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(54) LASER SCANNING MICROSCOPE AND ITS OPERATING METHOD

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(57) **ABSTRACT**

Laser scanning microscope and its operating method in which at least two first and second light distributions activated independently of each other and that can move in at least one direction illuminate a sample with the help of a beam-combining element, and the light is detected by the sample as it comes in, characterized by the fact that the scanning fields created by the light distributions on the sample are made to overlap mutually such that a reference pattern is created on the sample with one of the light distributions, which is then captured and used to create the overlap with the help of the second light distribution (correction values are determined) and/or a reference pattern arranged in the sample plane or in an intermediate image plane is captured by both scanning fields and used to create the overlap (correction values are determined) and/or structural characteristics of the sample are captured by the two scanning fields as reference pattern and used to create the overlap in which correction values are determined.

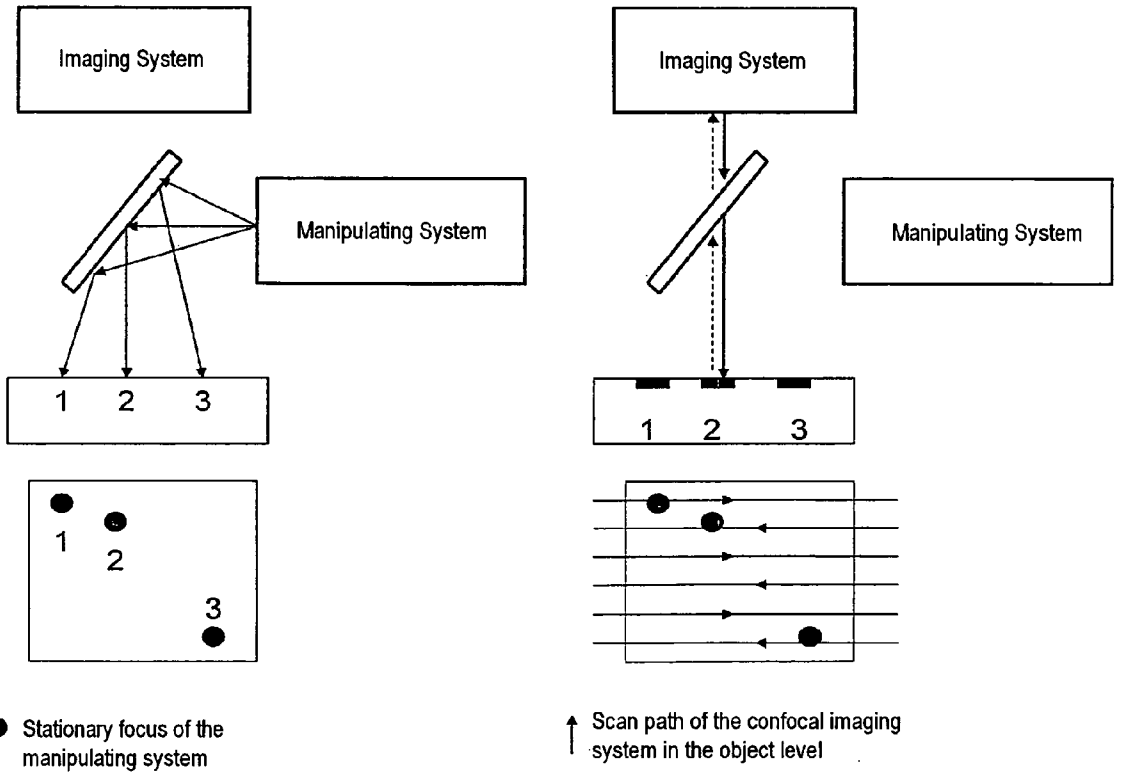


FIG. 1

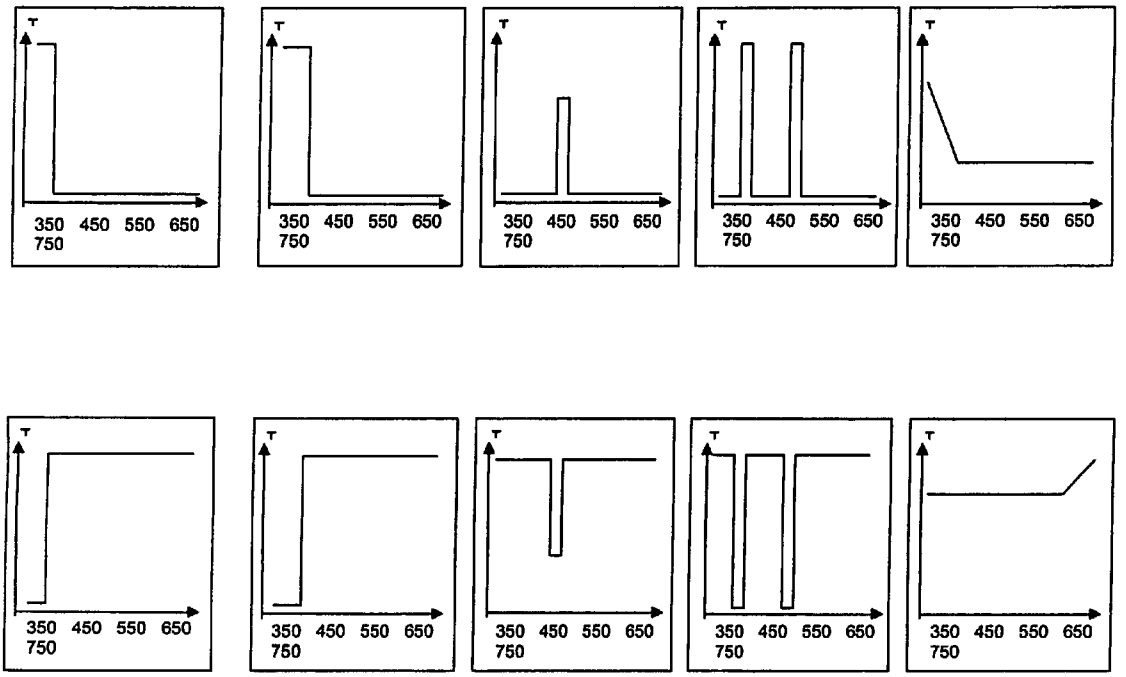
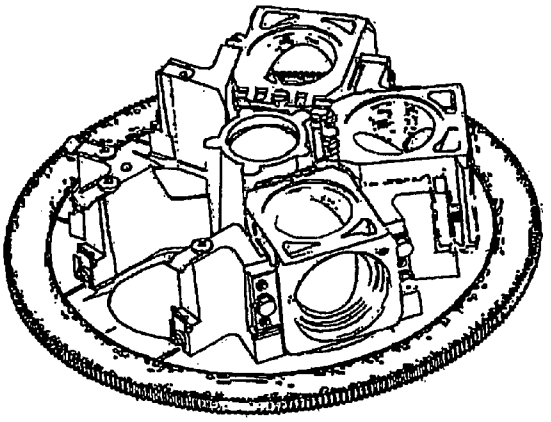



