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Biological imaging by super-resolution microscopy

Superrozlišovací mikroskopie a její využití při zobrazování biologických objektů

BACHELOR THESIS

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Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstrakt: Fluorescenční mikroskopie je jedna z nejvíce používaných zobrazovacích metod v biologickém výzkumu. I přes její mnohé přednosti ji však kvůli difrakčnímu limitu rozlišení způsobenému vlnovými vlastnostmi světla nelze uplatnit při zkoumání struktur menších než zhruba 200 nanometrů. To je nejlepší dosažitelná hodnota optického rozlišení, tedy nejnižší možná vzdálenost dvou bodů, které je možné od sebe odlišit pomocí tradičních optických metod. Až do konce 20. století tak nebylo možné pomocí fluorescenční mikroskopie zobrazit jemnější detaily buněk. V posledních letech se ale podařilo tuto bariéru obejít a byla vyvinuta řada zobrazovacích metod, souhrnně nazvaných superrozlišovací mikroskopické metody, které dokážou difrakční limit překonat a umožňují tak biologům pomocí fluorescenční mikroskopie zkoumat mnohem menší objekty (malé organely, viriony, proteinové komplexy či dokonce jednotlivé proteiny) než dosud, a to stále za použití viditelného světla. Tato bakalářská práce seznamuje s vybranými superrozlišovacími metodami, jejich principy a možnostmi využití v biologii.

Klíčová slova: fluorescenční, FPALM, mikroskopie, PALM, RESOLFT, SIM, SSIM, STED, STORM, superrozlišovací

Abstract: Fluorescence microscopy is one of the most widely used imaging techniques in biological research. Despite its numerous advantages, it can be used only for studies of structures larger than 200 nanometres, due to diffraction limit caused by a wave nature of light. The value of 200 nanometres is the best reachable value of optical resolution, in other words, the smallest distance of two objects, which can be separately recognized by conventional optical systems. Up to the end of the 20th century it was therefore impossible to observe finer details of cells. However, recently several breakthrough imaging techniques, named super-resolution microscopy techniques, managed to bypass the diffraction limit and enabled biologists to study much more delicate structures, such as small organelles, virions, protein complexes or even particular proteins, while still using a visual light. This thesis introduces some selected super-resolution methods, explains briefly their principles and presents some of their applications in biology.

Keywords: : fluorescence, FPALM, microscopy, PALM, RESOLFT, SIM, SSIM, STED, STORM, super-resolution

List of Abbreviations

AFM	Atomic Force Microscopy
CLSM	Confocal Laser Scanning Microscopy
DM	Deformable Mirror
ER	Endoplasmic Reticulum
iSCAT	Interferometric Detection of Scattering
FCS	Fluorescence Correlation Spectroscopy
FLIM	Fluorescence Lifetime Imaging
FPALM	Fluorescence Photoactivation Localization Microscopy
FRET	Förster Resonance Energy Transfer
FT	Fourier Transformation
GFP	Green Fluorescent Protein
GSD	Ground State Depletion
NA	Numerical Aperture
PALM	Photoactivated Localization Microscopy
PSF	Point Spread Function
RESOLFT	Reversibly Saturable Optical Linear Fluorescence Transitions
SIM	Structured Illumination Microscopy
SLM	Spatial Light Modulator
SPIM	Selective Plane Illumination Microscopy
SSIM	Saturated Structured Illumination Microscopy
STED	Stimulated Emission Depletion
STORM	Stochastic Optical Reconstruction Microscopy
TIRF	Total Internal Reflection Fluorescence

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Introduction

When using microscopy for object visualization, scientists highly value an assistance of a core facilities' staff in helping and explaining to them what is physically possible to achieve with their samples and how to interpret obtained data. Behind this assistance, there is a deep knowledge of optical systems and good understanding of physical aspects of biological imaging experiments.

In a remarkable development of recent decades, several optical methods have circumvented the resolution limit of a conventional optical microscopy. To emphasize their impact on natural sciences the Nobel Prize for chemistry was awarded in 2014 jointly to Eric Betzig, Stefan Hell and William Moerner. Citing the Stefan Hell Nobel Lecture (Hell, 2015): *"... What does it take to achieve the best resolution? Now let us assume one had asked this question in the 20th century. What would have been the answer? Well, the answer was unquestionably: good lenses. ... [but] Resolution is definitely limited by diffraction if one separates features by the focusing of light - no way to tell features, the molecules, apart, because everything overlaps on the detector. So what was the solution to this problem? Do not separate just by focusing. Separate by molecular states, in the easiest case by "on/off" states. If separating by molecular states, one can indeed distinguish the features, one can tell the molecules apart even though they reside within the region dictated by diffraction. ... The resolution game is not about lenses anymore. It is about molecular states, and molecular states are of course about molecules. The molecules determine now how well we can image; they determine the spatial resolution. And that is not optical technology — that is chemistry."*

The aim of this bachelor thesis is to deliver the relevance of the technical progress and consequent broadening of possibilities in biological research.

The first chapter attempts to bring to a reader a pedagogical description of the optical and fluorescence microscopy, detailed understanding of which might be for majority of life scientists, those out of the physical field, hidden or never explored. The conventional microscopy is based on the same physical principles as a human eye, so these microscopes are in a way just "enhanced eyes". On the other hand, the super-resolution techniques are based on mechanisms never invented by biological evolution.

The second chapter briefly introduces selected accessible super-resolution methods and explains their principles to show that by ingenious modifications of optical systems, many delicate details of biological structures can be observed, so far unseen in visible light. This chapter also mentions some selected applications of these methods.

The third chapter serves as a comparison of the previously described super-resolution methods, illustrating how each of them can facilitate different improvements of biological experiments.

In the last chapter, I briefly discuss some shortcomings of the super-resolution methods and their desired future enhancements. Finally, I give my summary in conclusions.

1. Background of traditional microscopy

1.1 Optical microscopy

To understand various cellular mechanisms, it is crucial to reveal the function and structure of the tiniest objects in cells. The most straightforward way to inspect an object is just to look at it – meaning to form its image from the emitted visible light. Of course, the naked eye is not powerful enough. It can register the light emitted from virtually point-like source, just consider the stars, but to learn more than the fact that some radiating object is at certain direction, in particular to assess its shape or structure, one has to be able to distinguish from which of its parts is the radiation coming. This desired information implies importance of the resolution. The resolution is the shortest distance at which two objects can be separately recognized. The focal length of a human eye lens and thus the viewing angle are modified by muscles attached to the lens so it can be focused on objects at various distances. The further the object is located, the smaller the visual angle becomes. The resolution of the naked human eye is essentially given by the minimal recognizable visual angle of about 0.01 degrees, which corresponds to about 26 μm object seen at distance of 15 cm (Wong, web 1).

The optical microscopy enables biologists observation of magnified object by increasing a visual angle at the retina making use of series of complex lenses, in the simplest type of compound microscope consisting of an objective and an ocular. The compound microscopes thus employ a two-stage magnification by projecting a magnified image by the objective into a body tube of the microscope and further magnifying the projected image by the ocular.
As a result, it effectively lets us to observe closer and larger image of the object and to discern its details that our bare eye could never see, which is of crucial importance in many scientific fields of these days, and the biology makes no exception (Fig. 1.1).

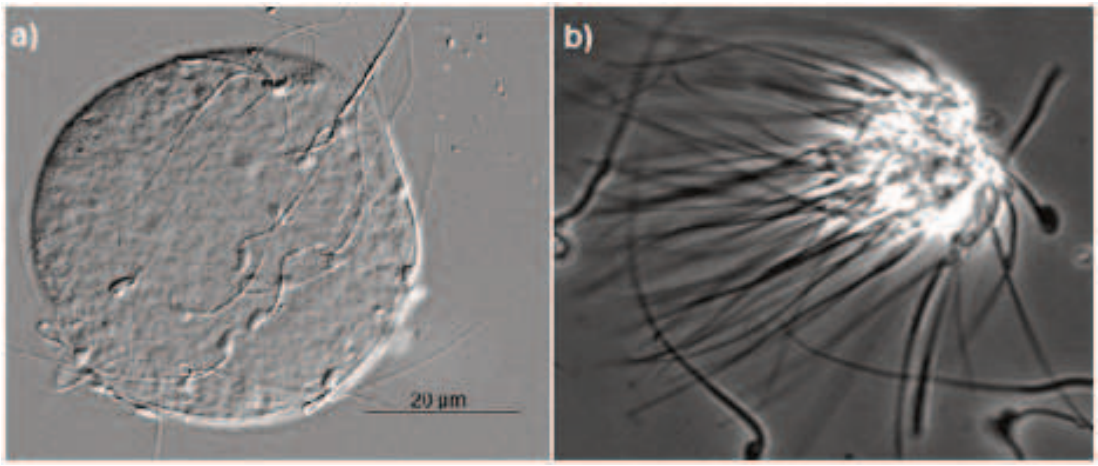

Figure 1.1: Use of the light microscopy. a) Human egg with attached sperm, scalebar 20 μm (Klinovska et al., 2014); b) sperm of *Apodemus sylvaticus* forming so called "sperm train" (Moore et al., 2002).

