Review article

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Fluorescence imaging with tailored light

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Abstract: Fluorescence microscopy has long been a valuable tool for biological and medical imaging. Control of optical parameters such as the amplitude, phase, polarization, and propagation angle of light gives fluorescence imaging great capabilities ranging from super-resolution imaging to long-term real-time observation of living organisms. In this review, we discuss current fluorescence imaging techniques in terms of the use of tailored or structured light for the sample illumination and fluorescence detection, providing a clear overview of their working principles and capabilities.

Keywords: fluorescence imaging; tailored light; structured light; beam shaping; optical microscopy.

1 Introduction

Fluorescence microscopy has been widely used in numerous biological applications because it provides molecular specificity via the labeling of target molecules with fluorescent probes [1, 2]. This allows one to systematically study the dynamic behavior of living cells and tissues, and to reveal fine structures of interest and their interactions with other biomolecules. Over the last two decades numerous fluorescence imaging techniques have been invented, expanding the capabilities of conventional imaging methods. For instance, subdiffraction spatial resolution was achieved by several far-field approaches, which enabled subcellular structures to be clearly imaged at the nanoscale [3]. Suppression of out-of-focus background light made it possible to directly observe and track single-molecules with high signal-to-background ratio (SBR), improving our understanding of molecular mechanisms [4] and providing an analytical means for quantitative measurements of gene expression [5]. The ability to perform long-term imaging with a low-light dose facilitated the investigation of developmental processes and neuronal activities on a large scale with unprecedented spatiotemporal resolution [6, 7].

One of the main factors that led to these advances in modern fluorescence microscopy is the great progress in tailoring the illumination beam and the light emitted from the samples. Manipulating the fundamental properties of light, i.e. amplitude, phase or polarization allows the generation of a user-defined spot, line or, plane depending on the imaging technique. Powerful optical devices such as deformable mirrors and spatial light modulators (SLM) offer a large degree of freedom to control beams [8] and especially make it possible to minimize aberrations induced by specimens and optical components [9, 10]. Specific arrangements of tailored beams can further enable desirable features by coherent/incoherent superposition, parallelization and time-averaging.

In this review, we will describe how tailored light has been applied to advanced state-of-art fluorescence imaging techniques regarding three main aspects: super-resolution microscopy, high contrast imaging with epi-illumination, and fast volumetric imaging. We will explain their underlying principles based on how the beams are prepared at the back focal plane (BFP) of the objective lens. This will guide more explicit understanding in applying them to specific applications.

2 Super-resolution

2.1 Stimulated emission depletion (STED) microscopy

STED microscopy was first proposed to break the diffraction-limited spatial resolution in far-field fluorescence imaging [11, 12]. In this method, a tightly focused excitation beam is overlaid with what is referred to as the STED beam, which is red-shifted in the wavelength. The two beams are scanned over the sample, and the emitted

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fluorescence signal is registered at each coordinate. The STED beam has a spatial distribution featuring a zero at the center of the beam such that it brings the excited fluorophores located in the peripheral regions of the excitation beam back to the ground state through stimulated emission before the spontaneous emission occurs. High intensity STED light induces saturation of the depletion of the fluorescent state, and this effectively narrows the point spread function (PSF).

Advancement of STED microscopy has been closely related to the use of sophisticated PSF engineering techniques. In the early days, an offset beam with a Gaussian profile was used to demonstrate that the STED mechanism indeed improves the spatial resolution [13]. In this case, the STED beam was displaced laterally with respect to the excitation beam, which provided a resolution improvement in one-dimensional (1D) images on the focal plane [13] and along the optical axis [14]. Before long, it was suggested to use focal intensity distributions with a dark spot at the center by modulating the phase of the wavefront. A semi-circular π-shifting phase plate was employed for transforming a Gaussian beam (Figure 1A) to produce a central minimum line (Figure 1B), yielding a robust 1D-STED where the polarization of the STED light was parallel to the dividing line [15]. This pattern has been used for maximizing STED resolution down to a few nanometers [16]. It was straightforward to attain a lateral (x, y) resolution enhancement by coherently or incoherently adding two orthogonal semi-circular π-shifting phase plates in a Mach-Zehnder interferometer [15].

Alternatively, a circularly polarized Laguerre-Gaussian beam called a doughnut pattern was suggested to be used in STED microscopy [17, 18]. A Gaussian beam with a planar wavefront was converted to the doughnut pattern by applying a phase vortex to an SLM and then imaged at the BFP of objective lens [19]. The SLM was either directly imprinted with a vortex phase distribution ranging from 0 to 2π [19] (Figure 1C) or configured with an off-axis hologram that minimized the contribution of the unmodulated 0th order beam [20]. Interestingly, this approach was shown to be immune to spherical aberration caused by refractive index mismatch in the sample [17, 21] and has a high depletion efficiency [22]. The availability of a polymeric vortex phase plate made the doughnut pattern widely used in STED microscopy [23]. This compact and affordable device exhibited high transmission efficiency (>95%) and a strong resistance to a high power CW laser, making it a standard beam shaping module in two-dimensional (2D)-STED microscopy. Additionally, a liquid crystal device [24] and an optical vortex fiber [25] can also generate a doughnut pattern, which may facilitate the implementation of STED microscopy if the intensity at the doughnut center decreases further.

In contrast, an axial (z) resolution enhancement in STED microscopy was realized very early by using a phase plate that had been used in optical trapping [26]. The central region of the phase plate imparted a phase delay of π, which induced destructive interference along the optical axis leading to what is called z-STED [27] (Figure 1H). While the z-STED technique primarily improves on-axis resolution, it also slightly improves the resolution in the lateral direction. Two incoherent de-excitation patterns, i.e. 2D-STED and z-STED can be superimposed upon each other [28] to yield a significantly reduced focal spot in three-dimensional (3D) imaging [29, 30].

More effective STED beams have been considered to improve the performance of STED microscopy in both 2D and 3D. The resolution of STED microscopy is described by an inverse square-root law, i.e. $d \approx \lambda/2\mathrm{NA}(1+\beta(I_m/I_S))^{1/2}$, where λ is the wavelength of fluorescence emission, NA is a numerical aperture of objective, I_m is the maximum intensity of the switch-off beam, and I_S is a characteristic intensity that can deplete half the fluorescence of fluorophores. Here, β is a geometrical factor and it displays a higher value when the slope near the center of the STED beam is steeper, i.e. it can deplete the fluorescence efficiently with a lower illumination intensity. Generally, the slope shows a quadratic dependence on x, y, and z [23, 31]. In particular, cylindrical vector beams [32, 33] have been extensively investigated. These beams have spatially varying polarizations, leading to polarization vortices in contrast to phase vortices. One suggested method to achieve a finer lateral resolution with less illumination intensity was by an azimuthally polarized beam (Figure 1D) [34], or by the superposition of two azimuthally polarized beams where a binary phase plate (Figure 1E) is applied to one beam and a quadrant $0/\pi$ phase plate (Figure 1F) is applied to the other [35]. However, the azimuthally polarized beam is undesirable to STED imaging because its depletion efficiency is strongly affected by the orientation of molecules [36], although this effect is often suppressed when the rotation of fluorophores is fast and/or many fluorophores reside within the subdiffraction spot. To generate uniform 3D de-excitation patterns, one approach was proposed by incoherently superimposing a radially polarized beam that is split and modulated with a quadrant $0/\pi$ phase plate (Figure 1G) along one path and with a π-shifted phase plate (Figure 1I) along the other path [37], but its effective PSF on the focal plane was non-uniform. Another approach was to use a higher-order radially polarized transverse electromagnetic mode (Figure 1J) and its variant modulated with a vortex phase plate (Figure 1K).

