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(54) Title: MICROSCOPE WITH AUTOFOCUS SYSTEM

(57) **Abstract:** A microscope apparatus (2) for imaging a sample (5) positioned on a substrate (3) of a sample holder (1), the microscope comprising a sample stage (16) on which the sample holder is mounted, a light collecting device (38), an imaging sensor (12), and an autofocus system (8) that serves to focus an image of the sample on the imaging sensor. The autofocus system (8) is positioned below the sample stage and comprises a light beam emitter (22) emitting an autofocus light beam (13), and a beam steering system (24) configured to direct and reflect the autofocus light beam (13) off a bottom side of the substrate (3) of the sample holder positioned on the sample stage (16) and onto a light sensor (40) comprised in the autofocus system or onto the imaging sensor (12), the beam steering system configured to adjust a translational position and an angle of the autofocus light beam output by the beam steering system.

FIG 1a

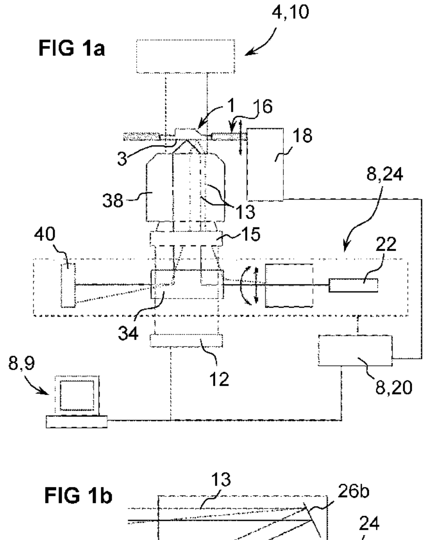
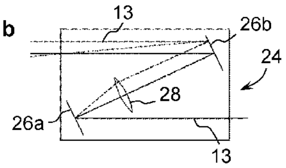


FIG 1b



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MICROSCOPE WITH AUTOFOCUS SYSTEM

The present invention relates to a microscope with an automated focus system.

It is known to provide microscopes with an autofocus system that adjusts the height of a sample under observation relative to a microscope objective collecting light from the sample under observation. It is known to utilize a separate beam for the autofocus system that is reflected off a substrate, in particular the substrate on which the sample under observation is positioned, and to adjust the height of the sample stage on which the sample substrate is mounted to bring the substrate surface into focus.

Ensuring a reliable and stable focus position in microscope imaging systems is a challenging task especially with the development of high resolution systems in which the precise positioning of the sample under observation is a key element for high quality measurements. Moreover, certain microscope imaging systems are used for imaging dynamic situations that may require dynamic autofocus. For instance, for the imaging of live cells under observation under a rotating sample illumination beam, a dynamic adjustment of the autofocus may be required.

Existing autofocus methods often suffer from a lack of flexibility in their ability to modulate dynamic range and sensitivity for different applications. While it is known to extend the usability range by employing methods with coarse and fine adjustments, this renders the adjustment mechanism complex and difficult to process rapidly. Also, known methods do not accommodate well for inaccuracies and tolerances in the angular positioning of the substrate which may also vary dynamically, for instance if the substrate on which the sample under observation is mounted on a rotating sample stage, or if the substrate profile is not perfect.

In view of the foregoing, it is an object of the invention to provide a microscope with an autofocus system, that provides very accurate and rapid autofocusing for imaging samples positioned on a substrate.

It is advantageous to provide a microscope with an autofocus system that allows rapid dynamic automated autofocusing, for instance in applications requiring the displacement of the substrate on which the sample under observation is mounted, for example due to rotation or translation of a sample stage on which the substrate is mounted.

It is advantageous to provide a microscope with an autofocus system that is reliable and compact.

It is advantageous to provide an autofocus system that allows integration in microscopes of various configurations, for instance microscopes for optical diffraction tomography, and microscopes including epifluorescence illumination and imaging modules.

Objects of the invention have been achieved by providing microscope with an autofocus system according to claim 1, and a method according to claim 14.

Dependent claims set out various advantageous features of embodiments of the invention.

Disclosed herein is a microscope apparatus for imaging a sample positioned on a substrate of a sample holder, the microscope comprising a sample stage on which the sample holder is mounted, a light collecting device, an imaging sensor, and an autofocus system that serves to focus an image of the sample on the imaging sensor.

The autofocus system is positioned below the sample stage and comprises a light beam emitter emitting an autofocus light beam, and a beam steering system configured to direct and reflect the autofocus light beam off a bottom side of the substrate of the sample holder positioned on the sample stage and onto a light sensor comprised in the autofocus system or onto the imaging sensor, the beam steering system configured to adjust a translational position and an angle of the autofocus light beam output by the beam steering system.

In an advantageous embodiment, the microscope apparatus is an optical diffraction microscope comprising an illumination and imaging system comprising an illumination light source, generating a collimated light beam which is split into a sample beam that is transmitted through the sample, and a reference beam that follows a reference path and is re-combined with the sample beam before impinging upon the imaging sensor, the light collecting device being positioned below the sample stage.

In an advantageous embodiment, the microscope apparatus comprises a positioning mechanism coupled to the sample stage or to the light collection device, configured to adjust a height of the sample stage relative to the light collection device, and thereby the height of the base of the sample holder relative to the light collection device, based on an input from the autofocus system.

In an advantageous embodiment, the microscope apparatus of any preceding embodiment is in combination with a computing system forming part of the microscope apparatus, or connected to the microscope apparatus, wherein the autofocus system and imaging sensor are connected to the computing system comprising a module configured to control the autofocus system statically or dynamically.

In an advantageous embodiment, the beam steering system comprises a light beam emitter, and electrically actuated pivotable mirrors configured to pivot about at least one axis, including at least a first electrically actuated pivotable mirror and a second electrically actuated pivotable mirror.

In an advantageous embodiment, the beam steering system further comprise one or more lenses positioned between the first and second pivotable mirrors configured to adjust a translational position of the autofocus light beam.

In an advantageous embodiment, the electrically actuated pivotable mirrors are connected to the computing system that controls the angle of the pivotable mirrors for adjusting the direction and position of the autofocus light beam.

In an advantageous embodiment, the electrically actuated pivotable mirrors are DMDs (digital mirror devices) or MEMS (micro electromechanical system) mirrors.

In an advantageous embodiment, at least one of the electrically actuated pivotable mirrors are configured to pivot about two orthogonal axes.

In an advantageous embodiment, the beam steering system further comprises an intermediate reflector configured to allow the beam from the first pivotable mirror to pass through to the second pivotable mirror, but to reflect the beam returning from the second pivotable mirror towards the light collecting device.

In an advantageous embodiment, the light collecting device is configured to function both as a microscope objective for a sample illumination beam generated by the microscope, and as an objective for the autofocus light beam.

In an advantageous embodiment, the the beam steering system is configured to direct and reflect the autofocus light beam off a bottom side of the substrate of the sample holder positioned on the sample stage and onto the light sensor comprised in the autofocus system, the light sensor being a component separate from the imaging sensor, the imaging sensor configured to receive light from the sample under observation.

In an advantageous embodiment, the the beam steering system is configured to direct and reflect the autofocus light beam off a bottom side of the substrate of the sample holder positioned on the sample stage and onto the imaging sensor, the imaging sensor further configured to receive light from the sample under observation.

In an advantageous embodiment, the autofocus system further comprises a light sensor (40) configured to receive the autofocus light beam reflected or emitted from the sample, the light sensor connected to a computing system (9) configured to analyse and compute the captured focus beam and to control an actuator adjusting a position of the sample relative to an imaging sensor to bring the system into focus.

Also disclosed herein is a method of controlling a microscope apparatus of any preceding embodiment comprising a procedure to find a focused position of the sample under observation, comprising:

- performing a scanning procedure controlled by a computing system including displacing the sample stage relative to the light collecting device in a Z direction transverse to the substrate bottom surface over a first pre-defined distance, and obtaining in the computing system from the light sensor or the imaging sensor a set of measurements of positions of a spot of the autofocus beam impinging upon the light sensor or the imaging sensor;
- calculating in the computing system an estimation of a Z direction position for said focused position of the sample;
- iteratively repeating, at least once, a step of performing a detection procedure from a second estimated ideal Z direction position until a difference between a previous and a subsequent estimated ideal Z direction position is below a chosen measurement threshold corresponding to an acceptable focused position.

In an advantageous embodiment, the method comprises a procedure controlled by the computing system to change a sensitivity of the autofocus system, the sensitivity corresponding to the ratio of the Z direction displacement relative to the displacement of the spot of the autofocus beam impinging upon the light sensor or the imaging sensor, the procedure comprising changing an inclination angle of the autofocus light beam output by the beam steering device.

