Breaking the resolution limit in light microscopy

Rainer Heintzmann and Gabriella Ficz

Abstract

Fluorescent imaging microscopy has been an essential tool for biologists over many years, especially after the discovery of the green fluorescent protein and the possibility of tagging virtually every protein with it. In recent years dramatic enhancement of the level of detail at which a fluorescing structure of interest can be imaged have been achieved. We review classical and new developments in high-resolution microscopy, and describe how these methods have been used in biological research. Classical methods include widefield and confocal microscopy whereas novel approaches range from linear methods such as 4Pi, I^5 and structured illumination microscopy to non-linear schemes such as stimulated emission depletion and saturated structured illumination. Localization based approaches (e.g. PALM and STORM), near-field methods and total internal refraction microscopy are also discussed.

As the terms 'resolution', 'sensitivity', 'sampling' and 'precision' are sometimes confused, we explain their clear distinction. Key concepts such as the point spread function and the Abbe limit, which are necessary for an in depth understanding of the presented methods, are described without requiring extensive mathematical training.

Keywords: *fluorescence microscopy; high resolution; Abbe limit; point spread function; sensitivity; sampling; localization precision; nonlinear microscopy*

INTRODUCTION

Fluorescent imaging microscopy has been an essential tool for biologists over many years, especially after the discovery of the green fluorescent protein and the possibility of tagging virtually every protein with it. Recent advances in fluorescence microscopy have dramatically enhanced the obtainable optical resolution [1], enabling the users to inspect structures of interest at finer and finer level of detail. The aim of our review is to describe some of these methods (Table 1), and how they break the resolution limit.

Usually, if the aim is to see the structure of a cell down to molecular detail, one has to reside to electron microscopy or electron tomography [2]. However this requires sample fixation which by itself can alter the micro-structure, even when preparative methods such as shock freezing or high-pressure freeze substitution are used. In addition, it is not easily possible to label and distinguish multiple targets inside the cell. The common approaches such as tagging with metal particles of different sizes [3] help to some extent, but these sparse markers usually serve as mere indicators that in their vicinity the structure of interest can be expected. Finally, but most importantly, electron microscopy at this level of resolution is not possible for living samples.

Light optical microscopy and especially fluorescence microscopy does not suffer from the above problems. The labelling of targets such as individual genetic loci, specific proteins or organelles is possible inside living cells, which led to the extensive use of fluorescence microscopy in life sciences [4]. Other microscopic modes usually lack this high specificity, but do sometimes provide other useful information such as the orientation of molecular species in polarization microscopy [5]. Modes such as differential interference contrast (DIC), phase contrast or dark field are useful to discriminate and follow

Corresponding author. Rainer Heintzmann, Tel: +44 20 7848 6519; E-mail: Rainer.Heintzmann@kcl.ac.uk

Rainer Heintzmann (1970) studied Physics in Osnabrück, Germany. After his PhD in Heidelberg (Supervisor Prof. Dr C. Cremer) he continued his work on fluorescence microscopy development for 5 years in the laboratory of Dr Thomas Jovin, MPI for Biophysical Chemistry, Göttingen, Germany. He now heads a research group, the Randall Division, King's College London, U.K.

Gabriella Ficz (1978) studied Biology in Iasi, Romania. After her PhD in Göttingen, Germany (Supervisor Dr D. Arnd-Jovin) she now works on mammalian epigenetics and reprogramming within the group of Dr. W. Reik, The Babraham Institute, Cambridge, U.K.

© Oxford University Press, 2006, All rights reserved. For permissions, please email: journals.permissions@oxfordjournals.org

