected with high contrast under polarized light including various filament systems (actin, microtubule, intermediate filaments and collagen), membrane boundaries including those of the plasma membrane, cellular vesicles and various organelles and cellular structures that show crystalline-like organization. Membrane boundaries exhibit edge birefringence that can be determined with better precision than predicted by the traditional resolution limits. Indeed, preliminary data suggest that polarized light microscopy may be applied to image viral particles in cells. Furthermore, nanoparticles offer a unique approach for labeling proteins and observing protein interactions using polarized light. Finally, unlike with fluorescence, cells can be imaged for long periods of time.

In order to realize the full capabilities of polarized light microscopy and visualize circular or linear birefringent structures, one cannot simply image the cell using crossed polarizers. The small retardances of biological structures are mostly masked by scattered light.

Contrast in polarized light images arises from changes in phase and amplitude due to differential retardation or attenuation of orthogonally polarized beams as they travel through the

specimen. Form-birefringence is exhibited in cells and tissues by various polymers including collagen and the cell cytoskeleton. The electric field of incident light oscillating perpendicular to the fibers ($\mathbf{E}_\perp$) induces surface charges that create an induced field ($\mathbf{E}_o$) within the fiber. The induced field ($\mathbf{E}_o$) anisotropically modifies forward scattered light so that phase and amplitude of $\mathbf{E}_\perp$ is altered relative to the electric field component polarized parallel to the fibers ($\mathbf{E}_\parallel$). The incremental **phase retardation** (δ_i) incurred by the perpendicular component ($\mathbf{E}_\perp$) results in slower light transmission and larger refractive index ($\mathbf{n}_s$) than that experienced by light polarized parallel to the fiber axis ($\mathbf{E}_\parallel$) with refractive index $\mathbf{n}_f$. The numerical difference between indices of refraction of light oscillations polarized along fast and slow axes is the *form-birefringence* ($\Delta \mathbf{n} = \mathbf{n}_s - \mathbf{n}_f$). Incremental phase retardations (δ_i) accumulate through fibrous structures and the composite phase retardation (δ) between components polarized parallel ($\mathbf{E}_\parallel$) and perpendicular ($\mathbf{E}_\perp$) to the fibers after propagating a distance Z is

$$\delta = \frac{360 \cdot \Delta n}{\lambda_o} \cdot Z \quad (\text{Eq.1})$$

where δ is given in degrees. In addition to phase retardation between $\mathbf{E}_\parallel$ and $\mathbf{E}_\perp$, forward scattered light may have a scattering anisotropy resulting in differential attenuation of light amplitudes. This quantity is given by the *form-biattenuance* ($\Delta \chi$). Similarly, the composite relative attenuation (ε) between components polarized parallel ($\mathbf{E}_\parallel$) and perpendicular ($\mathbf{E}_\perp$) to the fibers after propagating a distance Z is

$$\varepsilon = \frac{360 \cdot \Delta \chi}{\lambda_o} \cdot Z \quad (\text{Eq. 2})$$

where ε is given in degrees. Effect of form-birefringence ($\Delta \mathbf{n}$) and form-biattenuance ($\Delta \chi$) between parallel ($\mathbf{E}_\parallel$) and perpendicular ($\mathbf{E}_\perp$) field components may be accounted for, respectively, by real ($\Delta \mathbf{n}$) and imaginary ($\Delta \chi$) parts of the complex differential wavenumber (β):

$$\beta = \beta_{re} + i\beta_{im} = \frac{2\pi}{\lambda_o} (\Delta n + i\Delta \chi) \quad (\text{Eq. 3})$$

Relative amplitude and phase between perpendicular ($\mathbf{E}_\perp$) and parallel ($\mathbf{E}_\parallel$) field components can be expressed mathematically by the complex relative-amplitude ($\mathbf{E}_\perp/\mathbf{E}_\parallel$). After forward scattered light propagates through a distance (Z), complex relative-amplitude is given by

$$E_{\perp}(z)/E_{\parallel}(z) = \exp(-\beta_{im} \cdot Z) \cdot \exp(i\beta_{re} \cdot Z)$$

Here, β_{re} is proportional to form-birefringence (Δn) and β_{im} is proportional to form-biattenuance ($\Delta\chi$). Accurate quantitative measurements of $\Delta\chi$ in cells have not been reported.

Another type of birefringence seen in cells is known as edge birefringence, which can be seen at the boundary between dielectric interfaces such as between water and cell membranes. Edge birefringence is an incompletely understood phenomena thought to be due to interference at boundaries where light from three different paths mix. It has been noted that edge birefringence can allow for determination of boundaries with greater accuracy than is obtainable with other types of microscopy. This feature of edge birefringence is consistent with our own observations and of notable value in experimental studies. Certain crystalline structures such as bone, glycogen granules are intrinsically birefringent. For reasons that are not clear at present, large T cell secretory vesicles are highly birefringent and may be an example of intrinsic birefringence. Another type of birefringence change is seen during neuronal action potentials. There seem to be several different cellular sources of this birefringence and they have not been well characterized. At least some of the signal change seems to arise from responses of membrane proteins and/or lipids to the change in potential. Based on the kinetics of the birefringence change, a second component has been attributed to calcium release from the sarcoplasmic reticulum. Finally, although circular birefringence is believed to be present in various cellular constituents (e.g. glucose) previous forms of circular birefringence have not provided contrast to observe these sorts of structures.

While there are many cellular sources of birefringence, these signals are typically quite small and are obscured by background light and optical aberrations. Furthermore, the brightness of the birefringent object depends on the orientation of the specimen with respect to the polarization angle or phase. To separate sample birefringence from the background, it is necessary to modulate the polarization state of light illuminating the specimen and then determine the birefringence quantitatively from the measured changing amplitudes. We refer to this imaging methodology as modulated polarization microscopy.

Any suitable camera with suitable speed, resolution, and low noise may be used to record the images. In order to take advantage of the fast modulation rate, the camera frame rate also must be proportionally fast. At the same time, since calculation of birefringence depends on

small changes in light intensity, the numerical precision (bits per pixel) and resolution (number of pixels) is also important. The camera should also have low noise and high sensitivity.

Exemplary cameras in the contemporary art that may be used include the Hamamatsu Orca Flash 4, the Andor "Zyla" and similar cameras.

In a previous system, we chose to modulate plane polarized light using two matched Faraday rotators to rotate the plane of polarized light through 90 degrees but in opposite directions. Faraday rotators are capable of modulating polarized light at speeds better than 1 KHz and glass-based systems like ours maintain a high degree of extinction ($\sim 50,000$) whereas liquid crystal retarders are relatively slow (requiring 0.1 seconds to settle down before an image is acquired) and they give low birefringence contrast corresponding to poor polarization extinction ratios (< 1000).

