Recent advances in structured illumination microscopy

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Abstract

Structured illumination microscopy (SIM), is a wide-field, minimally-invasive super-resolution optical imaging approach with optical sectioning capability, and it has been extensively applied to many different fields. During the past decades, SIM has been drawing great attention for both the technique development and applications. In this review, firstly, the basic conception, instrumentation, and functionalities of SIM are introduced concisely. Secondly, recent advances in SIM which enhance SIM in different aspects are reviewed. Finally, the variants of SIM are summarized and the outlooks and perspectives of SIM are presented.

1. Introduction

Super-resolution (SR) optical microscopic techniques revolutionize the history of optical microscopy by providing significantly enhanced resolving power surpassing the optical diffraction limit, and hence have been playing a vital role in biomedical and chemical sciences. In 2014, the Nobel Prize in chemistry was awarded to Stefan Hell, Eric Betzig, and William Moerner for the development of SR microscopic techniques, which overcome the classical resolution barrier proposed by Ernst Abbe about 150 years ago \cite{1,2}. Among these SR techniques, stimulated emission depletion microscopy (STED) reduces the size of effective focus (or point spread function (PSF)) of traditional laser scanning confocal microscopy \cite{3,4} by superimposing the excitation focus with an additional doughnut-shaped, red-shifted light, which silences the fluorescence in the periphery of the focus. Another SR technique is single-molecule localization microscopy (SMLM), which provides super-resolved images via precise positioning of individual fluorescent molecules that are lighted up sparsely in a time sequence. The core idea behind SMLM is to chemically or physically govern molecules (fluorescent dyes or proteins) in such a way that in each frame only a small fraction of molecules emit fluorescence signals \cite{5}. The lateral location of these sparsely distributed molecules is then determined by Gaussian peak fitting with high precision to realize SR. To reconstruct an SR image with a typical resolution around tens of nanometers, SMLM always needs to record thousands of images. SMLM can be implemented with organic dyes, e.g. photoswitchable Cy3–Cy5 dye pairs in stochastic optical reconstruction microscopy (STORM) \cite{6} and direct STORM \cite{7,8}. Meanwhile, analogous methods have been proposed and implemented by using fluorescent proteins to achieve the lateral resolution of ~20 nm. For instance, photoconvertible fluorescent protein named EosFP in photoactivated localization microscopy (PALM) \cite{9} and photoactivatable green fluorescent protein in fluorescence PALM \cite{10} have been explored. Theoretically, both STED and SMLM have an unlimited resolution, even to the atom scale. However, in practice, the optical aberration, background noise, autofluorescence, as well as the brightness, photo-stability, and labeling density of fluorophores used, often reduce the signal-to-noise ratio (SNR) of STED and SMLM, and hence restrict the achievable resolution of both.

Structured illumination microscopy (SIM) is an alternative SR optical microscopic approach, which has been more extensively used in biomedical research subsequently. In SIM, the fringe patterns of different
orientations and phase-shifts are used to illuminate samples, and the moiré patterns generated are recorded in sequence. SR images can be reconstructed from these intensity patterns. Compared with STED and SMLM, SIM has several excellent characteristics, such as simple sample-preparation, fast imaging speed, and minimally-invasive. Along with the development of the electron devices, like detector and wave-front modulator, SIM has undergone a vigorous development, and a variety of extensions in both conception and implementations have been reported [11–18].

In this review, we focus on recent advances in SIM, which includes but is not limited to, multi-color SIM, 3D-SIM, total internal reflection SIM (TIRF-SIM), grazing-illumination SIM (GI-SIM), phase-type SIM, and spot-scanning SIM, etc. The review will also briefly introduce the fundamental principles, instrumentation, and functionalities of SIM. This review will serve broad readers (both optical and biological orientated readers) as a roadmap for the development of SIM technology.

2. Basics of SIM

The basic schematic diagram of SIM is shown in figure 1(A). A grating controlled by a precision displacement stage is projected to the sample through the telescope systems (L₁–L₃ and L₅–L₆). In the middle focal plane of L₂, the spectrum of the illumination beam is filtered so that only the ±1st orders can be passed, generating sinusoidal stripes at the sample plane. When a sample is illuminated by such structured illumination, a moiré pattern (figure 1(B)) is generated. The moiré pattern is then imaged by two telescope systems L₆–L₅ and L₃–L₄ to a charge coupled device (CCD). Upon the grating being rotated and laterally shifted along its diffraction direction, the moiré patterns are recorded by the CCD. Utilizing similar structured illuminations, SR and optical sectioning (OS) can be achieved.

The SR capability of SIM originates from the moiré effect, which downshifts the high-frequency information of a sample into the supporting area of the system's optical transfer function (OTF), which is always missed in conventional fluorescence microscopy. As a consequence, the spatial resolution can be effectively enhanced up to twofold even more, as shown in figure 1(C). With SR SIM, one could observe finer structures (smaller than the diffraction limit) of samples, revealing more valuable structural information. While, OS generally benefits from an incoherent illumination source, of which the stripe's contrast is only preserved in a narrow section near the in-focus plane. Through a simple recovery phase-shifting operation (e.g. using equation (1)) the defocus light can be easily suppressed, yielding a sectioned image, as shown in figure 1(D). Eventually, a 3D sectioned image can be obtained by translating the sample along the axial direction and calculating sectioned image for each axial position.