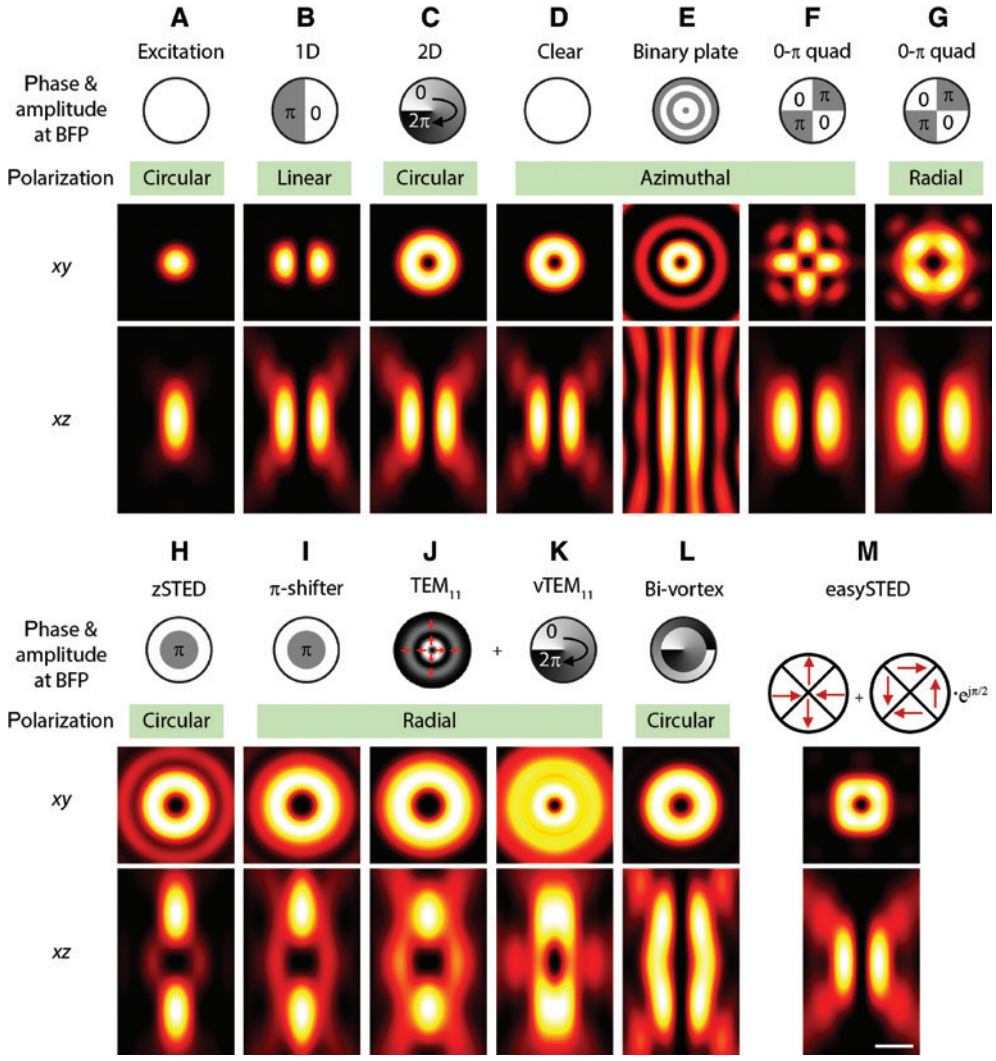

Figure 1: PSF engineering of tightly focused STED beams.
Beam intensity distributions in the *xy* and *xz* planes with various phases and polarization states at a pupil plane of objective. (A) Gaussian beam with circular polarization. (B) Linearly polarized 1D-STED pattern generated by a semi-circular π-shifting phase plate, where the linear polarization is parallel to the dividing line of the phase plate. (C) A doughnut pattern (2D-STED) through a phase vortex using a circularly polarized beam. (D–G) Depletion patterns using cylindrical vector beams. Azimuthally polarized beams through a clear aperture (D), binary phase plate (E) or quadrant $0/\pi$ phase plate (F). A radially polarized beam through a quadrant $0/\pi$ phase plate (G). (H,I) Axially confined patterns through a π-shifter with a circularly (H) or radially (I) polarized beam. 3D de-excitation patterns can be generated by incoherently superposing C and H as well as G and I. (J,K) Optical cage patterns for isotropic 3D-STED using a higher-order radially polarized transverse electromagnetic mode (TEM_{11}) in (J) combined with a vortex phase plate (K). (L) Coherent hybrid depletion pattern generated by two out-of-phase vortices. (M) Alignment-free easySTED using an achromatic quarter waveplate and a chromatic segmented half waveplate. Polarization states are displayed at two different wave cycles. A wavelength of STED beam = 775 nm, objective NA = 1.4, refractive index (n) of medium = 1.518. Scale bar, 500 nm.

This technique can potentially generate optical bottles or cages [38, 39] with isotropic 3D resolution.

Two interesting methods hybridized previous techniques to provide new features and capabilities. The first is a coherent hybrid depletion pattern called CH-STED [40]. The phase pattern consisted of two vortices, a disc and a ring, which had a same helicity but were out of phase (Figure 1L). They were reminiscent of an enlarged 2D-STED [41] and an annular vortex [42], respectively. CH-STED enabled high contrast STED imaging even at low or intermediate STED power by suppression of incomplete depletion of the out-of-focus background [43]. The second method is called easySTED and uses a different approach to create a 2D doughnut beam by using a quarter wave plate and a segmented half wave plate (Figure 1M) to manipulate the polarization of the STED beam [36].

The beam then becomes radially polarized by a quadrant $0/\pi$ phase plate for $\phi = 0$ (Figure 1G) and azimuthally polarized for $\phi = \pi/2$ (Figure 1F), where ϕ is the phase of STED beam. The segmented half wave plate was designed to be chromatic so the excitation beam was unaffected. easySTED facilitated the use of pre-aligned excitation and STED beams emanating from a single fiber without a degradation of the imaging performance, which made it possible to have an alignment-free STED system. A similar approach was demonstrated for easy z-STED [44].

A sharper excitation beam can also enhance the performance of STED microscopy. A radially polarized annular beam [45] or a circularly polarized beam with a binary amplitude filter [46] was known to show a laterally or axially tighter focus, respectively (Figure 2A,B,C). A recent study applied the former to STED microscopy and demonstrated that less STED power was required than the conventional approach to attain a similar resolution [47]. However, as this beam shows a significantly elongated excitation profile along the optical axis, it may suffer from a poor contrast due to incomplete depletion for 3D samples. Similar problems are expected if a super-oscillation based excitation beam [48, 49] is used in STED microscopy.

