

Modern Optical Microscopy. Part 2

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Abstract. The subject of this article which is the second part of the review published earlier, is the methods of fluoresce microscopy which make it possible to avoid the diffraction restrictions and obtain image resolution with sub-diffraction accuracy. These methods provide new opportunities for biomedical research. In the review some hybrid or mixed microscopy methods combining the capabilities of different approaches are also described. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: optical microscopy; optical microsphere nanoscopy; fluorescence microscopy; reversible saturable optical fluorescence transition (RESOFT); Stimulated Emission Depletion (STED); single-molecule localization microscopy (SMLM); Photo-Activation Localization Microscopy (PALM); Stochastic Optical Reconstruction Microscopy (STORM); Fluorescence Photo Activation Localization Microscopy (FPALM); Total Internal Reflection Fluorescence (TIRF) microscopy; 3D nanoscopy; Double-Helix Point Spread Function (DH-PSF); tetrapod PSF; hybrid microscopy methods.

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1 Introduction

Since the end of the seventeenth century, optical microscopy has become an irreplaceable tool for biologists. The problem of increasing spatial resolution beyond the diffraction limit remains relevant to the present time and is actively studied by many scientific groups [1–3]. This review covers the currently existing optical microscopy methods that provide images of better quality and higher information content. In this second part of the review the following methods are considered: the microsphere nanoscopy, fluorescence microscopy of ultrahigh resolution and hybrid systems whose design combines various approaches aimed to obtain the most complete information about the test sample. Here we will consider the possibilities, features and limitations of these methods. In the previous part of our review [4], the first one, the subject of research was the methods that have already found their application in practice. These are laser scanning confocal microscopy, near-field optical microscopy and digital holographic microscopy.

2 Optical Microsphere Nanoscopy

While studying the laser radiation scattering on quartz micro cylinders, an effect was revealed, later called “photonic nanojet”. The extensive studies of this effect, both theoretical and experimental, were carried out in the

near field zone of various micro-objects. A photonic nanojet (PNJ) is a narrow light beam of high-intensity formed near the shadow surface of transparent dielectric microobjects: spheres, hemispheres, cylinders, ellipsoids. The curvature diameter of these microobjects is approximately equal to the wavelength of the incident radiation or slightly greater than it (Fig. 1) [5–7].

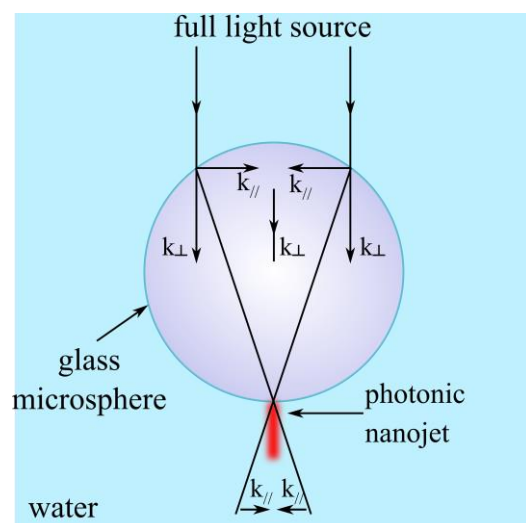


Fig. 1 Schematic diagram of the photonic nanojet emergence.

This effect originates from the interference of the scattered light wave and the wave that passed through the particle. Its peculiarity consists in the sub-wavelength dimensions of the light field localization. The length of such object is $\sim 2\lambda$ by full width at half maximum (FWHM), and its width is $\sim 0.5 \pm 0.2\lambda$ by the same criterion. Such dimensions of the light field localization can be used in nanostructuring and nano-lithography with sub-diffraction resolution. The width and height depend on the environmental parameters. The mechanism of influence of the above-mentioned parameters on photonic nanojets and their application aimed to increase the resolution of the generated image has been circumstantially studied in the last decade [8].

To describe the structure of the light field near the illuminated spherical particle, the Mie theory is used [9, 10]. Within the framework of this theory, the field is conditionally divided into 3 components with respect to the particle: incident, internal (diffracted), and scattered. These fields are represented as a series of eigenfunctions of a ball, which are described by vector spherical harmonics. In a spherical coordinate system, vector spherical harmonics $\vec{M}_{hon/odd\ nm}^{(l)}$ and $\vec{N}_{hon/odd\ nm}^{(l)}$, are characterized by indices m and n , which determine the azimuthal and polar harmonics, respectively. The index l determines the type of spherical Bessel function that is included in the harmonics, and the *hon/odd* notation takes into account the parity of the harmonic with respect to a change in the azimuth angle. The coefficients of the fields expansion in the rows are found in the usual way, that is from the conditions of continuity of the tangential components of the electric field of an optical wave on the particle surface. For a homogeneous spherical non-magnetic particle with a_0 radius, with a complex refractive index $n_a = n'_a + in''_a$ and located in a non-absorbing ($n''_m = 0$) medium with a refractive index $n_m = n'_m + in''_m = n'_m$, the following expressions are found for the complex envelope of the electric fields of the scattered $\vec{E}_{sc}$ and diffracted $\vec{E}_1$ waves for the case of a flat linearly polarized monochromatic wave (azimuthal index $m=1$) incident on a particle:

$$\begin{aligned}\vec{E}_{sc}(\vec{r}) &= \sum_{n=1}^{\infty} E_n \left[ia_n(m_r, x_a) N_{eln}^{(3)}(\vec{r}) - b_n(m_r, x_a) M_{olin}^{(3)}(\vec{r}) \right]; \\ \vec{E}_1(\vec{r}) &= \sum_{n=1}^{\infty} E_n \left[c_n(m_r, x_a) M_{eln}^{(1)}(\vec{r}) - id_n(m_r, x_a) N_{olin}^{(1)}(\vec{r}) \right],\end{aligned}\quad (1)$$

where $\vec{r}$ is the radius vector of a point with spherical coordinates $\{r, \theta, \varphi\}$, $m_r = \frac{m_a}{m_m}$; $E_n = E_0 i^n \frac{2n+1}{n(n+1)}$ are the coefficients of expansion by spherical harmonics of an incident wave $\vec{E}_i = E_0 \exp\{ikr \cos \theta\} \vec{e}_x$. The amplitude of this wave E_0 is propagating along the Z axis and is polarized along the X axis. The partial coefficients of the rows (Mie coefficients) a_n, b_n, c_n, d_n depend on the relative refractive index of the particle n_r and the

parameter of its optical size, i.e. diffraction parameter, and they are calculated by the recurrent schemes. In the Mie theory, the optical field inside and outside a weakly absorbing sphere illuminated by a light wave, is described by the presence of spatial focusing zones called internal and external field focuses. They are caused by the curvature of the spherical particle surface that deforms the wave front of the wave incident on the particle. The peak intensity of the field in the focusing zones depends on the particle size and can by several orders exceed its initial value, and the focuses spatial dimensions can be equal only to fractions of the wavelength of the incident radiation. Thus, a spherical microparticle behaves like a microlens that focuses light within an extremely small volume. A special combination of the particle material optical properties and its size provides a special ratio between the length and width of the waist of the radiation external focus. The focusing zone is stretched in the direction of the radiation incidence, and a light jet is formed of a narrow subwavelength size in the transverse direction. Provided that the particle is illuminated by radiation from the visible or UV range of the spectrum, then the jet transverse dimensions will be of nanometer size.

The results of experimental research on photonic nanojets created by single latex microspheres illuminated by a plane wave at a wavelength of 520 nm, are presented in Ref. [11]. The 3D structure of photonic nanojets was measured using a fast-scanning confocal microscope under detection mode. On the base of the accumulated set of 2D images, a 3D structure was reconstructed. The photonic jets were formed by polystyrene spheres of 1-, 3- and 5- μm diameters. The spheres were applied to a glass substrate placed in air or water. The experimental results were then compared with calculations carried out using the Mie theory. This work proved the existence of photonic nanojets. It was also demonstrated that several microspheres are able to form nanojets simultaneously and independently.

The use of microspheres as lenses is related to microscopy techniques based on the use of photonic nanojets with solid immersion microscopy, which was proposed in 1990 [12]. The essence of this method is to increase the microscope resolution by raising the refractive index of the medium located between the lens and sample. This is reached by placing on the surface of the sample an extra lens with a refractive index greater than the refractive index of traditional immersion media. In Ref. [12], it became possible to resolve lines of 100 nm-thickness through using a lens with $n = 2$ and radiation at a wavelength of $\lambda = 436$ nm. The disadvantage of the method is that an additional lens with a high refractive index must be in direct contact with the test sample, and therefore the field of view of the microscope is smaller than the field of view of a conventional microscope. The field of view can definitely be increased by scanning the sample, but in this case, it is impossible to observe dynamic processes in the scanned area under study. An alternative method

proposed in 2000, suggests the use of materials with a negative refractive index [13].

