

Modern Optical Microscopy. Part 1

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Abstract. The review presents to the reader the physical principles of various techniques of modern optical microscopy providing better quality and contrast of the image of the object under study and even overcoming the limitations caused by diffraction. These methods have proven to be a suitable tool for biomedical research. The first part of the review is devoted to the discussion of the diffraction limit of resolution, and to the description of laser confocal scanning microscopy, near-field optical microscopy, structured illumination microscopy, light-sheet microscopy, and digital holographic microscopy. © 2023 Journal of Biomedical Photonics & Engineering.

Keywords: optical microscopy; laser scanning confocal microscopy; photon tunneling microscopy; near-field scanning optical microscopy; structured illumination microscopy; light-sheet microscopy; digital holographic microscopy.

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1 Introduction

Since the end of the XVII century, optical microscopy has been an irreplaceable tool for biologists. In this review the authors consider known to date optical microscopy techniques providing better quality and informative value of the object images. The first part of the review is a discussion of the problem of resolution of a classical optical microscope and its constraints. Next, the method of confocal microscopy is considered offering the opportunity to get a better contrast and image quality for the studied samples with diffraction resolution. And fundamentals of near-field optical microscopy are also described. Then the approaches are examined where special lighting of the test sample is used. In the final fragment of part 1 of the review, the method of digital holographic microscopy is outlined, that is extremely useful for the cells study. This method makes it possible to obtain three-dimensional information about a transparent object, i.e., phase object. In the second part of the review, attention is paid to the techniques of optical microsphere nanoscopy, super-resolution fluorescence microscopy and hybrid systems combining various approaches in their structure in order to get the most complete information about the test sample.

2 Diffraction Limit of Resolution

Optical microscopy dates back to the end of the XVI and the beginning of the XVII centuries, exactly when the

first microscopes appeared. They quickly acquire the look familiar to us: there are two main optical systems in their design, the eyepiece (ocular) and the objective. In 1674, Anthony van Leeuwenhoek created a microscope with magnification, which makes it possible to distinguish and study unicellular organisms. Since then, the optical microscope has become and remains an indispensable tool for biological and medical research, due to the fact that light allows us to examine living cells without any damage.

The researchers needed to observe smaller and smaller details, and therefore the magnification of the designed microscopes increased. As magnification increases, the quality of the image obtained with a microscope stops improving after a certain value. The image of the object becomes blurry and fuzzy. The concept of optical system resolution is introduced as the ability of the system to separately represent closely located objects. The first quantitative limitation caused by the diffraction of light waves was given by Ernst Abbe in 1873 [1, 2], who worked for the company of Carl Zeiss producing optical devices. He determined the value of the minimum distance (Δx or Δy), where the two points can still be resolved:

$$\Delta x = \Delta y = \frac{\lambda}{2NA} = \frac{\lambda}{2n \sin \alpha}, \quad (1)$$

where λ is the wavelength of light, NA is the numerical aperture, n is the refractive index of the material between

the object and the objective, α is half the angle of view of the objective. In addition, there is an axial resolution limit (or the depth of field (Δz)), which is determined by the same values:

$$\Delta z = 2 \frac{\lambda}{NA^2}. \quad (2)$$

Thus, it is basically impossible to focus the electromagnetic radiation into a spot smaller than half the wavelength of light (even for the best modern immersion lenses for visible light, the transverse resolution is $\Delta x = \Delta y = 0.2\text{--}0.25\ \mu\text{m}$ and the depth of field is $\Delta z = 0.6\ \mu\text{m}$). A decrease in the radiation wavelength leads to photodamage to biological samples by ultraviolet radiation [3, 4] and requires the use of more expensive optical components [5]. The resolution problem can be solved by using electromagnetic waves with wavelengths less than the wavelengths of the optical range (electronic and X-ray microscopy). But we are not going to consider these methods here, since the aim of the review is to discuss optical microscopy techniques.

Since the end of the XIX century, a search has been carried out for methods of obtaining images with better sharpness and clarity, as well as for the ways to overcome the diffraction limit. Here it is appropriate to recall Ernst Abbe's statement regarding the expansion of the resource of the microscope as a tool for scientific research: "Modern science recognizes that the potential of our organ of vision is limited by the nature of light itself, and this limit cannot be overcome even with the help of the whole arsenal of modern natural science... The human mind will probably be able to subdue the processes and forces which will allow us to overcome in completely different ways the obstacles that now seem unconquerable. I share the hope that this will happen someday. However, I believe that the devices that will help us more than modern microscopes in our cognition of the elements of the material world, will have nothing to do with these modern microscopes, except for their name" [1, 6]. As the matter of fact, the capabilities of modern optical microscopes would not be so impressive without the development of computer technologies and new types of devices for recording images and their subsequent computer-aided processing, that makes modern microscopes hybrid systems [5, 7]. The techniques of optical microscopy capable of raising the quality and resolution of images and overcoming the diffraction limit are discussed in detail below.

3 Confocal Microscopy

Our consideration of ways to improve the quality of the image formed by an optical microscope begins with the method of confocal microscopy. Marvin Minsky, the author of the first patent (1957) for a confocal microscope [8], proposed the method. The method of optical confocal microscopy provides a more contrasting image of the test sample in comparison with a classical microscope. This method offers the most successful

results in case of relatively thick samples under study [9]. The thickness of the sample is tens of micrometers. These are sections of brain tissue, embryos, whole organs and small organisms. An increase in the image contrast is achieved by using a small aperture that filters the light, its diameter ranges from tens to hundreds of micrometers. As a result, only a small volume near the focus point of the lens is displayed, since the aperture cuts off the illumination of the sample layers located above and below. This method is clearly seen in Fig. 1, which shows the trajectory of the rays in the classical and confocal scheme of microscope. In order to obtain a high-contrast image of the whole sample, it is necessary to scan it while re-configuring the system or moving the sample.

