Extended resolution fluorescence microscopy

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Fluorescence microscopy is an essential tool of modern biology, but, like all forms of optical imaging, it is subject to physical limits on its resolving power. In recent years, several exciting techniques have been introduced to exceed these limits, including standing wave microscopy, 4Pi confocal microscopy, I^{5}M and structured illumination microscopy. Several such techniques have been definitively demonstrated for the first time during the past year.

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Abbreviations

2D	two-dimensional
3D	three-dimensional
CCD	charge coupled device
I^{2}M	image interference microscopy
I^{3}M	incoherent interference illumination microscopy
I^{5}M	I^{2}M and I^{3}M combined
I^{n}M	I^{2}M, I^{3}M and I^{5}M, referred to collectively
NA	numerical aperture
SLTM	single-lens theta microscopy
STED	stimulated emission depletion microscopy
SWFM	standing wave fluorescence microscopy

Introduction

Fluorescence microscopy is a ubiquitous and irreplaceable part of modern biology and has been made more so by recent advances in immunolabeling and *in situ* hybridization and by the advent of the green fluorescent protein. The development of computational deconvolution [1–3] and confocal methods [4,P1] has turned it into a powerful 3D visualization tool. The primary limitation of the classical microscope is its finite spatial resolution, which is limited by diffraction to about 0.2 μm in the lateral direction (in the image plane) and about 0.6 μm in the axial (focus) direction.

Resolution extension concepts

Even though the classical resolution limits are imposed by physical law, they can, in fact, be exceeded. There are loopholes in the law or, more precisely, the limitations are true only under certain assumptions. Three particularly important assumptions are that observation takes place in the conventional geometry in which light is collected by a single objective lens; that the excitation light is uniform throughout the sample; and that fluorescence takes place through normal, linear absorption and emission of a single photon. The negation of these assumptions leads to three basic concepts for resolution extension:

1. Gathering light over a different, preferably larger, set of angles around the sample.

2. Using excitation light that varies with positition.

3. Using a fluorescence process that involves two or more photons nonlinearly.

The first of these concepts is able to increase the resolution by much the same reason that the resolution of any objective lens increases with increasing light-gathering angle (or numerical aperture, NA) [5,6]. The second concept works because a spatial structure in the excitation light modulates the emitted light and encodes into it higher resolution information than it normally can carry [7]. To understand the third concept, consider a line profile through a conventional microscope image of an ideal point source. Because of the finite resolution, the profile will not be an infinite spike, but will be a smooth peak with some width. The corresponding profile for an imaging process that requires multiple photons may equal the square or higher power of that conventional profile and therefore have a narrower width, corresponding to higher resolution.

In this review, I outline the capabilities and limitations of a number of resolution enhancement methods. Each of these methods is based on one — or several — of the above three concepts.

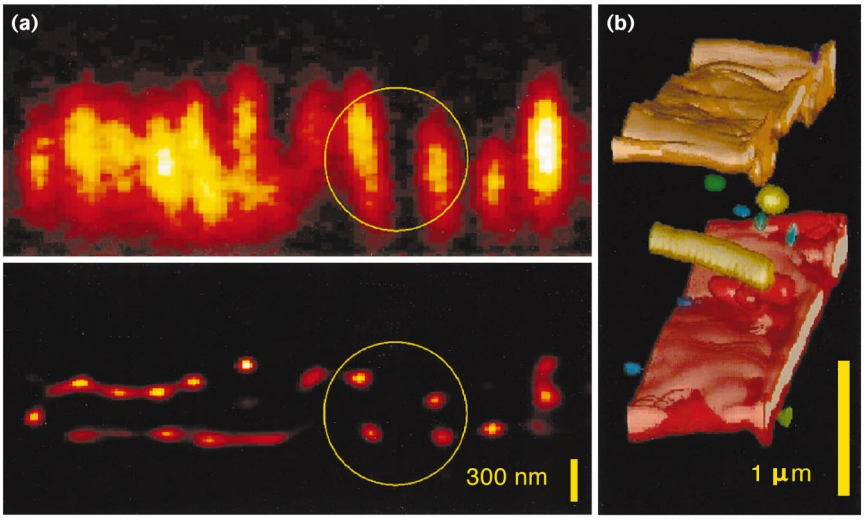
Extended resolution microscopies

Confocal microscopy

Confocal microscopy is, of course, not a new technology; I include it here to put the other techniques into a proper context. Conventional microscopes that use charge coupled device (CCD) cameras or film are called ‘wide-field’ to distinguish them from confocal microscopes.

