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REVIEW ARTICLE

Resolution and contrast enhancements of optical microscope based on point spread function engineering

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Abstract Point spread function (PSF) engineering-based methods to enhance resolution and contrast of optical microscopes have experienced great achievements in the last decades. These techniques include: stimulated emission depletion (STED), time-gated STED (g-STED), ground-state depletion microscopy (GSD), difference confocal microscopy, fluorescence emission difference microscopy (FED), switching laser mode (SLAM), virtual adaptable aperture system (VAAS), etc. Each affords unique strengths in resolution, contrast, speed and expenses. We explored how PSF engineering generally could be used to break the diffraction limitation, and concluded that the common target of PSF engineering-based methods is to get a sharper PSF. According to their common or distinctive principles to reshape the PSF, we divided all these methods into three categories, nonlinear PSF engineering, linear PSF engineering, and linear-based nonlinear PSF engineering and expounded these methods in classification. Nonlinear effect and linear subtraction is the core techniques described in this paper from the perspective of PSF reconstruction. By comparison, we emphasized each method's strengths, weaknesses and biologic applications. In the end, we promote an expectation of prospective developing trend for PSF engineering.

Keywords super-resolution, optical imaging, point spread function (PSF) engineering, non-linear effects, linear subtraction

1 Introduction

Imaging of the cellular world using optical microscopes has experienced a great development along the last decades. Since far-field light microscopy has great demand in biologic investigations due to its non-destructive and real-time properties, improvements of the performance of optical microscopes has attracted an international cadre of pioneering researchers.

To enhance resolution and contrast of optical microscopes, confocal fluorescence microscopy was first invented by using pupil filters at the illumination or detection side of the microscope to reject out-of-focus light [1]. The size of the illumination spot in a confocal microscopy, which determines the resolution of the microscope as the spot blurs all structures, actually depends on the point spread function (PSF) of the microscopy system. As Abbe's limit of resolution exists [2], no method can be obtained to make one light beam focused more tightly than the diffraction limit. However, this diffraction light barrier was broken by carefully choosing contexts in which Abbe's law did not apply [3]. Quite a lot of work has been done by using two beams to make the effective PSF sharper for enhancements of resolution and contrast.

Since the inception of nonlinear optics a few decades ago [4], modeling a nonlinear relationship between the applied intensity and the measured fluorescence signal possibly would expand the capabilities of resolution or sharpen the PSF. However, this notion had remained vague until the early 1990s when the first valid concrete physical concept called reversible saturable optical fluorescence transitions (RESOLFT) appeared for breaking the diffraction resolution barrier with focused light [5]. In fact, RESOLF can be viewed as a family of concepts or methods such as stimulated emission depletion (STED) [6], ground-state depletion microscopy (GSD) [7], quantum dot switching [8], and FPs [9] and organic dyes [10] that utilize reversible saturable optical (fluorescence) transitions [11–14]. The grow-up of the nonlinear-based methods allow scientists peer inside micro-world with unprecedented level of resolution and sensitivity [6,7,15].

In contrast, a simple linear subtraction technique can also achieve resolution enhancement by linear subtraction

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of two images from each other, saving the trouble of complex nonlinear process in RESOLFT-type microscopy. In the past decades, a lot of efforts have been made in the subtractive methods. Armed with the traditional confocal microscopy, the subtraction result of images taken under different pinhole sizes has been demonstrated capable to improve the lateral resolution and signal-to-noise (SNR) [16–19]. Other novel subtraction approach known as fluorescence emission difference microscopy (FED) [14] and switching laser mode microscopy (SLAM) [20,21] are based on the intensity difference between two images acquired with fundamental Gaussian solid PSF and a doughnut-shaped PSF, respectively. Furthermore, the linear subtractive approach can also be combined with the nonlinear effect-based STED/time-gated stimulated emission depletion (g-STED) microscopy to achieve superior resolution and contrast [22,23].

In this review, we tried to give a complete description of each method mentioned above theoretically and practically from the perspective of the so-called PSF engineering. According to their distinct approaches to reshape the PSF, we classified all these methods into three categories: nonlinear PSF engineering, linear PSF engineering, and linear-based nonlinear PSF engineering. This review is divided into four parts. First, after describing the limitations of traditional light microscopy, we conclude common unifying principles of how PSF engineering generally could be used to break the diffraction limitation and conclude that the common target of PSF engineering-based methods is to get a sharper PSF. Secondly, we choose STED, g-STED and GSD microscopy to stand for nonlinear PSF engineering based on the concept of RESOLF and discuss them in detail with emphasis on nonlinear PSF deformation. In the third part, to which most attention has been paid, we take a PSF-engineering perspective to explore how each linear subtractive approach reconstructs the system's effective PSF in order to enhance resolution and contrast. In the last part, we introduce the methods in the category of linear-based nonlinear PSF engineering, which combine nonlinear effects with linear subtraction technique to pursue new advances.

2 Breaking diffraction barrier: point spread function (PSF) engineering

In an optical microscopy system, the details that can be observed are not determined by its resolution. The resolution is limited by the properties of diffraction which make sharp point-like objects appear blurry. When observed through any optical imaging system, a single-point emitter is blurred and two close point-like objects are unresolvable, which is illustrated in Figs. 1(a) and 1(b) clearly. The blur is a reflection of an imperfect focus of the optics, but an inherent property of the wave-like nature of light [24].

