8
Super-Resolution Fluorescence Imaging

8.1 Diffraction Barrier of Optical Microscopy

Optical microscopy enables the non-invasive investigation of optically transparent material, such as cells, to be made under a variety of conditions in three dimensions (3D). Through the development of efficient fluorescent labels that can be covalently attached to antibodies and other biomarkers, and the associated possibility to specifically label cellular structures, the expansion of fluorescence microscopy in laboratory applications and research has been accelerated substantially [1–5]. Today, fluorescence microscopes are essential in biological and biomedical sciences for 3D non-invasive imaging of the interior of cells. Thus, diverse confocal and wide-field optical fluorescence microscopes are in operation at most major research institutes.

However, conventional fluorescence microscopy only enables two neighboring emitting objects to be spatially resolved when they are separated by approximately the wavelength of used light. The ability to spatially resolve a structure has a physical limit, which is caused by the wave nature of light. Because of diffraction by lenses, focusing of light always results in a blurred spot, the size of which determines the resolution achievable [6]. Already at the end of the nineteenth century Abbé had shown that the diffraction limit is proportional to the wavelength and inversely proportional to the angular distribution of the light observed [7]. Therefore, any lens-based microscope can not resolve objects that are closer together than half the wavelength of the light in the imaging plane, that is, for visible light in the region of ~200 nm. The resolution along the optical axis is even worse, that is, fluorescent objects can only be distinguished if their axial distance is larger than approximately 700 nm. Analogously, the resolution limit for optical microscopy can be described by the point-spread function (PSF), which describes the response of an imaging system to a fluorescent point object. Owing to diffraction of light passing through an aperture, the fluorescence signal of a single fluorophore produces an Airy disk in the image plane of a fluorescence microscope, a bright region in the center with a series of concentric rings called the Airy pattern. The dimensions of this image are much larger than the fluorophore itself, and are determined by the wavelength of the light and the size of the aperture (Figure 8.1).
Owing to this “spreading” of the emission, the images of adjacent fluorophores overlap, and the ability to distinguish between them is lost. On the other hand, with the introduction of low-noise high-quantum yield charge-coupled device (CCD) cameras, it is possible to localize the center of mass of the PSF of a single fluorophore at sub-wavelength scales [8–10]. This enables colocalization or distance determination between individual emitters, that is, single fluorophores that are separated by a distance less than the diffraction limit, provided that their emission can be separated in some way. This can be achieved using any optically distinguishable characteristic [11] either by spectral [12–14] or time-resolved [15, 16] detection, and also by temporal means via subsequent photobleaching of the fluorophores [17, 18].

Unfortunately, the decryption of structural information with nanometer resolution is challenging, relying solely on colocalization methods. What is challenging scientists the world over is the development of techniques to improve the spatial resolution of far-field fluorescence microscopy, using alternative methods capable of resolving the structural details of sub-cellular structures. This might pave the way towards a refined understanding of how biomolecules are assembled within cells to form the fundamental molecular machines supporting living organisms [19–21]. As was suggested in 1928 by Synge, near-field techniques are qualified to enable high-resolution optical microscopy [22]. However, the use of a nanometer-sized mechanical tip to excite molecules or to detect their fluorescence signal has the disadvantage that it can only be used on surfaces and cannot image the interior of cells [23]. Although the spatial resolution cannot compete with that of electron microscopy, optical far-field microscopes allow us to study living samples, for example, cells or tissue, with minimal perturbation. However, compared with the biomolecular length scale, which is in the range of a few nanometres, one can immediately determine that structural details or the organization of biomolecular assemblies can not be adequately resolved by light microscopy. This fact motivated researchers worldwide to overcome the limit imposed by the diffraction barrier and to find ways to report on important details on biomolecular structures and interactions at the molecular level and inside the cell.
8.2
Multi-Photon and Structured Illumination Microscopy

The first concepts developed to improve axial resolution are based on the quadratic dependence of the fluorescence signal on excitation intensity. Two-photon microscopy as introduce by Denk et al. in 1990 [24] does not lead to a resolution enhancement per se: although the size of the excitation point-spread function (PSF) is reduced, the trade-off is that the wavelength has to be doubled to excite the fluorescent probes. However, and more importantly, two-photon microscopy reduces out-of-focus light considerably, and allows for true optical sectioning in the axial direction. At the same time, the imaging depth of two-photon microscopy is much larger because of the reduced scattering of the infrared light used to excite the sample. Therefore, two-photon microscopy is an ideal tool to study deep tissue and even living animals [25]. Two main concepts capable of effectively improving the axial resolution have been introduced independently, these are, 4Pi microscopy [26] and I^3M microscopy [27]. Both concepts use a set of opposing microscope lenses that sharpen the PSF along the optical axis through interference of the counter-propagating wave fronts. At the same time, the efficiency of collecting light is increased by the presence of two objectives. 4Pi microscopy is a spot-scanning method that achieved a four- to sevenfold increase in axial resolution using different experimental configurations [28]. On the other hand, I^3M microscopy is a wide-field method but it achieves a similar axial resolution of 100 nm [27, 29, 30]. Both 4Pi and I^3M microscopy have mainly been used to study the structure of sub-cellular organelles in 3D [27–32], but they have also been combined with other microscopic techniques that improve the lateral resolution. A concise comparison of both methods can be found in reference [33].

