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Considerations on a laser-scanning-microscope with high resolution and depth of field

Christoph Cremer1) and Thomas Cremer

Institute of Human Genetics, University of Freiburg, Federal Republic of Germany

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Summary

In conventional light microscopy, the depth of focus is severely limited. This limitation might be overcome by a light optical scanning procedure. In this procedure, the specimen surface is scanned point for point by a focused laser beam. The image of the specimen surface is generated by an electronic system, similar to the procedure used in the scanning electron microscope.
Possibilities to develop a "laser-scanning-microscope" on the basis of available techniques (laser microirradiation, miniprocessors, light detecting systems, automatic focusing, holographic focusing etc.) are discussed.
On account of its possibility to form images of high resolution and depth of focus, a laser-scanning-microscope might become a valuable tool in addition to conventional light microscopy and scanning electron microscopy.

Überlegungen zu einem Laser-Scanning-Mikroskop mit hoher Auflösung und Schärfentiefe

Die bei der herkömmlichen Lichtmikroskopie unvermeidliche, starke Beschränkung der Schärfentiefe konnte durch ein lichtoptisches Scanning-Verfahren überwunden werden, bei der die Objektoberfläche von einem fokussierten Laserstrahl punktweise ,,abgetastet" wird und das Bild der Objektoberfläche ähnlich wie beim Rasterelektronenmikroskop auf elektronischem Wege zusammengesetzt wird.
Der konstruktive Plan eines ,,Laser-Scanning-Mikroskopes" auf der Grundlage der heute gegebenen technischen Voraussetzungen (Lasermikrobestrahlung, Miniprozessoren, Lichtnachweissysteme, automatische Fokussierung, holographische Fokussierungsverfahren etc.) wird beschrieben und Probleme einer technischen Realisierung werden erörtert.
Aufgrund seiner besonderen Darstellungsmöglichkeiten konnte das Laser-Scanning-Mikroskop-Verfahren eine wertvolle Ergänzung herkömmlicher lichtmikroskopischer sowie rasterelektronenmikroskopischer Verfahren werden.

1. Introduction

One of the fundamental limitations of conventional light microscopy is due to the fact that with increasing magnification and numerical aperture the depth of focus becomes very low [10]; at high magnification and numerical aperture it is

') Dipl.-Phys. Dr. Christoph Cremer, Institut für Humangenetik der Universität Freiburg, Albertstrasse 11, D-7800 Freiburg i. Br., Bundesrepublik Deutschland / Federal Republic of Germany.

only a fraction of a micron. This limitation results from the laws of geometrical and wave optics and therefore principally cannot be surmounted in conventional light microscopy. If it is desired to obtain an image of a specimen surface at a magnification corresponding to a high numerical aperture in light microscopy, but with high depth of focus, nowadays a scanning electron microscope is used. In this case, however, an observation of living biological objects usually is not possible. Furthermore, the light microscopical image of an object contains important informations, which are only difficult or even not obtainable by using the scanning electron microscope.

Our contribution deals with the problem whether the severe limitation of the depth of focus which is inevitable in conventional light microscope, might be removed by a light optical scanning procedure in which the image of the object is formed point by point whereas in conventional light microscopy all points of the image are formed simultaneously.

The present state of laser technique and of electronics suggests that the realization of such a laser scanning microscope might be feasible. Recently, scanning methods have been used to obtain images with high resolution (1 µm) of biological objects by means of focused acoustic waves ("scanning acoustic microscope") [2, 19].

The laser-scanning-microscope-method proposed here is intended to solve two problems:

1) A high resolution image of a specimen surface characterized by a high degree of unevenness has to be formed, the unevenness being too large to allow the forming of a sharp image of the whole specimen surface by conventional light microscopy.

To save trouble, in the following only objects with a fluorescent surface are considered. Furthermore, it is assumed that the fluorescence of the surface differs in at least one wavelength 2ri from the fluorescence emitted by the interior of the object. This assumption does not restrict very much the class of objects which are accessible to an imaging in the laser-scanning-microscope, because in objects without a sufficient specific natural fluorescence in many cases fluorochromes may be attached to the surface. E. g., in biological objects fluorochrome-conjugated antibodies may be used [20].

2) Simultaneously with the imaging of the topographical details of the specimen surface (problem 1), the fluorescence distribution on the surface is to be recorded. In addition to an imaging of the surface, this gives light optical informations which are in principle difficult or even not obtainable with scanning electron microscopy.