A microscopy system's resolution can be quantified by analyzing its PSF, which effectively describes how blurry a point-like object (a single molecule or small fluorescent bead) will appear. A cross section of the lateral PSF generates a pattern that is often in the shape of an Airy disk shown in the left of Fig. 1(b). Evaluating the PSF's full-width at half-maximum (FWHM) is always a simple way to characterize the resolution in each direction. The Raleigh criterion states that two points are resolvable if their distance d is at least the radius r_{Airy} of an Airy disk-shaped PSF (Fig. 1(d)) described by the formula:

$$d \geqslant r_{\mathrm{Airy}} \approx 0.61\lambda_0/\mathrm{NA} \approx \mathrm{PSF}_{\mathrm{FWHM}},$$

in which λ_0 represents the wavelength and NA is the numerical aperture of the objective. Since λ_0 and NA are both have their limitations, the PSF dimensions of conventional microscopes are limited to FWHMs of 200–250 nm in the lateral directions, and 500–700 nm in the axis [3,25]. Therefore, challenges to break diffraction barrier convert to efforts to reconstruct the microscopy system's PSF dimension by exploiting the so-called PSF engineering.

PSF engineering has pushed the envelope of what can be resolved by optical microscopy. As we know, if the lateral PSF's pattern could be reduced to the scale as the left pattern in Fig. 1(c), the two point emitters that are unresolvable in conventional light microscopy (right in Fig. 1(a)) would obviously be split clearly (right in Fig. 1(b)). PSF engineering's intention is to reshape the microscope's PSF by reducing its PSF's lateral section pattern (Fig. 1(c)) in order to create a sharper PSF profile marked in red rather than the convention's marked in black in Fig. 1(d). What should be recommended here is the invention of confocal laser scanning microscopy (CLSM), which rejects a lot of stray light but is still diffraction-limited as its PSF is equivalent to a conventional light microscopy's. However, this pioneering promotion has pushed a surge of innovative methods based on PSF engineering appearing successively which share a common destination: the acquisition of the red-marked targeted PSF [16,26–28]. According to their distinct approaches to reconstruct the PSF, we classify all these methods into three categories, nonlinear PSF engineering, linear PSF engineering, and linear-based nonlinear PSF engineering.

1) Nonlinear PSF engineering category is composed of a family of methods based on RESOLFT using optical nonlinear processes, whose key point is the creation of a nonlinear response of marked molecules within interested illuminated spot.

2) Linear PSF engineering category has a host of methods required by the linear subtraction of two images scanned by different PSF patterns. The resultant PSF of the subtractive image is equal to the linear subtraction of the

PSFs of the two original images. And all these strategies aim to form two suitable PSFs which can model a sharper equivalent PSF by linear subtraction.

3) Linear-based nonlinear PSF engineering category is a combination of linear PSF subtraction and nonlinear optical effects. In other words, linear subtraction techniques are applied to RESOLFT-type microscopy to step forward in optical imaging resolution.

3 Nonlinear point spread function (PSF) engineering

Nonlinear PSF engineering breakthrough occurred in mid 1990s accompanied by the first viable concept to break the diffraction barrier called RESOLFT appeared along with a series of methods coming after. These methods reconstruct the PSF relying on a nonlinear relationship between the applied intensity and the measured signals. A common formalism of these methods has been explained briefly [29].

Supposing a fluorescence molecule has two distinct states A and B, and the transition from A to B is light-driven at a rate k_{AB} which equals the light intensity I multiplied by the transition cross-section σ_{AB}, while the transition B to A at a rate k_{BA} is driven optically, chemically, spontaneously, or by any other means. After the populations of the two states have reached a dynamic equilibrium, the population of state A is

$$N_A^{\infty} = k_{BA}/(\sigma_{AB}I + k_{BA}).$$

Defining $I_{sat} = k_{BA}/\sigma_{AB}$, $I \gg I_{sat}$ means $k_{AB} \gg k_{BA}$ and further increasing I renders $N_A^{\infty} \rightarrow 0$.

We assume state A is fluorescence state which can emit fluorescence and state B is non-fluorescence state which cannot emit fluorescence. And nonlinear PSF engineering is purposed to take advantage of this analysis to create an arbitrarily sharp emission PSF of 'state A'. It can be realized by a doughnut-shaped light intensity distribution which drives molecules at the periphery from A to B and leave the central area in state A. Profiles (i)–(iv) in Fig. 2 show the doughnut cross section profiles with different light intensity. Figure 2 indicates that by making the power of the light beam sufficiently strong, the area of state A is squeezed down and can be calculated as

