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Image scanning microscopy

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Image Scanning Microscopy (ISM) has emerged as a successful and robust technique which nearly doubles the spatial resolution of a confocal microscope by simple means. Meanwhile, it has found implementation into several highly successful commercial systems, and it has branched into different experimental realizations. The review gives a brief introduction into the basic principles of ISM, and it reports about recent progress and applications of this new microscopy technique.

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Introduction

A conventional confocal microscope is based on two ingredients: raster-scanning a diffraction-limited excitation laser focus over a sample for recording an image, and confocal detection (focusing the collected fluorescence light through a confocal pinhole) for rejecting out-of-focus light. With this, true three-dimensional images can be recorded (z-sectioning). In a well-adjusted confocal microscope, the confocal pinhole is chosen large enough so nearly all the light from a point emitter on the optical axis in the focal plane can pass. This assures maximum sensitivity for emitters in the center of the excitation focus. In that case, the lateral resolution of the microscope is determined by the lateral extension of the excitation laser focus. Under ideal aberration-free conditions, this lateral resolution is given by Abbe's famous formula $\Delta x = \lambda_{exc}/(2\ \text{N.A.})$, where λ_{exc} is the excitation wavelength, and N.A. the numerical aperture of the microscope's objective. A similar relation holds for the lateral resolution of a wide-field microscope, where the excitation wavelength has then to be replaced by the detection wavelength of the fluorescence λ_{det}.

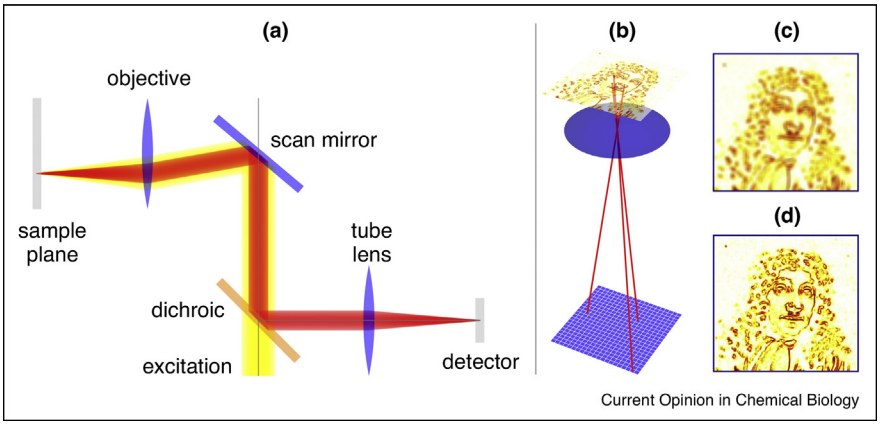
From the early days of confocal microscopy, it was known that the lateral resolution can be improved by decreasing the diameter of the confocal pinhole. Theoretically, the extreme case of a fully closed pinhole would nearly double the resolution. However, in that case no light would pass to the detector. Even a moderate reduction of the pinhole diameter drastically reduces light throughput, so that this idea is almost never used in practice. However, in 1988 Colin Sheppard proposed an elegant solution to achieve doubled resolution without sacrificing light-throughput and sensitivity [1••]. In what follows, we will briefly describe his solution which is the basis for all experimental realizations of Image Scanning Microscopy (ISM) and related commercial products.

The main idea is to replace the point detector of a conventional confocal microscope by an array detector (camera) consisting of many pixels, as shown in Figure 1(a). This array detector records, at each scan position, a small image of the illuminated region. That is why the method is named Image Scanning Microscopy. Furthermore, the effective pixel size (pixel size when back-projected into the sample space) approximates the zero-size confocal pinhole limit (typically, effective pixel size should be $\sim$ 50 nm to 80 nm). *Each* pixel of the array acts as union of confocal pinhole and detector and records its own confocal scan image. Thus, after one scan with the laser focus, one did not record just a single scan image, but as many scan images as the array detector has pixels. Due to the small pixel size the spatial resolution of these scan images corresponds to that of a confocal microscope with zero-size pinhole. However, there are no light losses because all collected fluorescence light will hit somewhere on the array detector.

It remains to combine the multitude of scan images into a single high-resolution image. However, simply adding all images into a single final image will not work: As visualized in Figure 1(b), each pixel has a slightly different *viewing angle* onto the sample (parallax), which leads to shifts between scan images recorded by different pixels. Thus, a simple addition of all scan images leads to a blurred final image without improved resolution, see Figure 1(c). For obtaining a high-resolution final image, Figure 1(d), one has to shift each scan image with respect to a common frame before adding it to the final image. For simplicity, let us consider the situation in just one dimension, as shown in Figure 2(a).