Structured illumination microscopy (SIM), on the other hand, is a concept that combines wide-field imaging and illumination of a sample with a known pattern of excitation light, and achieves a twofold resolution improvement [34]. Experimentally, a periodic illumination pattern of parallel stripes of excitation light is projected onto the sample with the help of a fine grating. A series of images is recorded where the light pattern is moved along the sample laterally and rotated into different angles. Structural features with spatial frequencies that are higher than the frequency of the illumination pattern are modulated by the latter, resulting in so-called Moiré fringes, and can be extracted mathematically (Figure 8.2). As such, a reconstructed image with increased spatial resolution can be obtained.

As a purely physical approach, SIM does not depend on any particular fluorophore properties, such as high photostability, or on any particular transitions between orthogonal states, and can therefore be applied generally. For example, multicolor SIM has been used to study the nuclear periphery of mammalian cells [35]. Furthermore, the acquisition time of SIM as a parallelized imaging method is substantially shorter than spot-scanning methods. As such, SIM is a well suited method to study dynamic processes in living cells [36].

The principle of SIM has been extended to three dimensions by two different experimental configurations. In the first approach, three coherent beams were used
to record an interference pattern varying both laterally and axially, yielding a twofold enhancement in resolution in all three dimensions after image reconstruction [three-dimensional SIM (3D-SIM)] [37]. Employing a nonlinear structural illumination scheme, saturated SIM (SSIM) experimentally demonstrated a lateral resolution of ~50 nm and is essentially unlimited [29]. The key feature that is exploited in SSIM is the nonlinear response of the fluorescence intensity with respect to the excitation intensity.

Alternatively, multi-photon absorption of semiconductor quantum dots and subsequent generation of multiple exciton states can be used for resolution enhancement [38, 39]. As opposed to two- or multi-photon microscopy, the generation of multi-excitonic states in quantum dots does not require the use of infrared light and high excitation intensities, such that a ~twofold resolution enhancement can be easily realized on any confocal microscope system equipped with a continuous-wave laser light source providing appropriate excitation wavelengths, with low excitation intensities (Figure 8.3). As a pure physical process, this so-called quantum dot triexciton imaging (QDTI) operates under any experimental conditions and in particular in living cells [39]. Although the resolution is less than that afforded by other techniques, QDTI is currently the only sub-diffraction–resolution imaging technique that can provide images of whole fixed and living cells with enhanced resolution in both axial and lateral directions, using standard fluorescence

Figure 8.2  (a) Structured-illumination microscopy (SIM) illuminates an unknown structural feature of a sample with a known periodic pattern. The spatial frequencies of the original structure appear as a beat pattern with lower spatial frequencies and can be resolved.  (b) Total internal reflection fluorescence (TIRF) (left) and reconstructed SIM image (middle) from the microtubular network of a mammalian cell (scale bar 1 µm). (Reprinted from Ref. [36]; reproduction with kind permission from Nature Publishing Group.)
8.3 Stimulated Emission Depletion

The ever-growing demand in biology for improved resolution has stimulated the development of novel fluorescence microscopy techniques that overcome the fundamental diffraction limit and achieve theoretically unlimited resolution. To attain sub-diffraction–resolution fluorescence imaging, methods have been implemented that are based on the fact that the emission of a fluorophore can have a nonlinear dependence on the applied excitation intensities, determined by the excitation wavelength(s) and intensity (e.g., saturation of the emission), and on its position (by applying a spatial intensity distribution) [40, 41]. One way to obtain this is to make use of saturable transitions of a fluorophore between two molecular states, for example, transitions to states with different emission properties, such as intersystem crossing to the triplet state [42]. These strategies have been generalized under the acronym RESOLFT [43], which stands for reversible saturable optical fluorescence transitions. The first realization of RESOLFT with far-field optics was stimulated emission depletion (STED) microscopy [40, 41, 44, 45].

In STED microscopy, the optical resolution is dramatically improved by de-excitation of excited fluorophores via stimulated emission using a red-shifted light beam featuring a local intensity zero in the center. In practice, this is accomplished by overlapping the diffraction-limited spot of the excitation beam of a scanning microscope with a red-shifted donut-shaped beam (generated by a zero-node phase

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**Figure 8.3** QD TMD using QDot655 (a) Triexciton fluorescence emission is centered around 590 nm, and is thus well separated from the monoexciton emission centered at 655 nm. (b) Immunofluorescence images of the microtubulin network of COS-7 cells stained with QDot655 labeled secondary antibodies, recorded with a confocal microscope with a resolution of 50 nm per pixel. The monoexciton image was recorded with 20 W cm\(^{-2}\) excitation intensity and the triexciton image measured on the short-wavelength detector between 550 and 600 nm applying an excitation intensity of 200 W cm\(^{-2}\) at 445 nm (scale bar 5 μm; 1 ms integration time per pixel). The line profiles shown for the monoexciton (red) and triexciton (green) emission channel demonstrate the improvement in resolution.
mask) for stimulated emission (Figure 8.4). Consequently, excited molecules in the outer parts of the focus are turned off by stimulated emission. Thus, depending on the illumination conditions (intensity and irradiation time) and the quality of the shape of the donut-mode beam (i.e., the stimulated emission beam), theoretically an arbitrarily high resolution can be achieved. The theoretical achievable resolution can be approximated by Equation 8.1 [40].

