

Title

MICROSCOPE WITH ADAPTIVE OPTICS

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Field of the Invention

The invention relates to the expansion of current microscopes by adaptive optics in the observation beam path and/or illumination beam path of a microscope. By adaptive optics is meant optically active component assemblies for wavefront modulation. The adaptive optics purposely change the phase and/or the amplitude of the light in such a way that a displacement and shaping of the focus in the object space and a correction of possible aberrations is achieved. The possible areas of use include confocal microscopy, laser-assisted microscopy, conventional light microscopy, and analytic microscopy.

Prior Art

The present invention proceeds from the following prior publications:

- U.S. 4,408,874; W. Zinky, L. Rosenberg; 1981/1983: "Mechanically or pneumatically deformable optical element for astigmatic magnification adjustment for imaging systems in lithography".
- EPO 0098969 B1; J. Arnaud; 1983/1987: "Deformable optical element for astigmatic correction". The thickness of the mirror membrane varies over the surface, so that the membrane adopts a previously calculated shape when subjected to external bending forces.
- EPO 0307354 B1; H. Choffat; 1988/1992: "Ring arrangement of bimorphic piezo layers for axial precision adjustment of components, e.g., microscope objectives".
- U.S. 5,142,132; B. MacDonald, R. Hunter, A. Smith; 1990/1992: "Adaptively controlled optical system for wafer fabrication (stepper)". The adaptive element controls the focus and corrects aberrations. The



error signal for correction is obtained from the light reflected back from the wafer through interference with the original light. An exact method for correction of aberrations is not indicated.

- DP DE 3404063 C2; A. Suzuki, M. Kohno; 1984/1993: "Curved transparent membrane in the beam path of an imaging system for correction of imaging errors, especially lateral focus offset".
- U.S. 5,504,575; R. Stafford; 1993/1996: "Spectrometer based on spatial light modulator and dispersive element". Fibers and optical switches/flexible mirrors are used to switch the light to the detector after passing through the dispersive element.
- EPO 167877; Bille, Heidelberg Instruments; applied for 1985: "Ophthalmoscope with adaptive mirror".

The description of the present invention is based on the following terminological definitions:

- "Wavefront modulator", within the meaning of the invention, is a device for deliberately influencing the phase and/or the amplitude of a light wave. Based on a reflecting optical element (deformable mirror, electrostatic control, or controlled by a piezo array, or as a bimorphic mirror) or a transmitting optical element (LCD or similar unit). It can be built in a continuous or segmented manner. In particular, the segments can be adapted for controlling the respective problem.
- "Aberrations in the microscope": The aberrations in the microscope objective occurring in defocused operating mode can basically be categorized as correctable or not correctable. Causally, the aberrations can be divided into aberrations caused by the objective,



aberrations caused by the additional imaging optics of the microscope, and, finally, those caused by the preparation itself.

— "Controlling the wavefront modulator": The controlling of the wavefront modulator is carried out by a computer with appropriate software. The required correcting variables are either calculated beforehand (offline) or are calculated from measured quantities (online, e.g., through a wavefront sensor or by measuring the point brightness in the intermediate image).

Description of the Invention:

In conventional light microscopy, as well as in laser-assisted microscopy, the focus of the objective must be displaced with high precision along the optical axis as well as laterally. In conventional microscopes, this is carried out by mechanical displacement of the object stage or objective. In addition, in case of illumination by laser radiation, displacements are also necessary in the object space. Consequently, there is a need for three-dimensional focus control in the object space.

It is the object of the invention to achieve the axial displacement of the focus in the object space without changing the distance between the objective and the object.

According to the invention, this displacement is carried out at the wavefront of the beam path. In this respect, the axial displacement of the focus in the object corresponds to a spherical change in the wavefront; the lateral displacement corresponds to a tilting of the wavefront. According to the invention, aberrations in the beam path are also compensated by changing the wavefront. These manipulations are carried out in a *pupil plane of the beam path*.

