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The present invention relates to a method for observing the emission of light from a sample by dynamic optical microscopy.

The light from the sample may result from diffusion or fluorescence. Fluorescence microscopy is a technique that takes advantage of the phenomenon of fluorescence in order to observe various compounds. Fluorescence is the property that certain bodies possess to emit fluorescent light by themselves.

The fluorescence of an observed compound can be primary if this compound is fluorescent itself (e.g., chlorophyll, oil), or secondary if the observed compound is labelled with a fluorescent substance known as a fluorochrome or fluorescent marker.

In particular in cell biology, a large number of molecular events occurring at the surface of the cells are studied by fluorescence microscopy, such as cell adhesion, the binding of hormones to receptors in the plasma membrane, the secretion of neurotransmitters as well as membrane dynamics (endocytosis, exocytosis).

A fluorescence microscopy device usually comprises a light source for excitation, means for separating the excitation photons from the emission photons, a system of lenses for capturing the photons and, in general, imaging means.

Fluorescence techniques can be used with different types of microscopes, in particular:

- a conventional optical microscope in which the excitation light can pass through the sample or the lens. In the latter case, this is termed epifluorescence microscopy;

- a confocal microscope, for example by laser scanning, which in particular enables three-dimensional images of the sample to be obtained;

- a total internal reflection fluorescence microscope (usually called TIRF), which uses an evanescent wave to excite the fluorescence only over a very shallow depth, immediately adjacent to the interface of the sample substrate (usually glass) and the liquid medium (usually water) in which the sample is contained. The lighting is achieved by a laser beam incident at a supercritical angle to create an evanescent wave (exponentially decreasing orthogonally to the interface). In the present application the terms “overcritical” and “supercritical” have the same meaning.

TIRF microscopy, although currently widely employed and allowing precise observations, nevertheless has some disadvantages. In fact, the use of an adapted laser source is costly and the excitation field thus generated may not be homogeneous (due to interference from the coherence of the beam). In addition, it is found that lighting by the lens does not allow a homogeneous excitation and the resulting depth of penetration is not constant over the whole of the field to be observed. Moreover, there are containment losses of the excitation field related to the intrinsic light scattering by the cells.

The document FR-A-2943428 discloses a method for observing by fluorescence microscopy a sample comprising fluorescent components in a liquid medium of refractive index n_L arranged on a transparent support of refractive index n_s, which is greater than n_L and less than or equal to 1.55, and an observation device comprising a full-field immersion lens, whose numerical angular aperture, ON, is greater than or equal to 1.33 and less than or equal to n_s, and a set of lenses for forming an image in at least one image plane, and which further comprises a mask arranged in the rear focal plane of the immersion lens or in a conjugate plane of said rear focal plane so as to obscure the fluorescence emission components of the sample in the angular directions in which the angle θ is less than or equal to a critical angle θ_c, where $\theta_c = \arcsin(n_L/n_s)$, and the angle θ is defined as the angle of an angular direction of the fluorescence emission relative to the perpendicular direction of the surface of the support on which the sample to be observed is arranged.

This observation method enables high-quality fluorescence images to be obtained with a low-cost device or the quality of images obtained by TIRF microscopy to be improved.

The device and the method described above provide very satisfactory images with a good resolution. Nevertheless, in certain circumstances, it may be desirable to improve the resolution.

The object of the present invention is to provide an alternative method enabling in particular the resolution of the images to be improved significantly at the cost of some changes.

The solution of the invention relates to a method for observing an emission of light from a sample in a medium of refractive index n_L, the sample being placed against a surface of a transparent support of refractive index n_s greater than n_L, wherein the light emission comprises luminous components of a given amplitude and phase, oriented toward the support and forming an angle θ with a direction perpendicular to the surface, including on the one hand supercritical luminous components for which the angle θ is strictly greater than a critical angle $\theta_c = \arcsin(n_L/n_s)$, and on the other hand critical or subcritical luminous components for which the angle θ is less than or equal to the critical angle θ_c, the method employing an observation device capable of:

- capturing at least part of the light emission from a region of interest of the sample and obtaining a captured luminous signal that comprises luminous components originating from the supercritical luminous components of the light emission;

- applying filters to the captured luminous signal in order to selectively decrease the amplitude and/or change the phase of certain luminous components of the captured luminous signal in order to obtain a filtered light signal; and

- transforming the filtered light signal into an image zone of the region of interest of the sample;

the method being characterised in that:

- a modulation of the filtered luminous signal is carried out, in which luminous components originating from the critical or subcritical luminous components of the light emission are allowed to pass through in order to obtain image zones of one and the same region of interest of the sample, the modulation affecting all or some of the captured luminous components of the captured light signal originating from the supercritical luminous components of the light emission; and

- at least one useful image zone of the sample is produced by combining image zones, the combination revealing differences between the image zones associated with the modulation.

The light emitted by the sample may result from fluorescence (after an appropriate excitation) or from diffusion. A portion of this light is captured by an observation device, then filtered and converted into an image zone.

The region of interest of the sample is the part of the sample that is to be observed. It can be an extensive or a point-like region. In the latter case it is possible to reconstruct a larger image by scanning the sample. An image zone is an image of a region of interest of the sample. It may thus be a full image of the sample, an image of a portion of the sample, or even an image of a point of the sample. The modulation applies to image zones originating from one and the same region of interest of the sample.

By means of the device according to the invention it is possible to obtain microscopy images with an improved resolution, enabling for example valuable information for the study of biological materials to be obtained.

The improvement of the resolution is obtained not by working directly on a luminous signal purged of all or part of the components arising from the light emitted by the sample at critical or subcritical angles, that is to say a signal rich in components

arising from the light emitted by the sample at supercritical angles (the method described in the document FR-A-2943428), but by working indirectly, by a modulation of the signal relating to the components arising from the light emitted by the sample at supercritical angles. The method is indirect since it requires taking several image zones of the sample and combining them to obtain a useful image zone that reveals the differences between the image zones created by the modulation of the luminous signal at the origin of the image zones.

Since the modulation relates to all or some of the luminous components of the captured luminous signal arising from the supercritical luminous components of the light emission, the combination enhances these supercritical luminous components in the useful image zone. In contrast to this, since the components of the captured luminous signal originating from the light emitted by the sample at critical or subcritical angles are not altered by the modulation, the combination reduces these critical or subcritical components in the useful image zone. The combination can be seen as a demodulation. It reveals in the useful image zone the contribution of the modulated supercritical components.