$$\Delta x \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + 1/I_{\text{sat}}}}$$

(8.1)

In Equation 8.1, which can be regarded as an extension of Abbe’s equation [7], \(I\) is the peak intensity (photon flux per unit area) of the donut beam and \(I_{\text{sat}} = (\sigma \times r)^{-1}\).
8.3 Stimulated Emission Depletion

gives the intensity at which the fractional population of the excited state is depleted by stimulated emission to \(1/e\). Here \(\sigma\) denotes the cross-section for stimulated emission and \(\tau\) the fluorescence lifetime of the first excited singlet state. For commonly used fluorophores \(I_{\text{sat}}\) corresponds to about 30 MW cm\(^{-2}\) in the visible range. Thus \(\Delta x\) approaches very small values, ideally \(\Delta x \to 0\), for \(I \gg I_{\text{sat}}\), that is, using very high excitation intensities for the donut-shaped stimulated emission beam. In combination with fast laser scanners, video-rate (28 Hz) far-field optical imaging of small areas (~1.8 \times 2.5 \mu m^2) of synaptic vesicles by STED with a focal spot size of 62 nm can be performed [46].

Importantly, STED microscopy does not always require pulsed excitation and has been demonstrated by solely applying continuous-wave lasers [48]. With decreasing complexity, STED microscopy can nowadays, likewise, be used for live cell imaging. This is simplified by the fact that STED microscopy is compatible with some photostable variants of fluorescent proteins, reaching a lateral resolution of ~50 nm in a living cell [49]. However, one should be aware of the fact that STED microscopy requires relatively high illumination conditions for efficient stimulated emission, due to the short lifetime of the first excited singlet state, of a few nanoseconds \([I_{\text{sat}} = (\sigma \times \tau)^{-1}]\). Therefore, many standard fluorophores are not amenable for use in STED microscopy, and live cell applications have to be carefully controlled with respect to light-induced damage. On the other hand, very photostable fluorescent nitrogen-vacancy defects in diamond can be used as ideal probes to demonstrate the resolving power of STED microscopy. In this context, PDFs of 5.8 nm have been measured for single nitrogen-vacancy defects [50].

It has to be pointed out that in practice any reversible and saturable molecular transition that effectively changes the fluorescence intensity of a fluorophore can be used advantageously to improve resolution. In this context, switching between a dark and a bright state has been identified as an essential element to separate adjacent objects in time. The ultimately appropriate saturable transition represents light-induced switching of fluorescence between two thermally stable states, an off and an on state. These requirements are fulfilled by molecular optical switches, that is, photoswitches that exhibit two stable and selectively addressable states, a fluorescent and a non-fluorescent one, which can be reversibly interconverted upon irradiation with different wavelengths of light under low illumination intensity conditions. Analogous to STED microscopy, optical switches can be selectively switched off upon illumination of the sample with a donut-shaped beam of appropriate wavelength superimposed on the regular beam used to excite fluorescence [40, 51, 52].

Alternatively, the temporal behavior of fluorescence intensity controlled by the local excitation intensity, that is, the nonlinear response of the fluorescence signal with respect to the excitation intensity, can be used for resolution enhancement applying concepts such as, for example, dynamic saturation optical microscopy (DSOM) [53, 54]. Another very promising method that exploits the temporal information of fluorescence fluctuations and mathematically extracts high-resolution spatial information constitutes super-resolution optical fluctuation imaging (SOFI) [55].
8.4
Single-Molecule Based Photoswitching Microscopy

To achieve sub-diffraction–resolution fluorescence imaging, fluorophore emission has to be separated in time. Whereas in the STED/RESOLFT case, the phase mask defines the coordinates of fluorescence emission (predefined in space by the zero-node), alternative methods have emerged that use stochastic activation of individual fluorophores and precise position determination (localization). These approaches randomly separate the emission of stochastically activated individual fluorescent probes in time. Provided the distance between the individual fluorophores enables the analysis of the different emission spots to be unaffected, that is, individual fluorophores are spaced further apart than the distance resolved by the microscope (>\(\lambda/2\) on a CCD camera), the standard error of the fitted position is a measure of localization and it can be made arbitrarily small by collecting more photons and minimizing noise factors. The error in the position determination of detected photons can be approximated as Equation 8.2:

\[
\left\langle (\Delta x)^2 \right\rangle = \frac{\sigma^2}{N} \tag{8.2}
\]

where

\(\Delta x\) is the error in localization
\(\sigma\) is the standard deviation of the point-spread function
\(N\) is the number of photons collected [8–10, 56, 57] (Figure 8.5).

Thus, depending on photon statistics fluorescence imaging with one nanometer accuracy (FIONA) can be used, for example, to monitor the migration of single fluorophores conjugated to myosin proteins along actin filaments [58, 59].