While producing good contrast static images, liquid crystal rotators were not useful for providing images of cytoskeletal elements in living fast moving cells. Faraday rotators could be used to visualize small structures in living cells, but are limited in that they only rotate plane polarized light and thus cannot sample the entire Poincaré sphere. Faraday rotators also require input of large amounts of electrical power into the magnets where heating becomes a problem for consistent operation of the instrumentation. High-current Faraday rotators must be water cooled and, even with cooling, one must constantly compensate for temperature changes.

Given the many problems with previous designs, we devised a novel approach to perform modulated polarization microscopy. A number of important features guided this design that enables harnessing the power of polarized light microscopy not demonstrated heretofore. The first feature was the ability to modulate the polarization state of incident light over the entire Poincaré sphere and record images at high speed. Secondly, it was important to capture digital images with high numerical precision and high resolution. Thirdly, it was important to use an optical train and components that maintain high polarization purity. Fourthly, it was important that the modulation approach allows for a null measurement. By this we mean that regardless of how the polarization state is modulated before the specimen, after the specimen it is demodulated so that one views the specimen as if between crossed polarizers. Fifthly, we provide support that an improvement in signal-to-noise ratio is obtained if the polarizer and analyzer are not orthogonal. Rather, signal to noise may be improved if the polarizer and analyzer are oriented off

from the orthogonal configuration resulting in a partially null state. Finally, we sought a system that could be readily commercialized and made available to many users.

A schematic diagram of a polarization microscope **100** capable of performing modulated polarization microscopy is depicted in FIG. 1. Polarization microscope **100** includes a variable wavelength light source **110**. Variable wavelength light source **110** is capable of emitting light at multiple wavelengths in a spectral range of about 300 nm to about 1000 nm. In some embodiments, variable wavelength light source **110** is capable of producing light having a wavelength from between about 350nm to about 800nm. Variable light source **110** may have a fast switching time between spectral emissions. In some embodiments, the switching time is shorter than the blanking interval between successive frames. In some embodiments, variable wavelength light source **110** is capable of changing emission wavelengths at a maximum speed of between 1 microsecond/wavelength and 5000 microseconds/wavelength. Variable wavelength light source **110** may be rapidly switched between emission wavelengths and provide sufficient radiant flux incident on the specimen over a narrow band of wavelengths. Examples of variable wavelength light sources that may be used include, but are not limited to: supercontinuum sources; arc lamps; plasma lamps; induction lamps; combination of diode lasers; tunable lasers, light emitting diodes (LED); Digital Light Projection (DLP) based devices; etc.

Variable wavelength light sources that may be used include two types of light sources: 1) light sources that can simultaneously emit a multiplicity of wavelengths combined with a spectrally tunable filter; or 2) discrete emission wavelength emitting light sources that are switched or tuned over time. For sources that emit a multiplicity of wavelengths simultaneously, a tunable spectral filter (e.g., monochromator, Fabry-Perot filter, acousto-optic filter) is used to select specific emission wavelengths. Light sources that simultaneously emit a multiplicity of wavelengths include supercontinuum sources, arc lamps, light emitting diodes (LEDs). For laser sources that switch emission wavelengths, an exemplary embodiment would be a rapidly tunable laser or light source composed of multiple diode laser elements each with a small M^2 number that are combined in for example a multimode optical fiber. The purpose of the multimode fiber is to spatially decorrelate or render light spatially incoherent. An exemplary variable wavelength light source that uses a lamp and tunable filter is the OL490 Agile Light Source from Optronic Laboratories (Orlando, FL). Similarly, a bright green LED in combination with a tunable spectral filter can serve as a light source. Exemplary supercontinuum sources are manufactured by NKT. An acousto-optic filter can be used to rapidly select a narrow band (1-5nm) of spectral

emission. Since the light emitted by the supercontinuum source has a high degree of spatial coherence, light may be coupled into a multimode fiber to provide spatially incoherent light incident on the sample. An exemplary light source that uses discrete laser diodes that are combined using dichroic elements is manufactured by Lumencor (Beaverton, OR). Tunable laser sources may also be used. In this embodiment, a laser source with a gain media that covers the spectral range of interest, and includes a tunable element in the laser cavity. The various light emission wavelengths are selected by the tunable element in the laser cavity. Light emitted by the tunable laser is coupled into a multimode optical fiber to reduce spatial coherence.

Polarization microscope **100** includes a first polarizer **120** having a first polarization axis. First polarizer **120** is optically coupled to variable wavelength light source **110**, and thus functions as the polarizer for the light source. First polarizer **120** receives light from the variable wavelength light source and converts the light into linear polarized light having an orientation equal to the first polarization axis (arbitrarily depicted in FIG. 1). First polarizer **120** may be a polarizing prism whose performance, preferably, does not depend on the wavelength of light used. Examples of polarizing prisms include, but are not limited to, Glan-Thompson prisms, Glan-Taylor prisms, and Glan-Foucault prisms. A second polarizer **160**, having a second polarization axis (arbitrarily depicted in FIG. 1), functions as the analyzer. Second polarizer **160** is oriented such that the second polarization axis is orthogonal to the first polarization axis. Second polarizer **160** is also a wavelength independent polarizer. Second polarizer **160** may be a polarizing prism. Preferably, first polarizer **120** and second polarizer **160** are matched polarizers having similar construction and optical properties.

Between first polarizer **120** and second polarizer **160** are two light retarders **130, 150**. . A first retarder **130** is optically coupled to first polarizer **120**. Second retarder **150** is an opposite-signed retarder with respect to the first retarder.

In one embodiment, the retarders are rotators (a circular retarder) that cause a phase delay between left and right circular polarized light. In another embodiment, an optical element composed of a rotator (circular retarder) and a waveplate (linear retarder) may be used as an elliptical retarder. In another embodiment, an optical element composed of two waveplates oriented at 45 degrees, with respect to each other, may be used as an elliptical retarder.

In an exemplary embodiment, retarders may be crystal polarized light rotators fabricated from quartz or TeO₂ crystals. In an embodiment, the crystal polarized light rotators are formed from left and right rotating version of TeO₂ crystals. Crystal polarized light rotators may rotate linearly polarized light independent of the angular orientation of the crystal such that circularly polarized light remains circularly polarized. The angle of rotation ($\Delta\phi$) by the crystal polarized light rotator about the pole on the Poincaré sphere is a function of wavelength (λ), thickness (d) and circular birefringence ($\Delta n(\lambda)$) of the rotator according to the equation below.

$$\Delta\phi(\lambda) = \frac{2\pi \cdot d \cdot \Delta n(\lambda)}{\lambda}$$

The first and second crystal polarized light rotators are matched light rotators (equivalent thickness (d) and circular birefringence ($\Delta n(\lambda)$)) such that the rotation produced by the first rotator is cancelled by the second rotator.

