Optical nanoscopy tools for biologists: advancements of fluorophores and optics for high resolution and live imaging

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Optical nanoscopy has emerged as an important tool for live cell imaging at the nanoscale resolution in the field of life sciences. The 2014 Nobel Prize in Chemistry for this invention proves its importance in multidisciplinary areas of science. Several optical nanoscopic methods have been introduced in the past decade to achieve diffraction-unlimited resolution by implementing new optical setup or utilization of unique photoswitchable fluorophores, or both. In this review we extensively discuss the biological importance of nanoscopy and the latest advancements and types of fluorophores needed for imaging. This review will be a starter-kit for biologists working in the field of bioimaging.

Keywords: Fluorophores, live cell imaging, optical nanoscopy, optical setup.

Background

To understand intracellular activities in detail, exploration of all their components such as cell activity, molecular interaction, structural insights, molecular dynamics are required. From the very beginning, microscopy-based cellular study played an important role in cell biology1–7. Fluorescence bioimaging helped realize many groundbreaking, biologically important discoveries in the living systems in several studies such as neurobiology8, cellular dynamics9–13, molecular interactions14,15, biomolecule counting16–18 and in vivo imaging19–23. It is well known that the fluorescence microscopy can allow imaging different cellular structures labelled with fluorophores with good signal-to-noise ratio and high specificity. This makes fluorescence imaging an indispensable tool in life sciences. However, fluorescence microscopy techniques are limited in their lateral and spatial resolution because of the wave nature of light, which restricts the separation of structures located closer than the half wavelength of light used for imaging24,25. This phenomenon is called the diffraction limit of light, and was introduced by Ernest Abbe in 1873. Hence, it is also known as Abbe’s rule of diffraction limit.

Abbe’s diffraction limit \( D = (\lambda/2\text{NA}) \)

where \( \lambda \) is the wavelength of light and ‘NA’ is the numerical aperture of the objective lens.

The light distribution from a focal spot in Fourier space is bigger than the actual size of the fluorescence spot in both \( x-y \) and \( x-z \) directions. This is because the generated image in the focused image plane consists of fluorescent signals from several points in the specimen, and is represented by the formation of the Airy diffraction pattern. The resolution limit criteria are given based on the closeness of the two Airy discs generated from different imaging point sources is called the Rayleigh criterion5,25,26 (Figure 1). If the distance between the two Airy disks or point spread function (PSFs) is greater than this value, the two point sources are considered to be resolved (and can readily be distinguished).

Rayleigh resolution \( = (0.61\lambda/\text{NA}) \).

Due to the diffraction limit, excitation beam used in the visible light domain result low spatial resolution. An

Figure 1. Illustration of lateral resolution of light microscopy explained by Rayleigh limit for two objectives close to each other.

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early and powerful approach to obtain more detailed resolution was the use of electrons instead of photons to form the image. Electron microscopy (EM) achieves 100 times superior resolution than light microscopy due to its relative (i.e. 105 times) smaller wavelength\(^{27,28}\). However, both transmission and scanning EM techniques are technically challenging, expensive, less user-friendly and time-consuming. To overcome this challenge, many fluorescence microscopy techniques were invented to achieve spatial resolution far better (30–40 nm) than the diffraction limit of light (>200 nm) (Figure 2 and Video 1, see online). This resolution is not as good as that using EM, but there is scope to constantly improve it at the molecular scale\(^{29,30}\). These techniques are collectively known as ‘nanoscopy’ that has profound impact on biology and other fields in which sub-diffraction-limited resolution of fluorescently labelled samples are desired. Considering the importance of nanoscopy techniques, the 2014 Nobel Prize in Chemistry was awarded to Stefan W. Hell, Eric Betzig and William E. Moerner.

Nanoscopy roughly includes three categories based on the light pattern used: (i) defined illumination pattern-based, (ii) wide-field illumination based and (iii) polarized illumination-based. Popular techniques in the first category are stimulated emission depletion (STED) microscopy\(^{31}\), reversibly saturable optical fluorescence transition (RESOLFT) microscopy\(^{31}\), saturated structured illumination (SSIM) microscopy\(^{32}\), Bessel-beam plane illumination nanoscopy (BBPIN)\(^{33}\) and lattice light sheet nanoscopy (LLSN)\(^{34}\). The second category includes photoactivatable localization microscopy (PALM)\(^{35}\), direct stochastic switching microscopy (DSSM) and stochastic optical reconstruction microscopy (STORM)\(^{36}\). The third and most recent technique based on the polarization of light is called super-resolution by polarization demodulation-exactly perpendicular to the polarization of the exciting light (SPoD-ExPan) nanoscopy\(^{37}\).