For imaging thick biological samples, it is crucial to properly manage aberrations that can reduce the resolution and signal-to-noise ratio (SNR) of images. To attain high quality STED images, it is important to keep the intensity at the center of the STED pattern close to zero; otherwise, the residual STED light adversely depletes the fluorescence. Typically the center needs to be <0.3% of the peak intensity of STED beam [16, 23]. z-STED has been known to be susceptible to aberrations, as contrasted with 2D-STED [17, 21]. For example, aberrations induced by a refractive index mismatch in the sample yield a considerable amount of residual light at deeper imaging depths with z-STED (Figure 3). A simple remedy is to use glycerol [50], silicone or water immersion objectives [51] for tissues or aqueous samples to minimize the refractive index difference between the immersion fluid and the sample. Adaptive optics has been used to fully compensate for specimen induced aberrations. For example, two SLMs have been used to correct the excitation and z-STED beams [52], and fluorescence detection was improved by an SLM and a deformable mirror [53]. This enabled the use of STED microscopy for imaging thick, strongly aberrating samples [54]. Furthermore, a single SLM has provided a convenient way to realize 3D-STED [55] and correct both the excitation and STED beams [56].

2.2 Structured illumination microscopy (SIM) and parallelized reversible saturable optical fluorescence transitions (RESOLFT) microscopy

SIM can achieve two-fold enhancement of the spatial resolution in a wide-field fluorescence microscope and is compatible with most standard fluorophores [57, 58]. The sample is illuminated with a series of known excitation patterns, such as sinusoidal grids with a corresponding phase shift for each different pattern orientation [59, 60].

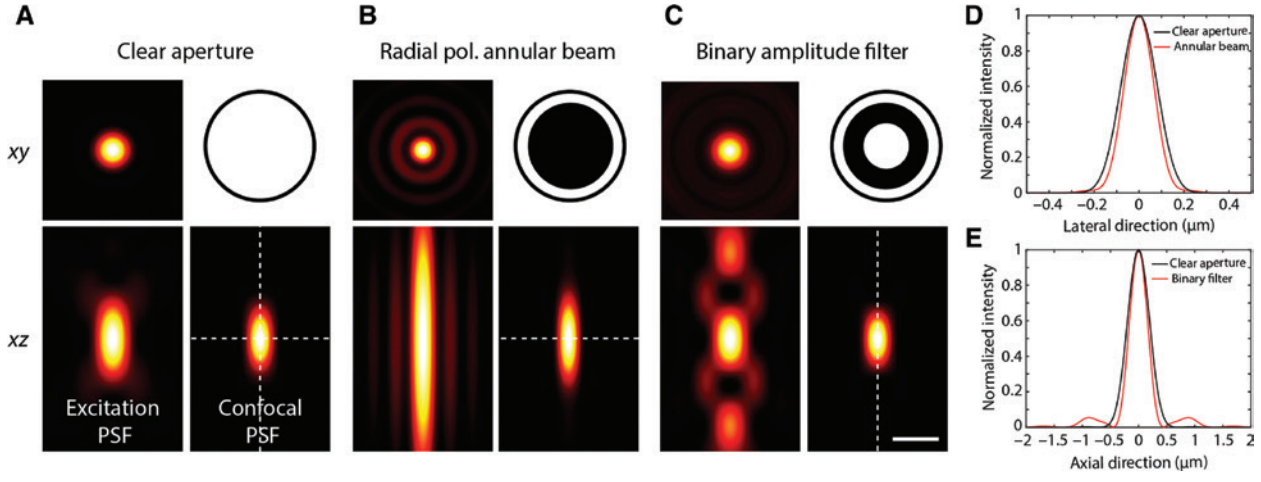

Figure 2: Sharper excitation beams for STED microscopy.
Excitation and confocal PSF of a circularly polarized beam through a clear aperture (A), a radially polarized annular beam for lateral resolution enhancement (B), and a circularly polarized beam through a binary amplitude filter for axial resolution enhancement (C). (D) Lateral profiles of confocal PSF with the clear aperture (black) and radially polarized annular beam (red). (E) Axial profiles of confocal PSF with the clear aperture (black) and binary amplitude filter (red). 1 Airy unit is used as a confocal pinhole size. Scale bar, 500 nm.

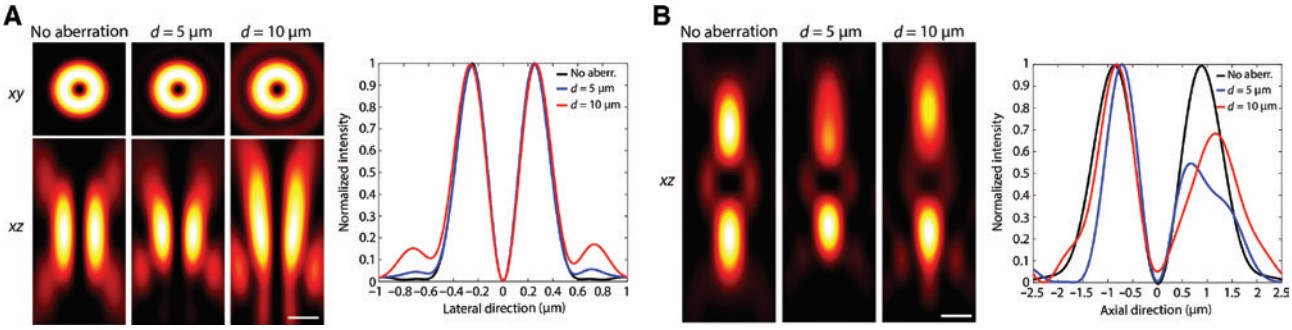

Figure 3: Aberration in STED beams.
Influence of the aberration induced by refractive index mismatch in the sample for 2D-STED (A) and *z*-STED (B). Intensity distributions in the *xy* and *xz* planes, and line profiles for an aberration-free case and different imaging depths indicated by *d*. Clearly, compared to 2D-STED, *z*-STED is more susceptible to aberrations, including the degradation of the symmetricity of PSF shape and considerable amount of the residual intensity at the center as the increasing imaging depths. As the index mismatch causes the focus shift, for better comparison, all profiles are shifted accordingly to keep the minimal intensity at the origin. Scale bar, 500 nm.

The high spatial frequency information is encoded into the observed images and can be recovered in post-processing.