The modulation can affect the amplitude and/or the phase of the luminous components of the captured luminous signal. In other words, the filters used modify the amplitude and/or phase of the light waves. This is also the meaning to be given to the verb “to act,” when it is used in relation to the filters in this application. To act differently, for two filters, means “to carry out a modulation.” The change of the amplitude can be a more or less strong attenuation, ranging up to occultation. Some of the filters used (but not all) can be neutral and not act; in this case the resulting luminous signal is still called a “filtered signal”, even if it is identical to the captured luminous signal. Allowing luminous components “to pass” means that after filtering, the components remain, even in an attenuated form (reduced amplitude) or with a modified phase.

The image zones, like all images formed using a sensor such as a camera, contain information on the luminous intensity related to the square of the module of the filtered luminous signal.

Unexpectedly, the obtained resolution of the useful image zone can be improved by a factor of the order 20 to 25%, especially if the modulation relates to all the luminous components of the captured signal originating from the supercritical luminous components of the light emission (see the explanation of Fig. 7 below) with respect to an image zone obtained by the method of the document FR-A-2943428.

Taking image zones in order to obtain a useful image zone can be carried out simultaneously, for example by duplicating the captured luminous signal and applying a filter to each duplicate. Taking image zones can also be done successively. A luminous signal is then captured and filters are successively applied to it, thereby producing the modulation. An image zone is stored corresponding to each filtered luminous signal obtained.

The method provides at least one useful image zone, but it can be applied as often as necessary, at a rate permitted by the observation device and the filters used, so as to obtain useful image zones at given time intervals. If the image zones correspond to points of the sample, a spatial scanning of the sample can be achieved.

The invention allows image zones to be obtained with a high sensitivity, especially zones localised at the interface between the support of refractive index n_s and the medium of refractive index n_L. Events occurring near this interface can thus be visualised, down to a certain depth. For a glass/water interface (n_L = 1.33 approximately and n_S = 1.51 approximately) the depth may be about 1/6 of the wavelength, or less if only the steeper luminous components (the most supercritical ones) of the emitted light are collected, i.e. of the order of 50 nm (nanometres) to 100 nm for wavelengths in the visible spectrum.

The supports commonly used in microscopy, particularly fluorescence microscopy, are made of glass, and it is possible to choose conventional types of glass, of refractive index less than or equal to 1.55, the cost of which is low, or types of glass having a higher index.

A fluorochrome behaves as an antenna capable of emitting a signal. This emitter has in its immediate environment (a few tens of nanometres) electromagnetic components that are evanescent when it is placed in a homogeneous medium. These components may become propagative when the fluorochrome is positioned in the proximity of an interface. They then propagate at angles that are larger than the critical angle in the medium with the highest refractive index.

Fig. 5a shows the fluorescence emission components of an emitter 12 located at the interface between a support 20 and a liquid medium 11.

The fluorescence emission of the transmitter 12 comprises a component 14 emitted for θ between 0 and θ_c, and a component 15 emitted for θ greater than θ_c, so-called “prohibited light” or “supercritical light” and corresponds to the evanescent components in the liquid medium 11 that have become propagative in the transparent support 20.

For example, for a glass/water interface it is found that the supercritical light can be up to about a third of the intensity of the total light emitted.

The value of the critical angle is given by the Snell-Descartes laws of refraction, where $\theta_c = \arcsin (n_L/n_s)$. In the case where the liquid is water ($n_L = 1.33$) and the support is regular glass ($n_s = 1.52$), θ_c is 61°.

Fig. 5b shows the components of fluorescence emission from an emitter 12 located at a distance of more than about 100 nm.

It is found that the emitted light comprises the same component 14, emitted for an angle θ between 0 and θ_c, but no longer comprises components emitted at an angle greater than θ_c, corresponding to the supercritical light.

The method according to the invention enables the information contained in the supercritical light to be recovered and thus enables useful image zones to be produced containing information on structures and their possible evolution in the 100 nm depth range with respect to the liquid medium/support interface.

The numerical aperture, ON, of a lens is defined by $ON = n.\sin(\alpha_{max})$, where n is the index of the operating environment of the lens and α_{max} is the maximum collection angle of the lens.

Current commercial lenses of large numerical apertures with oil immersion (n = 1.51) have been developed. They have ON values of, for example, 1.45 and 1.49. The maximum captured angles are greater than the critical angle θ_c. Thus, these lenses enable the majority of the supercritical light to be collected. Preferably, these lenses are corrected for spherical and chromatic aberrations and thus enable a full-field image of excellent quality to be obtained.

According to particular embodiments, the invention can implement one or more of the following characteristics:

- the luminous components of the captured luminous signal that are the object of the said modulation are emitted from supercritical luminous components of the light emission for which the angle θ is within a predetermined range according a range of depths to be explored in the sample.

- the image zones obtained by means of the observation device and that produce, by combination, the useful image zone of the sample are successively obtained by successively applying filters to the captured luminous signal.

- the method may comprise the following steps:

a) taking a plurality of image zones of one and the same region of interest of the sample by means of the observation device and a plurality of filters, each filter being used to take an image zone of the said plurality of image zones, the plurality of filters being such that:

+ a filter of the said plurality of filters allows the passage into the filtered luminous signal of the luminous components arising from supercritical luminous components of the light emission;

+ the filters of the said plurality of filters allow the passage of the luminous components of the captured luminous signal arising from the critical or subcritical components of the light emission and act in a substantially identical manner between them on the luminous components of the captured luminous signal arising from critical or subcritical luminous components of the light emission, and

+ at least two filters of the said plurality exist that act in a substantially different manner between them on the amplitude or the phase of at least part of the luminous components of the captured luminous signal arising from supercritical luminous components of the light emission; and

b) production of a useful image zone of the sample by a calculation combining the plurality of image zones taken in step a) in order to reveal differences between the image zones of the plurality of image zones of the sample.

- the method may comprise the following steps:

a) taking at least two image zones of one and the same region of interest of the sample by means of the observation device and two filters, each filter being used to take one of the two image zones, the two filters being such that:

- one of the two filters allows the passage of luminous components arising from the supercritical luminous components of the light emission into the filtered luminous signal; and

- the other filter acts in a substantially identical manner to the said one of the two filters on the luminous components of the captured luminous signal arising from the critical or subcritical luminous components of the light emission and it reduces, to a substantially greater degree than the said one of the two filters, the amplitude of at least part of the luminous components of the captured light signal arising from the supercritical luminous components of the light emission; and

b) producing a useful image zone of the sample by a calculation combining the two image zones of the sample taken in step a), the calculation comprising an algebraic difference between the two image zones of the sample.