**Nanoscopy with defined illumination pattern (STED, RESOLFT, SIM and SSIM)**

A direct approach to constraining the fluorescence focal spot to a diffraction unlimited spot consists of a selective reduction of a part of the circumference fluorescence. This simple but powerful concept was used in STED, where the fluorescence was switched using a deterministic nanometric interference pattern of STED beam\(^{31,38}\). Increasing the STED beam intensity erases the fluorescence signal at the periphery of the spot, reducing its size. Ideally, the fluorescence signal will be liable at the centre of the doughnut hole. A modified Abbe’s equation describes this sub-diffraction resolution as

\[
D = \frac{\lambda}{2n\sin\alpha \sqrt{1 + \frac{I}{I_{\text{sat}}}}},
\]

where \(\lambda\) is the wavelength, \(n\sin\alpha\) is the numerical aperture of the microscope, \(I\) is the applied intensity of the STED pulse and \(I_{\text{sat}}\) is the STED intensity that gives 50% depletion of the emission\(^{39}\). The laser power used for STED beam is strong (>MW) to consider its applicability for live cell study. As reported earlier, strong laser power can cause cell death and photobleaching of the fluorophore; thus the high power is neither useful for live cell imaging nor fixed cell imaging. Hence, the STED nanoscopy is limited to short-time imaging of a photo-insensitive sample.

The more biocompatible version of STED, called RESOLFT has been implemented with a combination of unique photochromic fluorophores (Figure 3.a). RESOLFT is an advanced version of the STED principle utilizing

**Figure 2.** Spatial resolution of optical microscopies in x–z. Scale bar = 100 μm.

**Figure 3.** Schematic of the principle of patterned illumination-based super-resolution imaging. a, RESOLFT imaging and b, SSIM imaging.
photoswitchable fluorescent probes rather than a strong STED beam, and allows achieving a resolution (30–40 nm) far better than the diffraction limit of light (>200 nm) \(^{40-42}\). In contrast to STED, RESOLFT achieves super-resolution at very low laser power (<10\(^{-6}\) times) by altering the point-spread function of ensemble photoswitchable fluorophores. RESOLFT proved its superior in several live cell-imaging studies, attaining a resolution up to ~20 nm (refs 40–44).

SSIM is based on the nonlinearity principle. Here, a sample is illuminated with high-frequency sinusoidal striped light, which can be generated by laser light passing through a movable optical grating and projected via the objective onto the sample \(^{32,45-49}\) (Figure 3b). However, SSIM usually requires high laser power to achieve nonlinearity for improvement of resolution \(^{32,45,46}\). The latest SSIM techniques are now using photochromic fluorophores to achieve nonlinearity at low laser power because the power of light needed for switching them on/off is much lower than that used in conventional SSIM microscopy \(^{21,47,49,50}\).

**Recent addition to defined illumination-based nanoscopy**

The above discussed fluorescence nanoscopy techniques used for 3D imaging expose entire samples due to their dependency on epi-illumination optics (wide field, SSIM, 3D-PALM, STED) which compromises with loss of large amounts of fluorescence signal. Especially live cell imaging is hampered due to whole area illumination proceeding to continuous photobleaching. Light sheet fluorescence microscopy (LSFM) has a relatively intermediate optical resolution than the above discussed nanoscopy techniques. It uses a thin sheet of light to optically section the imaged object and a fluorophore. In contrast to other nanoscopy techniques, LSFM only illuminates a very thin section of the imaged object perpendicular to the direction of observation. Therefore it drastically reduce photobleaching of fluorophore. Several LSFM techniques have been developed with continuous improvement of spatial and temporal resolution. The most recent one, Bessel beam plane illumination microscopy (BBPIM), is based on a thin light sheet illumination that achieves ~300 nm resolution in 3D with a conventional fluorophore \(^{33,51}\). BBPIM uses a special Bessel light beam, which has highly non-reflective property. The resolution of BBPIM could be further improved using photocontrollable or photoswitchable fluorophores. Recently, BBPIM combined together with a structured illumination approach lead to great improvement in resolution (both x–y and x–z directions) with reduced phototoxicity. This new nanoscopy approach is known as lattice light sheet nanoscopy (LLSN) \(^{34}\). Both BBPIM and LLSN are best suited for long-time 3D imaging with very low phototoxicity.