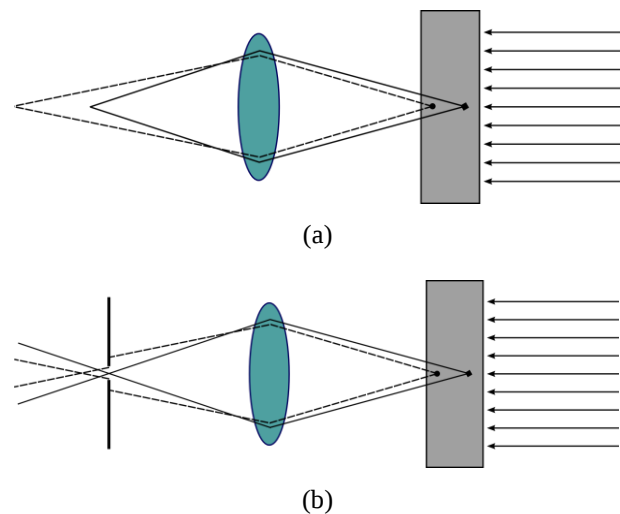


Fig. 1 Sample illumination: (a) ray path in the classical scheme of the microscope, (b) ray path in a confocal microscope.

Another distinctive feature of the confocal microscope is that it illuminates only the volume to be studied [9]. This can be achieved by equipping the confocal microscope scheme with a second focusing system placed behind the sample (Fig. 2(a)). But this scheme requires an additional restriction on the test samples – they must be transparent. Objectives are rather expensive optical elements, and the use of a second focusing system for the sample illumination raises the cost of the entire system. To avoid this, an alternative scheme for the sample illumination was found where a beam-splitting plate was used. In this scheme, a single objective is used to illuminate and look the sample (Fig. 2(b)). This configuration was proposed by M. Minsky [8]. It is more accessible than the previous one and besides it facilitates the adjustment of the confocal system.

The beginning of serial production of confocal microscopes dates back to the 80s of the XX century, being associated with the development of laser and computing technologies. Replacing the lamps, the laser becomes the source of the radiation that excites fluorescence in the sample. To obtain three-dimensional information on the sample, its scanning is carried out.

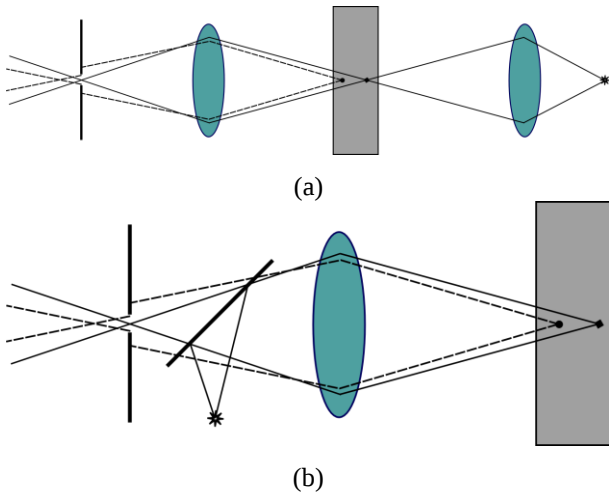


Fig. 2 The sample illumination in confocal microscopy: (a) with use two focusing systems, (b) with use of one focusing system.

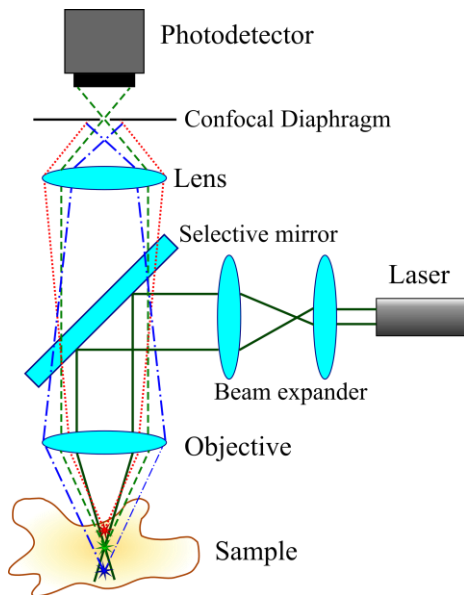


Fig. 3 Principle of the confocal signal filtering.

These features are reflected in the name of the method – Laser Scanning Confocal Microscopy (LSCM) [10–13]. Let us consider a fundamental scheme of a modern LSCM-system. To illuminate the sample, a scheme is developed that uses an objective both to obtain an image and simultaneously to illuminate the sample (Fig. 2(b)). Laser radiation is directed onto the sample through the objective using a dielectric selective mirror (Fig. 3). The use of a selective mirror allows spectral filtering of radiation, the light from the laser will not penetrate into the channel where the sample fluorescence is registered. The laser radiation is focused by the objective and is exciting fluorescence within the volume of the test sample under study (solid lines in Fig. 3). Induced fluorescence of the sample (dotted lines in Fig. 3) is collected by the objective, the same one used for the sample illumination. The fluorescent radiation excited in the layers located above and below the focal plane is cut

off by confocal diaphragm. Only the part of the sample fluorescence that is emitted from a small volume of the sample close to the focus area of the laser beam under the objective-passes through the diaphragm (Fig. 3) [12].

The diameter of the confocal diaphragm D determines the thickness of the optical layer of the sample Δz near the focus of the objective, within the boundaries of which the signal is measured. The thickness of the optical layer Δz near the focus is defined by the expression [12]:

$$\Delta z = \sqrt{\left(\frac{0.88\lambda_{em}}{n - \sqrt{n^2 - NA^2}} \right)^2 + 2n^2 \frac{(D/\Gamma)^2}{NA^2}}, \quad (3)$$

where λ_{em} is the wavelength of fluorescence emission, n is refractive index of the medium between the objective and the sample under study, D is confocal aperture diameter, NA is numerical aperture of the objective, Γ is magnification of the optical system of the microscope between the objective focal plane and the conjugate focal plane where the confocal diaphragm is located. By changing the size of the confocal diaphragm aimed to increase the longitudinal resolution, a situation can be achieved in which a decrease in the diaphragm diameter no longer causes an increase of resolution in the longitudinal direction. The optimal ratio between the light power and resolution is achieved in case of equality of the confocal diaphragm diameter to the first diffraction maximum, while the best resolution is obtained when the diameter of the confocal diaphragm is equal to 5–10 times the size of the Airy disk. Diaphragms of this size are used in modern confocal microscopes [11, 12]. The confocal diaphragm on the fluorescence path from the sample to the detector changes the point spread function (PSF) of the optical system. The PSF becomes narrower.

