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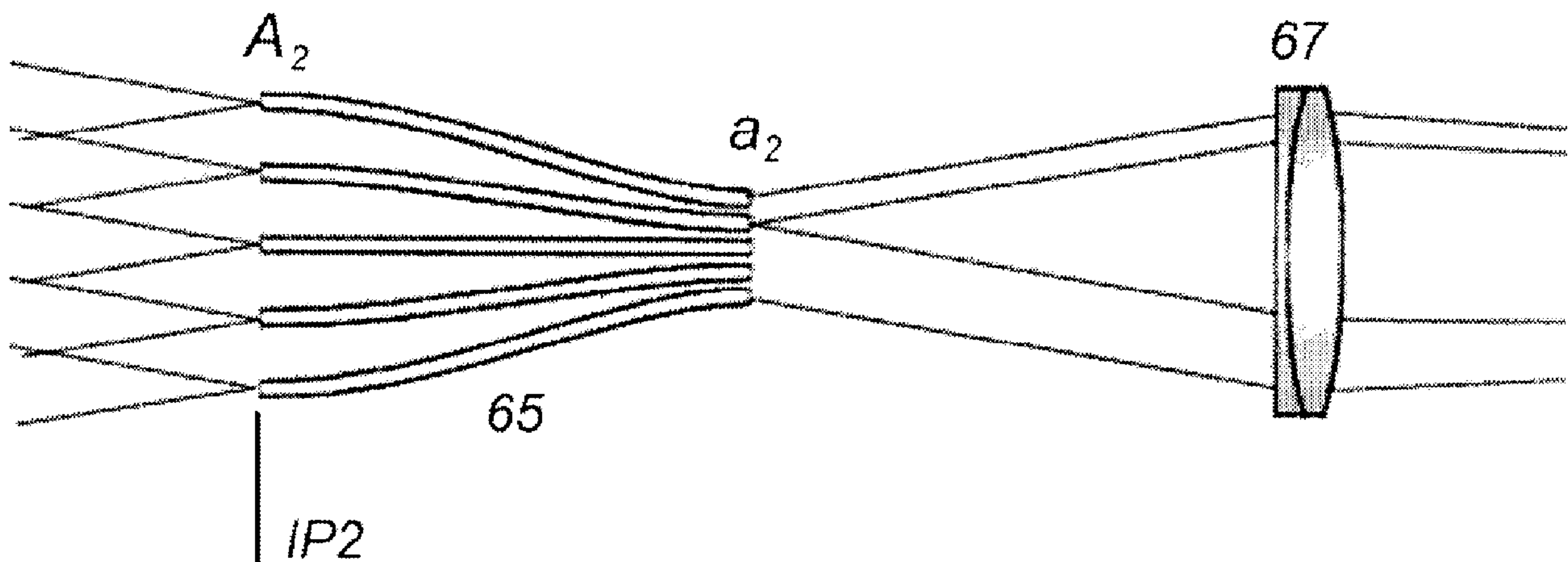
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(54) Titre : SYSTÈME DE MICROSCOPIE STED MULTICANAL EN TEMPS REEL

(54) Title: REAL TIME MULTICHANNEL STED MICROSCOPY SYSTEM



(57) Abrégé/Abstract:

The invention discloses a multichannel real time STED microscopy system providing high imaging frame rates and wide field of view super-resolution STED microscopy capable of resolving details below the Abbe diffraction limit. The multichannel real time

(57) Abrégé(suite)/Abstract(continued):

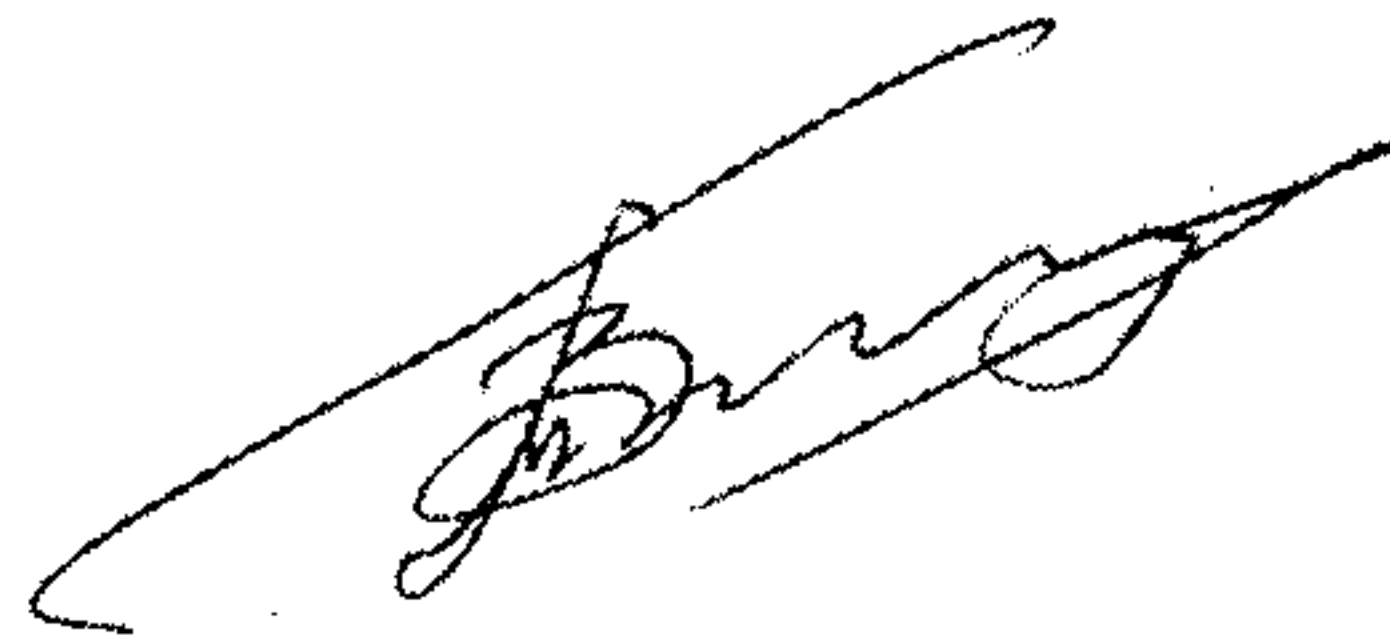
STED microscopy is a technique whereby observations of fluorophore-labeled samples is realized by scanning the sample ROI with a plurality of focused excitation light beamlets and spatio-temporally overlaying coaxial doughnut-shaped depletion light beamlets, having zero intensity central points, and synchronous scan of a digital camera image sensor face with a corresponding plurality of fluorescence light spots zoomed out so that their diameters are not exceeding STED super-resolution spot diameter, which is significantly smaller than the diffraction limited spot diameter. An optically sectioned STED super-resolution image of the sample ROI may be provided directly without any pre-processing, as a single frame of the camera.

Real Time Multichannel STED Microscopy System

ABSTRACT

The invention discloses a multichannel real time STED microscopy system providing high imaging frame rates and wide field of view super-resolution STED microscopy capable of resolving details below the Abbe diffraction limit. The multichannel real time STED microscopy is a technique whereby observations of fluorophore-labeled samples is realized by scanning the sample ROI with a plurality of focused excitation light beamlets and spatio-temporally overlaying coaxial doughnut-shaped depletion light beamlets, having zero intensity central points, and synchronous scan of a digital camera image sensor face with a corresponding plurality of fluorescence light spots zoomed out so that their diameters are not exceeding STED super-resolution spot diameter, which is significantly smaller than the diffraction limited spot diameter. An optically sectioned STED super-resolution image of the sample ROI may be provided directly without any pre-processing, as a single frame of the camera.

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Real Time Multichannel STED Microscopy System

FIELD OF THE INVENTION

The present invention relates to an optical microscopy system for stimulated emission depletion (STED), and a corresponding method for performing microscopy with such a system.

More particular, the present invention relates to a real time multichannel STED microscopy imaging method and devices providing high imaging frame rates and wide field of view super-resolution microscopy methods capable of resolving details below the Abbe diffraction limit.

BACKGROUND OF THE INVENTION

Fluorescence confocal microscopy is one of the most extensively used tools for our understanding of how cells function. Its popularity has steadily grown despite the fact that it notoriously fails to image structures smaller than about half the wavelength of light (~200 nm), i. e. that it is limited by the so-called 'diffraction barrier'.

Development of STED microscopy, which is one of the techniques that makes up the so-called 'super-resolution microscopy', highlighted the fact that the diffraction barrier to the spatial resolution can be effectively overcome in a regular far-field visible light microscope [1]. STED microscopy currently allows the nanometer scale imaging of the structures of a sample, which are marked with fluorescent labels, with a spatial resolution below the diffraction limit, while retaining most of the advantages of far-field optical operation, such as the ability to non-invasively image cells in 3D [2]. From the beginning of the process, STED has allowed fluorescence microscopy to perform tasks that had been only possible using electron microscopy.

In a STED microscope, the excitation beam is spatially overlapped on a second de-excitation or depletion doughnut-shaped beam (also called a STED beam) which can de-excite, by stimulated emission, the fluorescent markers previously excited by the excitation beam. Since the doughnut-shaped STED beam has at least one zero intensity point only the fluorescent labels in the proximity of the zero intensity points can actually emit fluorescence when they return to the ground state.

The probability of de-exciting the fluorescent species by stimulated emission increases with the increase of the depletion beam intensity, and the volume from which the fluorescent species emit decreases below the diffraction limit. When the two co-aligned beams are moved and the spontaneously emitted fluorescent light is recorded, the structures of interest marked with the fluorescent species are detected with a spatial resolution below the diffraction limit.

In the prior state of the art, a single point scanning STED system is made to scan a sample region of interest (ROI) point by point with increments smaller than the optical resolution limit of the optical system, and an image is captured at each stationary beam scan position producing an array of pixel intensities. The main disadvantage of single point scan STED microscopy, which has prevented its widespread use, is that the image acquisition speed

is relatively slow for large fields of view because of the need to scan the sample in order to retrieve an image. Thus, a frame rate of 200 frames per second (FPS) was achieved [3], but for a very small image size of $(1.8 \times 1.5) \mu\text{m}^2$ or 50×60 pixels only. Due to the sinusoidal movement of the resonant scanner, corrections to the image brightness and dwell times were necessary. Furthermore, super-resolution requires small pixels, which leads to a longer acquisition time and multiple exposures of the specimen. This is particularly critical in living biological tissue, which is easily damaged by excessive excitation light, and with fast bleaching fluorescence dyes.