**Nanoscopy with wide-field illumination (PALM, STORM)**

In this category, nanoscopy uses the principle based on high-precision localization of a single fluorescence emitter (Figure 4a). To avoid overlapping of adjacent fluorophores, the optimum concentration of the fluorescence molecules is necessary. The localized positions of emitting fluorophores from the set of image frames can be combined to yield a nanoscopy image for some defined cellular structure. The Poisson process of photon detection determines the precise localization of the single molecules; thus the most crucial factor is the detected photon from the emitter relative to the background \(^{35,36,52}\).

Basically, the localization nanoscopy aims to keep a limited number of detectable fluorophores sparsely distributed in each frame for their precise localization in diffraction-limited spots. Hence, the intentional control of the emitting single molecules is the key to these methods. The switching on/off of the fluorophore molecules in random or stochastic methods should be controlled so that a small subset of labelled fluorophores switch on in each frame. This process can be controlled either by repeatedly switching the fluorophore on and off or by switching the fluorophore on and then bleaching it \(^{53-56}\). Using this approach, the achievable resolution in stochastically switching methods is down to 10–20 nm, far better than the diffraction limit of light (~200 nm). This improvement caused much excitement and made the single molecule-based nanoscopy methods popular among researchers that eventually led to the 2014 Nobel Prize in Chemistry. Many types of photoswitchable fluorophores are used in this method, these are reversibly or irreversibly switchable from one fluorescence state to another \(^{56,57}\). Both photochromic chemical dyes and genetically encoded fluorescent proteins (FPs) can be used, depending on the purpose. FPs are commonly used for live cell nanoscopy; however, with some special tag, dyes can also be used for live cell imaging.

For superresolution imaging PALM and STORM are widely applicable nanoscopy techniques based localization precision principles. Other similar methods with different names have been invented such as fluorescence-PALM (f-PALM), spectral precision distance measurement (SPDM), direct-STORM (d-STORM), PALM with independent running acquisition (PALMIRA), etc. \(^{58-60}\). Many cellular events have been explored with high resolution using localization precision microscopy techniques. The localization precision for these methods can be calculated as described earlier using the following equation \(^{61,62}\):

\[
(\sigma^2_{x,y})_n \approx \frac{s^2}{N_m} + \frac{a^2}{aN_m^2} + \frac{4\sqrt{s^3}s^3}{aN_m},
\]

where \(s\) is the standard deviation of the PSF, \(a\) the pixel size in the image (taking into account the system
Figure 4. Stochastic blinking-based super-resolution imaging. a, Schematic of PALM imaging principle. b, (i)–(iv) Wide-field image of pcDronpa2-labelled β-actin in HeLa cells made by averaging the 700 frames used in pcSOFI. (v)–(vii) pcSOFI image of the same cell. (viii), (ix) PALM image of the same cell. Scale bar in frames (i), (iv) and (vii) is 10 μm. Frames (ii), (v) and (viii) show details of the β-actin structure (scale bar = 1 μm). Frames (iii), (vi) and (ix) are further zoomed in (scale bar = 0.3 μm). Absence of some actin fibres at the outer edges of the cell in the pcSOFI image is due to the increased z-sectioning in pcSOFI combined with imperfections of the 488 nm illumination, and also these structures being slightly out of focus²⁷.
magnification), $N_m$ is the total number of photons measured from molecule $m$ and $b_m$ is the number of background photons collected in the fitting window used for molecule $m$.