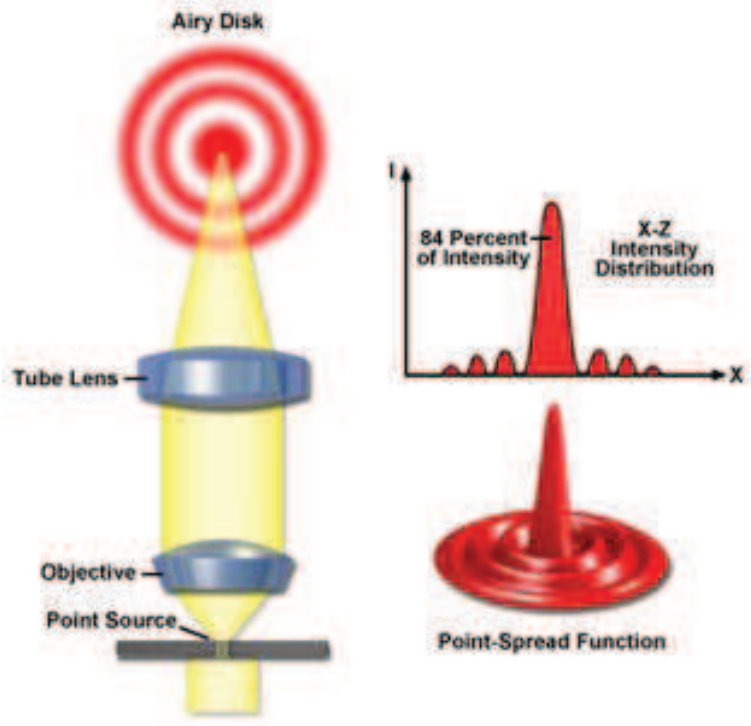

Figure 1.2: Airy disk and PSF (Rottenfusser et al., web 2)

1.1.1 Resolution limit

So far the description of optical microscopes was oversimplified, based on a geometrical optics, which does not consider the wave nature of light and therefore implies that it is possible to increase the resolution infinitely. However, the wave nature of light brings a limit on the resolution because of the diffraction. (Thus, when visualizing a structure, we can see that, e.g., tubulin network is spread across the cell cortex, but we cannot distinguish details of this filamentous structure (see Fig. 1.4b) or its interaction with other proteins, due to the resolution limit, unless we use an electron or super-resolution microscopy.)

To grasp the origin of the diffraction limit, consider a light from a point source passing through a circular hole of a size comparable to its wavelength and perpendicular to the optical axis. Behind the hole, one detects the diffraction rings: the objective is not able to focus all beams into a single point, but instead there is a slightly blurry spot surrounded by ring formations, known as the Airy disk (Airy, 1835). To describe properties of the optical system and to generalize the situation described above, the concept of the Point Spread Function (PSF) was developed (Hecht, 2002). Assuming that the light is emitted by the point source, the PSF describes its distribution after passing through the optical system. In the example above, the PSF is 3D pattern composed from the Airy patterns in particular image planes (Fig. 1.2). The PSF tells us, how blurry is the image of the point light source. The image of some extended object then results from the superposition of the PSFs of its points, so the PSF is the smallest unit of the formatted image. To recognize two separate objects, the intensity loss between the maxima of their PSFs has to reach at least twenty percent. This

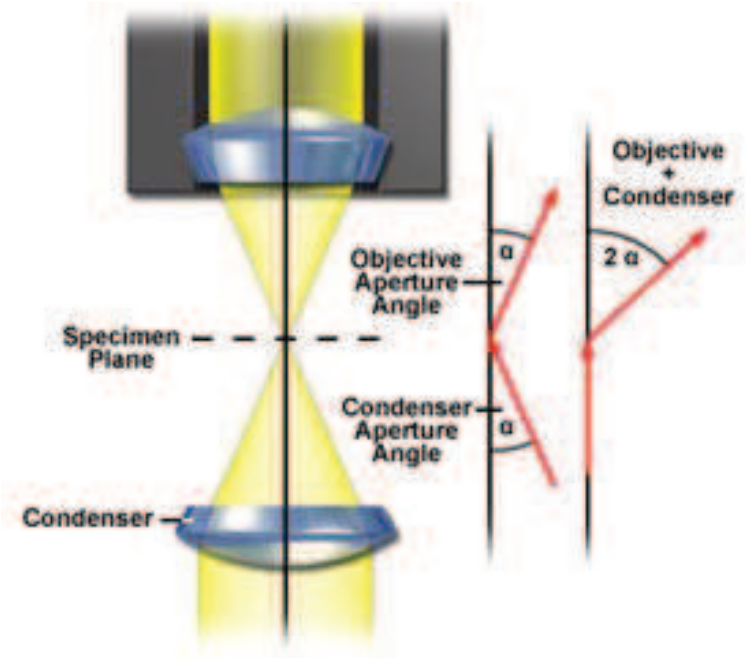

Figure 1.3: The concept of numerical aperture of objectives and condensers (Rottenfusser et al., web 2)

condition, from which a simple estimate $d \simeq \lambda/2$ for the resolution d follows, is known as Rayleigh criterion (Lord Rayleigh, 1879). As the light propagation is reversible, one can also interpret PSF as a probability distribution of a photon emanating from the sample area and reaching the single point in the focal area (Hell, 1997). This interpretation is especially useful when dealing with confocal optical setups (see below).

In 19th century, Ernst Abbe published the formula of the microscopy resolution d based on the diffraction (Abbe, 1873 , as cited in Schermelleh et al., 2010):

$$d = \frac{\lambda}{2\,NA} \ , \qquad \text{with } NA = n \cdot \sin\theta \ , \tag{1.1}$$

where λ is wavelength, NA stands for a numerical aperture, n is a refractive index of an immersion medium located between the objective and the cover slip and θ is a limit half-angle in which the light is able to enter or exit the lens, in other words the angle between the ray perpendicular to the objective and the most angled passing ray. The numerical aperture basically tells us how much light the objective is able to gather at the specimen, hence NA affects brightness of an image. To enhance the resolution, the condenser is added to the microscope between the specimen and the source of light to create a cone of light, which illuminates the specimen. In the half-angle limit both the objective and the condenser are considered (Fig. 1.3).

1.1.2 Resolution improvements

The Abbe equation (1.1) shows that one way to improve the resolution (that is, to decrease d) is to increase the numerical aperture. When increasing NA, maxima of the PSFs coming from two close points can be distinguished one from the other on a smaller

distance due to smaller radius of the Airy disk and thus thinner and steeper profiles of PSFs. Therefore, the intensity between the maxima decreases faster. The NA can be increased by using the immersion oil placed between the objective and the cover slip so that the refractive index n of the immersion oil is matched with the refractive index of the glass. Nevertheless, the angle θ is also limited since its theoretical limiting value is 90 degrees and so the maximal value of $\sin\theta$ equals one (Hecht, 2002). These values are only theoretical, they are not reached by the real not ideally adjusted microscopes. In practice, the maximal commonly achieved NA values are from 1.0 to 1.35 when using the immersion oil (Davidson, web 3). Thus, the theoretical diffraction limit for the optical microscopy is very difficult to approach in practice.

Thanks to the link between the resolution and the PSF, properties of the imaging system can be evaluated if the PSF for the used imaging system is determined. If the PSF is known, the blur of an image can be reduced. The blur is an out-of-focus light which is a consequence of the diffraction. To decrease it, it is appropriate to apply a deconvolution on obtained pictures. The deconvolution is a mathematical operation allowing a postprocess removal of the out-of-focus signal from an acquired image leading to its refinement and so to the resolution improvement (Wallace et al., 2001).

To enhance the resolution, one can also decrease the wavelength of light. However, by decreasing the wavelength, the energy of radiation E is increased, because:

$$E = \frac{h \cdot c}{\lambda} \simeq \frac{1240\,\mathrm{eV} \cdot \mathrm{nm}}{\lambda}\ , \tag{1.2}$$

where h is the Planck constant, c is the velocity of light.

For all the mentioned above, it is probably obvious that the optical systems for the visual light cannot be used for the shortwave radiation: as we know from daily life, X-rays pass through materials and cannot be focused. Electrons also demand an entirely different focusing and imaging systems. As much as maybe wished, the usage of the shortwave microscopy for biological samples is not possible due to the fact that the shortwave light or electrons can damage specimen by ionization or radiation. Besides, these methods usually suffer from lack of contrast for biological samples. Hence, the biology puts a stress on developing visualization tools and methods that would enable to examine specimens in a less or no damaging way.