Compared with other SR techniques, such as localization microscopy and STED, SIM has a few merits. The first one is its high imaging speed due to the nature of wide-field imaging. Hence, SIM is suitable for capturing the fast dynamics in live samples. The second merit of SIM is that it can obtain the super-resolved information of samples prepared for conventional fluorescence microscopy, i.e. no need for a complicated/dedicated sample preparation process. The third merit of SIM lies in its low photo-toxicity to bio-samples, which enables imaging live samples for a longer time. Considering several raw images are needed to reconstruct one super-resolved SIM image, SIM is still limited to samples with slower movements.

2.1. OS SIM

When imaging a thick sample, the signal from the out-of-focus region often induces a blurring to the sharp image captured from the in-focus plane. As a result, the spatial resolution and the image contrast are reduced accordingly. Several OS methods have been proposed to suppress such blurring. Confocal laser scanning microscopy (CLSM) rejects the out-of-focus signal (blurring) by scanning a focused light across a sample and filtering the excited signal with a pinhole, providing an axial resolution of about 600 nm [23]. Alternatively, standing wave fluorescence microscopy (SWFM), such as 4π microscopy, was then introduced, possessing a higher OS capability of about 50 nm and at the same time remedying the temporal resolution [24]. This technique effectively opens the door for 3D imaging of thick samples using standing-wave illumination. The complexity of the system, however, limits its practical application in industrial inspection and biomedical research. Meanwhile, due to the point-by-point scanning scheme, both the SWFM and CLSM have a lower imaging speed.

SIM possesses the OS ability, notably fast imaging speed and simple system [25]. The basic idea of SIM lies in the fact that the structured stripes have only high contrast in the regions near the focal plane, while the structured illumination tends to become a uniform illumination like Kohler illumination in the region far away from the in-focus plane. In short, the out-of-focus region is not modulated by the structured illumination patterns. Therefore, the sectioned images can be obtained by modulating and demodulating the in-focus region with three structured illuminations having phase shifts of 0, 2π/3 and 4π/3. Then, the optically sectioned image can be reconstructed by
Figure 1. The principle, instrumentation, and imaging results of SIM. (A) Basic schematic diagram of SIM [19]. DM, dichroic mirror; L1−L6, lenses; QWP, quarter-wave plate. (B) The moiré pattern generated by projecting a periodic pattern on a sample with dense structures [20]. (C) The images of microtubule cytoskeleton in HeLa cells obtained by using conventional fluorescence microscopy (top) and SR SIM (bottom) [21]. (D) The images of a pollen grain obtained by using conventional bright-field microscopy (top) and OS SIM (bottom) [22].

\[ I_{sec} = \sqrt{(I_0 - I_1)^2 + (I_1 - I_2)^2 + (I_0 - I_2)^2}, \]  

where \( I_0, I_1 \) and \( I_2 \) represent the intensity distributions captured on the image plane under the structured illumination having relative phase shifts of 0, \( 2\pi/3 \) and \( 4\pi/3 \), respectively. The operation in equation (1) rejects the contribution from the unmodulated component from the out-of-focus regions, as shown in figure 2. It is worth mentioning that OS SIM was originally developed for bright field microscopy with partially coherent illumination [25], and was then extended to fluorescence microscopy, providing an OS thickness of \( \sim 400 \) nm or even less [26–28].

2.2. SR SIM

Apart from the OS capability, SIM enables providing super-resolved images. The initial utilization of structured illumination in optical microscopy for resolution enhancement can trace back to 1966 [29, 30].
that time, Lukosz proposed and implemented coherent scattering imaging, in which several coherent waves illuminated the sample simultaneously along different incident angles, and resolution enhancement was achieved via a synthetic aperture process. However, in theory, the spatial resolution achieved cannot surpass the diffraction limit \[31\].

Extending the lateral resolution beyond the diffraction limit using structured illumination was implemented in fluorescence microscopy successively by Heintzmann in 1999 and Gustafsson in 2000 \[12, 13\], in which the samples were illuminated successively by a series of patterns projected by a movable diffraction grating through optics. Based on the moiré effect between the excited stripe and sample’s structural distribution, the frequency information of the sample, which is beyond the support of the OTF of the fluorescence microscopy, can be down-shifted and passed through the imaging system. In the linear region, the fluorescence emission signal generated is linear to the excitation intensity, and the fluorescence distribution transmitted by the detection path onto the image plane can be written as

\[
D(r) = [(I(r) \otimes h_{\text{illu}}(r)) \cdot S(r)] \otimes h_{\text{det}}(r),
\]

(2)

where \(r = (x, y, z)\) is the Cartesian coordinates in sample space, \(D(r)\) represents the intensity distribution on the image plane under structured illumination, \(I(r)\) represents the intensity of the structured illumination on sample plane, \(S(r)\) represents the structural information of the sample with full-spectrum information, \(\otimes\) represents the convolution operation, \(h_{\text{det}}(r)\) represents the PSF of the detection path which determines the supporting region of the system’s spectra, and \(h_{\text{illu}}(r)\) represents the PSF of the illumination path that limits the maximal frequency of the structured illumination that can be propagated on the sample plane. Generally, \(h_{\text{det}}(r) = h_{\text{illu}}(r)\) when the same objective is used for both illumination and detection. For simplicity, the structured stripe can be expressed as

\[
I(r) = I_0 \cdot \{1 + \mu \cdot \cos(2\pi k_g \cdot r + \theta_0)\},
\]