In an advantageous embodiment, the method comprises a procedure controlled by the computing system to change the position of the spot of the autofocus system, impinging upon the substrate, which directly determines the position of the spot of the autofocus beam impinging upon the light sensor or the imaging sensor, the procedure comprising changing the position of the autofocus light beam output by the beam steering device.

Further advantageous features of the invention will be apparent from the following detailed description of embodiments of the invention and the accompanying illustrations.

Brief description of the figures

Figure 1a is a simplified schematic representation of a microscope with an autofocus system according to an embodiment of the invention;

Figure 1b is a simplified schematic representation of elements of a beam steering system of the autofocus system of figure 1a;

Figure 2 is a simplified schematic representation of a microscope with an autofocus system according to another embodiment of the invention;

Figure 3 is a view similar to figure 1a of a variant;

Figure 4 is a simplified schematic representation of a microscope with an autofocus system according to yet another embodiment of the invention;

Figure 5a is a cross-sectional view of a microscope with an autofocus system according to an embodiment of the invention based on the schematic system of figure 4;

Figure 5b is an enlarged view of a portion of the microscope of figure 5a showing the autofocus system;

Figure 5c is a cross-sectional view through lines 5c-5c of figure 5b;

Figure 6 is a schematic representation of an autofocus system according to embodiments of the invention showing the effects of a change in sensitivity and orientation;

Figure 7 is a schematic simplified cross-sectional through a substrate with sample under observation positioned thereon;

Figure 8 shows representations illustrating the effect of a vertical displacement of the sample on the position of the focusing beam;

Figure 9 are schematic illustrations showing the effect of a tilt angle of a sample substrate on the position of the focusing beam of the autofocus system.

Referring to the figures, starting with figure 4 and 5a, a microscope apparatus 2 for imaging a sample 5 positioned in or on a sample holder is illustrated. The sample holder may be in the form of a container, for instance a single container or a container of well plate, or the sample holder may for instance be in the form of a coverslip, or any other known holder for supporting a sample to be observed with the microscope.

The sample holder comprises a base 3 and typically the sample 5 will be fixed onto or lying on and against a top surface of the base. The base or substrate 3 is in most applications made of a light transparent material, however this may depend on the type of microscope and sample under observation, whereby for transmission microscopy the base must be transparent in order to allow the sample illumination beam to pass through the sample under observation and the substrate, for collection by a wave collection device (for instance a lens) positioned below the sample.

In applications where the sample to be observed is observed from a top side, for instance by light reflection or diffusion off the sample to be observed, the substrate could optionally be made of an opaque, light absorbing or light reflective material.

In the embodiments illustrated in figures 4 and 5a, the microscope apparatus 2 is an optical diffraction microscope, for instance for optical diffraction tomography, for instance as described in WO 2016046714. In this microscope embodiment, the microscope apparatus comprises an illumination and imaging system 4 comprising an illumination light source 10, generating a collimated light beam which is split into a sample beam 10a that is transmitted through the sample 5, and a reference beam 10b that follows a reference path and is combined with the sample beam. An intensity and phase of the resultant beam impinging upon an imaging sensor 12 may be measured to provide information on the refractive index distribution of the sample under observation, which allows 3D reconstruction of cells and cellular matter, as per se well known.

The microscope apparatus comprises a sample holder device 6 having a sample stage 16 on which the sample holder or container is mounted, and a positioning mechanism 18 coupled to the sample stage 16, comprising a driver 20 configured to adjust at least the height of the sample stage 16 and thereby the height of the base 3 of the sample container or holder 1.

The microscope apparatus 2 comprises a light collection device, for instance comprising an optical lens, for collecting the sample beam 10a that is transmitted through the sample 5, and directing the sample beam to the imaging sensor 12, whereby in the illustrated embodiment the sample beam 10a is combined with the reference beam 10b prior to impinging upon the imaging sensor 12.

The microscope apparatus comprises an autofocus system 8 that serves to focus the sample beam in the microscope objective (wave collection device) 38 and concomitantly to focus the sample beam on the imaging sensor 12.

In this example, the sample stage 16 is displaced relative to the wave collection device 38 in order to focus the sample beam on the imaging sensor 12, whereby either the sample stage may be displaced and the wave collection device remain static, or the wave collection device displaced and the sample stage remain static, or both the sample stage and both collection device may be moved. Positioning and adjusting mechanisms for moving sample stages relative to microscope objectives are *per se* known and need not be further described herein.

The autofocus system 8 is positioned below the sample stage 16 and comprises a light beam emitter 22 and a beam steering system 24, configured to direct and reflect an autofocus light beam off the substrate 3 of the sample container or holder positioned on the sample stage 16. The reflected autofocus light beam is captured by a light sensor, that may either be a light sensor 40 comprised in the autofocus system 8, independent from the microscope apparatus imaging sensor, or the light sensor may consist in the imaging sensor 12 that functions both for capturing the beam from the illumination light source transmitted through the sample and for capturing the autofocus light beam transmitted by the light beam emitter 22 of the autofocus system 8. This depends on the embodiment as will be described in more detail hereinafter.

The autofocus system 8 may further comprise a light beam splitting system 34, for instance comprising a beam splitter 36 and a light collecting device 38 such as a lens.

The light collecting device 38 may be the microscope objective, such that the microscope objective serves both as a wave collection device for the autofocus light beam and for the microscope sample illumination beam. However, in certain embodiments the autofocus system may be independent from the microscope apparatus illumination and imaging system 4, and thus comprises a dedicated light collecting device 38 that is independent from the microscope apparatus illumination and imaging system 4. This may be the situation for instance in embodiments where the microscope is configured to image samples on a substrate with imaging sensors positioned above the substrate in non-transmission microscopy, the autofocus system however being positioned below the sample stage and configured to measure and adjust the position of the substrate 3 from below.

The microscope apparatus further comprises a computing system 9 or is configured to be connected to a computing system 9, that may have modules to perform various functions such as for control of the illumination and imaging system 4 of the microscope apparatus, as well as for control of the autofocus system 8. In particular, computing system comprises a module to control the driver 20 moving the positioning mechanism 18 to adjust the focus statically or dynamically.

The computing system may form part of the microscope apparatus, or may be an external computing system connected to the microscope apparatus, or may have a computing portion integrated in the microscope apparatus and a computing portion being external and connected to the microscope apparatus.

In an embodiment of the autofocus system, as best illustrated in figures 5a to 5c, the beam steering system 24 comprises a light beam emitter 22, and electrically actuated pivotable mirrors 26a, 26b, including at least a first electrically actuated pivotable mirror 26a and a second electrically actuated pivotable mirror 26b. The beam steering system may further comprise an intermediate reflector 36 configured to allow the beam from the first pivotable mirror 26a to pass through to the second pivotable mirror 26b, but to reflect the beam returning from the second pivotable mirror 26b towards the light collecting device 38. The electrically actuated pivotable mirrors may be configured to pivot about one axis, or about two orthogonal axes. Single axis pivoting enables the autofocus beam 13 to be steered (change direction) in a plane, whereas two axis pivoting enables the autofocus beam 13 to be steered within a conical space / volume.

The light beam emitter 22 emits a collimated light beam on the first pivotable mirror 26a that reflects the emitted light beam towards the second pivotable mirror 26b, that further reflects the autofocus light beam towards the substrate 3, optionally via a beam reflector 36 and possibly further reflectors for directing the autofocus light beam towards the sample substrate.

The electrically actuated pivotable mirrors are coupled to the computing system that controls the angle of the pivotable mirrors for adjusting the direction and position of the autofocus light beam.

As best seen in figure 1b, the autofocus system may further comprise one or more lenses 28 positioned between the first and second pivotable mirrors 26a, 26b configured to adjust a translational position of the autofocus light beam.

The beam steering system 24 according to an aspect of the invention is configured to adjust both an angle of the autofocus light beam as well as a translational position of the autofocus light beam, such that the autofocus light beam impinging upon the substrate of the sample holder positioned on the sample stage 16 can be adjusted both in the angle of incidence on the substrate as well as the translational position on the substrate. As will be described in more detail, this advantageously allows to adjust the sensitivity of the autofocus system as well as to measure the tilt angle of the substrate relative to a reference position, for instance the plane of the sample stage 16.

The adjustment of the translational position of the autofocus light beam is configured to translate the beam incident on the sample stage without a change of the angle of incidence of the beam impinging upon the sample support, whereas the adjustment of the beam angle is configured to change the angle of incidence of the beam impinging upon the sample support.

Going now through the embodiments illustrated in the figures, Fig. 1 presents a schematic view of the autofocus system 8 integrated in a microscope apparatus 2. An illumination light source 10 transmits light through a sample 5 which is positioned on a sample holder 1 mounted on a sample stage 16 coupled to a positioning mechanism 18 that controls the position of the sample holder. The light scattered by the sample 5 is collected by a light collecting device 38 and focused and imaged by a focusing device 15 onto an imaging sensor 12, which transfers the information to a computing system 9 that processes the information and enables it to be displayed to an operator. The focusing device 15 may comprise for instance optical lenses, curved mirrors, or diffracting elements (such as for instance Fresnel lenses or gratings).