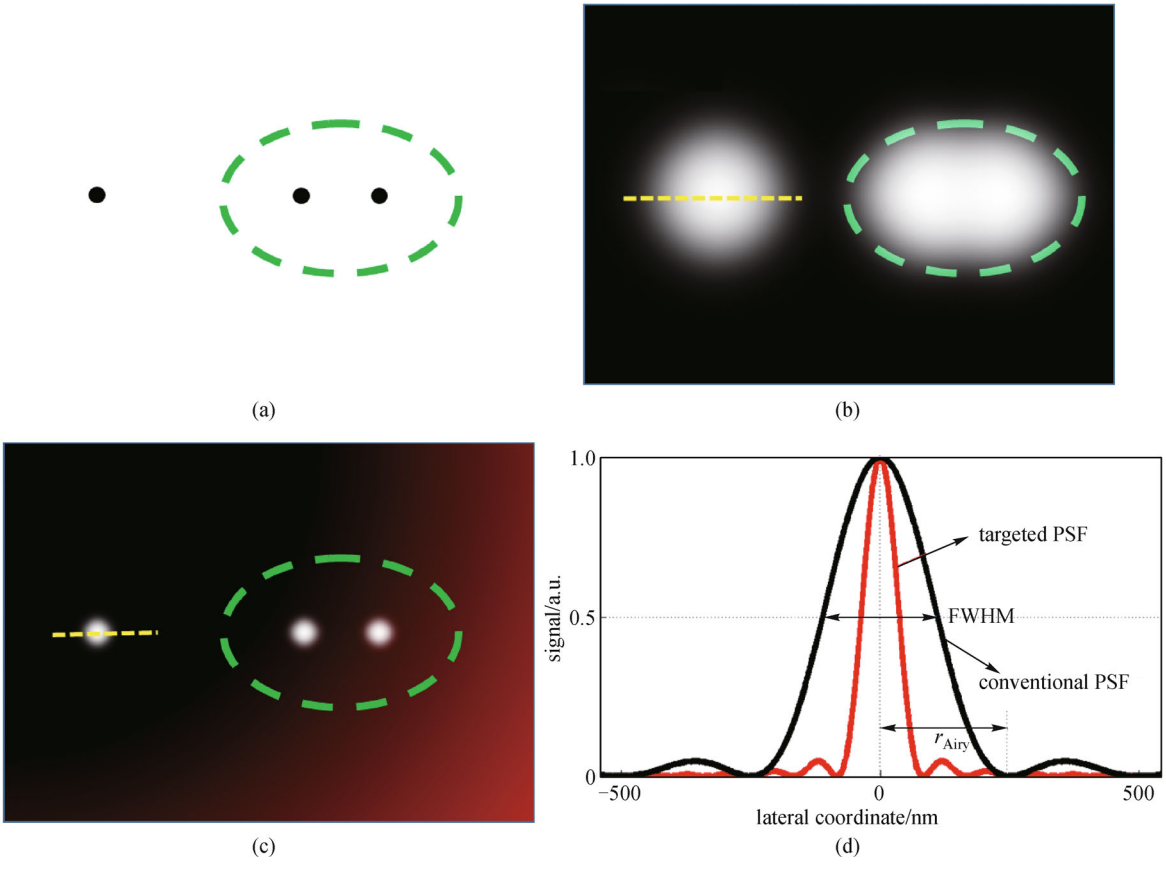

Fig. 1 (a) Schematic image of a point-like object (left) and two close point-like objects (right); (b) image of point-like objects of (a) taken with a conventional (confocal) microscopy; (c) targeted image of point-like objects of (a); (d) conventional (confocal) light microscope's PSF's profile and targeted PSF's profile

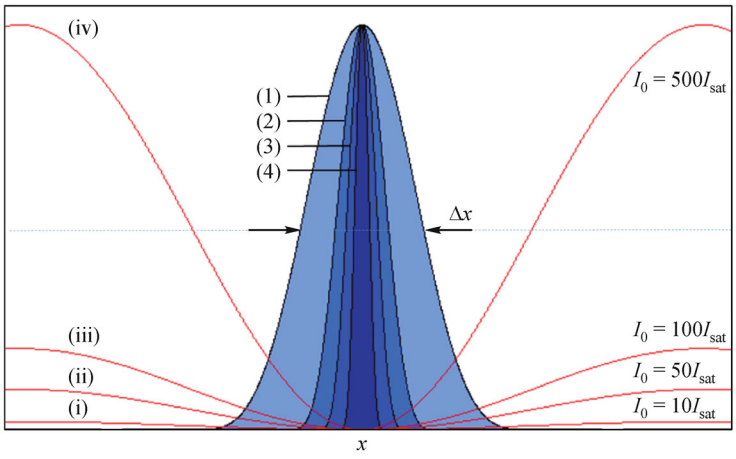

Fig. 2 Profiles (1)–(4) show the spatial region in which the molecules are allowed to be state A (fluorescent state), if the region is illuminated with a doughnut-shaped patterns focused by a standing wave of light which drives molecules from A (fluorescent state) to B (non-fluorescent state) with peak intensities $I_0 = 10I_{sat}$, $50I_{sat}$, $100I_{sat}$ and $500I_{sat}$ respectively whose corresponding cross section profiles are (i)–(iv) [30]

$$\Delta x = \lambda/\pi n \sin\alpha \sqrt{I_0/I_{sat}},$$

$$I_0/I_{sat} \rightarrow \infty \text{ yields } \Delta x \rightarrow 0.$$

In conclusion, two beams are necessary here. One is doughnut-shaped depletion beam to force the molecules at the periphery of the common illuminated spot transmit from fluorescent state to non-fluorescent state, and the other is a solid beam to excite only the doughnut-hole's region to emit fluorescence. The increasing power of the doughnut-shaped beam squeezes down the central emitting-fluorescence region [9,12,13,29–32].

3.1 Stimulated emission depletion (STED) microscopy

STED microscopy was the first implementation of RESOLFT demonstrated from theory to practice in 1994 by Hell & Wichmann. The nonlinear PSF engineering applied in STED microscopy (Fig. 3) is to reduce the fluorescent emission PSF's size by employing stimulated emission to inhibit the fluorescence process in the outer regions of the illuminated PSF [6,15].

In detail, two PSFs are needed, of which one is a solid (blue, Fig. 3(a)) and the other is a doughnut-shaped (yellow, Fig. 3(a)). The probe molecules in the illuminated spot are first excited to a higher energy state S_1 by the solid PSF focused directly by a laser beam with wavelength of about 640 nm. Before spontaneous emission of fluorescence occurs, which takes a few nanoseconds, the second doughnut-shaped PSF focused by a STED beam (e.g., 740 nm) modulated by a 2π phase mask illuminates the same spot to de-excite the probe molecules. The de-excitation process is to force molecules in periphery area of the excitation PSF from their excited electronic state back to their ground/non-fluorescence state by stimulation emission, and only molecules in the doughnut hole stay in S_1 (fluorescence state) and could emit fluorescence [3,15]. A band-pass filter is essential in collecting useful light as it stops the stimulated photos and only lets the fluorescence pass through. Therefore, a sharper effective PSF of fluorescence is generated (green, Fig. 3(a)). STED imaging is relatively fast for small fields of view and requires no data post processing, while it has a resolving ability of about 30 nm. However, STED has disadvantages that it requires precise special dyes and expensive setups, and the high laser power which is required to totally de-excite the unwanted molecules may cause an easy photo-bleaching and phototoxicity.