In a confocal fluorescence microscope, the idea of spatially varying excitation light (concept 2) is carried to an extreme — the excitation light consists of a laser beam that is focused ‘to a point’. The emitted light is detected through a pinhole located at the image of that point. Either the sample or the beam is then scanned in a raster pattern and a 3D image of the sample is built up point by point [4,P1]. As unwanted light from out-of-focus planes is largely rejected by the pinhole, the confocal microscope has true axial resolution, even for some samples (such as thin layers perpendicular to the optic axis) that wide-field microscopes cannot resolve [8]. The lateral resolution is also improved over that of wide-field microscopes (by a factor of about 1.4 for the raw data or up to a factor of 2 if computer processing can be applied), although this only occurs if the pinhole is substantially smaller than the image of the focus spot, that is, if much of the ‘good’ in-focus emitted light is discarded together with the ‘bad’ out-of-focus light [9–11]. The lateral resolution increase is, therefore, rarely realized in biological fluorescence,

Figure 1



A demonstration of 4Pi(A) confocal microscopy. The axial direction is shown as vertical. **(a)** Microtubules in a mouse fibroblast cell. A comparison of single-section side (xz) views of the same area, as seen using conventional confocal microscopy raw data (top) and 4Pi(A) confocal microscopy with two-photon excitation and full deconvolution (bottom). The improved axial resolution is obvious. **(b)** 3D double-surface rendering of a 4Pi(A) confocal microscopy reconstruction of actin structures in a fibroblast. Color only indicates separate objects. Adapted with permission from [45,47•].

microscopy, in which photons are usually too precious to discard — particularly so for confocals, as their typical detectors, photomultiplier tubes, already have a lower quantum efficiency (by a factor of 3–30, depending on the wavelength) than the CCDs used in wide-field microscopes [12].

Multiphoton methods

In two-photon absorption microscopy [13], the sample is illuminated by a focused beam of light of approximately twice the wavelength (half the photon energy) of the normal excitation light, but of such high intensity that two photons can be absorbed simultaneously, and together supply sufficient energy to excite the fluorophore. (Three-photon absorption microscopy [14] similarly involves the absorption of three photons at a time.) The necessary high intensity is typically achieved by using an (expensive) ultrafast pulsed laser. A confocal pinhole is optional; optical sectioning occurs even without one.

Two-photon absorption microscopy has important strengths — including the facts that out-of-focus planes are not subject to photobleaching and that the longer wavelength allows deeper penetration into scattering samples — and is likely to play a major role in biological research. It is not, however, by itself a resolution extension method.