FIG. 2



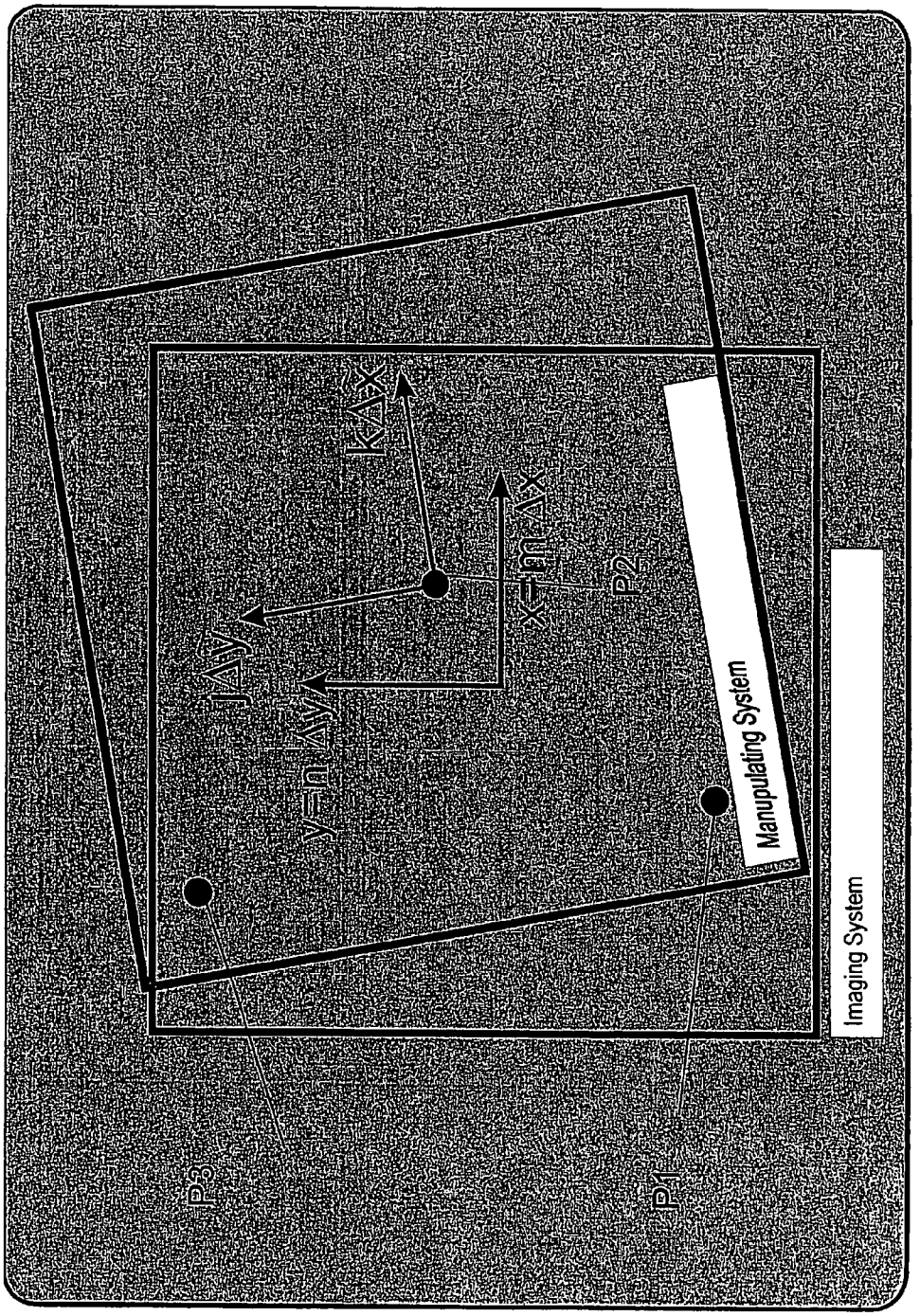

P3
P1
P2
Manupulating System
Imaging System
y=nΔy
Δy
KΔx
x=mΔx

FIG. 3a

FIG. 3b

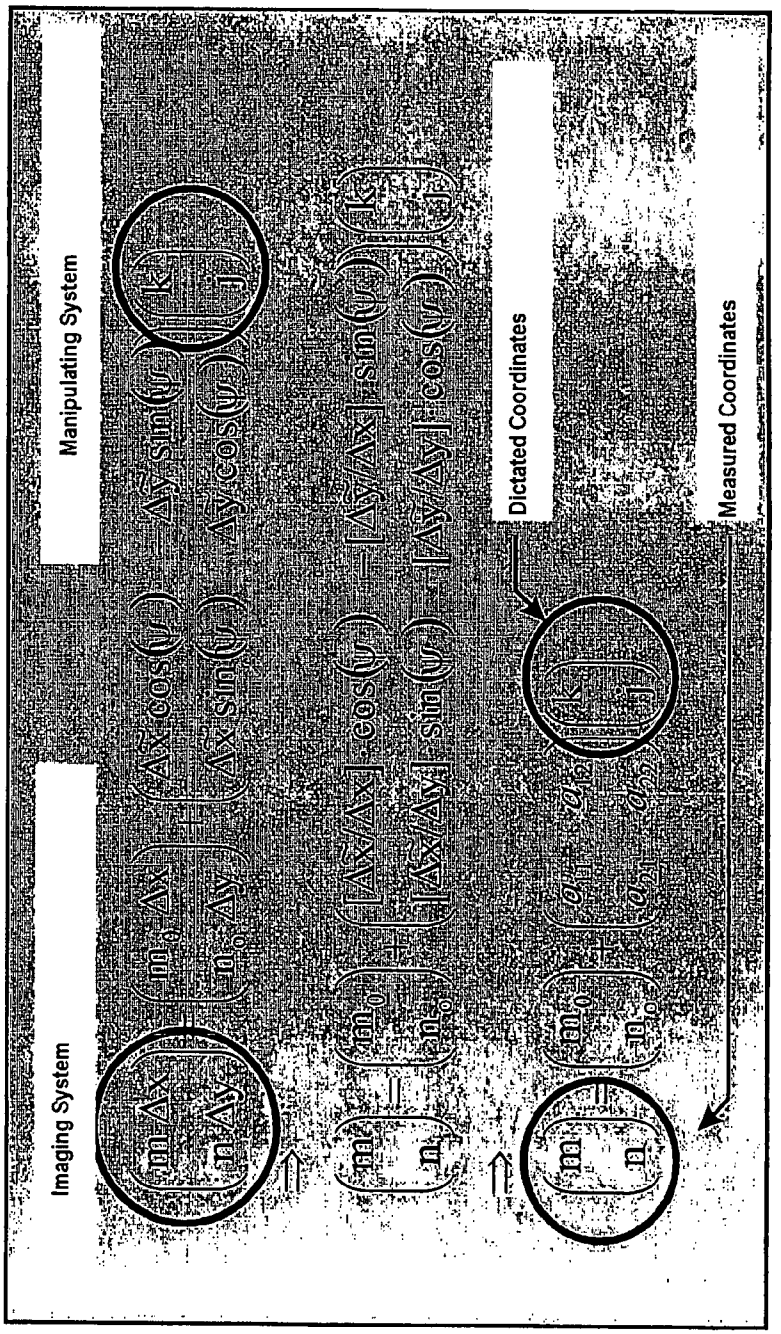

Affine Transformation:
Minimum Number of Items: 3
Imaging System
Manipulating System
Dictated Coordinates
Measured Coordinates

FIG. 4

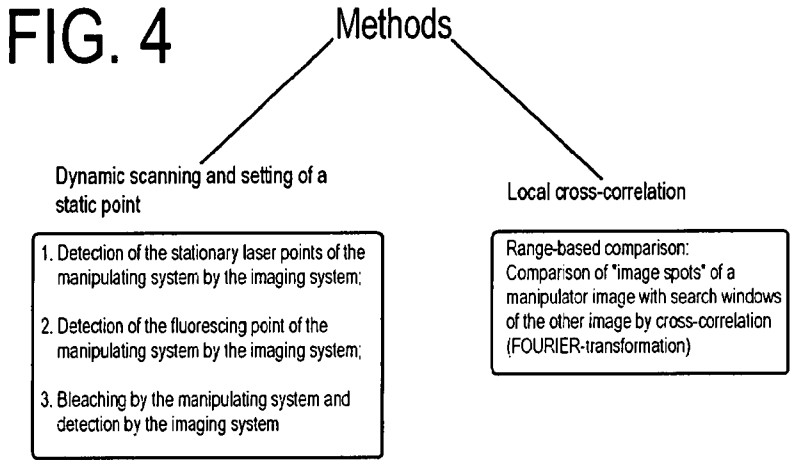



FIG. 5a

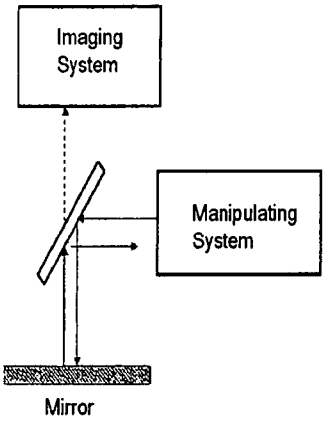



FIG. 5b

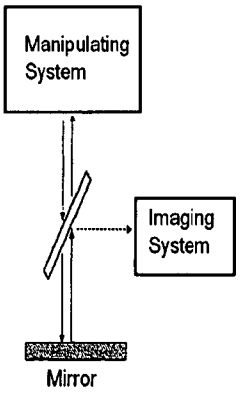
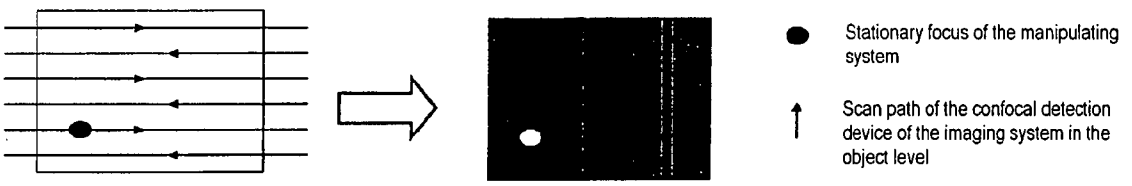