From the resolution of human eye which is 26 μm we got as low as to resolution of ~ 250 nm by the optical microscopy. The diffraction limit d summarized by a simple rule of thumb to $\sim \lambda/2$ was for almost the whole century considered to be unsurpassable obstacle on a way to better resolution. Nevertheless, in last decades the scientists developed several approaches to overcome this limit and to achieve better resolution. Generally speaking, the super-resolution techniques are based on a radical redesign of an optical system and/or on utilizing some additional information. Some of these methods developed for improvement of the resolution beyond the Abbe diffraction limit and in particular for more efficient examining of biological specimen will be subject of my thesis. Of course, several other methods with a significantly improved resolution were developed, such as an atomic force microscopy (AFM) (Binnig et al., 1986) or near-

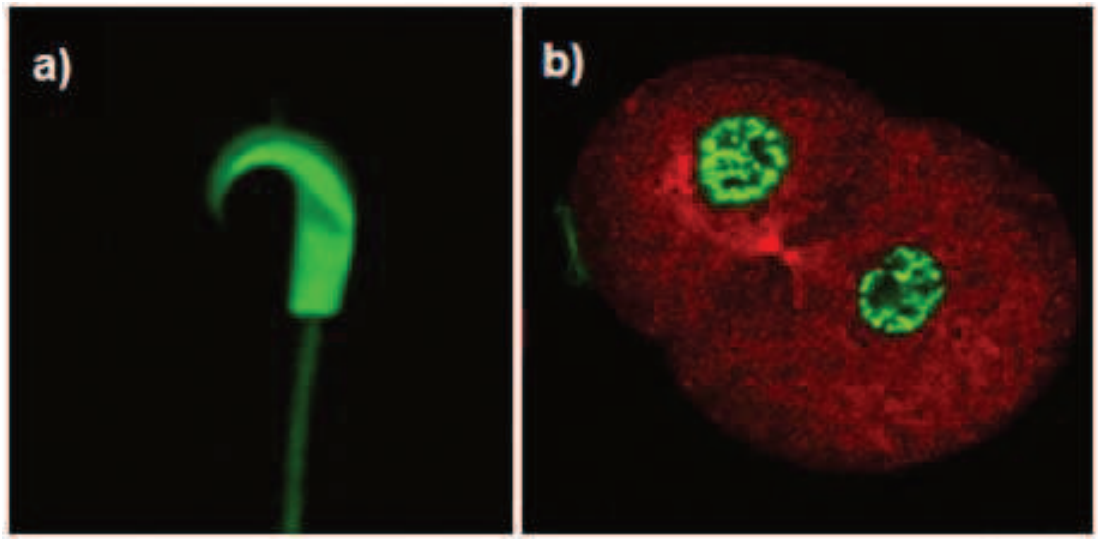

Figure 1.4: Fluorescence imaging. a) Hamster sperm head stained for actin (green) (Dvořáková et al., 2005); b) Two-blastomere embryo stained for DNA (green) and Anti-α-tubulin (red) (Milani et al., 2011).

field methods (Dürig et al., 1986), however, my thesis will be focused on techniques more suitable for biological purposes, especially for life imaging of cells and tissues.

Before getting to this point, I would like to briefly mention the physical basics of fluorescence microscopy and explain why it represents the crucial concept of biological imaging.

1.2 Fluorescence microscopy

To better localize structures within the specimen, the fluorescence microscopy employs emission by dedicated markers. These markers specifically label the gene product of interest, usually a protein, and allow to observe its position within the whole examined structure, while the rest of the specimen is left unseen. The marker, which is called the fluorophore, emits a radiation of a distinct wavelength. The fluorophore molecules absorb the light and few picoseconds later emit a part of its energy in a form of radiation of lower energy and thus longer wavelength. This emission of light is called fluorescence. For represented examples of cell fluorescent labeling see Fig. 1.4.

1.2.1 Process of fluorescence

Figure 1.5 represents possible energetic levels of an excitable molecule. S_0 is a ground singlet state (with a band of vibration excitations just above it), the lowest energy level in which molecule appears when it is not excited by light. S_1 and S_2 are different singlet states in which molecule may appear when its electron in an outer orbital is shifted into a different one due to an extra energy which the molecule receives. Black horizontal lines in each of those states represent different vibrational bands (the molecule can also occur in different rotational states, but these are not drawn in the scheme).

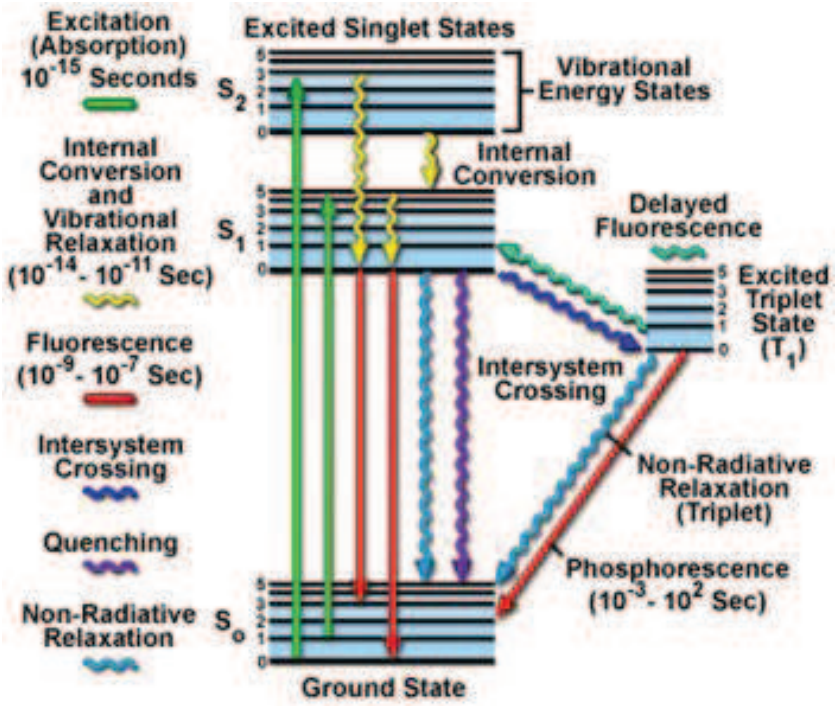

Figure 1.5: Jablonski diagram (adopted from: Herman et al., web 4).

The amount of the energy E gained by a fluorophore depends on the wavelength of the photon: $E = h/\lambda$. The molecule absorbs only the exact amount of energy equal to a difference of some of its energy levels. Since above S_1 and S_2 there are several vibrational states, the fluorophore can be exited by a variety of wavelengths, some of those transitions are more probable than others. The excitation of the fluorophore occurs in order of femtoseconds (10^{-15} seconds).

After the fluorophore absorbs a photon and reaches the excited state, there are several different possibilities of what can happen with the yield energy. If the fluorophore attains higher vibrational state of S_1 or S_2, then it cascades to its lowest vibrational level. The molecule may exhaust that redundant energy by vibrational relaxation (decreasing its vibrational state by passing energy to neighbouring molecules) and in case of S_2 it may undergo internal conversion – the energy of fluorophore stays the same, but it transits into higher vibrational level of different orbital state S_1. These processes usually take picoseconds (10^{-12} seconds). When the lowest energy level of S_1 is reached by vibrational relaxation, the fluorophore may reach the ground state by non-radiative relaxation, or it emits fluorescence (which lasts for nanoseconds) or it may undergo intersystem crossing and then emit phosphorescence. The intersystem crossing appears when the lowest S_1 energy level overlaps with a triplet band T_1. These transitions are improbable and are established slowly, so after the excitation it takes longer time to the phosphorescent molecule to emit radiation than in case of the fluorescent one. It may take even hours until the phosphorescence radiation fades out.

While emitting fluorescence, the fluorophore may lose part of its fluorescence energy in several ways collectively called quenching. One of those processes appears when a molecule emitting fluorescence is in close proximity of another fluorophore and their

emission and absorption spectra overlap. In this case, the emitting molecule may pass part of its energy in non-radiative way to the other one in act of the Förster resonance energy transfer (FRET). This mechanism allows analysis of a precise distance among observed fluorophores (Wu and Brand, 1994).

Particular types of fluorophores may differ in its fluorescence lifetime. This fact is exploited by fluorescence lifetime imaging (FLIM) applicable to various imaging methods (Lakowicz et al., 1992).

The knowledge of energy levels of particular fluorophores enables their effective usage not only for the fluorescence emission itself, but also for a depletion of fluorescence, as will be further discussed below.

1.2.2 Employment of fluorescence

Now let me briefly describe the practical use of fluorescence for biological imaging. Traditionally, fluorescent probes can be fused with a protein of interest and expressed by the cell itself or they can be specifically bound to distinct structures with the aid of antibodies. When using different fluorophores absorbing and emitting light of different wavelengths, it is possible to observe different types of molecular structures in a given specimen simply by changing the wavelength of the exciting light. In case of a multicolour imaging, fluorophores excitable by the shortest wavelengths are used especially for the structure of the highest interest. The most frequently used type of a fluorescence microscope is epifluorescence microscope (Lakowicz, 2006), where the objective not only illuminates but also images the specimen (Fig. 1.6). In practice, after a beam of light is created by the source of light it reaches the excitation filter which lets through the light of a particular wavelength. The excitation beam is then reflected by a dichroic mirror and after passing through an objective it ends in molecules of the specimen. After absorption of the exciting light the specimen emits a red-shifted light which passes through the objective and is transmitted by the dichroic mirror straight to the emission filter (it selects only the light emitted by the sample). Finally, after leaving the filter cube, the emitted light is detected.