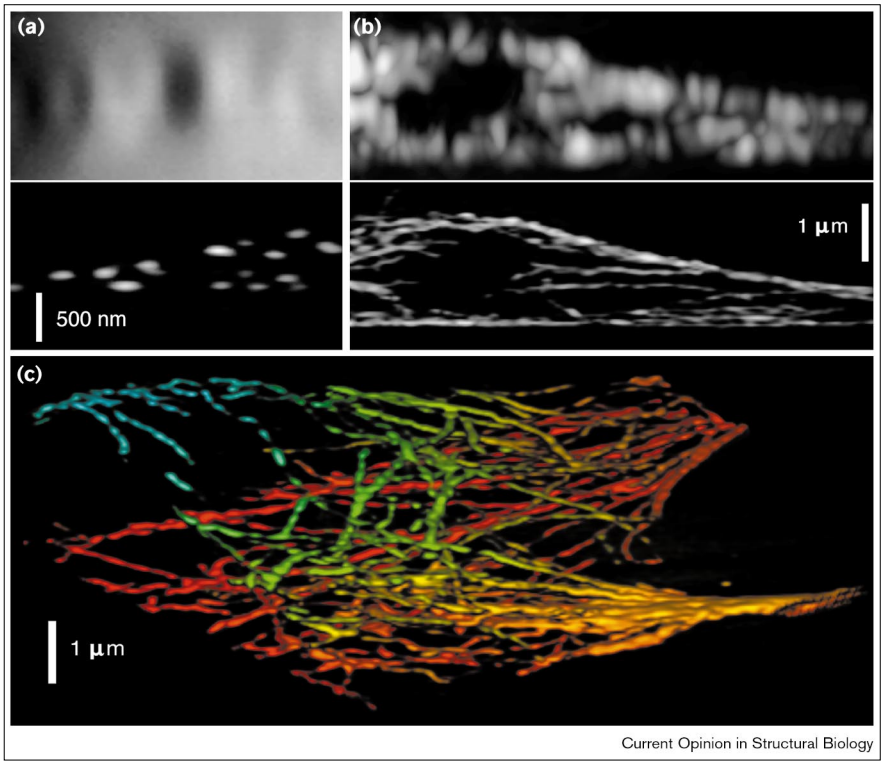
The two-photon absorption is proportional to the square of the local light intensity, so most of the excitation takes place in the center of the focused illumination spot. Thus, one would think that the resolution would be improved and this is indeed the case in a comparison in which the excitation wavelength is kept constant, that is, if one has a given light source and is choosing between exciting either a long-wavelength dye by one-photon absorption or a short-wavelength dye by multiphoton absorption. In practice, one is more likely to have a given fluorescent dye and be choosing between exciting it with single- or multiphoton absorption. In such a comparison, in which the emission wavelength is kept constant, the resolution is, in fact, made somewhat worse by multiphoton excitation [15,16], because of the longer excitation wavelength.

Other nonlinear schemes exist, however, that can improve resolution. These typically involve multiple photons through separate single-photon processes. One proposed method is pump-probe microscopy [17,18], which is based on stimulated emission and should yield a substantial increase in 3D resolution over that of confocal microscopy [18] (while simultaneously providing fluorescence lifetime information). Photon efficiency may be its weak point. The same phenomenon is used in a different regime in stimulated emission depletion microscopy (STED) [19,20••], which has been recently shown to yield a 30% improvement in resolution over that of confocal microscopy in one lateral direction on a test sample [20••]. It could, in theory, yield more, although at an increasingly severe cost in signal. More speculative ideas include frustrated energy transfer [21•], which relies on a hypothetical new type of clustered fluorophore, and repetitive excitation [22•].

Tilted-view microscopy

A conceptually simple way to alleviate the problem that the resolution is worse in the axial direction, both in wide-field and confocal microscopes, is to simply rotate the sample until what was the axial direction is now lateral and acquire a second data set in the new orientation [23–26]. The two data sets can then be combined computationally to yield a reconstruction with equal axial and lateral resolution [23,24,26]. Unfortunately, a microscope slide under

Figure 2



A demonstration of I^{5}M. The sample is microtubule cytoskeleton in PtK2 tissue culture cells. The axial direction is shown as vertical. **(a)** A comparison of single-section side (xz) views of the same area, as seen using conventional wide-field microscopy raw data (top) and I^{5}M after deconvolution (bottom). **(b)** Maximum intensity side (xz) view projections through a 1.2 µm wide slice of a cell, comparing conventional microscopy with deconvolution (top) with I^{5}M with deconvolution (bottom). In both (a) and (b), individual microtubules that are obscured by axial blurring in the conventional microscope are clearly distinguished using I^{5}M, as a result of its superior axial resolution. **(c)** 3D view of I^{5}M reconstruction. This cell has just divided and is still connected to its sister cell by the mid-body (lower right). Also note the nuclear cavity on the left. The color hue varies from red to blue with increasing distance from the substrate on which the cell was grown. The view angle is 70° off axis. Adapted with permission from [41••].

a high NA objective can only be tilted a few degrees before physically hitting the lens. Therefore, tilted-view microscopy requires either using a low NA objective, the inherently lower resolution of which would defeat the purpose, or mounting the sample on (or in) a narrow rod or tube that can be rotated. The method is, of course, also restricted to sample areas that can be viewed without obstruction from all the angles involved.