Paper [13] presents a theoretical approach to amplification of decaying waves and transmission of their encoded information to the far field. The main problem with this method is its limitation to a certain frequency, since materials with a negative refractive index are created in the spectrum microwave range, and to special materials required for the lens and medium.

The interest in the photonic nanojet effect is due to the potential practical applications of photonic nanojets in studying the properties and creating nanoparticles of various compositions, such as metallic, dielectric, and fluorescent materials. These nano particles can be used to create waveguides with low losses and record and store optical data with high density. When a nanoparticle enters an area of increased intensity created by a photonic nanojet formed by a dielectric sphere, backscattering occurs due to the interaction between the photon jet and the nanoparticle [10]. This will lead to a significant increase in the backscattering power of a microsphere with a diameter of 3 μm , when a nanoparticle with a diameter of 30 nm is placed in a photonic nanojet emerging from the microsphere. Despite the small size of the nanoparticle compared to the cross-sectional area of the microsphere, the backscattering characteristics change significantly compared to an isolated microsphere. This allows for a more accurate determination of the presence of certain substances at lower concentrations, making it possible to use this phenomenon for developing new methods of ultra-high-resolution optical microscopy.

The first results of research into improving the resolution of images formed in a PNJ-based microscope were published in 2004 [14]. The photonic nanojet was generated by dielectric micro-objects with a high refractive index. Since then, a number of articles have appeared on photonic nanojets and their use in microscopy [15–18]. A schematic diagram of the operation of such a system is shown in Fig. 2. Radiation from a source, such as a laser, light-emitting diode (LED) or lamp, is reflected by a beam-splitting cube and illuminates the sample (Ob). The radiation then passes through a micro lens (microobjective, MO) and microsphere (MS) before reaching the detector. Then, the micro lens displays an imaginary image of the object formed by the microsphere on the detector. For microspheres with a diameter not exceeding 10 μm , a model was created to study the dependence of photonic nanojet shape and size on the generating microobject and the illuminating beam. The study of two-layer microparticles as objects generating photonic nanojet showed that this phenomenon can be the basis for detecting inhomogeneities with nanometric sizes [15]. Experimental studies of the photonic nanojet phenomenon have also been conducted. It has been shown that not only microspheres but also hemispheres can form photonic nanojet and be used to visualize objects [16]. Between 2011 and 2012, extensive studies were conducted to establish a relationship between the

parameters of photonic nanojet and illumination of the generating micro-object [18–20]. The relationship between the shape, size, and layout of photonic nanojet and the wavelength of radiation illuminating a glass sphere has been established, as has the dependence of photonic nanojet's location relative to the surface of a microsphere on the radius of curvature of an illuminating spherical wave [18]. Some unusual or extraordinary photonic nanojets have been obtained [19, 20] under illumination of glass and polymer spheres with a field that has a complex spatial distribution or polarization structure, and polymer spheres have been proposed as an alternative to glass spheres.

The simulation showed a non-monotonic dependence of the resolution obtained on the wavelength, due to the amplification of the field caused by resonances. This phenomenon can be used to visualize broadband point sources, such as dye molecules or fluorophores. In Ref. [21], the fundamental limit on resolution was determined when using photonic nanostructures, which corresponds to a transverse resolution of the order of $\lambda/6$ to $\lambda/7$. However, there are several challenges associated with the practical implementation of this method.

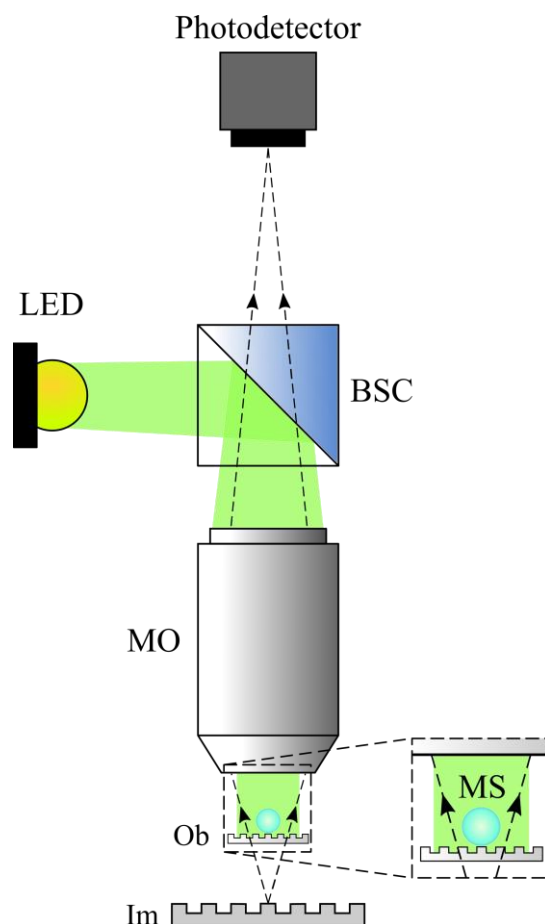


Fig. 2 Schematic diagram of the operation of a super-resolution microscope based on the use of photonic nanojets formed by transparent micro-objects. LED – light-emitting diode, BSC – beam splitter cube, MO – microobjective, Ob – sample, MS – microsphere, Im – imaginary image.

The main challenge lies in the difficulty of manufacturing and handling micro-objects. This can create obstacles for experiments, as without precise control over the microspheres processing, it will be impossible to isolate the part of the sample that researchers want to study. Another challenge is that the photonic nanostructures created by these microspheres are located very close to the surface (within a distance not exceeding λ). This issue was addressed by creating two-layer microspheres that can project photon jets up to a distance of about 4λ . These microspheres have a similar diameter to those made from a single material, but with two layers [22–26]. The two-layer microspheres are SiO₂ spheres coated with gold. This concept was applied to larger diameter microspheres [27], which demonstrated a 2.4-fold increase in the length of the generated photonic nanojet when a two-layer and single-layer microsphere were placed in the same medium and illuminated by light with the same wavelength. The development of this concept continued in the investigation of the possibility of forming photonic nanostructures using hollow microparticles [28]. Alongside studies on two-layered microspheres, various other geometries of microparticles were investigated for their potential application in generating photonic nanostructures [29] and the effect of surface roughness [30].

The second area of research conducted with microspheres focused on the possibilities of visualizing different microspheres under various conditions [31–36]. In Ref. [32], a comparison was made between the techniques of solid-state immersion microscopy and the use of microspheres, demonstrating the significant advantages of the latter method. This comparison inspired further research into the potential applications of photonics nanojet based microscopy. As a result, in Refs. [33–35] various biological samples were visualized, and in Refs. [36], this technique was used for metrological tasks.

One more problem related to these methods is the limited field of view. This can be addressed by increasing the size of the microspheres used, but this would result in a decrease in resolution. To solve this issue, several approaches have been proposed, including the formation of an array of micro-objects for monitoring the test sample [37–39] and shifting the microsphere relative to the sample, or vice versa, to scan it [40–45].

The most impressive results from this method of improving resolution are described in Ref. [46]. A technique is presented that allows for a spatial resolution comparable to that of an atomic force microscope, using microspheres made from barium titanate (BaTiO₃). These unique results were achieved through the use of an interferometer. Due to the use of these microspheres, the field of view for the microscope was expanded and, as a result, the scanning time for the sample was significantly decreased. The authors of this work reported a transverse resolution of 50 nm and an axial resolution of 10 nm for the created system.

The advantage of the above methods for increasing the resolution of optical microscopes by using photonic nanojets formed by micro-objects is their economic

feasibility compared to analogues [47]. The scope of application for these methods is wide and includes metrology, biological observation, robotics, environmental monitoring, and others. However, due to the lack of a universal method for evaluating the resolution of these systems it is difficult to analyze the results obtained from different groups. In Refs. [21, 48] standardization of measurement processes was proposed. The following basic elements are necessary for a precise description of the method: 1) a clear description of a priori knowledge about the system, including illumination, wavelength, filtering, polarization state, and the main components of the optical system such as the lens type and post-processing stages of the resulting image; 2) obtaining images of test mires; and 3) considering gold-coated substrates or fluorescent samples as specific cases rather than general ones.