To enhance the resolution and mainly the contrast of fluorescence imaging, the epifluorescence microscope can be further adjusted. Instead of simultaneous imaging of the full field of view (called wide-field imaging), it can form an image of one point of a specimen at a time. This can be achieved making use of excitation laser light scanning the specimen line after line. To reduce the out-of-focus light, the laser beam passes through a pinhole aperture and illuminates a tiny spot described by a diffraction-limited PSF. If the laser is aimed at fluorophores, they emit light which is filtered as described above and then passes through another pinhole situated in front of the detector (it selects only light emitted from the targeted point of a specimen), which results in squaring and thus effectively narrowing a profile of PSF (Hell, 1997). This method is called Confocal Laser Scanning Microscopy (CLSM). As Marvin Minsky, the inventor of this method, stated in his patent: *"This high degree of selectivity afforded by the optical system results in a minimum of blurring, increase in signal-to-noise ratio,*

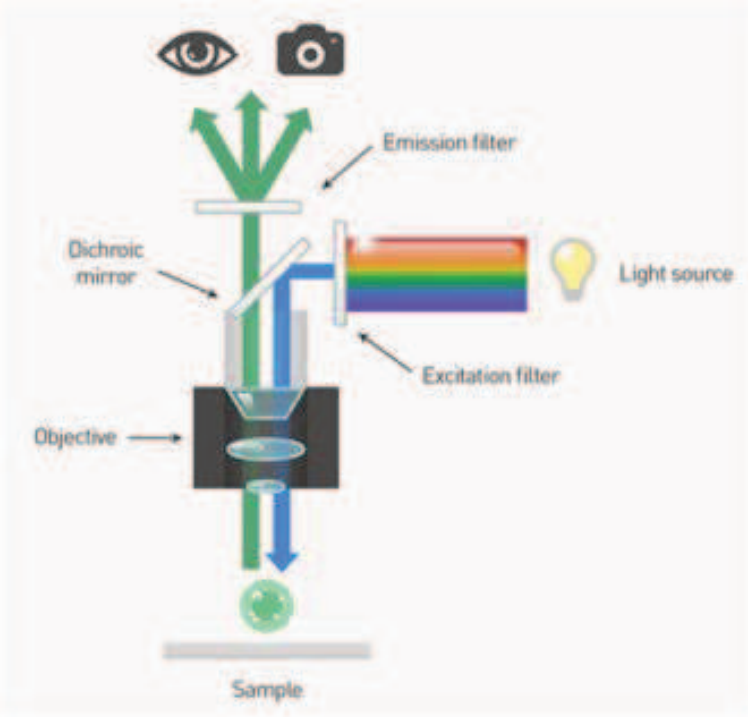

Figure 1.6: Scheme of the epifluorescence microscope (from: Anonymous, web 5).

increase in effective resolution, and the possibility of high resolution light microscopy through unusually thick and highly-scattered specimen." (Minsky, 1961). CLSM thus enables to restrict the signal from out-of-focus molecules and so to significantly enhance the final image contrast, which is, besides resolution, another important aspect of image quality. CLSM can be used for mapping the 3D structure of the specimen by a process of optical sectioning, when images of different planes of the specimen are collected, due to well controlled depth of focus of CLSM. The laser beam of CLSM brings into the setup an additional information about the position of recorded molecule by pointing at the sample. But since both excitation and emitted light are diffraction-limited, the final lateral point-resolution improvement over wide-field microscopy is not significant (Sheppard and Choudhury, 1977).

The confocal microscopy is also exploited in a 4Pi microscopy method (Hell and Stelzer, 1992), where two microscopes are placed in opposing position to simultaneously scan the same area of the specimen from both sides at a time, leading to increase of numerical aperture and axial resolution. However, this method requires precise configuration of both microscopes and also very thin specimen, so its application in biology is limited.

Despite the possibility of specific imaging with the fluorescence microscopy, its resolution is still bounded by the diffraction limit, so its potential is not fully exploited. When using light, those physical limitations given by wave propagation cannot be broken. As mentioned above, there were developed several so-called super-resolution methods which are able to bypass them. In the following chapter, some selected super-resolution methods will be introduced.

2. Super-resolution methods

To summarize the previous chapter, the fluorescence light microscopy is one of the most versatile methods of imaging for biological purposes, due to the employed non-invasive radiation, suitable for observations of living specimen, a reasonable time resolution and an option of simultaneous studying of more types of cellular components at a time thanks to specific fluorescent probes. The biggest limitation of the light microscopy resides in its diffraction-limited resolution. In this chapter, I will discuss methods which have all the advantages of the optical fluorescence microscopy but also push the resolution towards a molecular scale and therefore are suitable for obtaining biological information about structures smaller than 200 nm, such as small organelles or proteins and their complexes. The chosen super-resolution methods can be divided into three groups:

1. The first group (PSF engineering techniques, according to (Hell, 1997)) is based on squeezing the size of the emitting area and thus of its PSF profile. This group contains STimulated Emission Depletion (STED) microscopy and its generalizations within a REversible Saturable Optical Linear Fluorescence Transitions (RESOLFT) approach (Hofmann et al., 2005).

2. The second group is based on employing structured illumination to generate additional information about the sample. It includes Structured Illumination Microscopy (SIM) (Gustafsson, 2000) and Saturated Structured Illumination Microscopy (SSIM) (Gustafsson, 2005;Heintzmann et al., 2002).

3. The third group is based on single molecule localization by random activation of few fluorescent molecules within a diffraction-limited area at a time and following compilation of the final image. This group consists of PhotoActivated Localization Microscopy (PALM) (Betzig et al., 2006), Fluorescence PhotoActivation Localization Microscopy (FPALM) (Hess et al., 2006) and STochastic Optical Reconstruction Microscopy (STORM) (Rust et al., 2006).

2.1 Point Spread Function engineering techniques

2.1.1 Principles of Stimulated Emission Depletion (STED) microscopy

The very first super-resolution method overcoming the diffraction barrier was STED microscopy (Hell and Wichmann, 1994;Klar and Hell, 1999). Even though the exciting beam is still diffraction-limited and excites all molecules in the area of approximately $200 - 250$ nm, STED succeeds in eliminating the number of emitting fluorophores at a time. To this end, STED employs an addition depletion beam (called a STED beam), which "turns off" most molecules from their excited state down to their ground state by the process of stimulated emission: before the photon is released by the excited molecule, another photon from the STED beam forces the de-excitation. The STED

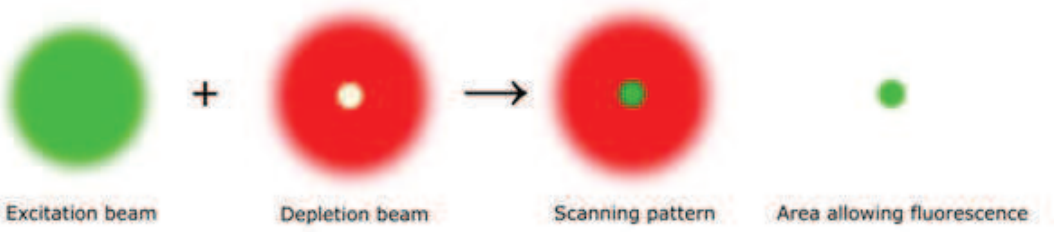

Figure 2.1: Illumination pattern of STED microscope. Scheme of the excitation and depletion beam overlay causing reduction of the area, where the fluorescence emission is allowed.

beam is shaped as a doughnut (i.e., its cross section is similar to that of a doughnut), so when it surrounds the excitation beam and both pulsed picosecond beams are carefully synchronized, the fluorescence emission is possible only for molecules situated in a small central area of the excitation beam (see Fig. 2.1). When using STED method, coordinates of signal are always well known from the position of the STED beam.

The depletion efficiency strongly depends on the STED beam intensity. To achieve nearly complete depletion, rather high pulsed STED beam intensities of the order of GW/cm^2 are necessary. STED exploits non-linear dependence of the saturation level of fluorophores on the STED beam intensity to squeeze a profile of the PSF. Then, the Abbe equation (1.1) does not any longer determine the resolution. The STED resolution is instead given by (Westphal and Hell, 2005):

$$d \simeq \frac{\lambda}{2\,NA\sqrt{1 + I/I_{sat}}}\ , \tag{2.1}$$

where I stands for maximum intensity of the STED beam and I_{sat} is the saturation STED beam intensity at which the fluorescence is reduced by $1/e$. *"For $I/I_{sat} = 100$, the theoretical resolution improvement over Abbe's one is by about a factor 10"* (Hell et al., 2006), so in practice, the lateral resolution of STED is of the order of tens of nanometres. With lower intensity of the STED beam, the area of emitting molecules increases. As a result, one can decide whether the stronger signal or lower photobleaching with shorter acquisition time is preferred in a planned experiment and accordingly tune the intensity of the depletion laser.

Now let me briefly mention several applications of the STED method. My selection is, of course, very far from being complete and fully representative. Nevertheless, I was trying to select some well-known examples to illustrate its versatility and contribution to biological studies. I will proceed analogously with other methods considered below.