Table I: Fluorescence Microscopy Methods

Method	Common abbreviations	Abbe applies	Remarks	Best resolution in nm	Ref.
Widefield	WF	Yes	The resolution depends on the numerical aperture, NA (not the magnification!). High NA is achieved by immersion objectives. The sample has to be immersed in the same refractive index as immersion medium.	~230 (XY) ~1000 (Z)	[46]
Confocal	CLSM, LSM	Yes	In theory: Twice the resolution of widefield microscopy. Local dose of light is very high for a short time. Potentially more photo-damage. Photons are blocked by the pinhole, wasting useful information. Fine with living cells.	~180 (XY) ~500 (Z)	[46]
4Pi	4Pi (Type A,B,C)	Yes	A second lens is used and adjusted to coherently participate in the imaging. Demonstrated with living cells, multiple foci preferred.	~200 (XY) ~90 (Z)	[27, 46]
I^5M	I^5M	Yes	Similar to the above except no scanning needed and an incoherent full widefield illumination is used from both sides. Potentially faster.	~230 (XY) ~90 (Z)	[26]
Localization	Pointillism PALM, STORM	Yes	Optics within the Abbe limit, but the task (localizing single particles) is not restricted to it. PALM and STORM managed (for the first time) to reconstitute full images from many thousand localized molecules. Particle discrimination by colour, fluorescence lifetime, blinking, bleaching, photo-activation followed by bleaching. Not demonstrated in 3D, no living cells yet.	~20 (XY)	[6–9, 12–23]
Structured illumination	SIM, PEM, LMEM, HELM, Lattice Microscopy	Yes	Currently ~10 CCD images required for a slice. So far, no 3D data published. Requires extensive image processing. Potentially fast. No living cells demonstrated yet.	~100 (XY)	[28–30, 46]
NonLinear structured illumination	SPEM, SSIM	No	Currently ~100 CCD images required for one single reconstructed plane. Sample must not move within this time. No living cells demonstrated yet.	~50 (XY)	[31, 32]
2 Photon	2P	No	Nonlinear gain in resolution is cancelled by longer wavelength. Inherent sectioning without a pinhole. Only in-plane bleaching. Deep tissue possible due to long illumination wavelength. Fine with living cells.	~200 (XY) ~ 400 (Z)	[46]
Stimulated emission depletion	STED	No	The saturation of the stimulated emission circumvents the limit. Combination with 4Pi possible. Not yet demonstrated in 3D. No living cells demonstrated yet.	~16 (X) ~20 (XY) ~50 (Z)	[35–40, 46]
Evanescent Wave	TIRFM	No	Resolution improvement only along Z. Can be combined with structured illumination. Contact to surface of different refractive index required. Fine with living cells. 3D stacks not demonstrated.	~230 (XY) ~100 (Z)	[30, 44, 46]
Near Field	SNOM, NSOM	No	Only applicable if scanning tip is in close proximity (<10 nm) to the sample. Living cells possible. 3D stacks not yet demonstrated.	~30 (XY) ~10 (Z)	[42, 43]

WF, widefield; CLSM, confocal laser scanning microscopy; LSM, laser scanning microscopy; 4Pi, stands for the full solid angle of a sphere; I^5M, incoherent interference illumination microscopy image interference microscopy; PALM, photoactivated localization microscopy; STORM, stochastic optical reconstruction microscopy; SIM, structured illumination microscopy; PEM, patterned excitation microscopy; LMEM, laterally modulated excitation microscopy; HELM, harmonic excitation microscopy; SPEM, saturated patterned excitation microscopy; SSIM, saturated structured illumination microscopy; 2P, two photon; STED, stimulated emission depletion; TIRFM, total internal reflection microscopy; SNOM, NSOM, scanning near field optical microscopy.

cells or structures within them without the need for specific labelling.

However, classically the resolution of all of these light microscopic modes used to be far below that of the electron microscope, and only some recent approaches have made significant progress in resolution increase.

WHAT IS RESOLUTION?

The term ‘resolution’ has to be discriminated from ‘sensitivity’, ‘sampling’ and ‘precision’. Subsequently we will try to clarify the meaning of these terms.

One can have a very sensitive light microscopic system which makes it possible to see single virus particles or even single fluorescent molecules [6–9]. This only means we would have single molecule sensitivity, but the size of such a molecule in the image could, for example, still correspond to 0.5 μm in the sample coordinate system, which would be a relatively poor optical resolution.

Another common confusion is the difference between resolution and sampling, which relates to magnification. It is easy to magnify an image of the sample by optical means to any desired degree, however, the process of magnification does not increase the optical resolution; at best it preserves it. When an image of the sample is detected by our eyes or for example a CCD camera, it gets ‘sampled’ into a discrete set of measured intensity values, one for each detector element (such as a photosensitive cell in the brain). These sampling points correspond to nominal positions in the coordinate system of the object under investigation, without a limit to their density. The magnification has to be adjusted such that the finest level of detail present in the image is still measured (sampled) by at least two such detector elements, but any denser sampling will not yield new information about the sample. For a detailed discussion on sampling in optical microscopy see [10, 11]. Magnification significantly beyond this limit is sometimes called ‘empty magnification’. When there is a noise attributable to the readout process of the detector (‘read noise’), such empty magnification should be avoided as it deteriorates the image quality.