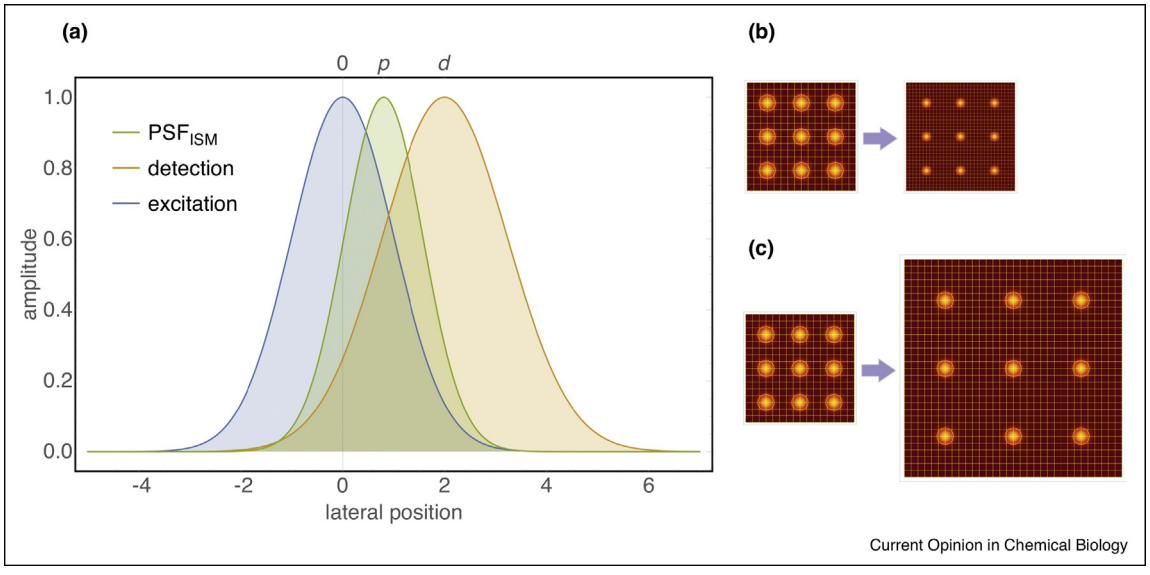
In this figure, the left curve depicts the excitation intensity distribution $E(x)$, which is centered on the optical axis (lateral position zero). It describes the excitation intensity

Figure 1



(a) Principal schematic of an ISM: An excitation laser beam (yellow) is deflected by a scan mirror and sent to the microscope's objective. In sample space, the beam deflection translates into a lateral scanning of the sample with a diffraction limited focal spot. The induced light signal (fluorescence, Raman scattering, etc.) shown here in red is collected by the same objective (epi-fluorescence microscope) and send back to the scanner, which de-scans the collected light. This light is then coupled out from the excitation path via a dichroic mirror and then imaged by the tube lens onto an array detector (camera, multi-diode array, etc.). Thus, in this simplest realization of an ISM, the main difference between a conventional confocal microscope and an ISM is that the pinhole and point detector of the former is replaced by an array detector in the latter. (b) When scanning the sample with the focus excitation spot, each pixel on the array detector records its own scan image. However, due to the different positions of the pixels on the array, each pixel sees the sample under a slightly different angle (parallax) so that the scanned images recorded by different pixels are shifted against each other. (c) When simply adding all scan images recorded by all pixels, the parallax effect would blur the resulting sum image. (d) The core theoretical idea of ISM is to propose an optimal algorithm for correctly adding all scan images recorded by all pixels to finally obtain a high-resolution high-contrast ISM image.

Figure 2



(a) Schematic of excitation, detection and PSF of an ISM in one dimension. In blue (left curve) is shown the excitation intensity distribution, which is centered on the optical axis at zero. The yellow curve (right) shows the detection efficiency function of a single pixel located a distance d away from the optical axis. Here, it is assumed that the pixel position and the shape of its detection efficiency function are back-projected into sample space. The final PSF for the scan image recorded by this single pixel is the product of the excitation intensity distribution and the detection efficiency distribution, and is shown by the green middle curve. (b) Schematic of image processing in ISM: At each focus position, the pixel array detector records a small image of the illuminated region (left). Each of these images is scaled down by a factor $p/d \approx 1/2$ and then placed back at the same focus position onto the final image (right). (c) Alternative way how to look at ISM pixel re-assignment: Instead of shrinking each recorded image at one excitation focus position and then to place it back at the same position onto the final image one can leave the images as they are but place them approximately two times farther (more correctly, by a factor d/p) from each other in the final image. Mathematically, the procedures in (b) and (c) are completely equivalent. However, the all-optical experimental realization of these procedures is fundamentally different, compare Figure 4(a) and (b).