(3)

where \(I_0\) is the intensity value of the uniform zero-frequency component, \(\mu\) is the modulation depth or the contrast of the excited stripe, \(k_g\) is the lateral frequency vector of the illumination stripe whose value is always limited to the maximal value of \(2\text{NA}_{\text{illu}}/\lambda_{\text{ex}}\) (\(\text{NA}_{\text{illu}}\) is the numerical aperture (NA) of the illumination objective and \(\lambda_{\text{ex}}\) is the wavelength of the excitation light) and \(\theta_0\) is the initial phase shift of the structured illumination. Substituting equation (3) into equation (2) and performing the Fourier transform operation on both sides we can obtain
Figure 3. Frequency spectrum extension and SR imaging of SIM. (A) Spectrum supporting region of the traditional fluorescence microscopy whose cut-off frequency is determined by $k_0 = 2NA_{\text{det}}/\lambda_{\text{em}}$. (B) The grating vector $k_g = 2NA_{\text{illu}}/\lambda_{\text{ex}}$ of the structured illumination. (C) Extended spectrum along the grating vector direction $k_g$ shown in (B). (D) Extended spectrum after assembling the spectrum carried by the structured illumination with different rotations. (E) Comparison of the images obtained by traditional fluorescence microscopy and SIM (image courtesy of Talley Lambert, Harvard Medical School, referred to https://sim.hms.harvard.edu/).

$$
\tilde{D}(k) = I_0 \cdot \left\{ \frac{m}{2} e^{j\theta_0} \tilde{S}(k-k_g) + \frac{m}{2} e^{-j\theta_0} \tilde{S}(k+k_g) \right\} \cdot H_{\text{det}}(k),
$$

where the symbol $\sim$ represents the Fourier transform operation and $H_{\text{det}}(k)$ is the OTF of the detection path. Clearly, the first term in the right of equation (4) corresponds to the spectrum along with the uniform illumination in traditional fluorescence microscopy, and it has a lateral resolution of $\sim2NA_{\text{det}}/\lambda_{\text{em}}$. Here, $NA_{\text{det}}$ is the NA of the detection objective and $\lambda_{\text{em}}$ is the wavelength of the emission light. The second and third terms are the super-resolved spectra, whose frequency spectrums have been shifted by the structured illumination for the values of $-k_g$ and $k_g$. These three frequency components can be solved out by translating the structured illumination along the grating vector direction, resulting in the phase shifts $\theta_0$, $\theta_0 + 2\pi/3$, $\theta_0 + 4\pi/3$. The components $\tilde{S}(k-k_g) \cdot H_{\text{det}}(k)$ and $\tilde{S}(k+k_g) \cdot H_{\text{det}}(k)$ can be resolved and then shifted to their original positions. After taking the same procedure for the structured illumination with different orientations (having an interval of 120°), a synthesized spectrum is generated, as shown in figure 3. After an inverse Fourier transform, a super-resolved SIM image with the resolution twice that of traditional fluorescence microscopy can be obtained. It is worthy to notice that, many factors, such as the stripe contrast, the direction, and the phase shift values of the structured illumination, should be accurately estimated to realize a reasonable SR reconstruction with minimal artifacts. Cross-correlation and iterative minimization algorithms were proposed to resolve these parameters required for high-quality reconstruction [32]. Meanwhile, the joint reconstruction strategy was proposed, such as, total variation based algorithm [33–38]. Alternatively, Chu et al proposed an image processing technique to deal with the raw images with low SNR and to reduce the photobleaching and phototoxicity induced by the illumination light [37]. With this method, the temporal resolution of 2D SIM has been successfully improved by 15 times while keeping the spatial resolution and image quality comparable to the conventional methods. Chen et al subsequently developed a novel deconvolution algorithm for 2D SIM using Hessian matrices to improve the spatiotemporal resolution to 88 nm under an imaging frequency of 188 Hz by reusing the raw data [38, 39]. Armed with these powerful reconstruction methods, 2D SIM with minimal invasion and destruction will fulfill its potential in long-term live-cell imaging.

It is worthy to point out that SR and OS can be performed in the same SIM setup. The difference for implementation of SR and OS is that the SR-SIM adopts much higher frequency fringe patterns and multiple orientations of illumination, while the OS-SIM just needs one-directional fringe illumination. The image reconstruction methods for SR-SIM and OS-SIM are quite different. The data processing of SR-SIM is conducted in the frequency domain and needs Fourier transform operations, while the image recovery of OS-SIM needs to be performed only in real space with a simple formula (equation (1)).
3. Extensions of SIM

Despite being already powerful, conventional 2D SIM needs to be extended to address the enhanced requirement of bio-imaging. Firstly, it lacks the ability to image thicker samples or deep inside live tissues due to the missing cone problem and the strong scattering of the sample. Secondly, SIM with one excitation/detection channel is not sufficient to capture the complex biological dynamics inside live cells, since there are more organelles inside a cell, and even many different components in one organelle, involved in one life event under investigation. Thirdly, beyond the 2D SR along lateral orientation, resolution enhancement in the axial direction is also necessary to detect the finer structures like proteins. As a consequence, further improvements to 2D SIM are needed to render it better applied in more research fields.