FIG. 5c



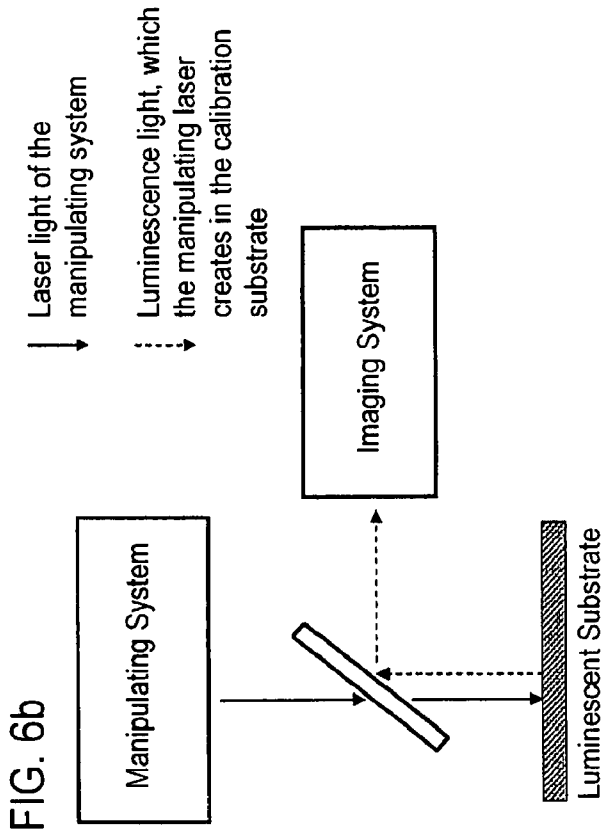



FIG. 6b

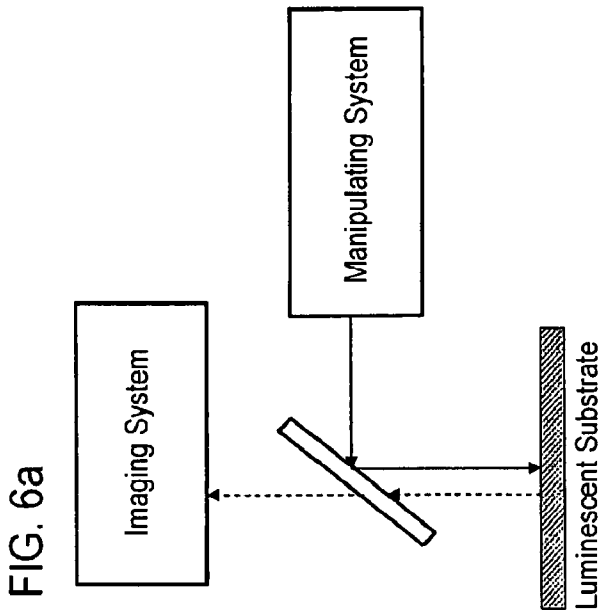
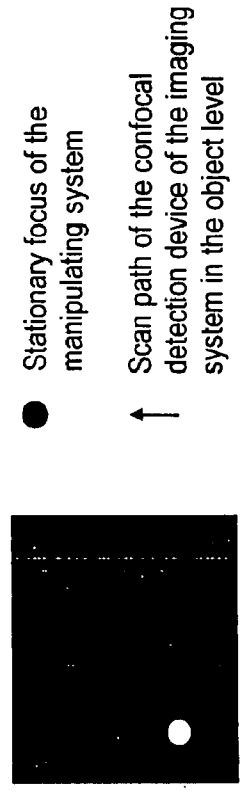