Theta microscopy

Theta microscopy [27–29] is a variation of confocal microscopy in which the sample is observed through a second objective lens that is placed at right angles (or approximately so) to the illuminating objective. As in confocal microscopy, the emitted light is detected through a pinhole that is confocal to the illumination focus. As the axial (lower resolution) direction of the illuminating objective is a lateral (higher resolution) direction of the observation objective and vice versa, the system resolution becomes less anisotropic.

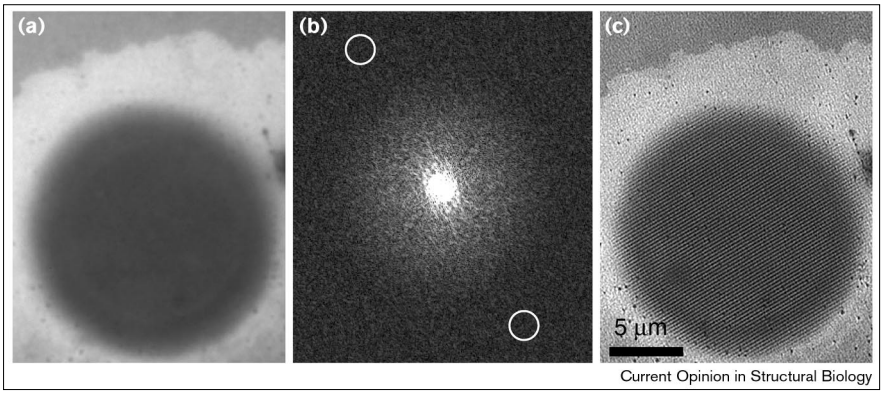
Whereas the tilted-view method has a choice between low aperture objectives or rod-mounted samples, theta microscopy suffers from both these weaknesses simultaneously. It requires low NA objectives, as high NA objectives would physically overlap if two of them were placed at right angles. In practice, the maximum NA for 90° viewing is around 0.7 or about half that of a high-resolution objective. This fact should be kept in mind when evaluating articles on this technique, which often misleadingly compare the performance of theta microscopy with that which other microscopes would get with the same low NA objective lenses. Compared with a confocal microscope with a high NA objective, theta microscopy improves the axial resolution at best slightly, but lowers the lateral resolution severely as a result of the lower NA. It is subject to the same sample limitations and awkward sample mounting as the tilted-view method, but yields lower resolution. It does have the advantage that no sample rotation and only a single image stack are needed; unfortunately, this does not translate into any speed advantage, as theta microscopy requires sample scanning, which is inherently slow.

Single-lens theta microscopy (SLTM) is a variant of theta microscopy that uses a small mirror unit in front of a single objective to deflect light into the 90° theta geometry [28,29]. SLTM can accommodate a somewhat higher NA, but it imposes further sample size restrictions [29].

Axially structured illumination: standing wave microscopy

The idea of spatially varying excitation light (concept 2) can also be applied to the wide-field microscope. One example is the standing wave fluorescence microscope (SWFM) [30–36,37••,P2–P4], in which the excitation light consists of two counter-propagating, nonfocused laser beams. The two beams interfere to form a standing wave, creating an excitation intensity that varies rapidly in the axial direction. The

Figure 3



Laterally structured illumination microscopy. The test sample is a thin layer of fluorescent dye, in which an area (the dark circle) has been patterned with stripes spaced 0.23 µm apart. The stripes were manufactured through photobleaching by two interfering laser beams. **(a)** The stripe pattern is not visible using the conventional microscope. In fact, no sign of the stripes can be detected, even in the **(b)** Fourier transform of the image, in which even a weak residual stripe pattern would generate a pair of dots at the circled locations – no such dots are seen. Thus, computer enhancement alone could not make the stripes visible. **(c)** The structured illumination technique, however, easily resolves the stripe pattern. Also note the improved definition of the dark background defects and the dye layer edge (MGL Gustafsson, DA Agard, JW Sedat, unpublished data).

