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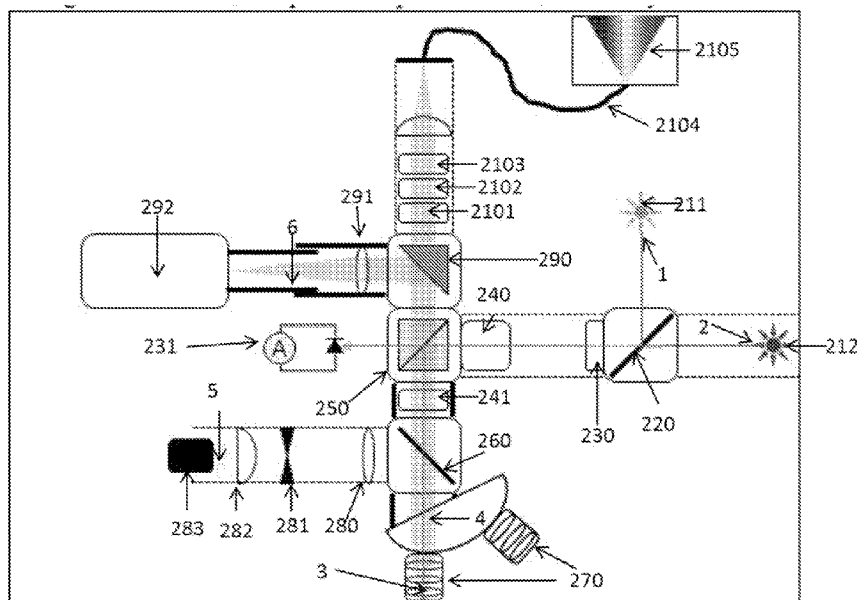
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(54) Title: METHODS AND APPARATUS FOR FLUORESCENCE MICROSCOPY

Fig. 2



(57) **Abstract:** A fluorescence microscope is provided having a linearly polarized excitation beam. The excitation beam is reflected by a beam splitter, and then focused onto a sample by a lens assembly. The sample emits emission radiation, and a portion of the emission radiation is transmitted back through lens assembly as an emission beam to the beam splitter. The beam splitter directs the emission beam along a different path from the incoming excitation beam, towards a measurement device. Both the emission beam and excitation beam pass through a quarter-wave plate which converts the linearly-polarized excitation beam into circularly polarized light, and converts circularly-polarized components of the emission beam into corresponding linearly-polarized components.



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Methods and Apparatus for Fluorescence Microscopy

Field of the invention

The present invention relates to apparatus and methods for fluorescence microscopy.

5 Background of the invention

Fluorescence microscopy is a technique for characterizing fluorescent samples, which absorb excitation radiation and emit photoluminescence (also referred to here as emission radiation) during de-excitation.

10 Conventionally, a light source generates a light beam (which may be referred to as an excitation beam) which is linearly polarized, for example because it passes through a linear polarizer. The excitation beam is reflected by a beam splitter, and then focused onto a sample by a lens assembly. The sample emits emission radiation, and a portion of the emission radiation is transmitted back through lens assembly as an emission beam to the beam splitter. There the beam splitter directs
15 the emission beam along a different path from the incoming excitation beam, towards a measurement device.

The photoluminescence response of fluorescent samples generally varies as a function of the input frequency and input polarization of the excitation radiation. For instance, chiral molecules, magnetic materials, low-dimensional materials, and spin-
20 polarized materials may preferentially absorb, reflect or emit either left-circularly-polarized or right-circularly-polarized electromagnetic radiation. To characterize such materials, techniques comprising excitation of samples with circularly-polarized radiation are desirable. Alternatively, instruments which measure the emission spectra of such materials as a function of polarization state may be used.

25 The proportion of left-circularly-polarized radiation and right-circularly-polarized radiation in a beam may be quantified using the degree of circular polarization (DOCP):

$$DOCP = \frac{I_{RHC} - I_{LHC}}{I_{RHC} + I_{LHC}}$$

30 where I_{LHC} and I_{RHC} are the intensities of radiation in the left- and right-circularly-polarized states respectively.

Conventionally, a long-pass dichroic mirror is used as a beam-splitter in a fluorescence microscope, due to its high transmission efficiency. However, dichroic mirrors frequently behave as linear polarizers, altering the polarization state of both the excitation and emission beams. The polarizing effect of a dichroic mirror is
35 wavelength-dependent, and particularly pronounced near the cut-off wavelength of

the dichroic mirror (i.e., the wavelength separating the transmission and reflection bands of the dichroic mirror).

To compensate for the polarizing effect of the dichroic mirror, post-measurement, a full spectral characterization of the dichroic mirror must be carried out for both left- and right-circularly-polarized light, to permit post-measurement compensation. As well as requiring some effort, this post-measurement compensation also tends to degrade data quality – particularly near the cut-off wavelength of the dichroic mirror. Consequently, fluorescence microscopes using a dichroic mirror to separate excitation and emission light are likely to be unsuitable for ‘near-resonant’ measurements, i.e. measurements for wavelengths near the cut-off wavelength of the dichroic mirror.

Statement of Invention

The present invention aims to provide a new and useful method and apparatus for fluorescence microscopy.

The present inventors have observed that beam splitters such as dichroic mirrors frequently introduce shifts in amplitude and phase between the orthogonal linear polarization components of any incident light. In the case of fluorescence microscopes in which samples are excited using circularly-polarized light, or emit circularly-polarized light, this may result in complex changes to the polarization state of the excitation or emission light. Such changes are difficult to correct for during post-measurement data analysis.

A first aspect of the present invention proposes in general terms that an apparatus for fluorescence microscopy includes a single quarter-wave plate through which both excitation beam and emission beam are directed.

The quarter-wave plate converts the linearly-polarized excitation beam into circularly polarized light, and converts a circularly-polarized component of the emission beam into corresponding linearly-polarized components before they reach the beam splitter. This has the effect that any phase shifts induced by the beam splitter do not affect the polarization state of the light. (Relative amplitude changes may arise, but are more easily compensated for during data analysis.) Post-measurement data analysis is therefore simplified, and data throughput increased.

Furthermore, by using the quarter-wave plate to convert left-circularly-polarized and right-circularly-polarized components of the emission beam into the corresponding orthogonal linear polarization components, it is possible to determine, by detecting the emission beam with a measurement device, the relative proportions of left- and right-circularly polarized light which were emitted by the sample.

One specific expression of the invention provides an apparatus for performing fluorescence microscopy on a sample, comprising:

a sample holder for receiving a sample;

a first linear polarizer for receiving an excitation beam from a light source;

a beam splitter arranged to receive the excitation beam from the first linear polarizer, and direct the excitation beam onto a path to a sample holder, and to direct an emission beam received along the path from the sample holder to a measurement device; and

a quarter-wave plate located on the path;

whereby the quarter-wave plate is operative to convert a linearly polarized component of the excitation beam into circularly polarized radiation, and convert a circularly polarized component of the emission beam into linearly polarized radiation.

In an embodiment, the first linear polarizer and quarter-wave plate have respective transmission axes, and are rotatable with respect to one another about their respective transmission axes. By changing the relative angular position of the two components, the degree of circular polarization of the excitation light may be varied. Such a configuration also allows for ease of alignment.

Preferably, the quarter-wave plate is rotatable with respect to the rest of the apparatus (e.g. with respect to the beam splitter), since in this case the degree of circular polarization (DOCP) of the excitation light may be controlled by rotating the quarter-wave plate only. Furthermore, linear polarizers frequently do not have a constant thickness in the direction parallel to the linear polarizer propagation axis. For example, a linear polarizer may be wedge-shaped. Rotating the linear polarizer may therefore result in deflection of an incident beam and misalignment of the beam focus. Rotating only the quarter-wave plate allows optical alignment to be maintained for different values of the excitation DOCP. This configuration also provides greater ease of use, as only the quarter-wave plate need to be rotated to adjust the excitation DOCP.