The sinusoidal pattern is typically generated by a transmissive phase grating with an order selection mask, and the ±1 diffraction orders are focused onto the BFP of the objective (Figure 4A) [59, 60]. The illumination pattern is projected on the sample as the grating is translated and rotated. For high contrast SIM images, it is crucial to have a full modulation depth of the pattern, which is ensured by a linearly polarized beam whose direction is parallel to the stripe (*s*-polarization) [61]. This minimizes the residual light at the trough and prevents unwanted background noise. When total internal reflection fluorescence (TIRF) illumination was used to image near the surface (Figure 4B), SIM readily demonstrated ~100 nm resolution [62–64] and even sub-100 nm resolution with a high NA objective [65]. The principle has been extended in 3D-SIM to double the axial resolution as well as the lateral resolution, where a 3D interference pattern was generated by the 0 and ±1 diffraction orders (Figure 4C) with different illumination intensities [66]. It is desirable to use a slightly (spatially) incoherent light source to help

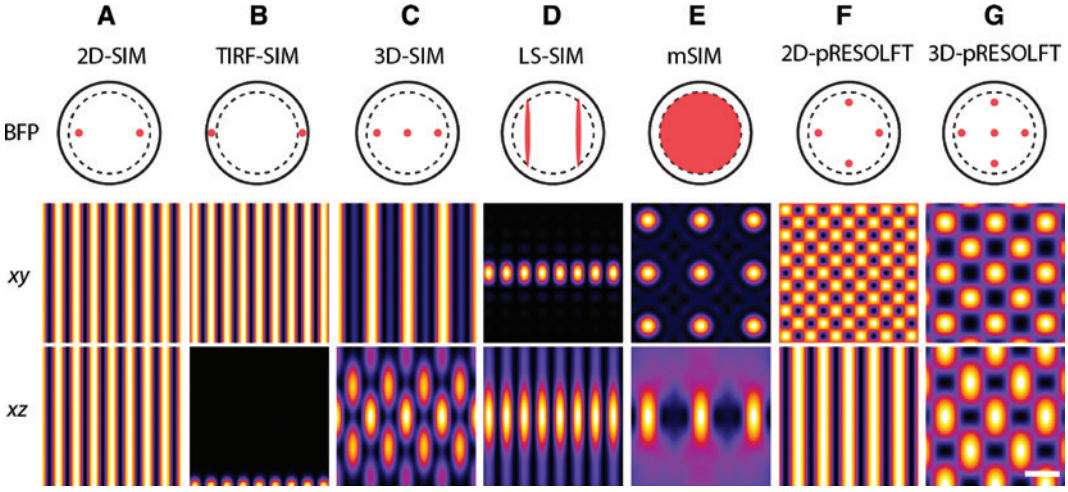

Figure 4: Beam shaping for SIM and parallelized RESOLFT.
Comparison of beam positions at the back focal plane (BFP) and intensity distributions on the *xy* and *xz* planes for each method. At the BFP, the dashed circle denotes the critical angle position assuming a glass/water interface. The red dots indicate focused beams at BFP. (A) 2D-SIM with a sinusoidal illumination pattern on the *xy* plane. (B) TIRF-SIM, SIM with total internal reflection fluorescence illumination. (C) 3D-SIM, 3D illumination pattern generated by interference of three beams. (D) LS-SIM, SIM with a line-scan pattern generated by interference of two lines. (E) mSIM with multiple focused spots. (F) 2D-pRESOLFT microscopy. Two orthogonally structured illumination patterns are incoherently superimposed on the sample plane. (G) 3D-pRESOLFT, three dimensional parallelized RESOLFT generated by interference of five coherent beams. All the beams are *s*-polarized except E and G, which are circularly polarized. Scale bar, 500 nm.

time-average the speckle pattern and to phase out the 3D SIM pattern along the *z*-axis. This was accomplished by coupling light sources to a multimode fiber with a phase scrambler. Whereas random speckle patterns showed several merits in SIM [67, 68], they demanded a substantially large number of raw images compared to the standard SIM pattern and were not suitable to 3D biological imaging.

The mechanical movement of the physical grating slows down the imaging speed of SIM. However, this problem was solved by employing an SLM [69]. This could rapidly generate, translate and rotate the pattern, and a liquid crystal device was used to change the polarization state of the output beam from the SLM [64]. This approach demonstrated video-rate 2D-SIM [64] and fast volumetric SIM [70] in living cells. Recently, a segmented azimuthal polarizer was proposed to replace the liquid crystal phase retarder to provide a more viable method of preparing the *s*-polarization for all illumination patterns [71].

When imaging a thick fluorescent sample, wide-field SIM illumination generates considerable out-of-focus light which highly increases the background noise. As a result, the reconstructed images suffer from strong noise artifacts. Recently, SIM with sparse illumination patterns has been proposed to reject out-of-focus fluorescence, including line-scanning [72] (Figure 4D) and multifocal illumination [73, 74] (Figure 4E) systems. The latter was conveniently generated by a digital micromirror device or a spinning disk system without considering polarization state, and moreover enabled video-rate imaging [75, 76]; however, its resolution was not as good as traditional SIM [58]. Adaptive optics has seen limited use in SIM [77], but will enhance the resolution and contrast of multifocal SIM, especially when combined with two-photon excitation [78].

Structured illumination patterns can be applied in parallelized RESOLFT microscopy. RESOLFT uses fluorescent probes exhibiting reversible photoswitching between a bright and a dark state, and STED is one of its mechanisms [79, 80]. As RESOLFT requires much lower light intensities than STED, it is possible to adapt a parallelized scanning scheme that fully benefits from fast, large field-of-view (FOV) imaging. Initially an 1D sinusoidal pattern was generated by an interferometer for proof-of-principle experiments of RESOLFT [81]. Recently, parallelized RESOLFT with more than 100,000 donuts has been achieved [82]. To implement this scheme, two orthogonally structured illumination patterns were incoherently superimposed on the sample plane (Figure 4F), serving as a fluorescence depletion pattern. Similar to SIM, the pattern was generated by a diffraction grating and it was suitable for multi-color imaging due to the independence of the grid pattern from the illumination wavelengths [83]. Parallelized STED microscopy with a 2D grid pattern was also demonstrated [84, 85] and in this case each standing wave pattern was formed from the interference of two inclined plane waves which is more efficient than a diffraction grating. Selective photoactivation by TIRF [86] or multifocal illumination [87] (Figure 4E) can enhance the contrast of parallelized RESOLFT images. A donut array [88] and a 3D depletion pattern based on the interference of five coherent beams (Figure 4G) [89] are likely to extend applications of parallelized STED/RESOLFT to fast 3D nanoscopy.

2.3 Single-molecule localization microscopy (SMLM)

A single fluorophore can be localized with high precision proportional to the inverse of the square root of the collected photons from the isolated single emitter [90]. Several techniques are able to isolate individual single-molecules within biological samples that are densely labeled with many fluorescent probes so that localization is possible. For example, a sparse subset of fluorophores can be activated by light that switches fluorophores between a fluorescent state and a dark state [91–93], or a subset of molecules can be sparsely targeted via the binding and unbinding of fluorescently labeled molecules [94, 95]. The SMLM image is then reconstructed from numerous subset images with nanometer localization accuracy [96].

In SMLM, the sample is typically illuminated by a large area beam such as epi- or TIRF-illumination. Other excitation schemes to guarantee high SNR in 3D single-molecule samples will be discussed in detail in Section 3. A few SMLM studies have used adaptive optics in the detection path to correct sample-induced aberrations [97, 98] and recent studies reported a significant increase of the localization precision in thick brain samples [99, 100].