If the emission of fluorophores can be separated in time, then the high localization precision can be used for sub-diffraction–resolution fluorescence imaging. One way to achieve this relies on targeting the surface of the object using the diffusion and transient binding of fluorescently labeled probe molecules [62]. Because the diffusion of the fluorophores is too fast to be followed on a CCD camera, the fluorophores only appear when they bind or adhere to the target object. This has two advantages: because the fluorophores, in general, all bind at different time intervals, the localized emission is spread out in time. Moreover, because binding can, ideally, occur anywhere on the target object, the probe molecules will eventually map out the entire structure. A high-resolution image of the target object can then be reconstructed using the localized positions.

With immobilized fluorophores a similar approach is possible if the spatial density of emitting fluorophores can be adjusted in a way that ensures that the majority of the fluorophores resides in a non-fluorescent state. In principle, this can be achieved by exploiting random fluorescence intermittencies of semiconductor quantum dots due to charge separated states [63] or organic fluorophores entering long-lived triplet states in the absence of oxygen [64]. However, for the well defined control of the density of active (fluorescent) fluorophores, molecular optical switches or photo-
switches are most promising [65]. Today, many fluorescent probes that exhibit the necessary photoactivation or photoswitching properties are available [65, 66], including conventional organic fluorophores, such as carbocyanine, oxazine, and rhodamine dyes [60, 61, 67–74], caged fluorophores [75], photochromic compounds [76–78] and a large variety of fluorescent proteins [79–83]. All these photoswitchable
fluorophores have in common the existence of at least two different states that are distinguishable in their fluorescence emission properties (Figure 8.5).

Photoswitchable fluorophores populate a fluorescent “on”- and a non-fluorescent “off”-state (dark state), and the interconversion between these states can be controlled by light or the chemical nanoenvironment. Photoactivatable fluorophores are initially found in a dark state and require activation to become fluorescent, typically achieved by irradiation with light. All methods that use photoswitchable fluorophores for sub-diffraction fluorescence microscopy employ a temporal confinement of the fluorescence signal. This is achieved by first turning off all fluorophores in a sample. In the next step, only a small subset of fluorophores is transferred into a fluorescent “on”-state. Here one has to make sure that activated fluorophores can be detected as individual emitters that are spaced far enough away from their nearest neighbor. The fluorescence emission signal of a fluorophore is read out, and the position of the fluorophore is determined by approximating the PSF with a Gaussian function (Figure 8.5). This procedure is repeated many times, and the ensemble of coordinates collected from localizing single fluorophores is used to generate (“reconstruct”) an artificial image, which provides sub-diffraction or super-resolution information.

Prominent examples of concepts that rely on photoswitchable fluorophores are photoactivated localization microscopy (PALM) [84], fluorescence photoactivation localization microscopy (FPALM) [85], stochastic optical reconstruction microscopy (STORM) [86], PALM with independently running acquisition (PALMIRA) [87], direct STORM (dSTORM) [60, 61] (Figure 8.5), and other alternative methods [73, 74]. Furthermore, faster variants of PALM have been reported using a stroboscopic illumination scheme [88]. A related method is ground-state depletion followed by individual molecule return (GSDIM), which switches off fluorophores by populating the non-fluorescent triplet state out of which the spontaneous return to the ground state occurs [64].

All the photoswitching-based methods mentioned above are being continuously improved. For example, multi-color imaging with ~20 nm lateral resolution has been demonstrated [69, 79, 89–91], setting the prerequisite to study biomolecular interactions at the molecular level. A variety of concepts that allow 3D-imaging have been developed, either by introducing astigmatism [92] or a helical shape [93] into the beam path, or by recording two imaging planes simultaneously and approximating the PSF to a three-dimensional model function [94]. Alternatively, an interferometric arrangement (iPALM) enables an axial resolution of only a few nanometers (Figure 8.6) [95]. Although the alignment of iPALM is very demanding, it can close the gap between electron tomography and light microscopy.

A challenge for all methods is compatibility with live cell imaging. Whereas methods that rely on the use of fluorescent proteins have the advantage that these probes can easily be implemented in living cells [96, 97], organic fluorophores cannot be used so easily in live cell super-resolution imaging, because they typically require very special buffer conditions for photoswitching [67, 68, 73]. However, a refined understanding of the impact of redox reactivity on photoswitching has paved the way for the use of organic fluorophores even in living cells [61, 70]. Finally, it also has to be mentioned that unsymmetric dimeric cyanine dyes, such as YOYO-1 and others that
are known to intercalate into double stranded DNA, can be used for super-resolution imaging, applying conditions as used for “classic” carbocyanine switching [67, 68]. Under these conditions some intercalating fluorophores act as reversible photo-switches and can be used to image DNA with a resolution of better than 40 nm [98].

### 8.5 Background and Principles of Single-Molecule Based Photoswitching Microscopy Methods

The experimental procedure for all single-molecule based photoswitching methods is similar: a sample has to be densely labeled with a photoswitchable fluorophore, for example, via immunocytochemistry or co-expression of a fluorescent protein, and prepared such that most fluorophores are in their off-state, and only a subset of fluorophores remains fluorescent at any time (Figure 8.5b). As described in the previous section, the emission profiles of single emitters can be localized through the approximation with a Gaussian fit with a precision that is determined by the number

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**Figure 8.6** Operating principle of iPALM [95].