Preferably, the quarter-wave plate is a broadband or achromatic quarter-wave plate, i.e. the retardance of the quarter-wave plate shows only limited spectral dependence within a pre-determined wavelength range of interest. For example, the retardance of the quarter-wave plate may be $0.25 \pm 0.03\lambda$ for radiation having a wavelength λ , where λ lies in the range 400-800nm. Preferably, the retardance of the quarter-wave plate is $0.25 \pm 0.003\lambda$ for radiation having a wavelength λ , where λ lies in the range 450-900nm.

Furthermore, the configuration is compatible with a temperature control module to maintain the temperature of a sample in operation. This allows samples to be maintained at low (e.g. colder than 265k) or cryogenic temperatures, so the apparatus may be used to study low-temperature effects (for example, quantum states). For example, a cryostat may be used to hold the sample under vacuum and at low temperature, preventing moisture from the air from condensing on the sample

at low temperatures. Preferably, a cryostat is used to maintain samples at temperatures below 250K. Alternatively, for sample temperatures above 250K, samples may be flushed with streams of dry air or nitrogen. In such an embodiment, microscope objectives having long working distances (distances from the front of the lens to the sample) are required. In an embodiment, the microscope objective lenses have working distances of at least 9mm from portion of the sample holder where a sample is to be located.

In an embodiment, the temperature of the sample may be varied between less than 10K and room temperature using the cryostat. In another embodiment, other components of the apparatus may also be maintained at low temperatures.

In an embodiment, the light source may be a laser. It, and a lens assembly of the apparatus, generate a highly focused excitation beam to address a portion of a sample, or, in a case in which multiple samples are present in the sample holder, individually address one of the samples.

In an embodiment, the apparatus may comprise a third linear polarizer configured to receive excitation light from the light source and transmit the excitation light to the first linear polarizer. The third linear polarizer is rotatable with respect to the first linear polarizer. Hence, by altering the relative angular position of the transmission axes of the first and third linear polarizers, the intensity of excitation light transmitted through the first linear polarizer (and therefore the intensity of light incident on a sample) may be varied. In still another embodiment, the apparatus may further comprise a module configured to obtain data indicative of the power of the excitation beam following transmission by the first linear polarizer. Hence, the power of the excitation beam may be controlled and monitored by a user.

In an embodiment, the apparatus may comprise a visible light source configured to illuminate a sample held in the sample holder. Optionally, the visible light source may be a white LED.

In an embodiment, a filter may be located between the beam-splitter and the measurement device, to substantially block any light of the excitation beam which is combined with the emission beam by the beam-splitter. As the excitation signal is likely to be significantly higher in intensity than the emission signal, this allows improved measurement of weak emission signals. This is possible because the excitation beam is typically at a different light frequency from the emission beam.

Optionally, the filter may be a long-pass filter (i.e., a filter that transmits wavelengths longer than a threshold wavelength, and attenuates wavelengths below the threshold wavelength), or a band-pass filter (i.e. a filter that allows wavelengths within a given wavelength band to pass, and attenuates wavelengths outside the wavelength band).

In a second specific expression, the invention provides a method for performing fluorescence microscopy on a sample, comprising:

receiving a sample;

receiving, by a first linear polarizer, an excitation beam from a light source;

5 receiving, by a beam splitter, the excitation beam from the first linear polarizer;

directing, by the beam splitter, the excitation beam onto a path to a sample holder;

10 directing, by the beam splitter, an emission beam received along the path from the sample holder to a measurement device; and

converting, by a quarter-wave plate located on the path, a linearly polarized component of the excitation beam into circularly polarized radiation; and

converting, by the quarter-wave plate, a circularly polarized component of the emission beam into linearly polarized radiation.

15 Optionally, the beam splitter may be a non-polarizing beam splitter. Non-polarizing beam splitters, i.e. beam splitters having substantially equal transmittance and reflectance for different polarization states, generally have a lower efficiency than the dichroic mirrors which are conventionally used in fluorescence microscopes. However, the use of a non-polarizing beam splitter reduces the changes to the
20 polarization state of the emission and excitation beams upon passing through the beam splitter. This yields improved control of the polarization of the excitation radiation, and more accurate measurement of the polarization of the emission radiation. Consequently, a higher-quality spectrum will be measured for the emission beam.

25 Preferably, the non-polarizing beam splitter has a substantially flat spectral dependence within its operating range. In an embodiment, the non-polarizing beam splitter may be a broadband, or achromatic, non-polarizing beam splitter. The use of a broadband or achromatic non-polarizing beam splitter allows the light source to be exchanged for another light source having a different excitation wavelength without
30 necessitating an modification to the beam splitter (e.g. replacing the beam splitter with another beam splitter having different frequency behavior), thereby increasing the ease of use when carrying out excitation of a sample at multiple wavelengths.

The concept of a fluorescence microscope using a non-polarizing beam-splitter provides an independent second aspect of the invention, freely combinable with the
35 first aspect.

In a first specific expression of the second aspect of the invention, the invention provides an apparatus for performing fluorescence microscopy on a sample, comprising:

a sample holder for receiving a sample;

5 a first linear polarizer for receiving an excitation beam from a light source;

a non-polarizing beam splitter arranged to receive the excitation beam from the first linear polarizer and, direct the excitation beam onto a path to a sample holder, and to direct an emission beam received from the sample holder to a measurement device.

10 In a second specific expression of the second aspect of the invention, the invention provides a method for performing fluorescence microscopy on a sample, comprising:

receiving a sample;

receiving, by a first linear polarizer, an excitation beam from a light source;

15 receiving, by a non-polarizing beam splitter, the excitation beam from the first linear polarizer;

directing, by the non-polarizing beam splitter, the excitation beam onto a path to a sample holder;

directing, by the non-polarizing beam splitter, an emission beam received along the path from the sample holder to a measurement device.

20 Optionally, in either aspect of the invention, the measurement device may be a spectrometer. A second linear polarizer may be provided, configured to receive the emission beam before the emission beam enters the spectrometer. A half-wave plate may be provided on the propagation path of the emission beam between the beam-splitter and the second linear polarizer. The second linear polarizer has a
25 propagation axis and may be in a fixed angular position with respect to the beam-splitter about the propagation axis, and the half-wave plate may be rotatable with respect to the second linear polarizer about the propagation axis.

By rotating only the half-wave plate, deflection of an incident emission beam may be reduced, as the thickness of a half-wave plate parallel to its propagation axis should
30 not substantially vary (i.e., it should have a high degree of parallelism). Hence, the beam focus may be better preserved than if, instead, the linear polarizer were rotatable. Thus, by arranging for only the second linear polarizer to be rotatable, optical alignment is maintained for different settings of the apparatus. Additionally, the above configuration provides ease of use, as a user need only rotate the half-
35 wave plate to change the polarization component of the emission beam which is transmitted to the spectrometer.

Furthermore, as the linear polarizer is in a fixed angular position with respect to the beam-splitter (and therefore normally with respect to the spectrometer), the same linear polarization of light will enter the spectrometer each time a measurement is taken. Hence, the emission spectrum may be measured for varying DOCP values, but there is no need to compensate for the spectrometer's polarization-dependent efficiency. Consequently, instrument reliability is improved, higher measurement accuracy is achieved, and data analysis is simplified.

Optionally, the apparatus may further comprise an optical fiber operative to direct the emission beam to the spectrometer after passage of the emission beam through the second linear polarizer. Use of an optical fiber to couple the light transmitted through the second linear polarizer to the spectrometer simplifies the process of optical alignment, as a user may adjust the position of the fiber end rather than altering the position of the spectrometer. Furthermore, the use of the optical fiber effectively reduces the size of the aperture through which light is transmitted to the spectrometer, reducing contamination of the signal by stray light and hence leading to an increased signal-to-noise ratio and improved data quality.

The concept of a second linear polarizer in combination with a rotatable half-wave plate, provides an independent third aspect of the invention, freely combinable with the first or second aspects.