The resolution in the transverse direction (XOY plane) for a confocal microscope is determined by the FWHM parameter (Full Width at Half Maximum of the point spread function) [11, 12]:

$$\Delta x = \Delta y = \frac{0.51\lambda_{em}}{NA}. \quad (4)$$

The following values of the parameters included in Δx and Δz , are typical for systems with the immersion lens: $n = 1.5$, $NA = 1.3$, the wavelength of the sample fluorescence $\lambda_{em} = 540$ nm. The estimation of the transverse and longitudinal resolution gives the following values: $\Delta x = 0.21 \mu m$ and $\Delta z = 1.06 \mu m$. That is, they have the order of values obtained by Eqs. (1) and (2), and do not exceed the diffraction resolution limit.

A successive scanning of the sample by re-adjusting of the optical system and/or by moving the sample allows you to restore the three-dimensional distribution of fluorescent labels over the sample. The sensitivity of modern microscopes and the scanning speed are high enough to study the dynamic and kinetic transformations occurring in living samples. Under intensive

fluorescence, modern scanning microscopes are able to measure an image of 512×512 pixels size in 200 ms. Lower resolution images are registered faster. For experiments with alive samples, special incubators have been developed that are mounted directly into the microscope system and permit to conduct hours-long research at specified temperature, concentration of substances and elements needed for the sample [10].

LSCM is a method of multidimensional microscopy since a three-dimensional XYZ data array is formed during the registration of the spatial distribution of fluorophores in the sample. When repeating XYZ-data measurements, a four-dimensional XYZt-data array is obtained. It is possible to add a fifth dimension if a spectral approach is used to analyze the received fluorescence signals [10].

The fluorescence of the sample in combination with the confocal system allowed for three-dimensional visualization of living cells and tissue slices [6]. This combination has turned the optical microscope into one of the most powerful and multifunctional tools for solving the problems in modern cell biology, for example, for studying bacterial biofilms [13, 14]. But even the use of high-quality optics does not eliminate the limitation imposed on the resolution of a fluorescent confocal microscope by the wave nature of light.

Confocal microscopy methods and equipment continue to evolve. To increase the field of view with homogeneous illumination it was proposed in Ref. [15] to use an array of micro-lenses with a spatial light modulator. A prototype of the system was built with the field of view of $3.5 \text{ mm} \times 3.5 \text{ mm}$ -size, illuminated by 2500 confocal points. The system provides a lateral resolution of $\sim 0.82 \mu\text{m}$, and the resolution increase is about 1.6 times. The unevenness across the total image area is 3%.

To increase the resolution in the axial direction, 4Pi scanning confocal microscopy was proposed [16–18]. The basic idea of the confocal fluorescence 4Pi-microscope is to use two microscope lenses with a common focus providing the increase in the microscope aperture [16–20] (Fig. 4). The radiation from the exciting laser source is divided in two beams and directed to micro-objectives. A standing wave is formed in the focus area. By changing the length of the optical path in one of the arms of the interferometer, it is possible to achieve the coincidence of the phases of the two waves, i.e., constructive interference. This results in a narrowing of the point spread function in the longitudinal direction and, accordingly, in an increase of resolution.

In the first version of the 4Pi fluorescence microscope system, the obtained resolution value was 110 nm according to the FWHM criterion, which is 4 times lower than the resolution of the confocal fluorescence microscope. Thus, it becomes possible to resolve smaller details if compared with the confocal fluorescence microscope. It was also shown that the 4Pi-microscope is capable of operating in two-photon excitation mode, thus further improving the achievable resolution [19]. The overall advantage of the 4Pi microscope is that, due to the

formation of interference, only half the light intensity is required to expose the sample, so it provides better protection against unfocused bleaching of fluorophores. Besides, it can achieve the same intensity and signal-to-noise ratio as a confocal fluorescence microscope system, but with a smaller volume of the fluorophore under study. Since the solid angle of 4π is not practically attainable, the term 4Pi was chosen to denote the basic idea of the method. In addition to working as a confocal fluorescence microscope, the confocal fluorescence 4Pi-microscope can form three different types of images with a higher resolution. The first type is the interference of two illumination waves; the second type is the interference of two recorded waves on the detector; and the third type is interference of illuminating waves and recorded ones. There was also available a similar I⁵M-microscopy technique [20, 21]. Due to the sophisticated technology, subsequent mathematical processing of images, and inconvenience of working with biological samples placed between close-standing objectives, these microscopic systems are not widely used.

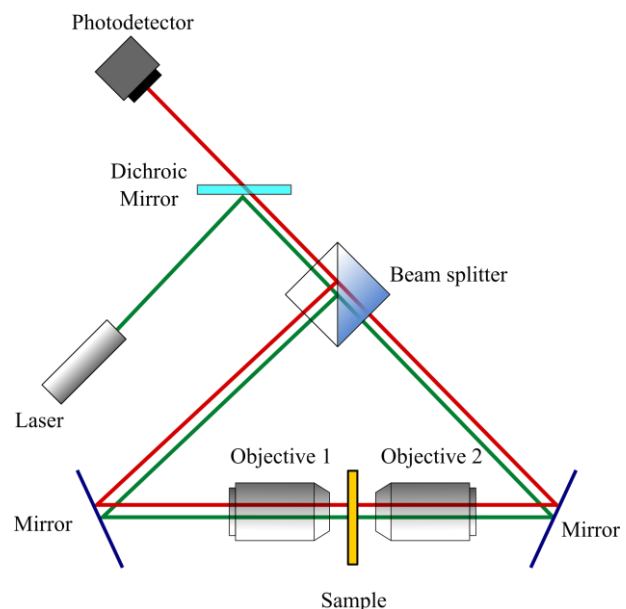


Fig. 4 Optical layout of a confocal 4Pi-microscope.