In parallel to this operation, the autofocus system 8 employs a light beam emitter 22 to deliver a collimated autofocus beam 13 to the beam steering system 24 which is configured to control and adjust the position and angle of propagation of the output autofocus beam 13. The autofocus beam passes through a light beam splitting system 34, and then through the light collecting device 38 that magnifies the incoming angle of the beam, which reflects on the sample substrate 3 to be sent back to the light collecting device 38, so that the light beam splitting system 34 separates the light used to image the sample and the light from the light beam emitter 22 which is sent to a separate light sensor 40. The autofocus system 8 is connected to a driver 20, itself connected to the computing system 9 to control and synchronize the beam steering system 24, the light beam splitting system 34, the sample stage positioning mechanism 18 and the light sensor 40.

The autofocus system relies on the autofocus beam impinging on the sample substrate 3 with an angle large enough so that a vertical (z axis) displacement, which is performed during a dedicated scanning procedure with the sample stage 16 and positioning mechanism 18, translates into a lateral autofocus beam 13 displacement that can be sensed by detecting the autofocus beam 13 position on the light sensor 40. This is illustrated in Fig. 6 c), where the displacement in the vertical (z) direction moves the autofocus beam 13 on the light sensor 40. The focus position can then be deduced from the current position of the autofocus beam 13 through calculations in the computing system 9, such as by finding the center position to then allow the movement into a focussed position.

Conventional autofocus systems, based only on a fixed position autofocus beam from the beam emitter and emitted from a top side of the sample substrate, suffers from a lack of flexibility, in that one can detect focus only within a fixed range with a pre-defined sensitivity. Furthermore, such a fixed beam system would be sample-dependent, as it is implemented by relying on the reflection on highly reflective samples in upright microscopes.

The improvements emanating from embodiments of the present invention render the autofocus system independent from the type of sample as it relies on a reflection from the bottom surface of the sample substrate 3, ideally made of a flat surface such as glass, although this is not a requirement of the invention, as explained below.

Embodiments of the invention implement a multi-sensitivity system, where the beam steering system 24, in conjunction with the light beam splitting system 34 and the light collecting device 38, can change the angle and position of the impinging autofocus beam

13 on the substrate 3 of the sample holder 1 in a rapid manner with automated control of the driver 20 of the positioning mechanism 18 by the computing system 9.

As illustrated in Fig. 6 b), the beam steering system 24 is configured and can be operated to increase or decrease the sensitivity: a control of the output autofocus beam 13 to decrease the impinging angle on the substrate 3, reduces the autofocus beam displacement on the light sensor 40, which reduces sensitivity and consequently increases the detection range for a constant sensor size; and a control of the output autofocus beam 13 to increase the impinging angle on the substrate 3, has the opposite effect of increasing sensitivity within a shorter scanning range.

As illustrated in Fig. 6 a), this autofocus system also provides full flexibility in terms of the orientation of the lateral displacement. Similarly, the beam steering system 24 can adjust the autofocus beam 13 position, in order to calibrate the center position, or, as illustrated in Fig. 6 c), to compensate for an imperfect alignment and perform beam centering with complete flexibility for various sample conditions.

The light beam splitting system 34 used to separate the light from the illumination light source 10 and the light from the light beam emitter 22 can be a passive device, such as for example a beam splitter, a polarized beam splitter or a dichroic mirror, allowing simultaneous detection on both the autofocus system light sensor 40 and the imaging sensor 12 (possibly at the cost of some light efficiency), or an active device such as a flipping mirror, which can enhance light efficiency (at the cost of a sequential detection).

In a specific embodiment, the functions of imaging the sample and of detecting the autofocus beam position can be fulfilled with one sensing component, as illustrated in Fig. 2, where the imaging sensor 12 can detect both. In this embodiment case, the light beam splitting system 34 is employed solely to combine the autofocus beam into the system.

One advantageous embodiment of the described beam steering system 24 comprises employing digitally controlled mirrors, such as DMDs (digital mirror devices) or MEMS (micro electromechanical system) mirrors.

One example is illustrated in Fig. 1b, where two electrically actuated pivotable mirrors 26a, 26b are located at conjugated planes, in order to independently control the position and angle of the autofocus beam 13. The first pivotable mirror 26a, conjugated by a lens 28, can control the output position of the autofocus beam, while the second pivotable mirror

26b can control the output angle. The control of the pivotable mirrors and thus of the output of the beam steering system 24 is fully flexible, and can be used to adjust position of the autofocus beam in the imaging system. While the principle is shown only in one dimension in Fig. 1b, it can of course be applied in multiple dimensions.

Based on the implementation described above, the detection of the focus can be performed directly through the relation between the height of the sample substrate 3 and the position of the autofocus beam 13 detected on the light sensor 40.

As illustrated in Fig. 8 a) and b), a height displacement Delta ***Z*** of the sample substrate 3 is translated into a lateral displacement Delta ***Xo*** in the object space. The light collecting device 38 then images this displacement onto the light sensor 40, giving a displacement Delta ***Xi*** of the image of the light spot. For example, in the case of a light collecting device 38 implemented as a microscope objective with 60x magnification and 0.8 NA (numerical aperture), it is possible to assume an effective NA of 0.75 for the autofocus beam 13, which implies that a displacement Delta ***Z*** of 1 mm would translate into a beam displacement of 136 mm on the light sensor 40. For a light sensor 40 having a resolution of 1024x1024 with a pixel pitch of 6 mm, this would imply a sensitivity of 22.67 px/mm and a search range of 45 mm, which could be then adjusted through the multi-sensitivity function of the beam steering system 24.

According to an embodiment, a method to find the focus would use a calibration of the system obtained by a limited set of measurements during a vertical (***Z*** direction) scan in a chosen range. The resulting scan provides a set of images, in which each image can be processed to detect the position of the beam on the light sensor 40. The position detection can be performed for example through thresholding and centroid detection, which leads to the detection of an ***X*** position for a given frame, as illustrated in Fig. 8 c). This procedure is then repeated for multiple frames during a ***Z*** (vertical) displacement of the sample stage 16, giving multiple light spot positions for each frame. These light spot positions can be used to determine the response of the system in terms of delta ***Xi*** displacement (horizontal displacement) for a given ***Z*** movement (vertical displacement), for example through a linear regression procedure, as illustrated in Fig. 8 d). During the autofocus procedure, the current ***Z*** (vertical) position can therefore be directly deduced from one detection of the light spot on the light sensor 40 based on the knowledge of the system response, allowing then to readily move the system into focus.

As it can be expected in the case of a limited scanning range far from the actual focus position as illustrated in Fig. 8 d), the extracted focus will be only an estimate of the ideal ***Z*** (vertical) position. An iterative procedure can therefore be employed, where the ***Z*** (vertical) scanning procedure – and subsequent focus position detection procedure, as described above – is performed iteratively, in order to reach a ***Z*** position closer to the ideal focus in an iterative way.

Autofocus systems based on the method described above can be sensitive to the tilt of the sample substrate 3, which can change the position of the autofocus beam 13 on the light sensor 40 position. As illustrated in Fig. 9, a tilt of the sample substrate 3 will directly add to the impinging angle $\boldsymbol{\alpha}$ of the autofocus beam, leading to an additional lateral displacement deltal ***X'***, which translates into an error in the estimation of the focus position. In most cases, the displacement is rather small, and may not require compensation. For example, In the case of a sample of around 100 mm of horizontal size with a 1 mm (1%) of height error in its alignment, this error would relate linearly into the error in displacement measurement. Furthermore, the tilt not only changes the absolute value of the estimated focus position, but also the slope of the relation between the height of the sample substrate 3 and the measured displacement on the light sensor 40.

The multi-sensitivity approach provided by embodiments of the present invention can therefore be used to compensate for the tilt of the sample substrate 3 and reach the exact focus position. By performing multiple scans (minimum 2) with different sensitivities that correspond to different grazing angles, as illustrated in Fig. 9, it is possible to estimate the tilt angle $\boldsymbol{\gamma}$ based on the change in slope of the scan curves. The exact focus position can then be deduced by subtracting the known additional displacement delta ***X'*** induced by the tilt.

In addition to global tilts generated for instance by an imperfect horizontality of the sample holder substrate, the tilt compensation procedure described above can also be used to compensate the non-perfect planarity of the sample substrate 3, which can appear as a local tilt that may induce a displacement of the spot position on the light sensor, as described above.

Several applications can require the temporal monitoring of a sample, which implies to continuously record images of a given location. It can often happen that the focus changes during such measurements, for instance due to mechanical or thermal drifts, or dynamic changes in the sample substrate. Such cases would therefore require the adjustment of the focus position during the course of the temporal acquisition, and sequential procedures can limit the speed of imaging.