One specific expression of the third aspect of the invention provides an apparatus for performing fluorescence microscopy on a sample, comprising:

- a sample holder for receiving a sample;

- a first linear polarizer for receiving an excitation beam from a light source;

- a beam splitter arranged to receive the excitation beam from the first linear polarizer, and direct the excitation beam onto a path to a sample holder, and to direct an emission beam received along the path from the sample holder to a spectrometer;

- a second linear polarizer configured to receive the emission beam before the emission beam passes to the spectrometer,

- wherein the second linear polarizer has a propagation axis and is in a fixed angular position with respect to the beam-splitter about the propagation axis; and

- a half-wave plate which lies on the propagation path of the emission beam between the beam-splitter and the second linear polarizer, and which is rotatable with respect to the second linear polarizer about the propagation axis.

A second specific expression of the third aspect of the invention provides a method for performing fluorescence microscopy on a sample, comprising:

receiving a sample;

receiving, by a first linear polarizer, an excitation beam from a light source;

receiving, by a beam splitter, the excitation beam from the first linear polarizer;

5 directing, by the beam splitter, the excitation beam onto a path to a sample holder;

directing, by the beam splitter, an emission beam received along the path from the sample holder to a spectrometer;

10 receiving, by a second linear polarizer, the emission beam before the emission beam passes to the spectrometer, wherein the second linear polarizer has a propagation axis and is in a fixed angular position with respect to the beam-splitter about the propagation axis; and

receiving, by a half-wave plate, the emission beam before the emission beam passes to the second linear polarizer,

15 wherein the half-wave plate is rotatable with respect to the second linear polarizer about the propagation axis. The method may further comprise rotating the half-wave plate with respect to the second linear polarizer.

Throughout this specification, the term 'light' should here be understood to refer to electromagnetic radiation having any wavelength, rather than visible light only, unless otherwise specified. Stating that "light", "radiation", or a beam "passes through" or is "transmitted by" an optical component does not necessarily imply that the properties of the "light", "radiation" or "beam" (for example, the polarization state or amplitude of the "light" or "beam") are unchanged by 'passing through' or 'being transmitted by' the optical component, unless otherwise specified.

25

Brief description of the drawings

An embodiment of the invention will now be described, for the sake of example only, with reference to the following drawings, in which:

30 Figure 1 shows the conversion of linearly-polarized radiation to circularly-polarized radiation resulting from passage through a quarter-wave plate.

Figure 2 shows the optical layout of an apparatus which is an embodiment of the invention.

Figure 3 shows the layout of the portion of the apparatus of Figure 2 used to excite a sample in operation.

Figure 4 shows the layout of the portion of the apparatus of Figure 2 used to measure the emission of a sample in operation.

Figure 5 shows the conversion of circularly-polarized radiation to linearly-polarized radiation resulting from passage through a quarter-wave plate; and

- 5 Figure 6 shows the layout of the portion of the apparatus of Figure 2 used to image a sample.

Detailed description of the embodiments

Figure 1 shows radiation 101 having a first linear polarization, and radiation 102 having a second linear polarization incident on a quarter-wave plate 110 ("quarter-wave retarder"). The radiation 101 has a plane of polarization rotated $\theta_{ex} = +45^\circ$ from a fast axis 120 of the quarter-wave plate 110, and consequently is converted to right-circularly-polarized radiation 130 ('right-handed circularly-polarized radiation', 'RCP', 'RHC') upon passing through the quarter-wave plate 110. As can be seen in Figure 1, the plane of polarization (131, 132, 133, 134) of the right-circularly-polarized radiation 130 rotates clockwise when viewed along the direction of propagation. The radiation 102 has a plane of polarization rotated $\theta_{ex} = -45^\circ$ from the fast axis 120 of the quarter-wave plate 110, and consequently is converted to left-circularly-polarized radiation 140 ('left-handed circularly-polarized radiation', 'LCP', 'LHC') upon passing through the quarter-wave plate 110. As can be seen in Figure 1, the plane of polarization (141, 142, 143, 144) of the left-circularly-polarized radiation 140 rotates counter-clockwise when viewed along the direction of propagation.

Therefore, any electromagnetic radiation incident on the quarter-wave plate 110 will have its component of polarization in the direction of the first linear polarization converted to right-circularly-polarized radiation 130, and its component of polarization in the direction of the second linear polarization converted to left-circularly-polarized radiation 140. Thus, a linearly polarized light beam may be converted to a light beam in the circular polarization basis (i.e. a light beam made up of left-circularly-polarized and right-circularly-polarized components) by passing through a quarter-wave plate 110.

The degree of circular polarization (DOCP) of the light beam in the circularly polarized beam as a function of the angle θ_{ex} may then be calculated as follows:

$$DOCP(\theta_{ex}) = \frac{I_{RHC} - I_{LHC}}{I_{RHC} + I_{LHC}} = \sin(2\theta_{ex})$$

where θ_{ex} is the anticlockwise angle between the fast axis 120 of the quarter-wave plate 110 and the plane of linear polarization of the incident light (101, 102), as viewed along the direction of propagation.

By varying the angle θ_{ex} , e.g. by rotating either the quarter-wave plate 110 or plane of linear polarization of the incident light (101, 102) with respect to the other, the degree of circular polarization of the beam (130, 140) transmitted through the quarter-wave plate 110 may be varied.

- 5 Figure 2 shows an apparatus according to an embodiment of the invention. The apparatus comprises an 'excitation' section (Figure 3) for generating radiation to excite a sample held in sample holder 3, and a 'detection' section (Figure 4) for measuring any radiation emitted by a sample held in sample holder 3 using measurement device 2105. Optionally, the apparatus may also include an 'imaging'
- 10 section (Figure 6), for imaging a sample held in sample holder 3 with an imaging device 292.

Figure 3 shows a possible configuration of the 'excitation' section of the apparatus according to an embodiment.

- 15 An excitation beam (1, 2) ("excitation light") may be generated by at least one light source (211, 212). In an embodiment, the light source (211, 212) may be a laser. The use of a laser light source provides a highly focused excitation beam (1, 2), allowing small samples or individual regions of a sample to be addressed. For example, two light sources (211, 212) shown in Figures 2, 3 may be present in the apparatus, allowing excitation beams (1, 2) to be generated at multiple respective
- 20 excitation wavelengths in operation, e.g. simultaneously or at different times. Both light sources (211, 212) may be laser sources, having distinct wavelengths (for example, 633nm and 594 nm respectively). In variants of the embodiment, more than two light sources may be accommodated in the apparatus, or only a single light source may be present.
- 25 The radiation from the two light sources (211, 212) is combined onto a single path by a first beam-splitter 220 in operation. This first beam-splitter 220 is a long-pass dichroic mirror. In a variant of the embodiment, a plurality of first beam-splitters 220 may be present, allowing for more than two light sources (211, 212) to be accommodated in the apparatus.
- 30 As discussed below, the apparatus includes a first linear polarizer 240. The excitation beams (1, 2) pass through a third linear polarizer 230. By rotating the third linear polarizer 230 with respect to the first linear polarizer 240, the power of the excitation beam (1, 2) incident on the sample may be adjusted in operation. A portion of the excitation light (1, 2) transmitted through the first linear polarizer 240 in
- 35 operation is directed to a module 231 configured to obtain data indicative of the power of the excitation beam (1, 2). The module 231 may be a photodiode. Optionally, the photodiode may be a silicon photodiode. Using known properties of the module 231 (e.g. a known or pre-calibrated photocurrent responsivity for the photodiode), and of any optical components the excitation beam (1, 2) passes

through on the path to the module 231, the power of the excitation beam (1, 2) may be determined.