- the filters employed also partially reduce the amplitude of all or some of the luminous components of the captured luminous signal arising from the critical and subcritical luminous components of the light emission.

- luminous components of the captured luminous signal arising from the luminous components of the light emission forming the same angle θ are processed in a substantially identical manner by a same filter for the reduction of the amplitude or the modification of the phase.

- the sample displaying a phenomenon to be observed having a given characteristic time, the image zones are successively taken at time intervals of less than or equal to half the characteristic time.

- one of the image zones of the sample is obtained using a neutral filter that allows the passage into the filtered luminous signal, without any decrease of amplitude, of all the luminous components of the captured luminous signal arising from the supercritical luminous components of the light emission; and another image zone of the sample is obtained using a total filter, which cancels in the filtered luminous signal all the luminous components arising from the supercritical luminous components of the light emission.

- the observation device comprises a full-field immersion lens and the filters are located in a rear focal plane of the immersion lens and/or in a conjugate plane of the said rear focal plane.

- the filters comprise a diaphragm which, in an open position, allows the passage of luminous components from the captured luminous signal arising from the supercritical luminous components of the light emission and that, depending on the degree of closure, allows the occultation of the luminous components of the captured luminous signal arising from the supercritical luminous components of the light emission having an angle θ greater than a limit value related to the said degree of closure.

- the sample to be observed is biological in nature.

According to a particular embodiment, the modulation does not affect all the luminous components of the captured luminous signal arising from the supercritical luminous components of the light emission. It may affect only those for which the angle θ is within a predetermined set, for example, the interval $[\theta_a, \theta_b]$, θ_a and θ_b being both strictly greater than θ_c. This enables the range of corresponding depths in the sample to be investigated (see explanation for Fig. 1).

According to another embodiment, the method includes a step a) of taking a plurality of image zones corresponding to modulated luminous signals. In order to obtain the modulation it is necessary that at least one of the filters used allows the passage of luminous components arising from the supercritical part of the light emission. It is also necessary that the other filters act differently from this filter on all or part of the supercritical components of the captured luminous signal. “Differently” can mean that the filters in question attenuate more, or less, the amplitude of the luminous components involved. The difference may also relate to the phase. The actions on the amplitude and phase can be combined.

On the other hand, the filters must act in a substantially identical manner on the luminous components arising from the critical or subcritical part of the light emission.

The luminous components arising from the critical or subcritical part of the light emission can be modified by the filters used to obtain the image zones, but this modification should be substantially the same for all the combined image zones in order to obtain the useful image zone. “Substantially the same” means that according to a particular embodiment there is no difference, but that according to another embodiment there may be minor differences with regard to the desired modulation (this relates to the luminous components arising from the supercritical luminous components of the light emission). Preferably, luminous components arising from the critical or subcritical luminous components of the light emission are allowed to remain in the filtered luminous signal.

The method comprises a step b), in which image zones taken in step a) are combined. The combinations are performed by a calculation based on the light intensity. This calculation depends on the filters used and serves to reveal the differences between the image zones caused by the modulation.

The advantage of taking a plurality of image zones is that it enables any background noise to be reduced, for example by introducing averages into the calculation.

For example, three successive image zones of a same region of interest of the sample can be taken. The first is an image zone of the sample that contains information related to all the luminous components arising from the supercritical part of the captured signal, that is to say, the filter that is used has not substantially modified these components. The second is an image zone that contains no information related to the luminous components arising from the supercritical part of the captured signal, that is to say the filter used has blocked out these components. The third image zone is taken under conditions identical to the first image zone. A possible combination of these three successive image zones is to calculate the absolute value of the difference between, firstly, an average of the first and third image zones and, secondly, the second image

zone. This can allow the reduction of noise in the useful image zone (phenomenon of photo-bleaching, changes in excitation intensity, movement of the sample).

The images contain information related to the (positive) intensity of the overall electric field. A difference between two pixels can thus be positive or negative, which is why an absolute value is used to obtain a positive result that represents an intensity, namely that of the useful image zone.

According to another particular embodiment, in step a) two image zones are taken of one and the same region of interest of the sample. The first one is taken using a filter that allows the passage of luminous components from the supercritical part of the light emission. The second one is taken using a filter that acts differently from the first filter on all or part of the supercritical components of the captured signal and acts substantially identically on the critical or subcritical components of the captured signal. In step b) the two image zones are combined by calculating for each pixel the absolute value of the difference between the first and second image zones. This result represents an intensity, namely that of the useful image zone. The advantage of this embodiment lies in the simplicity of the combination of the image zones, that is to say the simplicity and speed of the calculations.

According to a particular embodiment, in an identical manner all or part of the luminous components of the captured luminous signal arising from the critical or subcritical luminous components of the light emission can be attenuated in the two image zones. This is important when the light originating from the subcritical rays is very intense compared to that originating from the supercritical rays.

In general, the filters used respect the symmetry of revolution and treat in the same way the luminous components of the captured luminous signal arising from the luminous components of the light emission forming the same angle θ. In fact, an azimuthal modulation of the components of the light emitted is not of great interest. On the contrary, the fact of not performing an azimuthal modulation prevents the introduction of astigmatism into the useful image zone.

If the sample changes over time (for example a cell membrane) with a given characteristic time, successive image zones (more representative of the sample in depth) are taken with a period shorter than half the characteristic time (video frame rate) so as to be able to monitor these changes. The method normally allows useful image zones (more representative of the sample interface) to be obtained at the same rate as that of the successive image zones, i.e. at the video frame rate and not at half the video frame rate as in the method described in the document FR-A-2943428.

The addition of a diaphragm in the rear focal plane of the lens and/or in a conjugate plane can be effected easily and in particular can be implemented with commercial microscopes. The result is a device whose cost of the improvement is very modest.

The function of the diaphragm is to block out the luminous components of the captured luminous signal arising from the luminous components of the light emission from the sample in the angular directions θ greater than or equal to a certain angle depending on the aperture of the diaphragm. Figs. 5a and 5b recall the principles of fluorescence emission. An advantage of the method is that the subcritical information is always available in the image zones used for the combination. This information is often interesting, since it is connected with the innermost regions of the sample.