In *conventional light microscopy in the observation beam path*, in order to achieve an axial displacement of the focus in the object space without changing the distance from the objective to the object, the wavefront in the pupil of the objective or in a plane equivalent to the pupil plane must be spherically deformed.



Such deformation can be achieved through a wavefront modulator, namely a wavefront-phase modulator.

Fig. 1 and Fig. 1a show a schematic imaging beam path of an optical light microscope with an observed object, an objective, and a tube lens for generating an intermediate image which can be viewed by eyepieces, not shown. A wavefront modulator, according to the invention, is arranged between the tube lens and objective. The wavefront which is curved after the objective is corrected by the wavefront modulator by compensating for the aberrations of the objective.

Calculations have shown that with radii of curvature of the wavefront in the pupil of between -3.0m and 1.5m , the focus can be displaced by more than 1.5mm . This depends on the objective that is used; in the present case, the data refer to the Epiplan-Neofluar $20\times/0.5$. Displacements in the range of several tens of micrometers are sufficient in most cases. As mathematical calculations have further shown, the interval of a possible focus displacement decreases as the magnification of the objective increases. However, since the objective is not calculated or designed for this spherically deformed wavefront in the entrance pupil, aberrations through the objective during defocusing cannot be prevented.

A focus displacement of the kind mentioned above without mechanical influence of the objective has several advantages. First, any mechanical influencing of the object by the microscope objective is eliminated by the fixed working distance between the front lens of the objective and the object. Accordingly, it is possible for the first time to carry out sectionwise image recording with different depth positions of the observation plane with a static water-immersed object. Previously, a technique of this kind failed as a result of the mechanical deformation of the object and its surrounding medium through mechanical pressure on the preparation.

The fixed working distance at the microscope also offers advantages in the analytic examination of specimens in the biomedical field. When using microtiter plates, a correction of aberrations caused by the microtiter plate can be compensated. The microtiter plate can be included optically in the beam path and the microscope objective can be partially (e.g., the front lens) integrated therein.

Fig. 1b shows a construction of an optical light microscope with deformable mirrors which correct the wavefront in the direction of the tube lens. A



first modulator arrangement and a second modulator arrangement are included in the imaging via a beam splitter between the objective and tube lens. In addition, optics for pupil adaptation are provided in front of each modulator arrangement. The above-mentioned arrangements will be described more fully in connection with Fig. 7.

A correction of aberrations due to the preparation and the medium surrounding the specimen is also possible by means of a suitable deformation of the wavefront through the wavefront modulator. This is shown in Fig. 2. The wavefront which is distorted by aberrations is corrected by the wavefront modulator arranged between the objective and the tube lens. However, the spherical components in the wavefront correction are not sufficient for this purpose; aspherical components must be included. Annular actuators are sufficient for rotationally symmetric aberrations (all terms of higher-order spherical aberration). For angle-dependent aberrations, segmented actuators must be used (Fig. 4). These segmented actuators can either be integrated together in the same wavefront modulator or two independent modulators can be used in different pupil planes. In the first case, the number of actuators is quadratically scaled, in the latter case linearly scale, with the required resolution, which means a reduction in the complexity of control electronics.

Currently obtainable phase modulators are limited with respect to amplitude and with respect to the maximum phase gradients that can be generated. This in turn limits the possibilities for correction far away from the working point of the objective. A conceivable alternative consists in combining adaptive optics with conventional glass optics. The latter serve to generate a large phase gradient or large wavefront amplitudes, and precision tuning is achieved by means of the adaptive optics.

When displacing to a greater focus distance, the required convex wavefront of the pupil results in a vignetting which leads to lower light efficiency and a reduction in usable aperture. This limitation is design-related and, in principle, can be taken into account in the future in the optical design of an objective.

Further, aberrations occurring in the beam path when the focus is displaced can result in distortions of the image. In order to correct these aberrations, non-spherical components can be superposed on the wavefront as was



indicated above. According to mathematical calculations, a considerable improvement can be achieved in the image (Strehl ratio greater than 98%) even with small rotationally symmetric components of orders r^4 and r^6 (spherical aberration of higher order) at the wavefront.