In many cases there can be very specific questions in biology which are to be answered by microscopic imaging. Such questions could be: ‘What is the spatial distance between two specific genetic loci in the nucleus or between two molecules on the cell surface?’ To answer these questions, there is no need for a resolution in the order of this distance, but the error of localization (the reciprocal of the localization precision) needs to be below this expected distance. That means that the distance between the estimated and the true centre position of the object has to be below the distance between the two objects. Localization precision can be far higher than the optical resolution [12, 13] (e.g. the localization error of single molecules can be smaller than 10 nm on a system of 200 nm optical resolution), and we can use this even for relative position determination between multiple loci, as long as we are able to discriminate between the target genes (or other small structures) in our image. Such discrimination can be achieved by using multiple colours [14–16], differences in fluorescence lifetime [17], photo-bleaching [12, 18], the individuality of statistical blinking events [19] and photoactivation [8, 9]. The higher the resolution the better the localization precision, but how much better than the optical resolution depends strongly (with a square-root dependence) on the number of photons collected from each target. An impressive application of the localization idea was to observe the molecular steps of Myosin V [20, 21]. Another interesting application was to follow the kinetics of the receptor-mediated entry of NTF2 and transport of NTF2 and transportin 1, with and without transport substrate, into the nucleus [6]. The method of speckle microscopy [22] uses precise localization for the tracking of molecular clusters to reveal their temporal behaviour. Nanosizing [23] can be used to address the question of the size of biological particles of known shape.

Even when objects are not point-like structures, the position of features like straight edges (e.g. tubulin fibres) or planes (e.g. plasma membrane) can often be determined very precisely by microscopy methods. All of these methods are based on localization precision, which should not be confused with the term resolution as discussed subsequently.

When we talk about high resolution of an optical instrument in this article, we mean the ability to see a structure at a high level of detail. There are various ways to define the term resolution more precisely which are discussed in the two separate boxed sections.

Although, the definitions of resolution [FWHM (Full Width of the point spread function measured at Half its Maximum) see Figure 1, Rayleigh and

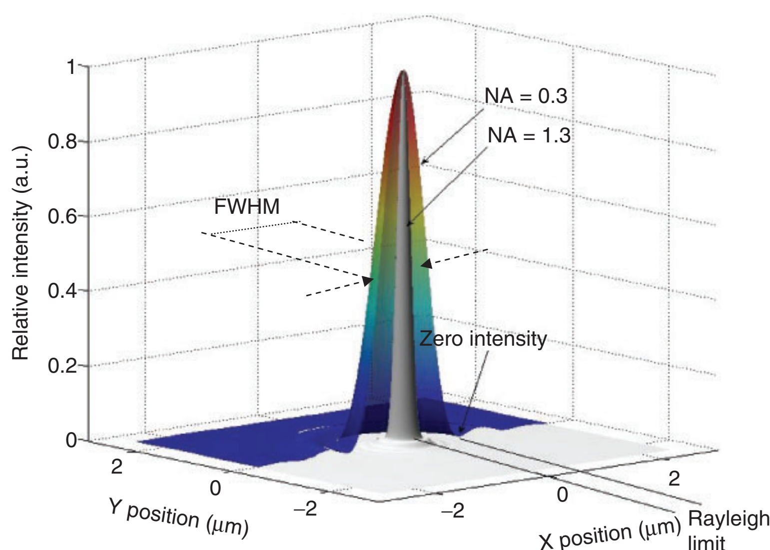

Figure 1: The Point Spread Function (PSF). Two PSFs are shown, one for high numerical aperture (NA = 1.3 in grey) and one for NA = 0.3 (slightly transparent shades). As can be seen the low NA PSF is wide and has well-defined positions of zero intensity, leading to the definition of the Rayleigh limit. For this PSF also the definition of full width measured at half the maximum (FWHM) is shown. The high NA PSF (uniformly grey peak in the middle) is much finer, but does not have the rings of zero intensity. The colour version of this figure can be found in www.bfgp.oxfordjournals.org

Sparrow limit] mentioned in the boxed section 'The PSF' may be useful in some cases, we would like to use a different limit called the Abbe limit (see other boxed section) within this article, as this limit has a very direct relationship to which light rays are captured by the objective lens of the microscope.

It is interesting to note that the ability to precisely localize can be turned into a genuine 'resolution' when many closely spaced particles can be discriminated. From the precise localization of these particles a picture can be constructed by painting dots at each localized position. A method termed 'Pointillism' [19]. A very successful way of discriminating closely spaced molecules is using a repetitive cycle of faint photo-activation followed by bleaching of the activated molecules [8, 9]. In every cycle only very few molecules get activated, so the chance of PSF overlap is small, and the localization thus very precise. In fixed samples it was possible to reconstruct impressive images of cryo-prepared thin section from a COS-7 cell expressing CD63 (lysosomal transmembrane protein) tagged with Kaede and dEosFP-tagged cytochrome-C oxidase import sequence in mitochondria of COS-7 cells down to a resolution of about 20 nm [8]. Currently a problem is the rather long imaging time well above an hour for a single slice image, but further development should obviate this.