Other features and advantages of the present invention will become apparent from the following description of non-limiting examples, with reference to the accompanying drawings, in which:

- Fig. 1 shows a schematic view of a microscopy device enabling the implementation of a method according to the invention;

- Fig. 2 shows a variant according to the invention of the device shown in Fig. 1, suitable for confocal microscopy;

- Fig. 3 shows a variant according to the invention of the device shown in Fig. 1, suitable for TIRF microscopy;

- Fig. 4 shows an example of a filter for the implementation of a method according to the invention;

- Figs. 5a and b show the fluorescence emission components according to different configurations and have been discussed above;

- Figs. 6a and 6b illustrate image zones of a same cell respectively with and then without the luminous components of the captured luminous signal from the critical or subcritical luminous components of the light emission;

- Fig. 6c shows a useful image zone of the same cell obtained by combining the image zones of Figs. 6a and 6b;

- Fig. 7 is a graphical representation of the light intensity of the spots obtained from a same test sample, firstly by the method described in the document FR-A-2943428 (occultation of critical or subcritical components), and secondly by a method according to the invention (modulation applied to the supercritical components, followed by demodulation).

For reasons of clarity, the dimensions of the various elements shown in these figures are not necessarily in proportion to their actual dimensions. In the figures, identical references correspond to identical elements.

Fig. 1 shows a schematic view of a fluorescence microscopy device 100. This comprises an immersion lens 110, the ON of which is greater than or equal to 1.33. A glass support 20 is arranged above this immersion lens 110. Oil is present between the immersion lens 110 and the glass support 20.

A sample 10 to be observed is arranged on the glass support 20. This sample 10 comprises, for example, fluorescent components dispersed in water.

The rear focal plane of the lens is identified by the reference numeral 400. Excitation light is generated by a beam 200 originating from a light source, which passes through an excitation filter 210 and is reflected by a dichroic mirror 120 to illuminate the sample 10 after passing through the transparent support 20. An example of the path of the incident excitation light is indicated by the arrows pointing to the top of the figure. The incident excitation light may be partly reflected and is then filtered by an emission filter 130 so that the image formed on an image plane comprises only the fluorescence light emitted by the sample 10.

The fluorescent light emitted by the sample 10 passes through the transparent support 20, the dichroic mirror 120, and the emission filter 130.

According to the embodiment shown in Fig. 1, this light is reflected at a mirror 140 and the rest of the device works with the reflected light.

A lens 150, a so-called tube lens, allows the light to be focused onto an intermediate image plane 410.

The light is then rendered parallel by a lens 160 and next focused by a lens 180 onto the image plane 430, where the image is acquired by a suitable device, in particular a camera 300. The planes 430 and 410 are conjugate image planes of the observation plane.

The lenses 160 and 180 are arranged so that a conjugate plane 420 of the rear focal plane of the immersion lens 110 is located between the lenses 160 and 180.

A variable aperture diaphragm 170 is arranged in this rear focal plane of the immersion lens. This diaphragm acts as a filter for the luminous components of the captured luminous signal. It can be in open position and allow the passage of all the luminous components of the captured luminous signal arising from the luminous components of the light emission. It can be in a partially closed position and block off part of the luminous components of the captured luminous signal.

More specifically, the light rays that are emitted according to a certain angle by the fluorescent emitters of the sample 10 located in the observation plane intercept the rear focal plane 400 of the lens (or any conjugate plane 420 of the plane 400) at a certain distance r(θ) from the centre (defined by the optical axis) of this plane. r(θ) is an increasing function of θ. For aplanatic lenses, for example, r(θ) is approximately proportional to sin(θ). Thus, all the rays emitted at the angle θ (forming a cone) describe a circle of a radius of r(θ) in the rear focal plane.

When the diaphragm 170 is arranged in the rear focal plane 400 of the lens 110, the relationship between r(θ) and sin(θ) is:

$r(\theta) = n_i \times f_o \times \sin(\theta)$, where f_o is the focal distance of the immersion lens 110 (usually of the order of a few millimetres) and n_i is the index of the immersion medium used for the lens (usually oil).

According to one embodiment an immersion lens with a magnification G = 100 is used and the focal distance of the tube lens 150 is f_t = 200 mm.

We then have $f_o = f_t/G$ = 2 mm. In this configuration we have: $r(\theta_c)$ = 2.66 mm.

If the diaphragm 170 is arranged in the conjugate plane of the rear focal plane, the magnification factor related to the optical system should be taken into account. For example, a multiplication factor G' = f160/f150 should be introduced in the plane 420, where f150 is the focal distance of the tube lens 150 and f160 is the focal distance of the lens 160.

The luminous components of the captured luminous signal arising from the critical or subcritical luminous components of the light emission intercept the rear focal plane along a closed centred disk of radius of $r(\theta_c)$. The luminous components of the captured luminous signal arising from the supercritical luminous components of the light emission, for which $\theta_c < \theta < \theta_{max}$, form an open ring in the rear focal plane $r(\theta_c) < r(\theta) <$

$r(\theta_{max})$. The implementation of a diaphragm 170 centred on the optical axis and having an opening $r(\theta_c)$ thus allows all the supercritical luminous components to be blocked.

The selection is thus made at the emission. As a result, the lighting system does not need to be changed from that of a standard epifluorescence observation device. It is thus possible to illuminate with a source of non-coherent light, for example a standard white light, in particular obtained using a mercury lamp. This results in several advantages, such as the absence of significant additional cost (compared to the TIRFM microscopy technique, where a laser lighting is required) as well as the possibility to obtain a homogeneous field (possibly allowing quantitative measurements).

According to one embodiment, actuation means of the diaphragm operate at the video frame rate (typically of the order of a few tens of Hertz) in order to pass alternately from the open position to the closed position at the speed of acquisition of the images. It is thus possible to have information on the volume and the surface simultaneously.

This imaging method is particularly suitable for imaging biological samples, in particular for the study of biological processes in living cells, such as cell adhesion phenomena, endocytosis/ exocytosis, ...

Fig. 2 shows a schematic view of a variant according to the invention of the device of Fig. 1, where the elements present before the image plane 430 are identical in both embodiments. In the device of Fig. 2 a pinhole-type mask 190 that comprises a hole 195 is arranged in the image plane. A mono-detector 350 allows a point-by-point acquisition of the light passing through the hole 195. It is thus possible to obtain a configuration allowing confocal microscopy to be carried out.