within the laser focus. Its width defines the lateral resolution of a conventional confocal microscope and its shape is the lateral cross-section (in the focal plane) of the Point Spread Function (PSF) of such a microscope (describing the scan image of a point emitter in the focal plane). The right curve depicts the detection efficiency function $D(x-d)$ of *one* pixel that is shifted by the distance d away from the optical axis (all variables refer to sample space). It describes how well this pixel, which is assumed to have close to zero size, detects fluorescence from a given position x in the sample. It is identical to the PSF of a wide-field microscope that uses the same optics. Please note that this curve slightly wider than $E(x)$, which reflects the fact that the fluorescence emission is generally red-shifted with respect to the excitation, and that the width of the PSF scales linearly with the wavelength. The final PSF for the scan-image recorded by this chosen pixel is the product of the excitation intensity distribution $E(x)$ (how good do we excite an emitter at lateral position x) times the detection efficiency function $D(x-d)$ (how good does the pixel at position d detect light from an emitter at lateral position x). This curve is shown in the middle and is denoted as $\mathrm{PSF}_{\mathrm{ISM}}$. The first thing to note is that the its center lies between $E(x)$ and $D(x-d)$, which reflects the resolution enhancement achieved by ISM. To make this all more quantitative, let us assume that both $E(x)$ and $D(x-d)$ can be represented by simple Gaussian functions (which is a quite fair approximation of the actual situation),*i.e.*

$$E(x) \propto \exp\left(-\frac{x^2}{2\sigma_{\mathrm{exc}}^2}\right) \tag{1}$$

and

$$D(x-d) \propto \exp\left[-\frac{(x-d)^2}{2\sigma_{\mathrm{det}}^2}\right] \tag{2}$$

where $\sigma_{\mathrm{exc}} \propto \lambda_{\mathrm{exc}}$ and $\sigma_{\mathrm{det}} \propto \lambda_{\mathrm{det}}$ are the widths of $E(x)$ and $D(x-d)$, respectively. Then, the product of both functions is again proportional to a Gaussian,

$$\mathrm{PSF}_{\mathrm{ISM}}(x-p) \propto \exp\left[-\frac{(x-p)^2}{2\sigma_{\mathrm{ISM}}^2}\right] \tag{3}$$

but with a reduced width σ_{ISM} given by

$$\sigma_{\mathrm{ISM}} = \left(\frac{1}{\sigma_{\mathrm{exc}}^2}+\frac{1}{\sigma_{\mathrm{det}}^2}\right)^{-1/2} = \sigma_{\mathrm{det}}\left[1+\left(\frac{\lambda_{\mathrm{det}}}{\lambda_{\mathrm{exc}}}\right)^2\right]^{-1/2}, \tag{4}$$

and a center position $p < d$ determined by

$$p = \frac{\sigma_{\mathrm{exc}}^2 d}{\sigma_{\mathrm{exc}}^2+\sigma_{\mathrm{det}}^2} = d\left[1+\left(\frac{\lambda_{\mathrm{det}}}{\lambda_{\mathrm{exc}}}\right)^2\right]^{-1}. \tag{5}$$

Thus, if we neglect any Stokes shift between excitation and detection and set $\lambda_{\mathrm{det}} \approx \lambda_{\mathrm{exc}}$, we find the simple relations $\sigma_{\mathrm{PSF}} = \sigma_{\mathrm{det}}/\sqrt{2}$ and $p = d/2$. In almost all cases of practical interest, these values are acceptable approximations close to the optimal values given by eqs. (4) and (5).

Eq. (4) demonstrates that the raw resolution of an ISM image is by a factor $\sim\sqrt{2}$ better than that of a wide-field or confocal microscope alone. The real resolution is even better and reaches a value close to two, but it requires additional image deconvolution. For technical details, we refer the reader to the original publications [1••,2••].

Eq. (5) gives us a recipe how to superimpose the scan images recorded by the various pixels of the detector array: If a pixel is a distance d away from the optical axis, then its scan image has to be shifted *towards* distance p before adding it to the final image. This corresponds to *re-scaling* the snapshot image recorded by the detector array at any position of the excitation focus by a factor of $p/d \approx 1/2$ before adding it to the final image at the position of the excitation focus. This is schematically depicted in Figure 2(b).

This simple procedure delivers a final ISM image that corresponds to one taken with a confocal microscope with fully closed pinhole. At the same time it improves the contrast and signal-to-noise ratio (SNR) by a factor of four due to the down-scaling of each snapshot recorded by the detector array at a given position of the excitation focus, see again Figure 2(b).

It should be noted that ISM is closely related to Structured Illumination Microscopy (SIM) [3–6], which uses similar physical principles to double the resolution of a wide-field microscope. In SIM, one starts from a wide-field microscope but then uses a non-uniform illumination pattern for excitation. After taking several images for different positions and orientations of this excitation pattern with respect to the sample, a final doubly-resolved image is calculated by applying a sophisticated reconstruction algorithm. In ISM, one starts with a non-uniform illumination (the excitation focus of a conventional confocal microscope), but uses a wide-field detection mode. Similar to SIM, from the many recorded individual images at each scan position, one finally calculates a high resolution image. However, there are several crucial differences between SIM and ISM. Theoretically, SIM delivers images with a higher signal-to-noise ration because it better excites high spatial frequencies in a sample [6,7]. Thus, under optimal conditions, SIM will deliver images with higher resolution and signal-to noise

ratio at high spatial frequencies than ISM. In practice, this is often outweighed by the sensitivity of SIM to slightest aberrations and imperfections of the setup. ISM is both in hardware and in image reconstruction algorithm clearly simpler than SIM, and proves to be much more robust against sample or microscope imperfections. Moreover, it seems much easier to combine ISM with other techniques such as multi-photon excitation, or fluorescence lifetime imaging. Finally, the advent of all-optical realizations of ISM (see below) makes this technique extremely fast and easy to handle.