4 Near-Field Scanning Optical Microscopy

In this chapter the techniques of near-field optical microscopy providing the possibility of obtaining super-resolution with no fluorophores are reviewed. Near-field optical microscopy is one of the options of scanning probe microscopy [22–25]. And in turn, scanning probe microscopy (SPM) is one of the powerful modern techniques for studying the morphology and local properties of the solid surface with high spatial resolution [22]. Currently, it is a widespread and effectively used tool for studying the surface properties.

As already mentioned, the resolution of a classical optical microscope is restricted by the diffraction limit, (Eqs. (1) and (2)). In near-field optical microscopy, other principles of the object imaging are used that make it

possible to avoid the difficulties caused by the light diffraction and achieve the spatial resolution level as high as 10 nm and still better.

The work of near-field microscopes is based on the light passage through subwavelength diaphragms. The electromagnetic field within the area of the subwavelength diaphragm has a complex structure. Directly behind the aperture at distances $Z < 100 \text{ \AA}$ the near zone is located, where the electromagnetic field exists mainly in the form of evanescent waves localized close to the diaphragm surface. The evanescent waves are non-propagating electromagnetic waves with their energy concentrated near the source. Therefore, they exponentially decay while moving away from the Ref. [26] (Fig. 5). Owing to this behavior they can be only recorded by placing near the sample the probe that will act as a converter of evanescent waves into-emitting modes whose intensity can be measured by a photodetector. Being tiny, the probe limits the field of view, therefore, in order to examine a large sample, it should be scanned.

A near-field optical microscope was invented by D. W. Pohl, working at the laboratories of IBM in Switzerland, in 1982, following the invention of the tunneling microscope [26]. The small radius aperture, $r = 30 \text{ nm}$, was made on the tip of a quartz crystal rod of 100 mm-length and 2 mm-thickness. The resolution of the first near-field optical microscope using laser radiation at a wavelength of $\lambda = 488 \text{ nm}$ was 5–10 nm. The further development of the method led to the emergence of new types of transducers and techniques for scanning the sample [25–31]. There are several ways of the sample scanning (Fig. 6). In the initial version, a subwavelength aperture is used, the sample is placed on a hemispherical substrate (Fig. 6(a)), and a part of its surface is illuminated [25, 28]. The glass hemisphere acts as a converter and transmits the signal collected in the near field to the detector in the far field. The transverse resolution obtained for this configuration ranged from 10 to 100 nm. Other versions of the scheme configuration have also been proposed (Fig. 6(b)), they are aperture-free or Tip-Enhanced Near-field Optical Microscopy (TENOM) [26, 29–31]. The external illuminating field is

captured by the probe end and amplified, thus making it possible to convert evanescent waves in the sample into emitting modes with higher efficiency. The cross-sectional resolution is up to $1\div 20 \text{ nm}$, but in this case the useful signal is superimposed on the background of the illuminating beam. The third variant of this method consists in scanning with a dielectric tip without covering the near field in the immediate vicinity from the sample (Fig. 6(c)). This method is called Scanning Tunneling Optical Microscopy (STOM) [26, 31] or Photon Scanning Tunneling Microscopy (PSTM).

The photon tunneling microscopy is also referred to methods of near-field optical microscopy. The resolution of this method in the XOY plane is $\sim 0,29\lambda$ [26], which is almost half the diffraction limit.

Methods of near-field optical microscopy [26] are widely used to obtain thin-films images, diffraction gratings, and also in near-field Raman spectroscopy, in pulsed laser ablation through probes, etc. However, these techniques are characterized by a very small working distance (the distance between the probe and the test surface does not exceed $\lambda/2$, approximately 1–10 nm), an extremely shallow depth of field (150 nm) and a long scanning time for large areas of the sample, so, for example a $1 \mu\text{m}^2$ area is scanned in 16.7 min. These features limit the scope of the method application.

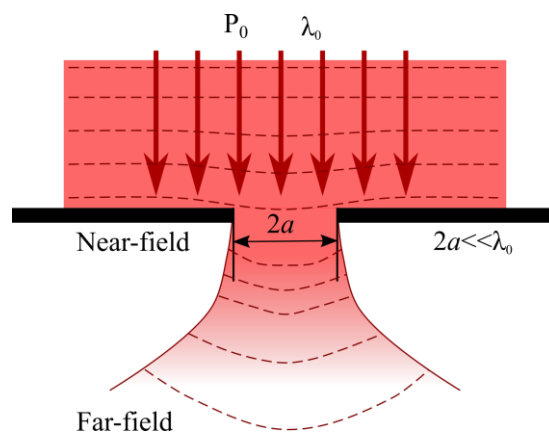


Fig. 5 Evanescent waves.