P. Bingen et. al [4] describe an STED scanning fluorescence light microscope in which a light beam of excitation light overlapped with emission stimulation light is divided by two Wollaston prisms into four nearly parallel partial beams. The emission stimulation light in these four partial beams is phase modulated by segmented chromatic phase plate so that these partial beams, being focused into the sample, form four partial intensity distributions with the intensity minima of the emission stimulation light. The luminescence light emitted out of the four local intensity minima is spatially separated and registered. By parallel scanning the sample with the four probing beams, the time required for imaging the structure of interest is reduced to a quarter as compared to imaging the structure only using one probing beam.

It is desirable to scan a sample with significantly higher number of probing points. US Pat. 9075013 and US Application 2016/0363751 disclose apparatus and method for super-resolution fluorescence microscopy in which two orthogonal interference-fringe patterns of an inhibiting wavelength are superimposed on the object along with light that induces fluorescence. The fluorescent light emitted by the object from sub-diffraction-sized emission areas of the object is imaged onto an imaging photo-detector. The major drawback of the method is that images of adjacent fluorescent areas of the object overlap at the photo-detector. Each image frame must be processed with a de-convolution algorithm to separate the fluorescent signals thus providing a sparsely sampled super-resolution image. Multiple sparse images are interwoven to form a complete super-resolution image of the object.

An increased RESOLFT imaging rate may be achieved by implementing a method and a system disclosed in US Pat. 7619732, wherein, an optical signal is simultaneously detected from a number of focal points. This system may be, in principle, adopted for the multichannel STED microscopy, but its imaging rate is strongly restricted by a frame rate of a high resolution digital camera, by virtue of the fact that thousands of full high resolution images must be detected and co-processed to form a complete RESOLFT image of the sample ROI.

SUMMARY OF THE INVENTION

Real time multichannel STED microscopy systems may employ a principle similar to one of single point scanning STED systems, but image multiple points in the sample plane simultaneously. While the principles of scanning STED microscopy do not rest on those of the confocal microscope, multiple illumination and multi-focal scanning methods, developed for confocal microscopy, may be applied for implementation of a multichannel scanning STED microscope to great effect. Multi-focal scanning systems offer several useful advantages over single spot scanners, for example, faster scanning of the sample ROI enabling higher image capture rates and/or a large

sample ROI to be imaged simultaneously. Since a series of probing points scans an area in parallel each probing point is allowed to hover over a specific area for longer time thereby reducing the accumulative excitation energy needed to illuminate a sample when compared to single point scanning STED microscopes. Decreased accumulative excitation energy reduces photo-toxicity and photo-bleaching of a sample often making it the preferred system for 2D and 3D imaging live cells or organisms in life science research. Multifocal specimen scanning makes it possible to employ longer excitation laser pulses of a few nanoseconds in duration against femto- and picosecond pulse durations in conventional single-point scanning STED microscopes, thus making it possible to use high frequency Q-switched lasers as excitation and depletion light sources, providing significantly higher fluorescence signals and signal-to-noise ratios, when applying excitation light pulses of lower pick power.

In the process of multichannel STED microscopy imaging of a sample, a plurality of fluorescence light focal spots is provided on the image sensor face. A diameter of these focal spots is not less than a diameter d_1 of a magnified image of diffraction limited focal spots on the microscope sample, defined by a first zero diameter d of the microscope point spread function (PSF):

$$d_1 = d \cdot M = \frac{1.22 \cdot \lambda}{NA_M} \cdot M \quad (1)$$

Here λ is an imaging light wavelength, NA_M is a numerical aperture of a microscope objective lens, and M is the microscope magnification value. The STED microscopy resolution may be expressed in a number of lines L_S :

$$L_S = a/d_s = \kappa \cdot a_1/d_1 = \kappa \cdot L, \quad (2)$$

where $L = a/d = a_1/d_1$ is a diffraction limited resolution of the microscope in a lines number, d_s is the STED super-resolution spot diameter, a is the sample ROI size, and a_1 is a size of the ROI image in the microscope image plane and κ is the STED super-resolution factor [5]:

$$\kappa \approx \sqrt{1 + I_D/I_S}, \quad (3).$$

here I_D and I_S are pick intensities of the depletion light and of the saturation light for the fluorophore label probed. A total scan by, for example, spin disk scanner of the sample ROI, fluorescence from which is captured by a single camera frame, will not provide a STED super-resolution image of the ROI, but rather an image that exhibits a regular confocal microscope resolution, defined by (1). For getting STED super-resolution images in the manner developed for multi-focal confocal microscopy, the imaging spot diameter d_2 must be scaled down to be equal to or less than the STED super-resolution spot diameter, which is substantially less than diffraction limited d_1 , while keeping unchanged the frame size a_1 , that is

$$d_2 \leq d_s \cdot M = d_1 / \kappa \text{ or } L_2 = a_1 / d_2 \geq \kappa \cdot a_1 / d_1 = L_S \quad (4)$$

For these purposes, each fluorescence imaging beamlet has to be additionally focused by individual lenslet having focal length substantially shorter than focal length of a light beam segmentation mean, disclosed hereinafter. The same result may be obtained by magnifying the image frame size a_1 while keeping unchanged value of imaging spot diameters d_2 , thus satisfying the conditions (4).

The major objective of the present invention is a provision of a real time multichannel microscopy system capable of providing a high STED imaging rate, desired for 2D and 3D real time imaging live micro-objects and/or other biological specimens.

Another objective of the present invention is the provisions of a real time multichannel STED microscopy system generating a desired 2D STED super-resolution image directly as a single frame of a camera, without summarizing and co-processing of multiple sparse STED sub-frames.

Yet another objective of the present invention is the provisions of implementations of the real time multichannel STED microscopy system showing wide field of view.

The present invention describes methods of realization of a multifocal scanning STED microscopy, and examples of implementation of a wide-field real time multichannel STED microscope to achieve this requirement. It is seen as a relatively simple, cost effective instrument that acquire images faster and with less damaging effects on samples than the STED point scanning systems.

Even if the invention will be described by particular reference to a STED microscope in which the STED beam is the beam of depletion light in the following descriptions, the invention is not limited to STED microscopes but also relates to all other fluorescence light scanning microscopes using a beam of suppression light in addition to a beam of excitation light, like, for example GSD-microscopy, up-conversion-depletion microscopy [6], and/or other RESOLFT techniques [7].

Other features and advantages of the present invention will become apparent to those skilled in the art upon examination of the following drawings and the detailed description. It is intended that all such additional features and advantages be included herein within the scope of the present invention, as defined by the claims.

SHORT DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the following drawings. The components in the drawings are not necessarily to scale, emphasis instead being placed upon clearly illustrating the principles of the present invention. In the drawings, like reference numerals designate corresponding parts throughout the several views.

FIG. 1 depicts a schematic representation of a first exemplary embodiment of a multichannel STED microscopy system in accordance with the present invention. In addition to the major components and units there are shown in FIG. 1 conjugate image planes, and conjugate pupil planes of the optical setup, marginal rays of aggregated probe light beam, and aggregated imaging light beam, as well as marginal rays of a single probe beamlet and a corresponding imaging beamlet, both highlighted with gray.

FIG. 2 schematically depicts a magnified view of one possible implementation of the pulsed laser light source. Here, the excitation light beam is illustrated by outlined arrows and doubled lines, while depletion light beam, illustrated by bold arrows and solid lines.

FIG. 3, where the same notations are used, and like reference numerals are applied to like parts, presents a simplified schematic diagram of a second exemplary embodiment of the multichannel STED microscopy system in accordance with the present invention.

FIG. 4 presents a simplified schematic diagram of a third exemplary embodiment of a multichannel STED microscopy system in accordance with the present invention.

FIG. 5, presents an alternative implementation of the third exemplary embodiment.

FIG. 6 presents a simplified schematic diagram of a fourth exemplary embodiment of a multichannel STED microscopy system in accordance with the present invention.

FIG. 7 schematically depicts a magnified view of one possible implementation of the image rescaling means, implemented in the form of a bundle of optical fibers. .

It will be appreciated that for the simplicity and clarity of illustration, elements shown in the figures have not necessarily been drawn to scale. For example, the dimensions of some of the elements may be exaggerated relative to other elements for clarity purposes.

DETAILED DESCRIPTION OF THE INVENTION

Embodiments according to the present invention will now be described hereinafter with reference to the accompanying drawings, where similar reference numbers indicate similar or identical features throughout the drawings.

In describing exemplary embodiments of the present invention illustrated in the drawings, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology selected, and it is to be understood that each specific element includes all technical equivalents that operate in a similar manner to accomplish a similar purpose.

As used herein, "multichannel STED microscopy" refers to the confocal scan super-resolution microscopy technique, whereby fluorescence observations can be made by scanning a sample ROI with a plurality of focused STED beamlets comprising spatially and temporally overlapped pulses of excitation and of phase-modulated doughnut-shaped de-excitation or depletion light of a different wavelength.

The terms "light" and "radiation" may be used interchangeably and refer to radiation in the ultraviolet-visible-infrared spectral range. The term "light source" and "radiation source" may refer to any source able to generate

and emit radiation, including but not limited to, lasers, light emitting diodes, super luminescent diodes, super-continuum light sources, gas discharge lamps, or any other suitable radiation sources as would be apparent to someone skilled in the art.

A “beamlet”, as used herein, refers to any one of plurality narrow beams provided by lenslet array. An “individual beamlet (spot, axis)” refers to one of plurality of corresponding beamlets (spots, axes).

A “probe beamlet” or “STED beamlet”, as used therein, may be used interchangeably and refer to a two wavelengths beamlet, which being focused provides diffraction limited excitation light spot and a doughnut-shaped spot of depletion light, in other words, each probe beamlet presents a single channel of the multichannel STED microscope.

An “aggregated excitation (depletion, probe, imaging) beam”, as used herein, refers to any beam comprised of a plurality of corresponding beamlets, i.e. of excitation, depletion, probe, imaging beamlets.

A “compound beam”, “compound beamlet” as used herein, refers to any aggregated beam and/or beamlet comprised of two co-aligned beams of pulsed laser radiation of different excitation and depletion wavelengths.

NA – “numerical aperture”, is the sine of the maximum angle of a light beam in the range of angles over which an optical system can accept or emit light. NA of a converging and/or diverging light beam is the sine of the half-angle of the beam cone multiplied by a refractive index of the medium.

PSF – “point spread function” describes the response of an imaging system to a point source or point object; it is used to express how a particular object smaller than the diffraction limit appears under specific imaging conditions;

ROI – “region of interest” is an analyzed part of an object to be imaged;

FWHM – “full width at half maximum” is a parameter commonly used to describe the width of a “bump” or “dip” on a curve or function.