(a and b) Schematic of the single-photon multiphoton fluorescence interferometer.

A point source with z-position $\delta$ emits a single photon both upwards and downwards. These two beams interfere in a special 3-way beam splitter. (c) The self-interfered photon propagates to the three color-coded CCD cameras with amplitudes that oscillate 120° out of phase, as indicated. The principle takes advantage of the wave-particle duality, which allows a single photon to form its own coherent reference beam. An emitted photon can simultaneously travel two distinct optical paths, which are subsequently recombined so that the photon interferes with itself. The position of the emitter directly determines the difference in the path lengths, hence the relative phase between the two beams. (With permission from [95].)
of photons detected and the background fluorescence, that is, autofluorescence of the sample and residual fluorescence of surrounding fluorophores in the off-state [10]. Thus, photoswitches with a high intensity contrast between the on- and off-states, together with high extinction coefficients and quantum yields in the on-state, are the key for nanometer accuracy.

For the case of a densely labeled structure, the dark state should exhibit a lifetime τ_{off} that is substantially longer than the lifetime of the fluorescent state τ_{on}. Ideally, the lifetime of the on-state τ_{on} should be very short, with a high photon yield in order to allow precise localization [10]. Upon irradiation the fluorophore is transferred to a metastable dark state at a rate of k_{off}, where it resides until it is converted into the singlet ground state with rate k_{on}. The ratio of these rates is defined as \( \lambda = \frac{\tau_{off}}{\tau_{on}} = \frac{k_{off}}{k_{on}} \) [99]. The denser the labeling of a structure, the higher the ratio \( \lambda \) has to be in order to ensure that fluorescence emission of single fluorophores is temporally well separated, to allow unambiguous localization of individual emitters. The on-time or lifetime of the fluorescent state τ_{on} can be shortened by applying high irradiation intensities, thus reaching a higher ratio \( \lambda \) and enabling fast acquisition with commonly available CCD cameras [64, 72, 73, 100]. This has the additional advantage that further effects such as mechanical drifts are reduced.

In this context, the Nyquist–Shannon sampling theorem becomes important, which requires that the average distance between neighboring molecules must be at least half of the desired resolution [101]. Therefore, next to the localization accuracy of single emitters, which is governed by the photons emitted, the achievable resolution is also controlled by the labeling density. In the extreme case, the density of photoswitchable fluorophores can become so high that the photophysical parameters τ_{on} and τ_{off} reach their limit and photobleaching must be applied to achieve the desired fluorophore density. Alternatively, the number of fluorophore labels (e.g., antibodies) can be reduced. However, both strategies fail at the expense of optical resolution according to the Nyquist–Shannon sampling theorem. The importance of \( \lambda \) for super-resolution imaging can be easily demonstrated if we imagine we want to image a PSF area with a typical diameter of 250 nm with a resolution of 20 nm. Hence, according to the Nyquist–Shannon criterion, the sample has to be labeled with photoswitchable fluorophores every 10 nm (Figure 8.7a). In order to achieve sub-diffraction–resolution imaging only one fluorophore is allowed to reside in the on-state at any time within a diffraction-limited area, that is, a ratio \( \lambda \geq 600 \) is required. Assuming a lifetime of the on-state τ_{on} of only 1 ms, a very stable off-state with τ_{off} ≥ 600 ms has to be realized.

Only under these conditions will the majority of isolated fluorophores residing in their fluorescent on-state be appropriately identified and localized (Figure 8.7b). In some instances, it can happen that more than one fluorophore is fluorescent at the same time in a diffraction-limited area because of the stochastic nature of the switching process, or due to inappropriate photoswitching conditions. In this case the shape of the PSF becomes unsymmetrical, which results in “irregular spots” that are usually sorted out during software analysis. However, if two or more fluorophores are very close, the asymmetry in their shape might be too small to be distinguished from single-molecule spots. Consequently they are fitted as one spot whose local-
Figure 8.7  (a) In order to image a 2D-area with an optical resolution of 20 nm, the Nyquist–Shannon criterion demands labeling with a photoswitchable fluorophore every 10 nm corresponding to ~600 fluorophores per PSF defined area. Thus, a switching ratio of $\tau_{\text{off}}/\tau_{\text{on}} \geq 600$ is required. (b) If the photoswitching rates are set inappropriately, two or more fluorophores are fluorescent at the same time and their PSFs may overlap significantly (multiple-fluorophore localizations). The approximation of the PSF of multiple emitters yields a position that does not correspond to the physical position of any of the fluorophores. If the fluorophores are arranged along a single filament (above), the mismatch has no consequences on the resolution enhancement. In the case of two adjacent fluorophores on different, for example, parallel, or crossing filaments (below), a false localization is generated and this will affect the ability to resolve the independent filaments. (c) Simulations on filaments. A network of straight adjacent filament pairs with neighbor distances of 50, 100, 300 nm was simulated. Every filament consists of a line labeled with a fluorophore every 8.5 nm. The simulated photoswitching properties are based on the experimental data of the photoswitchable fluorophore Cy5. The network was resolved for different $\lambda = k_{\text{off}}/k_{\text{on}}$. Cross-section profiles of three different filament pairs with distances of 50, 100, and 300 nm; within a structure the ratio required to resolve the filaments increases with the complexity of organization, that is, the denser an area is labeled the higher the ratio has to be. A low ratio of $\lambda = 33$ suffices to resolve single filaments with large distances (300 nm), whereas filaments with smaller distances or crossing areas need higher ratios. The image simulated with $\lambda = 100$ is a good demonstration of the influence of false localizations. The two filaments separated by 100 nm show a third artificial filament appearing in the interspace [99].