Fig. 3 shows a schematic view of a variant according to the invention of the device of Fig. 1 where the elements of the microscopy device are similar, but where the light source differs.

In the device of Fig. 3, the light 250 is coming from a laser and the illumination of the sample is produced by total internal reflection. It is thus possible to obtain an improved TIRF type device.

It should be noted that the rear focal plane of commercial lenses is usually located inside the lens and is therefore difficult to access. It is therefore often recommended to realise a system enabling this rear focal plane to be imaged so as to be able to insert the filter system 170 between the sensor and the lens.

According to one embodiment, an inverted fluorescence microscope of Ti type from Nikon is used, comprising a module (ref. TI-T-BHP, MEB55810) that enables the rear focal plane to be imaged and an annular mask to be positioned so as to enable the (external) phase contrast with large numerical aperture lenses. It is possible to put a diaphragm 170 of the device according to the invention into this type of module. The system for centring and adjusting the position of the plane are perfectly suitable for a diaphragm filtering supercritical angles. The system comprises multiple positions for different lenses.

Fig. 4 shows a schematic view of a diaphragm 170 to be arranged in the rear focal plane 400 of the immersion lens 110 or in a conjugate plane 420 of the said focal plane. The diaphragm 170 comprises a peripheral area 176 able to block out the light. This area 176 is either effectively mobile (as in a camera), or the diaphragm is replaced by another, for example by turning a motor-driven rotary filter wheel.

The diaphragm 170 may be an iris diaphragm, such as that marketed by Thorlabs. Its aperture is adjusted by the movement of mechanical moving parts (not shown).

Another possibility is to use a wheel with openings or semi-transparent materials distributed on sectors of the wheel and rotate it. In this case the diaphragm can for example be realised by a circular hole of a suitable diameter in an opaque material. This

enables very short transmission/shutter cycles to be obtained, which are able for example to keep up with the acquisition pace of images with a camera.

According to one example of an embodiment, a Nikon “Ti-U”® type inverted fluorescence microscope is used with a binocular tube base of phase “TI-T-BPH”®, an oil-immersion x100 lens with a numerical aperture of 1.49. A fluorescence filter cube containing a transmission filter, a dichroic plate, and an excitation filter, is used. The light source used is a fibre source of commercial reference “Nikon Itensilight”® with a 130 Watt Hg lamp and a “C-HGFI”® generator. The camera used is an EMCCD Andor Ixon+ camera, cooled to approximately -75°. The diaphragm 170 used is the iris diaphragm produced by Thorlabs.

The diaphragm is then positioned in the Nikon MEB55810 module (“TI-T-BPH”) instead of the phase ring. The position of the diaphragm is adjusted by means of the Bertrand lens of the microscope and by displacement of the module by means of screws for centring and axial positioning. The procedure followed is identical to that for the adjustment of the phase ring supplied by the manufacturer with the module.

Observations have been conducted on embryonic human kidney cells marked with Choleratoxin (which binds to glycolipids on the membrane and the constituents of lipid rafts) coupled to Alexa 488 and excited by the Nikon Intensilight 130 Watt Hg lamp (conventional lamp). The filter cube used consists of an excitation filter with a bandwidth from 450 to 490 nm, a 500 nm dichroic mirror, and an emission filter with a bandwidth from 510 to 550 nm.

Figs. 6a and 6b show two images obtained with an open diaphragm (6a) and with a closed diaphragm to block out all the supercritical luminous components (6b). The pause time (T = 300 ms) and the gain (G = 0) are identical for the images 6a and 6b. The image 6c is obtained as the absolute value of the difference between the images 6a and 6b (that is to say, between the intensity of the signals associated with these images).

It is found that the two images 6a and 6b appear to be identical. On the other hand the image 6c is well contrasted. Variations in intensity are advantageously observed that are associated with membrane phenomena that are difficult to distinguish in the other two images, because they are embedded among other information coming from the interior of the cell.

It should be noted that these observations were advantageously made with a “conventional” lamp, and that it was not necessary to implement a laser to obtain them.

Measurements of the lateral resolution were carried out with Fluosphere® fluorescent beads (marketed by Invitrogen), with excitation/emission: 580/605 nm, deposited by spin-coating on a standard glass slide (thickness 0.13 -0.16 mm), and then immersed in distilled water.

Fig. 7 illustrates the fluorescence intensity profile of these beads (normalized signal intensity on the ordinate as a function of lateral displacement on the abscissa, expressed in microns). It is found that the C2 profile, which corresponds to the useful image zone obtained by the method according to the invention, is narrower than the C1 profile, which corresponds to the image obtained by the method described in document FR-A- 2943428. The corresponding improvement in resolution is 20-25%.

The invention is not limited to these types of embodiments and should be interpreted in a non-limitative way, and understood to encompass all equivalent embodiments.

Patentkrav

1. Observationsapparat (100) til at observere en lysemission (14, 15) fra en prøve (10) i et medium (11) med brydningsindeks n_L, hvor prøven er anbragt mod en overflade (20a) af en transparent bærer (20) med brydningsindeks n_s, som er større end n_L, idet lysemissionen omfatter lyskomponenter, som hver især har en amplitude og en fase, er orienterede mod bæreren og danner en vinkel θ med en retning (20b) vinkelret på overfladen (20a), blandt hvilke på den ene side superkritiske lyskomponenter, for hvilke vinklen θ netop er større end en kritisk vinkel $\theta_c = \arcsin(n_L/n_s)$, og på den anden side kritiske eller subkritiske lyskomponenter, for hvilke vinklen θ er mindre end eller lig med den kritiske vinkel θ_c, idet observationsindretningen (100) er i stand til:

- at fange mindst en del af lysemissionen fra et interesseområde af prøven og opnå et fanget lyssignal omfattende lyskomponenter, der stammer fra de superkritiske lyskomponenter af lysemissionen;
- at anvende filtre (170) på det fangede lyssignal for selektivt at mindske amplituden og/eller at ændre fasen af nogle af lyskomponenterne af det fangede lyssignal for at opnå et filtreret lyssignal; og
- at omdanne det filtrerede lyssignal til et billedområde af interesseområdet af prøven;

idet opservationsapparatet (100) er **kendetegnet ved, at**:

- det tillader at udføre en modulation af det filtrerede lyssignal, idet det tillades lyskomponenter, der stammer fra de kritiske eller subkritiske lyskomponenter af lysemissionen at passere igennem for at opnå billedområder (6a, 6b) af et og samme interesseområde af prøven, hvor modulationen anvendes på alle eller nogle af lyskomponenterne af det fangede lyssignal, der stammer fra de superkritiske lyskomponenter af lysemissionen; og
- det tillader at frembringe mindst et brugbart billedområde (6c) af prøven ved at kombinere billedområderne (6a, 6b), hvor kombinationen fremhæver forskelle mellem de med modulationen associerede billedområder (6a, 6b).