In an advantageous embodiment of the invention, the light beam splitting system 34 is a passive element that allows simultaneous imaging on the imaging sensor 12 and the light sensor 40. In that case, it is possible to perform continuous focus tracking by monitoring small displacements of the autofocus beam 13 around the focus position, to compensate the *Z* position of the sample while continuously recording with the imaging sensor 12, without requiring a full scan to detect the focus position, as described above.

In other applications, it is advantageous to horizontally scan the sample to measure different locations of the sample, possibly in a continuous way. In that case, due to an imperfect horizontality of the sample substrate, it often happens that the focus position may be significantly different between various positions on the sample. An advantageous way to compensate for that is to perform focus tracking during the displacement to the new position. Similarly to the previous case, such tracking can avoid the need of a lengthy scan at the new sample position thanks to continuous tracking. Furthermore, it should be noted that this method is also applicable when the imaging sensor 12 is used for both the sample beam and the autofocus beam as illustrated in Fig. 2. As no image is taken during the sample stage displacement, it is indeed possible to employ the single sensor for focus tracking during this time by gradually compensating small displacements.

It should be noted that the autofocus system 8 is fully compatible with other imaging modalities, such as epi-illumination, as illustrated in Fig. 3. In that case, an epi-illumination device 42 combines the light emitted by a fluorescent light source 43 into the system, typically to excite the sample, as employed in fluorescence imaging, for example.

An advantageous method is to use infinity corrected light collecting devices 38 that allow for direct extension of the space between them and the focusing device 15, for instance implemented as a tube lens. However, the use of infinity-corrected elements is not necessarily required, as the autofocus system 8 can be fully adjusted to accommodate different incoming angles.

In several applications, the sample 5 under study cannot be installed directly on the sample holder 1, such as in the case of cells studied in vitro, for example, as illustrated in Fig. 7. In this specific application, the location detected by the autofocus system corresponds to the bottom surface of the substrate 3, on which the sample 5 is attached. The position that should be employed in such a case for imaging is different from the detected position. An offset distance delta *Zu* that relates the detected position to the desired image focus can therefore be defined, either through user input or with computational criteria, for example.

One particularly advantageous embodiment of the invention employs holotomography as an imaging modality, as illustrated in Figs. 4 and 5a. It is possible to take advantage of the particular optical configuration of holotomography that includes an optical recombining system for the sample and reference beams 12a, 12b. This system can be functionally used as the light beam splitting system 34, in an implementation conceptually similar to the one described in Fig. 2, where one imaging sensor 12 is used for both focus detection and imaging.

Literature reference

List of references

- **Sample holder / container 1**
  - Base 3
  - Container chamber
  - Sample 5

- **Microscope apparatus 2**
  - **Illumination and imaging system 4**
    - **Illumination Light source 10**
      - Sample beam 10a
      - Reference beam 10b
    - Beam splitter 14
    - **Imaging sensor 12**
  - Epifluorescence illumination device 42
    - Fluorescent light source 43
  - **Sample holder device 6**
    - Sample stage 16
    - Positioning mechanism 18
      - Driver 20

  - **Autofocus system 8**
    - Light Beam emitter (generator) 22
    - Beam steering system 24
      - Pivotable Mirrors 26a, 26b
        - 1st mirror
        - 2nd mirror
      - Lens 28
      - Mirrors support 30
        - Displacement mechanism 32
    - Focusing device 15
    - Light Beam splitting system 34
      - Beam splitter 36
    - Light collecting device (lens) 38
    - Light sensor 40

- **Computing system 9**

Claims

1. A microscope apparatus (2) for imaging a sample (5) positioned on a substrate (3) of a sample holder (1), the microscope comprising a sample stage (16) on which the sample holder is mounted, a light collecting device (38), an imaging sensor (12), and an autofocus system (8) that serves to focus an image of the sample on the imaging sensor, **characterized in that** the autofocus system (8) is positioned below the sample stage and comprises a light beam emitter (22) emitting an autofocus light beam (13), and a beam steering system (24) configured to direct and reflect the autofocus light beam (13) off a bottom side of the substrate (3) of the sample holder positioned on the sample stage (16) and onto a light sensor (40) comprised in the autofocus system or onto the imaging sensor (12), the beam steering system configured to adjust a translational position and an angle of the autofocus light beam output by the beam steering system.

2. The microscope apparatus of claim 1 wherein the microscope apparatus is an optical diffraction microscope comprising an illumination and imaging system (4) comprising an illumination light source (10), generating a collimated light beam which is split into a sample beam (10a) that is transmitted through the sample, and a reference beam (10b) that follows a reference path and is re-combined with the sample beam before impinging upon the imaging sensor (12), the light collecting device (38) being positioned below the sample stage.

3. The microscope apparatus of any preceding claim comprising a positioning mechanism (18) coupled to the sample stage (16) or to the light collection device (38), configured to adjust a height of the sample stage (16) relative to the light collection device (38), and thereby the height of the base (3) of the sample holder (1) relative to the light collection device (38), based on an input from the autofocus system.

4. The microscope apparatus of any preceding claim in combination with a computing system (9) forming part of the microscope apparatus, or connected to the microscope apparatus, wherein the autofocus system and imaging sensor are connected to the computing system comprising a module configured to control the autofocus system statically or dynamically.

5. The microscope apparatus of any preceding claim wherein the beam steering system (24) comprises a light beam emitter (22), and electrically actuated pivotable mirrors (26a, 26b) configured to pivot about at least one axis, including at least a first electrically

actuated pivotable mirror (26a) and a second electrically actuated pivotable mirror (26b).

6. The microscope apparatus of the preceding claim wherein the beam steering system (24) further comprise one or more lenses (28) positioned between the first and second pivotable mirrors (26a, 26b) configured to adjust a translational position of the autofocus light beam (13).

7. The microscope apparatus of claim 5 or 6 in combination with claim 4, wherein the electrically actuated pivotable mirrors are connected to the computing system that controls the angle of the pivotable mirrors for adjusting the direction and position of the autofocus light beam.

8. The microscope apparatus of any of the three directly preceding claims wherein at least one of the electrically actuated pivotable mirrors are configured to pivot about two orthogonal axes.

9. The microscope apparatus of any preceding claim wherein the beam steering system further comprises an intermediate reflector (36) configured to allow the beam from the first pivotable mirror (26a) to pass through to the second pivotable mirror (26b), but to reflect the beam returning from the second pivotable mirror (26b towards the light collecting device (38).

10. The microscope apparatus of any preceding claim wherein the light collecting device (38) is configured to function both as a microscope objective for a sample illumination beam (10a) generated by the microscope, and as an objective for the autofocus light beam (13).

11. The microscope apparatus of any preceding claim wherein the beam steering system (24) is configured to direct and reflect the autofocus light beam (13) off a bottom side of the substrate (3) of the sample holder positioned on the sample stage (16) and onto the light sensor (40) comprised in the autofocus system, the light sensor being a component separate from the imaging sensor (12), the imaging sensor configured to receive light from the sample (5) under observation.

12. The microscope apparatus of any preceding claim wherein the beam steering system (24) is configured to direct and reflect the autofocus light beam (13) off a bottom side of the substrate (3) of the sample holder positioned on the sample stage (16) and onto the imaging sensor (12), the imaging sensor further configured to receive light from the

sample (5) under observation.

13. The microscope apparatus of any preceding claim wherein the autofocus system further comprises a light sensor (40) configured to receive the autofocus light beam reflected or emitted from the sample, the light sensor connected to a computing system (9) configured to analyse and compute the captured focus beam and to control an actuator adjusting a position of the sample relative to an imaging sensor to bring the system into focus.

14. A method of controlling a microscope apparatus of any preceding claim comprising a procedure to find a focused position of the sample (5) under observation, comprising:
- performing a scanning procedure controlled by a computing system (9) including displacing the sample stage (16) relative to the light collecting device (38) in a Z direction transverse to the substrate bottom surface over a first pre-defined distance, and obtaining in the computing system (9) from the light sensor (40) or the imaging sensor (12) a set of measurements of positions of a spot of the autofocus beam impinging upon the light sensor (40) or the imaging sensor (12);
- calculating in the computing system an estimation of a Z direction position for said focused position of the sample;
- iteratively repeating, at least once, a step of performing a detection procedure over a second pre-defined distance encompassing the previously estimated ideal Z direction position until a difference between a previous and a subsequent estimated ideal Z direction position is below a chosen measurement threshold corresponding to an acceptable focused position.

15. The method of the preceding claim comprising a procedure controlled by the computing system (9) to change a sensitivity of the autofocus system, the sensitivity corresponding to the ratio of the Z direction displacement relative to the displacement of the spot of the autofocus beam impinging upon the light sensor (40) or the imaging sensor (12), the procedure comprising changing an inclination angle of the autofocus light beam output by the beam steering device (24).