Numerous SMLM studies have focused on the detection path, aiming to improve the axial localization precision by encoding the fluorescent emitter's depth information into the shape of PSF using phase aberrations. This includes an astigmatic PSF [101], rotating double-helix PSF (DH-PSF) [102], tetrapod PSF [103], and self-bending PSF [104]. The features of these PSFs vary distinctly as a function of defocusing depth [105]. The lateral and axial position of emitters can be extracted and characterized based on parameters specific to the tailored PSFs. For example, the variation of PSF width along the *x* and *y* axes for the astigmatic PSF (Figure 5A), the angle of two main lobes of the DH-PSF (Figure 5B) or relative stretching of the two lobes along the

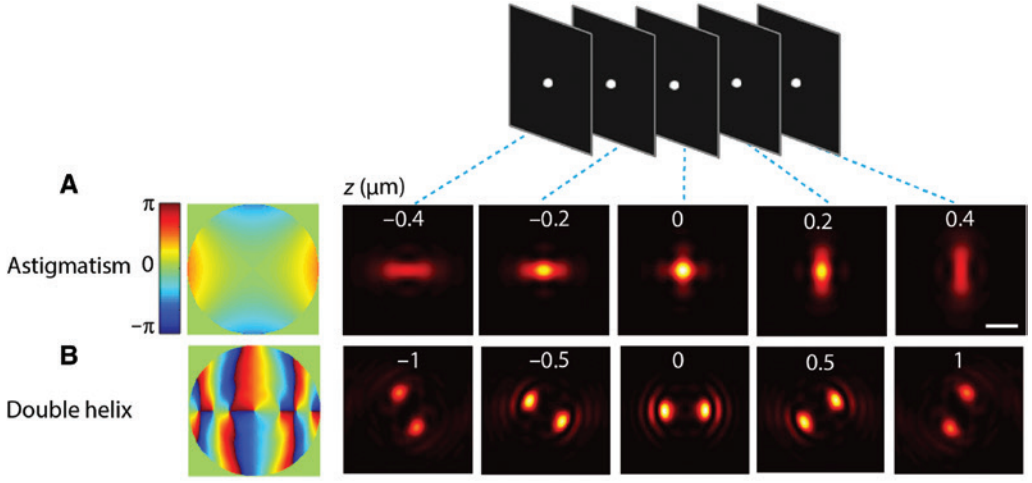

Figure 5: PSF engineering for encoding the fluorescent emitter's depth information.
(A) Astigmatic phase mask on the back focal plane and PSF intensity distribution at the different imaging depths, where the PSF widths along *x* and *y* axes vary as a function of defocusing depth. (B) Double-helix phase mask and PSF intensity distribution, where the angle of two main PSF lobes is characterized as a distinguishable parameter for encoded imaging depths. Scale bar, 1 μm.

lateral direction for the self-bending PSF. The axial position of the emitter is then determined by a calibration curve linked to these parameters, and the lateral position is calculated in the same manner as other localization methods. One should carefully select the proper PSF for their application according to the wide-ranging performance in terms of size and axial range. For instance, the astigmatic PSF has much smaller axial range than that of the DH-PSF (~2–3 μm), and the Tetrapod PSF has even larger range up to 6 μm at the expense of a larger footprint [105].

3 Background suppression for fast and high contrast imaging with epi-illumination

3.1 Widefield and total internal reflection fluorescence (TIRF) microscopy

Epi-illumination widefield fluorescence microscopy has been the most common imaging technique for biological and clinical applications. For example, many super-resolution imaging methods discussed in Section 2 including SIM, parallelized RESOLFT and SMLM indeed use a widefield approach. While for confocal microscopy a collimated beam is tightly focused by an objective (Figure 6A), for widefield microscopy the whole volume of the sample is uniformly illuminated (Figure 6B) by focusing a beam to the center of objective's BFP or pupil plane. This allows fast imaging with relatively gentle illumination. The main drawback of widefield microscopy is the lack of optical sectioning capability. Although its depth of field is <1 μm when a high NA objective is used, the out-of-focus background greatly degrades the SBR of targets of interest. Deconvolution [106] and incoherent structured illumination [107] are efficient means to achieve 3D imaging capability with widefield microscopy, but they are not suitable to study weakly fluorescent samples and thus limitedly used for thin and bright specimens.

A confined illumination near the sample surface can be provided by TIRF microscopy [108]. When the incidence beam reaches to a critical angle, total internal reflection (TIR) occurs at the glass/water interface which generates an evanescent wave that excites fluorophores within the penetration depth of ~100–200 nm. This feature makes TIRF illumination an ideal tool for monitoring cellular dynamics with high contrast at the cell surface [109] and performing single-molecule experiments [110]. For TIR generation, a beam is tightly focused close to the edge of the pupil of a high NA objective (Figure 6C). As the available annulus width for TIR generation at the BFP is relatively small, a light source with high spatial coherence is needed for objective type TIRF imaging. Note that there are other methods to generate TIRF, e.g. prisms [111] or waveguides [112, 113].

TIRF microscopy often suffers from inhomogeneous spatial illumination attributed to two main causes. The first is a speckle fringe produced by interference from the laser light that is scattered or reflected from the sample and optical components. This unevenness can be eliminated