Based on STED, another alternative method further using nonlinear effect of fluorescence to pursue higher resolution is g-STED microscopy. A phenomenon was noticed that lifetime of fluorophores can significantly be shortened when it was illuminated by the light with moderate intensity and wavelength. By illuminating the outer region of the interested point with a doughnut-shaped STED beam, the lifetime of the illuminated fluorophores by STED beam is shortened, while the fluorophores in the doughnut hole remain a relatively longer lifetime. Setting a time gate and collecting photons after a selected detection delay are equivalent to collecting the long-time fluorescence emitted only by the emitters within the doughnut's central-hole regio. Based on this principle, the effective collected PSF's size is reduced amazingly and sub-diffraction resolution is attainable. It is noteworthy that g-STED stands out for its significantly lower incident beam intensity when providing a similar resolution as that of the general STED microscopy. However, further enhancement of resolution by setting a longer time gate is infeasible because the residual signals would be too weak to be distinguished from the noise [23,33–35].

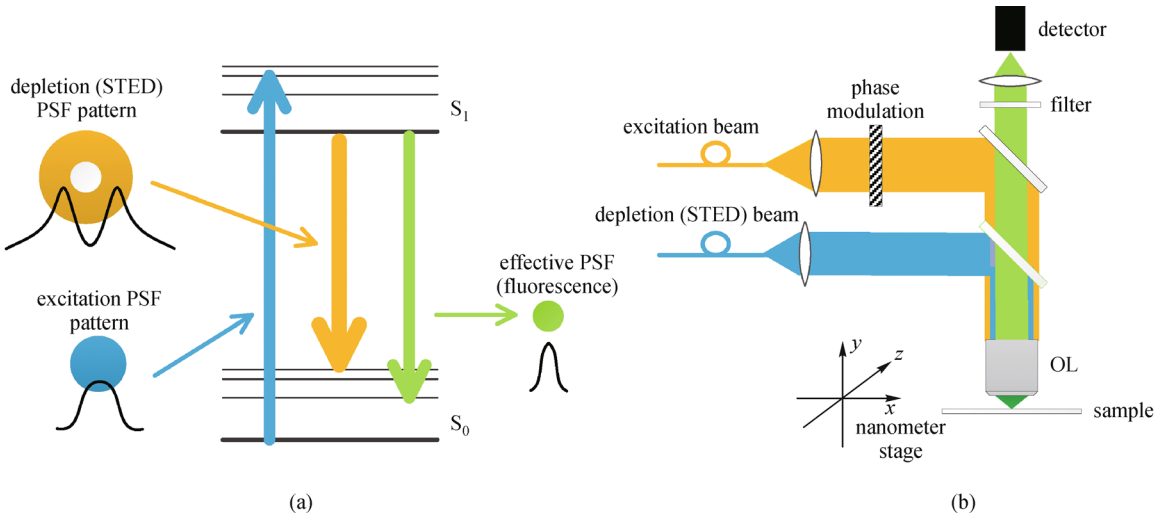

Fig. 3 (a) Energy diagram of STED microscopes; (b) schematic representation of a STED microscope. A phase modulation mask is used to create a doughnut-shaped depletion beam overlapping the excitation laser beam

3.2 Ground-state depletion microscopy (GSD)

Sharing the similar nonlinear PSF engineering with STED, GSD is another RESOLFT-type microscopy proposed by Hell together with Kroug in 1995 [7]. GSD accomplish super-resolution also by reducing the spatial extent of the focused PSF of a scanning fluorescence microscope and has the resolution potential as STED with relatively low-power continuous wave illumination.

Similar with STED, two PSF patterns are needed, of which one is solid and the other one is doughnut-shaped. Distinctive from STED, the time sequences of the two illuminating PSFs are different. Compared with STED in which the doughnut-shaped beam illuminates the specimen after the solid beam, in GSD the doughnut-shaped beam's nonzero fields are illuminated on the region surrounding the point of interest in advance to transmit the molecules within this area to a comparatively stable triplet state (non-fluorescence). Then the doughnut-shaped beam is switched off, and the solid beam centered at the point of the interest is focused to excite the molecules in the central field without the periphery which still stay in the ground (fluorescence) state to emit fluorescence. GSD is capable of attaining considerable resolution. However, the speed of imaging is limited since it is time-consuming to wait the de-excited molecules that stay in the stable triplet state to fall into the ground state, when these molecules are prepared to be scanned and imaged, resulting in limitations in the time resolution and restrictions of its developments.

4 Linear point spread function (PSF) engineering

The nonlinear PSF engineering has achieved a lot in super-resolution optical imaging, but in the meantime, has some inevitable bottlenecks. Enhancing the resolution relies on increasing laser power which easily causes fluorescence photo-bleaching or even damages to samples. To overcome these limitations, a simpler method called linear PSF engineering has been proposed to realize achieve super-resolution with common confocal laser scanning microscopy setups.

The principle of linear PSF engineering is to reconstruct the PSF by linear subtraction. The final image is obtained by linear subtraction of two images scanned by illumination patterns with different PSFs. A variety of novel approaches based on this principle have been proposed intending to provide simpler and low-cost super-resolution optical microscopy systems. According to the subjects of subtraction, we classify all the strategies into two types: linear subtraction of two solid PSFs and linear subtraction of a doughnut PSF from a solid PSF.