16. The method of either of the two directly preceding claims comprising a procedure controlled by the computing system (9) to change the position of the spot of the autofocus system, impinging upon the substrate (3), which directly determines the position of the spot of the autofocus beam impinging upon the light sensor (40) or the imaging sensor (12), the

procedure comprising changing the position of the autofocus light beam output by the beam steering device (24).

FIG 1a

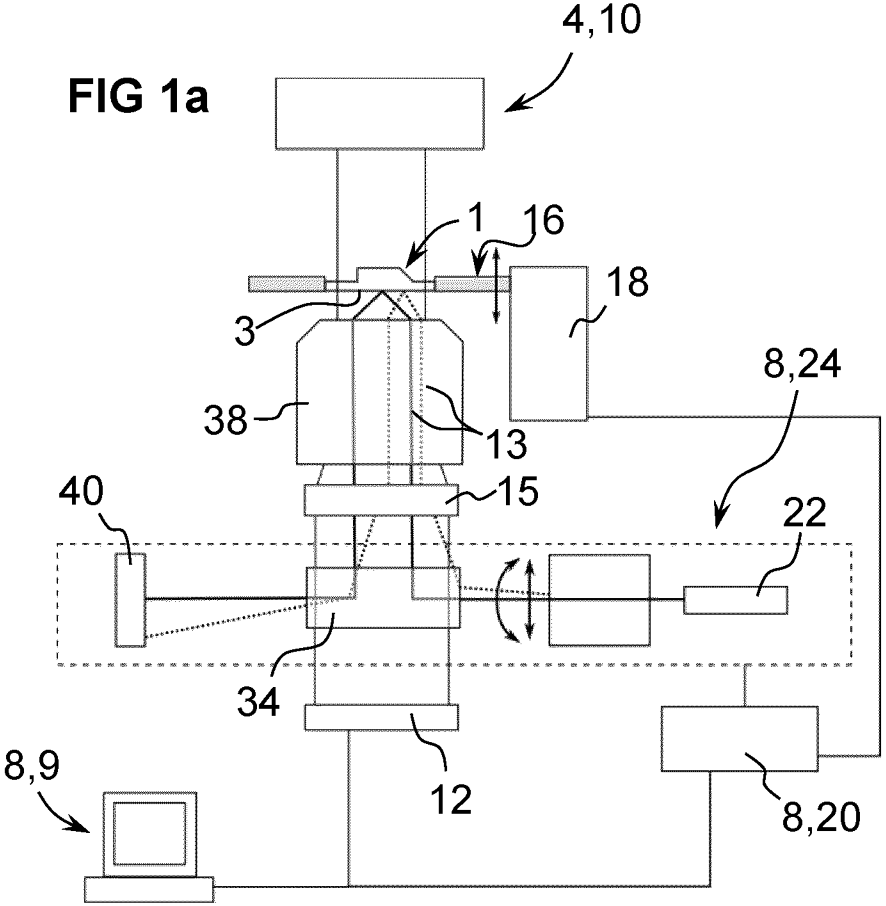


FIG 1b

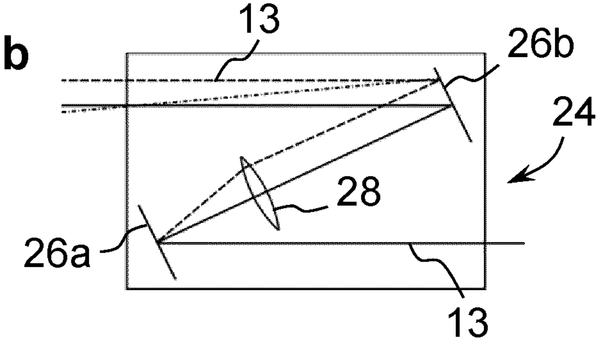


FIG 2

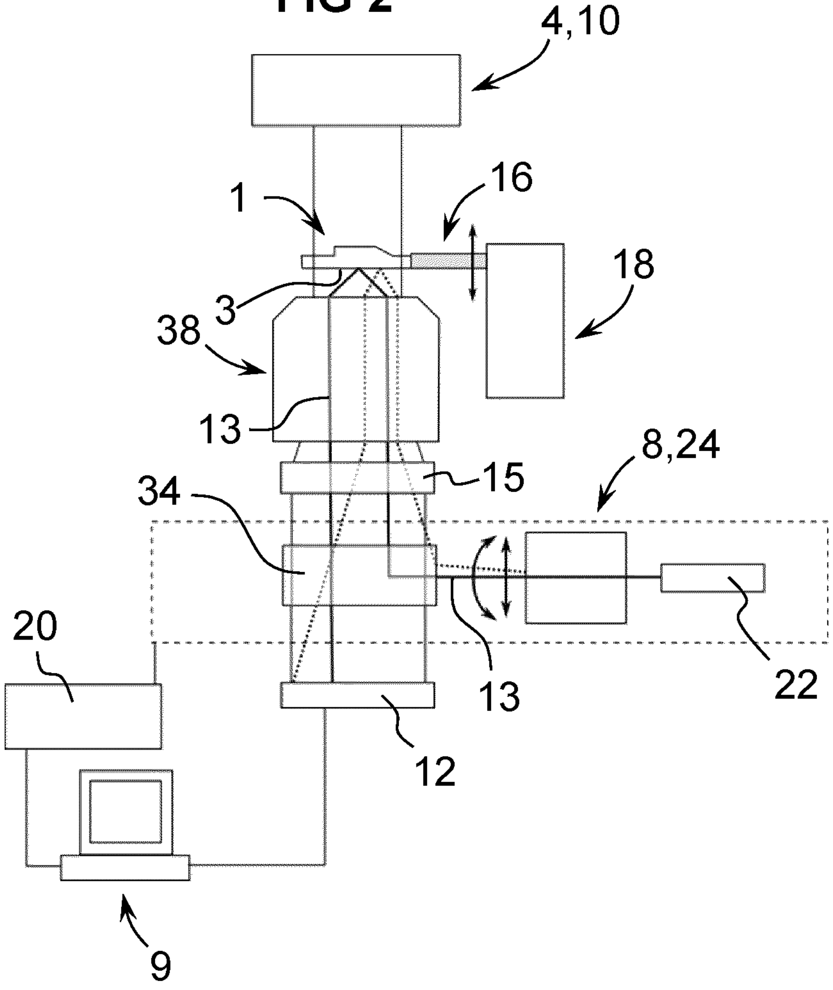


FIG 3

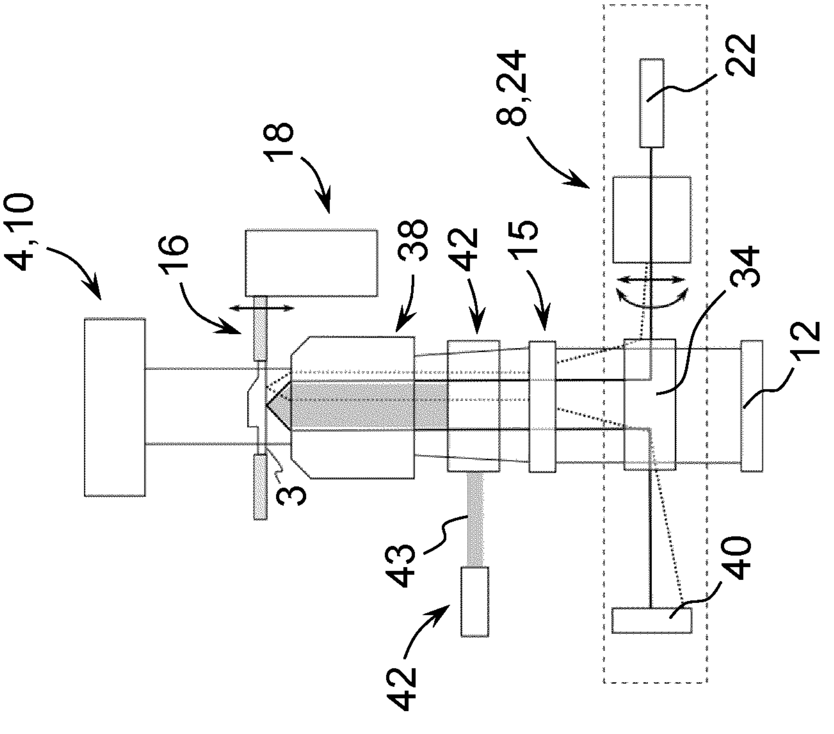
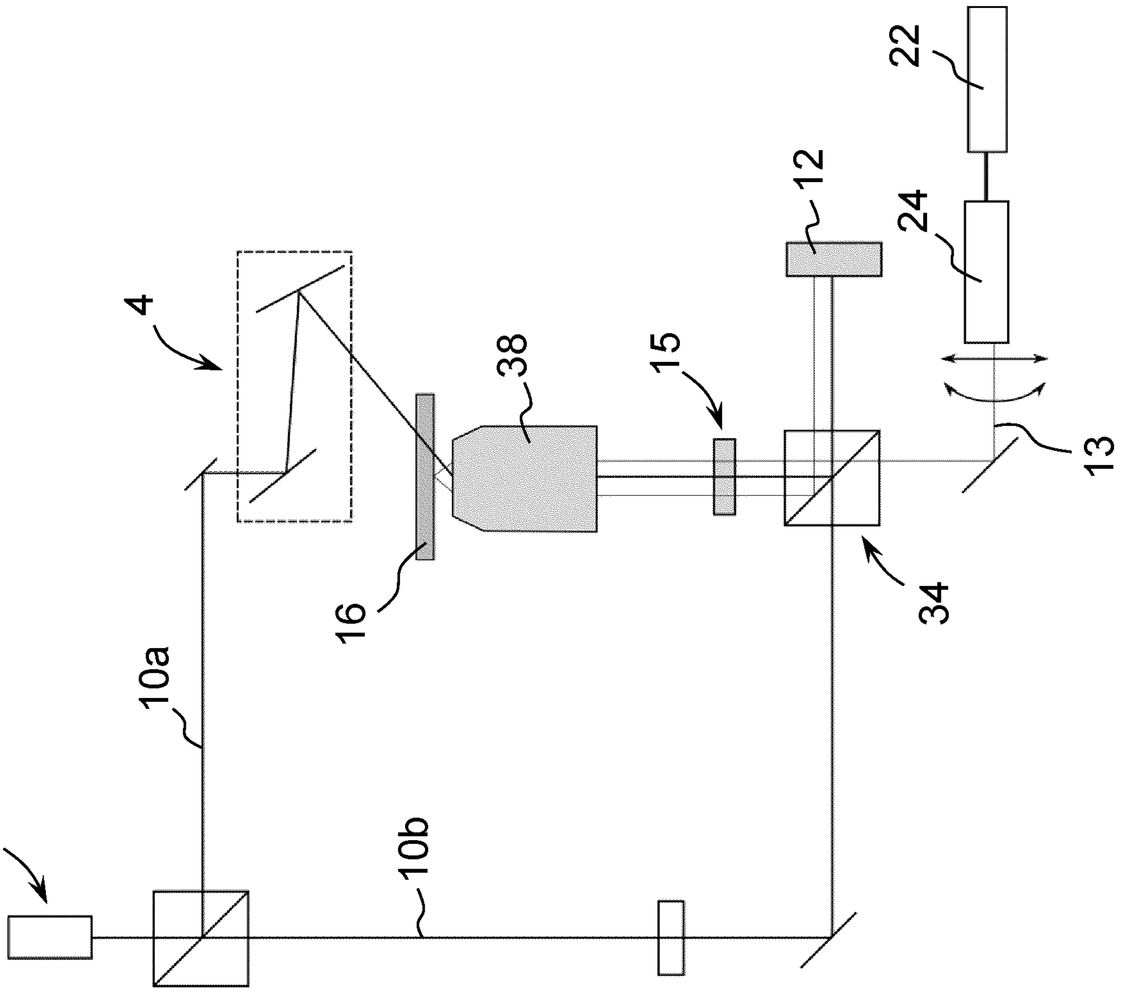