2.1.2 Applications of STED

In practice, the beam crosses the specimen line after line as described above for the confocal microscopy, so no further image processing is needed and also the specimen preparation is quite similar. Therefore, STED is appropriate method for improvement of confocal images, which was demonstrated for example on virions using green fluorescent protein (GFP) (Willig et al., 2006) or on syntaxin membrane clusters of the size

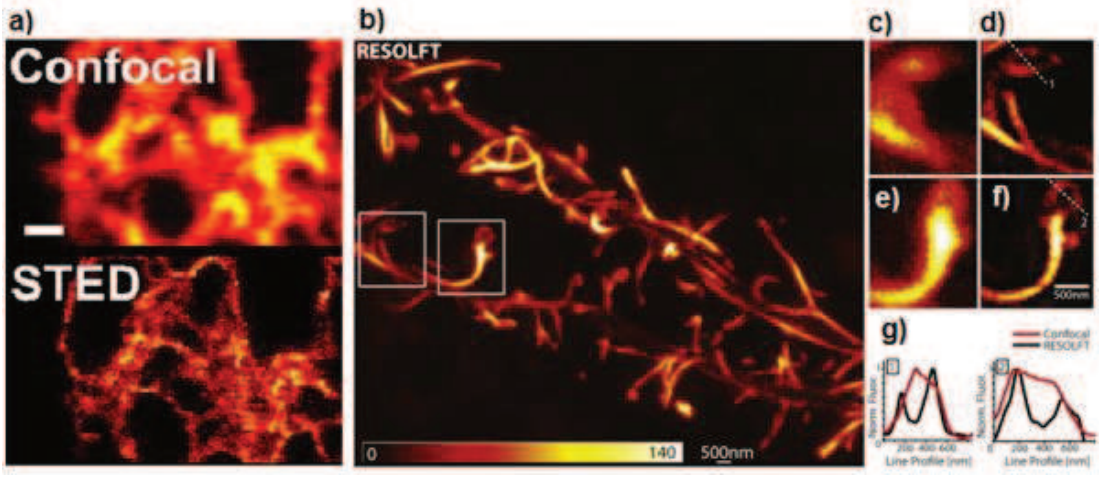

Figure 2.2: Images acquired by STED and RESOLFT methods. a) Comparison of confocal and STED images of ER (scale bar, 500 nm). By consecutive imaging of the same field of view, dynamics of ER were recorded in detail. ER was labelled by Citrine, variant of GFP targeted to ER. (Hein et al., 2008); b) Dendrites with labelled actin imaged by RESOLFT (scale bar, 500 nm); c)-f) the boxed areas of the figure b): comparison of the confocal (c, e) and RESOLFT (d,f) imaging of heads of dendritic spines; g) Comparison of intensity profiles of the confocal (red) and the RESOLFT (black) microscope, taken along lines 1 (d) and 2 (f). In contrast to the confocal microscope, RESOLFT is able to separate two objects in this area. (Testa et al., 2012)

of about 60 nm, not separately recognized by the confocal microscope (Sieber et al., 2007). In this study, STED enabled the quantification of number of syntaxin proteins per cluster, a size of those clusters and their density within the plasma membrane.

In addition, STED was used for high resolution imaging in different focal depths and thus for creating of 3D models, applied for example to distinguish individual microtubules or tubules of endoplasmic reticulum (ER) network (Fig. 2.2a) inside of a living mammalian cell (Hein et al., 2008). In this study, the 3-fold axial resolution improvement was demonstrated and ER dynamics was also recorded by a video. In fact, STED can be utilized as a fast recording method applicable for making videos and it was also used for example for studying movements of individual synaptic vesicles in living neurons (Westphal et al., 2008) or even for observing dynamics of dendritic spines in a living anesthetized mice (Berning et al., 2012).

Moreover, the system of two-colour STED have been developed for studying protein interactions. It exploits either two pairs of excitation and depletion lasers for each of two fluorophores (Neumann et al., 2010), or a single depletion beam (Tønnesen et al., 2011), utilized for both fluorophores thanks to similarity of their emission spectra. It was employed (Tønnesen et al., 2011) for examining dendritic and axonal segments in living brain slices of mice. Previously the concept of 4Pi-like arranged isoSTED was realised, where the prefix iso comes from isotropic (meaning of the same value in a lateral and an axial dimension). It was used for simultaneous imaging of proteins of a mitochondrial membrane and of a matrix in fixed mammalian cells (Schmidt et al., 2008). Later, it helped to clarify the cristae structure of the mitochondrial interior (Schmidt et al., 2009) with the isotropic resolution of 30 nm. Similar 4Pi-like set-up

was published one year later and was used also for single molecule imaging, which will be discussed below (Hell et al., 2009).

STED has also been combined with several other imaging methods: imaging of thick samples, such as 350 μm brain slices, was facilitated by the combination of two photon microscopy and STED (Bethge et al., 2013, Moneron and Hell, 2009). In another example, STED enabled to unravel a difference in diffusion dynamics of phosphoethanolamine and sphingomyelin within a living plasma membrane (Eggeling et al., 2009), thanks to the reduction of an observed area of the membrane to the size of $30-40$ nm, which could not be reached by a confocal microscope. In addition, when combined with fluorescence correlation spectroscopy (FCS), it was possible to calculate average diffusion time of those two types of lipids and to testify that sphingomyelin does not diffuse freely.

However, to use the STED method, the extra beam needs to be employed and precisely aligned with the excitation beam, so technical complexity of this imaging system is considerably higher than in case of conventional microscopes.

2.1.3 RESOLFT concept

The idea of STED, which is limiting the emission to sub-diffraction area, can be generalized by considering other ways of keeping the molecules dark. Soon after STED, there appeared a method of a Ground State Depletion (GSD, see (Hell and Kroug, 1995) which the molecules in the suppressed area by pushing them to their triplet state. In this technique it is sufficient to use much less invasive offset beam with intensity up to MW/cm^2, about three order of magnitude weaker than that of STED. Moreover, less expensive continuous wave lasers can be used. Another option is to employ cis/trans isomerisation fluorophores (Hofmann et al., 2005), which allow to decrease the intensity of the supporting beam even further, up into the range of MW/cm^2. These approaches, including STED, are often covered by a general concept of REversible Saturable Optical Linear Fluorescence Transitions (RESOLFT) (Schwentker et al., 2007). The light intensity can be decreased, because metastable conformations of fluorophores such as cis and trans survive longer than excited but not yet emitting form of fluorophores used by STED. The "off-turning" beam is again doughnut-shaped, but in this case, all molecules get turned on again after every switching cycle. That implies that molecules used for RESOLFT must be able to reversibly switch between on and off state for many times. Such proteins have been developed (Grotjohann et al., 2011;Grotjohann et al., 2012;Duwé et al., 2015;Tiwari et al., 2015). Also, two-colour RESOLFT imaging was realised (Lavoie-Cardinal et al., 2014).

RESOLFT technology enables gentle imaging of a living tissue for a long period of time, like the observation of long term structural changes of actin in living neurons (see Fig. 2.2b-g). When compared to the above mentioned *in-vivo* observation of dendritic spines in a living mouse by STED, the light intensity was decreased from 25–30 mW to 0.5–3 μW, so much more sensitive imaging was possible (Testa et al., 2012). Another

example is a recent experiment with drosophila living tissues and larvae (Schnorrenberg et al., 2016): RESOLFT succeeded in focusing through the larval cuticle into the tissue and enabled its recording despite the random movement of larvae. Very importantly, the dynamics of larvae microtubules were recorded for 2.5 hours with no signs of cellular distress.

Since the acquisition time in RESOLFT techniques is prolonged due to repeated on and off switching of molecules, a parallelized recording has been implemented (Chmyrov et al., 2013). In this case, the acquisition time is limited mainly by switching kinetics of the fluorophore. By this approach, outgrowing neurites of living neurons were recorded.

2.1.4 Implications of STED and RESOLFT methods

The main disadvantage of point-illumination based methods (STED, RESOLFT) resides in their small region of image acquisition. Since the image is recorded pixel after pixel by scanning the beam across the sample, the size of the observed area is reduced in order to optimize the acquisition time (Hell et al., 2006). Therefore, these methods are suitable for analysing details of examined structures. Because STED requires high intensity beams, for more delicate structures, such as living samples, another RESOLFT imaging methods are more appropriate. Nevertheless, the temporal resolution of RESOLFT is limited by switching kinetics of fluorophores.

Since the position of recorded molecules is known when using STED or other RESOLFT methods, no further image processing is needed apart from deconvolution for a contrast enhancement. Aside from high light intensities and demands on the fluorophores photostability, STED can be the convenient versatile method for detailed biological imaging on a daily basis.

2.2 Structured illumination microscopy (SIM) and saturated structured illumination microscopy (SSIM)

2.2.1 Principles of structured illumination

Another illumination patterning method called SIM is, unlike STED, based on a conventional wide-field fluorescence microscope. SIM also does not exploit monolithic light for the entire area of the specimen, but it excites fluorophores making use of an illumination grating. When the unknown pattern of specimen is overlapped with the screen of the grating, interfering patterns called moiré fringes are created in areas where those two patterns do not precisely match each other (Fig. 2.3). The grating is rotated by different angles and set of images is acquired. The Moiré fringes carry high frequency information which cannot be seen by lenses but can be computationally extracted, which leads to images of higher resolution.