The excitation beam (1, 2) is then incident on a second beam splitter 250. The beam-splitter directs at least a portion of the incident excitation light (1, 2) towards the sample holder 3. The second beam splitter 250 is a non-polarizing beam splitter – i.e., the transmittance values of the beam-splitter 250 for orthogonal polarizations of light (e.g. horizontal and vertical linear polarizations) lie within some predetermined tolerance value of one another, and the reflectance values of the beam-splitter 250 for orthogonal polarizations of light (e.g. horizontal and vertical linear polarizations) also lie within some predetermined tolerance value of one another. For example, the transmittance values of the beam splitter 250 for any two orthogonal polarizations may lie within 1-2% of one another. In another example, the reflectance values of the beam splitter 250 for any two orthogonal polarizations may lie within 3% of one another. Furthermore, the second beam splitter 250 is a broadband non-polarizing beam splitter – i.e., the transmittance and reflectance values of the second beam splitter 250 lie within respective transmittance and reflectance ranges over some pre-determined wavelength range of interest (the spectral dependence of the second beam splitter 250 is substantially flat within the wavelength range of interest). For example, the pre-determined wavelength range of interest may be 600-1000nm, and the transmittance value may be $45\% \pm 5\%$ for incident radiation having a wavelength within the pre-determined wavelength range of interest. The use of this second beam splitter 250 allows the excitation beam (1, 2) and emission beam 4 to share a common path in operation.

The portion of the incident excitation light (1, 2) which is directed towards the sample holder 3 by the second beam splitter 250 is then incident on a quarter-wave plate 241. The quarter-wave plate 241 is a broadband or achromatic quarter-wave plate – i.e., the phase shift which the quarter-wave plate 241 introduces between the polarization component of incident light parallel to its fast axis 120 and the polarization component of incident light orthogonal to its fast axis 120 (i.e., parallel to a slow axis of the quarter-wave plate 241) remains within some pre-determined tolerance value of 90° or $\pi/2$ radians over a pre-determined wavelength range of interest. For example, the pre-determined wavelength range of interest may be 400-800nm. In a further example, the phase shift introduced could be within 6% of $\pi/2$ within a wavelength range which includes both the excitation and emission wavelengths, such as 400-800nm.

The excitation beam (1, 2) which passes through the quarter-wave plate 241 is then received by a sample contained in a sample holder 3. Consequently, the sample contained in the sample holder 3 may be excited with excitation beam (1, 2). The excitation beam at the sample contains circular polarization components, even though the excitation beam (1, 2) when incident on the second beam splitter 250 is in a linear polarization basis. This reduces the complex changes to the polarization

state of the excitation beam (1, 2) which would result if the excitation beam (1, 2) were in a circular polarization basis upon reaching the beam splitter 250.

The sample holder 3 may be mounted on a translation stage, and the sample holder 3 may comprise a cryostat for maintaining the temperature of the sample below a threshold.

Figure 4 shows a possible configuration of the 'detection' section of the apparatus according to an embodiment.

The excitation light (1, 2) received by the sample (see Fig. 3) may excite at least a portion of the sample. Upon de-excitation, the sample or portion of the sample will then emit radiation. In an embodiment, the sample holder 3 may be located within, or proximate to, a module 270 configured to collect and collimate radiation emitted by the sample, generating an emission beam 4, and to direct the emission beam 4 to the quarter-wave plate 241.

The module 270 may comprise one or more (typically multiple) microscope objective lenses having different magnification values. The module 270 may comprise multiple sets of lenses, such that one set of lenses may be selected and placed on the path of the excitation beam, allowing a user to switch between magnifications. Preferably, the microscope objective lens or lenses are non-birefringent and strain-free, reducing unintentional polarization of the excitation beam (1, 2) when it passes through the module 270. Examples of suitable microscope objective lens or lenses are Nikon CFI TU Plan EPI ELWD series lenses, or Nikon CFI T Plan EPI SLWD series lenses. Since the sample is held within a cryostat, the microscope objective lens or lenses preferably have long working distances. Preferably, the working distance of the microscope objective lens (or in the case of a set of lenses arranged in series along a line, the working distance of the lens closest to the sample holder) is at least 9mm.

The emission beam 4 is then incident on the quarter-wave plate 241. Right-circularly-polarized radiation in the emission beam 4 is converted to linearly polarized radiation having a plane of polarization oriented $+45^\circ$, or 45° anticlockwise when viewed along the propagation direction of the emission beam 4, from the fast axis 120 of the quarter-wave plate 241. Left-circularly-polarized radiation in the emission beam 4 is converted to linearly polarized radiation having a plane of polarization oriented -45° , or 45° clockwise when viewed along the propagation direction of the emission beam 4, from the fast axis 120 of the quarter-wave plate 241. The emission beam 4 is hence converted to a linear polarization basis (see Figure 5 for further details).

The emission beam 4 in the linear polarization basis is then incident on the second beam splitter 250. As the emission beam 4 is in the linear polarization basis when it passes through the second beam splitter 250, any inadvertent linear polarization changes caused by passage through the second beam splitter 250 will be relatively simple to compensate for in post-measurement data analysis.

Beam splitters generally introduce shifts in both amplitude and phase between the vertical and horizontal polarization planes, which alters the polarization of both excitation and emission beams if they are in the circular basis. By putting the excitation beam (1, 2) and emission beam 4 in the linear polarization basis, such phase shifts no longer affect their polarization states. Relative amplitude changes are far simpler to compensate for.

The second beam splitter 250 then directs at least a portion of the emission beam 4 towards the measurement device 2105. The portion of the emission beam 4 which is directed towards the measurement device 2105 passes through a filter 2101 before reaching the measurement device 2105. Optionally, the filter 2101 is a long-pass filter, i.e. a filter configured to transmit radiation having wavelengths above a cut-off wavelength and to reject or attenuate radiation having wavelengths below the cut-off wavelength. Alternatively, the filter 2101 may be a band-pass filter, i.e. a filter configured to transmit radiation with a wavelength lying in some pre-determined wavelength band, and to attenuate or reject radiation with a wavelength lying outside the pre-determined wavelength band. Thus, any excitation radiation (1, 2) which, for example due to faults in the second beam splitter 250, has been combined into the emission beam 4, can be attenuated or rejected by the filter 2101.

The apparatus comprises a second linear polarizer 2103 lying between the second beam splitter 250 and the measurement device 2105, and a half-wave plate 2102 ("half-wave retarder") configured to receive the portion of the emission beam 4 which is directed towards the measurement device 2105. The relative angular position of the second linear polarizer 2103 and the half-wave plate 2102 is variable.

Specifically, the second linear polarizer 2103 has a propagation axis and has a fixed angular position with respect to the second beam splitter 250 about the propagation axis, and the half-wave plate 2102 is rotatable with respect to the second linear polarizer 2103 about the propagation axis.

By rotating the half-wave plate 2102 with respect to the second linear polarizer 2103 about the propagation axis, the position of the fast axis of the half-wave plate 2102 with respect to the polarization axis of the second linear polarizer 2103 is changed. This allows different linear polarization components of the emission beam 4 incident on the half-wave plate 2102 to be selected for output to the measurement device 2105. For example, the second linear polarizer 2103 may be configured to transmit vertically-polarized light only (where "vertical" refers to the direction perpendicular to the propagation direction of the emission beam 4 such that light linearly polarized in that direction is transmitted through the second linear polarizer 2103). When the fast axis of the half wave plate 2102 is oriented either vertically or horizontally (parallel or perpendicular to the plane of polarization of light transmitted through the second linear polarizer 2103), there is no change to the polarization of the emission beam 4 on passing through the half-wave plate 2102. When the fast axis of the half-wave plate 2102 is oriented at a 45° or 135° angle from the vertical, the polarization of the

emission beam 4 is rotated to the polarization direction orthogonal to the original polarization direction of the emission beam 4.

Optionally, the half-wave plate 2102 may be an achromatic or broadband half-wave plate, i.e., the phase shift the half-wave plate 2102 introduces between the polarization component of incident light parallel to its fast axis and the polarization component of incident light orthogonal to its fast axis (i.e. parallel to a slow axis of the quarter-wave plate 2102) is 180° or π radians (or equivalently the half-wave plate 2102 has a retardance value of $\lambda/2$), to within some pre-determined tolerance value, over a pre-determined wavelength range of interest. For example, the pre-determined wavelength range of interest could be 600-1000nm, or 400-800nm. The pre-determined tolerance value could be $\lambda/300$ for wavelengths λ lying in the pre-determined wavelength range of interest. As a further example, the retardance of the half-wave plate 2102 could remain $\lambda/2$, within the pre-determined tolerance value of $\lambda/300$, for wavelengths λ in the range 400-800nm, or more preferably wavelengths in the range 400-1000nm.