Referring now to FIG. 1, which is a simplified schematic diagram of the first exemplary embodiment of a real time multichannel STED microscopy system in accordance with the present invention. In addition to the major components and units there are shown in FIG. 1 a sample plane SP, conjugate image planes IP1, IP2, IP3 and conjugate pupil planes PP1, PP2 of the optical setup, marginal rays of aggregated probe light beam, comprising excitation and depletion laser radiation, and aggregated imaging light beam, as well as marginal rays of a single probe beamlet and of a corresponding imaging beamlet, both are highlighted with gray. Directions of the excitation and depletion light beams are illustrated by outlined and bold arrows respectively; a direction of the imaging light beam is illustrated by a stripy arrow.

The entire assembly of the first proposed embodiment is preferably comprising: a pulsed multi-wavelength light source 1 that provides excitation and depletion pulsed laser radiation; a multi-beam scanner module 2, based on twin spinning scan disks 21, 22 with a dichroic mirror 26, a phase modulation means 3, an optical microscope 4 incorporating a high magnification objective lens 41, a tube lens 42, a microscope stage 43, and a microscopy sample 44 fixed on said microscope stage 43 in a sample plane SP of the microscope 4, and a light detection and imaging module 6, which may preferably comprise a high resolution digital camera 61, relay optics, schematically presented in the form of two achromatic doublets 62, 63, and at least one blocking filter 64.

The pulsed multi-wavelength light source 1 provides collimated or nearly collimated preferably even ("top-hat") light beam of pulsed laser radiation, preferably comprising at least two coinciding light beams: a beam of pulsed excitation laser radiation of a first wavelength λ_1 , for exciting, in single- or multi-photon excitation mode, a fluorophore in a sample to be imaged, and a beam of pulsed depletion laser radiation of a second wavelength λ_2 for depopulation of excited states of fluorophore molecules and so suppressing spontaneous emission of fluorescence light by the fluorophore in the sample. Preferably, a temporal FWHM of the depletion light pulse exceeds a full duration of the excitation light pulse, and temporally overlays said excitation light pulse. The depletion light pulses may, for example, arrive at the sample plane synchronously with the excitation light pulses and proceed for at least 0.2 ns longer than the excitation pulses. The excitation and depletion laser pulse energies and/or pulse powers are functions of a number of the probe beamlets, physical properties of samples and used fluorophore labels, and optical properties of the STED microscopy system.

The pulsed multi-wavelength light source 1 may be built in a variety of forms, previously developed for illumination in confocal microscopy. This can be accomplished, for example, by coupling two separate lasers into a common optical fiber or by using a single multi-wavelength laser, a super-continuum light source, or another light source providing both the excitation and depletion light. One possible implementation of the pulsed multi-wavelength light source 1 is schematically depicted in FIG. 2. Said pulsed multi-wavelength light source may comprise at least two synchronized pulse light sources, for example, lasers: a pulsed excitation light source 11 for providing a beam of excitation light of a first wavelength λ_1 , and a pulsed depletion light source 12 for providing a beam of depletion light of a second wavelength λ_2 , light delivery optics schematically presented in the form of a dichroic mirror 13, a fiber coupling lens 14 providing coupling said excitation and depletion light into a fiber patch cord 15, and beam conditioning means schematically presented in the form a collimation lens 16, beam shaping element 17, and a projection achromatic doublet 18. The pulsed light sources 11, 12 may be synchronized with said spinning disk multi-beam scanner 2 and said light detection and imaging module 6.

Said beam conditioning means 16 – 18 provide a collimation and shaping of the delivered compound laser beam, in other words said means transform the diverging compound light beam outgoing from the distal end of said fiber 15 into collimated top-hat light beam of the two wavelengths laser radiation having millimeter-scale cross section and exhibiting high evenness of the transverse intensity distribution. Said beam shaping means 17 may be built in a variety of forms, previously developed for illumination in confocal microscopy, for example, those based on diffractive beam shaper elements, refracting optical components, such as crossed Powell lenses, filter means, or

beam shaping lens (for example GTH-5-250-4, by TOPAG), and may comprise some additional optical components, such as imaging and projection lenses, mirrors and similar.

A substantially even transverse distribution of the composed light beam exiting the pulsed multi-wavelength light source 1 may result in substantially equal intensities of the individual probe beamlets that results in uniform excitation and uniform depletion of fluorophore label molecules in the sample, which, after a complete scan across a sample to be probed by the proposed microscopy system, may result in a substantially uniform spatial resolution and brightness of a resulting STED image of the sample ROI and in avoiding apparition of undesired artifacts. Note that the power density of excitation light on sample plane is responsible for STED image brightness, while the power density of depletion light on the sample plane is responsible for STED image resolution and affecting its brightness.

Said multi-beam scanner module 2 may be described as a modified Nipkow-like disk setup; it may comprise the first spinning disk 21 and the second spinning disk 22, both mounted on a common shaft 20, and provided with a dichroic mirror 26. Said first spinning disk 21 comprises an array of positive lenslets 23, which are disposed in a pattern of several interleaved spirals, and have equal focal distances and numerical apertures NA_{23} , preferably proportional to the NA_{41} of the objective lens 41:

$$NA_{23} = NA_{41} \cdot F_{41} / F_{42} \quad (5)$$

Said first spinning disk 21 may be additionally provided with a metal coating mask for creating microlens circular apertures.

Said second spinning disk 22, mounted on the same shaft 20 as the first spinning disk 21, comprises a similar array of preferably positive lenslets 24, disposed coaxially with corresponding lenslets 23. It is designed to provide projection into image plane IP2 of the imaging fluorescence light spots, whose diffraction limited diameters d_1 are zoomed out by pairs of said lenslets 23, 24 to be equal to or less than d_2 , the STED super-resolution spot diameters $d_2 = d_1 \cdot F_{24} \cdot F_{36} / (F_{23} \cdot F_{37})$, i.e. to satisfy the condition (4), where the first zero diameter d_1 of an image of a diffraction limited PSF is defined by focal distances F_{41} , F_{42} and numerical apertures NA_{41} , NA_{23} of corresponding lenses 41, 42, 23:

$$d_1 = \frac{1.22 \cdot \lambda}{NA_{41}} \cdot \frac{F_{42}}{F_{41}} = \frac{1.22 \cdot \lambda}{NA_{23}}$$

Said lenslets 24 of the second spinning disk 22 may have focal distances F_{24} shorter and correspondingly numerical apertures NA_{24} higher than those of lenslets 23:

$$F_{24} \leq F_{23} / \kappa; \text{ and } NA_{24} \geq \kappa \cdot NA_{23} = \kappa \cdot NA_{41} \cdot F_{41} / F_{42} \quad (6)$$

Alternatively, said second spinning disk 22 may be implemented in the form of array of negative lenslets 24; absolute value of focal distances of such negative lenslet 24 and their numerical apertures must satisfy the same conditions (5). Image plane IP2 is the virtual image plane in this case.

Said multi-beam scanner module 2 may optionally comprise an array of pinholes 25, coaxial with corresponding lenslets 24 and, consequently, with corresponding lenslets 23, and disposed in the focal plane IP1 of said first spinning disk 21, or, alternatively, in the focal plane IP2 of said second spinning disk 22. Said pinhole array may be implemented in the form of an additional pinhole array disk fixed on the same shaft 20 or, rather, in the form of a pattern coating on the back side of said second spinning disk 22, as it is shown in FIG. 1, whereas said positive microlenses 24 are disposed on its front side. A thickness of said second spinning disk 22 may be equal to the focal distances of said lenslets 24 (in the disk material). The reasons for using the pinhole array are the rejection of stray and ambient light provided by this scheme. It is to be noted that confocality is not an ingredient of the concept of STED microscopy, because the STED microscopy resolution dominating factor is the depletion function $\eta(x,y,z)$, not the detection function of the confocal pinhole.

The dichroic mirror 26 is designed to reflect excitation and depletion radiation from said pulsed multi-wavelength light source 1 and to transmit fluorescence light from the sample to said light detecting and imaging module 6; it is placed between said microlens array disks 21, 22.

Said multi-beam scanner module 2 disposed so that a common focal plane of said lenslets 23 coincides with the image plane IP1 of said microscope 4. Said image plane IP1, the focal plane of said second microlens spinning disk IP2, and an image sensor plane IP3 of said camera 61 are arranged to be optically conjugate with each other, and further with the microscope sample plane SP.

Said phase modulation means 3 may be implemented in the form of a selective phase modulation means providing helical and/or radial phase modulation of depletion light in each of plurality of said probe beamlets, leaving the first radiation unaffected. It may be a chromatic beam shaping device implemented in the form of a diffractive lens that generates optical null (for example R. Menon et al [7]), birefringent chromatic optical element (for example US Pat. 8536543, US Pat. 8586945), a phase plate that relies on the different dispersion properties of different optical materials (for example, N. Bokor et al [8]), or of any other known or future type means. Said selective phase modulation means 3 in the form of said chromatic beam shaping device may be disposed in the pupil plane PP1 directly in the back of said microscope objective lens 41.

Alternatively, said phase modulation means 3 may be implemented in the form of a discussed hereinafter phase retardation plate 31 providing helical and/or radial phase modulation of said depletion radiation in each of said plurality of the probe beamlets, designed for the specific depletion wavelength λ_2 ; undesired partial modulation of said excitation radiation in each of said plurality of the probe beamlets may be avoided by implementation of said pulsed light source 11 in the form of a device providing said excitation radiation that have low spatial coherence.

Said phase modulation means 3 may be alternatively disposed in a pupil of optional relay optics, relaying said image plane IP1 onto intermediate image plane and vice versa, as it done, for example, in the second exemplary embodiment, depicted in FIG. 3.

The light detection and imaging module 6 of the first exemplary embodiment preferably comprises a high resolution imaging device 61 with at least one blocking filter 64, and relay optics, schematically presented in the form of two achromatic doublets 62, 63. Said imaging device 61 is to be preferably mounted so that the image sensor face is disposed in or close to the image plane IP3, provided by said relay optics 62, 63 and conjugate to IP2, IP1 and consequently to the sample plane SP. Said imaging device 61 may be preferably implemented in the form of high resolution, high sensitivity and low noise digital camera, such as EMCCD camera, or rather sCMOS camera (for example, *ORCA-Flash4.0 v2* by Hamamatsu, or *Neo 5.5* by Andor).