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increasingly important, imaging networks of crossing and adjacent filaments. Furthermore, when using fluorophore labeled antibodies as probes, the degree of labeling (DOL) has to be considered because a DOL > 1 reduces the off-time of the label. According to a binominal distribution for a sufficiently low (and realistic in terms of antibody labeling) DOL (2–6) and higher rate-ratios (λ > 50), λ is approximately reduced by λ/DOL.

The issue of resolving straight, single, and well isolated filaments is fairly simple. It does not matter whether a single fluorophore or a bulk is fitted. Each time a multiple-fluorophore localization is made along the filament, it contributes to the artificial reconstructed image in the same way as every regular localization (Figure 8.7c). In this case the localization no longer reflects the position of a single fluorophore, but this does not affect the resolution of the structure. Therefore, the rate-ratio λ can be relatively small, and also short-lived off-states of a standard organic fluorophore can be used under high excitation conditions for super-resolution imaging [64, 73, 102].

The resolution of crossing or adjacent filaments, however, requires a higher λ and a stable non-fluorescent state with a comparably long lifetime t_{off}, respectively. Localizing more than one fluorophore within a diffraction-limited area affects the resolution of the structure as it yields coordinates where no fluorophore actually resides. In Figure 8.7c, straight filaments were simulated using different λ values with an interfluorophore distance of 8.5 nm, predicting a theoretical resolution of 17 nm [101]. Three crossing pairs of filaments with neighbor distances of 50, 100, and 300 nm were simulated. Photoswitching parameters used in the simulation were derived from experimental values derived from carbocyanine dyes [60, 68, 71, 72, 99].

In case of low switching rates (λ = 33), adjacent filaments with distances of 50 and 100 nm are not resolvable and crossing filaments are blurred completely (λ = 33, 100). With an increasing ratio (λ = 300, 1000) adjacent and also crossing filaments can be resolved clearly. As can be seen for high λ, the bulk of the localizations are made from single molecules, whereas for low rate ratios most of the fluorophores lead to irregular spots. These spots are sorted out automatically due to their asymmetry during software analysis. Hence, for lower ratios the total number of localizations that contribute to the final image is reduced.

After demonstrating the importance of the generation of thermally stable long-lived off-states, the question arises as to how states such as these can be realized in standard organic fluorophores. The triplet state of organic fluorophores can only be used as the off-state when oxygen is efficiently removed in order to prolong the triplet lifetime from micro- to milliseconds in aqueous solvents [64]. This can be achieved by oxygen scavenging systems or embedment in a polymer matrix such as poly(vinly alcohol) (PVA) with low oxygen permeability. Furthermore, relatively high excitation intensities (>10 kW cm\(^{-2}\)) have to be applied to shorten t_{on}. However, besides the triplet states, radical ion states can be efficiently populated by the presence of appropriate redox partners. For example, the triplet state might be reduced by the presence of appropriate reducing substances in the micromolar range. Thus, a semi-reduced radical anion is formed that exhibits a surprising stability in aqueous solvents with a lifetime of up to several seconds, which is dependent on the oxygen concentration. The fluorescent singlet state can be easily
recovered by reaction with oxygen or other oxidizing substances; that is, $\tau_{\text{on}}$ can be controlled by the irradiation intensity and the concentration of the reducer, and $\tau_{\text{off}}$ by the concentration of the oxidizer. This reducing and oxidizing system (ROXS) was originally introduced to minimize photobleaching and blinking of fluorescent dyes (see Chapter 4) [103] but might, likewise, be used advantageously to control $\lambda$ [61, 69, 70, 73, 74].

In order to identify a general mechanism for the generation of reversible long-lived off-states in organic standard fluorophores, the photophysical properties are again in the spotlight. As has been demonstrated in Chapter 7, most rhodamine and oxazine derivatives are efficiently quenched by electron donors, in particular by aromatic amines such as the amino acid tryptophan. This finding can be used as a platform to develop a universal, widely applicable method for super-resolution imaging with small organic fluorophores. With the exception of some carbocyanine dyes such as Cy3, Cy5, and Alexa Fluor 647, most other commercially available fluorophores with absorption maxima between 480 and 700 nm (e.g., Alexa Fluor and ATTO dyes) belong to the class of rhodamine and oxazine derivatives. Because they feature the same basic chromophore structure they exhibit similar redox properties, that is, rhodamine and particularly oxazine derivatives have a high lying one-electron reduction potential, and because of this they are prone to reduction, whereas cyanine dyes exhibit a lower one-electron oxidation potential, and are therefore more easily oxidized than rhodamine and oxazine dyes (see Table 7.1 in Chapter 7).