4.1 Linear subtraction of two solid point spread functions (PSFs)

Actually, the technique of subtraction of two images with different solid PSFs by using different sized detectors in confocal microscopy was applied more than two decades ago [18]. In this kind of imaging methods which was also called difference confocal method, two optical configurations based on a confocal laser scanning microscopy are important in practice; One with a relatively larger pinhole in front of the photo-detector is used to acquire an image with resolution identical to that of the convention optical microscope and the other one with a limiting pinhole placed in front of the photo-detector is applied to obtain a confocal image. The subtractive image can achieve resolution enhancement by a linear subtraction of the

confocal image with a parameter α from the conventional image. The subtractive image's PSF is calculated as

$$\mathrm{PSF}_{\mathrm{sub}} = \mathrm{PSF}_{\mathrm{conventional\ (large\ hole)}} - \alpha \cdot \mathrm{PSF}_{\mathrm{confocal\ (small\ hole)}}. \quad (1)$$

The sizes of the two pinholes are usually chosen as follow: one is nearly the size of the diffraction limited Airy-disk, and the other corresponds to about the second dark ring of the Airy disc. The setups are shown in Fig. 4(a). The PSFs' profiles are shown in Fig. 4(b), which illustrates clearly that FWHM of the subtractive PSF is almost half that of the conventional system and narrower than that of the confocal system's [16–19,36–38].

The resolution enhancement of the method mentioned above is at the expense of an increased noise level and a decreased useful signal. A new linear subtraction approach has been proposed to improve this situation and get a high SNR. This method also needs a big-pinhole image and a small-pinhole image and its core is subtracting the out-of-focus part from the small-pinhole image. The out-of-focus part is acquired by subtracting small-pinhole detecting signal from big-pinhole detecting signal with a parameter β, thus the PSF of the final image is

$$\mathrm{PSF}_{\mathrm{sub}} = \mathrm{PSF}_{\mathrm{small}} - \beta \cdot (\mathrm{PSF}_{\mathrm{big}} - \mathrm{PSF}_{\mathrm{small}}). \quad (2)$$

The subtraction can achieve an enhanced lateral resolution without much loss of in-focus light when the parameter β is specially weighted [39].

Meanwhile, the out-of-focus light can also be eliminated from a large-pinhole image by a new method called Virtual Adaptable Aperture System (VAAS). In this method, two kinds of detectors are used to capture the light that passes through the pinhole and the light that is blocked by the pinhole. By subtracting the latter from the former with an appropriate parameter, it features an advantage that the signal of out-of-focus can be reduced without decreasing the signal of in-focus [40].

In view of all the subtractive methods mentioned above, it should be noted that the subtraction may lead to negative intensities in $\mathrm{PSF}_{\mathrm{sub}}$, which means that the subtractive parameter value is crucial for the result performance. A higher value suppresses more noise and results in sharper PSF but leads to more negative intensities. In some cases, the negative figures are just put into zeros under the condition of no image distortion. But in most cases negative intensities introduce artifacts. Hence, the value of the subtractive parameter should be set to maintain a balance between the achievable resolution and the negative appearance. In addition, in practical experiments, energy matching, prior knowledge and specific area of the sample are all needed to be considered when determining the optimal parameter's value [16,17]. This type's PSF engineering techniques was previously used for edge enhancement to measure object height and some non-fluorescence samples. Afterwards, this method was extended to the incoherent case such as fluorescence imaging and has been gradually applied widely in biologic imaging such as brainstem, cells and microtubule networks and has the potential to realize superior resolution with lower expense in the future [17,18,22,36,37,39,41].

4.2 Linear subtraction of a doughnut point spread function (PSF) from a solid PSF

Considering the disadvantages of the linear subtractive ways: the signals used to construct the final super-resolution image are those emitted at the periphery of the excitation spot, while the actually effective signals which are emitted at the center of the excitation spot, in fact, have been reduced during the subtraction process. An alter-

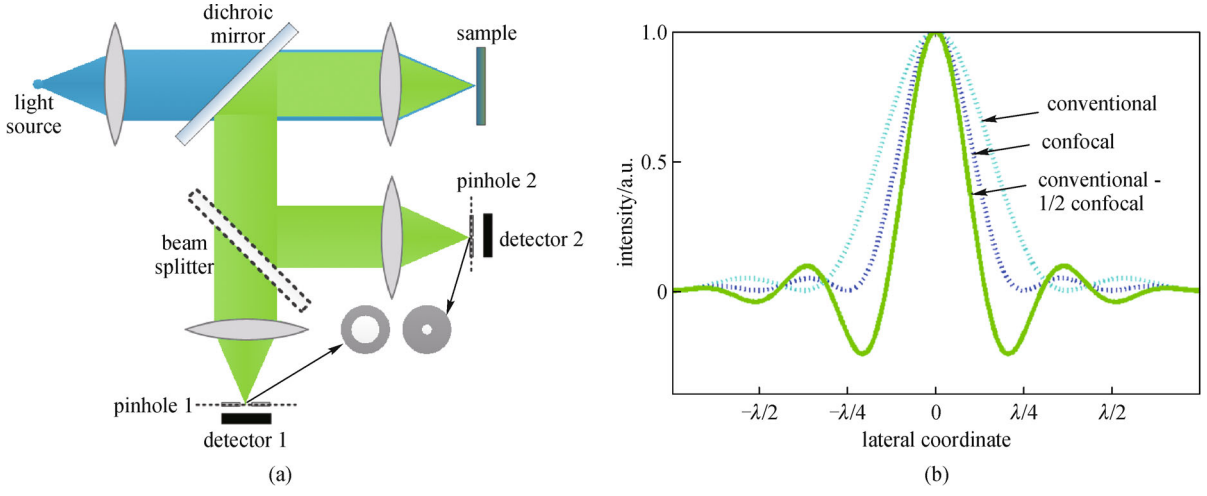

Fig. 4 (a) Schematic representation of a difference confocal microscopy; (b) PSF profiles of conventional (large hole) microscopy system, confocal microscopy system and the subtractive result with α of 1/2

native approach for enhancing optical resolution has been recently proposed by two independent groups. This method makes use of the linear subtraction of a doughnut PSF from a solid PSF in the confocal fluorescence microscopy.