In the first exemplary embodiment, the pulsed multi-wavelength light source 1 provides preferably collimated top-hat light beam of pulsed excitation and depletion radiation. Lenslets 23 of said first spinning disk 21 transform said collimated compound light beam into an array of focused beamlets having parallel optical axes, equal numerical apertures, and a common focal plane coinciding with IP1, and forming in said image plane IP1 a corresponding plurality of regular nearly diffraction limited coaxial focal spots of excitation light and depletion light. Said selective phase modulation means 3 provides appropriate phase modulation of depletion part of each compound beamlet. Said optical microscope 4, provides demagnified projection in the sample plane SP of said plurality of probe beamlets, collects fluorescence light spontaneously emitted by the fluorophore in said plurality of excited areas confined by coaxial doughnut shaped spots of depletion light, and magnified imaging the plurality of the fluorescence light emitting points on said image plane IP1. Lenslets 23 of said first spinning disk 21 converts diverging fluorescence imaging beamlets from the image plane IP1 into collimated beamlets that further are focused sharply by lenslets 24 of said second spinning disk 22 into image plane IP2, and transferred by relay optics 62, 63 onto image sensor plane IP3 of said digital camera 61.

Said two spinning disks 21, 22 being co-rotated on a common shaft 20 at a constant speed, provide simultaneous swept field scan of the sample ROI by the plurality of focused excitation light beamlets and overlaying coaxial doughnut-shaped depletion light beamlets, phase modulated by said means 3, and synchronous scan of the IP2 and image sensor face of said digital camera 61 with a corresponding plurality of zoomed out spots of fluorescence light. Therefore, an optically sectioned fluorescent image, that is a super-resolution STED image of the sample 44 ROI, is formed on the image sensor of the digital camera 61. Thus STED images are provided in a manner, similar to one of a conventional real-time spinning disk confocal scanning microscope.

An exposure time of said high resolution digital camera 61 continues a number of the laser pulse periods enough to get a required resolution of STED images. When a shutter of said digital camera is open, every pulse of excitation and depletion light provided by said pulsed multi-wavelength light source 1 generates on the image sensor of said digital camera 61 a number of imaging points, equal to the number of STED probe beamlets. The shutter of said digital camera may be closed, when a distance between centers of any two adjacent probed points

in the sample plane ROI becomes equal or substantially less than a half of the desired STED resolution. The camera 61 readout signal provides the full frame STED image of the sample ROI.

A simplified schematic diagram of the second exemplary embodiment of the multichannel STED microscopy system in accordance with the present invention is displayed in FIG. 3, where the same notations are used and like reference numerals are applied to like parts.

This second exemplary embodiment of the real time multichannel microscopy system is similar in general to the first one shown in FIG. 1, and comprises the same major modules: the pulsed multi-wavelength light source 1, the multi-beam scanner module 2, based on the twin spinning scan disks 21, 22 with a dichroic mirror 26, the optical microscope 4 with the microscopy sample 44 fixed on the microscope stage 43, the light detection and imaging module 6, but it has an alternative implementation of said selective phase modulation means 3.

Most of current phase modulation plates, using specific phase masks, cannot sufficiently distinguish between wavelengths and may forge the excitation beam into something close to a doughnut and are thus not suitable for a direct use with pre-combined probe beamlets. For this reason the proposed selective phase modulation means 3 may comprise a phase modulation plate 31, path-doubling means, preferably built in the form of interferometer-like setup comprising two dichroic mirrors 32, 33 and two folding mirrors 34, 35, and relay optics schematically presented in the form of two achromatic doublets 36, 37.

Said phase modulation plate 31 providing phase retardation to the depletion beams yielding optimum confinement of the fluorescence, is to be built in the form of a polymer optical vortice (for example manufactured by RPC Photonics), that imparted a helical phase retardation to the depletion beamlets and yielding optimum confinement of the fluorescence mainly in the lateral directions, and/or in the form of a phase mask with a phase retardation of π over a central circular area yielding optimum confinement of the fluorescence mainly along the axial direction, and/or of any other proposed type (for example, US Pat. 7709809, J. Keller et al [9], US Pat. 8536543, CN 101907766, and CN 102122080).

Said dichroic mirrors 32, 33 of said path-doubling means may be designed, for example, to reflect excitation light to the pupil plane PP3a, to transmit depletion light to the pupil plane PP3b, where said modulation plate 31 is disposed, and to transfer fluorescence imaging light. Said path-doubling means may be built with alternative beam splitting components, such as, for example, polarizing beam splitters, diffraction gratings, or other suitable optical means, as would be apparent for one skilled in the art.

Said relay optics 36, 37 and said path-doubling means 32 – 35 are arranged to reimage said array of compound focal light spots, provided by said first spinning disk 21 in the illumination aperture IP4, onto microscope image plane IP1 through a common pupils in twin pupil planes PP3a, PP3b, and to reimage fluorescence light spots form IP1 to IP4 via both twin pupil planes PP3a, PP3b without significant transformation because of non-coherence of the fluorescence light.

The second exemplary embodiment of the proposed real time multichannel STED microscopy system operates similarly to the first embodiment, with the difference that the compound probe beamlets provided by said first spinning disk 21 are focused into a common focal plane IP4 conjugate to the image plane IP1 of the microscope 4, and forming in said image plane IP4 a corresponding plurality of regular diffraction limited coaxial focal spots of excitation light and depletion light. Said selective phase modulation module 3 provides helical and/or radial phase modulation of said depletion part of each of the plurality of probe beamlets, leaving its excitation part unchanged, and forms in the image plane IP1 a corresponding plurality of overlapped coaxial regular nearly diffraction limited focal spots of excitation light and doughnut shaped spots of depletion light. The lens 36 converts all the plurality of the diverging compound beamlets into a converging bundle of collimated compound beamlets. Said dichroic mirror 32 splits every collimated probe beamlet into comprising its excitation and depletion collimated beamlets. Said turning mirror 35 directs said plurality of the collimated excitation beamlets through the pupil plane PP3a to the dichroic mirror 33, where each excitation beamlet recombines with its counterpart depletion beamlet directed there by the turning mirror 34 through said phase modulation plate 31 disposed in the duplicate pupil plane PP3b. Every individual depletion light beamlet is modulated by said phase modulation plate 31. The lens 37 projects recombined probe beamlets into the microscope image plane IP1 conjugate to the sample plane SP. Said optics 37, 36 relay fluorescence light spots from IP1 to IP4, which are further reimaged with scanning onto the digital camera 61 sensor face in the IP3. The STED super-resolution images of the sample ROI are provided by said digital camera 61 in the same manner, as in the first embodiment.

It should be noted that the first and the second exemplary embodiments may comprise multi-beam scanner module 2, alternatively implemented in the form of a single-axis or biaxial lateral translation scanner, comprising two properly aligned lenslet arrays: the first lenslet array similar to the first spinning disk 21 and the second lenslet array similar to the second spinning disk 22, and an optional pinhole array, all are fixed one relative to another, with the dichroic mirror 26 in-between. Said scanner that controls said the first and the second lenslet arrays may provide a conjoint translational single-axis or biaxial lateral movement thereof in predetermined manner resulting in synchronous scan of the sample ROI with said plurality of probe beamlets and of camera image sensor face with a corresponding plurality of said scaled down fluorescence imaging light spots.

A simplified schematic diagram of the third exemplary embodiment of the multichannel STED microscopy system in accordance with the present invention is displayed in FIG. 4, where the same notations are used and like reference numerals are applied to like parts.

This third exemplary embodiment of the multichannel microscopy system comprises the same pulsed multi-wavelength light source 1, the light beam segmentation means in the form of the first lenslet array 27, the same selective phase modulation means 3 comprising phase modulation plate 31, the path-doubling means 32, 33, 34, 35, and relay optics 36, 37, the same optical microscope 4 with the microscopy sample 44, the multi-beam scanner module comprising a scanner 51 with a scanning mirror 52, and alternatively implemented light detection and imaging module 6'.

The light detection and imaging module 6' of the third exemplary embodiment preferably comprises the same high resolution imaging device 61 with the blocking filter 64, the same relay optics 62, 63, an imaging lens 67, image rescaling means in the form of a second lenslet array 65, an optional pinhole array 66, and an image scanning means, schematically presented in the form of a scanning mirror 55 controlled by a scanner 54, similar or identical to and synchronized with said scanner 51. Said light detection and imaging module 6' may be coupled to a remaining part of the STED microscopy system with said dichroic mirror 32, as it is shown in FIG. 4, alternatively it may be coupled with an appropriate dichroic mirror, transmitting fluorescence light and replacing one of said turning mirrors 34, 35, or with an additional specific dichroic mirror.

Said light beam segmentation means 27 and said image rescaling means 65 may comprise linear or preferably 2D segmented arrays of focusing elements, each of which is described hereinafter as a "microlens array" or "lenslet array". This is for the sake of brevity and clarity only; it may refer to any plurality of optical elements that can be used to focus light onto a plurality of focal points in a corresponding focal plane. The optical elements may be microlenses, micromirrors, or any other focusing elements, as it would be apparent to someone skilled in the art.

Said lenslet array 27 is preferably built in the form of square or hexagonal packed array of identical positive spherical or aspheric microlenses 28 with equal focal distances F_{28} and numerical apertures NA_{28} , defined by the following condition similar to (5):

$$NA_{28} = NA_{41} \cdot \frac{F_{41}}{F_{42}} \cdot \frac{F_{37}}{F_{36}}.$$

It may be provided with a metal coating mask for creating microlens circular apertures. The common focal plane of said lenslet array 27 coincides with an intermediate image plane IP2 conjugate to the image plane IP1 and the sample plane SP.

Said multi-beam scanner module, schematically presented in the form of the scanning mirror 52, driven by piezo-scanner 51, may be built as step-by-step or continuous mode scanners in a variety of forms, similar to those proposed and developed for conventional confocal microscopes, for example, those based on 1-D array and/or 2-D array so called swept field (for example U.S. Pat. No. 6,856,457, or U.S. Pat. No. 2011/0002024), 2-D array bi-directional raster (for example, U.S. Pat. No. 4806004, or U.S. Pat. No. 7209287) or random-sampling stochastic (for example, U.S. Pat. No. 7990524) scanners employing single- or bi-directional angle scanning galvo-scanner or piezo-scanner, as well as single- or bi-directional translational scanning means of any kind.

Said scanner module 51, 52 provides a simultaneous scan of ROI of the sample 44 by the plurality of probe beamlets formed by said light beam segmentation means 27 and modified by said selective phase modulation module 3 and de-scan the corresponding plurality of fluorescent light beamlets originating from the focal points on the sample plane SP and collimated by said lens 37.

Said lenses 37, 67 relay the microscope image plane IP1 onto a conjugated virtual image plane IP2', in a vicinity of which said second lenslet array 65 is placed providing a real image plane IP2, where said optional pinhole array may be disposed. A reflection surface of said image scanning mirror 55 is disposed close to the pupil plane PP3 of said relay optics 62, 63.