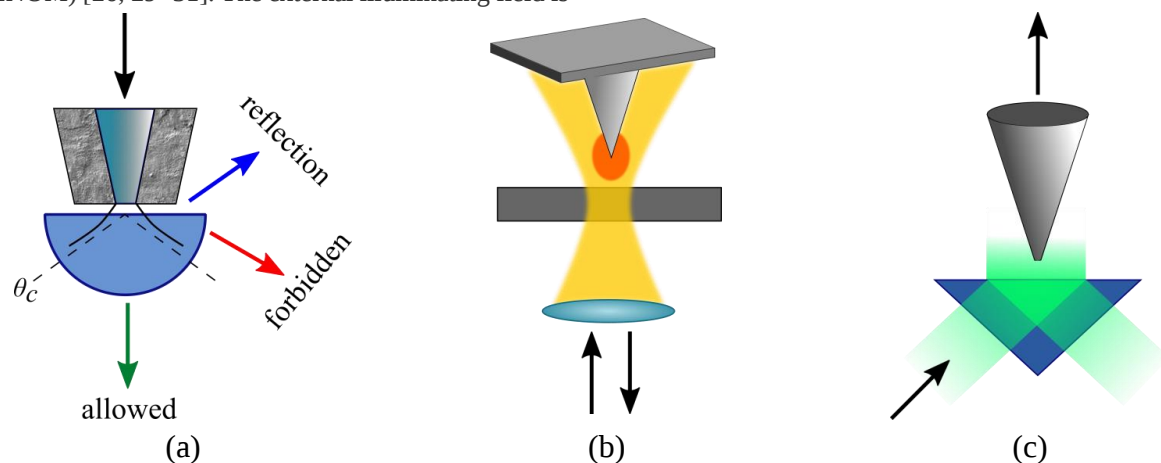


Fig. 6 Options of practical implementation for near-field scanning microscopy: a) based on a subwavelength aperture, b) tip-enhanced near-field optical microscopy, and c) photon scanning microscopy.

5 Microscopy with Special Illumination of the Sample

In order to increase the transverse magnification of microscopes, it was proposed to use special illumination of the studied samples. It is possible to achieve a transverse resolution greater than the classical Abbe's limit without reducing the field of view and without getting rid of background radiation, but instead using lateral illumination with a special structure of intensity distribution in a microscope with a wide field of view. The structure of the intensity distribution of the illuminating beam is set in a special way. In a method called Structured Illumination Microscopy (SIM), the sample is illuminated by spatially structured exciting light, which makes normally inaccessible high-resolution information visible on the image record in the form of moire fringes. The spatial structure of the beam illuminating the sample is formed by a diffraction grating and is a sinusoidal distribution with the period close to the resolution limit of the micro-objective used. A series of such images is then processed and as a result, the image of an object with an improved resolution is obtained [32–34].

To evaluate the functionality of the method, the Fourier transform should be considered. In the spatial spectrum, low-resolution information (low frequencies) is close to the center (zero frequency) while higher-resolution information (high frequencies) is further away. A conventional microscope can resolve structures of samples no smaller than the diffraction limit $\Delta x = 0.2 \mu\text{m}$. Therefore, the microscope can detect only the information located in the spectral zone enclosed by a circle of $1/\Delta x$ -radius centered at the origin of coordinates. This will be the observed region (Fig. 7). Red circle is the diffraction limit of $k_{em} = 2NA/\lambda_{em}$, λ_{em} is the wavelength of the fluorescence emission and NA is the numerical aperture of the microscope objective. The structured illumination of the sample will not change the size of this physically monitored area, but it will facilitate the movement of information from the high frequency region (smaller details) and make this information observable. As a concrete example, the structured exciting illumination in the form of a strip with a sinusoidal profile of the intensity distribution, obtained with a diffraction grating is considered (Fig. 7(a), setup diagram). Its Fourier transform will have three non-zero points (Fig. 7(b), spatial frequency $k_{ex} = 2NA/\lambda_{ex}$, λ_{ex} is the wavelength of the exciting illumination). One of these points is located at the origin of coordinates and the other two are shifted relative to the origin in the direction determined by the orientation of the stripe and by the distance inversely proportional to the width of the stripe. Under the sample lighting with structured illumination, the resulting image of the object will also contain high spatial frequencies (Fig. 7, reading in the limited frequency range, $k-k_{ex}$ and $k+k_{ex}$). The parts of these shifted circles that go beyond the normally observed region, provide new information that is not available via an ordinary classical microscope.

The observed image is the sum of three components, and they cannot be separated by using a single image. However, the coefficients by which they are summed up, depend on the known phase of the structured illumination which can be controllable. By recording three or more images of a sample with different lighting phases, the three components can be separated by calculations, and information on the sample structure can be restored (Fig. 7(b)).

Provided that the extremely large shift distance is selected and the lighting scheme is configured as accurately as possible, then it becomes possible to double the image resolution in the direction of structured illumination. By repeating this procedure with multidirectional lighting, it is possible to collect almost all the information within a circle twice as large as the physically observed area (Fig. 7(b)). The complete information obtained in this way will help to restore the image of the sample with a resolution twice as high.

Comparison of the resolution of structured illumination microscopy with other approaches shows a twofold gain [32]. When examining the sample, which is a cluster of fluorescent microspheres of 121 nm-diameter, the size of microspheres measured using three types of microscopies, i.e., classical microscope, confocal microscope and structured illumination microscopy was 290 nm, 210 nm, and 130 nm respectively. So, the last size measured using the structured illumination microscopy is closest to the sample parameters. Thus, the results confirmed the expected increase in resolution when using this method compared to conventional microscopy and confocal microscopy. And the confocal microscope worked in the maximum resolution mode.

The method of structured illumination microscopy is compatible with existing modern optical microscopes, which makes the technique accessible. This method has been further developed over the past two decades in the form of various approaches with their advantages and shortcomings; it has become a key illumination-method for optical slices, for ultra-high-resolution imaging, surface profiling and quantitative phase visualization of micro-objects in cell biology and engineering. The work [34] can be considered a practical guide for biologists; it attempts to answer the question: which approach is best suited for these studies.

Another approach of using special sample illumination is Light-Sheet Microscopy (LSM). The idea of such a visualization method appeared and has been brought to realization over the past 20 years. The name of the method reflects the basic concept of illuminating the sample planes with sheets or layers of light and using subsequent scanning of the sample to construct a three-dimensional image. This approach has a number of obvious advantages, such as a significant reduction in the dose of optical radiation per sample, and simplicity of large-format visualization at high speed [35]. The method has the capability to meet the criteria required to obtain a large-scale three-dimensional image with a perfect space-time resolution.