2. Elements of a laser-scanning-microscope

Fig. 1 shows the basic design of a laser-scanning-microscope. A laser system (1) emits coherent light of appropriate wavelength(s) $?_E$ [22] with small beam divergence [12]. The wavelength A].; has to be selected in such a way, that at least at a given wavelength $?_{FI}$ only the specimen surface contributes considerably to the fluorescence emission.

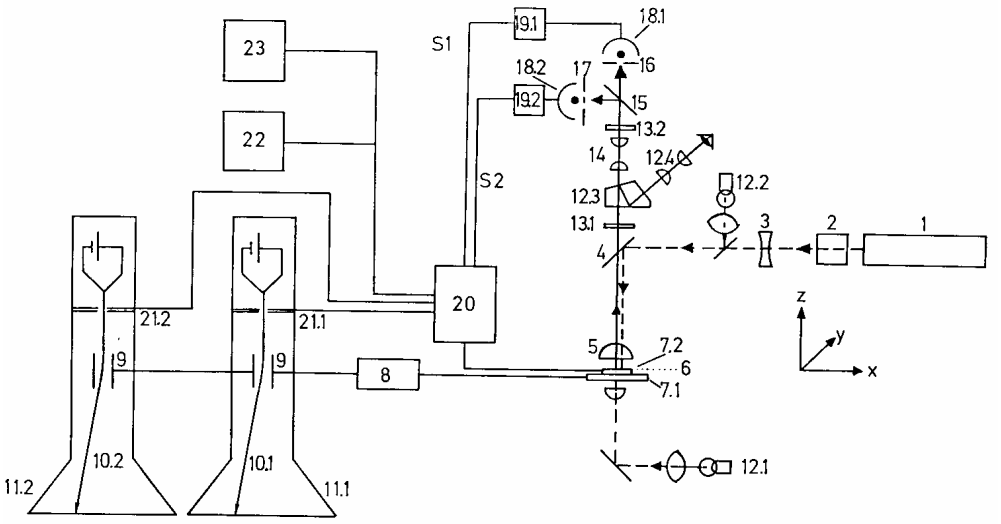


Fig. 1: Schematic design of a laser-scanning-microscope.

1) laser system (continuous wave laser); **2**) electro-optic modulator; **3**) adjusting lens; **4**) selecting mirror; **5**) microscope objective for focusing of the laser beam. The objective is simultaneously used for observation and collection of the fluorescent light; **6**) object plane-

7) scanning stage:

7.1: mechanism for horizontal displacement (X, Y) of the object,

7.2: mechanism for vertical displacement (Z) of the object;

8) scan generator; **9)** deflecting systems;

10) recording beams:

10.1: recording beam for topographical display of the specimen surface,

10.2: recording beam for display of the fluorescence distribution;

11) TV-screens:

11.1: TV-screen for topographical display of the specimen surface,

11.2: TV-screen for display of the fluorescence distribution;

12) optical system for visual observation:

12.1: system for transmitted light illumination,

12.2: system for incident light illumination,

12.3: beam splitter,

12.4: eye piece;

13) filters:

13.1: barrier filter for elimination of the exciting laser light (wavelength $?_E$),

13.2: narrow band filter for selection of the fluorescence light (wavelength $?_{Fl}$);

14) magnifying system; **15**) beam splitter (50%); **16)** circular measuring diaphragm (image plane Bl); **17**) annular measuring diaphragm (image plane B2);

18) photomultipliers:

18.1: photomultiplier for measuring the luminous flux F 1 in the circular measuring diaphragm **16** (signal Sl),

18.2: photomultiplier for measuring the luminous flux F 2 in the annular diaphragm **17** (signal S2);

19) amplifier circuits:

19.1: amplifier for photomultiplier **18.1,**

19.2: amplifier for photomultiplier **18.2;**

20) electronics of the Automatic Focusing System;

21) intensity control for recording beams:

21.1: control for beam **10.1,**

21.2: control for beam **10.2;**

22) facility for the twodimensional numerical display of the specimen surface and of the fluorescence distribution, respectively (facultative); 23) facility for the threedimensional display of the specimen surface and of the fluorescence distribution, respectively (facultative).

By means of gas lasers presently available it is possible to obtain a large number of discrete laser lines in the ultraviolet and visible spectral region. Dye lasers a low it to obtain coherent light of any wavelength in the whole visible and near ultraviolet region. For reasons of power stability, it is advisable to use a continuous wave laser system [12], the beam intensity being controlled externally with an electro-optic modulator (2).