Said image rescaling means may be built in the form of a second lenslet array 65 comprising an equal number of similarly arranged lenslets, as the first lenslet array 27. Lateral dimensions of individual microlenses and of whole lenslet array 65 are proportional to those of the lenslet array 27 with the proportionality coefficient $c = F_{67} / F_{36}$.

A focal distance F_{65} of the lenslet 65 and its distance z_{65} from said lens 67 have to provide an effective lenslet focal distance F_E that satisfies the conditions (4) and (6), the last may be rewritten as:

$$F_E \leq c \cdot F_{27} / \kappa ;$$

where

$$F_E = \frac{F_{65} \cdot F_{67}}{F_{65} + F_{67} - z_{65}}.$$

Alternatively, said image rescaling means may be implemented in the form of optical setup providing magnification of the image frame size from a_2 up to $A_2 \geq \kappa \cdot a_2$, while keeping unchanged value of imaging spot diameters d_2 thus satisfying the conditions (4). Such a setup may be realized, for example, by applying a bundle of optical fibers with close packed front fiber ends and widely spaced distal fiber ends, as it is schematically presented in FIG. 7.

Said relay optics 62, 63, said scanning mirror 55, and said high resolution digital camera 61 are arranged to relay with a desired scale the image plane IP4 via said scanning mirror 55 and via said blocking filter 64 onto the conjugate image plane IP5, where a sensor face of said digital camera 61 is disposed, thus providing imaging the multitude of magnified super-resolution fluorescence light spots originating from the STED-confined focal points on the sample plane SP onto the image sensor face of said digital camera 61.

Said scanner 51, 52 and said synchronized image scanner 54, 55 may be implemented as a single unit, for example, in the form of a bilateral mirror swept field galvo-scanner providing scanning deflection of probe beamlets, descanning fluorescence imaging beamlets, both by a front side of the mirror, and simultaneous rescanning deflection of imaging beamlets by the back side of the bilateral mirror (for example, US Pat. No. US7463396).

The Third exemplary embodiment of the proposed multichannel STED microscopy system operates similarly to the first and to the second ones. Said multi-beam scanner module 51, 52 provides a periodic simultaneous scan across the ROI of the sample 44 by the plurality of probe beamlets, while said image scanner 54, 55 provides a synchronous and proportional scan across the image sensor face plane IP3 of said digital camera 61 by the

plurality of imaging beamlets. The shutter of said digital camera 61 is open for the full scan period. After the shutter closing, readout signal of said digital camera 61 provides a STED super-resolution image of the sample ROI.

FIG. 5, where the same notations are used and like reference numerals are applied to like parts, presents one of multiple alternative implementations of said third exemplary embodiment, operating in the same manner and providing the same results.

Said light detection and imaging module 6' of the third exemplary embodiment, presented in the FIG. 4 and/or FIG. 5 may be alternatively implemented as an optical detector array comprising a number of individual detectors equal to the number of STED imaging beamlets, with their light detection planes disposed in the image plane IP3. Each individual light detector, synchronized with said pulsed laser light source 1 forms a single-channel STED microscopy setup. Said optical detector array may be implemented, for example, in the form of a unit 68 of a fourth exemplary embodiment. FIG. 6, where the same notations are used, and like reference numerals are applied to like parts, presents a simplified schematic diagram of a fourth exemplary embodiment of the STED microscopy system in accordance with the present invention. This fourth exemplary embodiment is similar in general to the third one, but has the two separated pulsed light sources, not aggregated into the single unit, namely: a first pulsed light source 7 providing a collimated beam of the first (excitation) radiation and a second pulsed light source 8 providing a collimated beam of the second (depletion) radiation, followed respectively by two identical lenslet arrays 27A, 27B, and identical projection lenses 36 38. Further entire assembly comprises the depletion light phase modulation plate 31, the dichroic mirror 32 reflecting excitation radiation and transmitting light of a fluorescence wavelength range, the dichroic mirror 33 transmitting depletion radiation and reflecting the the excitation and fluorescence light, the optical microscope 4 with the microscopy sample 44, the multi-beam scanner means 51, 52, and a multi-channel light detection module 6".

Said first pulsed laser light source 7 schematically presented in the insertion of FIG 6, may comprise a light source 11 providing a beam of pulsed excitation radiation of a first wavelength λ_1 , and light delivery optics comprising a fiber coupling lens 14, an optical fiber 15, a collimation lens 16, beam shaping means 17, and a projection lens 18; all identical to the corresponding components of FIG. 2. Said second pulsed laser light source 8 may comprise a second light source 12 providing a beam of pulsed depletion radiation of a second wavelength λ_2 and the same light delivery and conditioning components 14 – 18. Said pulsed light sources 11, 12 are synchronized with each other, with said scanner 51, and with said multi-channel light detection module 6".

Said projection lenses 36, 38 form, in combination with said lens 37, two coupled relay systems having a common image plane IP1, namely the excitation light relay system 36, 53 with its object plane IP2a and pupil plane PP2a, and the depletion light relay system 38, 53 with its object plane IP2b and pupil plane PP2b, where said phase modulation plate 31 is placed. Said pupil planes PP2a and PP2b are conjugate to the pupil plane PP1 of the microscope objective lens 41.

Said multi-channel light detection module 6" of the embodiment preferably comprises a 2-dimensional optical detector array 68, imaging optics, schematically presented in the form of an achromatic lens 63, and at least one blocking filter 64. It may be coupled to a remaining part of the STED microscopy system, for example, with said dichroic mirror 32, as it is shown in FIG. 5.

Said optical detector array 68 comprises a number of individual detectors 69 preferably equal to the number of individual STED probe beamlets and to the number of active microlenses in said lenslet arrays 27a, 27b, or may exceed these values. Detection planes of said individual detectors 69 are disposed close to the image plane IP3 provided by said lens 63, and conjugate to said image plane IP1 and to the sample plane SP. The optical detector array 68 may be built, for example, in the form of a low-resolution digital camera having high readout speed (for example *iXon 860* by Andor Technology, or *Evolve 128* by Photometrics). Each individual light detector 69, formed by a single pixel of the camera 68 or by a specified group of adjacent pixels, is meant for detection of fluorescence light spontaneously emitted by the fluorophore in a focal spot provided by a corresponding probe beamlet in the sample plane SP. Said optical detector array 68 may optionally comprise a corresponding pinhole array (not shown).

Alternatively, said multi-channel light detection module 6" may be implemented in the form of said light detection and imaging module 6' disclosed in the third exemplary embodiment, that is may comprise the same similarly arranged parts: the high resolution digital camera 61, the second lenslet array 65, the optional pinhole array 66, the image scanning means 54, 55, and the second relay optics 62, 63.

All other components of the fourth exemplary embodiment of FIG. 5 are the same, as of the third embodiment depicted in FIG. 4 and are operating in the same manner.

Said lenslet arrays 2A, 2B split excitation and depletion light beams provided by said pulsed light sources 7, 8 correspondingly into identical pluralities of beamlets and focus them into similar pluralities of diffraction limited focal points in corresponding illumination apertures IP2a, IP2b conjugate to the microscope image plane IP1 and the sample plane SP. The projection lenses 36, 38 convert said pluralities of the diverging excitation and depletion beamlets into two bundles of collimated beamlets crossing in the corresponding pupil planes PP2a, PP2b. Said phase modulation plate 31 placed in the pupil plane PP2b provides appropriate phase modulation of each depletion beamlet. Every phase modulated depletion light beamlet is coupled coaxially on said dichroic mirror 33 with a corresponding excitation beamlet directed there by dichroic mirror 32. The lens 37 projects said plurality of thus coupled probe beamlets into the microscope image plane IP1. Said optical microscope 4, provides scaled down projection in the sample plane SP of said plurality of probe beamlets, and magnified imaging the plurality of the STED confined fluorescence light emitting areas on the sample plane SP into said image plane IP1 conjugate to the image plane IP3.

Said scanner 51, 52 provides simultaneous scan across the sample 44 ROI by said plurality of probe beamlets and de-scan the corresponding plurality of fluorescent light beamlets originating from the focal points on the

sample plane SP and collimated by said objective lens 41 for being projected onto sensor array 68 of said light detection and imaging module 6". Each individual light detector 69 of said optical detector array 68 being synchronized with said pulsed lasers 11, 12, detects amplitude of optical fluorescence signals originating from the focal point, provided by a corresponding probe beamlet. Thus said light detection and imaging module 6" provides a sparsely sampled super-resolution image frame, consisting of a number of image points, equal to the number of probe beamlets, for every pulse of the probe excitation and depletion light.

This information, being pre-processed in combination with the scanner 51 data, provides intensities (photon counts) and coordinates of the corresponding number of image points of a desired STED image. In between two consecutive laser pulses, the scanner shifts the array of the STED probing points in the sample plane for a single scan step, defined by the desired microscopy system resolution, and the scan method used.. Multiple sparse images are interwoven to form a complete STED super-resolution image of the sample ROI.

The third and the fourth disclosed exemplary embodiments of the multichannel STED microscopy system may be built to provide a sample scanning with a plurality of linear STED projections, for which purposes said light beam segmentation means 27, 27a, and 27b are to be implemented in the form of cylindrical lenslet arrays and the phase modulation plate 31 is to be implemented in the form of linear π -step phase plate, oriented parallel to the axes of cylinder lenslets, and corresponding scanning means are to be implemented in the form of single-axis scanners.

All the exemplary embodiments of the multichannel STED microscopy system disclosed may be successively applied for realization of parallel STED imaging different fluorescent labels in different wavelength ranges by employment of probe beamlets comprising two or more different pairs of excitation-depletion pulsed laser radiation. For example, it may comprise pulsed excitation radiation of two different wavelengths and single wavelength pulsed depletion laser radiation. Such STED images of micro-structures labeled by different fluorophores may be produced by time or spectral separation of corresponding imaging signals.

The disclosed multichannel STED microscopy systems may be also successively used for Z-stack scanning providing multiple 2D slice super-resolution STED images in order to obtain 3D microstructure reconstructions. It may be done by utilizing an additional one-axis Z-scanner, for example a piezo objective focus scanner. The focal plane of the microscope is stepwise adjusted and a series of 2D STED slices are obtained with one of the phase modulation masks. One can then reconstruct the 3D model of the sample by combining and post-processing the 2D slices by use of appropriate software.

It should be noted that the specific preferred embodiments have been particularly shown and described heretofore only for the purpose of explaining and illustrating the present invention. It will therefore be apparent to those skilled in the art that various changes, modifications or alterations to the invention as described herein, may be made without departing from the spirit and scope of the invention and essential characteristics thereof.