Recent additions in wide-field nanoscopy

pcSOFI nanoscopy: Stochastic optical fluctuation imaging (SOFI) is an easy and effective nanoscopy imaging technique that uses the blinking signal of fluorophore for reconstruction of nanoscopic images\textsuperscript{65}. The extended approach of SOFI in combination with photochromic FPs proteins called photoconversion SOFI or pcSOFI is accessible in live cell nanoscopy imaging with fast and simpler approach\textsuperscript{64,66}. A few recently published studies show the potential of pcSOFI for fast, easy and live cell study well below the diffraction limit, using as few as 200–500 image frames for nanoscopic image reconstruction\textsuperscript{65,67}. Combination of pcSOFI with reversibly photoswitchable fluorescent proteins (RSFPs) provides robust blinking desired for reconstruction of high-resolution nanoscopic image of pcSOFI (Figure 4b).

Polarization light-based SPoD-ExPAN nanoscopy: Another nanoscopy approach reported recently is based on the polarization of light. The advantage of this approach is that the optical set-up does not require special photocontrollable or photoswitchable fluorophores\textsuperscript{68}. This nanoscopy technique is called SPoD and unlike single-molecule detection-based nanoscopy, is applicable to samples with a high density of fluorophores. Polarized excitation used for SPoD allows the collection of signals only from fluorophores with different modulation phases among the fluorophore population. Thus, conventional fluorophores are compatible with this nanoscopy technique. However, the study used another high-power laser exactly perpendicular to the polarization of the exciting light (ExPAN) to further improve resolution\textsuperscript{68}. This high laser power could be reduced several hundred-fold using photoswitchable FPs.

Fluorescent proteins for nanoscopy

Photocontrollable FPs are the key component for nanoscopic imaging, i.e. they can either switch between two fluorescent states repeatedly or irreversibly with light irradiation at a certain wavelength. Selection of a suitable PSFP is crucial in nanoscopic imaging. By their photoswitching properties, FPs can be categorized as: (i) irreversibly or one-way switching and (ii) reversibly or repeated switching.

One-way switching FPs

In this group, FPs are irreversibly switched from one state to another in response to irradiation (Figure 5a and b). Those FPs which are converted from one fluorescent state to another (with a different emission wavelength) receive the name photoconvertable, and the others, which switch from a dark to a fluorescent state are called photoswitchable. Some good examples of one-way photoswitching FPs, which have the ability to switch from the original colour to another red-shifted colour are PAmCherry, mKikGR, PSmOrange, PAtagRFP, PACFP2, mEos2, mEos3 and Dendra2 (Table 1). The commonly used photoactivatable FPs are PA-GFP, PAmRFP1, PAmCherry1, PAmCherry2, and PAmCherry3 (Table 1).

mEos2 (an improved variant of mEosFP) has good folding efficiency, superior spectral properties, high brightness, fast photoconversion and high contrast ratio, making it ideal for fusion tagging for live cell imaging at 37°C. In its thermal equilibrium state mEos2 emits green colour, which can be converted to red state upon violet–blue light irradiation. Several cellular phenomena have been explored using mEos2, such as protein counting within a diffraction-limited region\textsuperscript{69}, live cell super-resolution imaging of yeast\textsuperscript{70}, cell tracking, and protein dynamics\textsuperscript{71}. However, recently researchers have shown that mEos2 is an oligomer in cells at high concentration\textsuperscript{72}. They further engineered mEos2 to develop mEos3.1 and mEos3.2 with truly monomeric nature, high brightness,
### Table 1. PSFPs and their photo-physical characteristics