4,10
16
18
38
42
15
8,24
22
34
12
3
43
42
40

FIG 4


4,10
10a
10b
4
16
38
15
12
34
13
24
22

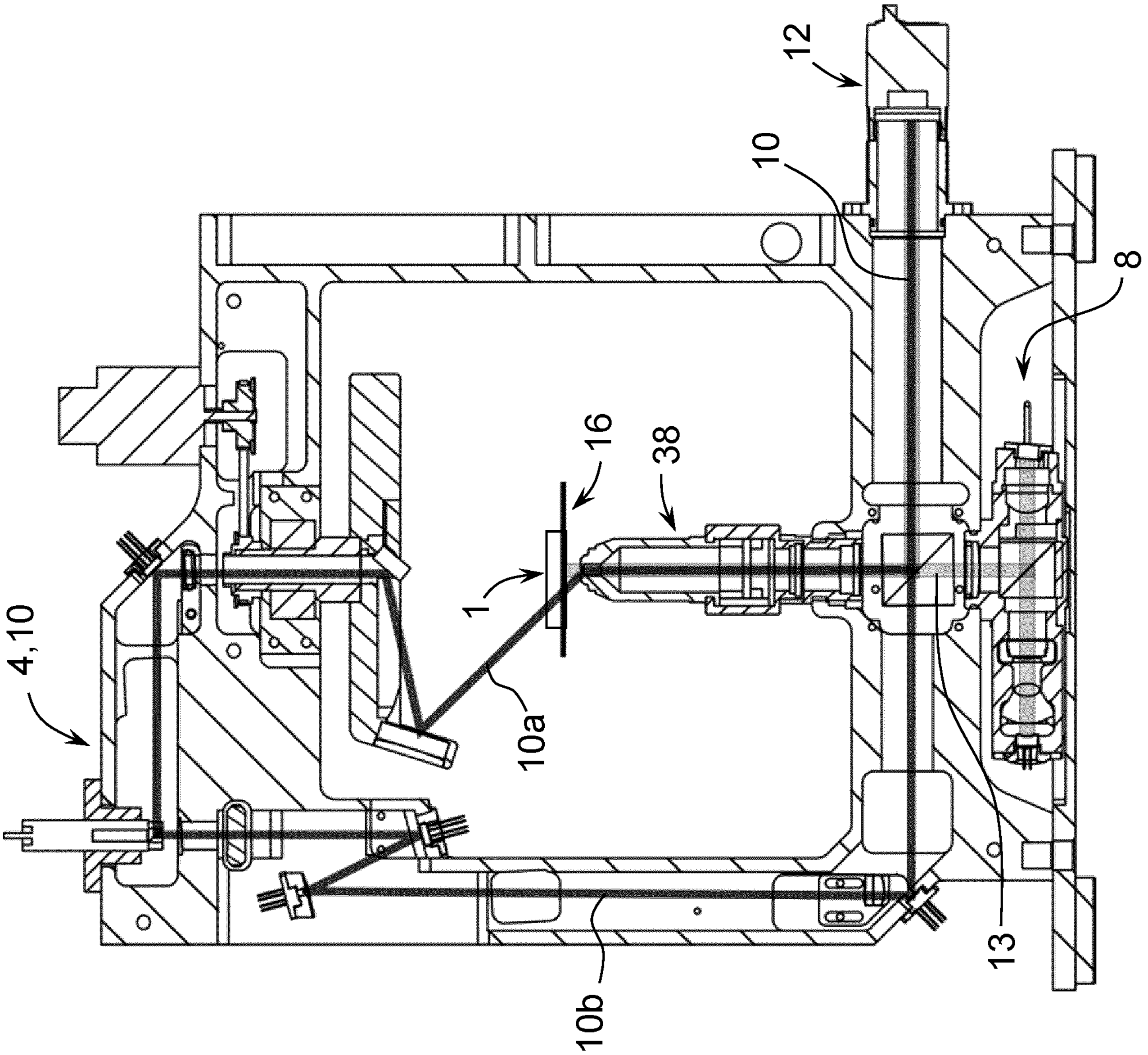


FIG 5a

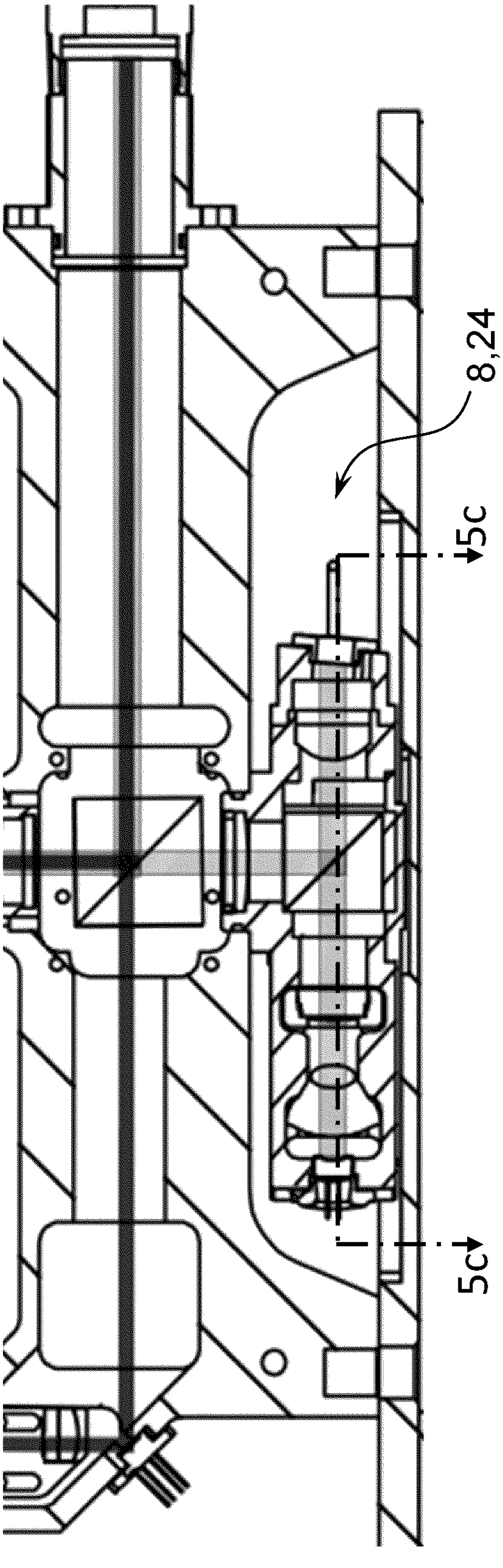


FIG 5b