spatially varying excitation intensity mixes high-axial-resolution information into the observed emitted light through a moiré-like effect. The new high-resolution information can be separated by acquiring three images at different phases of the standing wave [31,32,P2]. As SWFM is a wide-field technique, it requires no scanning and has the potential to rapidly acquire data with a high signal-to-noise ratio.

A weakness of SWFM is that there is an 'information gap' — a set of intermediate-resolution spatial frequencies about which no information is present in the data [33]. This can be thought of simply as an inability to tell which fringe in the excitation intensity is which. The problem can be alleviated by superposing many standing waves of different fringe spacing, a procedure known as field synthesis [34,P3,P4], but an information gap still remains and makes 3D reconstruction challenging. For very thin samples (less than 0.25 µm), however, there is no such fringe ambiguity. As a result of the particularly simple and well-characterizable structure of the excitation light, SWFM is capable of making precise quantitative measurements on this class of very thin samples [36,37••].

4Pi confocal microscopy and I^{n}M

The deepest meaning of the collection geometry concept (concept 1) lies in actually increasing the set of angles over which emission light is collected at any one time. There is a large and (in hindsight) obvious set of untapped angles on the entire back side of the sample slide. Light emitted in those directions can be collected by adding a second objective lens on that side. If the light beams from the two objectives are combined on a single detector, after having traveled equal distances, the two beams can interfere. New high-axial-resolution information that is not accessible in a single-lens microscope is revealed by these interference effects [7]. This idea can be used both in the confocal context, where it yields 4Pi(B) confocal microscopy [38,39], and in the wide-field context [7,40,41••,P5], where it yields image interference microscopy (I^{2}M).

Both 4Pi confocal microscopy and I^{2}M can also incorporate the idea of spatially varying excitation light (concept 2). As in SWFM, an axial variation can be introduced into the excitation light by dividing it with a beam splitter and sending it through both objectives into the sample. There, the two beams can interfere to form an axial fringe, which encodes high-axial-resolution information into the observed data. Used by itself, this effect is called 4Pi(A) confocal microscopy [38,42,43,44••,45,46•,47•] or, in the wide-field case [7,P5], incoherent interference illumination microscopy (I^{3}M). The excitation light interference used in 4Pi(A)/I^{3}M can be combined with the emission light interference of 4Pi(B)/I^{2}M. The combined techniques, known as 4Pi(C) confocal microscopy [38,48] and I^{5}M (I^{2}M and I^{3}M combined) [7,40,41••,P5], respectively, reach higher axial resolutions than any other form of 3D light microscopy.