The coherent beam of wavelength $?_E$ enters into the scanning microscope and passes an adjusting lens (3). Then the beam is reflected by a selecting mirror (4) in such a way that it becomes collinear with the optical axis (Z) of the microscope. The selecting mirror (4) reflects the excitation light (wavelength $?_E$) maximally while it transmits maximally the emitted fluorescent light (wavelength $?_{Fl}$). A microscope objective (5) of high numerical aperture focuses the laser beam into the object plane (6). Using a laser beam of small divergence and an appropriate adjustment of the adjusting lens (3), it is possible to obtain in the object plane an almost diffraction limited focus.

The optical arrangement described here is used in a number of laser microbeams [4, 5, 12] E. g. using coherent ultraviolet light of 257.3 nm we succeeded in limiting the object field irradiated to an area with a diameter of 0.4 µm.[12] In this case, an objective 100:1/1.25 was used. Using an objective 32:1/0.4, the minimum diameter of the irradiated area was approx. 1 µm [25].

The object is placed on a special scanning stage (7) (see below). The horizontal movement [(X,Y)-direction] (7.1) is coupled to the deflecting systems of two recording beams (10.1) and (10.2) of two TV-screens in connection with a scan generator (8). In addition to the scanning stages presently used [displacement in (XY)-direction only] there exists a mechanism (7.2) which allows it to displace the object also very rapidly in direction of the optical axis (Z) of the microscope.

The fluorescence excited by the laser microirradiation in the specimen surface is collected by the objective (5). A barrier filter I (13.1) eliminates the rest of the excitation light (wavelength $?_E$). A portion of the fluorescence light is used for visual observation by the help of a beam splitter (12.3). The visual observation is made through the eye piece (12.4). To diminish fading, the visual observation can be also done using transmitted light (12.1) or incident light illumination (12.2) with other wavelengths than /IE .

A narrow-band filter II (13.2) transmits only light of wavelength $?_{Fl}$. Having passed a magnifying lens system, the fluorescence light is splitted by a 50% beam splitter (15). The image planes of the lens system (14) are Bl and B2, respectively In the plane Bl a *circular* measuring diaphragm (16) is located the diameter of which is equivalent to the diameter (in the Bl-plane) of the principal maximum of the diffraction pattern of a self-luminous point source situated in the object plane (luminous flux F 1). In the plane B2 an *annular* measuring diaphragm (17) is located in such a way that only the luminous flux F 2 passes which corresponds to the first secondary maximum of the diffraction pattern of the self-luminous point source Having passed the measuring diaphragms (16) and (17), the fluorescence light enters into the photomultipliers (18.1) and (18.2), respectively. The signals Sl and S2 resulting after amplification units (19.1) and (19.2) are processed in the electronics (20) of the "Automatic Focusing System" (AFS).

The basic functions of the Automatic Focusing System (AFS) are-

1) By way of the signals Sl and S2 the Z-coordinate Z,(X,Y) is determined which corresponds to the exact focusing of the laser beam on the specimen surface at a given (X,Y)-site. Then the AFS generates a signal *S* by which the intensity of the recording beam (10.1) is controlled proportional to $Z_0(X,Y)—Z_M$, Z_M being a fixed intermediate position. If this procedure is performed for all coordinates (X,Y) of the specimen surface, then on the TV-screen (11.1) an image of the specimen surface is generated which is formed by different gray values. For visual observation, the display has a long-persistence phosphor coating; a higher resolution may be obtained with a short-persistence phosphor coating and photographic recording. The image contrast is generated only by electronic means on the basis of the Z_0-values determined by measurements of S1(Z) and S2(Z).

This procedure allows an optimal adaptation of the image contrast to the special requirements of the object.

• 2) If the laserbeam is focused exactly on the specimen surface, then the signal $Sl(Z_0)$ produced by the aid of the photomultiplier (18.1) corresponds to the fluorescence signal in conventional microfluorometry [20] of objects with an even surface.

After determination of Z_0, the AFS has to perform simultaneously the following procedures:

(i) to control the intensity regulation (21.1) of the recording beam (10.1) proportional to $Z_0 - Z_M$;

(ii) to control the intensity regulation (21.2) of the recording beam (10.2) synchronized with (10.1) proportional to $Sl(Z_0)$.

By this procedure the distribution of fluorescence on the specimen surface is shown on the TV-screen (11.2)

An alternative to the use of two seperated TV-screens to display specimen surface and fluorescence distribution is the use of a colour-TV-monitor on which specimen surface and fluorescence distribution are shown in different colours.