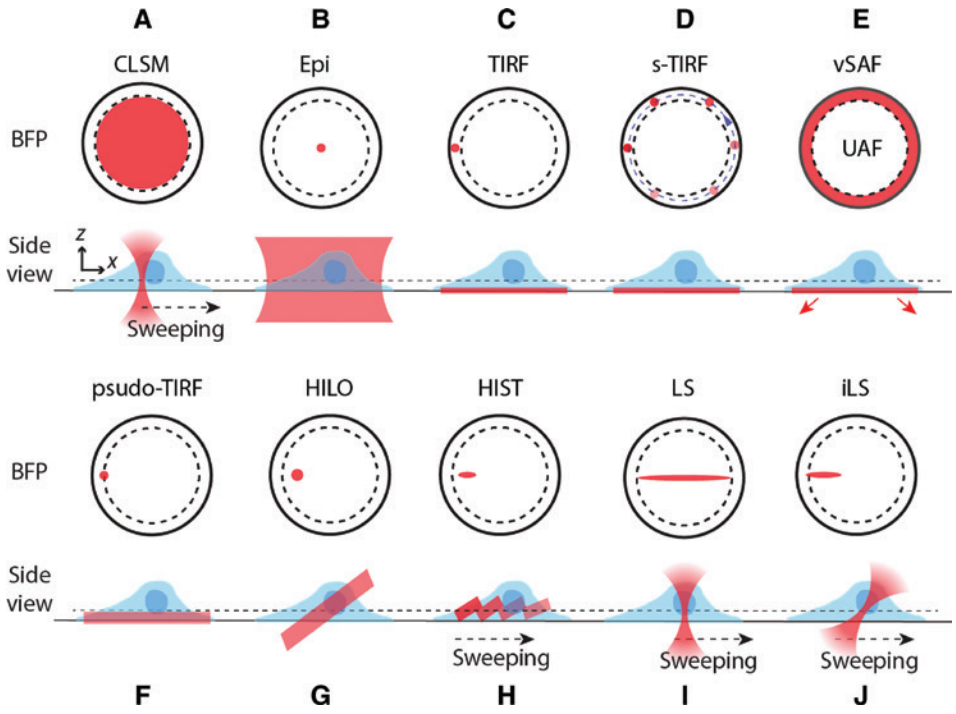

Figure 6: Background suppression schemes for different imaging systems.
(A) CLSM, confocal laser scanning microscopy. (B) Epi, epi-illumination widefield microscopy. A beam focused at the center of BFP excites an entire imaging volume. (C) TIRF, total internal reflection fluorescence microscopy. A beam is focused to the edge of BFP. (D) s-TIRF, spinning TIRF generates uniform illumination by rotating the focused spot. (E) vSAF, virtual supercritical angle fluorescence. TIRF-like imaging is achieved by the subtraction of undercritical angle fluorescence (UAF) from all emission components. (F) Pseudo-TIRF with a deeper penetration depth of ~1 μm. (G) HILO, highly inclined and laminated optical sheet. Inclined illumination with a small FOV is typically used for minimizing out-of-focus background. (H) HIST, highly inclined swept tile microscopy. Inclined tile beam ensures a thinner illumination and larger FOV than HILO. Full FOV imaging is achieved by sweeping the tile together with a confocal slit detection. (I) LS, line-scanning confocal microscopy. Out-of-focus background fluorescence is rejected by a slit. (J) iLS, inclined line-scanning confocal microscopy. Inclined focused line is used for sample illumination with much lower illumination intensity than LS.

by rotating a focused beam along the annulus of the BFP (Figure 6D) [114–116]. In this way, the illumination is time-averaged. The second cause is a Gaussian-shaped beam. Many approaches that improved the illumination flatness in epi-illumination [117, 118] are inadequate to be used in TIRF microscopy because of their poor spatial coherence. This has recently been resolved by refractive optics-based beam shaping tools [119, 120]. This showed significant improvements in quantitative single-molecule analysis [119] and uniform resolution in SMLM with large imaging areas [121].

Another challenge of TIRF is a nonevanescent excitation that comes from scattering of the evanescent wave due to the nonuniform refractive index in cellular environments [122]. To suppress background fluorescence by far-field excitation, it was suggested to monitor supercritical angle fluorescence (SAF) [123] which used the fact that the fluorescence emission near the sample interface preferentially orients to high angles on the BFP [124]. SAF is regarded as a counterpart of TIRF illumination [108]. As the PSF of the SAF is broader laterally, it is undesirable to use it directly. Instead, image subtraction of undercritical light from all the emission components provides high resolution TIRF, so-called virtual SAF microscopy (Figure 6E) [125]. Another approach to obtain clear TIRF images is to apply incoherent structured illumination patterns, yielding widefield microscopy with improved optical sectioning [126].

3.2 Highly inclined illumination

As TIRF microscopy has a limited excitation depth, it is highly desirable to seek alternatives that enable imaging inside cells and tissues with single-molecule sensitivity. When the focused spot under a TIRF condition is slightly shifted at the BFP such that the illumination gets very close to the critical angle, a pseudo TIRF or grazing incidence illumination is generated (Figure 6F) [127, 128]. Its illumination depth (~1 μm) is particularly useful to study structures and dynamics of subcellular organelles that are

positioned near the surface. Basically, this approach utilizes a leaky far-field evanescent wave for excitation. To obtain a deeper imaging depth, for example, to visualize the entire nucleus of mammalian cells, the focused spot is further shifted toward the center of the BFP. In this case, a collimated beam refracted at the glass/water interface is propagated with an inclined angle (θ) with respect to the z axis (Figure 6G). Specially, if the inclined angle is high, for example, $\theta > 75°$, a thin off-axis light sheet is generated, which significantly reduces out-of-focus background. This technique, called highly inclined and laminated optical sheet (HILO) microscopy [129, 130], has been widely used for 3D super-resolution imaging [131] and single-molecule analysis [132]. It is also possible to switch between TIRF and HILO illuminations by rapidly changing the beam position at the BFP [133]. Interestingly, despite its simple working principle, HILO imaging was reported more than two decades after TIRF microscopy was first demonstrated.

One drawback of HILO is that its beam thickness (Δz) is closely related to the imaging FOV. If aberrations are neglected, the beam thickness is roughly calculated as $\Delta z = D/\tan(\theta)$, where D is the beam diameter. It means that a thinner illumination unavoidably results in a smaller illumination area, which greatly limits its applications. Therefore the commonly used HILO beam is relatively thick, about 5–7 μm. This problem has been recently resolved by highly inclined swept tile (HIST) microscopy [134]. An elongated beam prepared by a pair of cylindrical lenses is focused on the BFP of the objective similar to HILO. Then a virtual light sheet is generated by laterally sweeping the tile beam and a confocal slit removes out-of-focus fluorescence (Figure 6H). In this way, HIST decoupled the beam thickness from the FOV and successfully demonstrated large area 3D single-molecule imaging in the presence of high background [134]. Note that an instantaneous illumination intensity of HIST is just 5–10 times higher than TIRF so it can be considered as gentle illumination. It is feasible to further decrease the beam thickness and extend the imaging depth by utilizing adaptive optics [9] and PSF engineering [105], which will enhance contrast and minimize unwanted excitation.

3.3 Parallelized illumination and line-scanning confocal microscopy

Confocal microscopy provides tightly focused illumination and its pinhole effectively removes out-of-focus background [135]. Nevertheless, due to its high illumination intensity, confocal microscopy has been used mostly when the sample is strongly fluorescent and relatively insensitive to photobleaching and photodamage, which depend on the peak excitation intensity given the same light dose [136]. To lower the excitation intensity and speed up the imaging acquisition time, it was proposed to use parallelized illumination instead of single-spot scanning. One approach called spinning disk confocal microscopy generates multifocal spots [137, 138] by a Nipkow-type pinhole array disk and a microlens array [139], and the generated signal is projected onto a camera. Its pattern is similar to multifocal SIM shown in Figure 4E, but has an equal-pitch spiral shape to ensure the beam uniformity. Video-rate imaging is accomplished by illuminating the sample with more than a few hundred foci [140]. Contrary to expectations, the optical sectioning of spinning disk system is substantially degraded by inevitable crosstalk between adjacent pinholes [141] unless multiphoton excitation is applied [142]. This drawback was mitigated by increasing the pitch between foci and decreasing the pinhole size, but this worsened the degree of parallelization [143].

Line illumination is another way to parallelize the excitation beam [144, 145]. A beam focused in one direction is scanned over the sample (Figure 6I), and a slit instead of a pinhole renders moderate optical sectioning, i.e. its out-of-focus background scales with ~$1/z$ as contrasted with ~$1/z^2$ for the single spot scanning confocal microscopy. Because of this, line-scanning (LS) confocal microscopy has not been popularly used in fluorescence imaging. However, a systematic comparison between spinning disk and LS revealed that LS indeed showed higher sensitivity and better depth discrimination (Figure 7) [146, 147].