METHODS WITHIN THE COMBINED ILLUMINATION AND DETECTION ABBE LIMIT

A number of modern microscopy techniques such as confocal, 4Pi and structured illumination microscopy achieve their high resolution (see boxed section 'The Abbe limit') by a spatially non-uniform illumination (Table 1). Non-uniform illumination implies that adjacent positions in the sample can receive different illumination intensity. Subsequently, we will describe in detail how the resolution enhancement is achieved for these methods.

In confocal microscopy [46] the sample is illuminated with a focused beam, which is then raster scanned over it. The emitted fluorescent light is imaged onto a pinhole, whose diameter can be adjusted. Due to the combined effects of illumination and detection, this procedure does indeed enhance the resolution beyond that of a standard widefield fluorescence microscope. If the pinhole is imagined to be very small, the light distribution used for illumination is very similar (except for the usually small difference in wavelength caused by the Stokes shift) to the map of how sensitive the detection system (with a small pinhole) can detect from each position in the sample (called the detection PSF). For light particles (photons) to make it to the detector a particular sample point needs to be illuminated

Box I: The point spread function—the sample as a sum of points

The point spread function (PSF) describes how a very small (in theory infinitely small) emitter gets imaged by an imaging system.
As a sample can be thought of consisting of many points (in the limit infinitely many) each with its own strength, the image can be described as an equivalent sum of corresponding PSFs. Most optical systems (called 'linear shift invariant') can be idealized as having a position-independent PSF, where emitters at every position in the field image to the same PSF shape.[6] This feature simplifies optical theory significantly as the imaging operation can then be described by a mathematical operation called 'convolution'.
In Figure 1, two examples of typical PSFs of a widefield fluorescence microscope are shown. As can be seen the PSF sizes are quite different for the numerical apertures[7] of 1.3 and 0.3. Imaging with a microscope is as if painting the object points with a brush of the size of the PSF. A useful resolution measure is thus this size as measured by the full width of the PSF measured at half its maximum (FWHM) (Figure 1). Thinking about the situation of 3D samples to 'paint', one will have to change the size of the brush (bigger and more disc-shaped when out-of-focus) for each object slice to paint, or envision the whole 'painting' operation as a 3D process with the appropriate 3D PSF which would then yield an entire 3D focus series.
In the in-focus PSF at low NA, the intensity reaches zero value at a well-defined closest distance (see arrow in Figure 1). The circle of the PSF inside the first zero position is called the Airy disc and its radius is called the Rayleigh distance.
The Rayleigh resolution limit uses the example of two point-like objects and defines the resolution as the distance, where the maximum in the image of each of these objects occurs at the position (Figure 1) where the image of the other object has its first intensity minimum.[8]
The Sparrow limit is the distance between such point objects of equal intensity at which a dip half way between them ceases to be visible in the superposition of their images (the first two derivatives of the intensity curve along the connecting line become zero).
All these limits can be to some degree changed by optical engineering approaches that vary the amount to which some light rays contribute to the image (sometimes referred to as apodization). Such modification usually introduces higher side-lobes (a side-lobe can be seen just right of where the arrow labelled 'zero intensity' points to in Figure 1), which are unwanted. Due to their non-absolute nature these limits could be called 'soft' whereas the Abbe limit as discussed in the other boxed section is a 'hard' limit in the sense that apodization approaches do not alter it. The Abbe limit makes an exact statement about which information about the sample is transmitted by the microscope.

(e.g. 1/10 probability) *and* light emitted from this point needs to be detected (e.g. 1/10 detection probability). This chance of illumination and detection (in the above example $1/10 * 1/10 = 1/100$) in combination with a linear response of the sample leads to the resulting PSF of the overall system to be the product of the illumination and detection PSFs. Discussed in terms of periodic distributions inside the sample, this confocal system is now in principle able to pick up a 2-fold finer periodicity.

However, in practice this is almost never achieved as the fine details (high spatial frequency[1]) are suppressed so much that they get lost in the noise. To make matters worse, a closed pinhole would not detect any light, which leads to pinhole diameters in the range of the diameter of an Airy disc (where the first theoretical minima occur in the image of a point) to be used in biological experiments. In this case, even features slightly finer than the standard uniform widefield illumination Abbe limit cannot be resolved as they get lost in the noise. This reduces the in-plane performance of a confocal microscope back to that of a uniform illumination system, however the increased sectioning ability of confocal microscopes is important and lead to their success.