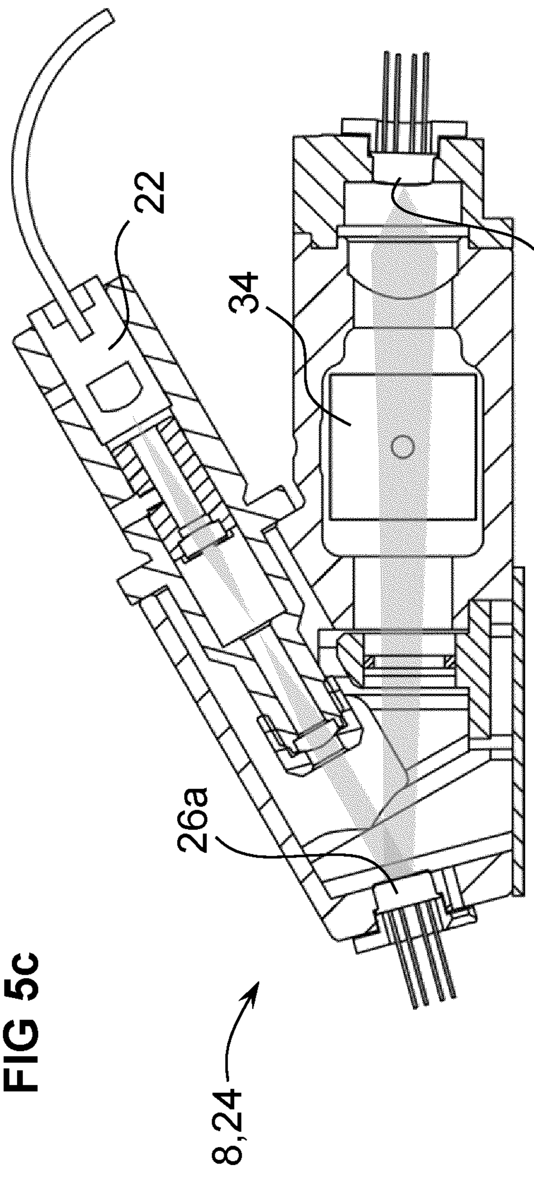


FIG 5c

FIG 6

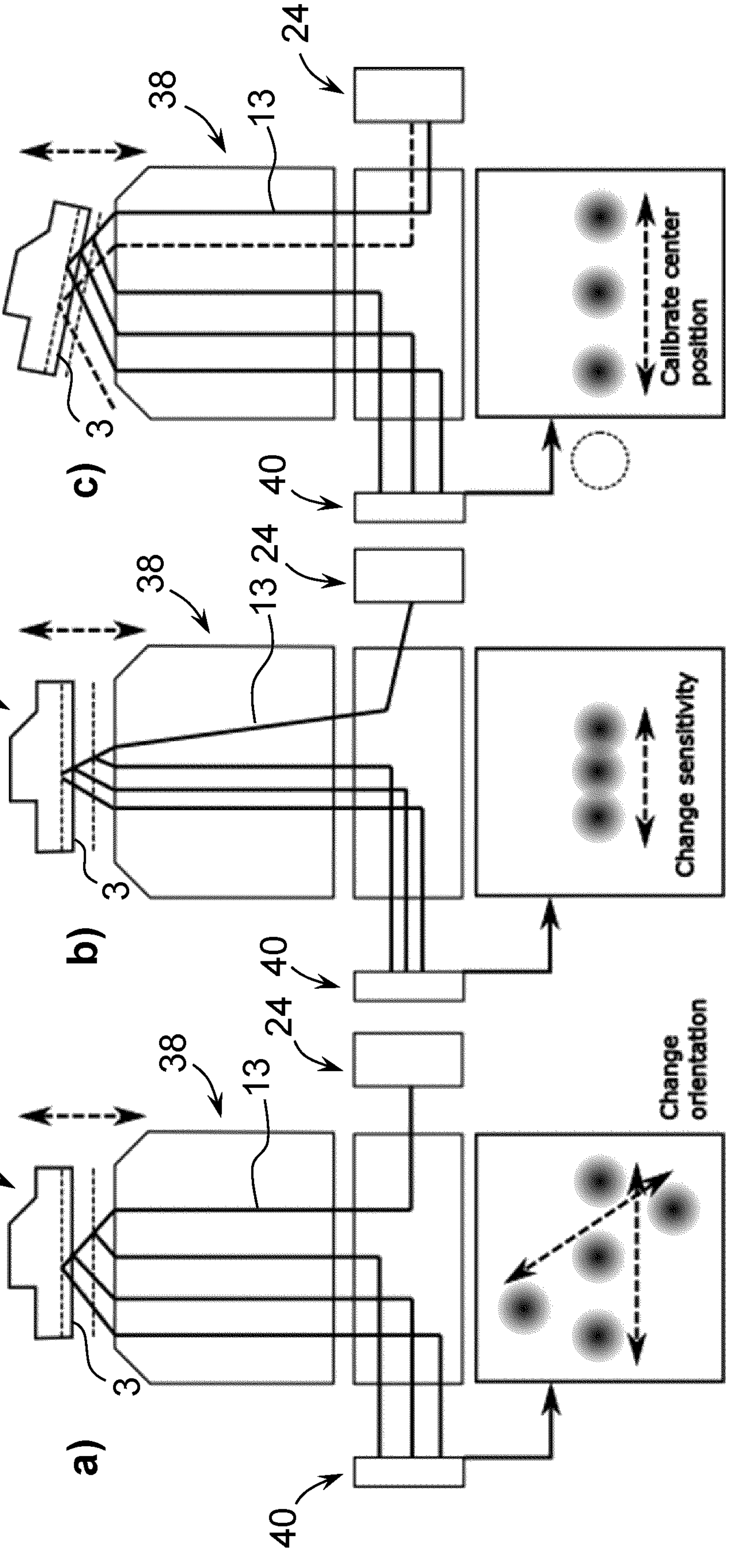

a)
1
3
38
13
24
40
Change orientation
b)
1
3
38
13
24
40
Change sensitivity
c)
1
3
38
13
24
40
Calibrate center position