A numerical representation of the specimen surface and the fluorescence distribution, respectively, may be obtained by means of the numerical display (22).

Furthermore, in connection with a computer system, it is possible to realize on a TV-sreen a perspective image of the specimen surface under various optical angles (23).

3. Considera tions on a technical realization

3.1. The "Automatic Focusing System"

As pointed out above, it is necessary to determine for each (X,Y)-site:

(i) the Z-coordinate $Z_0(X,Y)$ at which the laser microbeam is focused exactly on the surface;

(ii) the fluorescence signal Sl corresponding to the (X,Y, Z_0)-site.

This problem might be solved by means of an "Automatic Focusing System"- In addition to the displacement of the object in (X,Y)-direction, the object is moved in Z-direction m a continuous mode (sinusoidal vibration). The period of

this oscillation has to be short compared to the duration of the displacement in (X,Y)-direction from one point to the next: the amplitude has to be larger than the height of the maximum elevation of the specimen surface. At a given (X,Y)-site, for each Z-value the signals S1(Z) and S2(Z) are registered and stored in the electronics (20) of the AFS. It is assumed that S1(Z) and S2(Z) are proportional to the luminous fluxes F_1 and F_2 entering into the photomultipliers (18.1) and (18.2), respectively.

From optical considerations it follows that the quotient F 1/F 2= S1/S2 is a maximum if the laser microbeam is focused exactly on the specimen surface: If $Z=Z_0$, the photomultiplier (18.1) registers the luminous flux F 1 corresponding to the principal maximum of the diffraction pattern of the image of a small fluorescent spot induced by the laser microirradiation in the specimen surface. The photomultiplier (18.2) registers the luminous flux F 2 corresponding to the first secondary maximum. Under favourable conditions, F 2 is only a small percentage of F 1. For $Z=Z_0$ this results in a high contrast S1/S2. For $Z? Z_0$ (i.e. if the laser beam is defocused) the contrast is smaller.

From all Sl/S2-values obtained at a given (X,Y)-site, the electronics (20) selects the maximum value max. S1/S2 and determines the corresponding Z-value Z_0. Simultaneously, the Sl-value corresponding to max. S1/S2 is determined. Both informations are used in the way described in section 2 and represent the elevation and the fluorescence intensity, respectively, of the given (X,Y)-site. This procedure is performed for all (X,Y)-coordinates of the surface and eventually results in an image of the specimen surface with high depth of focus and of the fluorescence distribution on the surface, respectively.

In principle, also other ways of automatic focusing are conceivable. E. g., instead of the object itself one could displace the adjusting lens [(3), Fig. I], in order to change the relative position of the laser focus to the specimen surface. In this case however, the displacements of the adjusting lens necessary are much larger than if the object itself is moved. In consequence, mechanical difficulties occur because the displacement has to be performed very rapidly. Therefore we feel that a vertical displacement of the object itself would be considerably easier to realize.

We would like to point out that a determination of Z_0 only by means of the maximum of the luminous flux 01, using objects with large differences in the elevations of the surface, is only possible, if the differences in absorption and fluorescence efficiency at neighbouring sites of the specimen surface are small.

3.2. Electronic and mechanic requirements

It is decisive for the practical utility of the proposed scanning procedure, that the determination of $Z_0(X,Y)$ and $S1(X,Y, Z_0)$ and the horizontal displacement of the scanning stage [(7.1), Fig. 1] is performed so rapidly that the object may be scanned in a sufficient short time. The requirements to be satisfied are illustrated in the following example: The surface of an object with a 100 mm X 100 µim lateral extension and maximum differences in elevation of the surface of 10 μm has to be displayed with a resolution of approx. 0.5 µm. Then a focus diameter of 0.5 μm is required. The step length in (X,Y)-direction has to be 0.5 µm, too.

In Z-direction, for each (X,Y)-site 20 measurements of Sl and S2, respectively have to be made. $Z_0(X,Y)$ and $S1(X,Y,Z_0)$ have to be determined for a total of 4×10^4 (X,Y)-sites and to be transferred to the TV-screens.

If an image of the specimen surface has to be formed within 10 seconds the average velocity of the scanning stage has to be 2 mm/s. Compared to the velocities of scanning stages presently available for light microscopy, this velocity is very high. It may be attained, however, by means of a continuous vibratory motion In this manner, a scanning velocity of 5×10^4 measuring points within only one second has been realized in a scanning acoustic microscope [19].