FIG. 6a



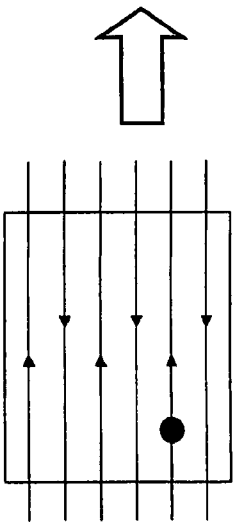



FIG. 6c

FIG. 7

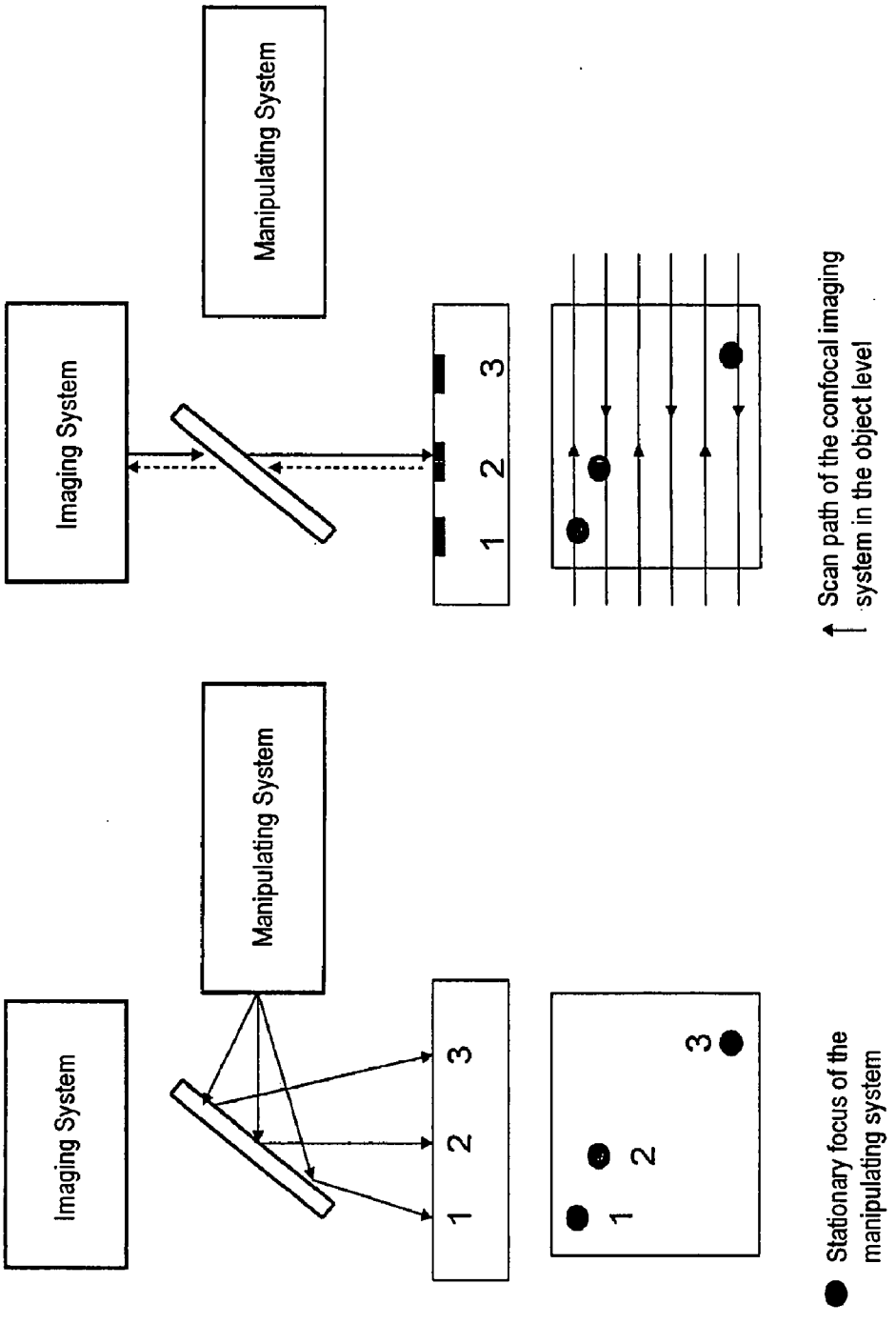
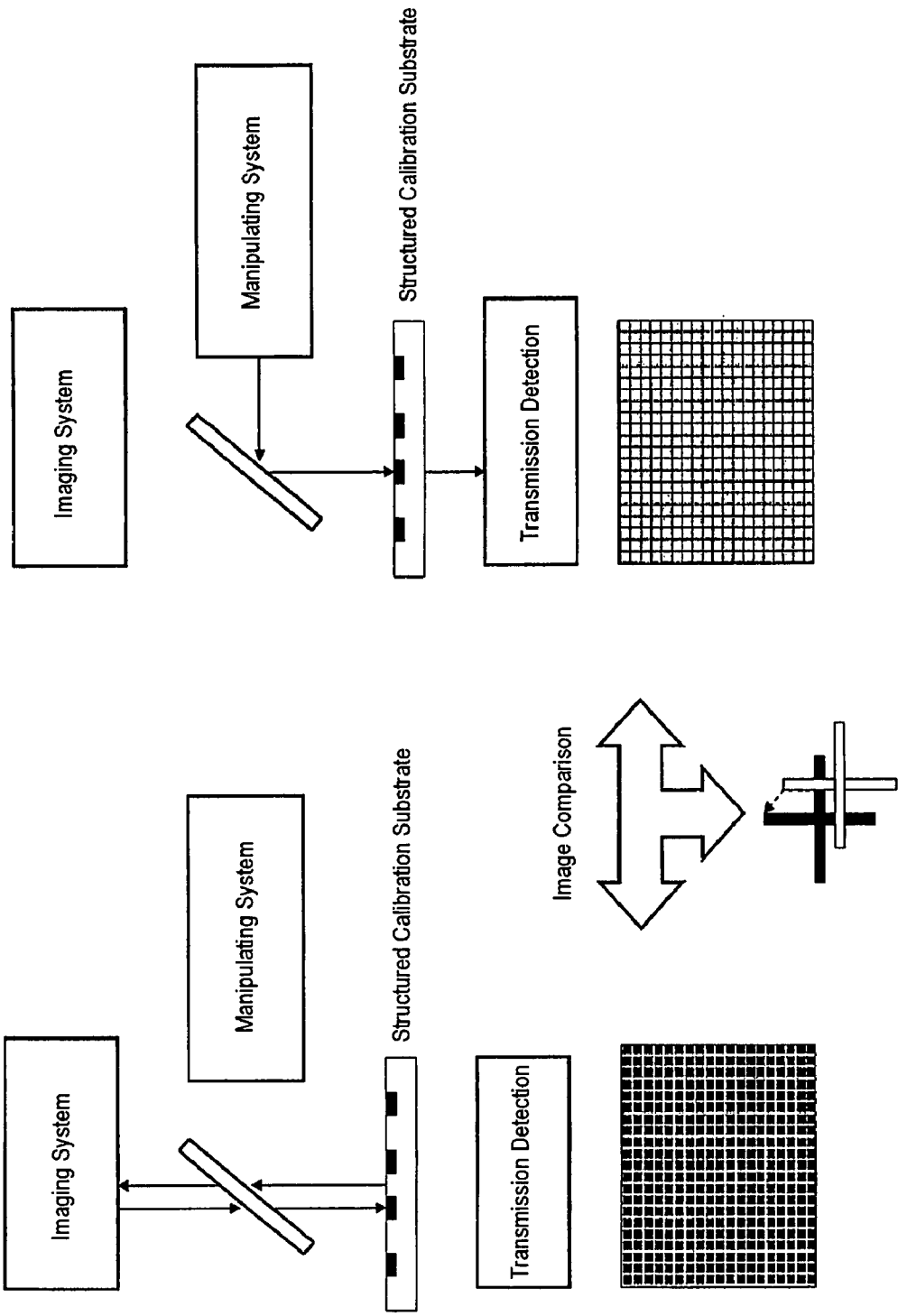

Imaging System
Manipulating System
1 2 3
Imaging System
Manipulating System
1 2 3
Scan path of the confocal imaging system in the object level
Stationary focus of the manipulating system

FIG. 8


Imaging System
Manipulating System
Structured Calibration Substrate
Transmission Detection
Image Comparison
Imaging System
Manipulating System
Structured Calibration Substrate
Transmission Detection