The image information from a real space can be converted into frequency information in a reciprocal space by the operation of the Fourier transformation (FT) (Hecht, 2002). Higher frequency information increases the resolution. In the FT scheme of

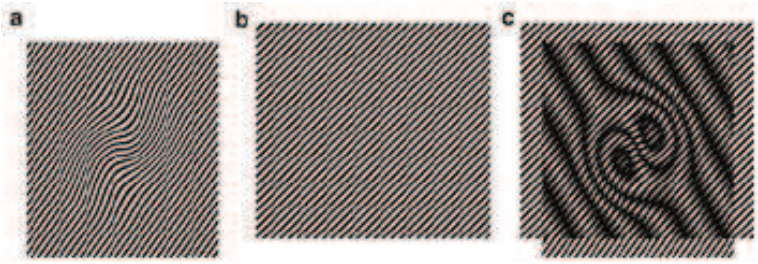

Figure 2.3: Moiré fringes. Creation of high frequency information (c) by overlapping two interfering line patterns (a and b) (Gustafsson, 2005).

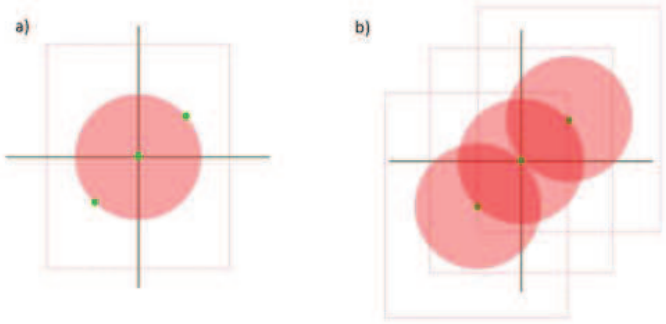

Figure 2.4: Gaining high frequency information by illumination grating pattern. Pink circle represents the observable region, green dots represent Fourier transform of the illumination pattern.

the reciprocal space, the diffraction limit is represented by a circle, which borders with frequencies low enough to be displayed by the conventional microscope (Fig. 2.4a). All the information outside the circle is not detected. However, when moiré fringes appear, *"generation of new spatial frequency of the object shifted exactly by the frequency of the structured pattern"* is created (Hirano et al., 2015), so the high frequency information relocates into the observable region (Fig. 2.4b). In brief, SIM enables computational acquisition of high resolution information from data gained by the diffraction-limited microscope.

2.2.2 SSIM applications

The original approach of SIM can achieve improvement of resolution by a factor of about 2 (Gustafsson, 2000) in all three dimensions when creating three coherent light beams using the diffraction grating (Gustafsson et al., 2008). In practice, lateral resolution of SIM usually reaches 120 nm. SIM enables significant reduction of the out-of-focus light and thus a fine optical sectioning. Later, with high intensities of illumination, the non-linear dependency of emission intensity on the illumination intensity was exploited with a saturated structured illumination microscopy (SSIM) (Gustafsson, 2005), leading to a diffraction-unlimited resolution. Still, to obtain high resolution images, the light intensity must be increased and so does the level of photobleaching. Thus, fluorophores usable for SSIM need to be photostable, although the problem of high intensity demand was bypassed similarly as in case of STED and alternative RESOLFT approaches: by using photoswitchable protein (Rego et al., 2012), which might

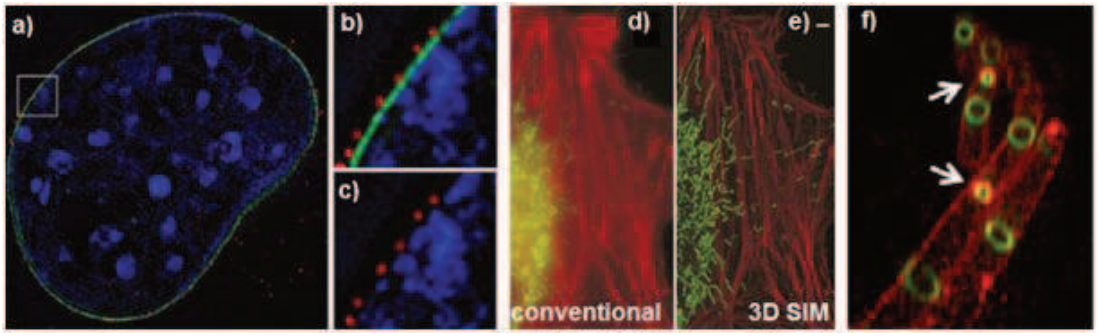

Figure 2.5: SIM images. a)-c) Imaging of cell nucleus with clearly separated nuclear lamina (green lamin B), nuclear pore complex (red) and DNA (blue) (Schermelleh et al., 2010). d)-e) Comparison of conventional and 3D SIM images of HeLa cell with labelled mitochondria (green) and actin (red) (scale bar, $2\,\mu$m) (Fiolka et al., 2012). f) Image of bacterial cells with labelled membrane (red) and cytokinetic Z rings (green). White arrows indicate cell division (Turnbull et al., 2014).

be further developed in the future. In addition, more images need to be acquired compared to the linear SIM, so the temporal resolution declines. In conclusion, the linear SIM is preferred over the super-resolution SSIM when used for biological purposes. To focus more on the imaging methods, which biologists these days may commonly employ, I will further describe some highlights of the linear SIM.

2.2.3 SIM applications

The linear SIM uses the wide-field microscope and does not demand high intensities of light, therefore a wide range of fluorophores can be applied and the sample preparation is no more demanding than for common fluorescent samples for conventional microscopes. The main advantage of linear SIM resides in easily performed multicolour imaging and thus the ability of simultaneous imaging of different subcellular structures and their interactions, often in a 3D version, as in case of labelling lamin B, chromatin and proteins of nuclear pore complex to simultaneously image (see Fig. 2.5a-c) their positions and to localize nuclear pores within the nuclear lamina (Schermelleh et al., 2008).

SIM was also combined with a total internal reflection fluorescence (TIRF) microscopy to enhance its resolution and a video of a mitotic spindle or kinesin dynamics in living cells has been obtained (Kner et al., 2009). In this experiment, instead of the rotation of the solid grating, a liquid crystal and a spatial light modulator (SLM) were used to achieve faster switching of the illumination grating position. Later, after further improvement of the SLM system, a multicolour 3D video was recorded (Fiolka et al., 2012) to simultaneously visualize a mitochondrial fusion/fission and a cytoskeletal network movement within the whole living cells, even a movement of clathrin-coated vesicles was captured. This experiment well demonstrated that since larger field of view can be observed, SIM is suitable for imaging of processes ongoing within bigger area (Fig. 2.5d-e), compared to those recorded by STED. In another interesting example, SIM enabled imaging of the whole bacterial cells with a labelled protein of cytokinetic

Z ring (Fig. 2.5f). SIM unravelled a heterogeneous and dynamically changing location of this protein within the cytokinetic Z ring during a process of bacterial cell constriction (Turnbull et al., 2014). Recently, SIM quantified density of synapses in a hippocampal stratum radiatum of transgenic mice and helped to bring new insights to the role of microglia and complement in a cognitive decline during the Alzheimer disease (Hong et al., 2016).

Similarly to STED, 3D SIM with opposing objectives was composed (Shao et al., 2008). Also, a multifocal adjustment was employed to image a thick specimen such as a living zebrafish embryo, so the structure of its proto-neuromast could be clarified (York et al., 2012). Promising combination of SIM with another imaging method is called lattice light-sheet microscopy, which allows, among others, an *in-vivo* imaging of a dynamic developmental processes in a living zebrafish embryos due to a combination of the illumination grating and a thin plane of light (Chen et al., 2014).

Even though linear SIM does not reach the resolution enhancement of other herein discussed methods, it can easily provide multicolour imaging in a wide field of view using light intensities in the order of W/cm^2, testifying its versatility in biological imaging. However, the user should beware of possible artefacts rendered by this technique, such as periodic stripes, caused by a low contrast or by a mismatch of refractive indices of a sample medium and an immersion oil (Wegel et al., 2016).

2.3 Single molecule localization microscopy

2.3.1 Principles of a single molecule localization

In a year of 2006, there were published three differently named methods – PALM (Betzig et al., 2006), FPALM (Hess et al., 2006) and STORM (Rust et al., 2006) – based on the same principle: to enhance the resolution, one can distinguish two neighbouring fluorescent molecules by randomly switching them to a fluorescent state one by one. After recording each molecule from the sample, the final image is composed (Fig. 2.6). By these methods one can determine the position of emitting fluorophore by finding a maximum intensity of the recorded signal. The coordinates of the molecule are thus directly acquired by its released photons. In contrast, when using STED, one obtains the position of the fluorescing molecule by knowing where the STED beam is, therefore, it is determined by the input photons and sufficiently high light intensities are required. In case of the single molecule imaging, the intensity of excitation light must be low enough to turn on only a sparse subset of molecules within the sample.