The object itself may be located in a special observation chamber [13] under defined ambient conditions, allowing the use of an objective with a small working distance (high numerical aperture). The chamber may be mounted on piezoelectric elements which allow a rapid displacement in Z-direction. In order to obtain stereopairs, such a chamber may be tilted between successive exposures

To determine $Z_0(X,Y)$ and $S1(X,Y,Z_0)$, to transfer these signals to the TV-screens, and to adjust the next (X,Y)-point a time of 250 µs is available, if the total scanning time is 10 seconds.

If the scanning stage is moved continuously, the time for determination of Z_0 and Sl (Z_0) has to be so short (e. g. 25 µs) that during this time the displacement of the object in (X,Y)-direction is negligibly small. If during this time 20 signals Sl, S2 as a function of Z have to be recorded, one signal Sl and S2 respectively has to be recorded within approx. 1 µs. To realize this requirement, the following procedure is proposed: The vibration of the piezoelectric crystal effecting the Z-displacement of the object is controlled by an alternating voltage with a frequency of 20 kc which generates a sinusoidal Z-displacement of the object with the same frequency and an amplitude of 6 µm measured from an intermediate value Z_M If sensitive objects have to be scanned, the frequency has to be reduced, if necessary, to avoid a damage due to the acceleration forces generated by the vibration In this case, of course, the duration of the horizontal scan has to be prolonged too

The twenty Sl and twenty S2-values measured during a half oscillation period (25 µs) are recorded (see above). To register and to store these signals a time of 1 µs is available for each S1/S2 pair. The present state of electronics allows to realize this requirement provided that the irradiation power density at the specimen surface is high enough.

Following the transfer of the signal *S* (corresponding to Z_0-Z_M) to the TV-screen [(11.1), Fig. 1] and of the signal $Sl(Z_0)$ to the screen [(11.2) Fig 1] the data (20 x Z, 20 x S1, 20 x S1/S2), from which the both signals *S* and $S1(Z_0)$ have been determined, may be erased. Therefore, only a small storage capacity is required in the AFS.

3.3. Requirements concerning the light source

The irradiation power density necessary depends on the energy absorbed per second, the quantum efficiency of fluorescence and the sensitivity of the photomultiplier systems. It is assumed that within a measuring time of 1 µs at least 10^3 quanta have to be received by the photomultiplier [(18.1), Fig. 1] to produce a sufficient high signal.

N_0 incident quanta per second yield $N_A = aN_0$ absorbed quanta per second. These N_A quanta induce the emission of $N_F = ßN_A = aßN_0$ quanta per second having the wavelength $?_{Fl}$. Furthermore, absorption, reflection and beam splitting in the microscope have to be considered. If the total transmission of the system between specimen surface and photomultiplier is assumed to be 10°/o, the condition $N_0 = 1/ß \times 10^{10}$ (s^{-1}) has to be satisfied. E. g., for $a = ß = 10^{-2}$ the condition $N_0 = 10^{14}$ quanta per second follows.

If light in the ultraviolet and visible spectral region is used, $N_0 == 10^{14}$ quanta per second are equivalent to a power of several 10^{-5} Watt in the focus. Assuming a spot diameter of 0.5 µm, the incident power density lies in the order of magnitude of 10^4 Watt/cm².

The typical power of continuous wave lasers with emission in the ultraviolet and visible region lies in the range of 1 mW to several Watt. **It** is possible to focus the greater part of this power to a spot with a diameter in the range of a wavelength [3, 21]. From this it follows that the focal power densities obtainable are as high as 10^5-10^8 Watt/cm². Even if more unfavourable conditions are assumed than in the example given above, a sufficient high power density can be provided. Much higher power densities are available by means of pulsed laser sources [5, 24]. They have, however, the disadvantage that at high power the pulse repetition rate is relatively low (up to several kc [24]). Furthermore, it is difficult to realize the high power stability necessary.

Similar problems occur if incoherent flash lamps [9] are used. On the other hand, using incoherent light sources with continuous emission [9], in general the power densities required are not available.

3.4. The problem of damage induced by the scanning procedure in biological objects

The power densities of $l0^5$ - 10^8 Watt/cm² which are available by using continuous wave lasers are far below the threshold for dielectric breakdown and frequency doubling [4].

Severe damage in the object by "microexplosions" as it is observed if pulsed laser sources of high power are used [15, 16], may be avoided. Therefore, in our discussion concerning the damage of the object by the scanning beam we shall consider only thermal and photochemical effects.