2. Observationsapparat (100) ifølge krav 1, **kendetegnet ved, at** lyskomponenterne af det fangede lyssignal, der er genstand for modulationen,

stammer fra de superkritiske lyskomponenter af lysemissionen.

3. Observationsapparat (100) ifølge et hvilket som helst af kravene 1 eller 2, **kendetegnet ved, at** det tillader successivt at anvende filtre (170) på det fangede lyssignal for successivt at opnå billedområder (6a, 6b), som i kombination giver det brugbare billedområde (6c) af prøven.

4. Observationsapparat (100) ifølge et hvilket som helst af kravene 1 til 3, **kendetegnet ved, at** det omfatter en flerhed af filtre (170), hvor flerheden af filtre er således at:

- i det filtrerede lyssignal tillader et filter blandt flerheden af filtre passage af lyskomponenter, der stammer fra de superkritiske lyskomponenter af lysemissionen;
- alle filtrene blandt flerheden af filtre tillader lyskomponenterne af det fangede lyssignal, der stammer fra de kritiske eller subkritiske lyskomponenter af lysemissionen, at passere igennem, og virker indbyrdes i alt væsentligt på en samme måde på lyskomponenterne af det fangede lyssignal, der stammer fra de kritiske eller subkritiske lyskomponenter af lysemissionen; og
- der er mindst to filtre af flerheden, der virker indbyrdes i alt væsentligt på forskellige måder på amplituden eller fasen af mindst nogle af lyskomponenterne af det fangede lyssignal, der stammer fra de superkritiske lyskomponenter af lysemissionen; og

observationsapparatet (100) tillader at tage en flerhed af billedområder af et og samme interesseområde af prøven ved hjælp af flerheden af filtre, idet hvert filter tjener til at tage et billedområde blandt flerheden af billedområder og tillader at frembringe et brugbart billedområde af prøven ved en beregning, der kombinerer flerheden af billedområder for at påvise forskelle mellem billedområderne blandt flerheden af billedområder af prøven.

5. Observationsapparat (100) ifølge et hvilket som helst af kravene 1 til 4, **kendetegnet ved, at** det omfatter to filtre, hvor de to filtre er således at:

- i det filtrerede lyssignal tillader det ene af de to filtre passage af de lyskomponenter, der stammer fra de superkritiske lyskomponenter af lysemissionen; og

- det andet filter virker i alt væsentligt på samme måde som nævnte ene af de to filtre på lyskomponenterne af det fangede lyssignal, der stammer fra de kritiske eller subkritiske lyskomponenter af lysemissionen, og det mindsker i alt væsentligt stærkere end det nævnte ene af de to filtre amplituden af mindst nogle af lyskomponenterne af det fangede lyssignal, der stammer fra de superkritiske lyskomponenter af lysemissionen; og

observationsapparatet (100) tillader at tage mindst to billedområder af et og samme interesseområde af prøven ved hjælp af to filtre, idet hvert filter tjener til at tage et af de to billedområder og tillader at frembringe et brugbart billedområde af prøven ved en beregning, idet beregningen omfatter en algebraisk differens mellem begge billedområder af prøven.

6. Observationsapparat (100) ifølge et hvilket som helst af kravene 1 til 5, **kendetegnet ved, at** de filtrene også delvist reducerer amplituden af alle eller nogle af lyskomponenterne af det fangede lyssignal, der stammer fra de kritiske og subkritiske lyskomponenter af lysemissionen.

7. Observationsapparat (100) ifølge et hvilket som helst af kravene 1 til 6, **kendetegnet ved, at** lyskomponenter af det fangede lyssignal, der stammer fra lyskomponenter af lysemissionen, som danner den samme vinkel θ, behandles i alt væsentligt på en samme måde med et og samme filter til at mindske amplituden eller ændre fasen.

8. Observationsapparat (100) ifølge et hvilket som helst af kravene 1 til 7, **kendetegnet ved, at** det omfatter:

- et neutralt filter, der i det filtrerede lyssignal tillader alle lyskomponenterne af det fangede lyssignal, der stammer fra de superkritiske lyskomponenter af lysemissionen, at passere uden nogen mindskelse af amplitude for at tilvejebringe et af billedområderne af prøven; og
- et totalt filter, der i det filtrerede lyssignal totalt ophæver alle de lyskomponenter, der stammer fra de superkritiske lyskomponenter af lysemissionen, for at tilvejebringe et andet billedområde af prøven.

9. Observationsapparat (100) ifølge et hvilket som helst af kravene 1 til 8, **kendetegnet ved, at** det omfatter et fuldfelt-immersionsobjektiv (110), og filtrene (170) er lokaliseret i et bageste fokusplan (400) af immersionsobjektivet (110) og/eller i et med det bageste fokusplan konjugeret plan (420).

10. Observationsapparat (100) ifølge krav 9, **kendetegnet ved, at** filtrene (170) omfatter en blænde (176), der i en åben position tillader passage af lyskomponenter af det fangede lyssignal, der stammer fra de superkritiske lyskomponenter af lysemissionen, og afhængigt af blænderens (176) lukningsgrad tillader at skjule lyskomponenterne af det fangede lyssignal, der stammer fra de superkritiske lyskomponenter af lysemissionen med en vinkel θ større end en grænseværdi relateret til lukningsgraden.