2.3.2 Resolutions of (F)PALM/STORM

From the difference between two general super-resolution approaches it follows, that switchable fluorophores usable for single molecule localization methods must comply with different requirements in comparison with those used for STED. The resolution is mainly limited by the precision of localizing a recorded molecule and the more photons

Figure 2.6: Illustration of a resolution enhancement by the single molecule localization. The single molecule image is composed from images acquired during switching cycles. The final image is rendered by analysing the centre position of the fluorophore.

are released by the activated molecule, the more precise can be its localization by the (F)PALM/STORM method. Its resolution is given by (Thompson et al., 2002):

$$d \simeq \frac{\lambda}{2\,NA\,\sqrt{m}}\ , \tag{2.2}$$

where m stands for a number of emitted photons by a fluorophore in the activated state. Therefore, fluorophores used for (F)PALM/STORM should be able to emit as many photons as possible before turning into an inactivated state, either reversibly inactivated or irreversibly inactivated by photobleaching. Also, the contrast in emission between activated and non-activated state must be high enough to suppress a background emission signal. The image of the emitting molecule is recorded and algorithmically matched with a Gaussian fit, so the centre of the fluorophore can be calculated as the intensity maximum. As a result, the final image is composed as a map of probabilities of individual molecules locations (Fig. 2.6). Therefore, the background fluorescence may cause worsening of the final resolution. To achieve a given resolution, a sufficient number of switching cycles must be realized to create this probability map dense enough, which affects a temporal resolution. Of note, there are two different quantities which describe efficiency of the method: precision and accuracy of the single molecule localization. The precision tells us about the difference between repeatedly estimated localizations of one molecule, whereas the accuracy compares the estimated position with the real one (Deschout et al., 2014). Consequently, even with a highly precise estimate of the fluorescent signal location, the final image may not be as realistic as it seems. The discrepancy can appear when the activated molecule donates part of its gained energy to another molecule in a close proximity in an act of the fluorescence resonance energy transfer. Another problem can be caused by two PSFs overlapping by an accident.

Even though the single molecule localization can be implemented with a conventional epifluorescence microscope (sensitive enough to discern single molecules), the use of the software analysing acquired data can be difficult, not to mention a huge amount of data acquired to gain one final super-resolution image, which leads to a problem of data storage and processing. Also, a specimen preparation requires very high labelling density to reach a high resolution together with a demand on a low background noise

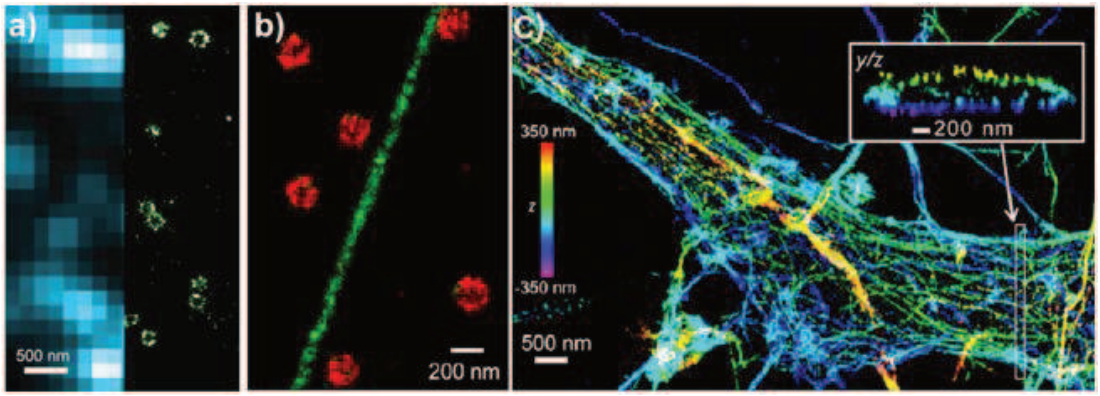

Figure 2.7: Single molecule localization images. a) Comparison of the conventional (left) and the x-y cross-section of the STORM image (right) on clathrin-coated pits (scalebar, 500 nm) (Huang et al., 2008); b) Simultaneous imaging of a microtubule (green) and clathrin-coated pits (red) by the two-colour STORM (scalebar, 200 nm); c) Image of a dendrite with labelled actin and a cross-section of the area (indicated by a white arrow) with a visible ring-like actin structure. Different colours indicate the z-positions of each actin molecule (scale bar, 500 nm) (Xu et al., 2013).

given by a diffusion of protein of interest or a non-specific binding of antibodies (Wegel et al., 2016). Nevertheless, the single molecule localization techniques can reach the resolution of the order of nanometres.

2.3.3 (F)PALM/STORM applications

The single molecule localization methods have found many implementations. Two-colour STORM was successfully used for simultaneous imaging (Bates et al., 2007) of clathrin-coated pits with microtubules in mammalian cells (Fig. 2.7b). Later (Huang et al., 2008), STORM was efficiently implemented to record clathrin-coated pits (Fig. 2.7a). In this study, the astigmatic lens was utilized for 3D STORM recording and enabled discerning the half-spherical 3D shape of those membrane pits of the size of $150 - 200$ nm, in other words, smaller than the diffraction limit. PALM unravelled details of an actin-integrin adhesion region (the membrane linkage between a cytoskeleton and an extracellular matrix) and clarified several specialized protein layers of a size of about 40 nm between the actin and the integrin (Kanchanawong et al., 2010). STORM was also implemented for imaging of the actin organization in neuronal axons and dendrites (Fig. 2.7c) and revealed differences in the actin distribution between these areas (Xu et al., 2013). Using STORM and PALM, a release of HIV-1 virion from infected cells was observed in detail (Prescher et al., 2015) by analysing the role of particular proteins of an endosomal sorting complex required for transport. 3D-STORM also enabled quantification of level of a DNA compactness and its spatial organization on a nanometre scale within regions of different epigenetic states of chromatin (Boettiger et al., 2016).

The single molecule imaging has a great potential in improving the resolution down to a scale of small molecules with a conventional fluorescence microscope. After handling the sample preparation and the image processing and interpretation, this imaging approach can facilitate many areas of biology research on a molecular scale.

3. Comparison of super-resolution methods

To summarize the previous chapter, I will draw a brief comparison of the discussed methods to demonstrate that each of these super-resolution techniques can provide different advancements to biological studies, therefore not every super-resolution method – despite its resolution enhancement ability – might be suitable for reaching an aim of some particular biological experiment. Although I mentioned several attempts to improve shortcomings of particular techniques, in the table below (Fig. 3.1) I summarize a comparison of concepts of various super-resolution techniques which a biology researcher might intend to use. To fully utilize all the benefits which super-resolution brings, one needs to gain a lot of experience and to consider, what is the aim of a planned experiment.

In general, there is a trade-off between a rapid resolution enhancement, beneficial mainly in case of quantification of detailed structures within subcellular regions of a sample, and adjusting light intensities compatible with imaging of living biological samples. Also, the improvement of resolution causes prolongation of the acquisition time, which may be inconvenient for video recording. Instead of a detailed analysis of small regions of specimen, one might be more interested in studying of particular protein dynamics in the whole-cell area with a good temporal resolution. Alternatively, the multicolour imaging ability plays crucial role when interactions of multiple types of proteins are investigated. Last but not least, a user-friendly specimen preparation and ease of data interpretation are important when choosing the right technique for a prepared experiment. In certain biological studies, a combination of multiple super-resolution methods can be also useful, as in case of a published work focusing on the reorganization of sperm proteins during acrosome reaction (Frolikova et al., 2016). In this study, detailed images of sperms were recorded by STED to analyse the movement of two types of proteins of interest within a sperm head (Fig. 3.2a), whereas observation of a mutual positions of those two protein types in the sperm with intact acrosome was realized using 3D SIM, due to its capability of higher axial resolution in all used colour channels (Fig. 3.2b).

As the field of biology itself offers many diverse conceptions of research, the super-resolution imaging methods are correspondingly various and each one has something to offer to upcoming biological studies, from a quantitative analysis of particular proteins within their complexes to the *in-vivo* recording of dynamics of whole-cell processes.

Method	STED	RESOLFT	SIM	(F)PALM/ STORM
Lateral resolution				
Axial resolution				
Image acquisition time				
Range of usable fluorophores				
Sample preparation difficulty				
Multicolour imaging				
Live-cell imaging				
Possibility of artefacts				

Figure 3.1: Super-resolution methods compared. Green indicates good, orange medium and red problematic.