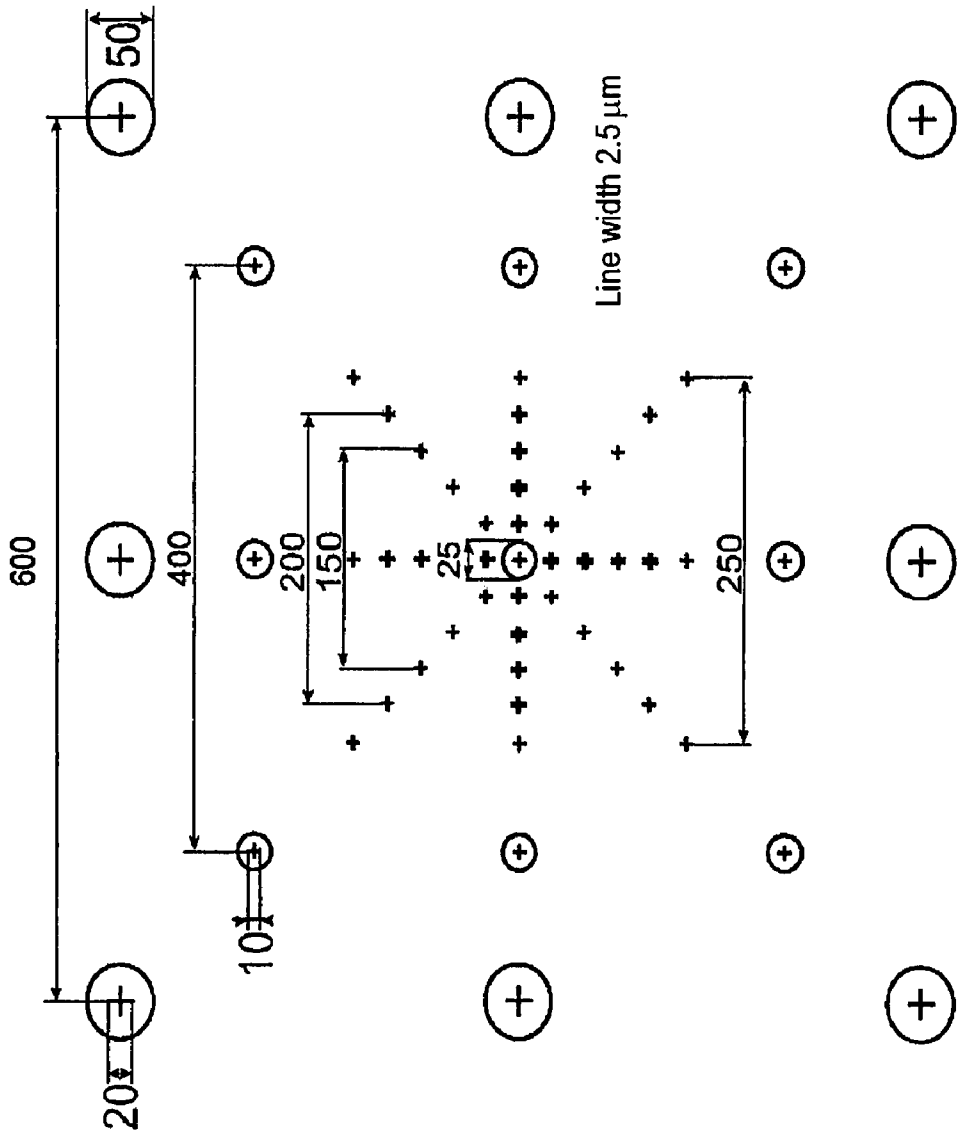



FIG. 9

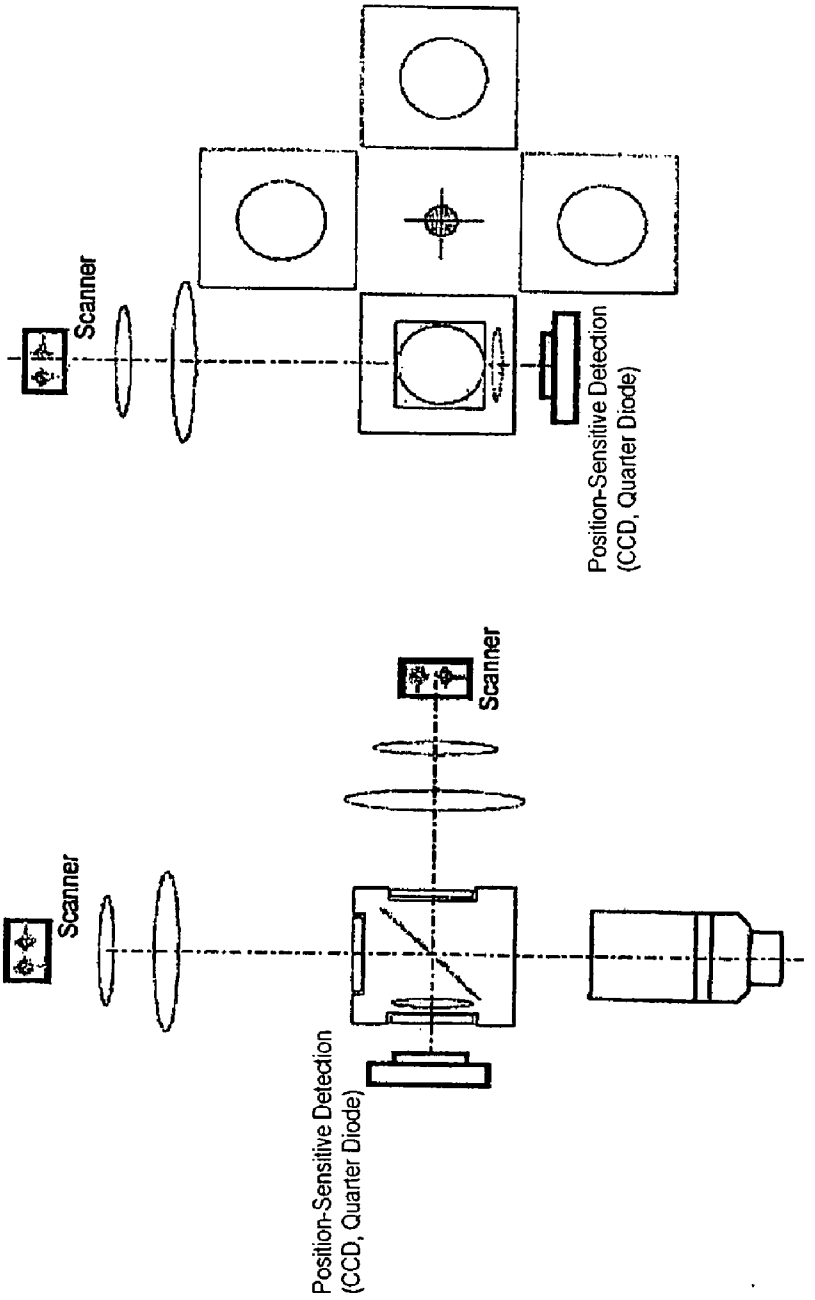

Scanner
Position-Sensitive Detection
(CCD, Quarter Diode)
Scanner
Scanner
Position-Sensitive Detection
(CCD, Quarter Diode)

FIG. 10

# LASER SCANNING MICROSCOPE AND ITS OPERATING METHOD

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** Not Applicable.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

**[0002]** Not Applicable.

## REFERENCE TO A SEQUENCE LISTING, A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISC

**[0003]** Not Applicable.

## BACKGROUND OF THE INVENTION

**[0004]** (1) Field of the Invention

**[0005]** The present invention relates to methods of operating a microscope, in general, and to a method of operating a laser scanning microscope having at least two independently controlled light distributions, in particular.

**[0006]** (2) Description of Related Art

**[0007]** Confocal laser microscopy is, among other things, the tool for the defined control of micro objects. Versatile methods of examining and influencing microscopic objects were recommended on this basis—e.g., Denk in U.S. Pat. No. 5,034,613, TPA, Liu in U.S. Pat. No. 6,159,749, Tweezer or Karl Otto Greulich in "Micromanipulation by Light in Biology and Medicine" 1999. A combination of a point-scanning or line-scanning imaging system and a "manipulator" system has evoked increasing interest in the specialized world.

**[0008]** Interest in observing and analyzing fast microscopic processes has created new devices and processes (e.g., line scanner LSM 5 LIVE), whose combination with the manipulation methods mentioned above leads to new insights. In this context, the simultaneous microscopic observation of a light induced, locally resolved sample manipulation with the help of a suitable imaging system occupies the foreground (U.S. Pat. No. 6,094,300 and DE 102 004 03 4987 A1). Modern microscopes therefore try to offer the maximum possible number of flexible and optically equivalent coupling and decoupling positions (DE 102 004 01 6433 A1).

**[0009]** The simultaneous availability of at least two coupling positions for independent scanning systems is very important in this context for avoiding limitations in time resolution due to slow mechanical control processes. In addition to tubular interface, there are other coupling positions on the sides of the microscope stands (preferably in an extended infinite space between the microscope objective and tube lens; "side ports") as well as on the rear side of the stand (typically optically modified reflected or transmitted light axes with suitable tube lens; "rear ports") as well as the bottom side ("base port"). In principle, arrangements with a common beaming direction (either reflected light or transmitted light) or the opposite beaming direction (reflected light and transmitted light) are conceivable. Apart from the applicative background, the technical instrument-based view of the common beaming direction is often preferred.

**[0010]** At least one element must be used in this case that combines the beam paths of the two instruments in the space between the scanners of the simultaneously operated scanning systems and the objective. According to the prior art, one can think of the most varied of beam-combining elements such as for instance, optical-mechanical components like suitably coated beam-combining, flat plates and beam-combining wedges, beam-combining cubes and a polarization splitter. Further, beam-combining acoustic-optical modulators and deflectors are also conceivable.

**[0011]** The mechanical requirements related to the precision of location and angle of this beam-combining element are very high. A faulty installation angle $\alpha$ causes a beam inclination in the reflection of $2\alpha$ For example, if the beam-combining element is in the infinite space between a tube lens of focal length $f_{TL}$=164 mm and an objective of the nominal foreground $M=f_{TL}/f_{Obj}=40\times$ then this leads to an angular deviation of $2\alpha$=1'(position deviation of the beam-combining element 0.5') to a deviation $\Delta=(f_{TL}/M)*\tan 2\alpha=1.2\ \mu m$ of both scanning fields in the object plane. In a field of view **18** (image diagonals) this already corresponds to a deviation of approximately 0.4% of the lateral length of the scanning field. In the usual image formats of 512×512 or 1024×1024, this corresponds to a deviation of 2-4 image pixels. In addition to the demanding mechanical requirements related to the mechanical positioning of the beam-combining element, there are similarly demanding tolerance specifications related to the mechanical interfaces of the imaging or manipulation scanning module (inclination errors and lateral shifting of interface, intermediate image position in axial direction, and rotation). Further, thermal influences (heating of the microscope system, and fluctuations in the environmental temperature) as well as undefined statistical effects, impose a condition that occurs especially in case of extremely precise measurements, the cover of the scanning fields in the manipulating and imaging systems must be adjusted repeatedly.

## BRIEF SUMMARY OF THE INVENTION

**[0012]** To compensate for the pixel displacement (x, y) between the manipulating and imaging scanning modules that cannot be controlled fully through the mechanical tolerance chain, this patent suggests calibration in such a way that, through various methods, the position deviations of the scanning fields of the two systems are determined and the coordinate transformations resulting there from (scaling, rotation, shift) are computed and considered in the control of at least one of the scanning systems.

**[0013]** In this context, it must be considered that the resulting image cover parameters are influenced by numerous device settings. An example of this would be the different main beam splitters of a confocal laser scanning microscope, which in several commercial devices is arranged on a motorized main beam splitter wheel. If the excitation beams are reflected on the main beam splitter at less than 90°, minor angular errors are already observable in the scanning field cover. Examples of other adjustable device parameters that can influence the scanning field cover crucially are movable optics (e.g. viewing field or pupil zoom) as well as non-linear factors and dynamic deviations of the beam deflecting devices used in the concerned scanning systems (e.g. selected scanning speed and scanning zoom in devices on the basis of galvo scanners). Add to this the fact that the wavelength dependency of the z-deposit is to be calibrated as a function of the excitation and manipulation wavelengths used in different applications as well as of the concerned used objective. The z-plane comparison can be conducted elegantly through

moveable collimator optics of the imaging and/or manipulating system under scrutiny of the color length fault of the concerned used objective.