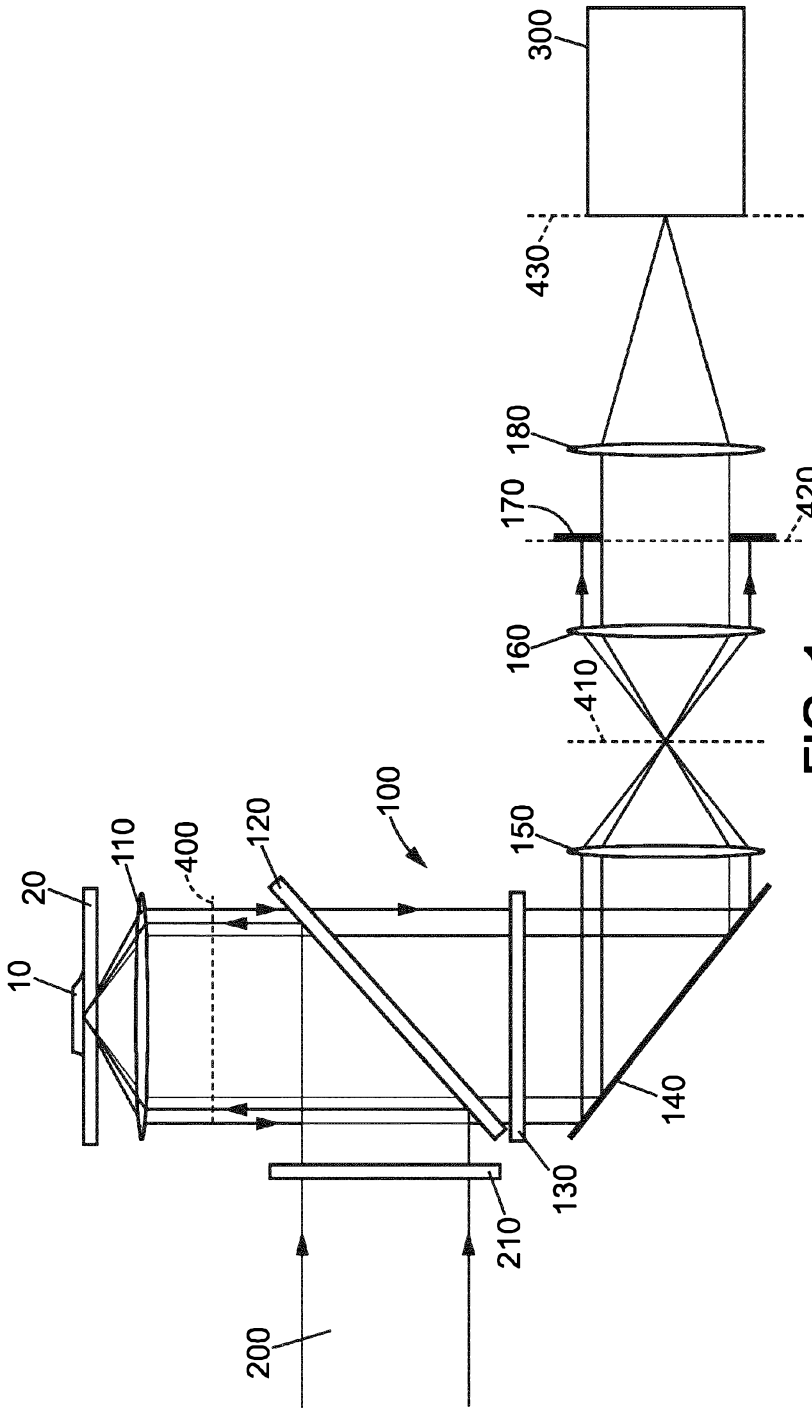

300
430
180
170
420
160
410
150
100
120
400
110
20
10
140
130
210
200

FIG. 1

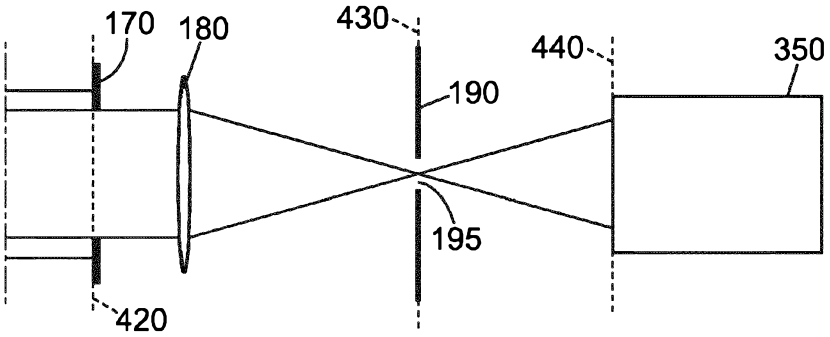


FIG. 2

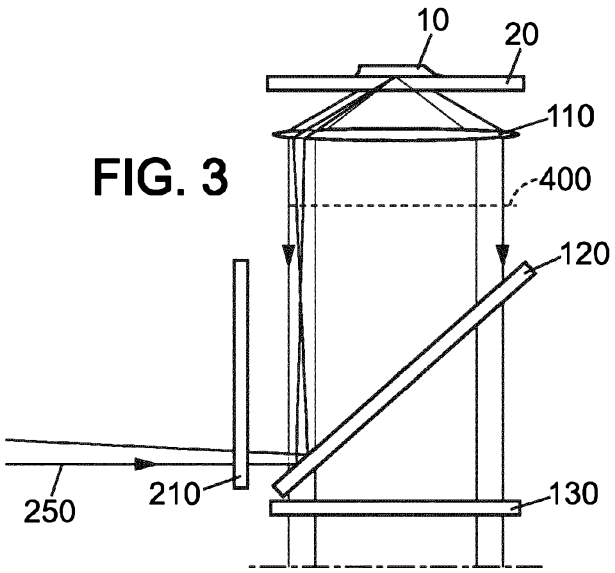


FIG. 3

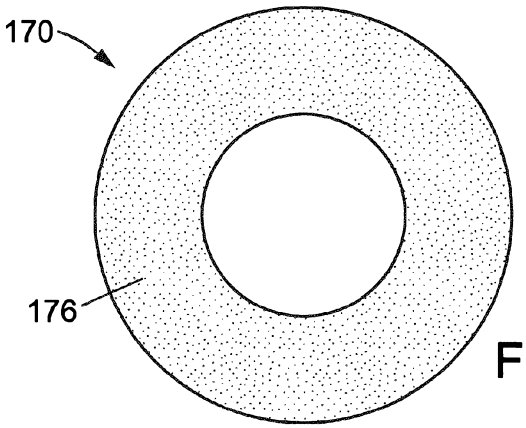


FIG. 4

FIG. 5a