A further advantage of the process consists in the achromatic behavior of a reflection-based wavefront modulator. With a suitable coating of the membrane mirror, the entire spectral range from low UV to far IR can be phase-modulated. Chromatic aberrations (with the exception of absorption effects) are ruled out. This results in new methods for chromatic correction in image generation. For this purpose, the illumination is adjusted sequentially to different wavelengths, wherein the wavefront modulator is adjusted to the suitable optical correction for each of the individual wavelengths. In this way, a set of images with optimum chromatic correction is obtained which, when superposed, give a white-light recording of high chromatic correction which cannot be achieved in the same way through the use of conventional glass optics. Accordingly, in principle, an objective with a wavefront modulator can be corrected in an optimum manner on any number of wavelengths in the optical spectrum.

The required wavefronts initially have only a rotationally symmetric character for displacement of the focus and for correction of spherical aberrations. In order to generate such wavefronts in the pupil of the microscope objective, the adaptive optics must have a distribution of actuators with spatial frequency increasing toward the edge (Fig. 4) because the largest gradient in the wavefront occurs at the edge.

Fig. 4 shows various actuator structures with increasing spatial frequency in Fig. 4a to Fig. 4c and with segments in Fig. 4d, e.g., for correcting astigmatism and coma.

In camera-assisted image generation, the effect of pixel mismatching occurs especially with high spatial resolution. In this case, the microscope image is displaced toward the camera so that the individual images of the video signal are spatially displaced. This problem can be eliminated by a variable tilt component in the wavefront of the imaging signal. By means of suitable regulation, the unsteady



movements of the image signal can be eliminated and a static image can accordingly be generated.

Another problem in camera-assisted image recording is field curvature. The field curvature can be improved during operation, at the expense of other parameters such as chromatic correction, through the use of a wavefront modulator in the imaging beam path.

In *conventional light microscopy*, a flexible configuration of optics, improved optical characteristics of the microscope, and new illumination techniques can be realized in the *illumination beam path* by introducing adaptive optics. In a similar way to the observation beam path, a wavefront phase modulator can optimize the imaging of the illumination burner (or of the laser, as the case may be) in the object plane. Likewise, in the case of critical illumination, an even illumination of the object space can be adjusted. Fig. 3 shows a wavefront modulator between the collector and condenser which are arranged following an illumination burner.

The illumination intensity in the object plane can be optimized spatially with respect to intensity and homogeneity by a wavefront amplitude modulator. In principle, a manipulation of the pupil is possible in this way. An oblique illumination of the object space can be achieved by purposely changing the tilt proportion of the wavefront.

In *confocal microscopy* and with the *laser scanning microscope*, the applications can be realized even more readily than in conventional light microscopy by using laser light for illumination.

When using a laser for illumination, the use of a wavefront modulator is advantageous already when coupling into the illumination fiber. In this respect, it is possible to realize variable adaptation optics whose focal lengths and imaging scale ratio are adjustable in dependence on the beam characteristics of the laser(s) and the utilized fiber(s) in order to achieve an optimum in-coupling into the fiber. Arrangements based on the same principle can also be used in coupling of illumination fibers to the microscope optics. Because of the rapidity of the modulators, time-resolved measurements and multiplexing procedures can also be realized in order to switch between one or more lasers and different fibers.



In confocal imaging, the transmission can be adapted dynamically through the defining pinhole. Both the position and diameter of the focus are variable within wide limits. The illumination laser, or lasers, can thus be adjusted in an optimum manner based on requirements. Further, the contour of the light distribution of the focus can also be adapted to the pinhole. Not only rotationally symmetric apertures but also those having other kinds of outlines or profiles such as lozenge-shaped or rectangular apertures of the type always occurring in pinholes realized in practice can accordingly be adapted and optimized to maximum transmission or minimum diffraction losses. An optimization of this kind can be initiated statically by parameters that are calculated beforehand on the one hand or can be regulated during operation to determined optimizing parameters.