The concept of ISM has been experimentally realized in different ways. In the following we will present the principal types of implementation along with their strengths and weaknesses.

Computer based pixel-reassignment ISM

Müller and Enderlein presented the first experimental realization of an Image Scanning Microscope in 2010 [2••]. Using an emCCD as detector in a home-built scanning confocal microscope, an image of the emission pattern was recorded for each scan position. Subsequently, the recorded data was processed as discussed in connection with Figure 2(a), namely the signal of each pixel was virtually shifted by half its distance from the optical axis and added to a final image at the position corresponding to the focus position in the sample. The final image was then additionally deconvolved for realizing the full two-times resolution enhancement of ISM. Despite nicely demonstrating that the method works, the principal drawback of this first ISM realization was its slow acquisition speed. This was due to the fact, that a camera image had to be recorded for each focus position. This required several tens of milliseconds (dwell time of the emCCD), which resulted in an total acquisition of several minutes for an image of only few micrometer size. Since just a small part of the camera chip was used to take record the images around the focal spot, it was obvious that the method could speed up by using a multi-focus excitation and a parallel read-out of all the excited focal spots. This was done by Andrew York and co-workers by employing a digital mirror device (DMD) to generate an array of excitation foci for sample scanning. Fast reconfiguration of the DMD allows to shift this multi-focus pattern over the sample. This method increased speed and efficiency of ISM drastically [8•]. In 2013 Schulz et al. presented a simple implementation of ISM into a confocal spinning-disk (CSD) microscope [9]. By pulsing the excitation laser with on-times of less than 10 μs, snapshots of the multi-focus array generated by the CSD system were taken while the disk was spinning at full speed.

Following these developments, the company Carl Zeiss released the first commercial ISM instrument in 2014 [10•]. The operating principle of this system is very similar to the first experimental ISM publication [2••]. The system is based on their standard confocal scanning microscope, but the usual point detector is replaced by a so-called AiryScan detector. This detector is based on a bundle of 32 optical fibers arranged in a hexagonal pattern. The end of each fiber is connected to one pixel of the 32 linear array multianode GaAsP-PMT. This detection scheme allows for fast readout with online signal processing. In this way, the super-resolved ISM image can be displayed in nearly real time, see Figure 3.

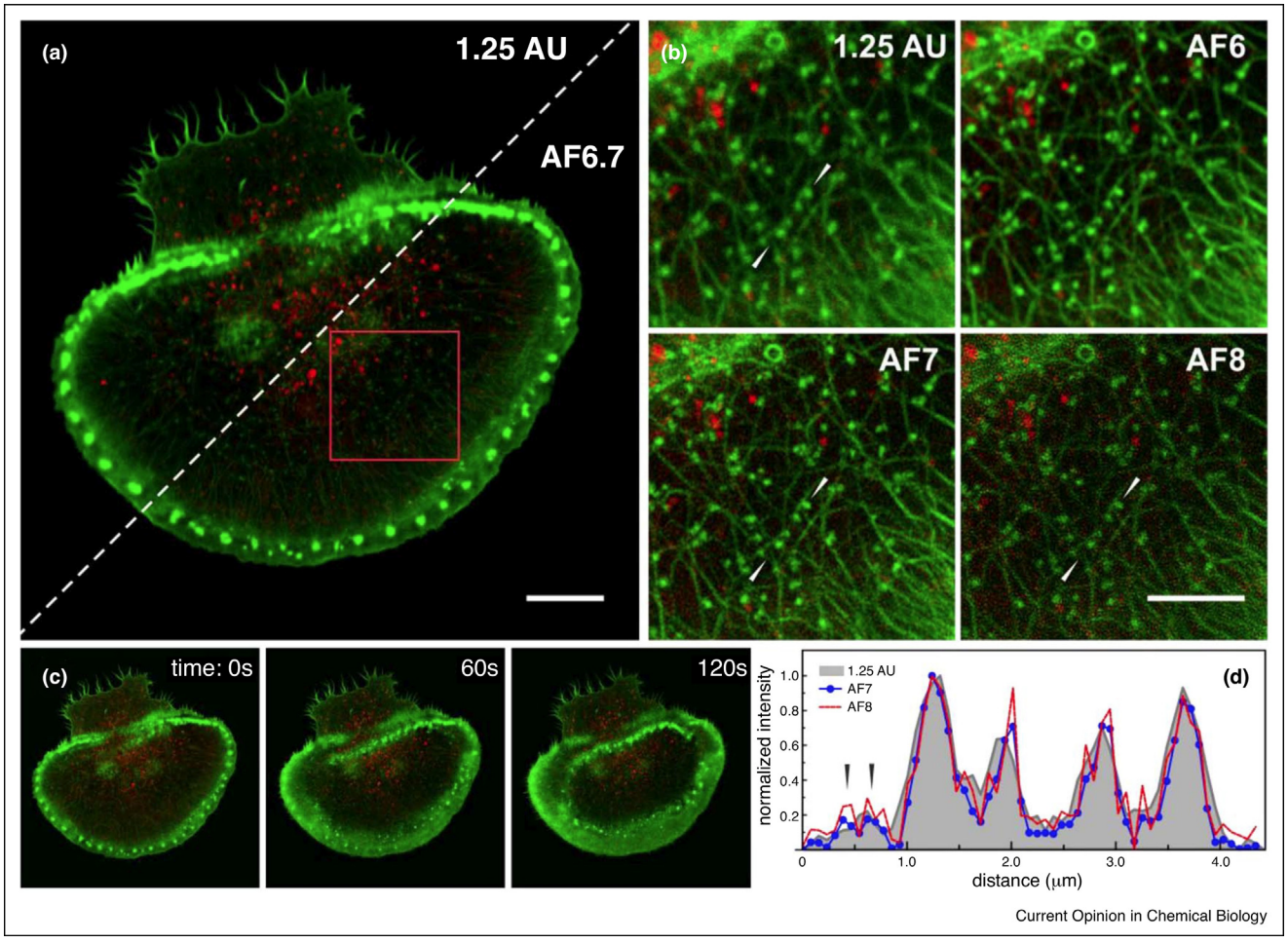
The group of Vicidomini presented a minimal ISM solution using a quadrant photo-diode [11]. This system did not reach the full resolution enhancement, but — given the simple and straightforward implementation — it performed remarkably well. However, at low signal strength photo-diodes show a significant lower signal-to-noise ratio (SNR) than PMT based detectors.