The issue of increasing the field of view of such nanoscopes also remains not finally solved yet. The works describing this problem have appeared in recent years.

All these factors prevent the classification of super-resolution nanoscopy methods based on the use of photonic nanojets formed by transparent microobjects, and their commercialization by now. Provided these issues are resolved, then micro lenses with microspheres attached to them and specialized software providing the super-resolution images will soon be available. In November 2020, OptoSigma presented a device for nanoscale imaging called OptoNano based on the principle of Optical Microsphere Nanoscopy (OMN) [23]. According to the developers, OptoNano provides a resolution as high as 137 nm with no need for sample preparation. OptoNano microscopes have not only overcome the optical limit, but also eliminated the barriers of high cost and high complexity of operations in ultra-high-resolution microscopy. This unlocks a new paradigm that allows the use of optical microsphere nanoscopy technology for users with a wide variety of training type and level: from research laboratories to biochemists and industrial enterprises managers.

The advantage of the microsphere nanoscopy method is that it does not require fluorescent labels, which can negatively affect the sample under study. There is also no need to use additional pulsed laser radiation, which can affect the sample. It can be used to study metal and silicon samples in cases where it is impossible to implement fluorescent methods. For further development of this technique, it is necessary to establish the production of special microspheres, methods for their positioning relative to the sample under study, and miniaturization of the technology. And although microsphere nanoscopy allows to overcome the diffraction limit to a lesser extent than ultrahigh-resolution fluorescent methods, it will be widely used in various fields, for example, for semiconductor control, where an ultrahigh-resolution fluorescent microscope does not work effectively [49]. A summary of the main characteristics of microsphere nanoscopy and other techniques considered in the review are presented in Table 1.

Table 1 Characteristics of modern super-resolution microscopy methods.

Technique	Resolution	Features	Advantages	Limitations
Optical microsphere nanoscopy	2D projection of the sample. Fundamental resolution limit $\Delta x = \Delta y = \lambda/6 - \lambda/7$; record value: 50 nm [46]. Commercially available solution: $\Delta x = \Delta y = 137$ nm ($\lambda = 460$ nm, $\Delta x = \Delta y = 154$ nm (white light) [23].	There is no need for special sample preparation and the use of fluorescent labels	Simple and accessible	Micro-objects of uniform size made with good accuracy are needed. A limited field of view
Stimulated Emission Depletion (STED)	2D resolution $\Delta x = \Delta y = 30 - 50$ nm Record resolution $\Delta x = \Delta y = 1$ nm [57].	The sample must contain fluorescent labels. In order for the molecules to be forced into the ground state, the sample is illuminated, in addition to exciting light, by a STED laser with a special spatial distribution of intensities in the form of a torus with zero intensity in the center.	Ultra-high resolution. Multiple types of labels can be tracked in a single experiment	Two lasers must be used to excite and quench fluorescence. Photobleaching of phosphors and phototoxic effects under intense laser exposure to the sample
Single Molecule Localization microscopy (SMLM)	2D resolution $\Delta x = \Delta y = 2.5 - 80$ nm	The sample must contain fluorescent labelsThe sample must contain fluorescent labels Stochastic activation of small groups of emitters and their separate localization. To achieve ultra-high resolution, post-processing of the obtained data is carried out, combining information about different groups into a single image of the sample. The accuracy of determining the coordinates of single molecules depends on the stability of the experimental setup and the signal-to-noise ratio	Ultra-high resolution.	Difficulty in processing images from closely spaced overlapping labels. Accuracy of determining the coordinates of single molecules depends on the stability of the experimental setup and the signal-to-noise ratio.
Total Internal Reflection Fluorescence (TIRF)	3D resolution $\Delta x = \Delta y =$ $=250 - 200$ nm Resolution $\Delta z = 100$ nm	The sample must contain fluorescent labels. Obtaining information about fluorescent labels located near the interface of media with different refractive indices.	Obtaining high-contrast images at the interface of media. Using different sources of exciting radiation	Resolution is limited by diffraction. Use of special systems for fluorescence excitation

Table 1 (Cont.)

		Short exposure time. Special image processing is required, since the intensity of the exciting radiation decreases exponentially with distance from the interface.	(lamps, lasers). Obtaining high-contrast images of cell structures located near its membrane adjacent to the cover glass of the sample under study, where total internal reflection occurs. Observation of fast processes.	(prisms or special objectives)
Nanoscopy with modified PSF	3D record resolution $\Delta x = \Delta y = \Delta z = 10 \text{ nm}$ [86]. Commercially available systems: $\Delta x = \Delta y = 20 - 30 \text{ nm}$ $\Delta z = 50 \text{ nm}$ ($\Delta Z = 1 \text{ }\mu\text{m}$) for the system N-STORM [64] with astigmatic PSF; $\Delta x = \Delta y = 20 \text{ nm}$ $\Delta z = 25 \text{ nm}$ ($\Delta Z = 2 - 20 \text{ }\mu\text{m}$) for the system SPINDLE from Double Helix Optics [100].	The sample must contain fluorescent labels. Additional elements are introduced into the microscope optical scheme to modify the PSF, and subsequent processing of the obtained images is necessary. By choosing the type of modernized PSF, it is possible to select different working depths and localization accuracies of labels in the sample	The processing provides access to 3D information with super-resolution without scanning. It is used to study samples of different nature	Special post-processing of the obtained images to determine the coordinates (x, y, z) of the emitting tags. The accuracy of determining the coordinates of a single object depends on the signal-to-noise ratio. The problem of detecting and processing superimposed images

3 Methods of Fluorescence Microscopy of Single Molecules

Fluorescence microscopy is a technique for detecting fluorescent micro-objects using an optical microscope. This method has found widespread application in materials science and the biomedical fields. The operation of a fluorescence microscope is based on the ability of atoms and molecules to absorb light quanta and transition to excited states. When a molecule returns to its ground state, it emits light or fluorescence. Absorption and fluorescence depend on the structure of an electron energy level of a molecule and are specific to each type of molecule. Biological samples fluoresce only weakly, but by using bright and diverse fluorescent molecules (fluorophores), which can stain various tissue and cell structures, fluorescence microscopy has proven to be a valuable technique in biomedical research.

In the 90s of the last century, new technologies for fluorescence microscopy were developed, such as the 4Pi and I⁵M methods (mentioned in the first part of this review). These technologies provide a better resolution than diffraction limits. One method for determining the exact location of a fluorescent molecule is by suppressing the spontaneous emission of surrounding molecules,

which is implemented in methods like reversible saturable optical fluorescence transition (RESOFT).

The first approach implemented in practice was the microscopy of spontaneous emission suppression, known as STED (Stimulated Emission Depletion) [50–52]. This method was developed by Stefan Hell in 1994 [50], and in 2014 he received the Nobel Prize for Chemistry for his development of the technique [53]. The first version of the STED microscope was a single-point system, which used two synchronized pulsed lasers to excite fluorescent molecules and suppress their fluorescence. When a molecule in the excited S₁ state interacts with a photon with an energy equal to the difference between the S₁ and S₀ states, it returns to its ground state (Fig. 3a). In order to force molecules to return to their ground state, the sample is additionally illuminated with a STED laser along with the exciting light. The STED laser has a special spatial distribution of intensity in the form of a torus, with zero intensity at the center. This results in only molecules located close to the region of zero intensity of the STED light emitting, narrowing the point-scattering function (Fig. 3b). Then a sequential scan of the entire sample takes place [51, 52]. The resolution achieved by the STED method is determined by an Eq.:

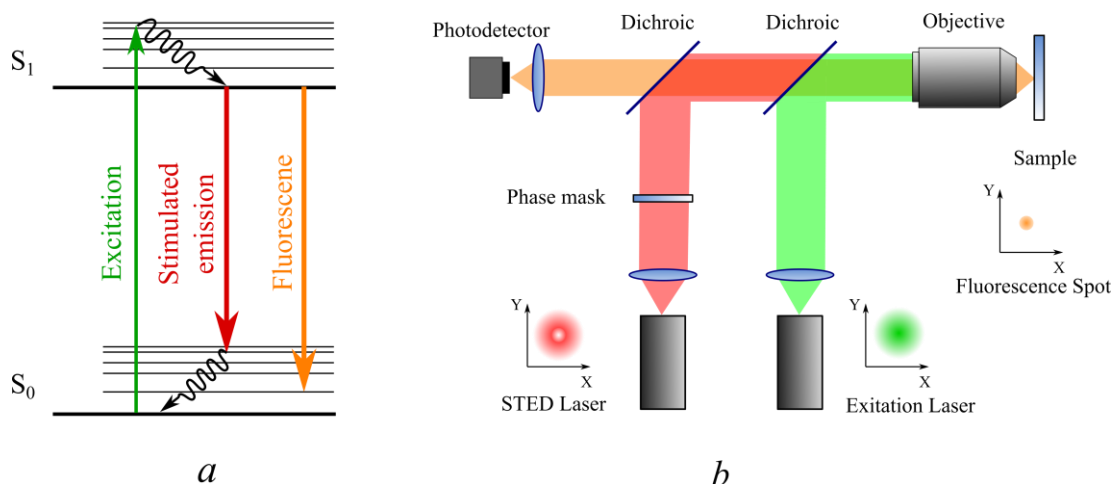


Fig. 3 STED-microscopy: (a) diagram of energy levels; (b) schematic diagram of a STED microscope, point source images obtained using confocal and STED microscopes.

$$\Delta x = \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I_{\max}}{I_{\text{sat}}}}}, \quad (2)$$

where $I_{\max}$ is the used intensity of the STED laser, I_{sat} is the intensity required for 50% of the forced emission. The enhancement of $I_{\max}$ to high values results in a fast photofading of the sample, therefore, to increase the resolution, I_{sat} , which is inversely proportional to the lifetime of the fluorophore, should be reduced. The achieved resolution in the XY- plane is 30–70 nm.

The advantage of this method lies in its high shooting speed, making it suitable for analyzing live, dynamic systems. However, creating multicolored STED microscopy can be challenging due to the undesirable overlap of wavelengths from the exciting and quenching lasers. Nevertheless, the selection of dyes allows us to solve this issue and perform two-color microscopy on living cells with a resolution of approximately 50 nm [54]. At the same time, achieving a resolution of 30–50 nm requires the use of very high intensities from a quenching STED laser, on the order of 10–100 MW/cm². When using a conventional STED laser that emits radiation in the red portion of the spectrum, such as 594 nm, this intense light flux can lead to rapid photobleaching of the dyes in the sample and cause phototoxic effects. These problems can be partially addressed by using pulsed lasers and time-gated detection methods (gated STED) [54]. Due to the registration of radiation with an expected lifetime of fluorescence, the intensity of the STED laser can be reduced by 3–10 times without losing resolution.

A reduction in the scanning area to smaller fields of view (subdiffraction in size) has proven to be an effective method for reducing photobleaching in STED microscopy [55]. With this approach (MINFIELD), the fluorophores inside the quenching laser ring are not exposed to the maximum light intensity, and therefore remain fluorescent for a longer period of time. In

addition, when scanning an area of 200 × 200 nm² corresponding to a single nuclear pore, this method provides an increase in photostability of up to an order of magnitude compared to scanning the entire nucleus using the same parameters. As a result, it is possible to obtain a high-resolution or dynamic image of fluorescent proteins in the nuclear pore complex. The most incoming results were obtained in the MINSTED (the doughnut position entailing minimal STED must be identical with the fluorophore coordinate, hence the name MINSTED) and MINFLUX (or minimal fluorescence photon fluxes microscopy) systems [56–58]. The localization accuracy is about 1 nm. This high accuracy can be achieved by localizing with the movement of the quenched STED beam in the form of a ring closer to the position of the fluorophore during localization. This increases information gain per detected photon and reduces the number of photons needed to obtain high accuracy.

Thus, STED microscopy allows obtaining images of ultra-high spatial resolution, an order of magnitude higher than the diffraction limitation. It is possible to implement tracking of several types of labels in a single experiment. Unfortunately, intense laser exposure can lead to photobleaching of luminescent labels and the occurrence of phototoxic effects. These drawbacks are overcome in the MINSTED and MINFLUX methods, which are the next stage in the development of this approach and increase the resolution to the size of an individual luminescent molecule of ≈1 nm.

In parallel, at the same time, methods of single-molecule localization microscopy (SMLM methods) are being developed. These methods operate by separately registering individual fluorescent labels (such as molecules, quantum dots, or fluorescent proteins) [59–63]. There is another name for these techniques, which are also referred to as SMACM (single-molecule active control microscopy) methods. For photo-switchable fluorescent proteins that undergo a reversible transition between fluorescent and dark states, fluorescence can be induced using radiation from a light source with an intensity that is one or two orders of

magnitude lower than a STED (stimulated emission depletion) laser, approximately 1–10 kW/cm² [62]. This corresponds to typical illumination levels in confocal microscopy.

Three independent groups of researchers have worked independently on the development and implementation of these techniques, and the results of their research are currently available in the form of three microscopy techniques. These techniques include Photo-Activation Localization Microscopy (PALM) [59, 62], Fluorescence Photo Activation Localization Microscopy (FPALM) [60], and Stochastic Optical Reconstruction Microscopy (STORM) [61, 62]. Single-molecule localization techniques rely on separately detecting individual fluorescent molecules. Typically, the sample being studied contains a large number of fluorescent tags. And if they all start emitting light at the same time, their images will overlap and it will be difficult to get information about their location. Therefore, it is necessary to provide conditions for the labels to emit in sparse groups, record their images, and determine the exact location of each individual label (Fig. 4). To achieve subdiffraction accuracy in determining the coordinates of the emitting labels, the type of radiation point source hardware is taken into account. The source image is an Airy disk, and its transverse coordinates can be determined by calculating the “gravity center” of the molecule image, or by approximating the distribution of radiation intensity recorded within the source using a 2D Gaussian function. The accuracy of evaluating individual molecule coordinates depends on the stability of the experimental setup and the signal-to-noise ratio. An image of the fluorescent label or fluorophore is formed by a number of photons emitted from it. Based on this, an image consisting of N photons can be considered as N measurements of the location of the fluorophore. And each measurement has an uncertainty determined by the point spread function (PSF), leading to a localization accuracy close to:

$$\Delta x = \frac{\Delta_{PSF}}{\sqrt{N}}, \quad (3)$$

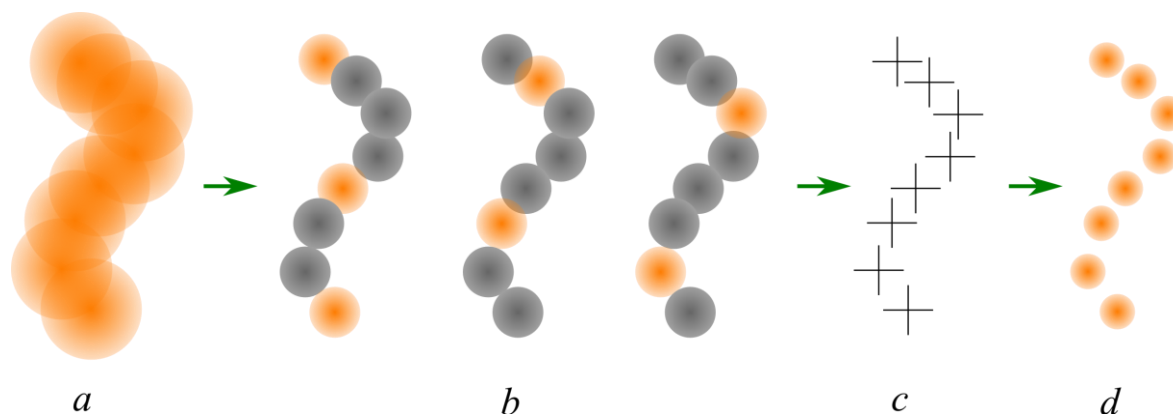


Fig. 4 Demonstration of the principle of single-molecule localization microscopy. (a) Standard fluorescence image. (b) Sequential stochastic activation of small groups of emitters and their separate localization. (c) Determining the location of individual emitters. (d) Reconstructed super-resolution image.

where Δx is the localization accuracy and ΔPSF is the size of the point spread function. It is necessary in these methods to bypass the group nature of fluorescent label emission by exposing them to laser radiation in order to study the entire sample. The labels are switched from the emitting to the non-emitting state. By sorting through the combinations of “switched-on” (i.e., emitting) labels and determining their location with accuracy exceeding the diffraction limit (localization accuracy ranging from 80 nm to 2.5 nm, depending on the level of useful signal), the sample image is compiled with higher resolution in the transverse plane [59–62].