Retarders may also be made from levo- and dextrorotatory optically active organic compounds (enantiomers), or enantiomorphs. Retarders may be made from fixed magnet or electromagnetic Faraday rotators. In some embodiments, retarders may be made from thin film polymeric coatings or from suitable nanopatterning of optically transparent materials.

Polarization microscope **100** also includes a specimen stage **140** optically coupled to first retarder **130**. Specimen stage **140** holds a specimen in the optical pathway of the light received from first polarized light rotator. An optical capture system is optically coupled to the second polarizer (analyzer) **160** to capture light passing through the second polarizer. The optical capture system includes a detector and a processor. The detector may be a sufficiently fast, sensitive, and low noise charged coupled device or scientific CMOS camera. A suitable detector is the Orca Flash 4.0 from Hamamatsu Photonics K.K. (Japan). Preferably the detector should be capable of capturing up to 100 frames per second at a resolution of up to 2048 x 2048. Higher frame rates and resolution would also be acceptable.

In the configuration of FIG. 1, as the wavelength from variable wavelength light source **110** is changed, linearly polarized light passing through first retarder **120** is rotated to a new polarization angle as a function of wavelength, such that small changes in emission wavelength move the polarization state around the equator of the Poincaré sphere (FIG. 2). Light then passes

through the specimen where it may be modified due to specimen birefringence. When the light passes through second retarder **150**, the rotation is reversed by an equal amount. If first polarizer **120** is oriented horizontally, after passing through second retarder **150**, light will be returned to the horizontal polarization state. Light then passes through the second polarizer (analyzer) **160** oriented vertically, which blocks horizontally polarized light that has not been altered due to birefringence of the specimen. This is the principle of what we herein refer to as a “null measurement”.

The maximum light intensity produced by a linearly birefringent object is obtained when the plane of linearly polarized light is oriented 45° with respect to the axis of the birefringent object. Since these objects can be oriented at any arbitrary angle in the cell, one must rotate the polarization angle through a minimum of 90° to realize the maximum birefringence light intensity signal. This 90° range of rotation can be achieved by recording images formed by light emitted from a variable wavelength source emitting shorter and longer wavelengths. For a TeO_2 crystal rotator, the rotation/mm thickness ($\Delta\phi/d$) drops steeply over the 450 – 600 nm range. Thus by switching from a longer to shorter wavelength one can change the amount of rotation more than 90° . Furthermore, as a TeO_2 crystal rotator gets thicker (d becomes larger), the wavelength range required to achieve a 90° rotation about the polar axis on the Poincaré sphere gets smaller. This allows some latitude to tailor the working wavelength range.

In one embodiment, using the configuration shown in FIG. 1, a user can choose a series of preset wavelengths and record an image at each wavelength. In one embodiment, using a switching time on the order of 20 microseconds, a user can easily switch wavelengths between recording each image. Processing the data may be accomplished using a single frequency Fourier filtering algorithm as set forth in Kuhn et al. “Modulated Polarization Microscopy: A Promising New Approach to Visualizing Cytoskeletal Dynamics in Living Cells” *Biophysics Journal*, 80 (2001) 972-985, which is incorporated herein by reference. Calibration may be necessary to keep the illumination intensity constant and to determine the exact rotation angle achieved for a given wavelength. In some embodiments, the variable wavelength light source will allow computer control of the illumination intensity. Determination of rotation angle may be done by using a rotating polarizer.

The proposed configuration represents one embodiment of a polarization microscope. One of the problems with simply rotating linearly polarized light using a circular retarder is that

one cannot measure and image circular or elliptical birefringent structures in the specimen. To measure arbitrary sample birefringence, it is necessary to present the specimen with both linearly and circularly polarized light or combinations thereof. In an alternate embodiment, schematically depicted in FIG. 3, the ability to present the specimen with both linearly, circularly and elliptically polarized light is added, while maintaining the ability to make a null measurement. In FIG. 3, the retarder is an optical element that is composed of a rotator (circular retarder) and a waveplate (linear retarder). As depicted in FIG. 3, first waveplate **225** is optically coupled to first polarizer **220** and the first rotator **230**. First waveplate **225** receives polarized light from first polarizer **220** and converts incident linearly polarized light into circularly polarized light and elliptically polarized light. First waveplate **225** variously passes linearly, circularly, or elliptically polarized light (depending on the wavelength) to first rotator **230**. First rotator **230** changes the orientation of the linearly or elliptically polarized light. A second waveplate **255** is optically coupled to second polarizer **260** and second rotator **250**. Second waveplate **255** reverses the changes created by passage through first waveplate **225** by being oriented at 90 degrees to first waveplate **225**. Second waveplate **255** receives circular or elliptically polarized light from second polarized light rotator **250** and converts the incident circular polarized light or elliptically polarized light into linearly polarized light. The linearly polarized light is passed to second rotator **250** which then rotates linear polarized light back to its original angle based on the first polarizer. In some embodiments, the waveplates are oriented at $+45^\circ$ and -45° with respect to the polarization axis of first polarizer **220**. The waveplates may be made from any suitable birefringent material, such as quartz, mica, and polymers. The first and second waveplates are matched waveplates (equivalent thickness (d) and circular birefringence ($\Delta n(\lambda)$)) such that the changes produced by the first waveplate is cancelled by the second waveplate.

Whereas, the polarization rotators (circular retarders) rotate the polarization state about the polar axis of the Poincaré sphere (as a function of wavelength) to give polarized light rotated at different angles, the effect of the waveplates is to rotate the polarization state about an equatorial axis on the Poincaré sphere. Action of the linear and circular retarder together, as the wavelength is changed, allow the polarization state to produce both azimuthal (about the poles) and longitudinal (about an equatorial axis) movement on the Poincaré sphere and spiral around the Poincaré sphere as it moves from one pole to the other. The total retardation and thus circularity of the polarization will be a function of wavelength and the thickness of the waveplates. For example, an approximately 0.5 mm thick quartz waveplate that retards 9.25λ at 500 nm will retard 8.5λ at 539.6 nm and 11.25λ at 420.9 nm. Given that one cannot detect

retardations of full wavelengths, we can arbitrarily refer to a 9λ waveplate for a given wavelength as retarding 0 at that wavelength. Therefore, at 500 nm a 9.25 waveplate is effectively a quarter wave plate. What can be seen then is that in moving from 539 nm (8.5λ retardation) to 500 nm (9.25λ retardation), the retardation changes from $\frac{1}{2}\lambda$ to $\frac{3}{4}\lambda$ to 0 to $\frac{1}{4}\lambda$. Notably at $\frac{1}{2}\lambda$ and 0 λ light is plane polarized whereas at $\frac{3}{4}\lambda$ and $\frac{1}{4}\lambda$ we have left and right circularly polarized light respectively. On the Poincaré sphere, changing from left to right circularly polarized light is represented by moving from one pole to the other. For wavelengths between those giving circular or linear polarization we would have elliptically polarized light with varying degrees of ellipticity. After light passes through the specimen, the remaining crystal rotator and waveplate reverse the polarization state back to linear polarized light oriented horizontally. The result is that in the absence of specimen birefringence, the field is dark. Thus this arrangement once again provides for a null measurement.