As in conventional light microscopy, the chromatic correction can also be adjusted in dependence on the utilized illumination laser. Sequential images can be recorded at different wavelengths, with optimum chromatic correction in each instance, through the use of fast, synchronously controlled wavefront modulators in the laser input coupling and in the illumination optics and recording optics.

Brief Description of the Drawings

The invention will be explained more fully with reference to embodiment examples. Shown in the accompanying drawings are

- Fig. 1 and Fig. 1a the schematic imaging beam path of a light microscope;
- Fig. 2 the correction of a wavefront distorted as a result of aberrations with a wavefront modulator arranged between the objective and the tube lens;
- Fig. 3 a wavefront modulator arranged between the collector and the condenser for adjusting an even illumination of the object space;



- Fig. 4 various actuator structures, with increasing spatial frequency in Fig. 4a to Fig. 4c and with segments in Fig. 4d;
- Fig. 5 various constructions of wavefront modulators, including those with electrostatic (Fig. 5a), piezo-controlled (Fig. 5b) and bimorphic membranes (Fig. 5c) as actuating elements;
- Fig. 6 wavefront modulator with electrostatic membrane mirror;
- Fig. 7 the principle of a laser scanning microscope with a short-pulse laser;
- Fig. 8 the basic construction of a pre-chirping unit.

Detailed Description of the Drawings

Fig. 5 shows various constructions of wavefront modulators as are currently obtainable. For example, transmitting modulators based on LCD, as shown in Fig. 5d, or reflecting modulators with movable membranes are available. These may be distinguished, in turn, according to their type of actuating elements: electrostatic (Fig. 5a), piezo-controlled (Fig. 5b), or bimorphic membranes (Fig. 5c) as actuating elements. Although the invention is directed generally to wavefront modulators, the electrostatic membrane mirror is especially emphasized in this respect in view of its numerous advantages.

A micro-fabricated monolithic membrane mirror of the type mentioned above, which is shown in more detail in Fig. 6a and Fig. 6b with membrane M and driving electrodes E, is distinguished by excellent flatness and good optical quality of the reflecting surface (less than $\lambda/20$), small physical size (2mm to 20mm), hysteresis-free control with low voltages (less than 100V), high mechanical cutoff frequency of the membrane (several MHz), large travel or lift ($\approx 100\mu\text{m}$), and therefore small radius of curvature (down to 1m), and an actuator structure that is



variable within wide limits and has a high spatial density. The minimum actuator size is ultimately only limited by the condition that it must be greater than the distance between the electrode and the membrane.

The great advantage of the electrostatic membrane mirror consists in the fact that only a constant potential need be applied to the actuator electrodes for adjusting a parabolic shape. The parabolic shape of the mirror is given at constant driving of the electrodes by the physical behavior of the membrane (constant surface force). Accordingly, high dynamics can be achieved in the correcting variable (mirror travel) with low dynamics in the control variable, that is, the applied voltage.

Fig. 7 shows a laser scanning microscope with a short-pulse laser, especially for multiphoton excitation. This will be explained more fully hereinafter.

In *nonlinear processes*, the detected signal depends on the n th power of the excitation intensity. High intensities are necessary for excitation. These high intensities are achieved through the use of short-pulse lasers and the subsequent diffraction-limited focusing with microscope objectives. Therefore, it is the aim of the arrangement to realize the smallest possible (i.e., ideal) focus and the shortest possible pulse length in the specimen. In this way, high intensities can be achieved in the specimen. Nonlinear processes are, for example, multiphoton absorption, surface second harmonic generation (SSHG), and second harmonic generation (SHG), time-resolved microscopy, OBIC, LIVA, etc.