Image Scanning microscopy works also for coherent imaging as was first demonstrated by Wang et al. for a reflection light microscope [12].

Ge et al. used the AiryScan detector to enhance also the axial resolution of the microscope [13]. This approach is based on the difference of the signals between the inner and outer rings of pixels on the hexagonal pixel array. The same group proposed a similar approach to combine signals from different radii of the AiryScan detector to further enhance resolution and SNR [14]. In a further work, the group proposed a combination of both ideas to enhance lateral and axial resolution of the AiryScan microscope [15]. Generally, these methods are delicate, since the corresponding PSF shows negative contributions which may lead to non-physical image artifacts. A simpler and more physical method was also presented by this group [16]. There, image reconstruction is based on a Fourier-transform of the scan images recorded by each pixel of the AiryScan detector. Instead of re-assigning pixels as in the original ISM concept, Figure 2(b), the phase of each Fourier image is shifted by a value corresponding to the pixel position. This can be done in a more flexible way as the direct pixel re-assignment because it allows for virtual pixel re-assignment shifts by non-integer values of pixel-size which can result in better ISM image quality.

The performance of ISM and especially its multi-focus variants depends strongly on the accuracy of the reference positions of the focal spots. These coordinates are crucial for the reconstruction algorithm. Wu et al. proposed a measurement-based learning algorithm to effectively determine these positions [17]. This eliminates the need for a reference measurement as well as ensures best resolution enhancement for a given data.

Figure 3



Example of ISM with the AiryScan microscope by Carl Zeiss: Time-lapse images of activating Rat Basophilic Leukaemia (RBL) cell. (a) Comparison of 1.25 AU confocal and AiryScan processed AF6.7 images. Scale bar is 10 μm. (b) Direct comparison of Region of Interest area (ROI; red rectangle in (a)) between confocal 1.25 AU and Airy processed images with AF4, AF6 and AF7, respectively. Scale bar is 5 μm. (c) Time-lapse of activating RBL cell at 0, 60 and 120 s, respectively. Green LifeAct-citrine (excitation at 488 nm), red SNAP-tag (excitation at 561 nm). (d) Intensity profiles from 1.25 AU (grey filled), AF7 (blue dots) and AF8 (red) images along the line indicated by white arrows in (b). Arrows indicate peaks from two separate actin fibres, which are only distinguishable at high AF strength and are not resolved at 1.25 AU. Image taken from Ref. [10•].