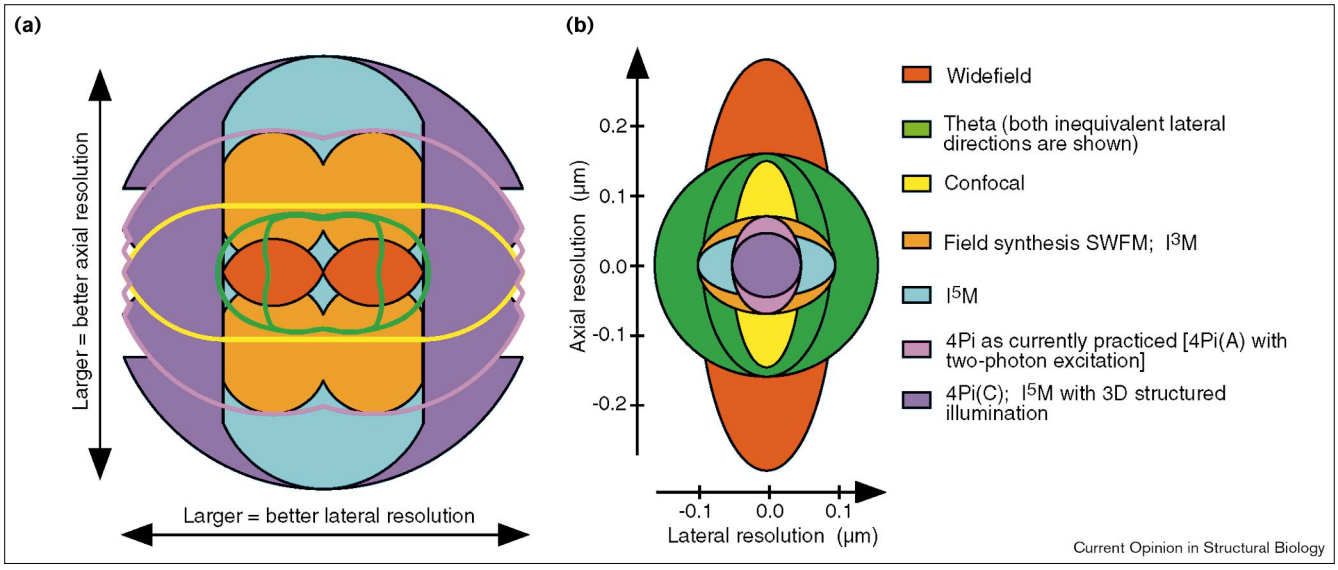
Figures 1 and 2 demonstrate the resolution abilities of 4Pi confocal microscopy and I^{5}M, respectively. Figure 1 uses the 4Pi confocal mode that appears to be favored at present, namely type A combined with two-photon excitation [42]. This mode does not have quite as high resolution as 4Pi(C), but it is simpler to align.

Reconstruction of I^{5}M or 4Pi confocal data is straightforward, because these methods do not suffer from the information gap problem of SWFM (though I^{2}M and I^{3}M do). Both methods, however, require precise optical alignment.

Laterally structured illumination

The idea of spatially varying excitation light (concept 2) is not limited to the axial direction. If a fine lateral structure is introduced into the excitation light of a wide-field microscope (e.g. with a grating or through interfering laser

Figure 4



Comparison of theoretical resolving powers. **(a)** Graphical depiction of the theoretical information-gathering ability of selected microscopies. (Technically, what is shown is the 'optical transfer function support'.) For clarity, some techniques are drawn as outlines. The larger solid-colored areas fully contain the smaller ones. All methods except theta are rotationally symmetric about the vertical axis; for theta, both the (inequivalent) lateral directions are shown. **(b)** Real-space resolved volumes corresponding to the limits in (a), for a wavelength of 550 nm. The volumes are shown as ellipses for simplicity; actual shapes are complex. For comparison, all the methods are assumed to use computer processing to fully utilize the information limits in (a). The figure assumes an objective NA of 0.75 for theta and 1.4 for all the other methods. Two-photon excitation wavelengths are assumed to be twice the emission wavelength; other excitation wavelengths are assumed to be equal to the emission wavelength.

beams), the lateral resolution can be extended using three phases, exactly as is done in the axial direction in SWFM [49••,P5]. If the illumination structure is a simple stripe pattern, it will enhance the resolution in one lateral direction at a time; to cover all directions, the procedure can be repeated once or twice with the pattern rotated to new orientations. More complex 2D patterns that cover all directions at once are also possible; these instead require more phases to decode.

This method can double the lateral resolution of a wide-field microscope, making it equal to that which is theoretically reachable using confocals. Importantly, it does so without discarding any emission light, as no pinhole is needed. Figure 3 demonstrates the power of this method on a 2D test sample (MGL Gustafsson, DA Agard, JW Sedat, unpublished data).

Laterally structured illumination can also — perhaps surprisingly — improve the axial sectioning ability [50,51••,52–54]. This is because purely axial sample structures, for which wide-field microscopy fails, are imaged as laterally patterned structures, for which it does well. This axial effect is maximal at a more slowly varying illumination, which yields only half the maximum lateral enhancement. Such a microscope has a similar optical sectioning ability to a confocal, but it produces real-time images and does not require a laser light source.