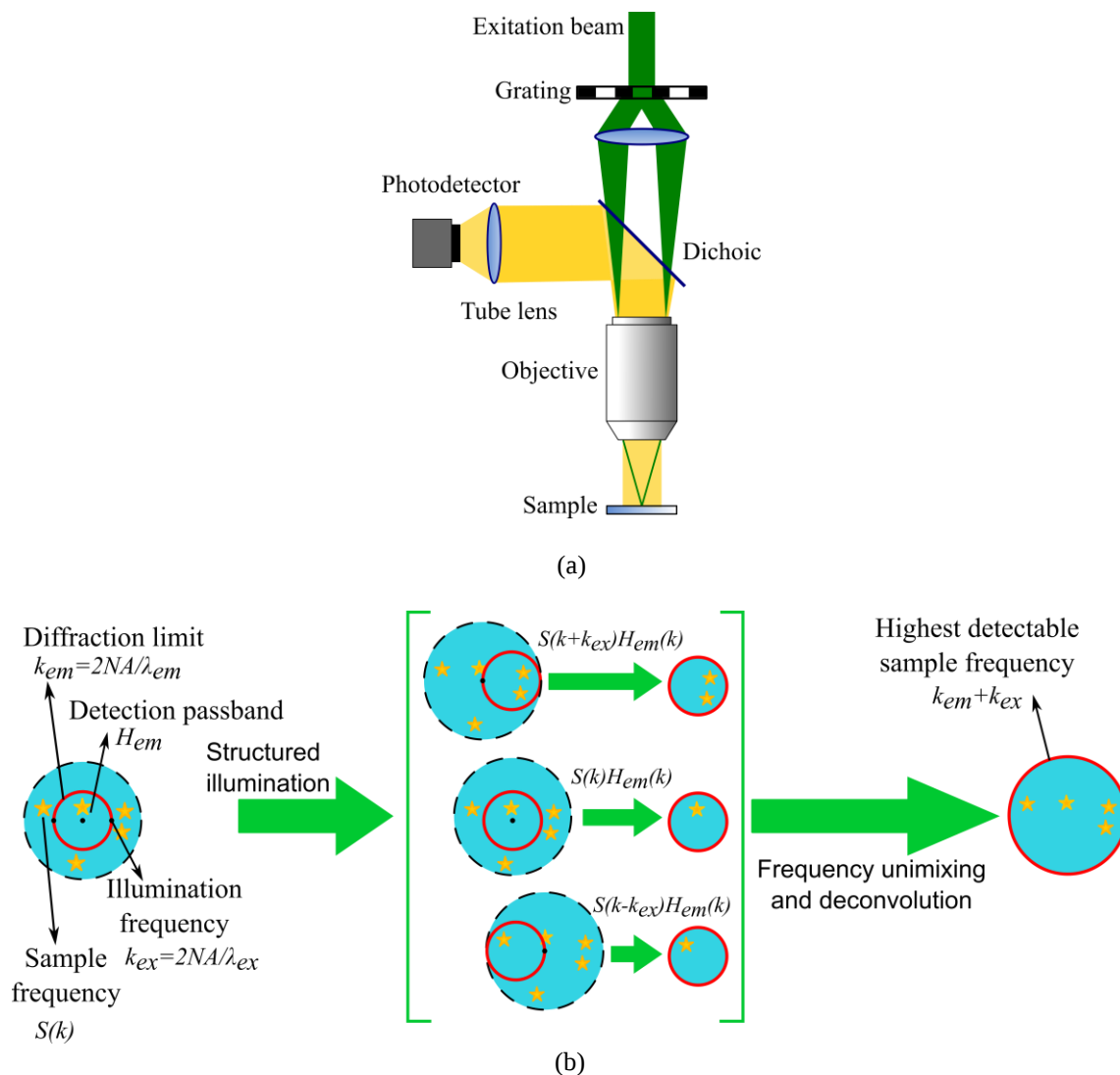


Fig. 7 The concept of the resolution increase as a result of the structured illumination. (a) Schematic diagram of Structured Illumination Microscopy. (b) The red circle in the figure on the left limits the resolvable spatial spectrum of the fluorescent emission of the sample. Maximum spatial frequency determined by diffraction limit is $k_{em} = 2NA/\lambda_{em}$. The diffraction grating creates an interference pattern in the plane of the sample with a spatial frequency k_{ex} . This leads to a shift in the spatial frequency of the sample spectrum $S(k)$ in $S(k+k_{ex})$ or $S(k-k_{ex})$. It permits to register higher frequencies in the detection bandwidth $H_{em}(k)$ of the system. After computational processing, the maximum frequency of the resulting image can be increased to $k_{em} + k_{ex}$.

In the most fundamental sense, the LSM-method is based on selective illumination of the sample [36, 37]. The fluorescence of labels lying outside the focal plane reduces the contrast of the formed image. For this reason, the sample is illuminated only within the focal plane. The sample is illuminated by a separate optical system placed perpendicular to the optics collecting the fluorescence radiation of the sample. A beam weakly focused by a lens, illuminates volume of an approximately cylindrical form with dimensions that remain relatively unchanged in the cross-section in the focal plane at a distance equal to the Rayleigh length of the illuminating beam [38]. To get an image from the entire focal plane at one go, the beam should be expanded along one axis. In the first systems of this type, it was achieved by means of a cylindrical lens that focuses the beam in only one

transverse direction. Such a system called microscopy of selective plane illumination, its schematic diagram shown in Fig. 8(A) [36, 37]. The light sheet in such a system has a Gaussian envelope along its entire length. A light quasi-sheet can be obtained by the sample fast scanning with a beam focused by a spherical lens. Such a system is called digitally scanned light-sheet microscopy [39, 40]. In case when the sample scanning by a beam takes less time than the integration time (i.e., exposure time) of the camera used to obtain the image of the label's fluorescence, then the scanning beam will be perceived by the camera as a continuous sheet [38]. In this configuration, the light sheet has a more homogeneous intensity profile all along its length, thus reducing the noise generated in the case of coherent light [40].