The team of Cuifang Kuang in Zhejiang University has proposed a novel physical mechanism for breaking the diffraction barrier in the far field, which is termed fluorescence emission difference microscopy (FED). This approach is based on the intensity difference between two differently acquired images. One is the conventional confocal image acquired when the sample is scanned by a solid excitation PSF pattern (Fig. 5(a)); the other, the negative confocal image, is obtained when the sample is scanned by a doughnut-shaped excitation PSF pattern (Fig. 5(b)). Both images are detected by the same pinhole. The final super-resolution FED image is constructed by intensity subtraction of these two images, and the resulting PSF can be calculated as

$$\mathrm{PSF}_{\mathrm{sub}} = \mathrm{PSF}_{\mathrm{solid}} - \gamma \cdot \mathrm{PSF}_{\mathrm{doughnut}}. \quad (3)$$

The subtractive PSF pattern is shown in Fig. 5(c) and the three PSF's profile curves are shown in Fig. 5(d), which illustrate the resolving ability of the subtractive result. It is necessary to mention that the doughnut-shaped excitation pattern can be achieved in various ways, such as modulation by a vortex $0-2\pi$ phase plate or the convergence of azimuthally polarized light. By tuning the amplitude distribution, polarization state, phase distribution and the size of the effective incident aperture of the illumination beam, the size of the corresponding PSF of FED can be optimized to achieve best resolution in FED [14,42–45].

Meanwhile, the team of Harold Dehez has proposed switching laser mode (SLAM) microscopy based on the similar principle with FED. This method relies on the subtracting images obtained with dark and bright modes, and exploits the smaller dimensions of the dark spot of the azimuthally polarized TE_{01} mode to enhance resolution and contrast in laser scanning microscopy. Primarily, both FED and SLAM's super-resolution is due to the under-diffraction-limitation size of the dark spot of a hollow PSF. It is necessary to note here that negative intensities still exist and the subtractive parameter γshould be chosen to make a balance between the achievable resolution and the negative appearance [20,21].

Compared to the nonlinear PSF engineering, linear PSF engineering's resolving ability is relatively weaker remaining above 100 nm, but this kind of methods employ a quite simple linear subtraction principle to replace complex nonlinear processes. Therefore, the laser power just similar to that used in conventional imaging is required. The flexibility of this kind of approaches enables retrofitting in commercial confocal microscopes, two-photon microscopes and Coherent anti-Stokes Raman Scattering (CARS) microscopy and open avenues for resolution enhancement in microscopy system with non-fluorescent imaging modalities such as second- and third-harmonic generation microscopy [21,41].

5 Linear-based nonlinear point spread function (PSF) engineering

It is obvious that from the point of the attainable resolution, nonlinear PSF engineering performs a bit better than linear PSF engineering. However, linear PSF engineering has many advantages in imaging process, expenses and is less harmful to samples. The combination of the two techniques gives scientists the inspiration to further enhance the resolution of nonlinear PSF engineering by using the linear subtraction method which helps to bypasses the nonlinear PSF engineering's bottlenecks. This new imaging strategy has been demonstrated experimentally and applied effectively both in continuous-wave STED (CW-STED) and g-STED microscopy [22,23].

Linear subtraction is a simple way to overcome STED's weakness. In this approach, a standard CW-STED microscopy system is used to obtain a confocal image and a STED image successively, and the confocal image is subtracted from the STED image. The subtractive PSF is calculated as

$$\mathrm{PSF}_{\mathrm{sub}} = \mathrm{PSF}_{\mathrm{STED}} - \delta \cdot \mathrm{PSF}_{\mathrm{confocal}}. \quad (4)$$

The principle of enhancing resolution from the perspective of shrinking PSF is illustrated in Fig. 6. And this new approach has been demonstrated to have abilities to obtain better resolution and contrast in imaging both fluorescent nanoparticles and microtubule networks, while the value of δ still needs to be chosen according to the experimental data [22].

Furthermore, the linear subtraction method has also been applied to another nonlinear effect-based method: g-STED microscopy. It is known that the doughnut-shaped spot in the focal plane focused by a STED beam can be regarded as differentiating the average fluorescent lifetimes at and around the interested point. A proper detection time gate is set to ensure that the fluorescence light with a longer lifetime from the fluorophores located at the doughnut center is mainly recorded, while the shorter lifetime signals can penetrate to the doughnut crest. The subtractive imaging is obtained from the subtraction of a suitably weighted short lifetime intensity-image from a long lifetime one, hence the PSF of final subtractive image is

$$\mathrm{PSF}_{\mathrm{sub}} = \mathrm{PSF}_{\mathrm{long}} - \varepsilon \cdot \mathrm{PSF}_{\mathrm{short}}. \quad (5)$$

The diverse fluorescent intensity distributions of $\mathrm{PSF}_{\mathrm{long}}$ and $\mathrm{PSF}_{\mathrm{short}}$ determines that $\mathrm{PSF}_{\mathrm{sub}}$ is sharpening. The optimal value of εcan directly be obtained from experimental data without a priori knowledge of the theory. And