3.1. TIRF-SIM

Similar to traditional fluorescence microscopy, conventional 2D SIM has a missing cone near the zero-frequency region that is the primary cause of lacking the OS ability. In other words, a certain axial resolution improvement can be achieved by filling the missing cone by lowering the frequency of the structured illumination strips. However, the lateral resolution enhancement will be decreased as a compromise. To circumvent this dilemma, total internal reflection based SIM (termed as TIRF-SIM) was proposed [40, 41]. The scheme of TIRF limits the excitation within a thin layer (∼100 nm thickness) above the coverslip. Or more specifically, the evanescent field generated when the illumination light transmits from an optically denser medium to an optically thinner medium at an angle larger than the critical angle of TIRF, only excites the region within about 100 nm depth of sample from the bottom surface [42, 43]. It is worthy to mention that TIRF-SIM has an excellent OS capability, while the stripe contrast in TIRF-SIM is often lower than the conventional 2D SIM, which in turn reduces the SNR of SIM imaging. Furthermore, the shallow imaging depth limits the detection range of TIRF-SIM to structures around the cell membrane that cannot reflect the real situation of the biological dynamic process in cells. In order to extend the detection range of TIRF-SIM, Jonathon et al proposed high-speed SIM with grazing incidence illumination (GI-SIM), resulting in an illumination depth comparable to the depth-of-field of the detection objective. Using GI-SIM, they have successfully captured the fast dynamics of organelles inside the live cells with the lateral resolution of ∼97 nm, the imaging depth of ∼1 μm above the sample surface, and the imaging speed of ∼266 frames per second with the help of interleaved reconstruction algorithm [39, 44, 45]. The comparison of the performance among TIRF-SIM, GI, and GI-SIM is shown in figure 4. The results show that with GI-SIM one can capture more information from an extended section.

3.2. Three-dimensional SIM based on multi-beam interference

As we know, the inner structures and their motions inside cells are always in 3D by nature, and therefore, it is difficult to provide a complete view of complex cellular systems with 2D SIM that lacks the axial discernibility ability. Consequently, it is of great importance for biology to develop SIM with 3D SR imaging capability. Aiming for this, several methods have been implemented. Firstly, standing-wave excitation significantly improves the axial resolution to ∼50 nm [24, 46], while the lateral resolution still stays at the diffraction-limit level. Secondly, two-beam interference was proposed to compromise the resolution enhancement and the OS ability. As an advance, three-beam SIM has been proposed, which has the same lateral support with the two-beam illumination case, but it fills the missing cone and has doubled the axial support [21, 47, 48]. The resulting high axial resolution and OS make three-beam SIM the method of choice for three-dimensional SR imaging, as shown in figure 5. Despite the fact that the axial resolution can be improved using three-beam interference compared to 2D SIM, the axial resolution is still much lower than, or at best half that of the lateral resolution. Such anisotropic resolution is highly detrimental for the accurate quantification of object sizes, shapes, volumes, and curvatures [49]. With the help of a mirror reflecting the central beam back to the space where the interference of three beams occurs, four-beam SIM generates more modulation points along the axial direction and extends the content of the final synthetic OTF impressively. With this method Manton et al improved the resolution from 200 nm × 200 nm × 545 nm to 135 nm × 135 nm × 135 nm [49]. PS technique [50–54], which is similar to 4π microscopy using two opposing objectives, can further push the imaging performance of SIM to an unprecedented level, yielding an isotropic resolution of 90 nm [50, 55]. Despite this magnificent feat, utilization of this kind of imaging technique has always been extremely limited due to the experimental difficulties in constructing and operating such a system, especially the strict requirement for optical alignment and the narrow environment for sample installation. The comparison among several three-dimensional SIM can be referred to [49], and the selection of SIM needs to be based on the actual application situation.
Figure 4. Comparison of performance among TIRF-SIM, wide-field illumination, grazing incidence illumination (GI), and GI-SIM. GI has the imaging depth of ~1 µm away from the sample surface, while TIRF has an imaging depth ~100 nm. GI-SIM can gain more information from an extended imaging depth [45]. (A) Difference of illuminations and imaging performance among TIRF-SIM, wide-field microscopy, and GI-SIM. (B) Combining GI with SIM powerfully enhances the resolution and contrast of the image.