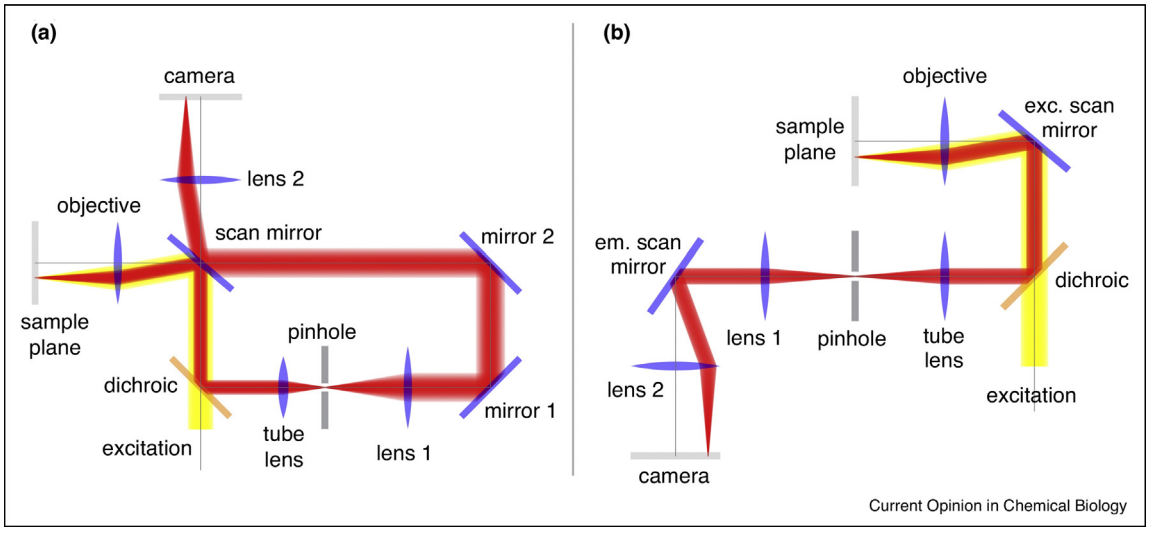
All-optical realizations of ISM

Soon after the first demonstration of ISM, it became evident that it can also be realized by purely optical means.

In 2013, York et al. presented a system which they termed Instant Structured Illumination Microscopy (ISIM) [18••]. Similar to their earlier publication [8•], it uses a microlens-array for generating a multi-focus pattern that is scanned over the sample. However, the revolutionizing new idea was that after de-scanning the emission light, it is passed through a matching pinhole array and a second microlens-array the expands the beam size two-fold. This light is finally sent back to the back-side of the scanner before being imaged onto the camera. A principal schematic of this idea for a single focus is shown in Figure 4(a). The beam size expansion by a factor two corresponds exactly to the focus image shrinking by one half as required by ISM. But now, after each scan, a full ISM image can be read out by the camera, without further need of recording individual images for each focus position and subsequent reconstruction of the final ISM image on a computer. Due to the peculiar 2D structure of the multi-focus excitation array, scanning could be restricted to one axis. This allowed frame-rates in the order of 100 Hz, see Ref. [19] and Figure 5.

In a similar vane, Roth et al. extended a single-beam scanning confocal microscope in such a way that after de-scanning of the fluorescence emission, the beam was contracted by a factor of two with a two-lens relay and then fed back onto the scanner (but on the same side as the

Figure 4

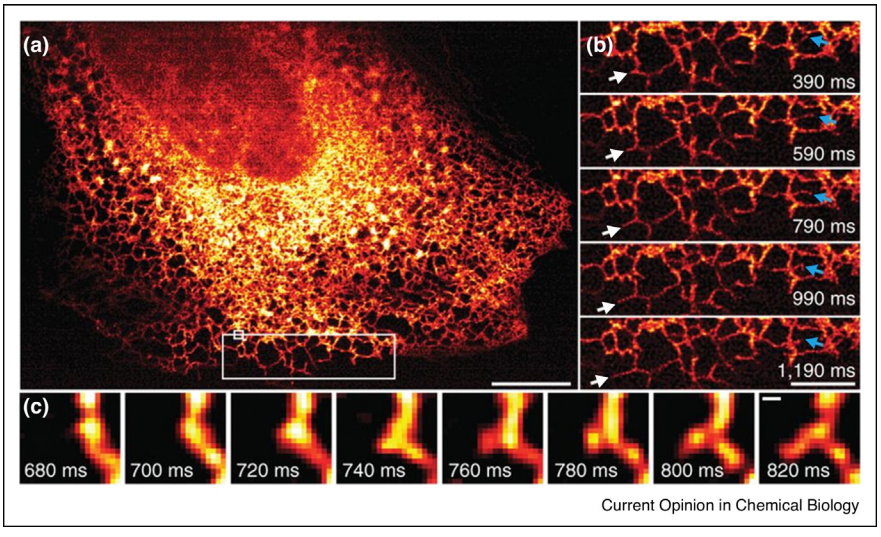


All-optical realizations of ISM. (a) The system uses conventional excitation focus scanning. The collected fluorescence is de-scanned and passed through the confocal pinhole. After the pinhole, lens 1 collimates the light in such a way that the beam size is doubled as compared to its size before the tube lens. This reduces the size of each image recorded at a given excitation position by a factor 1/2. After this, the light beam is sent back to the scan mirror before being finally focused onto the camera. After each full scan of the excitation focus, the camera has recorded an ISM image with enhanced resolution. (b) Realization of re-scan ISM as schematically shown in Figure 2(c). Up to the confocal pinhole, the re-scan microscope is identical to a conventional confocal microscope. After the pinhole the collimated light is sent over a second scan mirror which oscillates in phase with the excitation scanner, but with doubled scan amplitude (or, more exactly, larger by the factor d/p).

excitation light) [20]. The authors termed their method Optical Photon ReAssignment (OPRA) microscopy.