<table>
<thead>
<tr>
<th>PSFP</th>
<th>Excitation (max), (nm)</th>
<th>Emission (max), (nm)</th>
<th>Excitation coefficient</th>
<th>Oligomeric state</th>
<th>Switching pattern</th>
<th>Light for switching</th>
<th>QY (ON)</th>
<th>Initial colour</th>
<th>Colour change</th>
<th>Chrophophorophoto conversion</th>
<th>Application in nanoscopy</th>
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<tbody>
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<td>rSEGFP</td>
<td>493</td>
<td>510</td>
<td>47,000</td>
<td>Monomer</td>
<td>Reversible</td>
<td>405/488</td>
<td>0.36</td>
<td>ON</td>
<td>Dark/green</td>
<td>Hydration/dehydration</td>
<td>PALM, RESOLFT</td>
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<td>mGeos-X</td>
<td>501–506</td>
<td>512–519</td>
<td>51,609–69,630</td>
<td>Monomer</td>
<td>Reversible</td>
<td>405/488</td>
<td>0.72–0.85</td>
<td>ON</td>
<td>Dark/green</td>
<td>ND</td>
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<td>Dreiklang</td>
<td>515</td>
<td>529</td>
<td>83,000</td>
<td>Monomer</td>
<td>Reversible</td>
<td>365/405</td>
<td>0.41</td>
<td>ON</td>
<td>Dark/green</td>
<td>Hydration/Dehydration</td>
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<td>Padron</td>
<td>503</td>
<td>522</td>
<td>43,000</td>
<td>Monomer</td>
<td>Reversible</td>
<td>488/405</td>
<td>0.64</td>
<td>OFF</td>
<td>Dark/green</td>
<td>Cis-trans conversion</td>
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<td>405/488</td>
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<td>psmOrange</td>
<td>548, 636</td>
<td>565, 662</td>
<td>113,300, 32,700</td>
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<td>Irreversible</td>
<td>Blue-green</td>
<td>0.51, 0.28</td>
<td>Orange</td>
<td>Red</td>
<td>Chromophore oxidation</td>
<td>In-vivo imaging, PALM, STORM</td>
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<td>PSCFP-2</td>
<td>400, 490</td>
<td>468, 511</td>
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<td>Violet</td>
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<td>518, 582</td>
<td>98,800, 60,400</td>
<td>Tetramer</td>
<td>Irreversible</td>
<td>Violet</td>
<td>0.80, 0.33</td>
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<td>π-conjugation</td>
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<td>515, 591</td>
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<td>UV–violet</td>
<td>0.69, 0.63</td>
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<td>507, 573</td>
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<td>Irreversible</td>
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<td>0.5, 0.55</td>
<td>Green</td>
<td>Red</td>
<td>π-Conjugation</td>
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<th>Excitation (max), (nm)</th>
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<th>Light for switching</th>
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<td>π-conjugation</td>
<td>PALM, STORM, RESOLFT</td>
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<td>513, 580</td>
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<td>UV</td>
<td>0.83, 0.62</td>
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<td>595</td>
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<td>Red</td>
<td>Anionic formation of chromophore</td>
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<td>596</td>
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high photon budget, and fast maturation. Dendra, another green-to-red photoconvertable FP extracted from Octocoral dendronephthya is the only one that uses cyan light (480 nm) for photo conversion. The monomeric Dendra2 has been already developed with improved maturation and brightness. Several super-resolution imaging studies have been made using Dendra2, e.g. vesicle tracking in neuronal gap junctions, protein tracking using fluorescence imaging with 1 nm accuracy (i.e. FIONA) in Caenorhabditis elegans, neuronal branching studies, and multi-compartment protein trafficking studies in live cells.

Most of the photoconvertible FPs change from green to red emission states. PSCFP2 is the only available irreversible-PSFPs (Ir-PSFPs) that converts from cyan to green. Its initial and photoconvertible states have completely distinct excitation (excitation (ex.) max 468 and 511 nm respectively) and emission spectra (emission (em.) max 400 versus 490 nm respectively). This property of PSCFP2 makes it useful for multi-color imaging with other photoconvertible or photoswitchable FPs. Another photoconvertible FP, PSmOrange and its variant PSmOrange2 have great value in imaging because both FPs have initially orange state (ex. max 548 nm and em. max 565 nm), and upon blue-light excitation can be switched to far-red (ex. max 636 nm and em. max 662 nm). These are the only most far-red-excitable photoconvertible FPs that makes them especially useful for in vivo imaging and multi-colour super-resolution imaging.

PA-GFP was the first photoactivatable protein developed from wild-type GFP. PA-GFP has a green emission and is used in many studies like protein diffusion, single-cell tracking, protein dynamics study, and neuronal dynamics and developmental studies. Among the red photoconvertible FPs, PACherry1 and PACherry are the best with QY (0.46 and 0.38 respectively), superior contrast ratio, excellent photostability, fast photoactivation, fast maturation, more suitable pH stability, and monomeric nature that makes them ideal for nanoscopy imaging. These FPs have proven well for both dual- and triple-colour super-resolution imaging of thick samples in two- and three-dimensional imaging.