At first the temperature rise to be expected is estimated. It is assumed, that

a) the object is irradiated continuously,
b) the energy absorbed per second, Pabs, is uniformly distributed in the whole object and completely transformed into heat,
c) the object is cylindrical with height *H* and radius *R*,
d) dissipation of heat occurs only perpendicular to the cylinder axis, which is coaxial with the optical axis of the laser beam.

Then in the equilibrium, i. e. after a long irradiation time, the following temperature distribution may be calculated [13]:

$$T(r) = T(R) + \frac{\text{Pabs}}{4\pi k R^2 H}(R^2 - r^2) \text{ for } r \leq R$$

$$T(r) = T(R) + \frac{\text{Pabs}}{2\pi k H}\ln(R/r) \text{ for } r \geq R$$

(T = temperature, r = distance from the cylinder axis).

The heat conductivity *k* is assumed to be equal in object and ambient medium If the diameter of the object is 100 µm and the height is 10 *µm* and assuming the heat conductivity of water, a temperature rise, [T(0)-T(1 mm)]

$$?T = 7 \text{ x } lO^{-3} \text{ x Pabs}(°C), \text{ Pabs in erg/s,}$$

is calculated. In the derivation of this formula it is assumed that the temperature rise for r = 1mm is negligibly small. If an incident power density of 10^4Watt/cm² in a focus of 0.5 µm diameter and an absorption of l»/o (see section 3.3) is assumed the temperature rise of the whole object is 1.4 X 10^{-2} °C.

Of course, the temperature rise of the whole object says little about the local temperature rise induced at a given (X,Y)-site by the laser microirradiation. The present knowledge is small concerning the temperature distribution at the irradiation site in case of a short time impact of light with high power density [5]. A first estimate, however, may be obtained from measurements [14] of the temperature pattern of a focused ruby laser beam (? = 694.3 nm). If it is assumed that the temperature distribution obtained in these experiments using pulse durations of 2.2 x 10^3µs:

(i) is valid also for the shorter duration of irradiation [approx. 25 µs at a given (X,Y)-site] in the laser-scanning-microscope, at least concerning' the order of magnitude;
(ii) is approximatively proportional to the absorbed energy,

then for the example considered above a local temperature rise of only 10-4 °C per measuring site compared to the given ambient temperature follows Even if the actual local temperature rise induced should be higher by several orders of magnitude, this would not result in a damage in biological objects. Concerning this point, however, further systematic investigations have to be done.

At an incident power density of 10* Watt/cm^ in the focus (diameter 0 5 µm) the incident energy per second is 200 ergs. The irradiation dose can be diminished to 20 erg/s if the irradiation is not continuously performed, but only during the measuring times of 25 µs per (X,Y)-site. The rapid switching on and off of the irradiation required in this case may be realized by means of an electro-optical modulator [(2), Fig. 1]. A scanning of living objects with the irradiation power and the velocity indicated should be possible, if there is no enrichment of target molecules absorbing very strongly the exciting laser light, and if there is no induction of strongly damaging photochemical processes. E.g., the microirradiation of mammalian cells with ultraviolet light (? = 257 nm) induces cell death at much lower doses than assumed in our example [13]. On the other hand, using light in the ultraviolet region it may be expected that a sufficient fluorescence is induced at much lower irradiation power densities. Besides the selection of the most appropriate wavelength in the case to be investigated (perhaps in combination with vital

dyes), a very sensitive light detecting system for F1 and F2 (Fig. 1) is a prerequisite for a nondestructive scanning of a living object. One should consider the possibility to use photon-counting light detecting systems. For the scanning of non-living objects, in many cases a higher power density may be used, so that the use of expensive photon-counting systems is not necessary.

3.5. The resolving power of the laser-scanning-microscope

In principle, the resolving power of the proposed laser-scanning-microscope is given by the spot diameter of the laser microbeam: The fluorescence of two object sites can be measured separately from each other if the sites can be separately "illuminated" by the laser focus. In practice, spot diameters as small as 0.5 µm have been obtained by laser microirradiation in the ultraviolet and visible region, using objectives of high numerical aperture. In principle, this corresponds to a resolving power of 0.5 µm in the laser-scanning-microscope. Using plane aplanatic optical systems, a focusing to a diameter of less than approx. ?/2 is not possible for physical reasons [1]. The working distance of microscope objectives of high numerical aperture (N.A = 1) is small and usually lies in the range of several hundred micrometers at maximum. If a much larger working distance is required by the special features of the object, objectives of larger working distance but smaller resolving power have to be used. A possibility to avoid this difficulty in the laser-scanning-microscope is offered by the use of a point-hologram [17] instead of the microscope objective [(5), Fig. 1]. By means of point-holograms, a focusing to a spot diameter of approx. ?/2 has been realized [17]. Since it is possible to choose the distance of the focus from the hologram and the diameter of the hologram independently from each other, a much larger working distance can be achieved without reducing resolving power than it is possible with lens objectives of high numerical aperture. With regard to a use of point-holograms to focus coherent light, two interesting points may be mentioned:

(i) it is not necessary that the Abbe-condition [7, 8] is fulfilled in point-holograms,
(ii) if non-plane holograms are used, a considerably larger spherical angle may be used for focusing than in plane optical systems.