By altering the intensity and phase of the light which gets sent into or detected from the sample under different angles, a technique often referred to as 'apodization' or pupil plane filtering, the high spatial frequencies can be emphasized, getting the practical performance a bit closer to the theoretical

Box 2: The Abbe limit—theory of the sample as a sum of waves

The Abbe limit corresponds to the distance of the finest periodical structure which can be imaged by an optical microscope.
To understand the importance of the Abbe limit it is useful to first think of a sample being a periodical structure. The simplest case could be a series of lines (in 2D) or even planes of alternating brightness (in 3D) describing the distribution of fluorophores. Such a restriction to a specific sample structure may seem to be far fetched at first, but it can be shown (when we also allow negative values), that an arbitrary sample structure (e.g. an image with cells) can always be described in this picture as a simple additive superposition of such periodical structures (waves). This would normally imply an infinite amount of waves, each with an individual orientation, wavelength and phase shift. Mathematics shows that in a somewhat magical way these waves can cancel each other in the dark sample areas and in total sum up such that the exact brightness structure of the sample can be represented in this way. In two dimensions, each of these waves would be like a sinusoidal line grating, a series of positive and negative line-like features. If we can understand how images are formed of these wave structures, we can still represent the final image as the sum on these individual images of wave structures.
Why is such a periodic wave structure-sample especially useful for discussing the resolution of optical systems? All classical far field optical systems have a well-defined fixed limit to the distance between these periodical structures in the sample to be at all present in its image. In the case of methods based on imaging scattered, diffracted or refracted light from the sample, such as transmission, phase contrast, DIC, dark field and others, this fixed limit can be easily understood: The light leaving the sample needs to be captured by the objective lens of the microscopy system to contribute to the image by interference[9] (Figure 2a). Due to the laws of diffraction, a finer periodic (grating-like) structure will lead to the light leaving the object at a higher angle hitting the objective closer to its finite aperture limit and diffraction from a very fine periodic structure will miss the objective lens (Figure 2b). Thus the aperture of the objective defines this resolution limit. When altering the incident direction of the light away from normal incidence to the plane of focus, the diffracted light will also emerge at a different angle. In this way, under oblique illumination, light which would miss the objective lens at normal incidence can then be directed into the acceptance angle of the objective. However, also in this case a limit is placed by the available angular range (thus the $NA_{illumination}$) of the condenser optics. Finally, even if one could illuminate an object under all possible angles and detect the light leaving the sample under all possible angles, there would still be a very finite resolution to the image. As the diffracted angles relate to the period of the grating in relation to the wavelength of light in the medium the finest possible grating that could be observed in this way is spaced at half this wavelength. As the wavelength in a medium is smaller (by the refractive index of this medium) than in vacuum or air, the best resolution is achieved by embedding the sample and the front side of the objective lens in a medium of high refractive index. This is usually done with oil immersion (with $n = 1.518$). The combination of the refractive index of the medium and the angle of detection (aperture half angle α) is conveniently summarized in the definition of the numerical aperture ($NA = n \sin(\alpha)$). This NA, together with the illumination angles and the wavelength thus define the Abbe resolution limit:

$$d = \frac{\lambda}{NA_{illumination} + NA_{detection}} \tag{1}$$

In fluorescence the situation is slightly different, as the light from different fluorescent molecules has to be incoherently[10] added as intensity instead of being added as coherent amplitude (intensity and phase), since their emitted light has a random mutual phase relationship. The image of each such molecule will nevertheless be formed by the rays leaving this molecule and by their coherent interference for each wavelength in the image plane (Figure 2c). There is a spatial frequency limit to such an image of a point as defined by the limiting angles and thereby also here similar restrictions to the resolution do apply. For the detection of a widefield fluorescence microscopy the corresponding Abbe limit becomes:

$$d = \frac{\lambda}{2NA_{detection}} \tag{2}$$

(continued)

Box 2: Continued

This factor of 2 in equation 2, when compared with $NA_{detection}$ in equation 1, stems from the fact that in an incoherent fluorescence PSF, the finest detail stems from mutual interference of the highest angled rays (e.g. left with right side), whereas in transmission, the finest scattering structure would be defined by the difference of the incident illuminating ray (e.g. in the middle of the aperture) and the highest angled rays that are captured (e.g. at the side of the aperture).