Figure 5. Comparison of wide-field fluorescence microscopy and 3D SIM; (A) uniform illumination in wide-field fluorescence microscopy and the corresponding OTF. (B) Structured illumination under two-beam interference and the corresponding OTF with lateral resolution enhanced. (C) Structured illumination under three-beam interference and the corresponding OTF with lateral and axial resolution enhanced. (D) 3D imaging performance comparison between wide-field fluorescence microscopy and 3D SIM in imaging the panoramic information of the samples. Image stacks were acquired with a DeltaVision OMX prototype system (applied precision) [56].
3.3. Multi-color SIM
There are multiple organelles in one cell, and even multiple components in one organelle, are involved in one life event under investigation. Therefore, it is essential to develop multi-color SIM that has different excitation/detection channels to image different structures inside cells, separately or simultaneously [57]. Multi-color SIM was developed by equipping conventional SIM with two or three excitation/detection channels. With this method, three-color imaging of distinct mitochondrial regions, mitochondrial outer and inter membranes, as well as mitochondrial matrix, in living cells was demonstrated for the first time [58]. Later, Andreas et al. developed a video-rate, multi-color SIM with ~250 ms time resolution by incorporating multi-color SIM with a GPU-enhanced reconstruction software [59]. Moreover, Guo et al [45] proposed multi-color grazing incidence illumination (multi-color GI-SIM), and utilized it to investigate several important biological phenomena, such as ER-branching events, as shown in figure 4(B). This investigation found that tubular ER plays a vital role in mitochondrial fusion and fission, transporting and controlling the local concentration of intracellular organelles, like late endosomes or lysosomes. However, till now it is challenging to image four or more different structures at the same time due to the interaction or emission bleed-through among the fluorescent markers, the influence of the complex imaging environment on multi-color data acquisition, and the laborious coordination of optical filters.

3.4. Phase-type SIM
Conventional SIM is often used to observe fluorescent samples with high image contrast and functional specificity with a resolution beyond the diffraction limit, and it has been witnessed to be a pragmatic tool for capturing dynamics in live samples [13, 60]. Recently, structured illumination has also been extended to label-free quantitative phase microscopy to image transparent samples in their natural state with resolution enhancement [61–64]. Structured illumination has been applied in imaging coherently scattering samples over the past few years [65–73]. Gao et al. incorporated structured illumination into digital holography microscopy (DHM), which is entitled SI-DHM, to image transparent samples with improved spatial resolution and auto-focusing capability [74], as shown in figure 6. In SI-DHM, four groups of binary phase gratings rotated by $m \times 45^\circ$ generated by a spatial light modulator are projected onto the sample for illumination. For each rotation the structured illumination is shifted three times, generating phase shifts of 0, $2\pi/3$, and $4\pi/3$. Under the illumination of such sinusoidal fringe patterns, the object wave is generated and interferes with a reference wave. The resolution-enhanced amplitude and phase images can be obtained by reconstructing the complex amplitude of the object waves along different diffraction orders of the structured illuminations with different orientations and synthesizing their spectrum in the frequency domain.

Despite there are some similarities between phase-type SIM and SIM reconstruction, there are also some differences between the two. At first, in SIM the intensity distribution of the sample is linearly modulated by the stripes, rendering their spectrum shifted to improve the spatial resolution. Nevertheless, in phase-type SIM, the complex transmittance of the sample, or the amplitude of the light field, is linearly modulated by the stripe and the complex amplitudes of the object waves are manipulated throughout the reconstruction as the basics. Secondly, the structured illumination used in DHM is essentially the simultaneous illumination with two oblique plane waves at two oppositely tilted angles [31]. It means that this method cannot provide SR information, i.e. beyond the diffraction limit ($\sim \lambda/2$). On the contrary, conventional SIM that utilizes the moiré effect to extend the support of OTF can achieve two-fold resolution enhancement above the diffraction-limit resolution ($\sim \lambda/2$), and even higher when employing nonlinear SIM (NL-SIM) schemes.

3.5. Nonlinear SIM
As mentioned before, the higher frequency of the structured illumination has, the higher resolution enhancement can be achieved in SIM. However, the highest frequency of the structured illumination is often limited by the NA of the illumination objective. Therefore, it turns out two-fold resolution enhancement, when the illumination objective is the same as the imaging objective. A unique approach to achieving much higher resolution enhancement in SIM (e.g. beyond the factor of 2) is to utilize the nonlinear response of fluorescent emission to excitation intensity.

3.5.1. Nonlinear SIM based on saturated excitation
The simplest and easiest way to generate a non-linear relation between the excitation and the emission of fluorescent markers is to use saturated excitation. It was originally theorized in 2002 in nonlinear patterned excitation microscopy that the spatial resolution can be improved unboundedly by saturated excitation to fluorophore [75]. After that, nonlinear saturated SIM (SSIM) was experimentally implemented by Gustafsson in 2005 to achieve a 2D spatial resolution of $\sim 50$ nm [60]. The saturated excitation is always realized by applying a sinusoidal illumination with very high intensity. This wildish illumination approach is controversial due to the existence of unwanted effects, such as severe phototoxicity and photobleaching.
Figure 6. Imaging result of phase-type SIM [74]. (A) Recorded off-axis hologram. The recovered phase images upon plane wave (B) and structured illumination (C), respectively. (D) Curves along the red lines in (B) and (C) indicate the resolution enhancement using structured illumination.

influencing the normal status of biological samples [60]. Another weakness of this direct saturated illumination is the low SNR as the majority of fluorescent molecules emit photons and the intensity becomes almost uniform under such intense illumination, while only a few keep in dark state. This results in an image with low signal to background ratio, and it becomes the obstacle to achieve theoretically infinite resolution.