In this case, the conventional theory of the focusing of light by plane aplanatic optical systems [1] seems not applicable without modification. The theoretical and practical consequences of holographic focusing [17] with respect to the resolving power of a laser-scanning-microscope are difficult to predict. Some remarks on this problem will be given in the appendix.

Appendix

Some remarks on the holographic focusing of coherent light

The propagation and diffraction of light is described by Maxwell's electromagnetic theory. For homogeneous isotropic dielectric media the principal solutions are given by periodic electromagnetic fields which fulfill the Sommerfeld condition [23]. Then, according to the theory of partial differential equations [18], the following problem has only one solution: Consider a region G in the three-dimensional space. On the closed envelope F of G the electric $\overline{(E)}$ and magnetic $\overline{(H)}$

field strength is known for each point of F. $\overline{E}$ and $\overline{H}$ are continually differentiable functions and represent in G solutions of the wave equation. Then the original electromagnetic field in G *can* be reconstructed by a superposition of monochromatic waves of a fixed frequency <u. which are propagated from each element of the closed envelope F. This principal possibility of a single-valued exact reconstruction of an electromagnetic field is a consequence of the time invariance of the homogeneous Maxwell equations. A consideration of the quantized Maxwell field yields analogous results [6].

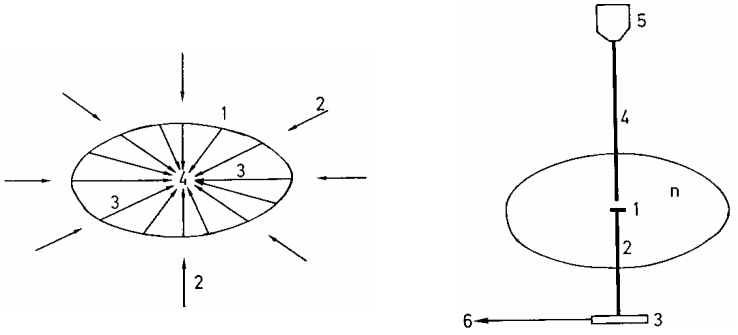
Light sources with a diameter of $0 < r = ?$ (decay of the intensity to a fraction $1/e$ in a distance r from the geometrical centre of the light source) are physically possible. The electromagnetic fields produced by these light sources obey the above mentioned conditions. Then the exact reconstruction of such a field by superposition of monochromatic waves of a fixed frequency, but with different incident angles should also be a physically possible process. In principle, this process requires the superposition of light waves with all incident angles possible in the three-dimensional space (closed envelope). It seems conceivable to realize this requirement by means of a holographic procedure [8, 17]. According to the above mentioned principles, a hologram may be regarded as the boundary condition the fulfillment of which yields the reconstructed waves.

At first, we consider the more simple case of a plane quadratic hologram with a lateral length D. It is assumed that the hologram has no image defects as spherical aberration, coma, astigmatism [17]. Then the lateral diameter of an image spot formed in the middle axis of the hologram in the distance b of the hologram is given [17] by

$$d \tilde{} 0.89 \; x \frac{b}{D} \; ? \qquad (1)$$

? being the wavelength of the reconstructed wave. Since it is possible to chose b and D independently from each other, the spot diameter as far as it is limited only by the diffraction at the edge of the hologram may be made smaller than the values obtainable in good lens systems [17]. According to formula (1) it would be possible to produce by means of such a point-hologram a spot with a diameter which would be considerably smaller than the wavelength used. We do not know, however, how far formula (1), which has been derived from scalar wave theory, describes the actual spot diameter appropriately at least in a qualitative respect for values $b/D << 1$, because using very high numerical apertures, considerable deviations are found between the scalar and the electromagnetic treatment [1]. But even if a calculation by means of Maxwell's theory should show that it is not possible to focus coherent light to a diameter $d << ?$ using plane holograms, it seems conceivable that this aim might be achieved by means of a so-called "4 p-point-hologram" [11]. This is defined as a point-hologram which is characterized by the postulate that it forms an envelope closed as far as possible.