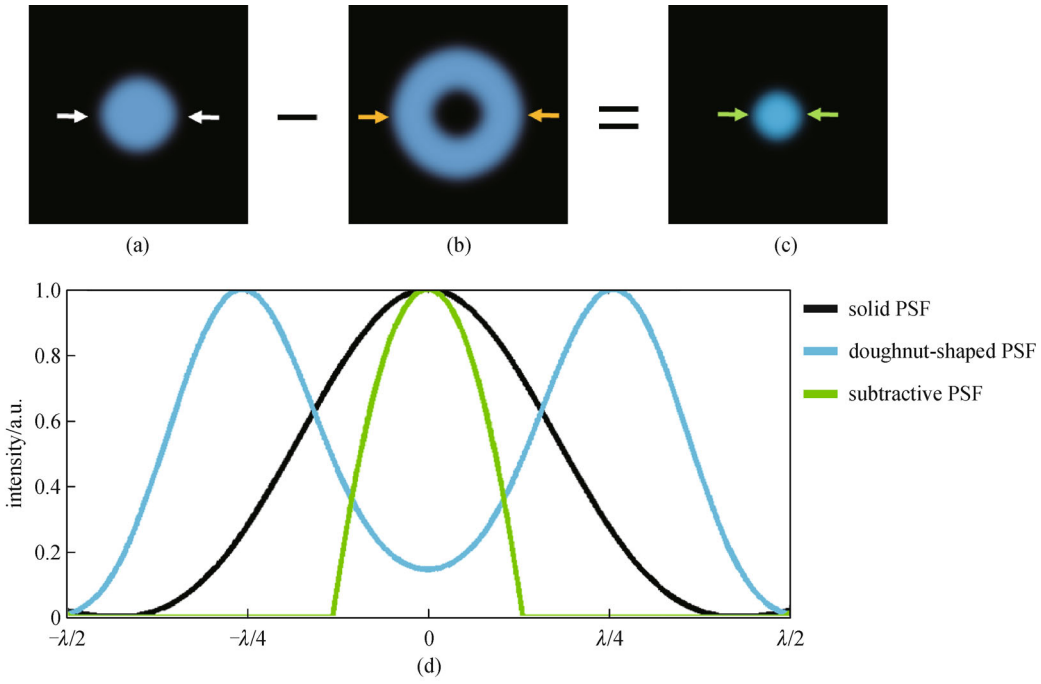

Fig. 5 Principle of linear subtraction of a doughnut PSF from a solid PSF. (a) Solid PSF pattern; (b) doughnut-shaped PSF pattern by a 2π phase modulation; (c) subtractive of the doughnut-shaped PSF from the solid PSF with a proper factor; (d) profile curves of the solid PSF, the doughnut-shaped PSF and the subtractive PSF

this approach has also been practically demonstrated in imaging human embryonic kidney (HEK) cells [23,33,34].

A group of images of 20 nm fluorescent beads, which have been processed by deconvolution, are presented in Fig. 7, while the function of nonlinear and linear PSF engineering is verified experimentally. Compared with the conventional confocal image (Fig. 7(a)), the image of STED (Fig. 7(b)) which is based on nonlinear PSF engineering shows great improvements in resolution and contrast. The subtraction image acquired by the linear subtraction of the confocal image from the STED image with the parameter of 0.5 is shown in Fig. 7(c). Compared to the STED image, it takes on stronger resolving ability and better SNR, which proves the advantage of linear PSF engineering. Figure 7(d) shows the image taken by a g-STED microscopy and it performs better than STED. Further improvements can be seen in the linear subtraction of the STED (a short lifetime image) from the g-STED (a long lifetime image) which is shown in Fig. 7(e). The b′, c′, d′ and e′ in Fig. 7(f) represent magnified view of the

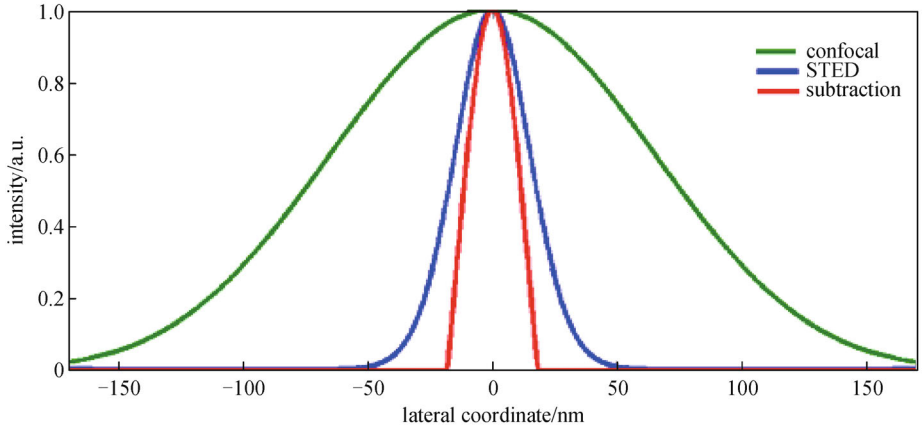

Fig. 6 PSFs of the confocal, the STED and the subtraction image ($\delta = 0.5$), and it can be seen the subtractive PSF is sharper than STED's PSF [22]

regions indicated by white box in Figs. 7 (b), 7(c), 7(d) and 7(e), respectively. It is denoted that the beads which cannot be separated in STED (b′) can be gradually split up in the subtractive image of the confocal image from the STED image but still blurred. The two beads in the g-STED image (c′) can be mainly resolved and can be completely divided in the subtractive image of the STED image from the g-STED image. It should be reminded here again that negative intensities are still unavoidable, hence the subtractive parameter should be determined not only with consideration to maintain a balance between the resolution enhancement and the appearance of negative intensities, but also with the reference to the specific region and prior knowledge.