**[0014]** Depending on the concerned application, the spectral use area can stretch basically from the ultraviolet to the infrared range for the imaging system as well as the manipulating system. Typical manipulation wavelengths used in applications are, e.g., 351, 355 and 364 nm (photo-uncaging), 405 nm (photo conversion, Kaede, Dronpa, PA-GFP), 488 and 532 nm (photo bleaching, FRET, FRAP, FLIP) as well as 780-900 nm (multi-photon bleach, e.g., MPFRAP, 2-photon uncaging; direct multi-photon stimulation). Depending on the combined wavelength as well as the coupling positions of the imaging system and the manipulating system, there are numerous types of dichroitic beam-combining elements that are meaningful from the application point of view. FIG. **1** shows a selection of spectrally possible properties of beam-combining element types that are relevant to applications in which the manipulation wavelengths of 355 nm, 405 nm, 488 and 532 nm can be used in the transmission as well as reflection direction. Neutral combining elements (e.g., T20/R80) can be used universally for different applications and they also enable simple applications in which the same laser wavelengths are used for the imaging system as well as the manipulating system (particularly FRAP).

**[0015]** Depending on the application under consideration, there is a typical requirement of using different beam-combining element types in a microscope system. A motorized replacement device is used for this purpose. It can be, e.g., a motorized reflector revolver in the area of the infinite space between the objective and tube lens, as illustrated in FIG. **2**. An alternative to the displayed reflector revolver is, e.g., an appropriate reflector disk. The replacement device for the different beam-combining element's conditions further influence factors that affect the coverage of the scanning fields of the imaging system and manipulation system. Thus, already negligible mutual deviations of the beam-combining element alignment lead particularly in the reflection direction to a measurable scanning field shift.

**[0016]** Another problem is the ability to mechanically reproduce (beam-combining element location and beam-combining element alignment) the scanning position of the replacement device. Thus, on the one hand, the precision and reproduction capacity requirements of the replacement device increase as compared to traditional light-microscopic systems, and, on the other hand, claims of the practical management of the calibration method mentioned above. Even the complete replacement of the revolver device displayed in FIG. **2** can lead to a deviation of the scanning field cover, requiring a fresh calibration due to residual errors of the mechanical record.

**[0017]** In short, there is a need for the very general requirement of the simplest possible calibration method that allows the correction of the scanning field cover of the imaging system and the manipulating system as a function of varying device settings. This calibration method should particularly be used by the device user and if possible, it should be possible to execute it automatically.

## BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

**[0018]** FIG. **1** shows a selection of spectrally possible properties of beam-combining element types;

**[0019]** FIG. **2** a motorized reflector revolver in the area of the infinite space between the objective and tube lens;

**[0020]** FIG. **3***a* illustrates the non-coinciding scanning fields of a mapped scanning system (imaging) and a manipulation system (manipulating) with orientations;

**[0021]** FIG. **3***b* illustrates an affine transformation with reference to the orientation points P**1**-P**3** and their position;

**[0022]** FIG. **4** provides a schematic overview of the different calibration methods for the determination of the scanning field cover;

**[0023]** FIG. **5***a*, the imaging system in transmission and the manipulating system in reflection are coupled or decoupled. In FIG. **5***b* it is just the opposite.

**[0024]** FIG. **5***c* displays a stationary focus of the manipulation system where at least three such focuses are captured directly in the direction of reflection in the imaging system.

**[0025]** FIG. **9** illustrates an exemplary embodiment of a suitably structured calibration sample.

## DETAILED DESCRIPTION OF THE INVENTION

**[0026]** The invention is described in greater detail in the following pages with the help of the following schematic diagrams:

**[0027]** FIG. **3***a* illustrates the non-coinciding scanning fields of a mapped scanning system (imaging) and a manipulation system (manipulating) with orientations, deviating from each other, of the schematically represented X/Y orientation as well as orientation points P**1**-P**3** whose position on both systems are used for overlapping.

**[0028]** FIG. **3***b* illustrates an affine transformation with reference to the orientation points P**1**-P**3** and their position.

**[0029]** FIG. **4** displays the different methods that are described subsequently schematically; at the left the creation of static reference points by one of the scanning systems, preferably of the manipulation system (in reflection, fluorescence or two photon conversion, in general, each sample modification through illumination (also, e.g., ablation)), at the right the cross-correlation with the help of image characteristics of the two scanners.

**[0030]** In FIG. **5***a*, the imaging system in transmission and the manipulating system in reflection are coupled or decoupled. In FIG. **5***b* it is just the opposite. FIG. **5***c* displays a stationary focus of the manipulation system where at least three such focuses are captured directly in the direction of reflection in the imaging system.

**[0031]** A luminescent light point or a light point created elsewhere through frequency conversion is captured and used, instead of the focus, in FIG. **6** *a-c*.

**[0032]** The light-inducing sample modifications created by the manipulation system are captured by the imaging system and used as points in FIG. **7**. This can happen statically one after another or even during the scanning movement of the two systems (through activation and deactivation of the manipulator light at different places).

**[0033]** FIG. **8** displays a structured calibration substrate detected by both systems and the position of the lines used for calibration, either through cross-correlation or interactively by the user (mutual displacement in the display).

**[0034]** FIG. **10** shows a separately arranged detector in both systems (quadrant diode or CCD receiver) directly in the beam-combining element that captures and evaluates the transmitted or reflected residual beam for calibration. If a programmable, automatically triggering beam deflection device is used for the imaging and/or manipulating system,

then the described problem of pixel-precise scanning field coverage can be solved elegantly with the help of a suitable coordinate transformation. Thus for instance, in the absence of an angular distortion corresponding to FIG. **3***a*, the coordinate systems of the two scanning systems can be exposed to the following shifts even in the two-dimensional case:

**[0035]** Parallel displacement along the translation vector ($m_0$, $n_0$)

**[0036]** Rotated around the angle Ψ

**[0037]** Narrowed or stretched along the x- or y-scaling factors.

**[0038]** In this case, a transformation of the k and j coordinates of the manipulating system in the concerned m and n coordinates of the imaging system is possible with the help of an affine mapping (compare FIG. **3***b*):

$$m=m_0+a11k+j \tag{1a}$$

$$n=n_0+a_{21}k+j \tag{1b}$$

**[0039]** Thus, if the coordinates of at least three points are defined in the two independent scanning coordinate systems within the framework of a suitable calibration, Equations (1a) and (1b) can be used to convert the coordinates of the two scanning systems into each other for random scanning field points. A total of six image cover parameters are to be determined in this calibration process: Offset (zero position), angle (mutual rotation) and three stretching parameters. This therefore enables control of the beam deflecting device of the manipulating system in such a way that a pixel-precise cover with the object field of the imaging system is possible (or vice versa).

**[0040]** This method of implementing a pixel-precise scanning field cover of the two independent scanning systems presupposes that at least one system has a programmable, automatically triggering beam deflecting device.

**[0041]** This can be based on one of the following scanning principles:

**[0042]** Galvo mirror or

**[0043]** Deflectable, especially rotary or tilting mirror, e.g., step motor controlled deflecting mirror

**[0044]** Polygon mirror

**[0045]** Acoustic-optical deflection devices, especially acoustic-optical deflectors (AODs)

**[0046]** Moved perforated mask, especially in the form of a Nipkow disk

**[0047]** Moved (mono-mode) fibers

**[0048]** Moveable objective or objective parts

**[0049]** Mechanical x- and y-displacement of a suitable part or of the whole scanning system, e.g., with the help of acoustic-optical modulators

**[0050]** (As the two scanning systems must be independent of each other in the sense of the invention, a mechanical x- and y-displacement of the sample is not permissible.)

**[0051]** In the case of the Galvo mirror that is used frequently in commercial systems, a transformation, for example, corresponding to Equation (1a, 1b) is possible through suitable adjustment of the gain and offset values of the associated triggering electronics.