3.5.2. Nonlinear SIM based on photoswitchable protein
Another effective method to extend the spatial resolution of SIM by nonlinear effect is utilizing photoswitchable fluorescent dyes or proteins, of which the saturated excitation turns their fluorescent state from on to off [19, 76]. With such photoswitchable protein based nonlinear SIM, photo-bleaching can be significantly reduced and SNR can be enhanced since the majority of fluorophores stay in their off-state. Furthermore, the illumination intensity required here is at least six orders of magnitude lower than that needed for SSIM [19]. Rego et al have experimentally achieved approximately 50 nm lateral resolution when imaging purified microtubules in fixed CHO cells using such nonlinear SIM based on the photoswitchable fluorescent protein called Dronpa, as shown in figure 7. Li et al [76] applied photoswitchable nonlinear SIM to capture dynamics inside live cells, achieving a lateral resolution of ~60 nm and a temporal resolution of ~40 frames per second. It is worthy to point out that this method relies on photoswitchable fluorescent proteins with extremely high stability, which should support at least hundreds repeated circulation between the fluorescent and non-fluorescent states.

3.5.3. Surface plasmon-resonance enhanced SIM
The third nonlinear SIM is a novel combination between surface plasmon polaritons (SPPs) and SIM, termed as PSIM [77]. The overall structure of PSIM is built on a traditional TIRF microscopy, but it requires a metal-coated coverslip to support samples on the top. When an electromagnetic wave travels along the metallic surface in the form of SPPs, their intensity decays exponentially with the penetration depth. Therefore, SPPs will be inherently confined to a layer of tens of nanometers above the substrate surface,
Figure 7. Nonlinear SIM based on photoswitchable protein and its application in imaging filament structures in actin network. Frequency spectrum evolution in linear SIM (A)–(C) and nonlinear SIM based on photoswitchable protein (D) and (E). The later one has more high-frequency components, greatly enhancing the lateral resolution to $\sim 50$ nm. (F) Comparison among traditional TIRF microscopy, linear TIRF-SIM, and nonlinear TIRF-SIM based on photoswitchable protein. (G) Characteristic of the stripes based on photoswitchable fluorescent protein. (H) Curves between the white triangles in (F) show that two filaments can be clearly distinguished using nonlinear TIRF-SIM based on photoswitchable protein while they are failed with the other two methods [19].

efficiently suppressing the background fluorescence, especially that from out-of-focus regions, compared to traditional two-beam interference SIM [77–80]. Besides the axial confinement, another merit of PSIM is the capability of enhancing the spatial resolution beyond two folds. This is due to the fact that the effective wave-number $k_{SPP}$ of SPPs is larger than that of the excitation light, generating finer stripes with the spatial frequency far beyond the diffraction limit.

3.5.4. Full-field STED-SIM

On the other hand, STED based nonlinear SIM can also enhance the spatial resolution above two folds. Utilizing four-beam interference, a patterned STED beam was generated and superimposed with a homogeneous excitation light, as is shown in figure 8(A). The STED pattern depletes (via a stimulated emission depletion process) the fluorescence excited by the excitation light, leaving only the fluorescence in the dark region of the STED pattern that forms a nonlinear structured light. In order to generate a nonlinear structured illumination with higher harmonic amplitude, a STED beam with considerably high power is needed. To circumvent this nearly-impractical requirement, surface plasmon resonance-enhanced STED-SIM was proposed, which provides a lateral resolution about 30 nm and an acquisition time of sub-second [81]. Benefit with the enhanced depletion efficiency by the surface plasmon, the power of STED required has been reduced significantly, as well as the phototoxicity. Surface plasmon resonance-enhanced STED-SIM has been applied to observe the biological processes in vitro or on the basal membrane of live cells due to its high spatiotemporal resolution. However, surface plasmon assisted STED-SIM is still limited to two-dimensional imaging as the light is bounded to the surface between metal and air.

4. Spot-scanning SIM

Conventional SIM techniques are wide-field imaging techniques, and therefore, they have the advantages of fast speed and low phototoxicity. However, they are often limited to thin samples, i.e. no thicker than 20 $\mu$m,
due to the contrast of the wide-field illumination fringes decays fast with the imaging depth, especially when imaging scattering tissues [82].

4.1. Basic idea of spot-scanning SIM
Recently, spot-scanning SIM was proposed to image thick and scattering samples, e.g. live tissues. The concept of spot-scanning SIM can date back to 30 years ago, but the experimental implementations are more recent [11, 82, 83]. In spot-scanning SIM, the structured illumination pattern is often generated by spatially scanning a focused light, while a 2D CCD/sCMOS camera is used to record the image of the sample under such illumination. Considering the fringes are generated with the focused light that has a much higher penetration depth compared with a wide-field illumination, spot-scanning SIM can achieve an imaging depth of ~500 µm. Notably, when combined with multi-photon excitation, spot-scanning SIM can achieve even larger imaging depth, better OS, and much cleaner background. For instance, Yeh et al [84] successfully integrated spot-scanning SIM with two-photon excitation to gain the lateral resolution improvement of about 1.4 times compared to traditional two-photon fluorescence microscopy while possessing the ability of imaging thick samples with suppressed out-of-focus background. And then Urben et al proposed resonant two-photon SR patterned excitation microscopy (2P-SuPER), as shown in figure 9(A), and investigated the nanoscopic neuronal architecture in the cerebral cortex of the mouse brain with a resolution of 119 nm at a depth of 120 µm in vivo and 210 µm ex vivo, as shown in figure 9(B) [85], creating a platform for investigating nanoscopic neuronal dynamics.