The optical elements in the microscope depicted in FIG. 3 can be each represented by a Jones matrix (Table 1) and when multiplied appropriately the matrices show the changes in polarization state as light passes through each element of the optical train. The polarization state was examined for several wavelengths with respect to the retardation plates, to give retardations of 0, 22.5° ($1/8\lambda$), and 45° ($1/4\lambda$). The results show that, regardless of the wavelength of illumination, after the last retarder, the light is horizontally polarized giving a null measurement.

Wavelength Dependent Retardation						
	0°		22.5°		45°	
Horizontal Polarizer	1	0	1	0	1	0
	0	0	0	0	0	0
Waveplate (45°)	1	0	.938	0	.707	0
	0	0	.346i	0	.707i	0
Rotator (L)	.707	0	.663+.245i	0	.5+.5i	0
	-.707	0	-.663+.245i	0	-.5+.5i	0
Rotator (R)	1	0	.938	0	.707	0
	0	0	.346i	0	.707i	0
Waveplate (-45°)	1	0	1	0	1	0
	0	0	0	0	0	0
Vertical Polarizer	0	0	0	0	0	0
	0	0	0	0	0	0

TABLE 1

The rationale for using a null measurement is that it allows for detection of weak signals without large swings in brightness of the background illumination. If, for example, one were viewing circularly polarized light through a linear polarizer the image would be very bright. On the other hand when viewing weak birefringence between crossed polarizers, the image would be relatively dim. To do both, it is desirable to limit the brightness of the illumination so that the brightest image is on scale. This in turn limits the sensitivity of the camera to the weak signals we need to detect. With a null measurement, the brightness of illumination is limited by the brightness of the weakly birefringent signals.

For detection of birefringence in various forms, linearly polarized light (0°) at wavelength (λ_i) is transformed to a pre-calibrated elliptical polarization state after propagation through a waveplate (45°)/ rotator (θ) combination (FIG. 3). Elliptically polarized light is incident on the sample after propagating through the condenser. The Poincaré sphere is utilized to analyze the polarization transformations in the microscope with an arbitrary polarization state denoted by azimuthal (ϕ) and polar (θ) angles on a sphere with radius 1. We consider an arbitrary elliptical sample birefringence (i.e., linear and circular birefringence) specified by (ϕ_o, θ_o) with phase retardation δ_i at wavelength λ_i . For a null measurement, sample positions with non-zero birefringence give a signal intensity (S_i) at wavelength λ_i that is proportional to square of phase retardation (δ_i), where (ϕ_o, θ_o) specifies orientation of the sample birefringence axis relative to the polarization state of light incident on the sample at λ_o and $\Delta\phi_i, \Delta\theta_i$ are orthogonal transformations on the Poincaré sphere that map the polarization state incident on the sample at wavelength λ_i into the reference state at wavelength λ_o .

$$\begin{aligned}
 S_o &= \frac{1}{4} \delta_o^2 (\sin^2(\theta_o) \cdot \sin^2(\phi_o) + \cos^2(\theta_o)) \\
 S_1 &= \frac{1}{4} \left(\frac{\delta_o \cdot \lambda_o}{\lambda_1} \right)^2 (\sin^2(\theta_o + \Delta\theta_1) \cdot \sin^2(\phi_o + \Delta\phi_1) + \cos^2(\theta_o + \Delta\theta_1)) \\
 S_2 &= \frac{1}{4} \left(\frac{\delta_o \cdot \lambda_o}{\lambda_2} \right)^2 (\sin^2(\theta_o + \Delta\theta_2) \cdot \sin^2(\phi_o + \Delta\phi_2) + \cos^2(\theta_o + \Delta\theta_2)) \\
 &\quad \cdot \\
 &\quad \cdot \\
 &\quad \cdot \\
 S_n &= \frac{1}{4} \left(\frac{\delta_o \cdot \lambda_o}{\lambda_n} \right)^2 (\sin^2(\theta_o + \Delta\theta_n) \cdot \sin^2(\phi_o + \Delta\phi_n) + \cos^2(\theta_o + \Delta\theta_n))
 \end{aligned}$$

These equations reveal a problem with carrying out a null measurement. Because the signal intensity (S_i) is proportional to the square of the phase retardation (δ^2), the sign of δ cannot be determined - or equivalently the direction of the birefringence axis is degenerate. When the sign of δ (positive or negative birefringence) is determined using a non-null measurement, as we show below, one can gain an 11-fold improvement in signal to noise.

In the proposed non-null measurement, the polarizer is misaligned from the analyzer by a small angle so that after propagating through the sample and rotator-retarder combination, polarization state (α_i, β_i) of light incident on the analyzer at each wavelength (λ_i) is slightly offset from the horizontal polarization state ($\alpha=0, \beta=\pi/2$). In the case of a non-null measurement, the signal intensity (S_i) for each wavelength (λ_i) is:

$$\begin{aligned}
 S_0 &= \frac{1}{2} \cdot \left[\frac{\delta_0^2}{2} \left(\begin{array}{l} 1 - \sin(\beta_0) \cos(\alpha_0) + \delta_0 (\cos(\theta_0) \sin(\alpha_0) \sin(\beta_0) - \sin(\theta_0) \sin(\varphi_0) \cos(\beta_0)) \\ \cos(\alpha_0) \sin(\beta_0) - \sin^2(\theta_0) \cos(\varphi_0) \sin(\beta_0) \cos(\varphi_0 - \alpha) \\ - \sin(\theta_0) \cos(\theta_0) \cos(\varphi_0) \cos(\beta_0) \end{array} \right) \right] \\
 &= \frac{1}{2} \cdot \left[\frac{(\delta_0 \cdot \lambda_0 / \lambda_1)^2}{2} \left(\begin{array}{l} 1 - \sin(\beta_1) \cos(\alpha_1) + (\delta_0 \cdot \lambda_0 / \lambda_1) (\cos(\theta_0 + \Delta\theta_1) \sin(\alpha_1) \sin(\beta_1) - \sin(\theta_0 + \Delta\theta_1) \sin(\varphi_0 + \Delta\varphi_1) \cos(\beta_1)) \\ \cos(\alpha_1) \sin(\beta_1) - \sin^2(\theta_0 + \Delta\theta_1) \cos(\varphi_0 + \Delta\varphi_1) \sin(\beta_1) \cos(\varphi_0 + \Delta\varphi_1 - \alpha) \\ - \sin(\theta_0 + \Delta\theta_1) \cos(\theta_0 + \Delta\theta_1) \cos(\varphi_0 + \Delta\varphi_1) \cos(\beta_1) \end{array} \right) \right] \\
 &= \frac{1}{2} \cdot \left[\frac{(\delta_0 \cdot \lambda_0 / \lambda_2)^2}{2} \left(\begin{array}{l} 1 - \sin(\beta_2) \cos(\alpha_2) + (\delta_0 \cdot \lambda_0 / \lambda_2) (\cos(\theta_0 + \Delta\theta_2) \sin(\alpha_2) \sin(\beta_2) - \sin(\theta_0 + \Delta\theta_2) \sin(\varphi_0 + \Delta\varphi_2) \cos(\beta_2)) \\ \cos(\alpha_2) \sin(\beta_2) - \sin^2(\theta_0 + \Delta\theta_2) \cos(\varphi_0 + \Delta\varphi_2) \sin(\beta_2) \cos(\varphi_0 + \Delta\varphi_2 - \alpha_2) \\ - \sin(\theta_0 + \Delta\theta_2) \cos(\theta_0 + \Delta\theta_2) \cos(\varphi_0 + \Delta\varphi_2) \cos(\beta_2) \end{array} \right) \right] \\
 &\quad \vdots \\
 &= \frac{1}{2} \cdot \left[\frac{(\delta_0 \cdot \lambda_0 / \lambda_n)^2}{2} \left(\begin{array}{l} 1 - \sin(\beta_n) \cos(\alpha_n) + (\delta_0 \cdot \lambda_0 / \lambda_n) (\cos(\theta_0 + \Delta\theta_n) \sin(\alpha_n) \sin(\beta_n) - \sin(\theta_0 + \Delta\theta_n) \sin(\varphi_0 + \Delta\varphi_n) \cos(\beta_n)) \\ \cos(\alpha_n) \sin(\beta_n) - \sin^2(\theta_0 + \Delta\theta_n) \cos(\varphi_0 + \Delta\varphi_n) \sin(\beta_n) \cos(\varphi_0 + \Delta\varphi_n - \alpha_n) \\ - \sin(\theta_0 + \Delta\theta_n) \cos(\theta_0 + \Delta\theta_n) \cos(\varphi_0 + \Delta\varphi_n) \cos(\beta_n) \end{array} \right) \right]
 \end{aligned}$$

The set of nonlinear equations for the non-null measurement must be solved to determine three parameters - phase retardation (δ_o) and axes of birefringence (φ_o, θ_o). The ratio of circular to linear birefringence ($\Delta n_{circular} / \Delta n_{linear}$) is given by $\Delta n_{circular} / \Delta n_{linear} = \tan(\theta_o)$ while direction of the

sample's linear birefringence in the laboratory frame is given by $2\varphi_o$. For example if N incident wavelengths (λ_i) are utilized, then real-time imaging requires computation of these three parameters at four-million pixels in N/F seconds where F is the camera frame rate in Hz. When $N=7$ and $F=100$, then this time is $N/F = 0.07$ seconds. We have shown that a single Radeon R9 - 290 graphics card can process data at the required rate. Given the number of camera pixels (for example 2048 X 2048) recording signal intensity (S_i) data at seven wavelengths, the amount of data that must be processed by the video card to compute one phase retardation and birefringence images is 2.8×10^7 samples. So, there are about 15,000 FLOPs per pixel to solve the non-null equations. An optimized Levenberg-Marquardt algorithm, for example, may be utilized to solve the signal intensity equations to provide real-time images of the birefringence properties of the specimen at each pixel location.

Equations for the signal intensity (S_i) at each wavelength contain terms that should be calibrated, including: the non-null state (α_i, β_i) at each wavelength λ_i and the orthogonal transformations ($\Delta\varphi_i, \Delta\theta_i$) on the Poincaré sphere that relate or map the polarization state incident on the sample at wavelength λ_i into the reference state at wavelength λ_o . For the calibrations, the goal is to determine the input state for light incident on the specimen so the second crystal rotator and the second waveplate will be removed from the path. In one embodiment, the input state of the second polarizer (analyzer) may be determined by rotating the polarizer through discrete angles by mounting it on a picomotor driven rotary stage (e.g., Newport AG-PR100, Newport Corporation, Irvine, CA) placed either in the second turret position or before the camera. Images obtained as the polarizer is rotated will be used for the pixel-by-pixel determination of ellipticity ($\tan(X_{i,j})$) and orientation ($\Psi_{i,j}$) of the major axis of the polarization ellipse. Other methods known in the art of light polarization may be used for determining the input polarization state of light. For circular polarization, the handedness should be obvious from the input state but it could also be easily determined by repeating the rotating analyzer measurement with an added achromatic $\lambda/4$ plate and determining the axis of the resulting linear polarized state. Each of these terms will be calibrated and fixed during operation of the microscope. One useful feature of the graphics cards is that they can be obtained with large amounts of memory for storing image data. These calibration images take into account and ultimately compensate for aberrations in the lenses that alter the polarization state.

For samples that exhibit rapid movements, current capabilities allows recording polarized light images at 100 frames/second with a 4 megapixel camera. In a previous modulated

polarization microscope (FIG. 1), we made null measurements from data recorded at 30 frames/second using an analog camera and a relatively weak light source. Even so, there was more than enough light. Since we could conceivably bin pixels to the equal that of the analog camera, it may be possible to find a point where there is enough light. However, the signal-to-noise depends on the brightness of the illuminating spot on the specimen.

In employing a non-null measurement, where the polarizers are not exactly crossed, the amount of light to the detector is increased. Furthermore, a non-null measurement actually increases the signal-to-noise by adding a signal term that is linear in phase retardation (δ). In the non-null case, the SNR_{linear} is given by,

$$SNR_{linear} = \sqrt{N_o T} \frac{|\delta(\cos(\theta_o)\sin(\alpha)\sin(\beta) - \sin(\theta_o)\sin(\varphi_o)\cos(\beta))|}{\sqrt{1 - \sin(\beta)\cos(\alpha)}}$$

Based on the above equation, SNR can be increased by using a brighter light source.