**[0052]** In confocal systems, coverage of the scanning coordinates of the imaging and manipulating system in three-dimensional space is possible. As in the plane, a transformation of the two scanning coordinate systems in space can be undertaken:

$$x=\Phi_1(u,v,w) \tag{2a}$$

$$y=\Phi_2(u,v,w) \tag{2b}$$

$$z=\Phi_3(u,v,w) \tag{2c}$$

**[0053]** Three-dimensional sample objects are captured in confocal imaging systems in which z microscopic images of the section planes x, y are recorded for each different sample depth.

**[0054]** Between recordings of the individual confocal split images, the sample depth z is varied in each case through a mechanical displacement of the sample, the objective or the entire microscope unit. In addition to the customary (micro) mechanical drive systems, one can also use acousto-optical modulators, especially in quick imaging systems for z-adjustment.

**[0055]** A preferred embodiment therefore uses two scanning systems that are independent in the x- and y-directions as the imaging system and manipulating system respectively, where at least one system has a programmable, automatically triggering beam deflection device so that a pixel-precise scanning cover is possible with the help of the affine mapping Equations (1a, 1b). In this preferred embodiment, the scanning process in the z-direction affects both systems identically; e.g., the sample or the common objective is displaced in the z-direction. In this case, it must be guaranteed that the scanning planes of the two independent modules overlap fully. A mutual adjustment ensures that scanning planes are not misaligned relative to each other. The comparison of the parallel scanning planes in the z-direction takes place preferably with the help of suitable motorized adjustable optics. The collimators described in DE 19702753 A1 are preferably used. The use of motorized optics for z-comparison of the two scanning planes enable the automated correction of chromatic longitudinal errors of the different objectives used, at the different excitation and manipulation wavelengths.

**[0056]** If, however, the two independent scanning modules do not have any common beaming direction on the sample, an independent scanning device is required for both systems in general in the z-direction. To implement a pixel-precise cover of the x, y, and z-scanning devices in a three-dimensional space in this case, one must use the generalized Equations (2a-c).

**[0057]** The determination of the concerned transformation equation with the help of which the two independent scanning systems can be superimposed with pixel precision requires a suitable calibration method. Hence, it has already been mentioned that the affine mapping Equations (1a, b) can be determined uniquely if the coordinates of at least three scanning field points are known in both the scanning coordinate systems.

**[0058]** FIG. **4** provides a schematic overview of the different calibration methods for the determination of the scanning field cover. It has already been explained at the beginning that the cover of the scanning fields of the two independent scanning systems depends on different adjustment dimensions. Thus for example, fine angle deviations between the different main and auxiliary beam splitters of the imaging system or between the different beam-combining elements used (compare FIGS. **1** and **2**) result in measurable differences in the cover of the two scanning systems. Especially in case of frequently used commercial scanning systems with Galvo mirrors as beam deflection device, the cover of the two scanning fields also depends on the scanning speed set in the two systems and the concerned selected scan zoom factor. In a design model of the invention, the calibration methods illus-

trated in FIG. **4** are determined for different setting combinations of the adjustable sizes of the system that influence the scanning field cover (e.g., determination of mapping equation (1a, b) for the different main and auxiliary beam splitters of the system and the different beam-combining elements of the replacement device displayed in FIG. **2**). This can be undertaken individually for the concerned adjustable combination by the device user, where suitable operating software is available. Another preferred design model of the invention enables the automatic determination of individual calibration records for all adjustable combinations of all relevant adjustable sizes, where the control software falls back on the concerned relevant calibration record as a function of the set device configuration.

**[0059]** In a calibration method according to the invention, the position of the stationary focus of the manipulating scanning system is determined with the help of the scanning imaging system. If this procedure is followed for a minimum of three focus positions of the manipulating system, it is possible to obtain a clear determination of the transformation equation (1a, b). Different practical embodiments of this calibration method are conceivable:

**[0060]** 1. In the simplest case, the stationary laser focus of the manipulating system is observed directly with the help of the confocal imaging system according to FIGS. **5***a* and *b*. In this calibration measurement, the imaging scanning module "scans" the object plane without beaming an exciting light. The manipulation focus appears in a dark image background (FIG. **5***c*) exactly when the stationary locus of the manipulating system is located within the detection volume of the imaging system. As the manipulating and imaging systems typically have the same beaming direction on the sample, a surface reflex of the manipulator focus is observed in a mirror located in the object plane, so that at least a small portion of this reflex (dotted line in FIGS. **5***a* and **5***b*) must pass the beam-combining element in the direction of the imaging system. This method is therefore ideally suited if a neutral splitter is used as a beam-combining element. Due to the typically very high sensitivity of imaging confocal systems, this calibration method is ideal in practice, but is also similarly suitable for any convenient dichroitic beam-combining elements in which even in the ideal case less than 1% of the reflected (drawn as a dotted line) manipulation light passes the beam-combining element in the direction of the imaging system. Further, this method requires an emission filter attachment in the imaging system which enables a direct observation of the manipulation wavelength. This is often not guaranteed in commercial systems, especially in the infrared and ultraviolet range.

**[0061]** 2. In a transformation of the calibration method 1 corresponding to FIG. **6** *a-c*, the stationary focus of the manipulating system is observed indirectly through the imaging system. In this context, the imaging system detects a frequency conversion such as luminescence, non-linear processes, or inelastic scattering such as Raman, which the stationary focus of the manipulating system creates in a suitable substrate located in the object plane or an intermediate image plane. Here too, the imaging system scans the object plane without beaming exciting light. As the wavelength of the manipulating system was not observed directly. Instead of the light produced by it in the range of the visible spectrum, this additional calibration method is often better adapted to the spectral properties of the beam-combining elements and the emission filter in the system than calibration method 1. Accordingly, calibration method 2 also allows an adjustment of the scanning field cover in the z-direction—even while using manipulation light in the ultraviolet or infrared range—i.e., a spectral range in which the detection optics (pinhole optics) of commercial imaging systems is typically not corrected. Ideally, the layer thickness of the calibration substrate in which the manipulating system creates the luminescent beam should be as small as possible because otherwise the spot observed in the imaging system becomes too large due to the absence of location discrimination with scattered light.

**[0062]** 3. In another transformation of calibration methods 1 and 2, a suitable unstructured sample substrate is modified through illumination with the stationary focus of the manipulating system, according to FIG. **7**. This light-induced sample modification can be, e.g., bleaching, photo activation or photo conversion of a fluorescent coloring substance, or even a thermally or mechanically induced sample change (e.g., laser ablation). Decisive for the calibration process is that this light-inducing modification is limited exclusively to the area of the stationary focus of the manipulating system, and it is stable at least sporadically. After this laser-induced sample modification is made at a minimum of three different scanning field positions, the thus structured sample substrate is measured with the help of the imaging system. The difference from the calibration methods 1 and 2 is that the calibration takes place in a two-phase process in which the image capturing after the sample structuring takes place with the excitation light of the imaging system, if necessary, also with the help of samples in which a modification, e.g., through optical switches can be reversed.

**[0063]** Decisive for the function of the three described calibration methods is a correct adjustment of the confocal opening of the concerned imaging system (e.g., pinhole for point scanners and slot opening for line scanners). In calibration methods 2 and 3, the signal light lies typically in the range of the visible spectrum (i.e., in the detection area typical in most applications). Hence, these calibration methods have the same requirements with regard to correct adjustment of the confocal opening as in the commercial confocal microscopes. In calibration method 1 however, the confocal opening is to be adjusted in such a way that a direct detection of laser light can take place in which the spectral range can lie, if necessary, even in the ultraviolet or infrared range.

**[0064]** Wavelength dependencies of the detection channel of the imaging system thus play the most crucial role in calibration method 1. In a design model of the invention, the three calibration methods 1-3 were combined with the automatic position optimization of the confocal opening. This automated adjustment of the confocal opening can be performed interactively by the device user—a suitable software interface is available for this—or it can also be undertaken fully automatically by microscope systems within the framework of the actual calibration method. The optimum adjusting positions for the concerned device settings can be stored in the corresponding calibration records.

**[0065]** In general, calibration methods 1 to 3 combine a dynamic scanning process of a module with a static focus positioning of the concerned other scanning module. In contrast, no spot bleaching is carried out in most of the applications described at the start. Instead, the bleaching process takes place within an extended "region of interest." All thus far explained calibration methods have the disadvantage that dynamic effects of the beam deflection device of one of the scanning modules cannot be determined during the calibra-

tion of the scanning field cover. As explained earlier, such dynamic effects are encountered especially in Galvo scanners in which the scanning field cover can depend, for example, on the concerned selected scanning speed and the concerned scanning zoom.