Repeatedly photoswitchable FPs

This group of FPs called reversibly photoswitchable fluorescent proteins (RSFPs) can be switched on/off repeatedly upon UV light irradiation (Figure 5c). Repeated photoswitching of RSFPs is useful for stochastic switching-based super-resolution imaging (e.g. PALM, STORM) and defined illumination-based RESOLFT imaging. Several RSFPs have been developed either from natural sources or by improvement of the existing FPs, e.g. Dronpa, rsFastLime, broad-spectrum Dronpa (bsDronpa), Padron, rsCherryRev, rsCherry, rsEGFP, rsEGFP2, Dreiklang, mGeosX and rsKame (Table 1). Almost all RSFPs show cis–trans isomerization during switching, but Dreiklang has a unique switching mechanism based on hydrolysis/dehydration.

Dronpa was the first discovered RSFP with on/off repetition between dark and bright states upon illumination at 405 and 488 nm light. It has very high quantum yield (QY) (0.68) and extinction coefficient (98,000 M⁻¹ cm⁻¹). Several other Dronpa variants have been developed, namely bsDronpa (bright spectrum-Dronpa, with a large Stork shift: abs. max 460 nm and em. max 504 nm), Padron (with a positive photoswitching behaviour), rsFastLime, and pcDronpa (photochromic Dronpa). Padron is unique among all RSFPs because of its positive-switching behaviour. Padron together with other negative switching RSFPs has the potential for dual-label imaging. Dronpa and its variant were used in several super-resolution imaging studies, e.g. cellular dynamics, dual-colour and 3D PALM imaging and photochromic stochastic optical fluctuation imaging (pcSOFI). EGFP-based RSFPs (rsEGFP and rsEGFP2) were the first with fast switching speed and a large number of switching cycles. Both can be repeatedly switched on/off upon illumination with 405 and 491 nm light. Both these RSFPs have proven well in RESOLFT imaging for data storage, rewriting and live cell dynamics tracking.

rsCherryRev, rsCherry and rsTagRFP are red-emitting RSFPs. However, the main drawbacks of rsCherryRev and rsCherry are their relatively high residual fluorescence and low brightness, perturbing super-resolution imaging. rsTagRFP is relatively brighter than both rsCherryRev and rsCherry, potentially making it a good partner for green PSFPs in nanoscopy imaging. rsTagRFP switches under blue and yellow light irradiation into a red fluorescent on state and a dark off state respectively. The available number of colour variants of RSFPs is limited; therefore, their development offers many opportunities.

Dreiklang is another unique RSFP that can be switched on and off with 365 and 405 nm light respectively, and uses another decoupled light (515 nm) from on/off for fluorophore excitation. Dreiklang displays a bright fluorescence equilibrium state (QY 0.41 and extinction coefficient 83,000 M⁻¹ cm⁻¹). Padron is better than Dreiklang owing to its high QY (0.64), but the extinction coefficient is nearly half (43,000 M⁻¹ cm⁻¹) that of the latter. Given its monomeric nature, Dreiklang is an ideal candidate for fusion tagging in live-cell imaging, allowing to achieve images with a resolution of up to ~35 nm (ref. 43). The only drawback with Dreiklang is the requirement of three laser lines in the microscopy system. A complete switching cycle for Dreiklang requires excess UV light, which always causes difficulty for live cell imaging. Compared to negative-switching rsEGFP (~1200 cycles), Dreiklang...
produces just one-tenth of the switching cycles, but its positive-switching characteristics are useful for time-lapse imaging super-resolution imaging. Negative switching RSFPs use similar wavelength for both excitation and off-switching; thus they are partially turned-off during excitation. To maintain the signal/noise ratio, high excitation power is required than that desired for excitation, which always causes photobleaching. Both rsEGFP and Dreiklang have homologies similar to that of GFP; therefore, the improvement in the switching cycle of Dreiklang is strongly advocated.

Conclusion

Nanosopic techniques such as STORM, PALM, LSFM, RESOLFT, SSIM and SOFI are capable of providing nanoscale resolution. All these techniques utilize photoswitching properties of fluorophores; therefore, the suitable fluorophores are the key factors for nanoscopic imaging. It is crucial to understand chemical structure and photobleaching chemistry for the development of new photoswitchable FPAs and their colour variants. PSFPs with multi-colour variants will provide several options for multi-colour super-resolution imaging.


41. Chmyrov, A. et al., Nanoscopy with more than 100,000 ‘doughnuts’. Nature Meth., 2013, 10, 737–740.


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