It is possible to obtain a 3D image using the microscopy technique of localization of single molecules, provided that a slight upgrade to the microscopic system is made. For example, weak cylindrical lenses can be added to the optical system [63]. As a result of using these extra cylindrical lenses, the image of the emitting molecule will depend on its position along the Z axis. The point spread function (PSF) of the system will also be modified. By analyzing the shape of the images obtained in this system, it will be possible to estimate not only the coordinates of the emitting object in the transverse directions (X and Y), but also its position in the longitudinal direction relative to the Z axis. The successful implementation of this system contributed to the fact that by the end of 2016 Nikon developed the N-STORM system, capable of building images with super resolution operating on this principle [64]. According to the manufacturer, the system provides a resolution of 20–30 nm in the transverse direction and 50 nm in the Z axis [61]. At the current stage of developing this technique, the ways to solve the issue of resolution and localization of overlapping source images are being studied. This solution is important, as it will accelerate data processing, and therefore – increase the speed of getting images with super-resolution. Currently, several algorithms for localization have been proposed that permit to resolve individual images of radiation sources even under significant overlap of their images [62, 65].

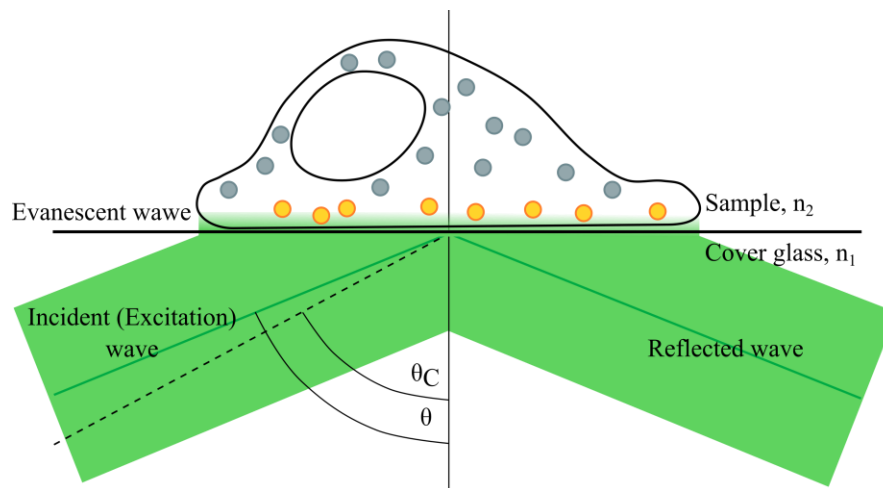


Fig. 5 Schematic diagram of total internal reflection microscopy.

The idea of separate activation, registration and subsequent processing of the received data images allows obtaining ultra-high spatial resolution in the SMLM method. However, it is necessary to choose a compromise between temporal and spatial resolution, since the accuracy of localization of single molecules depends on the stability of the experimental setup and the signal-to-noise ratio. As the ensemble of excited labels increases, difficulties arise in processing images of closely spaced and overlapping labels. Various approaches are proposed to solve this problem.

An increase in resolution can be achieved by using the phenomenon of total internal reflection. Total Internal Reflection Fluorescence (TIRF) microscopy is based on this principle. The phenomenon of total internal reflection occurs when light passes through the boundary between two media, provided that the first medium from which the light originates is denser than the second medium into which it enters. This condition is expressed as $n_1 > n_2$ where n_1 and n_2 are the refractive indexes of the two media. By increasing the angle of incident light on the interface between the two media, a critical angle of incidence can be reached, at which the angle of refraction becomes equal to $\pi/2$ and the reflected light becomes completely internal. At this point, the light no longer propagates in the second medium, but slides along the interface. The value of the critical angle depends on the refractive index of the media, and not on the wavelength of the light. In the region where the incident light experiences total internal reflection, there is a narrow area where attenuated, non-propagating (evanescent) light waves occur. These waves can excite fluorescence in a thin layer of material, as shown in Fig. 5 [66].

The size of a typical living cell is several microns. Radiation at the boundary between media with full internal reflection penetrates to a depth of about 100 nm [67]. Therefore, TIRF microscopy allows obtaining high contrast images of cell structures next to the membrane adjacent to the cover glass of the sample being studied, where total internal reflection occurs. The sample is illuminated in a thin layer, leading to the fact that there is no “illumination” of the image by

fluorescence excited in structures deeper than 100 nm from the cover glass. Using TIRF microscopy, fast moving processes can be observed because the exposure time for this method is short. The intensity of light in the area of fluorescence excitation decays exponentially with distance from where the light is incident. This feature must be taken into account when evaluating fluorescence at different depths – at the surface it will be more intense than at the edges of the attenuated wave region. With an equal content of fluorochrome, this fact will require special image processing [66, 67].

As the light source for fluorescence excitation, lasers and various lamps can be used. The choice of the light source depends on the specific parameters of a particular system. The use of white light is more convenient, since any component necessary for work can be extracted from it with the help of light filters [68]. But regardless of the type of light source chosen, its rays should illuminate the sample at an angle. There are 2 main methods for illuminating a sample at an angle: using prisms or through a specialized objective. When a prism is used, the light source should be placed above the test sample and there should be a system for the prisms configuration correction for each test sample [69]. And when using special objectives, the angle of light incidence on the sample is adjusted by focusing the objective. An important parameter of the objective is its numerical aperture, since it determines the ability of the objective to illuminate the sample at a certain angle. The higher the numerical aperture value, the greater the angle of incidence. For TIRF microscopy, the value of the numerical aperture must exceed the value of the sample refractive index. For instance, in biological tasks, this requirement is met by objective with a numerical aperture (NA) of 1.4. Prism-based systems allow operating in a wide range of wavelengths, and have larger sensitivity. The systems where lenses are used are easy to handle and therefore widely used, but their capabilities regarding the field of view size and sensitivity are smaller.

The interface between the media, where complete internal reflection of light happens, is located between the cover glass and the test sample, but for solving some

specific research problems, it can be deeper. Owing to focusing in deeper layers of the sample, it became possible to see what was previously considered inaccessible for observation, for example, a membrane of a plant cell hidden under the cell wall [69]. Detection of fluorescence in various parts of the spectrum can be fulfilled automatically, the TIRF-microscopy system can be combined with the visualization of fluorescence recovery after photobleaching (FRAP) or fluorescence resonance energy transfer (FRET).

Although TIRF microscopy does not provide super-high resolution imaging, it does visualize structures at the interface with high contrast inaccessible to other approaches. This feature opens up new possibilities. The combined use of TIRF microscopy and optical tweezers opens up new possibilities for studying the physical interactions between organelles. Specifically, it allows us to study the interaction between the endoplasmic reticulum and Golgi corpuscles in plant cells [70]. Currently, the TIRF microscopy method is being used beyond biological applications in other areas. For example, it is used for visualization and study of kinetics in nanocatalysis [71]. In Ref. [72], this technique was used to study the surface dynamics of polyethylene terephthalate hydrolase. This is important for the future development of polyethylene terephthalate (PET) hydrolases.

4 Microscopy with Increased Axial Resolution by Upgrading the Point Spread Function

In the methods of fluorescence microscopy of single molecules, wide-angle microscopes and 2D camera detectors are normally used to localize molecules with an accuracy significantly exceeding the diffraction limit. Due to the limited number of photons available from each emissive label (single molecule, nanoparticle, fluorescent protein, quantum dot), these methods require a scrupulous mathematical analysis and image processing. They allow

getting the information about the projections of the radiating labels location on the sample observation plane. Much more information on the test object can be obtained by extending it to the 3D localization of the emitters. While working with a 2D projection, when there is no information about the position of structures or their dynamics in the axial direction, it is easy to miss important information or make a mess of the data obtained. This prevents a correct understanding of the structure and processes taking place in the sample under study. By means of scanning, the 3D information about the test sample structure or position of a small luminous object can be obtained. And this is exactly what is done in the methods earlier described in the first part of the review: laser scanning confocal microscopy, near-field optical microscopy (by this method the information at a shallow depth can be obtained), light sheet microscopy. In order to obtain 3D information from a single 2D image, it is necessary to modify the PSF of the microscopic system so that it depended on the object position along the Z axis. Then the information about the axial object location will be encoded in its image [73]. To date, many options for the microscope PSF upgrading are known, but the schematic diagram of them is approximately the same (Fig. 6), and represents an inverted 4f microscopic system. The PSF of a standard microscope is changed by changing the phase of the electromagnetic field in the Fourier plane of the 4f system.