The invention will be explained more fully in the following with reference to *two-photon microscopy*. In this connection, the following prior art serves as a point of departure:

WO 91/07651 discloses a two-photon laser scanning microscope with excitation through laser pulses in the subpicosecond range at excitation wavelengths in the red or infrared region. The publications EP 666473A1, WO 95/30166, DE 4414940 A1 describe excitations in or above the picosecond range with pulsed or continuous radiation. A process for optical excitation of a specimen by means of two-photon excitation is described in DE C2 4331570.

Utility Model DE 29609850 describes coupling of the radiation of short-pulse lasers into a microscope beam path via light-conducting fibers. In this case, an optical arrangement for wavelength-dependent temporal change of laser pulses



is provided between the laser and light-conducting fiber and comprises at least two optical elements, for example, prisms or mirrors. This optical arrangement can be used to adjust the time difference of different wavelengths of the laser pulses by changing the distance between the optical elements.

As is well known, two-photon fluorescence microscopy basically opens up the following possibilities in contrast to conventional single-photon fluorescence microscopy:

- Realization of a nonlinear excitation probability $I_{2hv} = A \cdot I_{exc}^2$ with the following advantages: three-dimensional discrimination, i.e., depth discrimination, without the use of a confocal diaphragm; bleaching out and destruction of cells takes place - if at all - only in the focus; improved signal-to-noise ratio; use of new detection methods such as non-descanned detection;
- NIR excitation with femtosecond lasers has the following advantages for the examination of biological preparations: Working in the region of the optical window for biological preparations (700-1400 nm) due to low absorption; this method is therefore also suitable for the examination of living preparations; low loading of cells due to low mean excitation output; large penetration depths due to low scatter;
- The excitation of so-called UV dyes without the use of UV light means that no UV optics are necessary;
- In two-photon excitation, there are broad-band excitation spectra of the dyes and it is therefore possible to excite a wide range of different dyes with only one excitation wavelength.

When ultrashort pulses pass through a dispersive medium, e.g., glass or a preparation, the following effects take place in particular:

Group Velocity Dispersion (GVD): Femtosecond laser pulses have a spectral width of several nanometers. The red-shifted wavelength components propagate more swiftly through a positively dispersive medium (e.g., glass) than the blue-shifted wavelength components. There is accordingly a temporal widening of the pulses and thus a reduction in peak output or in the fluorescence signal.



A pre-chirping unit (pair of prisms, pair of gratings or a combination of the two) is a negatively dispersive medium, that is, blue-shifted wavelength components propagate faster than red-shifted wavelength components. The group velocity dispersion can accordingly be compensated by means of a pre-chirping unit.

Propagation Time Difference (PTD): The glass paths differ over the beam cross section (see Fig. 4). This causes a spatial magnification of the focus so that there is a reduction in the resolving capability and peak output or fluorescence signal.

This effect can be compensated by means of a wavefront modulator, for example, an adaptive mirror. With a modulator of this kind, the phase and amplitude of the light wave in the excitation beam path can be specifically influenced. A reflecting optical element (e.g., deformable mirror) or a transmitting optical element (e.g., LCD) are possible modulators.

Wavefront distortion through scattering and diffraction/refraction: This distortion can be caused by the utilized optics themselves or by the preparation. As in the second effect, the wavefront distortion likewise results in deviations from the ideal focus. This effect can also be compensated by a wavefront modulator (as was already shown).

The effects mentioned above are generally dependent on the depth of penetration into the preparation. In this regard, the arrangement according to the invention compensates for the GVD, PTD and wavefront distortion effects synchronously as a function of the depth of penetration into the preparation in order to achieve short pulse lengths and the most ideal small focus in the focus of the preparation even with large penetration depths.