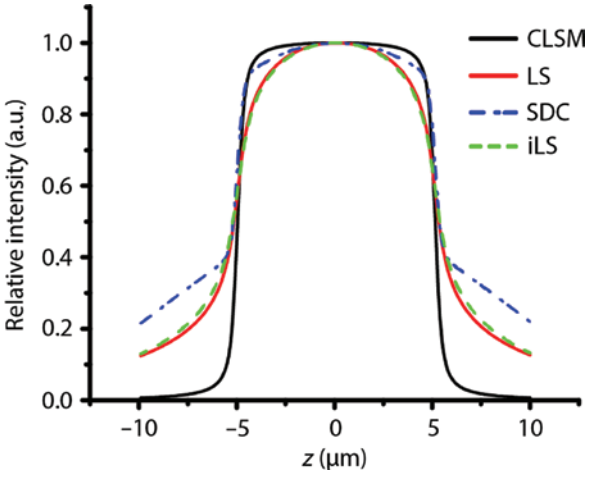

Figure 7: Optical sectioning of confocal microscopy and its variants. z-Responses of a fluorescent sample with 10 μm thickness for point-scanning (CLSM, solid black), line-scanning (LS, solid red), spinning disk (SDC, dashed blue) and inclined line-scanning (iLS, dashed green) confocal microscopy. SDC shows the highest offset background level due to a strong crosstalk between pinholes.

Since then many applications have been demonstrated, including SMLM in cells and thick tissues [148, 149] and fast imaging of large specimens for histopathology [150, 151]. The biggest advantage of LS is its simplicity [152]. Line illumination was also used in real-time two-photon imaging [153].

When the line illumination is titled with respect to the *z*-axis (Figure 6J), the excitation area gets wider, which slightly degrades spatial resolution but results in similar effects as parallelization. This technique showed reduced photobleaching while maintaining a similar optical sectioning to conventional LS [154]. The instantaneous intensity required to maintain the same SBR can be lowered by using multiple inclined beams distantly spaced from each other. This inclined line-scanning confocal microscopy can be regarded as a special case of HIST microscopy with a very thin illumination beam.

4 Fast volumetric imaging

4.1 Extended FOV and high resolution in light-sheet fluorescence microscopy (LSFM)

Confocal microscopy and other epi-illumination approaches provide good sectioning capability; however, they excite fluorophores in unwanted volumes, which leads to unavoidable photobleaching and phototoxicity in living biological samples. LSFM selectively excites the target of interest in the vicinity of imaging plane with an additional orthogonally placed objective lens [155–157], which maximizes the usable excitation photons for 3D time-lapse imaging [158]. Due to its capability of fast, gentle and long-term imaging [157], LSFM has shown remarkable success in diverse areas.

Typically a cylindrical lens was used to shape the Gaussian beam into a light-sheet for the sample excitation (Figure 8A), and the emitted fluorescence was detected by a large FOV camera [157]. To get a more uniform illumination a focused laser beam was digitally scanned to form a time-averaged virtual light-sheet (Figure 8B) [159], but this approach required at least two orders of magnitude higher instantaneous illumination intensity than the former. In LSFM, non-uniformity due to strong absorption and scattering often occurs in the illumination, resulting in image artifacts such as stripes and shadows. This problem was alleviated by multidirectional illumination and reconstruction of these images [160, 161]. Another shortcoming of LSFM is that the available FOV, governed by the Rayleigh length of a propagating Gaussian beam, is coupled with the thickness of the illumination beam, i.e. $y_R = \pi \cdot \omega_0^2/\lambda$, where y_R denotes the Rayleigh length, ω_0 the beam waist and λ the excitation wavelength. Therefore, it is not feasible to conduct high-resolution subcellular imaging across a large FOV with a tightly confined Gaussian beam (Figure 8A).

To overcome this problem an extended and uniform light-sheet was generated by scanning a Bessel beam [162], projected by an annular illumination at objective's BFP [163, 164] using an SLM or an axicon [165, 166] (Figure 8C). The Bessel beam features an invariant transverse profile as well as self-reconstruction that suppresses artifacts from scattering. Nevertheless, its narrow center peak is accompanied by strong side lobes, which generates substantial out-of-focus background [164]. This was reduced by structured illumination, two-photon excitation or a confocal slit [164, 167, 168]. Later, a lattice light-sheet was introduced by dithering a Bessel beam array that was carefully designed to be destructively interfered [169] to effectively suppress the side lobes (Figure 8D) [170]. Due to its thin illumination across large FOV, lattice light-sheet microscopy enables high-resolution long-term live-cell imaging with an extremely low light dose and is regarded as an ideal fluorescence imaging system for many biological studies. Alternatively, it was proposed to use pseudo-nondiffracting beams featuring attenuated side lobes by superimposing two coaxial Bessel beams [171, 172] (Figure 8E) or two cosine-Gauss beams [173, 174] (Figure 8F). However, they showed either a rather limited FOV or a relatively thicker beam as contrasted with the lattice light-sheet. Similarly, an Airy beam [175] was also used for LSFM with a cubic phase mask [176] but it is unlikely to be suitable to high-resolution imaging [158]. The second strategy to attain high contrast LSFM imaging with a large FOV is to sweep or tile Gaussian beams. For example, in axially swept light-sheet microscopy, a light-sheet with a small Rayleigh length was rapidly scanned over large specimens by defocusing the beam in conjunction with a confocal slit for background rejection, resulting in a thin virtual light-sheet [177]. Another approach is to stitch multiple images generated by light-sheets via the Gaussian beam [178].

Many LSFM techniques use virtually generated light-sheets. As discussed earlier, they require much higher peak excitation power. Similar to epi-illumination, parallelization of the beam can lower the peak power without sacrificing imaging speed [179]. Recently, a new approach called field synthesis was demonstrated which can generate any scanned or dithered light sheet [180]. Whereas conventional methods scanned the beam laterally on