4.2. Extensions of spot-scanning SIM
Another option to enhance the imaging depth of SIM is to combine CLSM with structured illumination (entitled confocal SR-SIM). Confocal SR-SIM can effectively improve the spatial resolution of CLSM while retaining its ability to reject the blurring signal. In confocal SR-SIM, a spatial light modulator is used to modulate the focus into the fringe patterns of different orientations and phase shifts. When a sample is scanned with these patterns in sequence, an SR image (with 1.8 times lateral and 1.7 times axial resolution improvement) can be synthesized by processing the obtained images [86]. Furthermore, this method has an enhanced imaging depth and improved SNR, and therefore, it is very beneficial for the biopsy of tissues. However, a sample under investigation needs to be scanned at least six times (with different focus profiles) to produce a final super-resolved image. Therefore, this method is not suitable for dynamic samples.

The emergence of multifocal structured illumination microscopy (MSIM) addresses the deficiency of low imaging speed in confocal SR-SIM [87, 88]. MSIM integrates a digital micromirror device (DMD) into a conventional wide-field microscope to generate sparse 2D multifocal patterns. After recording and processing the images under different illumination patterns, optically sectioned super-resolved images with 145 nm lateral and 400 nm axial resolution at 1 Hz frame rates can be obtained. In MSIM, sparse multifocal illumination patterns reject the out-of-focus light via the digital pinholes (similar to the physical pinhole in confocal microscopy), thus allowing live SR imaging at a depth of 50 µm from the coverslip surface [87]. Despite MSIM having several excellent features, the OS capability has not been maximized due to the diffraction size of the patterned spots. The combination of two-photon excitation with MSIM (termed as
two-photon MSIM) not only improves the spatial resolution compared with conventional MSIM but also further improves the OS, making MSIM more perfect [89].

To further enhance the temporal resolution, instant SIM was then proposed, synthetically improving the imaging performance with a lateral resolution of 145 nm and an axial resolution of 350 nm at acquisition speed up to 100 Hz [90]. This technology ingeniously uses two matched converging microlens arrays and a pinhole array to generate the multifocal patterns for exciting deep structures and rejecting the out-of-focus emissions. In addition, a galvanometric mirror is used to translate the excitation pattern and sum the fluorescence emissions during each camera exposure, producing an SR reconstruction. There are two main differences between instant SIM and two-photon MSIM; one is that the summing of emissions under multiple multifocal patterns is done optically for the former while with software for the latter, improving the imaging speed and reducing the contribution of camera noise using the former. The other difference is that the OS is achieved by optical filtering in instant SIM while reducing the excitation volume in two-photon MSIM.

Instant SIM can achieve an imaging depth comparable to spinning-disk confocal microscopy while inferior to point- or line-scanning confocal microscopy whose imaging depth is not enough for deep tissue imaging. Further, two-photon instant SIM was then developed that also performs optical rather than digital processing to permit the SR imaging with no additional cost in acquisition time or phototoxicity relative to the two-photon spot-scanning SIM. Two-photon instant SIM combines two-photon excitation with SIM to realize the three-dimensional interrogation of live organisms at a depth exceeding 100 µm from the coverslip surface, rendering whole nematode embryos and larvae, and tissues and organs inside zebrafish embryos [91].

Besides these techniques, there are several others to realize the purpose of resolution enhancement using scanned structured illumination. The ability of light-sheet microscopy to image the deep tissues is further improved by combining digital scanned laser light-sheet, i.e. structured illumination [92–94], as shown in figure 10. The blurred specimen-related signals are effectively rejected using the idea in [25] to improve the image contrast of in-focus structures. Using these methods, long-term imaging of zebrafish development for

![Figure 9. Setup for two-photon superresolution patterned excitation reconstruction microscopy (2P-SuPER) (A) and experimental comparison between two-photon laser scanning microscopy (2P-LSM) and 2P-SuPER (B) [85].](image)
58 h and fast multiple-view imaging of early drosophila melanogaster development have been implemented. Further, the structured illumination can also be used for enhancing the performance of the spectral measurement. Raman SIM achieves a well-balanced performance in spatial and spectral resolution with the capability of OS and spectral analysis, generating highly detailed spatial contrast from the ensemble of molecular information. This technique is expected to contribute to a greater understanding of chemical component distributions in organic and inorganic materials.

5. Discussion and outlook

SIM is a high-speed, wide-field, minimally-invasive SR optical imaging approach, and is hence widely applied in many fields. This review elaborates on the fundamental theory, instrumentation, basic capabilities, and especially the recent advances of SIM.

Generally, SIM has two basic functions, OS and SR imaging. Recently, many advances have been reported to enhance the performance of SIM in different aspects, as are summarized in table 1. In general, TIRF-SIM and 3D SIM enhance the axial-discernibility of SIM. Multi-color SIM endows SIM with the capability of imaging different structures inside cells when labeling them with spectrum-different fluorophores. Phase-type SIM images transparent samples with enhanced spatial resolution and in absence of any fluorescent labeling or other types of intervention. Nonlinear SIM further enhances the spatial resolution of SIM to surpass the factor of two. Spot-scanning SIM, which illuminates the samples by a series of spots and records an accumulated image with a 2D sensor, can penetrate much deeper in samples with significantly reduced background noise. In short, in virtue of these extensions, SIM has been successfully applied in many situations to achieve different imaging aims and becomes increasingly attractive and important in many research fields.