The rays striking the objective lens at the highest angle (being closest to the edge of the aperture in the back focal plane) define the finest possible fluorescent periodic structure which can be resolved. Finer such structures would only be imaged as a homogeneous level of brightness with no modulation what so ever. One may now wonder, whether changing the illumination method could help to further enhance the resolution. This turns out to be a good approach. However, just illuminating at an oblique angle in fluorescence microscopy will not change anything, since the emitted light of a molecule cannot interfere with the illumination or the light emitted from any other molecule. It is necessary to generate local intensity differences in the sample in order to gain resolution. In the main text, a few of these systems with a non-uniform illumination are discussed.

In analogy to the discussion on the Abbe limit previously mentioned, a similar limit exists to describe the finest possible intensity feature in the illumination light field that can be generated, when illuminating through the objective lens. Usually the fluorescent sample reacts linearly to the incident light intensity, which means its emitted light is proportional to the incident light at this specific sample position. This multiplication leads to the fact that the two Abbe limits (for illumination and detection) can simply be added yielding a new combined Abbe limit. Although some authors using these effects have called the new resolution limit 'beyond' the Abbe limit, we like to call this combined illumination and detection limit still within the Abbe limit, in the sense that the microscopy system adheres to a limit obtained by the addition of the illumination and the detection Abbe limit.

limit of twice the widefield resolution with uniform illumination [24, 25]. Nevertheless, to our knowledge, no such system has ever been successful enough to make it into the market.

The I^5M and the 4Pi type C microscope take both the illumination aperture and the detection aperture to their extreme [26, 27]. As the name 4Pi suggests (a full sphere has a solid angle of 4π), the idea is to get as close as possible to illumination (and detection) from all sides of the sample. For practical reasons the best approximation is achieved by combining two objective lenses opposed to another along the Z (optical axis) direction to act as a single lens. To do so, the two optical paths to the sample (through the top and though the bottom objectives) need to be identical in length, both for the illumination and detection wavelengths (4Pi type C microscope). From the discussion above it can be seen that the maximal possible numerical aperture is obtained along the optical axis (Z) to which the two objectives are perpendicular (becoming identical to the refractive index), whereas little changes along the X and Y directions. Thus for illumination the resolution limit is $\lambda_{ex}/(2n)$ and for detection it is $\lambda_{em}/(2n)$, with λ being the respective wavelengths of excitation and emission. This means the finest periodical fluorescent structure which can be detected in a 4Pi (type C with visible excitation) microscope is about $\lambda/(4n)$, which amount to approximately 100 nm along the optical axis for glycerol embedding and a wavelength around 500 nm. However, considering the in-plane directions (X and Y) this realization of the 4Pi concept has not increased the numerical aperture (and thus the resolution) as compared to a standard confocal microscope. Noticeably the lateral resolution, albeit in theory improved by a factor of $\sim$2, suffers also from the same problem as standard confocal microscopes, with practically no XY-resolution improvement in comparison to a uniform illumination microscope, detectable for reasonable pinhole sizes. Nevertheless the enormous improvement along the axial direction is worthwhile (Figure 3b). Features in only slightly different planes would normally blur together in one image plane, but are now separated nicely in a 4Pi microscope. This leads also to a much cleaner appearance of individual slices of a 4Pi data set. Similar arguments apply to the I^5M method, where incoherent illumination is used [26].

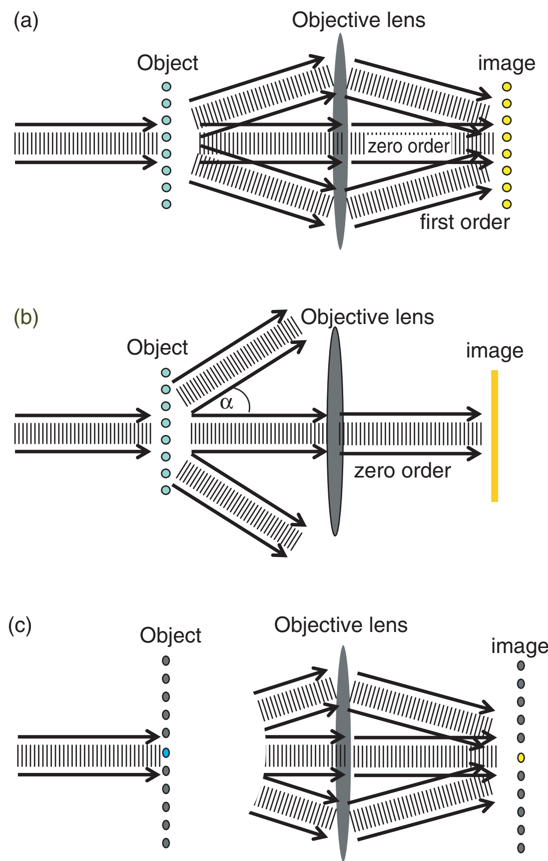

Figure 2: The Abbe limit (a) Diffraction of a parallel light beam from a periodic grating structure (only the zero and first diffraction order are shown) (b) Diffracted light from a fine periodical structure already fails to fall within the angular acceptance range of the objective lens. The image can thus only be formed from the undiffracted zero order yielding a flat image of equal intensity. (c) For fluorescence the emitters have to be considered independently and intensity will be added. Nevertheless, the finest resolution (constituent of the PSF) is still defined by the biggest angle of emitted light rays that were captured and refocused. The colour version of this figure can be found in www.bfgp.oxfordjournals.org