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Real Time Multichannel STED Microscopy System

CLAIMS

What is claimed is:

1. A fluorescence microscopy system, in particular a real time multichannel stimulated emission depletion (STED) microscopy system for imaging a structure of interest in a sample comprising:
 - an optical microscope comprising a microscope objective lens, a tube lens, and a microscope stage and having conjugate a sample plane and a first image plane;
 - a microscopy sample comprising structures of interest labeled with a fluorescent dye and placed on said microscope stage to be imaged;
 - a first light source that provides a collimated light beam of pulsed first radiation having a first wavelength λ_1 for exciting, in single- or multi-photon excitation mode, a fluorophore in a sample to be imaged;
 - a second light source that provides a collimated light beam of spatially coherent pulsed second radiation having a second wavelength λ_2 for depletion of an excited state the fluorophore in the sample, said second light source is synchronized with said first light source, and pulses of said second radiation temporally overlay pulses of said first radiation;
 - light beam segmentation means comprising at least one segmentation lenslet array and providing a plurality of compound light beamlets, each of which comprises said first radiation and coaxially aligned said second radiation; said plurality of compound beamlets is focused into a corresponding plurality of focal points in an illumination plane conjugate to said sample plane;
 - phase modulation means for doughnut-shape phase modulation of said second radiation in each of said plurality of compound light beamlets; said phase modulation means provides in tandem with said beam segmentation means a corresponding plurality of probe beamlets;
 - light detection and imaging means comprising an imaging detector with its image sensor plane disposed in a plane conjugate to said sample plane, and at least one blocking filter;
 - a beam sampling optics separating said probe beamlets and coming from the opposite direction imaging beamlets and guiding said imaging beamlets toward said light detection and imaging means;

- a first scanner meant for scanning across said microscopy sample with said plurality of probe light beamlets, and for de-scanning corresponding plurality of imaging fluorescence light beamlets originating from the sample;
2. The STED microscopy system according to Claim 1, wherein said first light source and said second light source are designed as a single unit that provides a collimated light beam of pulsed radiation of at least two wavelengths: said first radiation for exciting a fluorophore in a sample to be imaged, temporally overlaid by said second radiation, which is spatially coherent, for depletion of the excited state of the fluorophore in the sample.
 3. The STED microscopy system according to Claim 2, wherein said light beam segmentation means are implemented in the form of a single segmentation lenslet array.
 4. The STED microscopy system according to Claim 3, wherein said phase modulation means are selective phase modulation means providing helical and/or radial phase modulation of said second radiation in each of plurality of said probe beamlets, leaving the first radiation unaffected.
 5. The STED microscopy system according to Claim 4, wherein said selective phase modulation means are built in the form of a chromatic beam shaping device.
 6. The STED microscopy system according to Claim 1, wherein said first light source provides a collimated light beam of pulsed first radiation having low spatial coherence.
 7. The STED microscopy system according to Claim 6, wherein said phase modulation means are built in the form of a non-selective phase modulation plate.
 8. The STED microscopy system according to Claims 5, 7, wherein said phase modulation means are disposed close to the pupil plane of said microscope objective lens.
 9. The STED microscopy system according to Claim 1 that further comprises first relay optics having an intermediate pupil plane and relaying said illumination plane onto the first image plane of said optical microscope.
 10. The STED microscopy system according to Claim 9, wherein said phase modulation means are disposed close to said intermediate pupil plane.
 11. The STED microscopy system according to Claims 4, 10, wherein said selective phase modulation means comprise a phase modulation plate of Claim 7, disposed in said intermediate pupil plane, and path-doubling means, preferably built in the form of interferometer-like setup comprising two dichroic mirrors and two folding

mirrors, and arranged so that said second radiation traverses said phase modulation plate, and said first radiation is guided around said phase modulation plate.

12. The STED microscopy system according to Claim 1, wherein said light beam segmentation means comprise:

- a first segmentation lenslet array providing a plurality of excitation beamlets of said first radiation focused into a first illumination plane conjugate to said sample plane;
- a second segmentation lenslet array providing a plurality of depletion beamlets of said second radiation focused into a second illumination plane conjugate to said sample plane;
- beam coupling means in the form of a dichroic beam splitter for coaxial coupling said plurality of excitation beamlets and said plurality of depletion beamlets providing a corresponding plurality of compound light beamlets, each of which comprises said first radiation and coaxially aligned said second radiation.

13. The STED microscopy system according to Claim 12, wherein said phase modulation means are built in the form of a phase modulation array disposed close to a back focal plane of said second beam segmentation lenslet array.

14. The STED microscopy system according to Claims 7, 12, that further comprises two relay systems coupled to each other and relaying said first illumination plane and said second illumination plane into common image plane, which is said first image plane of said optical microscope, and having a first pupil plane and a second pupil plane, both conjugate to the pupil plane of said microscope objective lens; said phase modulation plate is placed in said second pupil plane.

15. The STED microscopy system according to Claim 1 wherein said imaging detector of said light detection and imaging means is built in the form of a high resolution digital camera, and said light detection and imaging means further comprise:

- image rescaling means providing in a second image plane fluorescence imaging light spots scaled down as compared with their inter-spot distances;
- relay optics reimaging said second image plane onto a sensor plane of said high resolution imaging camera;
- a second scanner identical to and synchronized with said first scanner and meant for scanning across said image sensor plane of said digital camera with a plurality of said scaled down fluorescence imaging light spots.

16. The STED microscopy system according to Claim 15, wherein said image rescaling means are built in the form of an imaging lenslet array having a positive or negative focal distance significantly shorter than a focal distance of said at least one segmentation lenslet array.
17. The STED microscopy systems of Claims 1, 15, wherein said scanners are selected from the group comprising a galvanometer-controlled or a piezo-controlled mirror, a tilting plate beam shift scanner, an acousto-optical deflector, polygonal reflecting and refracting scanners, a translation scanner, a spinning disk, and a microelectromechanical system.
18. The STED microscopy system according to Claim 15, wherein said first scanner and said second scanner are implemented in the form of a combined scan device.
19. The STED microscopy systems of Claim 18, wherein said combined scan device is implemented in the form of a bilateral scanning mirror, which provides a scanning deflection of probe beamlets and descanning fluorescence imaging beamlets, both by a front side of the mirror, and synchronous rescanning deflection of imaging beamlets by a back side of said bilateral mirror.
20. The STED microscopy system according to Claim 18, wherein said combined scan device is implemented in the form of translation scanner providing a conjoint single-axis or biaxial lateral movement of said segmentation and said imaging lenslet arrays.
21. The STED microscopy system according to Claim 20, wherein said segmentation lenslet array and said imaging lenslet array are fixed coaxially relative to each other.
22. The STED microscopy system according to Claim 21, wherein said beam sampling optics is implemented in the form of a dichroic beam splitter reflecting said first radiation and said second radiation, transmits said fluorescence imaging light, and is disposed between said segmentation and said imaging lenslet arrays.
23. The STED microscopy system according to Claim 22, wherein said segmentation lenslet array and said imaging lenslet array are built in the form of twin microlens array disks fixed on a common shaft, conjoint scanning is provided by co-rotation of said twin microlens array disks on said common shaft.
24. The STED microscopy system according to Claim 1 wherein said imaging detector of said light detection and imaging means is implemented in the form of 2-dimensional optical detector array comprising a number of individual detectors equal to the number of said probe beamlets.
25. A STED microscopy system according to Claim 1, wherein:
 - said segmentation lenslet array of said light beam segmentation means are built in the form of a first lenticular comprising one or more cylindrical or acylinder lenslets, and providing one or more

compound beamlets each of which is focused in a first direction and collimated in an orthogonal second direction;

- said phase modulation means are implemented in the form of a linear structure comprising one or more retarding areas, providing π -step retardation of said plurality of depletion beamlets of second radiation, which, being focused by said microscope objective lens, form in said sample plane a corresponding plurality of diffraction limited focal lines of excitation light overlaid with double-line shaped depletion light projections; and
- said first scanner implemented in the form of a single axis multi-beam scanner.

26. A STED microscopy system according to Claims 1, 25 further comprising a Z- scanning means for Z-stack scanning providing multiple 2D slice STED images in order to obtain 3D super-resolution reconstructions of a microstructure.

27. A method of real time multichannel STED microscopy imaging a structure of interest in a sample, the method comprising the steps of:

- providing a sample, comprising structures of interest labeled with a fluorescent dye;
- providing a plurality of probe beamlets each of which comprises pulsed first radiation having a wavelength λ_1 for exciting a fluorophore labels in a sample to be imaged and synchronized and spatio-temporally overlaying doughnut-shape phase modulated pulsed second radiation having a second wavelength λ_2 , for depletion of excited states of fluorophore molecules;
- directing and focusing said probe beamlets onto said sample, thus providing in the sample ROI a corresponding plurality of probe areas in the form of nearly diffraction limited focal spots of the first (excitation) radiation overlaid with coaxial doughnut-shaped spots of the second (depletion) radiation having zero intensity central points, thereby confining the volumes of effective molecular fluorescence,
- collecting fluorescence light spontaneously emitted by the fluorophore in said plurality of confined areas of effective molecular fluorescence , and magnified imaging thereof on an intermediate image plane;
- scaling down each of said plurality of diffraction limited fluorescence imaging light spots as compared with their inter-spot distances;
- directing said plurality of scaled down fluorescence imaging light spots toward an image sensor of a digital camera;

- synchronous scan of the sample ROI with said plurality of probe beamlets and of said digital camera image sensor face with a corresponding plurality of said scaled down fluorescence imaging light spots; and
- imaging detection of the provided optical signals during at least one full scan cycle; readout signal of said digital camera provides an optically sectioned STED super-resolution image of the sample ROI.