Despite being already powerful, SIM still needs further improvements to make it more functional and useful for complex biological problems in the future. The spatial resolution of SIM can be further improved by combining it with SMLM to capture the rapid dynamics of the structures at the molecular level. In addition, by combining SIM with label-free imaging methods, like SI-DHM [74], partially-coherent annular illumination-based quantitative phase microscopy [96], or Raman microscopy, multifunctional or multi-dimensional information can be obtained to achieve a comprehensive picture of biological processes. Further, it is also of great importance to miniaturize SIM systems to make them more portable, convenient and low-cost for users in different disciplines.
| Category                        | Technique/concept                  | Features/capabilities                                                                 | References |
|--------------------------------|------------------------------------|---------------------------------------------------------------------------------------|------------|
| Traditional SIM                | Optical sectioning SIM             | 1. Applicable to bright-field and fluorescence cases  
2. Simple structure and recovery process  
3. OS $\sim$ 400 nm |
|                                |                                    | [25, 26]                                                                              |            |
|                                | Super-resolution SIM               | 1. Maximum resolution enhancement limited to two folds, typical lateral resolution  
$\sim$ 88 nm  
2. Long-term SR imaging of thin samples  
3. Lacking axial sectioning ability |
|                                |                                    | [32–39]                                                                               |            |
| NL-SIM                         | Saturated SIM                      | 1. Theoretical infinite spatial resolution, the lateral resolution of $\sim$ 50 nm demonstrated  
2. High illumination intensity destroying samples  
3. Low SNR limits resolution enhancement |
|                                |                                    | [60]                                                                                  |            |
|                                | Photoswitchable fluorescent proteins based SIM | 1. Lateral resolution $\sim$ 45 nm  
2. Very low illumination intensity  
3. Require fluorescent proteins with extremely high stability |
|                                |                                    | [19, 76]                                                                              |            |
|                                | Surface plasmon polaritons based SIM | 1. Lateral resolution $\sim$ 30 nm  
2. High sensitivity and less phototoxicity  
3. Very clear background  
4. Limited to 2D imaging  
5. Need special coverslip, hard to be popularized |
|                                |                                    | [77–81]                                                                               |            |
| 3D SIM                         | 4$\pi$-alike SIM                   | 1. OS $\sim$ 50 nm  
2. Complicated structure by two opposite objectives  
3. Resolution limited along the lateral direction  
4. Laborious optical alignment and experiment |
|                                |                                    | [24]                                                                                  |            |
|                                | TIRF-SIM                           | 1. $\sim$ 97 nm along the lateral direction  
2. OS $\sim$ 100 nm  
3. Imaging depth $\sim$ 1 micron  
4. Limited to imaging of sample’s bottom surface |
|                                |                                    | [44, 45]                                                                              |            |
|                                | Multi-beam based SIM               | 1. Isotropic 3D resolution of $120 \times 120 \times 135$ nm$^3$  
2. Complicated configuration  
3. Susceptible to environmental disturbance |
|                                |                                    | [47–49]                                                                               |            |
|                                | I$^3$S                             | 1. 3D isotropic resolution $\sim$ 90 nm  
2. Complicated configuration due to the usage of two opposite objectives  
3. Susceptible to environmental disturbance  
4. Laborious sample installation and optical alignment |
|                                |                                    | [50, 55]                                                                               |            |
| Spot scanning SIM              | Single-spot scanning SIM           | 1. Lateral resolution $\sim$ 119 nm  
2. OS ability comparable to confocal microscopy  
3. Imaging depth $120 \mu m$ in vivo and $210 \mu m$ ex vivo  
4. Low imaging speed |
|                                |                                    | [84–86]                                                                               |            |
Table 1. (Continued.)

| Category          | Technique/concept          | Features/capabilities                                                                                     | References |
|-------------------|-----------------------------|-----------------------------------------------------------------------------------------------------------|------------|
| Multifocal scanning | 1. Spatial resolution: 145 nm (lateral) and 400 nm (axial)  
2. SR imaging depth of 50 µm demonstrated  
3. Fast speed, OS, and deep imaging  
4. Complicated structure and recovery process |                                                                                                           | [87–89]   |
| Instant SIM       | 1. Spatial resolution: 145 nm (lateral) and 350 nm (axial); acquisition speed up to 100 Hz  
2. Imaging depth of 100 µm demonstrated  
3. Relatively complicated configuration and optical alignment |                                                                                                           | [90, 91]  |
| Light-sheet SIM   | 1. Rejecting blurred specimen-related signals while possessing SR ability  
2. Long-term observation (~58 h) of dynamics  
3. Low phototoxicity due to the limited light exposure to a certain section  
4. Suitable for 3D imaging of thick samples |                                                                                                           | [92–94]   |
| Others            | Phase-type SIM              | 1. Enhancing spatial resolution for non-fluorescent imaging techniques  
2. Applicable to transparent sample  
3. Possessing autofocusing capability  
4. Resolution cannot surpass the diffraction limit | [74, 95]  |
|                   | Multi-color SIM             | 1. Capturing complex dynamics using multiple channels  
2. 3D imaging of multiple structures simultaneously  
3. Potentially influenced by the interaction among different fluorescent markers and complex imaging conditions | [45, 59]  |

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