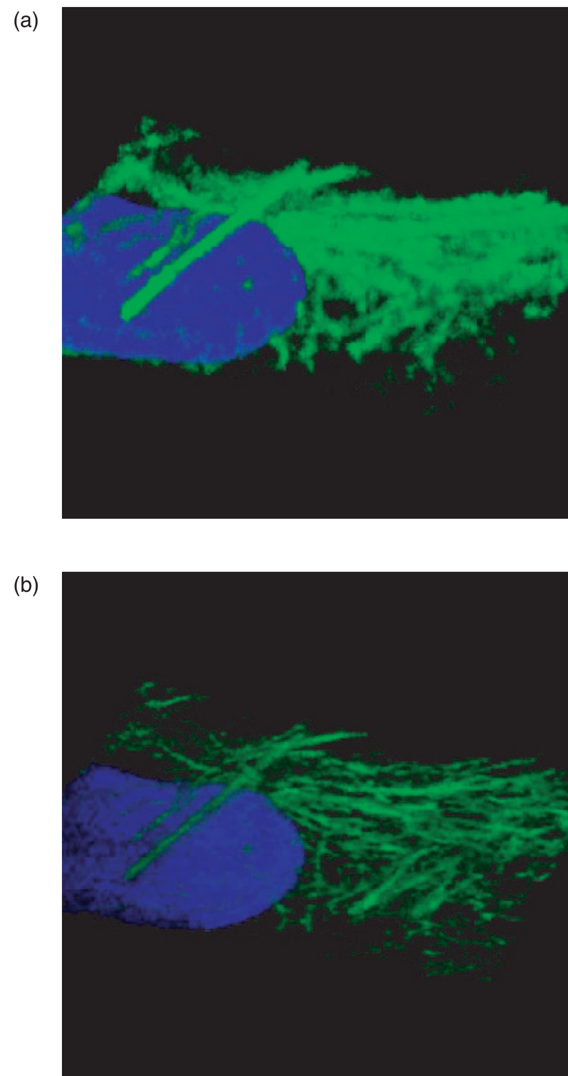

Figure 3: Confocal and 4Pi Image. Comparison of volume rendered images of the actin cytoskeleton and the nucleus of muscle support tissue endothelial cells. The nucleus was stained with DAPI and the actin cytoskeleton with Alexa488-phalloidin. (a) 2-photon confocal view of the sample (b) 2-photon 4Pi (type A) view (after 3-point deconvolution) of the sample. The improved resolution and better discrimination of biological structures is visible. This data set corresponds to a width of 23.4 μm, a height of 2.9 μm and a depth of 30.0 μm. Sample prepared by Elisabeth Ehler. The colour version of this figure can be found in www.bfgp.oxfordjournals.org

The methods of structured illumination [28–30] exploit the fact that the excitation and emission Abbe limit can be added. The highest possible spatial frequency is intentionally introduced during excitation via a fine sinusoidal illumination pattern in the sample volume. This is achieved by projecting a fine grating[2] into the sample under coherent illumination conditions, which allows the contrast of the excitation structure to be maximal. A high-resolution image can be reconstructed (Figure 4) from a series of images under different positions (and orientations) of the excitation pattern. In comparison to confocal microscopy, structured illumination captures high spatial frequencies of the sample much more efficiently, and thus allows for a true factor of two resolution improvement [29], albeit at the expense of requiring extensive computational reconstruction.