An alternative to the three-phase method of decoding the images is to do part of the decoding optically. Typically, this is done by passing the emitted light back through the same mask as was used in the illumination [52–54]. This makes operation very simple and allows the use of complex 2D illumination structures [52], at the cost of losing some light.

Three-dimensional structured illumination

There is no fundamental reason to limit the illumination structure to be purely axial or purely lateral [P2,P5]. Complex 3D patterns that satisfy certain conditions can be decoded using the same multiphase method described above [P5]. Such patterns will allow simultaneous axial and lateral resolution enhancement. A prototype microscope is now being completed (MGL Gustafsson, DA Agard, JW Sedat, unpublished data) that integrates 3D structured illumination into I^{5}M. This system is expected to combine the lateral resolution enhancement shown in Figure 3 with the axial enhancement of Figure 2, yielding a resolution of 0.1 μm in all directions without scanning and without discarding any emission light.

Discussion

Figure 4 attempts a graphical comparison of the theoretical resolution limits of some of the above techniques. To allow comparison, Figure 4b generously assumes that computer processing is fully applied in all methods — in practice,

not all of the methods may produce a sufficiently good signal-to-noise ratio to allow meaningful processing. It is also important to remember that each method has strengths and limitations that are not visible in such graphs.

It is clear from Figure 4 that three families of methods stand out — 4Pi confocal microscopy, I^{5}M (especially with 3D structured illumination) and SWFM. Of these, 4Pi confocal microscopy and I^{5}M are straightforward to reconstruct, as they do not suffer from the gap problem. All three share the element of illuminating and/or observing the sample from both sides using opposing objective lenses. Not coincidentally, they also share the limitation that the sample cannot be arbitrarily thick, as it must be possible to view each focal plane from both sides. In fact, the thickness limit is particularly stringent because the interferometric nature of these methods makes them sensitive to refractive index variations in the sample. This limitation can be countered using sample-mounting protocols specifically aimed at optical homogeneity and/or using adaptive reconstruction algorithms that allow the interferometric phase to vary with position. The ultimate thickness limit is yet to be established, but it is likely to be of the order of tens of microns [41••,45•], which should bring a large class of biological questions within reach of these methods. Live samples that must be mounted aqueously may face somewhat stricter limits.

Comparing 4Pi confocal microscopy with I^{5}M, the former is even easier to reconstruct because light from far out of focus planes is suppressed, whereas the latter is more light efficient. As it is a confocal scheme, 4Pi yields a higher lateral resolution in those situations in which one can afford to discard emitted light by using a small pinhole; the generalized structured illumination scheme described above promises to bring the same lateral resolution to an I^{5}M-like setup that discards no light. Perhaps the most important difference is that 4Pi confocal microscopy, as presently practiced, is a sample-scanning technique that acquires about 50 voxels/s [45], whereas I^{5}M exposes an entire image plane at once and reads it out at about 1 million voxels/s. The much higher speed of I^{5}M allows much larger areas to be studied. The original data set in Figure 3c (twice the area shown) was $512 \times 512 \times 160$ voxels; it was acquired in 12 min on an I^{5}M, but would have required almost 24 h on a sample-scanning 4Pi confocal microscope.

Conclusions

Several methods now exist that can extend the spatial resolution of fluorescence microscopy by impressive factors. Although no method is applicable to all samples, I believe that extended resolution fluorescence microscopy will soon play an important role in biological research. The I^{5}M and 4Pi confocal methods, in particular, will allow a large class of biological questions to be addressed by fluorescence microscopy at a previously unreachable 3D sizescale of 0.1 μm.

The near future should bring the first demonstrations of at least one major new technique — 3D structured illumination microscopy. This method, particularly in combination with I^{5}M detection, promises an unprecedented combination of 3D resolution and light efficiency. We may also see lateral resolution pushed to new ground using highly nonlinear schemes, such as STED.

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Patents

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