In principle, by rotating the second linear polarizer 2103 about the propagation axis and keeping the half-wave plate 2102 in a fixed angular position with respect to the second beam splitter 250, an output linear polarization component could be selected. However, linear polarizers are generally not constructed with sufficient parallelism to allow the second linear polarizer 2103 to be rotated about the propagation axis without causing slight angular deviation of the emission beam 4. Consequently, the focus of the emission beam 4 will drift away from the input to the measurement device 2105 as the second linear polarizer 2105 is rotated. As half-wave plates require a higher degree of parallelism (in order to introduce accurate phase shifts into an incident beam), rotating the half-wave plate 2102 about the propagation axis and keeping the second linear polarizer 2103 fixed, reduces the deviation of the emission beam 4.

Additionally, as the second linear polarizer 2103 is fixed, the polarization of the light entering the measurement device 2105 will always be the same. As the efficiency of a number of measurement devices 2105, such as spectrometers, is polarization-dependent, ensuring that the polarization direction of light which enters the measurement device 2105 is fixed removes the need to calibrate, or compensate for, the variation in efficiency with polarization. The measurement device 2105 may be a grating spectrometer. The light transmitted through the second linear polarizer 2103 may be transmitted to the measurement device 2105 through an optical path comprising an optical fiber 2104.

Figure 5 shows radiation 510 having right-handed circular polarization and radiation 520 having left-handed circular polarization incident on a quarter-wave plate 560 ("quarter-wave retarder"). As can be seen in Figure 5, the plane of polarization (511, 512, 513, 514) of the right-circularly-polarized radiation 510 rotates clockwise when viewed along the direction of propagation, and the plane of polarization (521, 522,

523, 524) of the left-circularly-polarized radiation 520 rotates counter-clockwise when viewed along the direction of propagation. The RCP radiation 510 is converted to linearly polarized radiation 540 having a plane of polarization rotated $\theta_{ex} = +45^\circ$ from the fast axis 530 of the quarter-wave plate 560 upon passing through the quarter-wave plate 560. The LCP radiation 520 is converted to linearly polarized radiation 550 having a plane of polarization rotated $\theta_{ex} = -45^\circ$ from the fast axis 530 of the quarter-wave plate 560 upon passing through the quarter-wave plate 560. Thus, any circularly-polarized components (510, 520) of radiation incident on the quarter-wave plate 560 are converted into corresponding linear polarization components (540, 550). The incident radiation is converted into the linear polarization basis.

Figure 6 shows a possible configuration of the 'imaging' portion of the apparatus according to an embodiment. An illumination source 283 generates an illumination beam 5. In an embodiment, the illumination source 283 is a white LED. Preferably, the white LED is of small size (for example, less than 1mm in diameter). The illumination beam 5 is collimated by a collimation lens 282 into a substantially parallel beam, and is then re-focused by a focusing lens 280 into a beam 5 having a focus at the back focal plane of the microscope objective 270. This arrangement allows the sample to be evenly illuminated.

The apparatus comprises a third beam splitter 260. The presence of the third beam splitter 260 allows the illumination beam 5 and imaging beam 6 to share a common path during the imaging process. The third beam splitter 260 may be a plate beam splitter. Preferably, the third beam splitter 260 has approximately equal reflectance and transmittance values in a predetermined wavelength range of interest, in order to yield optimum image brightness. However, plate beam splitters may cause unintended polarization changes to an incident beam (1, 2), and therefore should be removed when taking measurements with the measurement device 2105.

An iris 281 selectively restricts the width of the light shaft entering the instrument. Preferably, the iris 281 is positioned such that the distance between the iris 281 and the focusing lens 280 is the focal length of the focusing lens 280.

The optical components 280-283 are arranged according to the Köhler scheme, in order to evenly distribute light (i.e. the illumination beam 5) on the sample.

The illumination beam 5 is then incident on the sample received in the sample holder 3, and the imaging beam 6 generated from the illumination beam 5 is then directed towards an imaging device 292. The imaging device 292 may comprise a charge-coupled device (CCD). Specifically, the imaging device 292 may be a CCD camera, preferably a thermoelectrically cooled CCD camera in order to reduce background thermal noise and dark current. Optionally, the imaging device 292 may be shielded to reduce the stray background light input to the imaging device 292.

The imaging beam 6 may be directed towards the imaging device 292 using a fourth beam splitter 290. In a further embodiment, the fourth beam splitter 290 may be a

metallic mirror configured to reflect the image into the imaging device 292. Optionally, the imaging beam 6 may be focused by an imaging lens 291, for example a tube lens. The fourth beam splitter 292 is removed when taking measurements with the measurement device 2105.

CLAIMS

1. An apparatus for performing fluorescence microscopy on a sample, comprising:

a sample holder for receiving a sample;

a first linear polarizer for receiving an excitation beam from a light source;

5 a beam splitter arranged to receive the excitation beam from the first linear polarizer, and direct the excitation beam onto a path to a sample holder, and to direct an emission beam received along the path from the sample holder to a measurement device; and

a quarter-wave plate located on the path;

10 whereby the quarter-wave plate is operative to convert a linearly polarized component of the excitation beam into circularly polarized radiation, and convert a circularly polarized component of the emission beam into linearly polarized radiation.

2. The apparatus of claim 1, wherein the quarter-wave plate and the first linear polarizer have respective transmission axes, and are rotatable with respect to one
15 another about their respective transmission axes,

wherein, following passage of the excitation beam through the quarter-wave plate, the relative proportion of left- and right-circularly polarized radiation in the excitation beam is dependent on a relative angular position of the quarter-wave plate and the first linear polarizer.

20 3. The apparatus of claim 1 or claim 2, wherein the beam splitter is a broadband non-polarizing beam splitter.

4. The apparatus of any preceding claim further comprising:

the measurement device, the measurement device being a spectrometer,

25 a second linear polarizer configured to receive the emission beam before the emission beam passes to the measurement device.

5. The apparatus of claim 4, wherein the second linear polarizer has a propagation axis and is in a fixed angular position with respect to the beam-splitter about the propagation axis, the apparatus further comprising a half-wave plate which lies on the propagation path of the emission beam between the beam-splitter and the
30 second linear polarizer, and which is rotatable with respect to the second linear polarizer about the propagation axis.

6. The apparatus of claim 4 or claim 5, further comprising an optical fibre operative to direct the emission beam to the spectrometer after passage of the emission beam though the second linear polarizer.

7. The apparatus of any preceding claim, further comprising a module configured to:

- (i) collect and collimate radiation emitted by the sample; and
- (ii) direct the collimated light to the broadband quarter-wave plate.

8. The apparatus of any preceding claim, further comprising a third linear polarizer configured to receive the excitation light and transmit it to the first linear polarizer, the third linear polarizer being rotatable with respect to the first linear polarizer about the path of the excitation beam.

9. The apparatus of claim 8, further comprising a module configured to obtain data indicative of the power of the excitation beam after transmission by the first linear polarizer.

10. The apparatus of any preceding claim, further comprising a filter located between the beam-splitter and the measurement device.

11. The apparatus of claim 10, wherein the filter is a long-pass or band-pass filter.

12. The apparatus of any preceding claim, further comprising the light source, the light source being a laser.

13. The apparatus of any preceding claim, wherein the sample holder comprises a temperature control module for maintaining the temperature of the sample in operation.

14. The apparatus of claim 13, wherein the temperature control module comprises a cryostat.

15. The apparatus of any preceding claim, further comprising a visible light source configured to illuminate the sample.

16. A method for performing fluorescence microscopy on a sample, comprising:

receiving a sample;

receiving, by a first linear polarizer, an excitation beam from a light source;

receiving, by a beam splitter, the excitation beam from the first linear polarizer;

directing, by the beam splitter, the excitation beam onto a path to a sample holder;

directing, by the beam splitter, an emission beam received along the path from the sample holder to a measurement device;

converting, by a quarter-wave plate located on the path, a linearly polarized component of the excitation beam into circularly polarized radiation; and

converting, by the quarter-wave plate located on the path, a circularly polarized component of the emission beam into linearly polarized radiation.