6 Conclusions

In the past three decades, PSF engineering has played an important role in breaking diffraction barrier to enhance resolution and contrast. On this fundamental base, a surge of innovative ways has merged to challenge the Abbe's limitation. According to each method's characteristics, we expound them in classification of three categories: nonlinear PSF engineering, linear PSF engineering, and linear-based nonlinear PSF engineering. In the category of nonlinear PSF engineering, we examine the PSF shrinking principles of STED, g-STED, GSD microscopy which stand for a family of RESOLF-type microscopies based on the fluorescence nonlinearity processes. As for the category of linear PSF engineering which reconstructs PSF by a simple subtraction process, we further classify this kind of methods into two types according to their specific subtractive subjects and theoretically and practically describe them in detail. The restriction of the two methods discussed above push the combination of them, linear-based nonlinear PSF engineering, which applies linear subtraction method in nonlinear effect-based microscopy. By showing a group of experimental images with regard to imaging effects and resolution, we give a comparison among the confocal, STED, g-STED and the subtractive images and prove the advantages of the combination method.

We project that new technologies will continue to enable rapid growth of biologic imaging with two goals, spatial resolution and time resolution. The former relies on the sharpening of PSF and the latter calls for the actually real-time recording techniques. All the techniques based on the PSF engineering implies that scanning is indispensable, which means that the speed of scanning has always been becoming the limitation in the time resolution. Recent announced new parallelized RESOLFT fluorescence nanoscopy use two incoherently superimposed orthogonal standing light waves to form more than 100000 doughnuts and to provide isotropic super-resolution in parallel [46]. This concept reconciles major goals of super-resolution development: low-intensity operation, large fields of view and fast recording, at the resolution that is conceptually diffraction unlimited. This new conceptual advances are expected to applied in all of the PSF-engineering based techniques, especially in the simpler and more low-cost linear engineering-based methods described in this paper to realize the actually real-time imaging.

The new optical nanoscopes are still in their early days,

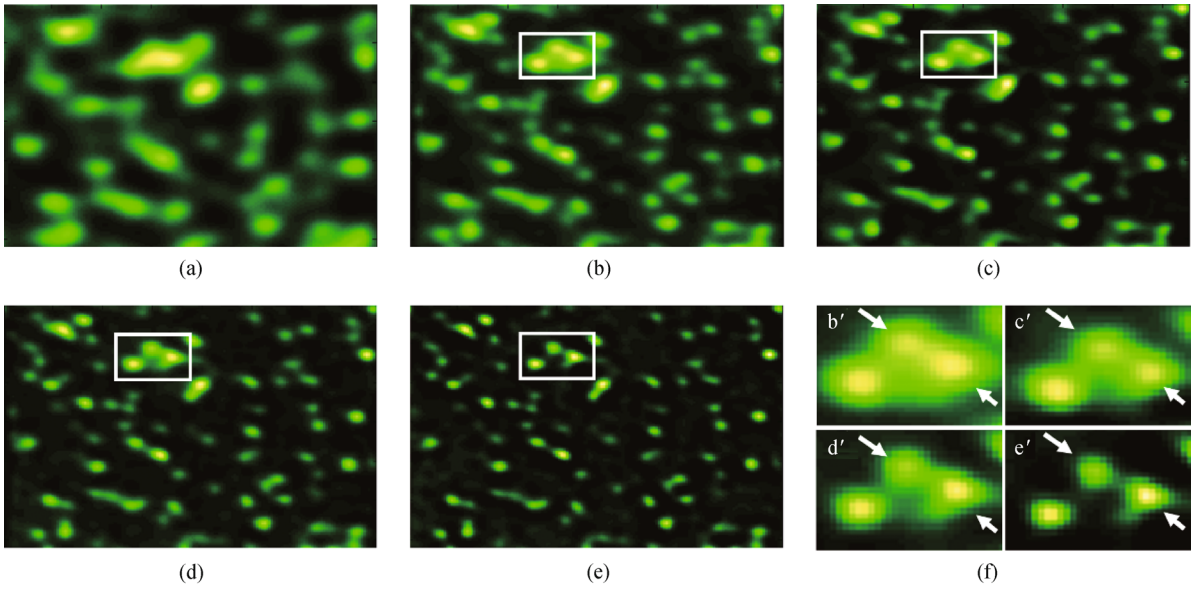

Fig. 7 (a) Confocal image of 20 nm fluorescence beads; (b) STED image; (c) subtraction of the confocal from the STED with δ of 0.5; (d) g-STED image; (e) subtraction image of the STED from the g-STED with ε of 0.08; (f) b′, c′, d′, e′: magnified view of the region indicated by white box in (b), (c), (d), (e)

and super-resolution optical microscopy is a rising new wave. Super resolution in real time by a simple way is always the final goal that we are always desperate for in the future.

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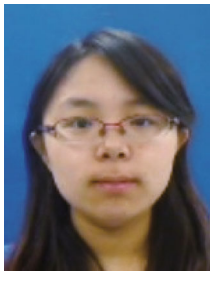

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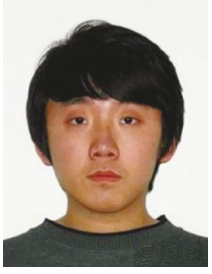

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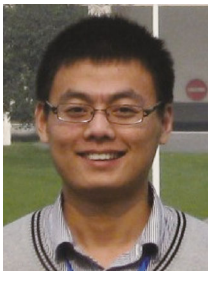

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