The most obvious and easily made PSF modification is the addition of astigmatism to the system using cylindrical lens [64]. Due to this, an astigmatic PSF can be obtained, and by the change of the source image shape, i.e., whether the ellipse is stretched along the X-axis or the Y-axis, (Fig. 7), the Z coordinate of the radiating label can be determined. However, the range for the Z coordinate estimation is limited to 1- μm depth. In section 2 the N-STORM system is described, functioning on this principle and able to restore the longitudinal coordinate with an accuracy of 50 nm.

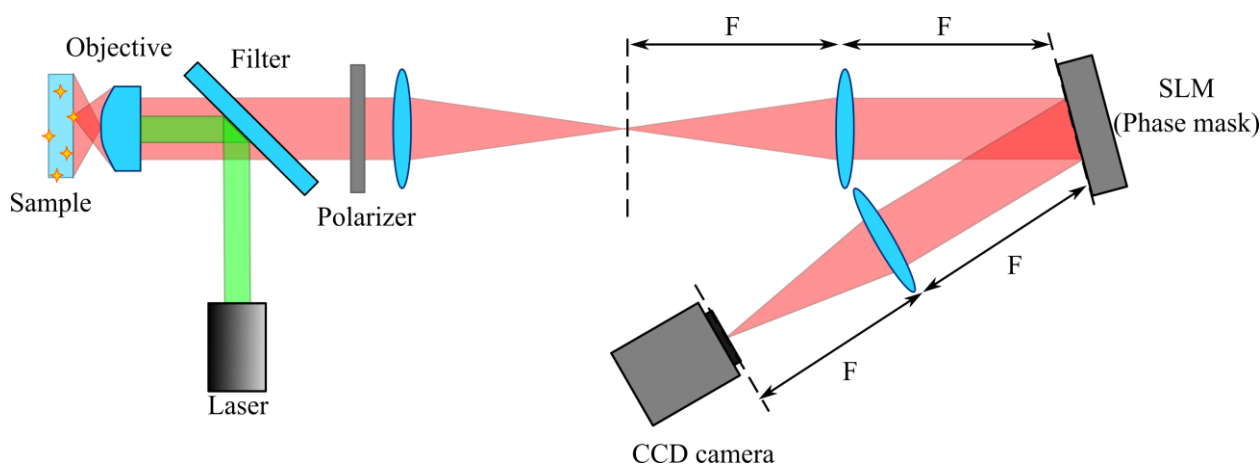


Fig. 6 Diagram of experimental setup for modifying the PSF based on spatial phase modulation of the back focal plane radiation. F – focal distance, SLM – spatial light modulator.

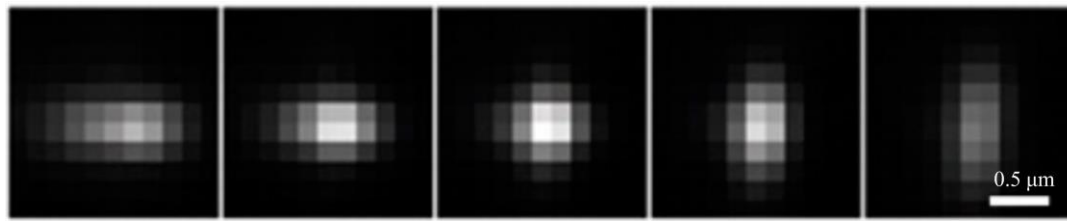


Fig. 7 Images generated due to the PSF modifying into an astigmatic PSF. Scale 0.5 μm (from Ref. [63]. Reprinted with permission from AAAS).

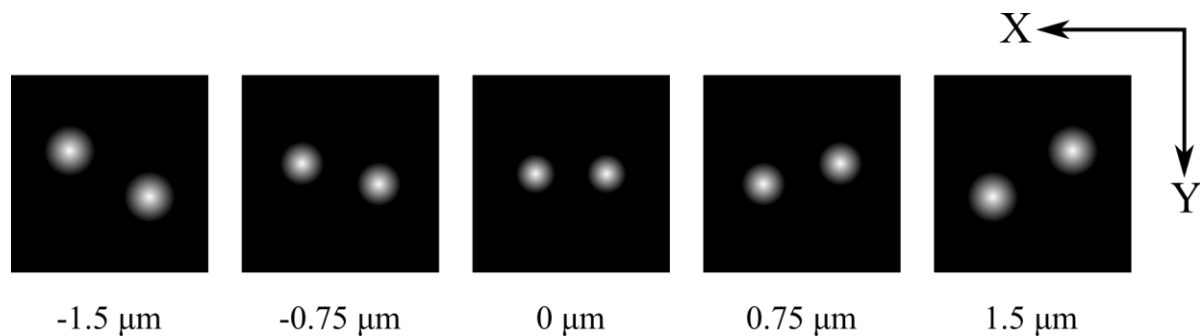


Fig. 8 Schematic images were obtained by converting PSF into an DH-PSF at different distances from the focus of the objective.

Another approach is based on the formation of a rotating light field collected from a point radiation source to determine the depth of the object by defocusing (Depth from Defocus, DFD). A rotating PSF can have various structure [74]. Thus, the PSF described in Refs. [75, 76], is a bright spot, rotating around the center under defocusing. For further processing, the easiest method is the one that allows transformation of the microscope classical PSF into two spots rotating during defocusing. In this case, it is possible to explicitly associate the longitudinal location of the radiating object with the gradient of the straight line passing through the centers of the rotating spots. The obtained two-lobe fluorescent images can be analyzed using algorithms and programs engineered for the analysis of fluorescent images, by the methods of fluorescence microscopy of single molecules described in the previous section. But these algorithms and programs should be slightly supplemented, since now it is necessary to analyze a pair of spots instead of one. To make this modification of the microscope PSF, several types of diffraction optical elements (DOE) were offered. Their development is based on various principles. A more detailed discussion of them is given below.

It was proposed in Refs. [77, 78] to use the 5 Laguerre-Gauss modes superposition in order to obtain a field with the intensity distribution in the form of 2 bright spots rotating around their common center during propagation (Fig. 8). The results of numerical modeling for a linear system along with the experimental obtaining of the proposed field using a calculated hologram are presented. A low efficiency, about 2%, is reported for this field formation. The authors draw a conclusion that the generated field can be used as a PSF system for the tasks of precise 3D-localization of luminous objects in optical

microscopy. But for this, an increased energy efficiency of its formation is necessary. It is shown in Ref. [79] that using rotating fields, the depth estimation accuracy is higher than that for classical microscopes with PSF, representing the Airy function. The authors offer to modify the microscope optical system in a way providing the microscope PSF transformation into a superposition of five Laguerre-Gauss modes, the same as in Ref. [78]). As for information about the object depth, it is proposed to obtain it by defining the transfer function of the system. The transfer function, in turn, depends on the value of the defocus parameter, which is related to the position of the object, since the received image is a convolution of the object field and the transfer function. The authors of this work assert that this method of getting information about the object depth is rather complicated. For these purposes it is reasonable to use a simpler and more efficient pre-built calibration curve for the depth estimation based on the image rotation. In the article the demonstration of the proposed method is also given. In Ref. [80], a solution to the problem of low efficiency of the two-lobe field generation is proposed. The authors propose an algorithm to increase the efficiency of the field formation, with the initial distribution of intensity and phase of the field being a superposition of Laguerre-Gauss modes. During the algorithm operation, the intensity distribution is replaced by a homogeneous one within the aperture boundaries. After this the field is decomposed by Laguerre-Gauss basis functions. To improve the efficiency of the field formation, 9 planes are selected in the space where the field under study rotates in the range from -90° to 90° through 2 Rayleigh lengths, or Rayleigh ranges. In each of the planes, the intensity distribution obtained due to the Fresnel transform, is multiplied by the weight function. The weight function is

two 2D Gaussian functions, it is located at the point of the intensity maxima. Parameters of the functions included in the weight function are chosen in such a way that the width of the Gaussian functions coincides with the maxima width. The reverse Fresnel transform is carried out and the phase in the plane of the phase mask is replaced with the received one. The efficiency of the field generation using the phase mask and obtained as a result of this algorithm was 56.8% according to the modeling results and 37% according to the data received in the experiment, where the phase profile was formed with a spatial light modulator. The modified PSF of the microscope is called a Double-Helix Point Spread Function, DH-PSF.