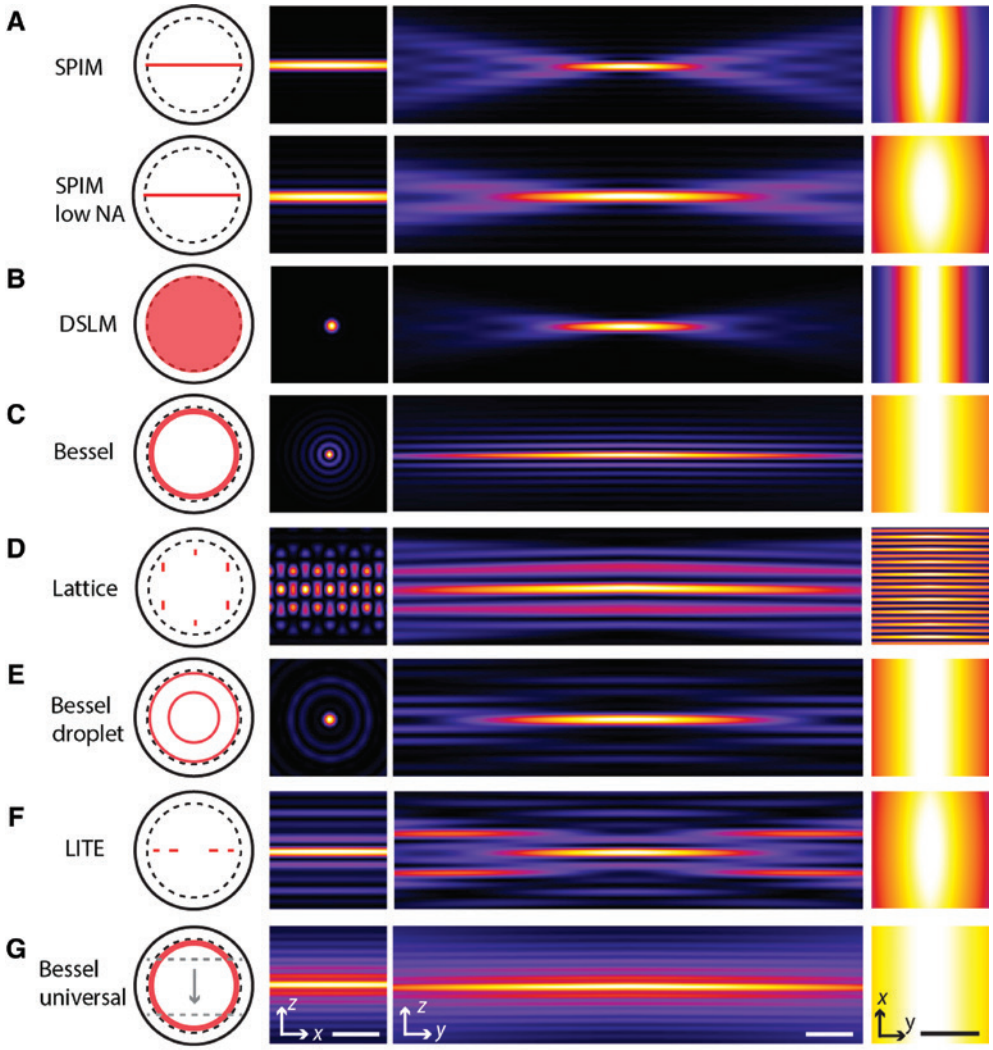

Figure 8: Beam shaping for light-sheet fluorescence microscopy.
(A) SPIM, selective plane illumination microscopy. A thicker beam is required to generate a larger FOV light-sheet with a low NA objective. (B) DSLM, digital scanned laser light-sheet fluorescence microscopy. A pencil-like excitation beam is scanned across FOV. (C) Bessel, Bessel beam light-sheet microcopy for generating thin illumination and large FOV. (D) Lattice, lattice light-sheet microscopy. (E) Bessel droplet, Side lobes of the Bessel beam are minimized by the interference of two beams. (F) LITE, lateral interference tilted excitation. (G) Universal light-sheet generation. Any scanned or dithered light-sheet illumination can be synthesized by scanning the aperture of BFP with a focused line. An example here is a synthesized Bessel beam. Scale bars, 2 μm (*xz* and *yz* planes), 5 μm (*xy* plane).

the sample plane, for field synthesis a focused line was scanned over the objective BFP, which generated instantaneous light-sheet patterns accordingly (Figure 8G). As the illumination profile at any moment covers almost the same excitation area, field synthesis ensured a much lower peak illumination intensity, or in other words, less photobleaching and photodamage to samples [180]. Similarly, it was also reported to use a line Bessel sheet using a slit and annular ring mask [181].

Other illumination schemes can be combined with LSFM to increase spatial resolution, such as SIM [65, 170, 182] and STED/RESOLFT [183, 184]. Adaptive optics has also been shown to be useful for correcting illumination and/or detection to clearly visualize thick specimens [185–187]. On the other hand, the requirement of two closely placed objectives has greatly limited the available NA for both the excitation and fluorescence detection, especially preventing the observation of single-molecules with high SNR [188, 189]. Many approaches have solved this problem by introducing new excitation arrangements [190], for example, the addition of a small reflective device in the sample chamber [191], the design of special sample holders [192–195], or the usage of auxiliary optics [174, 196] for delivering a

thin light-sheet into the sample. Alternatively, single objective-based LSFM has emerged to circumvent this issue. This approach used inclined illumination in a standard microscope, but an additional two objectives were used to relay an intermediate image with minimal spherical aberration and to set the image on the focal plane by tilting the last objective [197, 198]. Recently, the low detection efficiency of single objective LSFM has been significantly improved by extending the collection angle [199, 200].

4.2 Extended imaging depth for *in vivo* imaging

Although LSFM enables fast volumetric imaging with a minimal light dose, it has limitations when studying opaque samples, such as mammalian brains and when interrogating awake mice [201]. In this case, two-photon excitation laser scanning microscopy has provided a solution to suppress the strong scattering and facilitate its implementation to study freely behaving animals [202, 203]. However, as this is still a point scanning technique, various beam shaping approaches have been developed to achieve fast volumetric imaging, particularly toward less or no serial *z*-scanning.

One approach is to use an elongated excitation by generating a Bessel beam and collecting the fluorescence signal from the illuminated volume [204, 205]. As targets of interest in neuroscience are usually sparsely distributed, it is unlikely that each feature is excited by the elongated beam at the same time. Scanning the Bessel beam on the *x*-*y* plane gives a 2D projected image of the 3D sample at a video rate, which makes it unnecessary to obtain multiple *z*-stacks. Instead of the axially extended beam, it is possible to acquire several 2D images simultaneously at different focal planes by generating multiple beamlets at different depths [206]. In this case, slightly different temporal profiles of each beamlet made them distinguishable.

Other approach is to use temporal focusing [207, 208]. When ultrafast pulses broadened by a diffraction grating are imaged on the sample through a lens and objective, two-photon excitation occurs dominantly on the focal plane. This effectively sharpens the excitation beam along the *z*-axis and the depth of field can be tuned irrespective of the lateral PSF by changing the temporal profile of the pulses [207]. In addition, the temporal focusing has shown resistance to scattering in tissues [209, 210]. These great features have been combined with line-illumination [211–213] and wide-field illumination [207, 214] for fast volumetric two-photon imaging. If high spatial resolution is not required, for example, when observing mouse brains with a single neuron resolution, the excitation beam can be shaped to an isotropic PSF with a few microns in size, which was not attainable by a Gaussian beam [215]. This illumination extends the imaging depth and achieves considerable lateral parallelization.

An extended imaging depth can also be achieved by engineering the detection PSF. One simple implementation is to encode the wavefront in the BFP using a cubic phase mask which shows a defocus invariance [216]. The whole volume excited by epi-illumination [217] or light-sheet illumination [218] was projected to a single 2D image by this phase mask. However, the resulting images contained a substantial amount of side lobes, necessitating careful deconvolution and making the images immensely dim. Alternatively, it was proposed to image multiple focal planes in a single exposure of the camera [219]. A distorted diffraction grating [220] was designed to split fluorescence light depending on the degree of defocus. Whereas it enabled one to monitor nine focal planes simultaneously, the divided fluorescence signal lowered the SNR [221].

5 Conclusions

Myriads of new fluorescence imaging techniques have advanced imaging performance in terms of spatial resolution, SNR, 3D contrast, imaging speed and photodamage. Unfortunately, there are no magic bullets in fluorescence microscopy. Each method has its own pros and cons, and thus one needs to choose the proper imaging tool depending on their specific application. Notably, tailored beams have been critical drivers to success and often provided a breakthrough. Tailored beams have gained notable attention and have played important roles in other research areas [222], including material fabrication [223, 224], optical manipulations [225], microfluidics [226], optogenetics [227], etc. We envision that novel structured beams will provide fluorescence imaging with new functions and extend its applications to new research areas.

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