One possible effect of the approach as described above is that the spectral range of wavelengths needed to obtain all spectral measurements is broad enough to introduce artifacts into the data. These artifacts include differences in resolution, differential scattering, and differences in absorption. Therefore, it is desirable to minimize the wavelength range needed to obtain all spectral (e.g., 7) measurements. Our data show that a spectral bandwidth of 5nm for each wavelength is adequate for accurate calculation of birefringence. Larger bandwidths lead to less accuracy. Better accuracy may be achieved by using narrow bandwidths. This may be accomplished by using laser illumination where the spectral emission bandwidth (nm) of individual laser lines is on the order of 2 nm. Given the narrow bandwidth of laser illumination one could obtain all 7 measurements over a wavelength range of less than 20 nm. This narrow range of wavelengths mitigates against detectable differences in resolution, scattering or absorption.

The use of a narrow range of emission wavelengths, such as that provided by lasers, is preferred when the objective is to provide the added capability of imaging the same specimen by polarized light or by fluorescence. The bright illumination provided by the polarized light source can bleach fluorescent molecules that absorb in the wavelengths used for polarized light imaging. The use of a narrow range of wavelengths for polarized light imaging allows for fluorescence

imaging of molecules whose absorption spectrum lies outside the wavelengths used for polarized light imaging.

An additional capability the proposed polarization microscopy approach allows is structured illumination at each spectral emission wavelength. Some of the radiant sources (e.g. lasers), radiant emission can be separated into discrete emitters such as optical fibers that are positioned in the front plane of the condenser lens. By positioning the discrete emitters in the front focal plane of the condenser lens, the angle of incident radiation on the specimen may be controlled. Light emission from each of the discrete emitters can be shuttered with for example a MEMS switch so that any combination of incident light angles on the sample can be obtained at each wavelength. This approach allows for polarization microscopy with super-resolution. Although super-resolution microscopy approaches are known in the art, the combination of spectrally-encoded high-extinction polarization microscopy with super-resolution provides a number of unique capabilities. Alternative approaches to multiple discrete fibers may be utilized to control the spatial illumination of light incident on the specimen. For example, a DLP chip can be positioned or imaged to the front focal plane of the condenser lens to control the angle of illumination on the specimen.

In order to reap the full power of polarized light imaging, it may be useful to identify particular molecules and structures. As a consequence, either embodiment of the polarization microscope may have the capability of fluorescence imaging. In an embodiment, the optical polarizers, waveplates and rotators are compact and can be moved in and out of the optical path to allow the introduction of fluorescent imaging optical components (e.g. a dichroic mirror and suitable filter) into the optical pathway. In one embodiment, the polarization optical components can fit into the place of a dichroic filter set in microscopes designed to hold multiple dichroic filter sets. This can allow the use of the standard epifluorescence light path in, for example, the Nikon Eclipse Ti inverted microscope. The fluorescence illumination in this case is simply turned on or off through a shutter. Alternatively, rather than have a separate light source for fluorescence illumination, a switching mirror (e.g., available from Newport Corporation, Irvine CA) may be used to feed two alternative light paths, one for fluorescence excitation and one for transmitted (polarized) light. These may be switched according to which mode of imaging is desired by the microscope user.

Alternatively, one light source such as that provided by a series of lasers can be used for polarized light imaging and a second broad band light source (lamp or supercontinuum) can be used for fluorescence. Here the laser-based source would enter the vertical light or transmitted light path whereas as the broadband source would illuminate the standard epifluorescence light path (typically through the rear of the microscope). Suitable controls such as shutters or power input into the laser would allow for alternating the illumination between the sources. The camera, which may be mounted under the microscope in a linear path, may be used to collect both polarized light and fluorescence images. Alternatively two different cameras might be employed by diverting the output from one port of the microscope to another.

The use of a narrow band (or closely spaced set) of spectral emission wavelengths for polarized light imaging allows for some flexibility for choosing what band will be used. The same optics may be used for different spectral emission wavelengths but the system would have to be calibrated (as described above) for each set of wavelengths. Alternatively, the user could employ different retarders and rotators for different wavelength bands so as to tune the configuration. In either case, the use of particular wavelength bands allows for polarized light imaging at a set of spectral emission wavelengths that avoids exciting and thus bleaching fluorophores outside the wavelength band used for polarized light imaging.

The disclosed polarization microscope may be used to study biological events as they occur. For example, the novel polarization microscope may be used to study cytoskeletal dynamics in living cells. In one example, polarized light imaging has facilitated understanding of how T cells function. Helper and cytotoxic T cells function by directed and focused secretion of molecules towards another cell. This is largely accomplished by movement of the microtubule organizing center (MTOC) up to the site of contact between a T cell and its cognate target and focusing of secretory vesicles around the MTOC. In an embodiment, modulated polarization microscopy may be used to follow microtubules and the MTOC as well as secretory vesicles in T cells. The MTOC organizes the microtubule cytoskeleton in T cells. In cytotoxic T cells, for example, the CTL engages a target cell and signaling through the T cell receptor leads to T cell activation. This leads to a dramatic reorganization of surface molecules at the target contact site as well as the underlying cytoskeleton. These rearrangements define what is termed the immunological synapse. At some point during synapse formation, the MTOC is drawn up to the synapse. Either before or after MTOC movement, secretory vesicles move along microtubules towards the MTOC where they concentrated. This combination of MTOC translocation and

secretory vesicle movements ultimately concentrates secretory vesicles at the synapse where they are secreted. Understanding the mechanism of MTOC translocation is critical because it lies at the heart of T cell effector functions. Furthermore, studies have shown that factors in the tumor environment can block MTOC translocation despite the fact that T cells activate. This can lead to tumor escape.

Specific applications of the polarized microscopes disclosed herein include visualization of the cytoskeleton, visualization of vesicles, membranes, cell organelles, viral particles and organized protein assemblies such as collagen. All of these structures can now be visualized in real time. Polarized light also can enable the visualization of molecular interactions. Nanorods should be visible based on their interaction with polarized light. Nanospheres, although invisible as monomers, would become visible when dimerized. As labels for individual proteins or receptors, dimerization might be detected when two labeled proteins come together in a binding reaction or when cross-linked by other means.