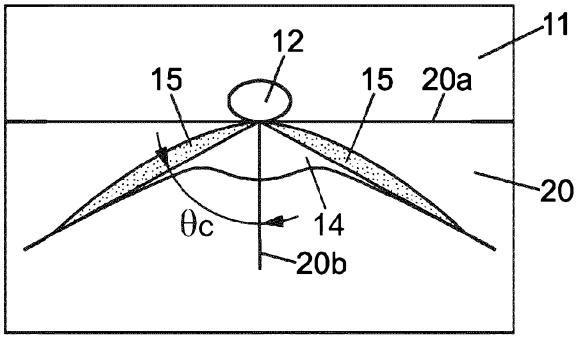
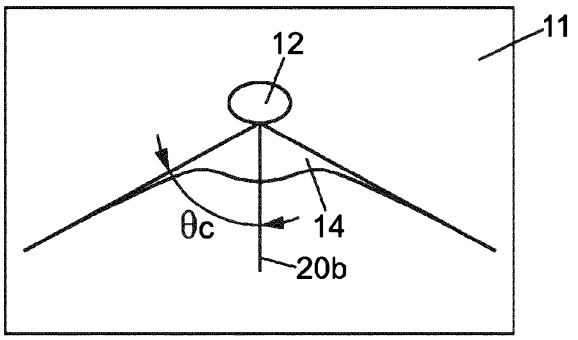


FIG. 5b



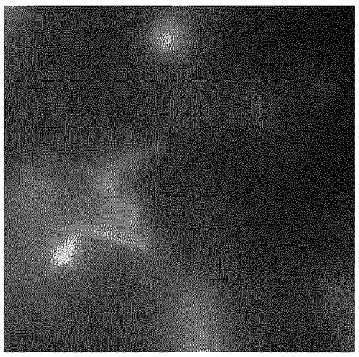


FIG. 6a

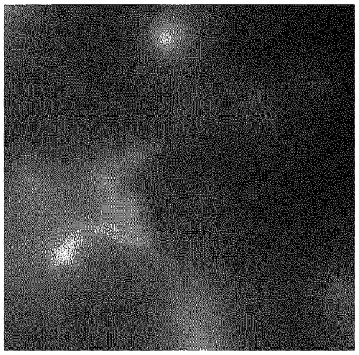


FIG. 6b

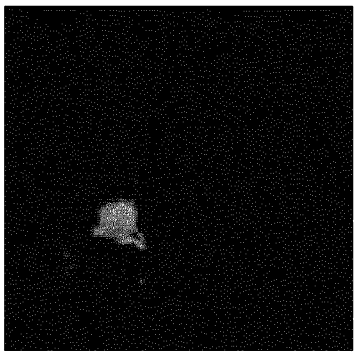


FIG. 6c

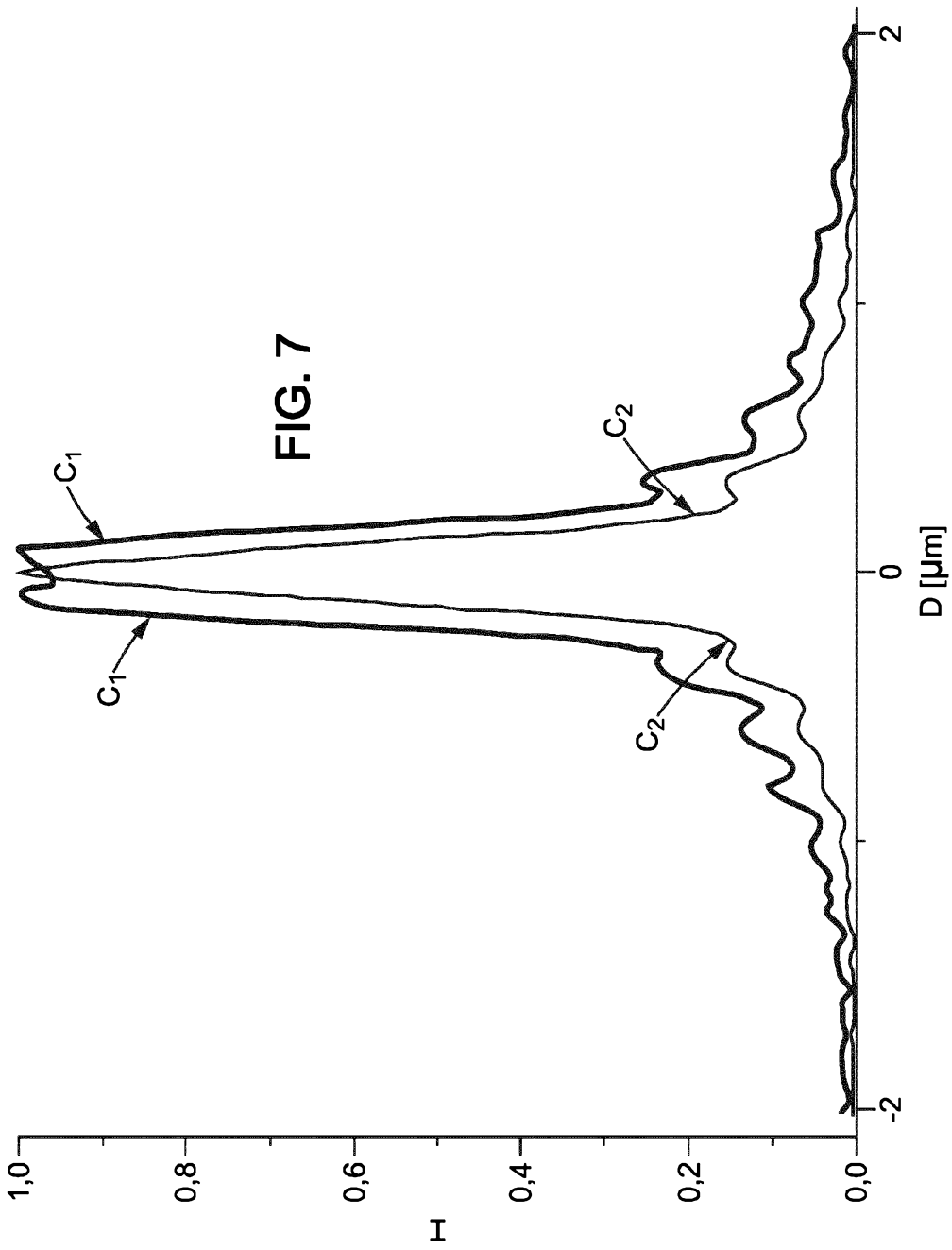


FIG. 7