Following these ideas it was discovered [61] that the triplet states of various Alexa Fluor and ATTO dyes are quenched by thiol containing reducing compounds such as $\beta$-mercaptoethylamine (MEA), dithiothreitol (DTT), or glutathione (GSH), that is, substances with slightly lower reduction power compared with aromatic amines [104]. The quenching efficiency depends on the pH of the solvent because most thiols (RSH) have a $pK_{a,\text{SH}}$ 8–9 [105, 106] and the reducing species is the thiolate anion (RS$^-$). Therefore, the reduction efficiency of compounds carrying one thiol group exhibits a plateau at pH > 9 with all relevant functional groups ionized, followed by a linear increase in reduction potential with pH decrease. Thus, the reducing power can be easily and sensitively controlled by the pH of the solvent.

Importantly, the first excited singlet state of dyes such as ATTO655 are quenched by electron transfer from various thiol compounds (RSH/RS$^-$) at pH values >9. The triplet state, however, has a longer lifetime and thus is quenched even at pH 7–8, that is, at lower RS$^-$ concentrations. Furthermore, the thiolate (RS$^-$) competes with oxygen, which is known to efficiently quench triplet states and is present at a concentration of $\sim$250 $\mu$M under standard conditions in aqueous solution. As a consequence, the pH value of the solvent and the concentration of the thiol compound are crucial experimental parameters, and efficient quenching of the triplet state under physiological conditions (pH 7–8) requires a concentration of 10–100 mM of the thiol compound. The reaction scheme for reversible radical anion formation by thiol compounds is presented in detail in Figure 8.8.

The semi-reduced radical anions formed in the first reaction step can be re-oxidized by molecular oxygen in a photoinduced process or can be further reduced to very stable non-fluorescent species that exhibits a lifetime of several seconds, even in
the presence of oxygen (Figure 8.8). The role of oxygen is twofold: (i) it quenches the triplet state in competition with the reducing thiolate and (ii) oxygen is responsible for generation of the on-state by oxidizing the reduced dyes produced. The stability of the reduced off-state is further corroborated by the fact that the fluorescent form cannot be recovered efficiently when oxygen is removed from the solution by purging with nitrogen [61].

According to these results, in the first step, the reaction of RS\(^{-}\) with the triplet states of fluorophores produces radical anions and thiol radicals (T\(^{+}\)RS\(^{-}\) \(\rightarrow\) T\(^{+}\) + RS\(^{+}\)). The main reactions of thiol radicals in aqueous solution are conjugation with thiols or thiolates, or dimerization to the corresponding disulfides or reaction with molecular oxygen [107, 108]. The free radical reactions can generate superoxide radicals and hydrogen peroxide. As the formation of superoxide radicals and hydrogen peroxide is less efficient, the main consequence of the reaction mechanism is oxygen consumption. Both thiol oxidation and oxygen consumption have been shown to increase with pH as a consequence of the increasing fraction of thiolate in the reaction medium [107, 108].

Consequently, direct stochastic reconstruction microscopy (dSTORM) using standard fluorophores in the absence of activator fluorophores has become feasible and allows super-resolution imaging with standard fluorophores under identical

![Figure 8.8] Underlying photophysical processes of reversible photoswitching of Alexa Fluor and ATTO dyes. Following excitation of the fluorophores (k\(_{\text{exc}}\)) into their first excited singlet state, S\(_{1}\), the excited-state energy is either released via fluorescence emission with rate k\(_{f}\), or the triplet state is occupied via intersystem crossing (k\(_{\text{ISC}}\)). The triplet state (T) is depopulated either via k\(_{\text{ISC}}\) by oxygen or reduced by thiolate (RS\(^{-}\)) with rate k\(_{\text{RED}}\) to form radical anions (T\(^{+}\)). The semi-reduced radical anions formed in the first reaction can be further reduced to yield the fully reduced species. Both the semi-reduced and the fully reduced form can be re-oxidized by molecular oxygen with rate k\(_{\text{OX}}\). Thus, molecular oxygen plays a crucial role in single-molecule based photoswitching methods according to the dSTORM principle [61]. In other words, the concentration of molecular oxygen and thiol have to be carefully balanced to adjust the desired switching ratio \(\lambda\).
experimental conditions by simply adding millimolar concentrations of thiols (Figure 8.9). Thus, the list of organic fluorophores that can be used for super-resolution imaging can be extended considerably by all common rhodamine and oxazine derivatives from the blue to the red part of the electromagnetic spectrum. The underlying mechanism can be described as a remarkably efficient cycling between a fluorescent and non-fluorescent state of the fluorophores in the presence of millimolar concentrations of thiols. Hence, the lifetime of the on-state can be adjusted by the excitation intensity, provided that the concentration of the reducing thiolate species ensures efficient quenching of the triplet state via formation of radical anions and subsequent secondary reactions to a stable off-state. The lifetime of the off-state, that is, the time it takes until the reduced species are oxidized by oxygen to repopulate the singlet state, is determined by the oxygen concentration. In the dSTORM experiments shown in Figure 8.9, the-laser intensities were adjusted to ensure that the lifetime of the off-state is substantially longer than the lifetime of the on-state. Thus, only a subset of fluorophores is activated at any time in the field of view. In order to reconstruct super-resolution images as shown in Figure 8.9, typically 10 000–20 000 frames at frame rates of 10–33 Hz were recorded to achieve an optical resolution of ~20 nm. Higher frame rates can be achieved using faster EMCCD cameras in combination with higher excitation intensities. Under the applied experimental conditions, all fluorophores tested exhibit fluorescence count rates of 10–30 kHz. Thus, 500–3000 photons can be used to calculate fluorophore localizations with a theoretical precision of 5–15 nm [10].