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Real Time Multichannel STED Microscopy System

DRAWINGS

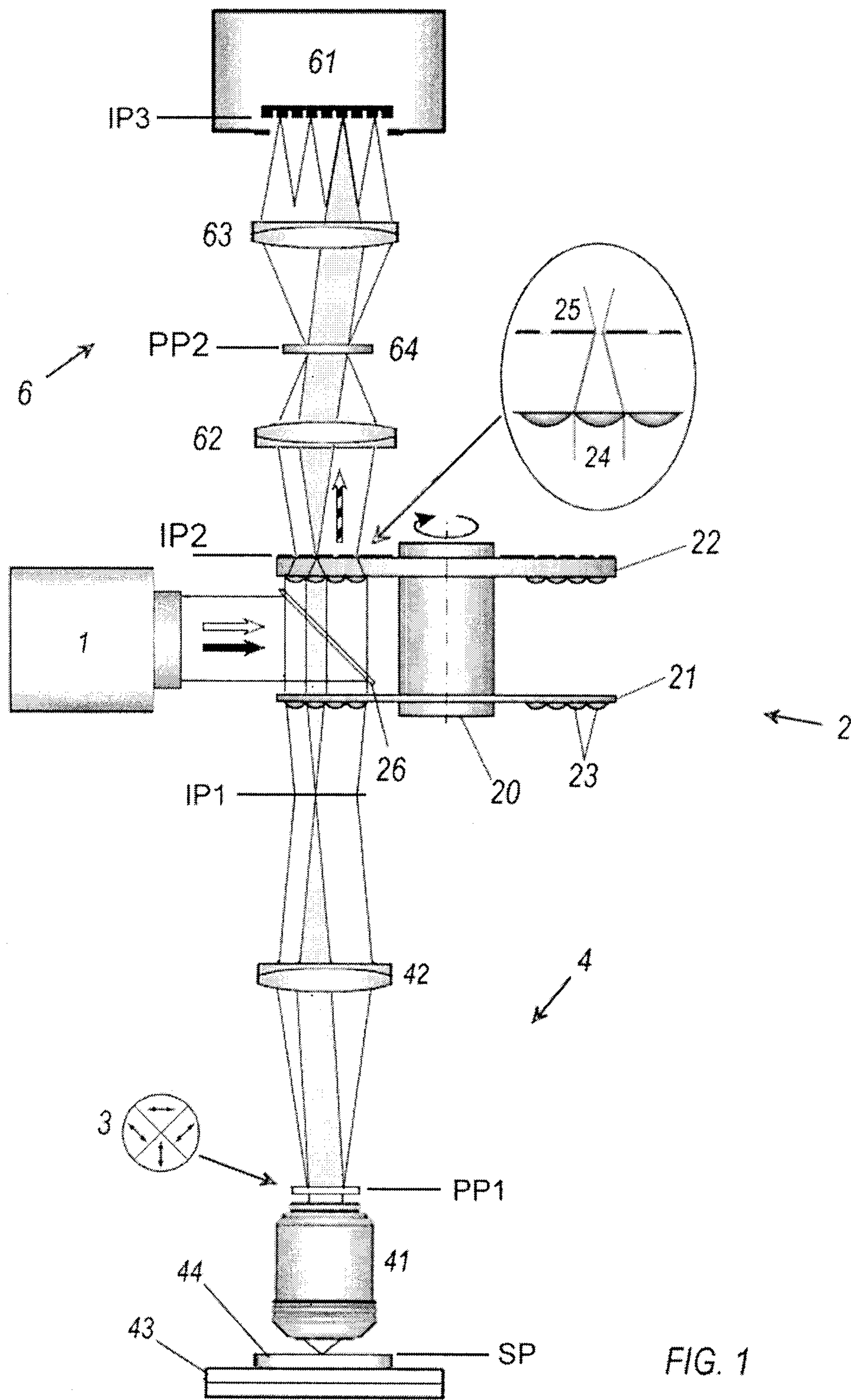
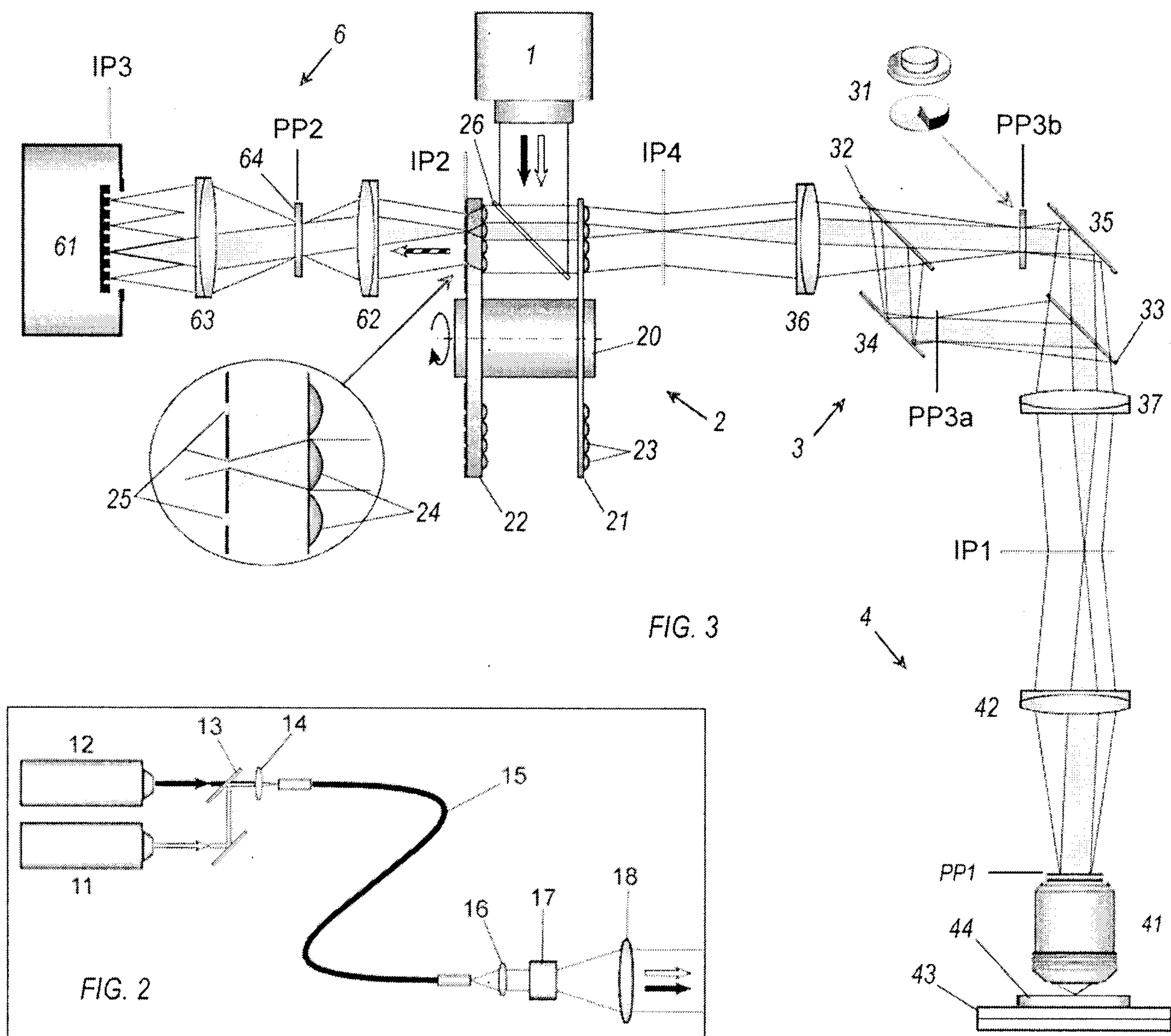
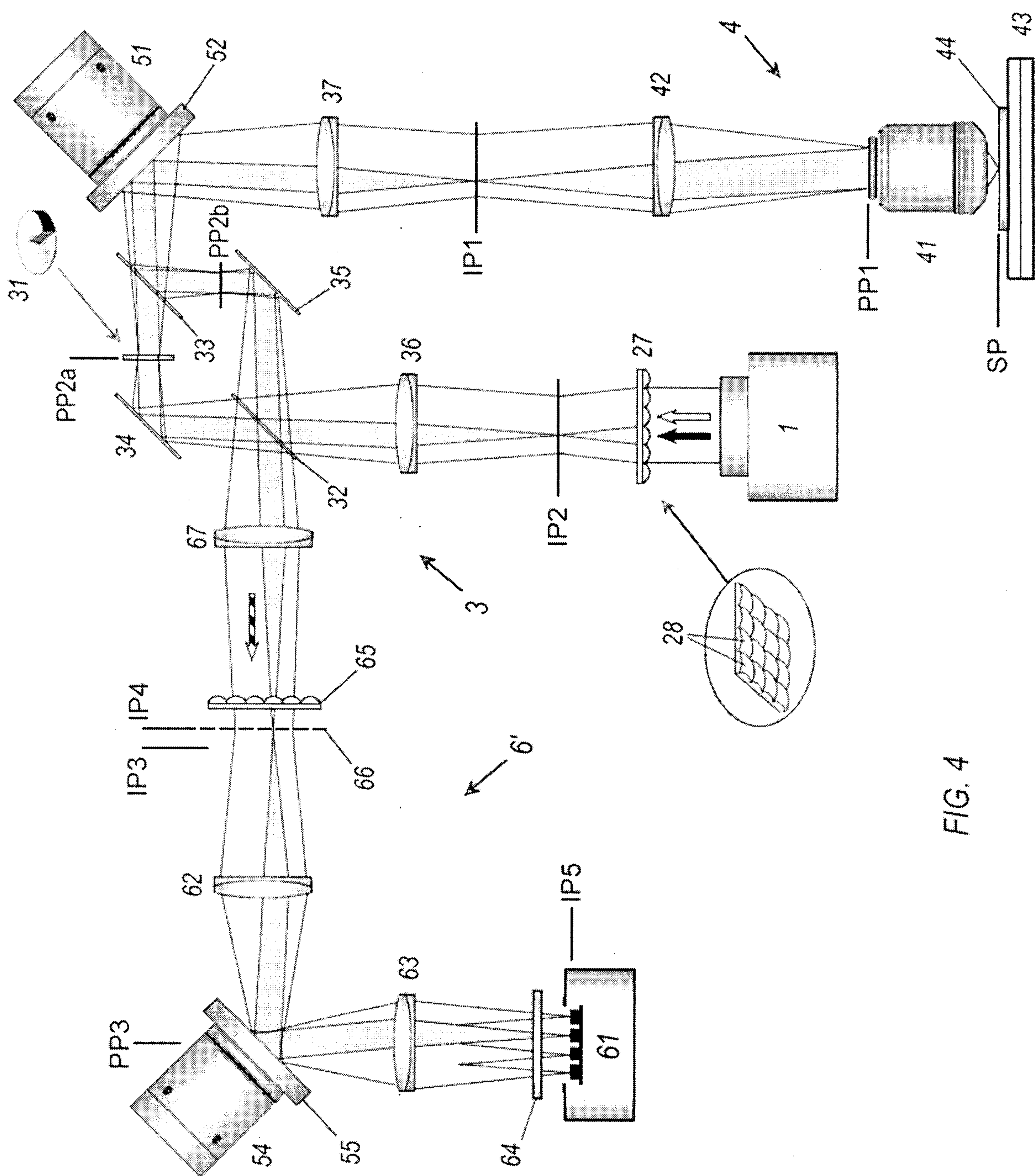