17. The method of claim 16, wherein the quarter-wave plate and the first linear polarizer have respective transmission axes, and are rotatable with respect to one another about their respective transmission axes, the method further comprising defining a relative angular position of the quarter-wave plate and the first linear polarizer,

wherein, following passage of the excitation beam through the quarter-wave plate, the relative proportion of left- and right-circularly polarized radiation in the excitation beam is dependent on the relative angular position of the quarter-wave plate and the first linear polarizer.

18. The method of claim 16 or claim 17, wherein the beam splitter is a broadband non-polarizing beam splitter.

19. The method of any of claims 16-18, wherein the measurement device is a spectrometer, and further comprising receiving, by a second linear polarizer, the emission beam before the emission beam passes to the measurement device.

20. The method of claim 19, wherein the second linear polarizer has a propagation axis and is in a fixed angular position with respect to the beam-splitter about the propagation axis, the method further comprising:

receiving, by a half-wave plate, the emission beam after passage through the beam-splitter and before passage through the second linear polarizer,

wherein the half-wave plate is rotatable with respect to the second linear polarizer about the propagation axis.

21. The method of claim 19 or claim 20, further comprising directing the emission beam to the spectrometer via an optical fibre after passage of the emission beam through the second linear polarizer.

22. The method of any of claims 16-21, further comprising:

(i) collecting and collimating light emitted by the sample; and

(ii) directing the collimated light to the quarter-wave plate.

23. The method of any of claims 16-22, further comprising:

receiving, with a third linear polarizer, the excitation light; and

transmitting the excitation light, by the third linear polarizer, to the first linear polarizer,

wherein the third linear polarizer is rotatable with respect to the first linear polarizer about the path of the excitation beam.

24. The method of claim 23, further comprising obtaining data indicative of the power of the excitation beam after transmission by the first linear polarizer.

5 25. The method of any of claims 16-24, further comprising receiving, by a filter, the emission beam after passage through the beam splitter.

26. The method of claim 25, wherein the filter is a long-pass or band-pass filter.

27. The method of any of claims 16-26, wherein the light source is a laser.

10 28. The method of any of claims 16-27, further comprising maintaining the temperature of the sample in operation using a temperature control module.