Independently, De Luca et al. realized an all-optical ISM in a completely different way. Instead of shrinking the image at each focus position and then placing it back in the final image at the corresponding focus position, as shown in Figure 2(b), one can alternatively maintain the image size and place them with double distance to each other, see Figure 2(c). Although both procedures are

Figure 5



Example of imaging ER dynamics with an all-optical ISM using scheme Figure 4(a) at 100 Hz image acquisition speed. (a) First image in series of 200 time points, showing ER labeled with GFP-Sec61A within MRL-TR transformed human lung fibroblasts. Data were acquired at the coverslip surface. Scale bar, 10 μm. (b) Higher-magnification view of the large white rectangle in a. White arrows mark growth of an ER tubule; blue arrows indicate remodeling of an ER tubule. Scale bar, 5 μm. (c) Higher-magnification view of the small white rectangle in a, indicating formation of a new tubule within 140 ms. Scale bar, 200 nm. Image taken from Ref. [18].

mathematically completely equivalent, they are experimentally quite different. In the re-scan system of De Luca et al. [21••,22], one uses a second synchronized scanner to re-scan the emission onto a camera, but with twice larger scan amplitude than used by the excitation scanner. The required precise synchronization of the scanners restricts this system to non-resonant scanners.

Azuma et al. transferred the concept of ISM to a spinning-disk confocal system by adding to the pinhole disk and additional micro-lens array that shrinks the focus image size in detection by a factor of two [23•].

Multi-photon and non-fluorescent variants of ISM

Without restrictions, the concept of ISM can be applied to all confocal imaging modalities. Ingaramo et al. used a microlens array to generate a 2D focus pattern from an expanded fs-pulsed NIR laser beam [24]. This pattern was then scanned over a sample for two-photon fluorescence excitation, and for each scan-position the emitted fluorescence pattern was recorded on an emCCD camera. Since two- or multi-photon excitation does not require detection through a pinhole for achieving optical sectioning, it was no longer necessary to de-scan the collected fluorescence light. This dramatically simplifies the setup. The same group used later also the re-scan principle to enhance resolution and contrast in two-photon excitation microscopy [25•]. The enhanced contrast of ISM enabled also deeper penetration into the sample. Finally, Gregor et al. implemented a re-scan microscope by using only one single resonant scanner, which allows for achieving higher frame rates than using non-resonant scanners [26]. However, in their re-scan realization, it is not possible to implement a pinhole for detection. Thus, it can only be used in conjunction with two- or multi-photon excitation when optical sectioning is required. The same system was also used successfully for non-fluorescent second-harmonic generation (SHG) imaging.

A two-photon ISM microscope based on the original ISM principle, Figure 1(a), was presented by Sun et al. [27]. Here, a bundle of seven fibers was used to map the fluorescence on an array of Avalanche Photo Diodes (APDs). The performance of this instrument was sub-optimal, because sampling of only seven points within the excitation focus is not fully sufficient. Additionally, the core diameter of the fibres used were not perfect for achieving an homogeneous sampling of the signal.

Recently, Tzang et al. reported on a multi-focus two-photon ISM microscope with an engineered double-helix PSF to encode depth information [28]. The multi-focus excitation pattern was generated via a spacial light modulator in the Fourier-plane of the tube-lens. In the detection path, a commercial phase-mask module created the double-helix PSF for the selected emission band. Scanning was performed using a 3D piezo-driven sample scanner.

Confocal Raman microscopy is a powerful technique to reveal chemical composition of a sample in three dimensions. Roider et al. demonstrated that this method can easily benefit from ISM [29•]. The authors used a hexagonal seven fiber bundle similar to the AiryScan system. The end of each fiber was aligned along a line and matched to the entrance slit of a spectrometer.

A special implementation of ISM was used to improve the spatial resolution of optical coherence tomography (OCT) [30]. This method uses virtual spatial modulation (VSD) of the PSF of the reflection signal of a rapidly scanned beam to enhance resolution. This paper provides also an overview on the principles and the progress that had been achieved.

Theoretical considerations

The advent of ISM has led to a series of publications that address specific theoretical questions. At first, Sheppard et al. examined the conditions for optimal performance of the method [31]. A corresponding study for non-linear scanning microscopy was presented by Laporte et al. [32]. Ingaramo et al. studied the performance of Richardson-Lucy deconvolution in the context of ISM (and other techniques) [33]. A broader study involving several strategies for post-processing of ISM data was presented by McGregor and co-workers [34]. Sheppard and colleagues discussed in detail the performance of ISM on the basis of the Optical Transfer Function (OTF) [35]. This paper and another one by Roth et al. showed that ISM and its variants does not only enhance resolution, but also increases the signal-to-noise ratio (SNR) [36].