**[0066]** This disadvantage is rectified with the help of a basically different calibration method illustrated in FIG. **8**. In this dynamic calibration method ("area-based image matching"), a structured calibration preparation located in the object or a common intermediate image plane is measured separately by the two independent scanning systems, in which the triggering of the beam deflecting device is adjusted by at least one of the two scanning systems according to Equations (1a, 1b) in such a way that the images of the structured calibration sample captured by the two systems are brought for cover. This calibration method can be executed interactively by the device user, in which a suitable software interface is used to superimpose the sample images captured with the help of the two scanning systems. However, a fully automatic calibration routine is also conceivable in which the optimal superimposition of the sample images captured with the help of the two scanning systems is determined through computation, e.g., with the help of the cross correlation method. If Galvo scanners are used as beam deflecting devices, the electronic gain and offset settings of at least one scanning system are adjusted during the calibration of the scanning field cover.

**[0067]** A precondition of this calibration method is that both scanning systems enable the image capturing of the calibration sample independently of each other. If no detector suitable for the image capturing is integrated in the manipulating system (e.g., a cost-effective diode with simple grab electronics), then an external detector according to FIG. **8** (preferably in the transmission beam path) must be used for this purpose.

**[0068]** FIG. **9** illustrates an exemplary embodiment of a suitably structured calibration sample. This can be, e.g., a reflecting structure on a glass substrate or vice versa—a transparent structure on a reflecting substrate. In the calibration, the laser light reflected or transmitted (during used of an external detector) from the concerned scanning system on this calibration sample is used for image capturing. If a line scanner with a bar mirror as a space filtering element (DE 10257237A1) is used as an imaging system, then neither the reflected laser light nor the transmitted laser light can be detected directly. In this case one can execute the described calibration method in which the calibration structure is brought in direct contact with a homogeneous fluorescence medium, where a dark sample structure is detected in a bright fluorescent background. Another option is to illuminate the sample structure with the help of a bulb in the wide field and to scan it with the help of a confocal scanning system.

**[0069]** Due to the parallel data capture, this method is ideal, particularly if a confocal linear scanner is used as the imaging system. This calibration method has the advantage of being a dynamic method, i.e., relative changes between the image field overlap between the two scanning modules can be determined directly as a function of the scanning speed and scanning zoom. Thus, the dynamic effects of the concerned beam deflecting device can be considered in the appropriate calibration records.

**[0070]** All the thus far described methods of optimizing the scanning field overlap can be automated with the help of suitable software in which a constant interaction of the device user is required. In contrast, the arrangement displayed in FIG. **10** enables a fully automatic calibration of the scanning field cover without involving the user. The second output of the beam-combining element is used here to determine the mutual scanning field cover of the two scanning systems. Thus, corresponding to FIG. **10**, even in case of dichroitic beam-combining elements, a small portion of the injected light is reflected or transmitted in the direction of the second output. If there is a locally triggered flat image detector (e.g., a CCD or CMOS camera or a quadrant diode), the relative position deviations between the two scanning modules can be determined directly and corrected automatically without requiring any further intervention of the user (such as e.g., the insertion of a calibration sample in the object plane). The arrangement displayed in FIG. **10** therefore is ideal particularly for automated rule processes that enable subsequent device-internal correction of the concerned optimum scanning field cover in case of fluctuating environmental influences (e.g., temperature) and variable device settings (e.g., beam-combining elements, main beam splitters, zoom optics, objectives, wavelengths).

**1**. Method of operating a laser scanning microscope, in which at least two independently controlled first and second light distributions that can move in at least one direction through a beam-combining element illuminate a sample and the light is detected by the sample as it comes in, the method comprising the steps of:

causing the two scanning fields created by the light distribution on the sample to overlap, such that

a reference pattern is created on the sample with one light distribution, which is then captured by means of the second light distribution and used to create the overlap and/or

a reference pattern arranged in the plane of the sample or an intermediate image plane is captured by both scanning fields and used to create an overlap and/or

structural characteristics of the sample are captured by both scanning fields as a reference pattern and used to create the overlap, so that correction values are determined.

**2**. Method of operating a laser scanning microscope according to claim **1**, in which the first light distribution is moved over the sample to capture a sample image, and the second light distribution is used to manipulate the sample.

**3**. Method of operating a laser scanning microscope according to claim **1**, in which the reference pattern is a point distribution.

**4**. Method of operating a laser scanning microscope according to claim **1**, further comprising the steps of creating reference points by a sample manipulation system and capturing the reference points with an imaging system.

**5**. Method of operating a laser scanning microscope according to claim **1**, in which the reference pattern consists of at least three points.

**6**. Method of operating a laser scanning microscope according to claim **1**, further comprising the steps of creating a light point on the sample.

**7**. Method of operating a laser scanning microscope according to claim **5**, further comprising the steps of employing light reflected by the sample points.

**8**. Method of operating a laser scanning microscope according to claim **1**, further comprising the steps of capturing frequency-converted light, wherein the frequency-con-

verted light is created through a non-linear or linear interaction of the illuminating light with the sample.

**9**. Method of operating a laser scanning microscope according to claim **8**, in which at least one luminescence point is created on the sample.

**10**. Method of operating a laser scanning microscope according to claim **8**, with creation through inelastic Light scattering.

**11**. Method of operating a laser scanning microscope according to claim **1**, further comprising the steps of creating reference patterns from points with light-inducing sample modification.

**12**. Method of operating a laser scanning microscope according to claim **1**, in which grids are used as reference patterns.

**13**. Method of operating a laser scanning microscope according to claim **1**, in which a statistical structure distribution of the sample itself serves as the reference pattern.

**14**. Method of operating a laser scanning microscope according to claim **1**, in which a coordinate transformation is used to determine correction values.

**15**. Method of operating a laser scanning microscope according to claim **13**, with an affine transformation having at least three reference points.

**16**. Method of operating a laser scanning microscope according to claim **4**, in which a point-scanning or line-scanning system, or a scanning point distribution system or a Nipkow system is used as the imaging system.

**17**. Method of operating a laser scanning microscope according to claim **4**, in which the manipulating system is a point scanning device, and the scanning takes place preferably in two directions.

**18**. (canceled)

**19**. Laser scanning microscope according to claim **27**, having an imaging system and a manipulating system.

**20**. Laser scanning microscope according to claim **19**, in which the imaging system is a point-scanning system, a line-scanning system, a scanning point distribution system or a Nipkow system.

**21**. Laser scanning microscope according to claim **19**, in which the manipulating system is mapped and the scanning takes place preferably in two directions.

**22**. Laser scanning microscope according to claim **27**, with at least one laser as the light source.

**23**. Laser scanning microscope according to claim **27**, in which a movement takes place over the sample in at least one scanning direction.

**24**. (canceled)

**25**. Laser scanning microscope according to claim **27**, in which at least one of the beam deflecting devices is provided with Galvo scanners.

**26**. Laser scanning microscope according to claim **23**, in which one coordinate transformation takes place through the modification of gain and offset values of the associated triggering unit.

**27**. A laser scanning microscope comprising:

first and second light distributions;

a beam-combining element for combining the first and second light distributions that are controlled independently of each other and that can move in at least one direction;

scanning means interposed between the beam combining element and the sample for causing first and second scanning fields to cause the combined light distributions to illuminate the sample;

at least one detector to detect the light coming from the sample; and

overlapping means for superimposing the first and second scanning fields on the sample through the use of at least one reference pattern by overlapping the first and second scanning fields.

**28**. Laser scanning microscope of claim **27**, wherein the overlapping means comprises:

means for creating a first reference pattern on the sample with one light distribution;

means for capturing the reference pattern with the second light distribution; and

means for determining and adjusting correction values and for creating the overlap of the first and second light distributions.

**29**. Laser scanning microscope of claim **27**, wherein the overlapping means comprises:

means for capturing a reference pattern arranged in the sample plane or in an intermediate plane of the two scanning fields; and

means for determining and adjusting correction values and for creating the overlap of the first and second scanning fields.

**30**. Laser scanning microscope of claim **29**, wherein the overlapping means comprises:

means for capturing the structural characteristics of the means for determining and adjusting correction values, and

means for creating the overlap with the first and second scanning fields.

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