29. The method of claim 28, wherein the temperature control module comprises a cryostat.

30. The method of any of claims 16-29, further comprising illuminating the sample with a visible light source.

Fig. 1

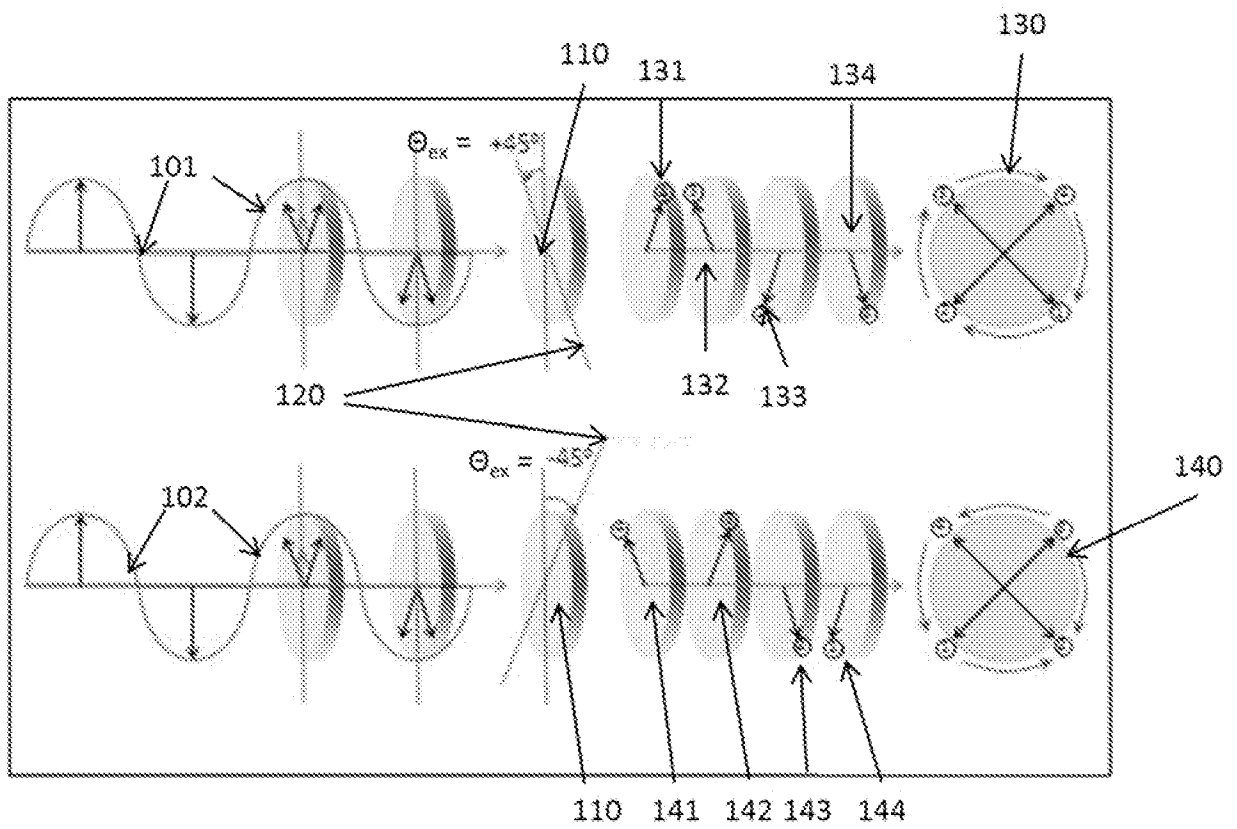


Fig. 2

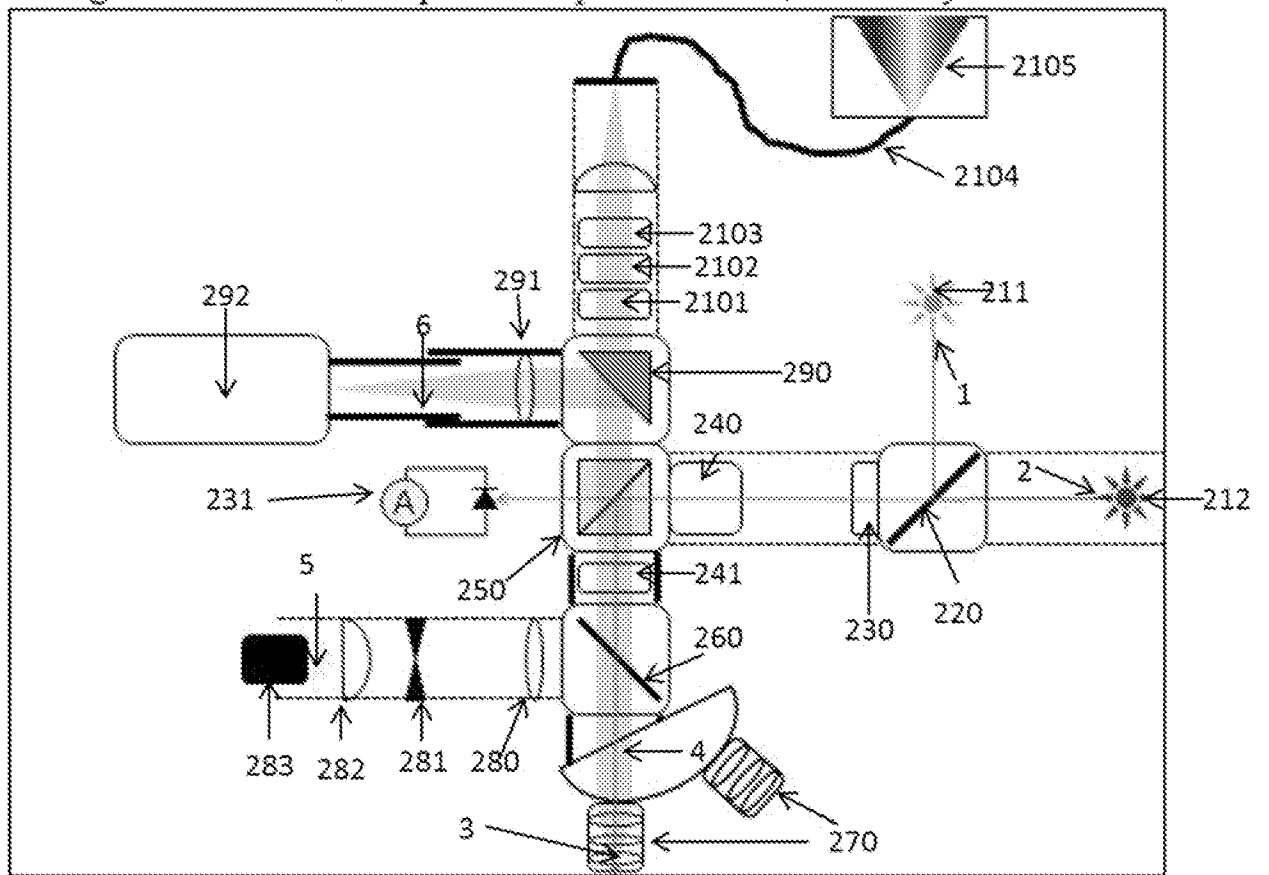


Fig. 3

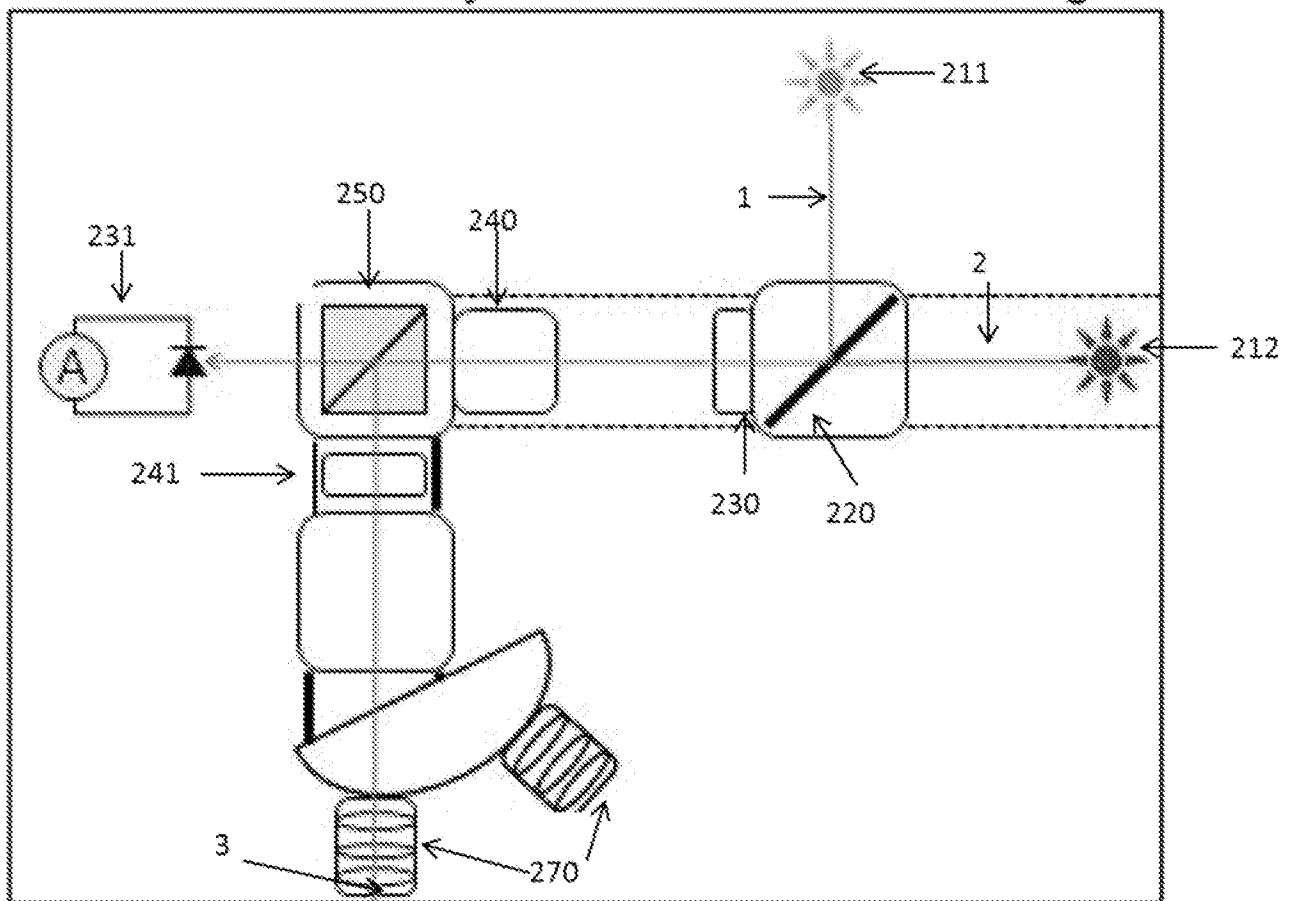


Fig. 4

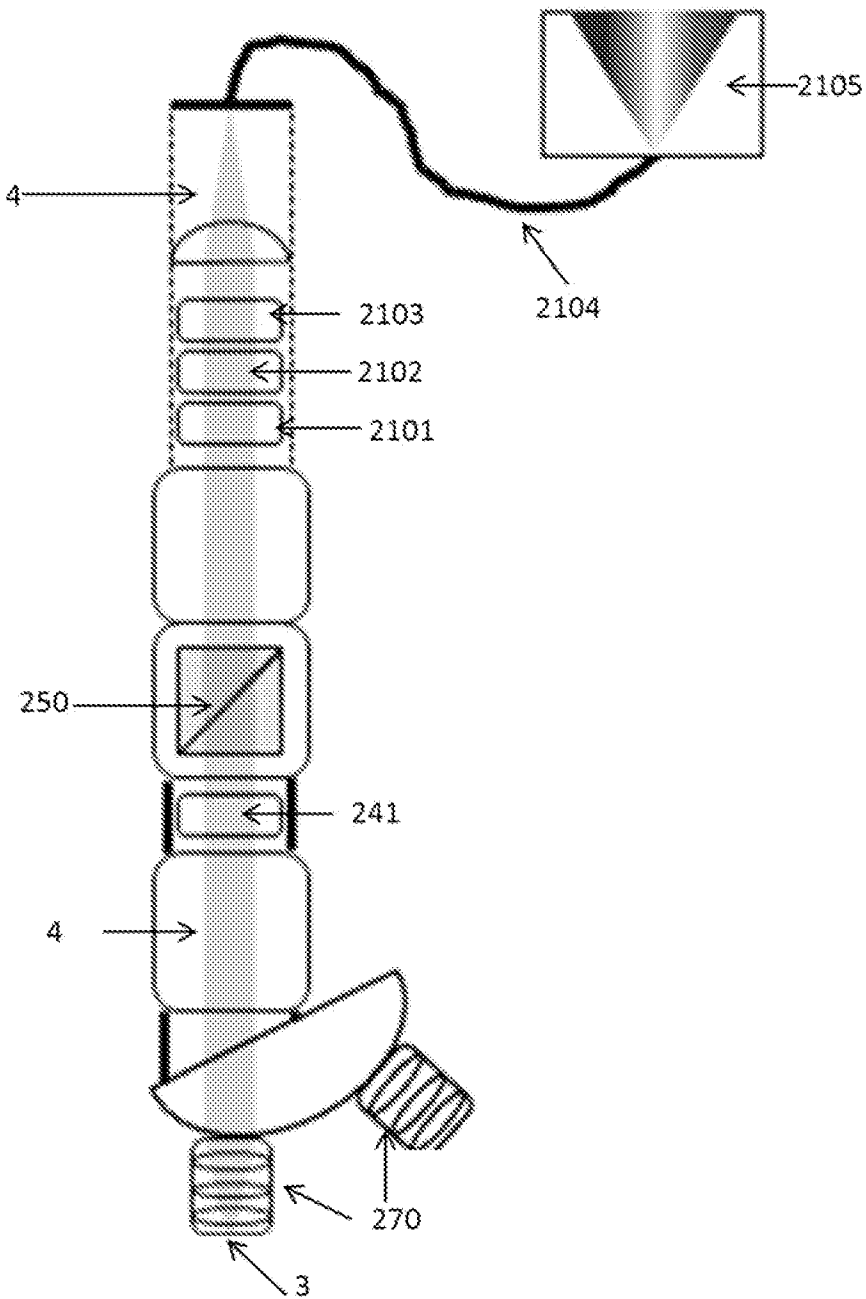


Fig. 5

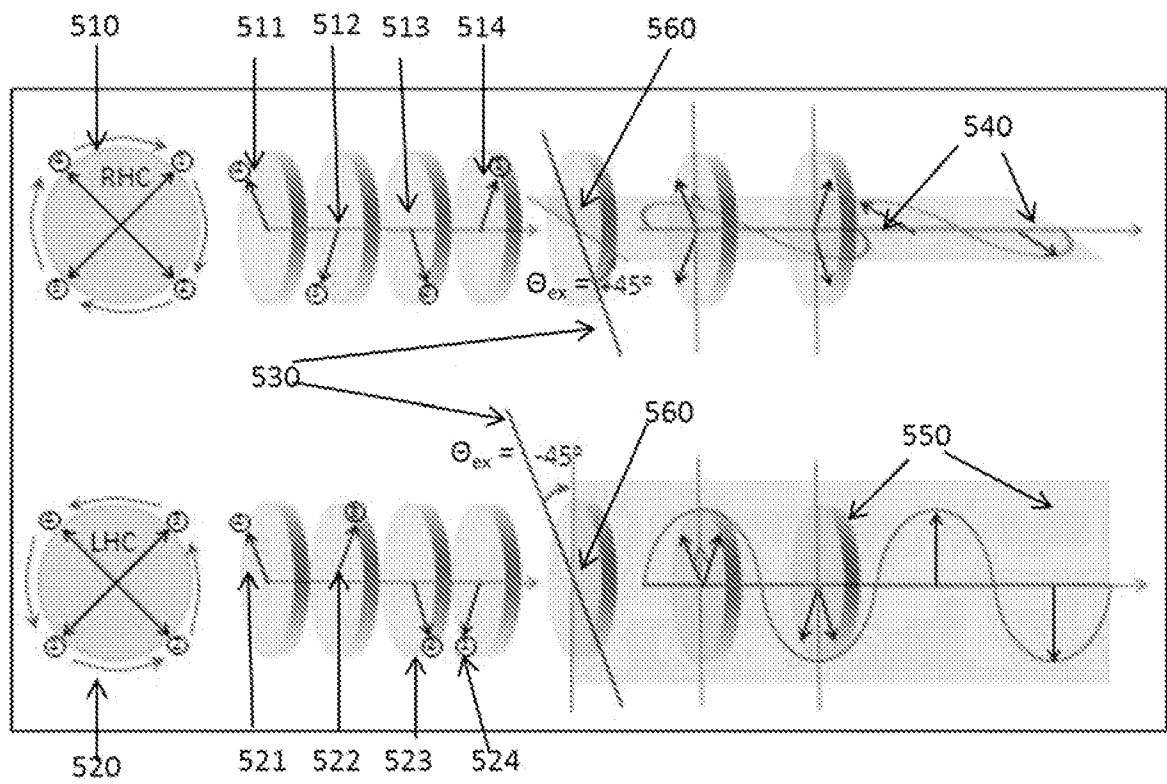
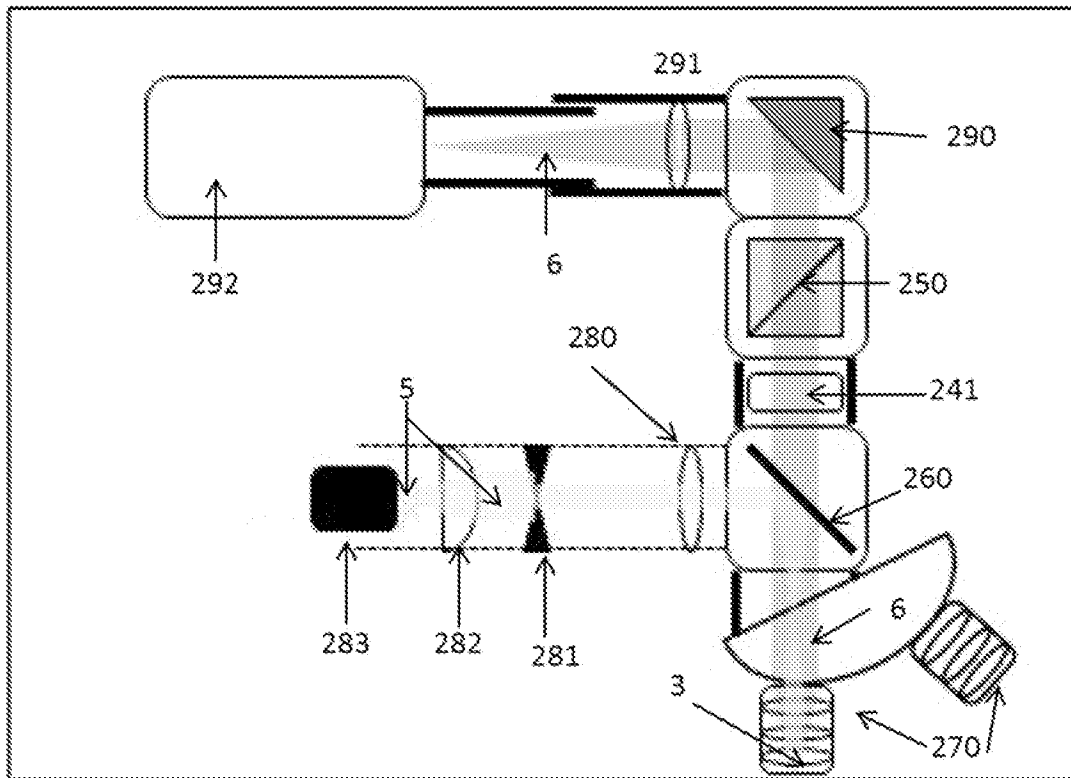


Fig. 6



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2019/050100

A. CLASSIFICATION OF SUBJECT MATTER

G01N 21/64 (2006.01)

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

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)

FAMPAT: Polarizer, polarizing plate, beam splitter, quarter-wave plate, phase retarder, circularly polarized, linearly polarized, s-polarized, p-polarized, non-polarizing, 起偏器, 偏光器, 分束镜, 分光器, 四分之一波片, 四分之一波板, 圆偏振光, 线偏振光, S 偏振光, P 偏振光, 非偏振光 and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	US 2010/0099583 A1 (CHO S-H.) 22 April 2010 Figure 1, paragraphs [0008], [0045], [0047], [0050]-[0056]	1, 2, 4-17 AND 19-30 3 AND 18
X A	US 2003/0197924 A1 (NAKATA T.) 23 October 2003 Figure 3, paragraphs [0047], [0053]-[0056]	1, 2, 4-11, 13, 14, 16, 17, 19-26, 28 AND 29 3, 12, 15, 18, 27 AND 30
A	JP 2015-007754 A (NIKON CORP) 15 January 2015 Whole document of the machine translation	
A	US 2002/0093648 A1 (NIKOONAHAD M. ET AL.) 18 July 2002 Whole document	
A	US 2016/0131885 A1 (NAKAYAMA H. ET AL.) 12 May 2016 Whole document	

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

☒ See patent family annex.

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"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

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Date of the actual completion of the international search

17/05/2019

(day/month/year)

Date of mailing of the international search report

23/05/2019

(day/month/year)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2019/050100**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2019/050100

Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.

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