FIG. 1





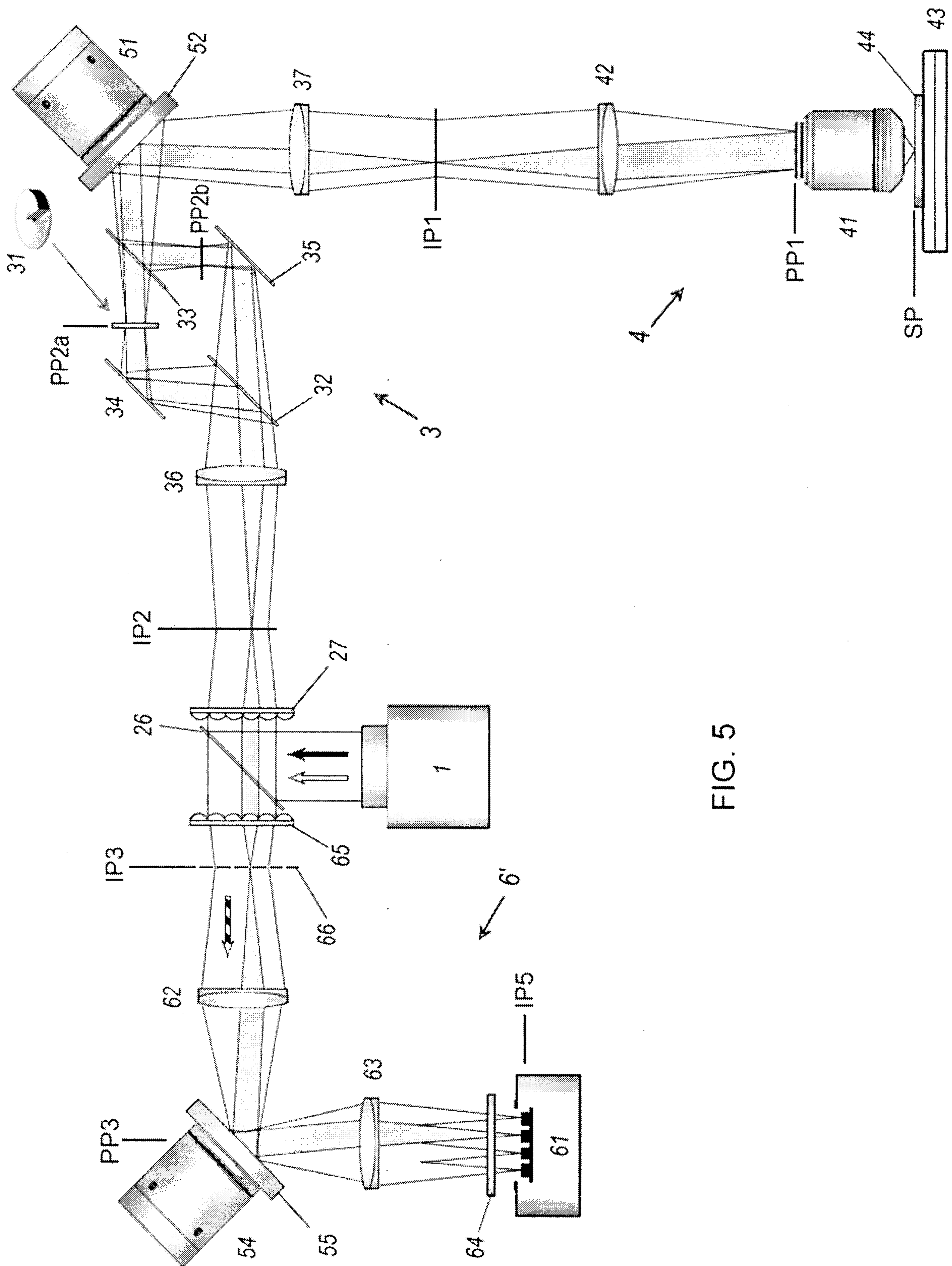
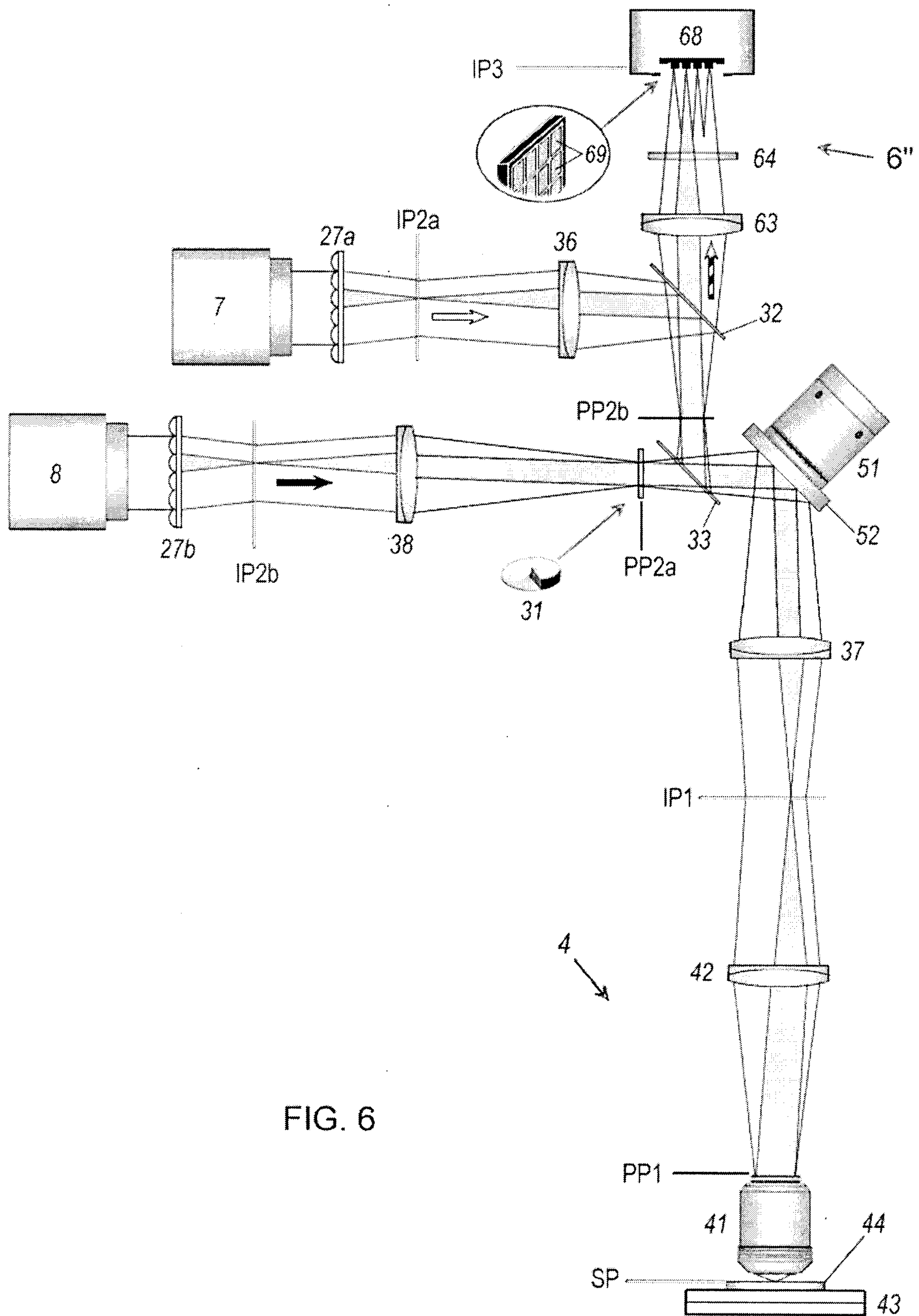


FIG. 5



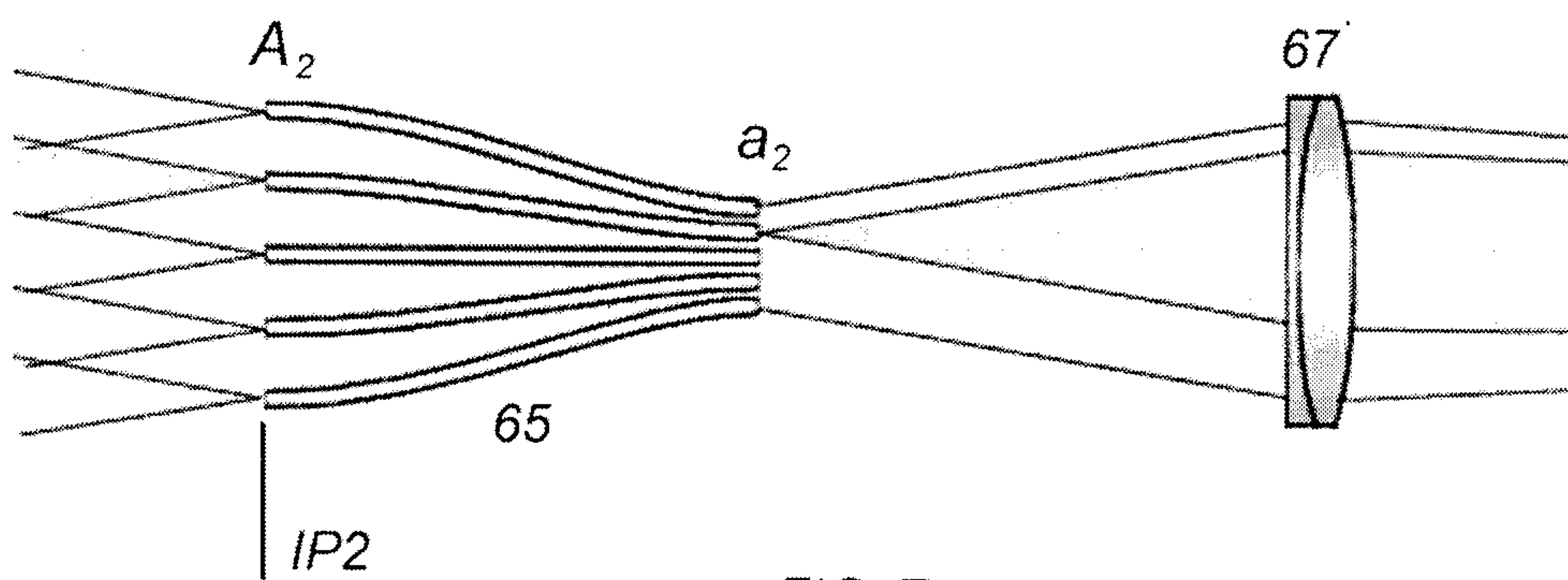


FIG. 7

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