After the publication of work [80], a series of papers followed testifying to the effectiveness of using a two-lobe field as the microscope PSF to get 3D information about the position of a small luminous object in a sample with sub-diffraction accuracy. The research described in Ref. [81], demonstrates the possibility of the DH-PSF application for visualization of weak emitters in biological systems for tracking the quantum dots motion in glycerin and in living cells. One more experimental demonstration of this mask use for precise localization of objects is given in Ref. [82]. A precise localization of the molecules of photoactivated fluorophore, DCDHF (2-dicyanoethylene-3-cyano-2,5-dihydrofuran fluorophore) residing in the polymer matrix, is carried out. The achieved accuracy is 10 nm in the transverse direction and 20 nm in the longitudinal direction for a sample of 2 μm thickness. This exceeds the diffraction limit by an order of magnitude. In Ref. [83], it is proposed to take into consideration the molecular radiation anisotropy. The polarization and intensity distribution of the field emitted by a molecule is related to its orientation in space. Disregard of this fact in designing systems for the precise localization of individual molecules, causes the errors of tens of nanometers in the localization estimation, which is unacceptable with an accuracy of the object position evaluating of 10–15 nm. The author's crew has proved that using a 2-lobe field as a function of the microscope PSF, it is possible to determine not only the exact location of the molecule, but also its orientation in space. Owing to the offered method use, the determination of the location of molecules was significantly improved: the dipole-induced location error was reduced, and the standard deviation for transverse and longitudinal localization was also lowered. The test object is localized within an elementary volume, $(\Delta x, \Delta y, \Delta z) = (5, 5, 34)$ nm; while previously proposed methods were limited to the volume $(\Delta x, \Delta y, \Delta z) = (25, 25, 116)$.

The probable wide use of two-lobe light fields in real optical systems influenced by aberrations inspired the studies of these fields for their resistance to aberrations. Thus, in Ref. [84] the influence of spherical aberration is studied, and in Ref. [85] the influence of coma and astigmatism is investigated. As a result, it became possible to develop a system capable of tracking RNA COVID-19 in a cell with an accuracy of 10 nm [86], to

identify nanoscale details of the MyosinH organization in the apical complex of *Toxoplasma gondii*, with localization accuracy of 12 nm in the transverse direction and 22 nm in the axial direction [87], to produce 3D localization of individual atoms in an optical lattice [88], imaging of whole mammalian cells [89].

Thus, in Ref. [86] a team of authors proposed to build an “unconventional” two-lobe point spread function (unconventional DH-PSF), with the controllable parameters, such as energy efficiency, transverse size and localization accuracy of the luminous source. By changing these characteristics, it is possible to obtain a PSF with the parameters required for a specific task and a certain sample. In the work, a 2-lobe field is generated, which is later used for 3-D localization, suitable for typical background conditions of the experiment. The development of a phase element providing the “unconventional two-lobe PSF” formation is based on a regularity revealed by the authors. If optical vortices of the first order are located along the axis, then the beam energy will be divided into 2 lobes. The impact of the number of vortices on the intensity distribution was studied by the authors and it was found that with an increase in the number of optical vortices, the efficiency of the 2-lobe field formation rises. But at the same time an increase in the image transverse size is observed. For an effective localization of molecules, a high efficiency of the field formation and a small transverse size of the resulting image are obligatory, therefore it is necessary to find the optimal efficiency-and-size combination, and consequently – the optimal vortices number. The search of these optimal values was fulfilled with the same unchanged number of vortices, $N = 9$, and the distance between them changed. The aperture of the pupil also remained unchanged. The localization accuracy was estimated using the Cramér-Rao inequality. It was illustrated that the field proposed by the authors of this work gives better results than the field proposed in article [79], but the depth of field (depth of focus) here is only 1.2 μm , whereas for the field from Ref. [79] this value is 2 μm .

The capabilities of the proposed phase mask and the “unconventional two-lobe PSF” obtained with its help are demonstrated by visualizing microtubules in mice cells, fulfilled using the obtained PSF. The tubes are marked with a fluorescent dye, excited by laser radiation. The re-emitted light passes through a system where the PSF is modified by means of the calculated element, and is recorded by an EMCCD camera (Electron Multiplying Charge-Coupled Device). This method used for getting the information about the fluorescent dye molecules position in 3-D space, is called Super-resolution Photon-efficient Imaging by Nanometric Double-helix-PSF Localization of Emitters (SPINDLE). By applying this algorithm, it is possible to localize the emitting object in an elementary volume $(\Delta x, \Delta y, \Delta z) = (2.5, 3.8, 16.5)$ nm when 6000 photons from the source are detected, and in a volume $(\Delta x, \Delta y, \Delta z) = (22, 29, 52)$ nm while detecting 1100 photons from the emitter. And the background noise of 30 photons per pixel was typical.

To form a light field with the required rotation parameters and intensity distribution structure, it is reasonable to refer to the structured light fields properties. Thus, in Ref. [90] a method was proposed to obtain diffractive optical elements (DOE) for the 2-lobe light field formation, based on the spiral beam's optics [91]. A further development of the method made it possible to develop a DOE optimized for a specific system with an increased efficiency [92, 93]. This allowed for upgrade of the spatial and temporal resolution characteristics. The capabilities of the obtained system are presented in Ref. [94]. A resolution about 10 nm for single colloidal quantum dots was achieved for all 3 spatial coordinates, and for an exposure time of about 100 ms.

An alternative method is proposed in Refs. [95, 96] for making a phase mask for the formation of fields rotating during propagation. In Ref. [95] a simple and effective method for generating rotating light fields is offered. Here a phase mask is used consisting of sectors (or spiral sections) filled with discrete phase levels ranging from 0 to 2π . The authors consider these spiral phase masks suitable for spatial modulation of light. The calculation algorithm for the mask phase was developed for a general selection of topological charges in radial and azimuthal zones, which provides controllability of the generated rotating field. The results of numerical modeling and experiments, demonstrating rotation of 3-lobe and 4-lobe fields during defocusing, are also presented. It is shown that during propagation, the dependence of the rotation angle of the field intensity distribution on the defocusing value corresponds to the arctangent function. Defocusing is understood as a displacement of the observation plane from the image plane along the axis of the beam propagation. The gradient of the linear part of the obtained dependence rises with the increase of both numerical aperture of the optics used and the number of phase levels of the spiral phase mask. The same researchers team reported on the obtainment of a phase mask capable of the formation of a two-lobe field in Ref. [96], and the algorithm for calculating the spiral phase used by this team was the same as in the previous work [95]. The rotation speed of

the field being generated was controlled by variation of two main parameters of the spiral phase mask: the number of radial zones and phase difference in the neighboring sectors. Characteristics of the fields generated by these masks were studied through numerical modeling. As a result of this numerical modeling, the influence of the number of radial zones and phase gradations on the resulting fields properties was determined. To prove the revealed dependences, a full-scale experiment was carried out, and its results were consistent with the numerical modeling results.

In works [97–99], for 3D localization it is proposed to use a tetrapod point spread function (Tetrapod PSF), the intensity distribution structure of which is more complex as compared to an astigmatic or double-helical PSF. The peculiarity of the tetrapod PSF is that it can provide detection of emitting objects at larger depths, compared to the above-described methods: 1 μm for astigmatic and 2 μm for double-spiral PSF. In Ref. [97] the use of tetrapod PSF is demonstrated for localization at depths of 20 μm (in the experiment carried out with a microchannel system) and 6 μm (in tracking of quantum dots attached to lipid molecules diffusing in the cellular membranes of mammalian cells). In a series of experiments at depths of 6 μm and 20 μm , the same optical system was used. The element forming the tetrapod PSF was obtained using a multi-element liquid crystal spatial light modulator (LC SLM). This enabled the researcher to dynamically control the structure of the generated field and change the PSF depending on the test sample. The phase distribution needed for the generation of tetrapod PSF was calculated using an algorithm. Accounting for the system parameters (magnification, numerical aperture, noise and signal levels), a numerical image model was constructed. The image model was used to solve the problem of finding the phase distribution in the Fourier plane that could provide the dependence of the system PSF on the depth of occurrence of the point source. This strict condition imposed on the PSF, forces researchers to solve a number of tasks for the parameter's minimization, therefore an optimization program was specially developed for these purposes.