Since ISM uses an intricate interplay of excitation and detection PSF, modulating the one or the other will influence its performance. In Ref. [37], A. Hamed studied the influence of different transmission profiles of the pinhole aperture (which modulates the detection PSF) on the resolution of ISM.

Sheppard et al. gave a detailed global theoretical analysis of ISM [38]. They derived optimal parameters for the ISM pixel reassignment as a function of Stokes shift, for multi-photon excitation, and as a function of excitation beam profiles, like e.g. Bessel beams.

Ward and Pal published a review on ISM and compared it to other super-resolution techniques [39]. The same group later proposed a method to enhance ISM resolution by sharpening the excitation focus [40]. The core idea is to measure the difference signal between a Gaussian focus and a corresponding doughnut focus, similar to what is used in STED microscopy.

Noteworthy applications of ISM

The relative simplicity and robustness of ISM inspired various companies to implement this feature into their products. As already mentioned, Zeiss introduced the AiryScan microscope in 2014 [10•]. The Re-scan confocal microscope (RCM) was introduced by Confocal.nl in 2015 [21••]. Finally, in 2017 Olympus presented their SpinSR10, based on a confocal spinning-disk microscope [41].

In this context, two studies assessed the performance of the AiryScan detector. Korobechevskaya et al. applied the AiryScan system for live cell microscopy and found a ≈1.4 times improved resolution and increased SNR [42]. Sivaguru and co-workers used pollen samples for comparing the performance of the AiryScan system with that of a SR-SIM microscope from the same company. They found higher resolution for the SIM, but a much better SNR for the AiryScan [43].

Since ISM does not improve the resolution along the optical axis, several methods have been developed to enhance its performance in this respect. A special approach was followed by Kuang et al. using the AiryScan detector [44]. For each acquisition point, the 'image' of the AiryScan detector was stored. With these, 144 copies of the image were computed, each multiplied with a distinct modulation pattern. A dedicated reconstruction procedure computes the final image which shows improvements in lateral as well as in axial resolution, and can also correct slight optical aberrations.

Roider et al. introduced a phase-mask to generate a helical PSF in the detection. In combination with a proper deconvolution, they could achieve ≈1.2 times improved axial resolution, but at the cost of a slightly reduced lateral resolution compared to ISM [45]. The same group later proposed more advanced schemes, by using phase masks to shape the excitation as well as the emission PSFs [46]. Using helical PSFs in both paths can enhance the resolution axially, but turned out to be not truly applicable in the general case. On the other hand, multi-plane excitation and emission can enhance the 3D capabilities of ISM in terms of sampling speed, but not so much for resolution. Using a double-helix phase-mask in the detection path in combination with multi-spot excitation was reported by Li et al. [47]. After retrieving the depth information from the rotation of the PSF, it was deconvolved into a Gaussian focus and then processed with the standard ISM algorithm. This method works well if the label density along the optical axis is sparse. In this case on can obtain decent 3D imaging with a good resolution along the z-axis.

Roth et al. increased the axial resolution using an improved OPRA microscope [48]. They scanned the beam in a striped pattern and shifted the offset of the stripes similar to what is done in a structured illumination microscopy (SIM). A rotation of the pattern is not required, since only depth information was intended to be obtained. For image reconstruction, different approaches were tested. In the best case, a scaled subtraction achieved a 1.4 fold sectioning improvement.

Because ISM is a technically rather simple method, it easily allows for combining it with other techniques. In a quite straightforward manner Zheng et al. used a deformable mirror to minimize aberrations in a two-photon excitation ISM [49]. The adaptive optics element was placed in the common beam path, such that optimization of the excitation focus as well as the detected PSF was achieved. Xiao and co-workers used radially polarized light to achieve tighter focusing and therefore better resolution than what can be obtained with linearly or circularly polarized light that is used normally [50].

Israel et al. reported on a home-built detector using a bundle of 15 optical fibers similar to the AiryScan detector [51]. The end of each fiber was connected to a single-photon avalanche photo-diode (SPAD). This detector shows high sensitivity, fast response, and low noise. This allows to evaluate correlation functions that give access to e.g. the number of emitters and sub-pixel localization of individual emitters.

Using a 2D array of SPAD detectors, Castello et al. built a similar ultra-fast ISM camera [52•]. Using pulsed laser excitation, they combined ISM with fluorescence lifetime imaging microscopy (FLIM).

Conclusions

We have presented a brief introduction and overview of the recently developed high-resolution Image Scanning Microscopy. It represents a natural and simple extension of conventional confocal microscopy, and due its technical simplicity it can be expected that in the near future all confocal microscopy systems will be ISM systems. Besides its simplicity, the hallmark of ISM is its robustness and versatility, which allows it to be combined with many other microscopy techniques such as multi-photon fluorescence microscopy, coherent imaging (Raman, OCT), or fluorescence lifetime microscopy. Thus, ISM will become a standard microscopy technique which provides all the known advantages of a conventional confocal microscope but with doubled lateral resolution and four times higher contrast.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

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