It is beyond the scope of this appendix to discuss further details concerning form and size of such a 4p-point-hologram, its production, material problems, or direction, polarization, amplitudes and coherence of the incident and the reconstructed waves, respectively. The schematic design in Fig. 2 a is only intended to indicate some points:



a) b)

Fig. 2: Focusing by "4p-point-holograms".

a) focusing by means of a 47r-point-hologram (principle):
1) surface of the 47r-point-hologram (ideally a closed envelope); **2**) incident waves; **3**) reconstructed waves; **4**) "focus" of the 4p-point-hologram.
b) application of a 4p-point-hologram in a laser-scanning-microscope:
1) specimen with a fluorescent surface located in the "focus" of a 4p-point-hologram;
2) holding device (same refractive index n as the specimen and the immersion fluid);
3) scanning stage to displace the specimen; **4**) light conductor; **5**) microscope objective for the collecting of the fluorescence light; **6**) connection to the scan generator and to the Automated Focusing System (see Fig. 1).
n: refractive index of the immersion fluid

1) The coherent light used for the reconstruction of the hologram, i. e. for focusing, has to be of an appropriate amplitude distribution, and ideally is to be propagated from all directions (total spherical angle 4 p). Since Abbe's condition [7, 8] has not to be fulfilled, the amplitudes and the incident angles of the coherent waves falling on the 4p-point-hologram can be varied almost independently from each other, if the same wave configuration has been used before in the production of the hologram or if the hologram has been produced for this wave configuration by synthetic methods.

2) The usable dimensions of the 4p-point-hologram have to be large. This implicates a high coherence of the incident waves.

3) Edges and strong curvatures have to be avoided as far as possible in order to reduce the influence of diffraction waves [23] occurring at these sites. To what extent this aim can be realized, remains to be investigated.

4) Possibly, it might be favourable, in analogy to formula (1) derived for a quadratic hologram, to choose also in the 4 p-point-hologram a small ratio of the focal length to the diameter of the hologram. For geometrical reasons, this is possible for one focal plane only. It would be already advantageous, however, for the application envisaged, if in this way only one focal diameter could be reduced to a minimum.

Fig.2b shows the principal design of a laser-scanning-microscope with a 4p-point-hologram in place of a conventional microscope objective. The object (1) with a fluorescent surface is located in the centre of the 4 p-point-hologram. The

diameter of the hologram is assumed to be large compared with the dimensions of the object. The object is placed on a holding device (2), the refractive index n of which is equal to that of the object itself. The interior of the hologram is filled with an immersion fluid of refractive index n. A small hole in the 4.71-point-hologram serves to connect the holding device (2) to a scanning stage (3). The fluorescent light emitted by the object is collected by a light conductor (4) which is connected to a microscope objective (5). The light signal is processed in the same way as in the laser-scanning-microscope designed in Fig. 1. It is of importance that the geometry of the light conductor is chosen in such a way that the part of the specimen surface which contributes to the luminous flux collected by the light conductor is as small as possible. It is assumed that by means of the holographic focusing the excitation light is focused on an area of the surface with a diameter which is considerably smaller than the wavelength used, and that the absorbance by the holding device and the object is small. Since the excitation light is incident from all directions, also the object surface outside the focus is excited to fluoresce. This background fluorescence is estimated to be small compared with the signal, if the thickness of the surface layer contributing to the fluorescence is considerably smaller than the "focus diameter".

The use of plane point-holograms for focusing the exciting beam in the laser-scanning-microscope seems to be realizable without difficulties and suggests to be advantageous at least with respect to the much larger working distance available compared with conventional microscope objectives of high numerical aperture. Whether it might be possible to produce point-holograms in which a larger spherical angle than approx. 2 n is used for focusing, and whether such holograms might be really used in a laser-scanning-microscope, remains to be investigated. Perhaps the use of non-plane point-holograms might result in a better focusing even if the spherical angle is considerably smaller than 4 n.

The above-said speculations on focusing by "4:7i-point-holograms" are only intended to allude to a method which perhaps might be used to enhance the resolving power of a laser-scanning-microscope by means of holographic focusing. To our knowledge, a detailed investigation of the principal theoretical and practical limitations of holographic focusing with non-plane point-holograms does not exist. We feel this to be an interesting subject, independently of the field of application envisaged here.

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