A possible construction of the arrangement is shown in Fig. 7 by way of example. The radiation of a short-pulse laser KPL passes into a pre-chirping unit PCU and then travels, via beam splitter ST1 and beam splitters ST2, ST3, to two adaptive optical elements AD1, AD2. The first element AD1 (coarse) is used for coarse adjustment of the wavefront. It is accordingly possible to shift the focus in the z-direction. The wavefront distortions and the PTD effects are compensated by the second element AD2 (fine). The laser light reaches the object via beam splitter DBS, x/y-scanning unit, optics SL, TL, and mirror SP and objective OL. The light



coming from the object travels back via beam splitter DBS, lens L, pinhole PH and filter EF to a detector, in this case a photomultiplier PMT, for example, which is connected in turn with a control unit as are the PCU, AD1 and AD2.

The adjustment of the adaptive elements AD1, AD2 and the pre-chirping unit, for example, can be effected in this way until a maximum signal is present at the PMT. The beam path shown in the drawing is particularly advantageous for an inverse microscope in which observation takes place "from below", wherein the advantage consists in that the specimen remains fully accessible for possible manipulation.

Fig. 6 already showed the basic construction of an adaptive mirror. It comprises a highly reflecting membrane (e.g., silicon nitrate) and a structure with electrodes. By specifically controlling the individual electrodes, the membrane situated above the latter can be deformed and the phase front of the laser beam can accordingly be influenced. The deformations of the phase front which occur when the pulses pass through the system and through the specimen can accordingly be compensated.

The pre-chirping unit can comprise one or more prisms or gratings or a combination thereof. Fig. 8 shows possible arrangements with four prisms in Fig. 8a, with four gratings in Fig. 8b, and with prisms and gratings in Fig. 8c. Its manner of operation is explained more fully in Fig 8a with reference to a prism compressor. The spectral width of a femtosecond laser pulse is several nanometers. When the laser beam passes through the first prism, the beam is broken up into its spectral components. Subsequently, the spectral components travel different glass paths in the second prism. Red-shifted wavelength components are accordingly retarded in relation to the blue-shifted components. The pre-chirping unit accordingly acts as a negatively dispersive medium and a compensation of GVD is also possible.

For the first time, through the use of the arrangement described above, the advantages of the excitation of nonlinear processes can be utilized to their full extent and the use of low-power femtosecond lasers is made possible even at greater depths of penetration in the specimen. High peak outputs can accordingly be realized with the use of low mean excitation outputs so that loading of the biological preparations or specimens can be kept low and a high signal-to-noise ratio



and high resolution can be achieved in the axial and lateral directions.

In all of the arrangements described above, the wavefront adaptation can be advantageously detected and monitored and adjusted in a defined manner by means of a wavefront sensor which communicates with the microscope beam path via a beam splitter (not shown).



Claims

1. Microscope, wherein adaptive optics are provided in the illumination beam path and/or in the observation beam path.
2. Microscope according to claim 1, wherein a transmitting wavefront modulator is located in the observation beam path between objective and tube lens.
3. Microscope according to claim 2, wherein at least one reflecting wavefront modulator is arranged in the observation beam path between objective and tube lens.
4. Microscope according to claim 3, wherein the coupling in of the reflecting wavefront modulator or reflecting wavefront modulators is provided via beam splitters.
5. Microscope according to claim 1, wherein a wavefront modulator is located in the illumination beam path between the light source and condenser.
6. Microscope according to claim 5, wherein a transmitting wavefront modulator is provided.
7. Laser scanning microscope, wherein the laser radiation source is followed by at least one adaptive optics.
8. Laser scanning microscope according to claim 7, with first adaptive optics for coarse adjustment and second adaptive optics for fine adjustment of the wavefront.
9. Laser scanning microscope according to one of claims 7 or 8, wherein the adaptive optics are formed as reflecting wavefront modulator.



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10. Laser scanning microscope according to one of claims 7 to 9 with a short-pulse laser as laser radiation source.

11. Laser scanning microscope according to one of claims 7 to 10, wherein the laser radiation source is followed by a pre-chirping unit for compensation of the group velocity dispersion (GVD).

12. Laser scanning microscope for multiphoton excitation with short-pulse laser according to claim 10, wherein the laser radiation source is followed by a pre-chirping unit and at least one adaptive optics.

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