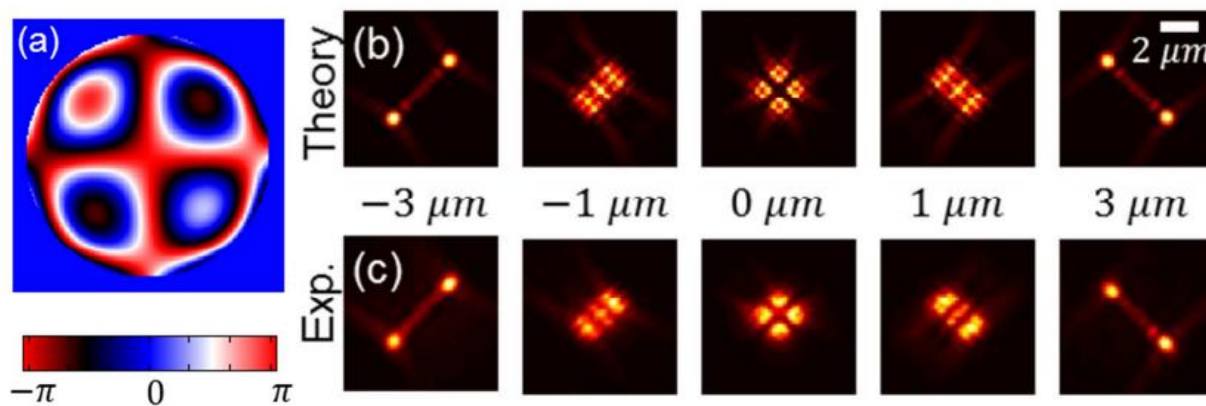


Fig. 9 (a) Phase distribution in the Fourier plane of the 4f system. Intensity distributions: (b) numerically calculated, (c) experimentally obtained (taken from Ref. [97]).

The optimization program was used for various depths of determining the point source location, from 2 to 20 μm . In the course of this program operation, a whole family of phase filters was obtained for the formation of tetrapod PSF needed for various conditions. The found PSFs consisted of 2 separate fractions, and the transverse distance between them raised while the emitter was moving away from the focal plane of the microscope lens. The type of phase distribution in the Fourier plane and the type of tetrapod PSF, found by the calculation and experimentally obtained in various sections along the Z axis, are shown in Fig. 9.

The modification of the microscopic system PSF provides new possibilities for making nanoscopes based on optical microscopes that are capable of displaying the 3D structure of the test sample. The PSF can be formed for a specific task, whether it is required to get a precise localization, ~ 1 nm, at a shallow depth >1 μm ; or vice versa – a less accurate localization, 40–50 nm, but at a greater depth, say up to 20 μm .

However, the use of microscopes with modified PSF has its own difficulties related to various factors: post-processing of images, choice of optimal algorithms for its obtainment, selection of concentrations of fluorescent labels, resolution of images overlapped from neighboring labels, the sample drift. Nevertheless, despite the difficulties these methods show imposing results. In 2019 in the USA, the company “Double Helix Optics” was established, that made a commercial offer for the set-top box attached to microscopes [100]. With this attachment it is possible to obtain 3D images with nanometric resolution due to replaceable phase masks that provide the PSF modification of the system. The localization depth declared by the manufacturer is 20 μm , and the accuracy of coordinate’s estimation is 25 nm. So, for a specific task it is possible to select a PSF required for the work in certain conditions. But manufacturers warn that the user will need to choose what is more important for him in each particular case: a larger localization depth or a higher accuracy in determining the point source location. The set of phase masks includes elements for getting DH PSF, tetrapod PSF, multi-color visualization, and elements providing larger depth. Besides, special software is also supplied complete with the set-top box for constructing a 3D image by the images obtained.

Thus, the modification of the PSF system allows one to obtain information about the position of luminous marks in 3D space from 2D images. Due to the large set of statistics, one can obtain ultra-high resolution. By choosing different types of modified PSF, one can localize one can localize luminous point sources at different depths or in a relatively thin layer, but with high accuracy. The accuracy of localization of single molecules depends on the stability of the experimental setup and the signal-to-noise ratio. The use-related approach continues to offer new DOE options for implementing 3D visualization [101] and to develop algorithms for working in a large field of view [102].

5 Hybrid Methods of Super Resolution Microscopy

Various methods of super resolution microscopy with their benefits and disadvantages have been considered in this review. Lately, works have been published describing systems where various methods are combined in order to obtain the most complete information about studied samples.

In Ref. [103], a system is described that combines the method of super resolution microscopy based on the use of photonic nanojets formed by transparent microobjects, with laser scanning confocal microscopy. To combine these two methods, the researchers placed microspheres in random order on the sample upper surface. To locate the microspheres on the sample upper surface, a solution of microspheres made of melted silicon dioxide ($d = 2.5$; 5 ; 7.5 μm , $n_{\text{sphere}} = 1.47$) and polystyrene ($d = 5$ μm , $n_{\text{sphere}} = 1.62$) was first poured onto the sample, and then evaporated. After the liquid component evaporation, the image of the sample was obtained with the aid of a confocal microscope using a laser light source with $\lambda = 408$ nm. In this way the image of an anode template made of aluminum oxide with holes of 25-nm diameter and a distance between them of 50 nm was recorded. In the resulting images holes are resolvable. Unfortunately, the authors did not provide any data on the intensity measurements or modulation for the fulfilled experiments, so it is problematic to compare the hybrid method with other methods and estimate the resolution. Besides, it is important to note that these impressively small-size elements were displayed with a laser light source, which provides the increased illumination power and reflected light intensity. One more quite important factor is the use of a confocal microscope, which ensures a higher resolution than a classical optical microscope due to the PSF change.

The construction of an ultra-sensitive nano immunosensor is described in Ref. [104]. The imaging system of this sensor is based on a joint use of methods of the microscope PSF modification for 3D localization of an object (the Airy disk is converted into an astigmatic PSF) and light-sheet microscopy. The researchers obtained images of plasmonic nanoimmunosenors consisting of specially processed couples of gold and silver nanoparticles of a certain size. Visualization was carried out in 1000 sections (500 sections above the focal plane and 500 – below the focal plane) with a pace of 10 nm. Basing on the difference in images of gold and silver nanoparticles, the distance between them was measured, it was 23 ± 3 nm for the concentration of 7.8 Zeptomol (zepto = 10^{-21}). The distances between the nanoparticles were estimated for different virus concentrations, and measurements were carried out from 23 to 48 nm. The earlier unknown concentration of the virus has been estimated in a sample of lettuce leaves. In the future the offered method may prove to be a reliable way for the virus molecules detecting at the level of a single molecule.

Another example of combining the capabilities of light-sheet microscopy with the PSF modification is given in Ref. [105]. The tilted light sheet microscopy with 3D point spread functions (TILT 3D) combines a new approach of sample illumination with an inclined light sheet and the use of PSF operating in a wide axial range (i.e. working depth along the Z axis) for 3D super-localization of individual molecules, as well as for 3D super-resolution of visualization in thick cells. Since the axial position of a single emitting particle is encoded in its image structure, and not in the position or thickness of the light sheet illuminating the sample, the requirements to the light sheet in this approach are not so rigid, and the extremely thin beam formation is also not required. The system described in this paper is based on a standard inverted microscope and has a small number of specially manufactured parts and assemblies, which makes it quite affordable. With this device, a 3D-visualization with super resolution (~10 nm) can be performed in thick mammalian cells.

A system presented in Ref. [106], allows for 3D mapping of the sample temperature. The PSF modification into a DH-PSF makes it possible to receive 3D information. Photoluminescence of quantum dots is closely connected with temperature, and a linear dependence has been established between the change in the wavelength of the fluorescence intensity peak and the temperature change. And this dependence was used by the authors in their work. This means that quantum dots can be used as nanoscale materials for the temperature measurements, while the temperature is estimated by the photoluminescence spectrum of quantum dots inserted in the sample. In the obtained system, the radiation collected from the sample was divided into 2 recording

channels, one was for determination of the spectral characteristics of their radiation, and the other – for the 3D localization of the emitting quantum dot. In the experiments a simultaneous measurement of temperature was demonstrated carried out in different points of the sample of living cancer cells of liver during the time $t = 300$ s.

6 Conclusion

The review presents modern methods for increasing the spatial resolution of optical microscopes. Table 1 summarizes the features of the high-resolution microscopy methods considered in this review. These methods make it possible to distinguish smaller details in the image of a sample, by an order of magnitude smaller than the diffraction limit. These approaches combine the latest scientific and technological achievements with the innovative ideas of their creators. By using the features of different methods, new opportunities are opened for studying samples. It is possible to obtain not only 3D information about the sample's structure, but also information about its spectral, thermal, and compositional properties, which is important for biomedical applications. This gives hope that the efforts of the scientific community to develop high-resolution imaging techniques will lead to the creation of more efficient and versatile microscopy methods that will allow obtaining more comprehensive information about the objects being studied.

Disclosures

The authors declare no conflicts of interest.

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