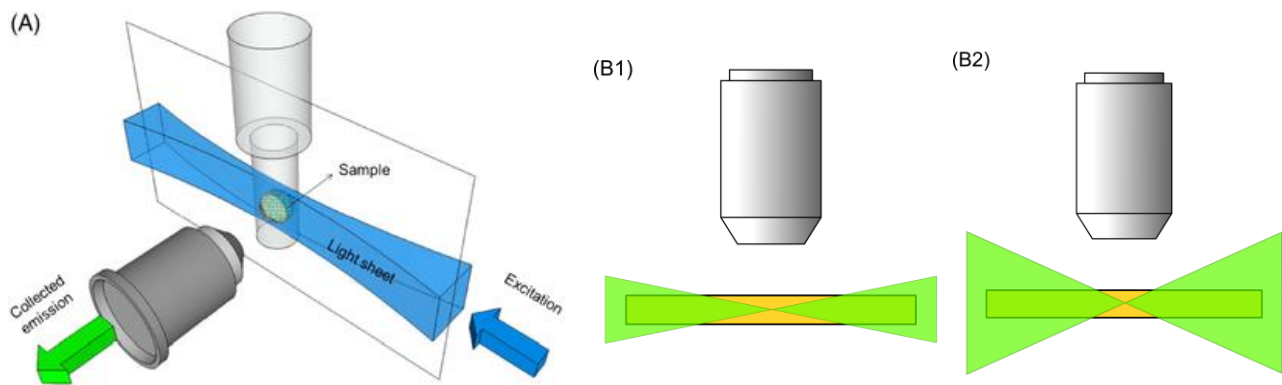


Fig. 8 Light-Sheet Microscopy. Schematic diagram. (A) Microscopy of selective plane illumination. Reprinted from Ref. [41], Copyright (2020), with permission from John Wiley and Sons. Sample lighting options: x-z views of low (B1) and high (B2) NA.

The resolution of a microscope with the light sheet's illumination will be determined by the Abbe criterion (1) and (2). However, there are two modes of operation that determine the axial resolution of the system. The first one consists in generation of a weakly focused and relatively thick light sheet illuminating the sample. The thickness of such a light sheet is approximately equal to the axial resolution of the micro-objective forming the image of the sample. Under such illumination the axial resolution will coincide with that of a standard microscope, but the generated image will have a high-quality contrast, since the fluorescent labels above and below the focal plane are not emitting light (Fig. 8(B1)) [35–37]. While increasing the thickness of the light sheet, you can save the resolution, but the contrast of the image will gradually decrease. Another running regime consists in making the thickness of the light sheet as narrow as possible compared with the axial resolution of the lens [42–44]. In this case, the axial resolution is determined by the light sheet thickness, Fig. 8(B2). With this configuration, a uniform high spatial resolution is achievable, but a sharper focusing of the light sheet is required. This configuration allows for uniform high spatial resolution, but requires sharper focusing of the light sheet. With sharp focusing, the natural divergence of the light sheet narrows the field of view where a homogeneous resolution can be achieved. This will also result in a change of the spatial resolution of the generated image across the field of view of the system.

On the contrary, by using a wide light sheet with low divergence (i.e., retaining its size), it is possible to get an image with the same spatial resolution and contrast all over the field of view. The light sheets microscopy is suitable for volumetric imaging, because it permits to obtain an optical cross-section of the sample by separating signals from z-shifted planes in the sample. The optical cross section can be also obtained by confocal microscopy and structured illumination methods described above. But for these techniques scanning of the sample (in case of confocal microscopy) or obtaining multiple images (in structured illumination microscopy) is required, which makes them slower

compared to the light sheet microscopy providing an optical cross-section of the sample in one measurement. In microscopy of light sheets, special beams are used that are invariant to propagation, for example Bessel, Airy beams owing to specific properties of structure of these fields. Such fields are also called non-diffractive. The Bessel beam amplitude is described by the Bessel function of the first kind, and its central peak of intensity does not expand during propagation, unlike a Gaussian beam. And Airy beams have similar properties. Their use makes it possible to obtain a narrow light sheet within a larger area, therefore a high spatial resolution is maintained in a large field of view. Besides, their use makes it also possible to improve the quality of the generated image while working with thick highly scattering samples [35].

The main drawback of this method remains its dissemination to a relatively small audience of end users. Most modern systems of light sheet microscopy require spatial light modulators inside the system, which complicate and raise the cost of the system design [45].

The problem can be solved by replacement of the controllable modulators with stationary elements. The development of methods for the aberration correction is also carried out. New promising opportunities for application of this method in studies of dim biological samples are opened up owing to multiphoton excitation of fluorescent labels.

There is still a great number of issues in microscopy of light sheets that need to be solved and worked out, but they are not mentioned in this review.

They are related to technical problems and constraints of the method. The most significant of these issues is the use of multiview systems, including polygonal and two-way illumination and/or fluorescence detection [46–49], which combine several data sets to obtain a high-quality image of the whole sample; oblique-plane illumination systems [50], capable of creating a light sheet and collecting fluorescence through a single lens. Although these system configurations provide important advantages, they still use the Gaussian beam and face the restrictions that it imposes.