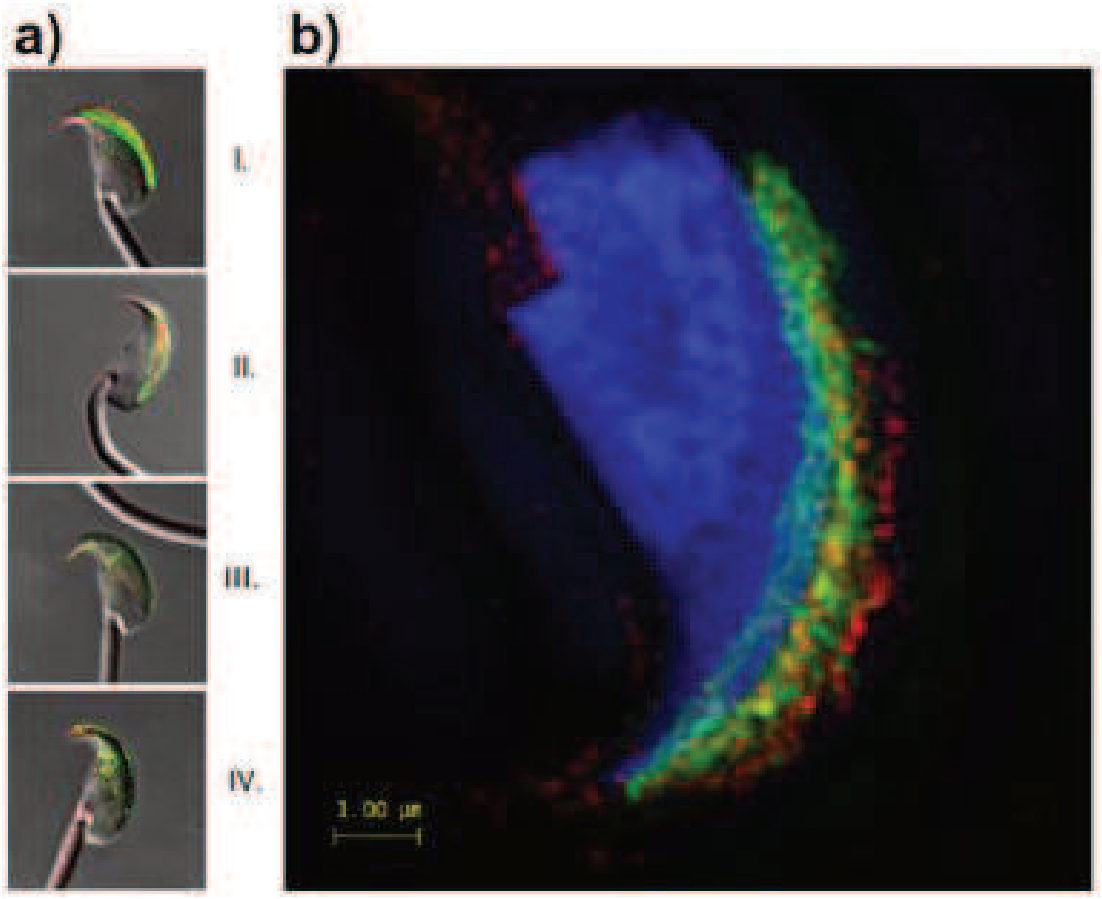

Figure 3.2: Sperm head visualized by two different super-resolution methods. a) Two-colour STED images of CD46 (green) and β1 integrin (red) in sperm in different stages of an acrosome reaction (I: intact acrosome, II: beginning of the acrosome reaction). b) 3D SIM image of a sperm head with labelled CD46 (green), β1 integrin (red) and DNA (blue), showing a co-localization of CD46 and β1 integrin at the outer acrosomal membrane (scale bar, 1 μm) (Frolikova et al., 2016).

4. Challenges and future enhancements

The super-resolution methods have already succeeded in defeating the biggest drawback of the fluorescence microscopy by pushing the resolution far beyond the diffraction limit. However, many other challenges still lie ahead. Currently, the super-resolution experiments can be considered rather demanding for untrained users. When not considering the need to effectively store and process large amount of data acquired by super-resolution microscopes, there are two main areas in which these techniques can be further improved: the hardware components and the sample labelling. From technical point of view, there is an effort not only to improve the performance and to create finer outcome (higher sensitivity of detectors, higher imaging speed, etc, which I will not further discuss), but also to make super-resolution systems more robust to enable their use on every-day basis. Since the resolution is highly affected by physical properties of fluorescent dyes, the aspect of sample labelling is crucial for further improvement of imaging quality. In this chapter, some innovations are mentioned in a context of promoting an easier use of the super-resolution for biological purposes.

To study biological processes in natural conditions, imaging of thick samples such as living tissues or even living organisms is desirable. However, since these samples consist of components of various refractive indices, the amount of light scattering increases with a growing imaging depth and leads to aberrations and thus worsening of the resolution and the image contrast. One way to restore a quality of imaging is to use a selective plane illumination microscopy (SPIM): a thin sheet of light is projected perpendicularly to an optical axis, scanning the specimen plane by plane, so each time only a thin part of the sample is illuminated (Huisken et al., 2004). This approach allows gentle imaging of living specimen while using low light doses and already has been implemented in super-resolution variation of the light-sheet microscopy for a living embryos time-lapse recording as mentioned in the second chapter. Another way to prevent aberrations is to implement adaptive optics (Booth, 2014), reconfigurable components such as a deformable mirror (DM) or a spatial light modulator (SLM), which are capable of immediate alteration of the microscope configuration during imaging and thus of correcting aberrations following from optically heterogeneous specimen. With further advances, the adaptive optics may enable imaging of wider range of biological samples.

Another important aspect of making the super-resolution microscopy more accessible is a possibility of calibrating microscopes easily. As one can notice from above mentioned studies, demonstrations of the achieved resolution have been done mostly on some "model structures", such as microtubules, nuclear pore complexes or mitochondria cristae. However, to provide better reproducibility of imaging experiments, some sort of reference samples should be available. For this reason, DNA origami-based standards have been developed (Schmied et al., 2014), the self-assembly DNA structures, which can be fluorescently labelled with a sub-nanometre accuracy, varying in offered distances of the labelled molecules and thus in the referenced resolution value.

These standards may be promising not only for the calibration and a comparison of different microscopes, but also as instructional samples when learning a proper sample preparation.

Obviously, super-resolution microscopy techniques could not be realized without specific fluorescent probes. Bulky antibodies, commonly employed for staining due to their high specificity and large selection, start to block biologists' view on studied structures. Also, it is challenging to reach sufficiently high labelling density due to their size, not to mention long distance between emitter itself and a target structure. With ongoing resolution improvements, demand on localization precision and on high labelling density keeps rising, so it is desired to place the emitter as near as possible to the structure of interest. Fortunately, it has been found that camels naturally produce single chain antibodies called nanobodies (Hamers-Casterman et al., 1993), which were utilized for the fluorescent labelling in combination with a GFP tagging (Ries et al., 2012) even in intact yeast cells by penetrating through the cell wall with no need of its digestion, demonstrating their high permeability. As an alternative to this study, single-stranded oligonucleotide chains called aptamers were utilized (Opazo et al., 2012) to also enable high-precision labelling and a fast live imaging. After developing a selection of nanobodies and aptamers against wide range of various cellular targets, their application promise easy-to-use labelling for the high-resolution imaging.

To empower further discoveries in biology on a molecular level, it is crucial to facilitate access to the super-resolution techniques as the regular laboratory tool. Among others, herein mentioned innovations will hopefully contribute to this mission.

So far I considered practical improvements of the fluorescence nanoscopy, but it is important to keep in mind that the progress of imaging methods is unstoppable and new ideas for resolution improvement keep coming. As an example of this progress, I would like to mention one super-resolution imaging technique without need to fluorescently label an examined sample. Using interferometric detection of scattering (iSCAT) (Piliarik and Sandoghdar, 2014), individual proteins and their binding were visualized. An interesting discussion of fluorescence-free super-resolution methods can be found in a recent review article (Weisenburger and Sandoghdar, 2015).

Finally, one has to keep eye on completely new ideas with yet unproven potential, for instance intriguing possibility of the holographic super-resolution technique based on exploiting the quantum information, requiring the information about a phase of the detected light (Paúr et al., 2016).

Conclusion

The main aim of this thesis was to introduce and review the current approach of super-resolution imaging, to explain how these techniques manage to bypass the diffraction limit and to imply their possible contribution to biological studies. I will further utilize this gathered knowledge and translate it into the practise when working on my master thesis.

The Abbe resolution limit effectively blocked the quest for higher resolution. Many discoveries were discouraged from being made, including those in a field of biology. Developments at the end of 20th century brought a completely new wind: with the new century the imaging became multi-disciplinary field receiving contributions from quantum optics, molecular physics, chemistry, informatics and structural biology. In general, the aim of new imaging methods is a sub-nanometre resolution.

In last decades, super-resolution microscopy techniques have brought new life to biological imaging. Their impact will continue to increase by their further expansion to biological laboratories. In order to fulfil the potential of the super-resolution, this imaging revolution may lead to higher demands on robustness of super-resolution microscopes, but also to steadily increasing requirements of extensive technical training of biologists. In particular, one should be prepared for a possibility that completely new concepts of imaging will deviate from "daily bread" of current fluorescent staining.

As improved or completely new techniques become available, they will keep opening new horizons for biological research and avalanche of new discoveries will inevitable follow. One thing is certain: the optical microscopy will not become stagnant for another century and there are many unexpected developments to look forward to.

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