Gold nanoparticles have tremendous potential as labels because as light scatterers, they are orders of magnitude brighter than fluorophores and they do not bleach. Thus one can easily image individual gold nanoparticles. Furthermore, molecule binding events can shift the emission, typically to longer wavelengths. In addition, using polarized light imaging, there is a difference between spherical and rod-shaped nanoparticles or when two spherical nanoparticles come close together. Rod shaped particles depolarize the illumination and exhibit anisotropy whereas individual spherical nanoparticles do not. However, when two spherical nanoparticles come together, with respect to polarized light they behave light rods and exhibit anisotropy. Thus individual molecules tagged with small nanoparticles may be imaged to determine if they are bound or free.

In this patent, certain U.S. patents, U.S. patent applications, and other materials (e.g., articles) have been incorporated by reference. The text of such U.S. patents, U.S. patent applications, and other materials is, however, only incorporated by reference to the extent that no conflict exists between such text and the other statements and drawings set forth herein. In the event of such conflict, then any such conflicting text in such incorporated by reference U.S. patents, U.S. patent applications, and other materials is specifically not incorporated by reference in this patent.

Further modifications and alternative embodiments of various aspects of the invention will be apparent to those skilled in the art in view of this description. Accordingly, this description is to be construed as illustrative only and is for the purpose of teaching those skilled in the art the general manner of carrying out the invention. It is to be understood that the forms of the invention shown and described herein are to be taken as examples of embodiments. Elements and materials may be substituted for those illustrated and described herein, parts and processes may be reversed, and certain features of the invention may be utilized independently, all as would be apparent to one skilled in the art after having the benefit of this description of the invention. Changes may be made in the elements described herein without departing from the spirit and scope of the invention as described in the following claims.

WHAT IS CLAIMED IS:

1. A polarization microscope comprising:

a variable wavelength light source;

a first polarizer optically coupled to the variable wavelength light source, wherein the first polarizer transmits incident light in a pure polarization state;

a first retarder optically coupled to the first polarizer;

a specimen stage optically coupled to the first retarder, wherein the specimen stage holds a specimen in the optical pathway of the light received from the first retarder;

a second retarder optically coupled to the specimen stage, wherein the second retarder is an opposite-signed retarder with respect to the first retarder;

a second polarizer optically coupled to the second retarder, wherein the second polarizer is oriented orthogonal to the first polarizer;

an optical capture device optically coupled to the second polarizer, wherein the optical capture device captures light passing through the second polarizer.

2. The polarization microscope of claim 1, wherein the variable wavelength light source is capable of producing light having a wavelength from between about 350nm to about 800nm.

3. The polarization microscope of claim 1 or 2, wherein the first polarizer and/or the second polarizer is a polarizing prism.

4. The polarization microscope of any one of claims 1-3, wherein the first retarder and the second retarders are rotators.

5. The polarization microscope of claim 4, wherein the first retarder and the second retarder are crystal polarized light rotators.

6. The polarization microscope of claim 5, wherein the first crystal polarized light rotator and the second crystal polarized light rotator are composed of tellurium dioxide.
7. The polarization microscope of claim 4, wherein the the first crystal polarized light rotator and the second crystal polarized light rotator are matched crystal rotators.
8. The polarization microscope of claim 4, wherein the degree of retardance of the rotators is based, in part, on the wavelength of the incident light.
9. The polarization microscope of any one of claims 1-3, wherein the first retarder and the second retarder are elliptical retarders composed of a rotator and a waveplate.
10. The polarization microscope of any one of claims 1-3, wherein the first retarder and the second retarder are elliptical retarders composed of two waveplates oriented at 45 degrees with respect to each other.
11. The polarization microscope of any one of claims 1-10, wherein the optical capture device is a charged coupled device.
12. The polarization microscope of any one of claims 1-11, wherein the light from the first retarder passes through the specimen held on the specimen stage.
13. The polarization microscope of any one of claims 1-11, wherein the light from the first retarder is reflected off the specimen held on the specimen stage.
14. A method of visualizing a specimen using a polarization microscope comprising:
 - placing the specimen on a specimen stage of a polarization microscope as described in any one of claims 1-13;
 - obtaining images of the specimen at one or more wavelengths.

15. The method of claim 14, wherein obtaining images of the specimen comprises periodically changing the wavelength of light impinging on the specimen and capturing images of the specimen after each change of wavelength of light.

16. The method of claim 15, wherein each change of wavelength occurs in between 10 and 500 microseconds.

17. The method of any one of claims 14-16, wherein imaging of the biological specimen is performed continuously over a time of at least about 2 seconds.

18. The method of any one of claims 14-17, wherein the method further comprises modulating the polarization state of the light over the entire Poincaré sphere by altering the wavelength of the light produced by the variable wavelength light source.

19. The method of any one of claims 14-18, further comprising:

adjusting the orientation axis of the first polarizer with respect to the second polarizer so that the first polarizer and the second polarizer are off axis; and

obtaining images of the specimen while the first polarizer and second polarizer are off axis.

20. The method of any one of claims 14-19, further comprising calibrating the polarization microscope by determining the orientation state of the light when the second polarized light rotator and the second waveplate are removed from the optical path.

21. The method of claim 20, wherein determining the orientation state of the light comprises rotating the second polarizer through discrete angles and analyzing the data collected by the optical capture device.