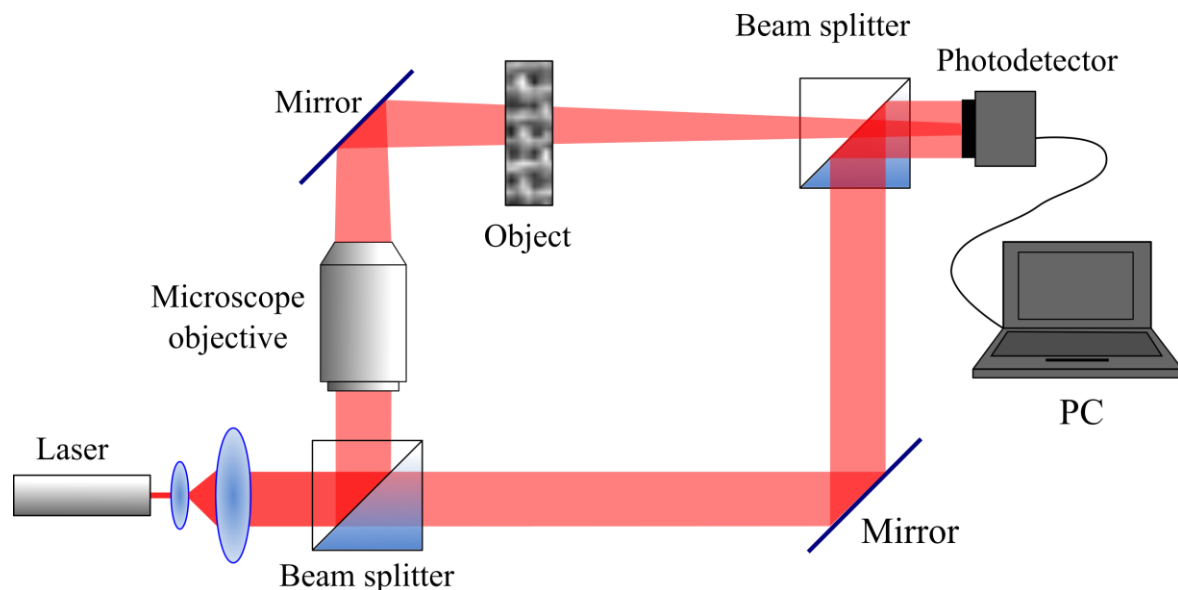


Fig. 9 Schematic diagram of the setup for recording holograms in holographic microscopy.

All these systems will acquire new advantages due to structured light approaches and use of Bessel or Airy beams in the near future. The article [51] discusses diverse applications of fluorescence microscopy of light sheets (Light sheet fluorescence microscopy (LSFM)) for visualization of individual cells and various multicellular samples, as well as a combination of LSFM with other imaging approaches that provide a larger working depth and ultra-high resolution, or the use of complex space-time manipulations providing observations in several directions.

6 Digital Holographic Microscopy

Recently, Digital Holographic Microscopy (DHM) is also successfully developed [52–55]. This method allows you to get a three-dimensional image of the sample being studied. Most of the components of a living cell are almost entirely transparent for the visible wavelength range, so the image obtained with a classical microscope has a poor contrast. However, the refractive index of different parts of the sample cell differs, and therefore the information about the sample will be contained in the change of the phase reflected from the sample or from the light wave that has passed through the sample. The hologram recording in digital holography is carried out using a photodetector device (digital camera), during registration of the resulting field, while this field is an interference of the reference wave and object waves. The hologram contains comprehensive information about the three-dimensional distribution of the optical field of the object wave in the form of the interference fringes. The process of recovery of the original object image in classical holography consists in illumination of the resulting interferogram (i.e. hologram) with a reference wave, and due to diffraction, the image of the object is restored. As for digital holography, here to reconstruct the object image, numerical methods are used, which make it possible to evaluate the amplitude and phase of

the object wave by means of the hologram processing. The main feature of digital holographic microscopy is the use of an additional optical system, i.e., micro-objective between the object and the photodetector (PD) so that to form an enlarged image of the interference area (Fig. 9). This is the main difference between the digital holographic microscopy and just digital microscopy. For the transparent samples study, the hologram is registered in a scheme with a Mach-Zehnder interferometer, whereas for the study of reflective samples – with Michelson interferometer (Fig. 9). Both lasers and sources with a low coherence, for example, super luminescent diodes, can serve as radiation sources.

For the method of digital holographic microscopy does not require precise focusing, which makes the system more accessible due to the reduction in the number of expensive optical and mechanical components in it. The hologram is recorded at a fixed position of the lens, and focusing at different distances is fulfilled using special computational algorithms. The image making is based on a new technique of computer data processing. The signal from the interferometer is processed in real time, and three-dimensional image of the object is concurrently built up.

With the development of inexpensive fast CMOS (Complementary metal–oxide–semiconductor) matrices, powerful algorithms for image reconstruction and phase search, in combination with lowering the cost of computation, the concept of lens-free holographic microscopy has become an attractive alternative to traditional digital holographic microscopy. Over the past 10 years, due to reduction in the cost and complexity of devices without any damage to resolution and image quality, the lens-free technology became more relevant for practical use, and adaptable for applied tasks. In Ref. [52], a lens-free holographic microscope is described, capable of distinguishing objects with the thickness ranging from 350 to 50 nm and with a signal-to-noise phase ratio of 23 dB. High resolution is

achievable due to the improvement of numerical algorithms for reconstructing a three-dimensional image of the object under study by a recorded digital hologram. Studies are underway to develop various ways to improve the spatial resolution of such systems.

7 Conclusion

The first part of the review is devoted to the consideration of optical microscopy techniques, widely used in biological and medical research as well-proven and reliable tools. Confocal microscopy opens an opportunity to obtain images with high contrast, and three-dimensional scanning makes it possible to restore the volumetric structure of the studied object. With the aid of near-field optical microscopy, images of surfaces and objects can be observed with a resolution below the diffraction limit via detecting of attenuated waves. Structured illumination microscopy provides an increase

in resolution of the resulting image due to special illumination of the sample and subsequent processing of the obtained images. Light sheets microscopy opens the way to obtain information about the volumetric structure of an object. Depending on the mode, either its axial resolution can be higher than diffraction limits, or the resulting image can have a better contrast compared to classical microscopy. Digital holographic microscopy has become a new well-proven method in biological applications, owing to which it became possible to carry out the volumetric reconstruction of transparent objects and analysis of their structure.

In the second part of the review, it is proposed to discuss optical techniques that give spatial resolution above the diffraction limit.

Disclosures

The authors declare no conflict of interest.

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