FIG 7

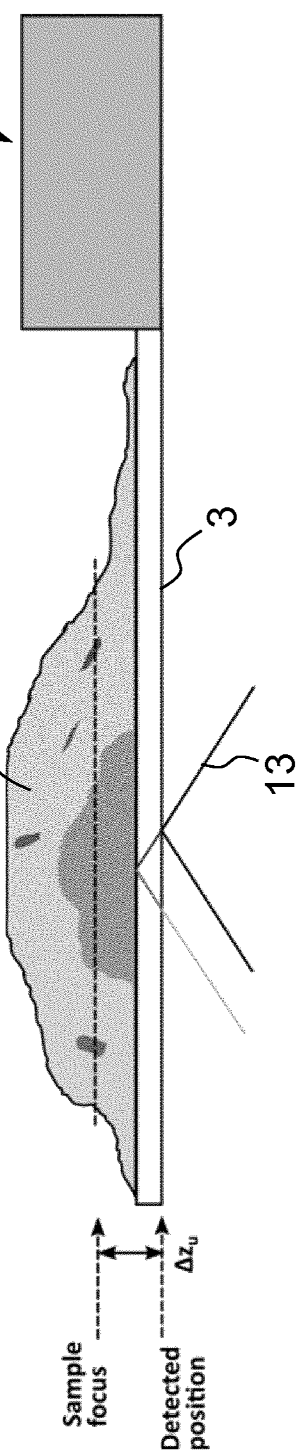
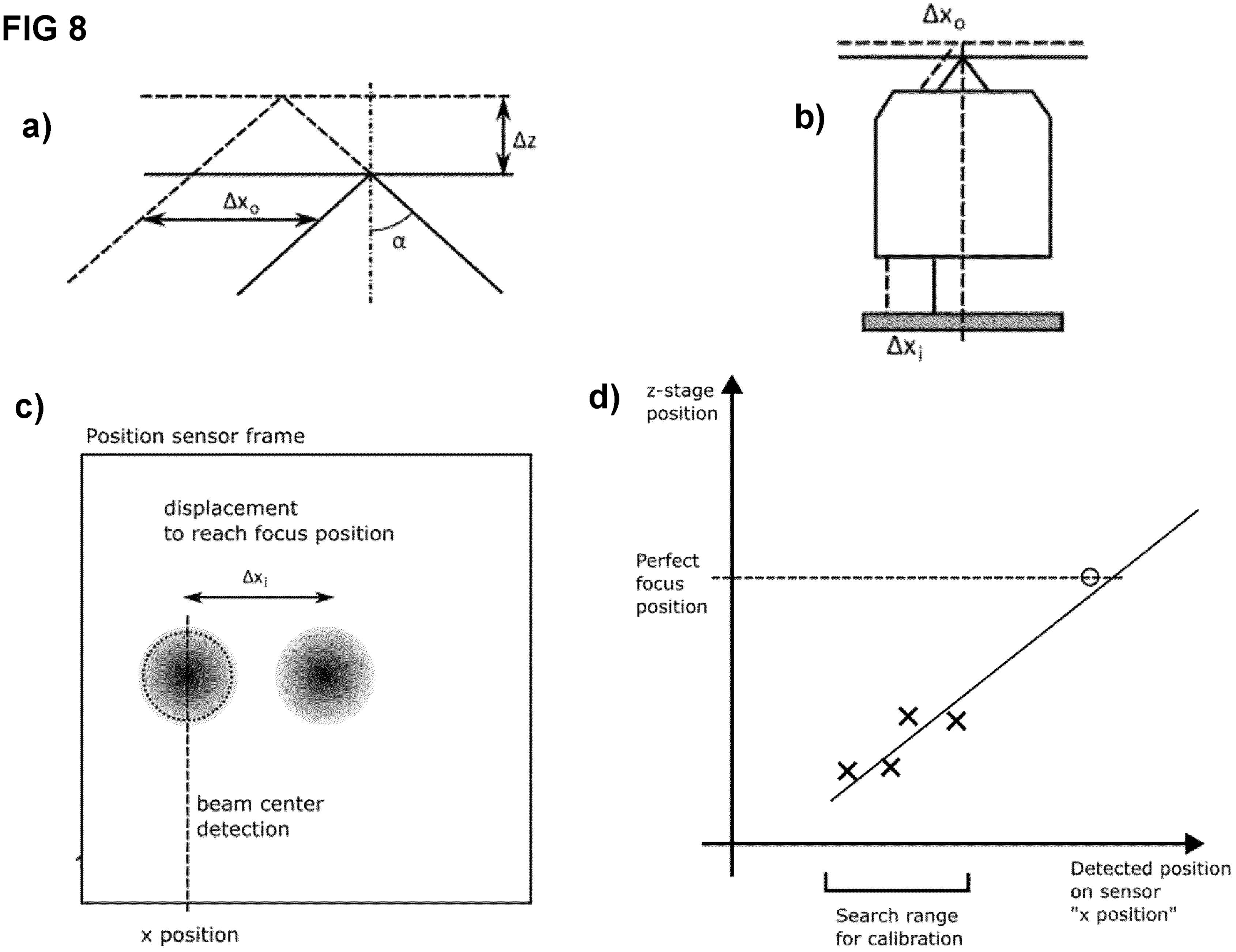

16
5
3
13
Sample focus
Detected position
Δz_u

FIG 8


a)
Δz
Δx_o
α
b)
Δx_o
Δx_i
c)
Position sensor frame
displacement
to reach focus position
Δx_i
beam center
detection
x position
d)
z-stage
position
Perfect
focus
position
Search range
for calibration
Detected position
on sensor
"x position"

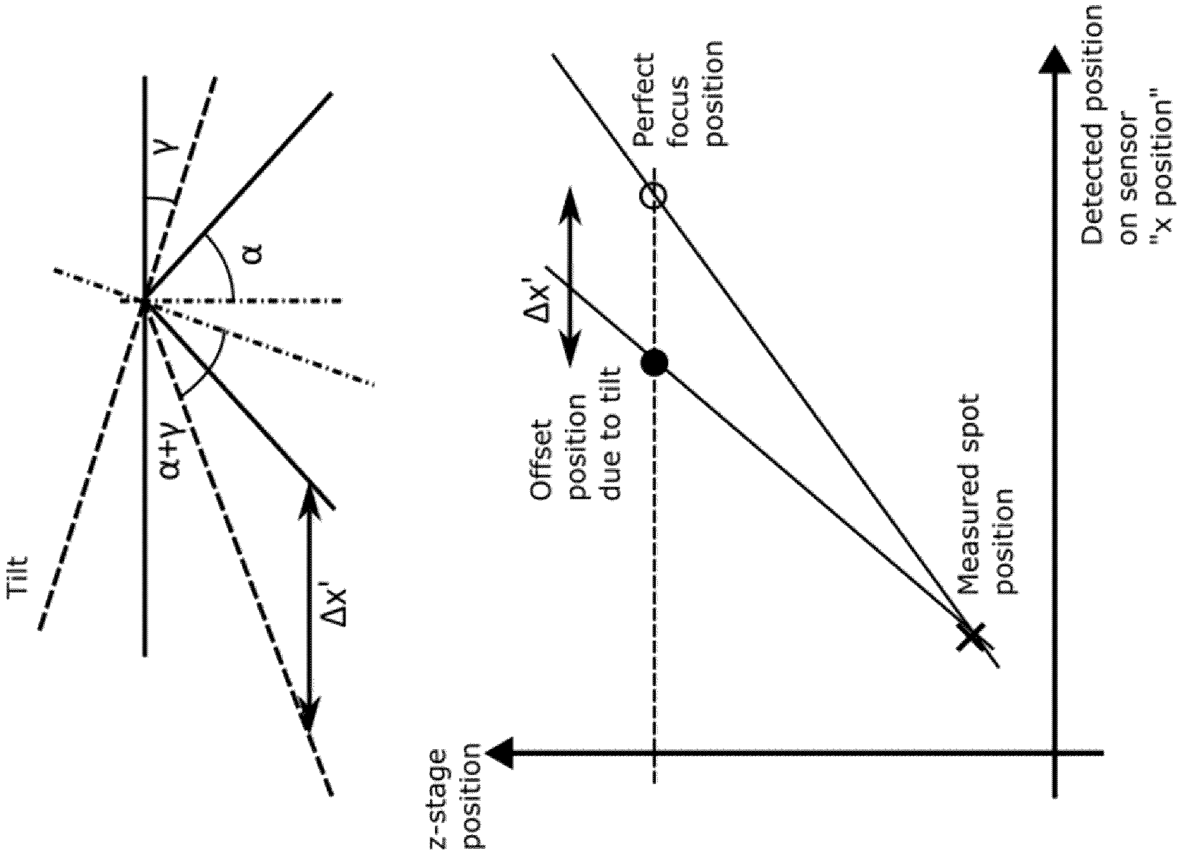

Tilt
γ
α
α+γ
Δx'
Δx'
Perfect focus position
Offset position due to tilt
Measured spot position
z-stage position
Detected position on sensor "x position"

FIG 9

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/057637

A. CLASSIFICATION OF SUBJECT MATTER

INV. G02B21/24 G02B21/26 G02B21/36

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G02B

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2011/127406 A1 (SASE ICHIRO [JP]) 2 June 2011 (2011-06-02) paragraphs [0001], [0037], [0040] - [0061], [0069] - [0079], [0101] - [0106], [0118]; figures 1,2,5,8-10 -----	1,3-16 2
X Y	US 2012/193511 A1 (OKABE KENJI [JP] ET AL) 2 August 2012 (2012-08-02) paragraphs [0041] - [0058], [0094] - [0105]; figures 1,3-6 -----	1,3-10, 13-16 2
	-/--	

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

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 April 2024	13/05/2024

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Weinberger, Thorsten

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C(Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEI-CHEN HSU ET AL: "Tomographic diffractive microscopy of living cells based on a common-path configuration", OPTICS LETTERS, OPTICAL SOCIETY OF AMERICA, US, vol. 39, no. 7, 1 April 2014 (2014-04-01), pages 2210-2213, XP001589333, ISSN: 0146-9592, DOI: 10.1364/OL.39.002210 [retrieved on 2014-03-31] the whole document -----	2

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/057637

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		US 2011127406 A1	02-06-2011
		WO 2009031477 A1	12-03-2009
US 2012193511 A1	02-08-2012	CN 102621658 A	01-08-2012
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