Interestingly, dSTORM is not restricted to the use of MEA but works similarly with other thiols, such as GSH, under physiological conditions. The tripeptide GSH is the most abundant low molecular weight thiol protectant and antioxidant in mammalian...
biology. The thiol groups are kept in a reduced state at millimolar concentrations in animal cells [109]. Thus, super-resolution imaging in living cells is also possible with small organic fluorophores such as ATTO655, ATTO680, ATTO700, and ATTO520, which exhibit the most pronounced electron accepting properties and require only low millimolar concentrations of thiols to be efficiently transferred to the long-lived off-state [70]. Although the pH value and GSH concentration of cells varies considerably between the various cell types and conditions, the method can offer live cell experiments using selected fluorophores [61]. Importantly, the method enables screening for suitable live cell fluorophores by simple ensemble cuvette experiments (see Figure 8.9). The intriguing simplicity of the method facilitates its application and opens avenues for multicolor super-resolution imaging with combinations of small organic fluorophores. The results indicate that the development of new methods combining the genetic labeling approach with small, bright and photostable organic fluorophores represents an elegant method for super-resolution imaging and precision colocalization experiments. Therefore, dSTORM is ideally suited to the study of subcellular structures and cluster analysis of protein heterogeneity (distribution) in fixed and living cells with, so far, unmatched resolution [110–112].

8.6 Temporal Resolution of Super-Resolution Imaging Methods

A major drawback to all photoswitching methods is that a large set of individual images has to be recorded, typically several thousands, which drastically reduces the temporal resolution. Even though the lifetime of the on- and off-states can be reduced in combination with higher excitation intensities, to enable higher imaging speeds, one should be aware of the fact that higher excitation intensities likewise promote light induced cell damage. Temporal resolution in STED microscopy, for example, is limited by the scanning process. The use of fast beam scanners made STED microscopy at video rate possible, fast enough to observe the dynamics of synaptic vesicles inside the axons of cultured neurons [46]. However, even the fastest beam scanner restricts the observable area to a rather small area of $1.8 \times 2.5 \mu m^2$. Furthermore, fast scanning limits the achievable resolution, which was determined to about 62 nm. In contrast to sequential spot-scanning in STED microscopy, SIM, as a parallelized imaging approach, allows fast imaging of larger areas of a size that is essentially determined by the imaging optics and the camera chip. SIM has been used to study tubulin and kinesin dynamics in living cells with a lateral resolution of 100 nm and a frame rate of up to 11 Hz [36]. Here the constraint is the resolution limit of about 120 nm, which is inherent to linear SIM, and the number of images that have to be recorded at different experimental settings.

Very different constraints have to be considered in single-molecule based photoswitching methods. First of all, images with sub-diffraction-resolution are obtained by reconstruction from individual localizations that were determined from thousands of individual imaging frames. In other words, the temporal resolution is, at first order, determined by the number of imaging frames that are required to obtain a
satisfactorily reconstructed image with sub-diffraction-resolution. Typically, thousands of images are required, recorded in an experimental time of tens of seconds to minutes. Other constraints lie in the nature of the photoswitchable fluorescent probes themselves. On the one hand, the fluorescent probes need to exhibit photoswitching under the particular experimental conditions, for example, in living cells, and on the other hand, the kinetics of the photoswitching process determines the temporal resolution. Fluorescent proteins can readily be handled in living cells, but exhibit slow photoswitching kinetics. Live-cell imaging with sub-diffraction-resolution using fluorescent proteins has therefore only been demonstrated for relatively slow processes, such as the dynamics of adhesion complexes [97]. Organic fluorophores are brighter than fluorescent proteins and at the same time are less prone to photobleaching, but their photoswitching requires specific chemical conditions. However, as has been demonstrated by the dSTORM method, the refined understanding of photophysical and photochemical processes that drive the transition of a fluorophore between a fluorescent and a dark state enables the identification of suitable fluorophores, even for live-cell imaging using the natural reducing redox buffer present in all cells.

An important advantage of organic fluorophores is that very fast photoswitching cycles can be achieved. Taking the example of carbocyanine fluorophores, such as the commercial derivatives Cy5 and Alexa Fluor 647, both the on- and the off-switching of the fluorophores is controlled by the irradiation intensity of a green and a red laser, respectively [60]. Using these carbocyanine fluorophores, rapid photoswitching with an imaging frame rate up to 1 kHz and a lateral resolution of ~30 nm has been demonstrated [72]. As such, switching cycles of ~1 ms can be realized with organic fluorophores that are about one hundred times faster than those reported for live cell PALM [97] or FPALM [96] experiments. Sliding window analysis of dSTORM data taken with frame rates of ~100 Hz allow the generation of video-like (~10 Hz) super-resolution movies [100]. Here just 100 consecutive frames are sufficient to generate a single high-resolution image with a lateral resolution of ~30 nm.

References