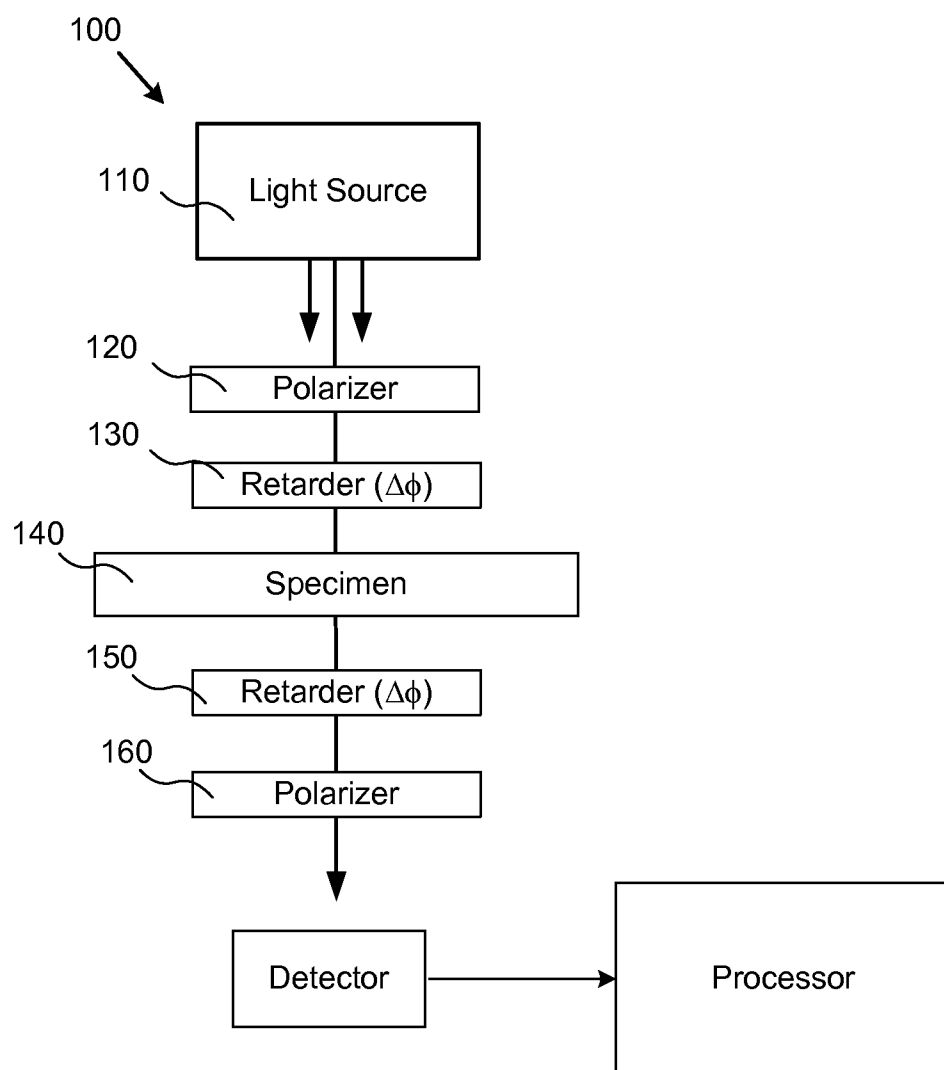


FIG. 1

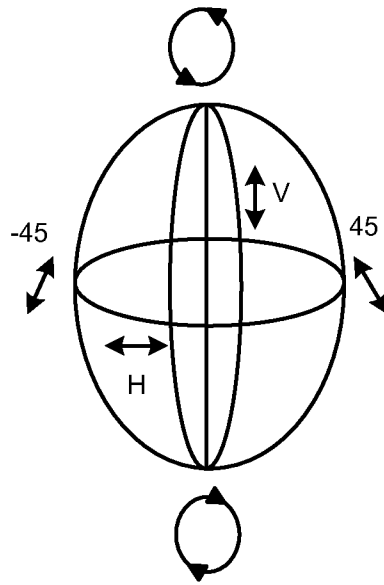


FIG. 2

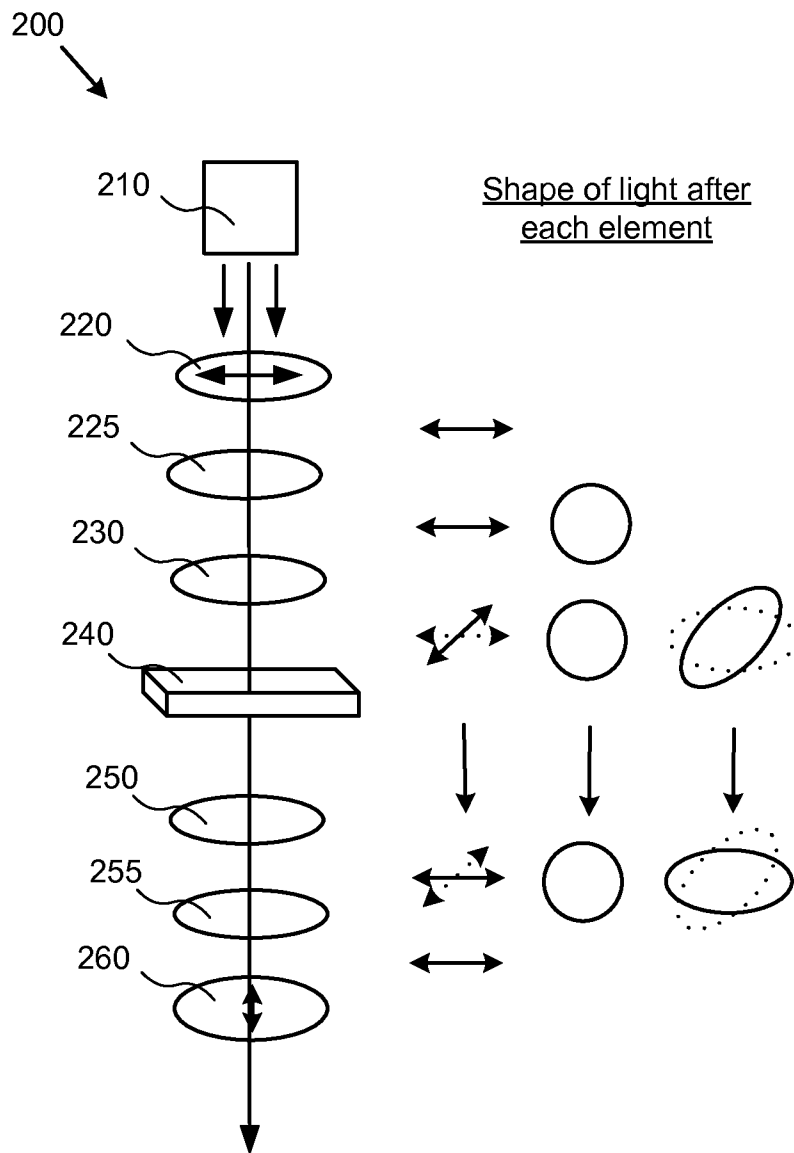


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/041570

A. CLASSIFICATION OF SUBJECT MATTER
INV. G02B9/04 G02B21/00 G02B27/28
ADD.

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

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 420 717 A (TABATA SEIICHIRO [JP]) 30 May 1995 (1995-05-30) figure 21; example 6 column 9, line 44 - column 10, line 30; figure 17	1-21
A	----- OLDENBOURG R ET AL: "NEW POLARIZED LIGHT MICROSCOPE WITH PRECISION UNIVERSAL COMPENSATOR", JOURNAL OF MICROSCOPY, BLACKWELL SCIENCE, GB, vol. 180, no. PART 02, 1 November 1995 (1995-11-01), pages 140-147, XP009045629, ISSN: 0022-2720 figure 1 ----- -/-	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/041570

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUHN ET AL.: "Modulated Polarization Microscopy: A Promising New Approach to Visualizing Cytoskeletal Dynamics in Living Cells", BIOPHYSICS JOURNAL, vol. 80, 2001, pages 972-985, XP002729543, cited in the application figure 1 -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2014/041570

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5420717 A	30-05-1995	JP H05232384 A US 5420717 A	10-09-1993 30-05-1995