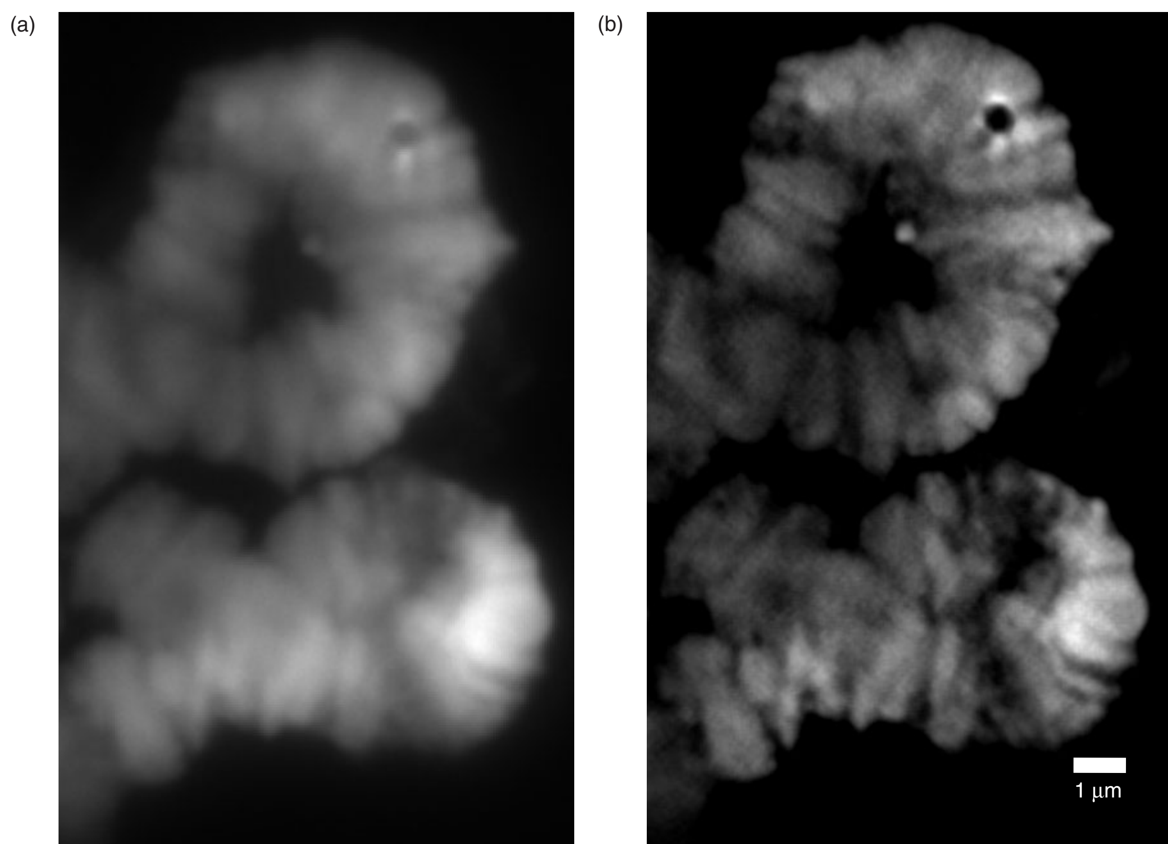

Figure 4: Linear structured illumination of *Drosophila* Polytene chromosomes stained with Sytox Green (a) Widefield image obtained by summing all the individual images under structured illumination. (b) Reconstruction of the sample from the structured illumination data (5 phases, 2 angles).

METHODS CIRCUMVENTING THE ABBE LIMIT

The most advanced high-resolution microscopy methods use some form of nonlinearity to avoid the restrictions of the Abbe limit. In the following section, the importance of the non-linearity is stressed and exemplified with two-photon microscopy, nonlinear structured illumination and stimulated emission depletion microscopy.

As mentioned in the boxed section 'The Abbe Limit', the linear[3] response of the sample led us to define the combined illumination and detection Abbe limit as a fundamental limit in far field microscopy. This limit, however, does not apply if this linear relationship can be violated. Abbe's theory has to be modified if the emitted light can only be described by a nonlinear[4] dependence of emission on the local excitation intensity. As it turns out, the highest exponent present in a polynomial expansion of the nonlinear dependence between excitation and emission defines the resolution limit [31]. Every exponent in such an expansion of the nonlinear dependence of emission on local excitation can be thought of a number of additional multiplications, each adding another excitation Abbe limit to the dependence.

The simplest non-linear dependence is encountered in two-photon microscopy [46]. Due to the probabilistic nature of the two-photon absorption process (both photons need to interact at the same time) the chance for a fluorescent molecule to be in the excited state is proportional to the square of the incident intensity at a specific position in the sample. Thus also the emitted light intensity is proportional to this square of the excitation intensity. As a result two-photon microscopy indeed circumvents the Abbe limit by a factor of two in the excitation pathway. However, this improvement is bought at the expense of a roughly 2-fold longer excitation wavelength leading to essentially no additional gain, when compared with a standard single photon excitation in a confocal microscope. However, the significant advantage of the two-photon microscopy is the reduced absorption and scattering, allowing to

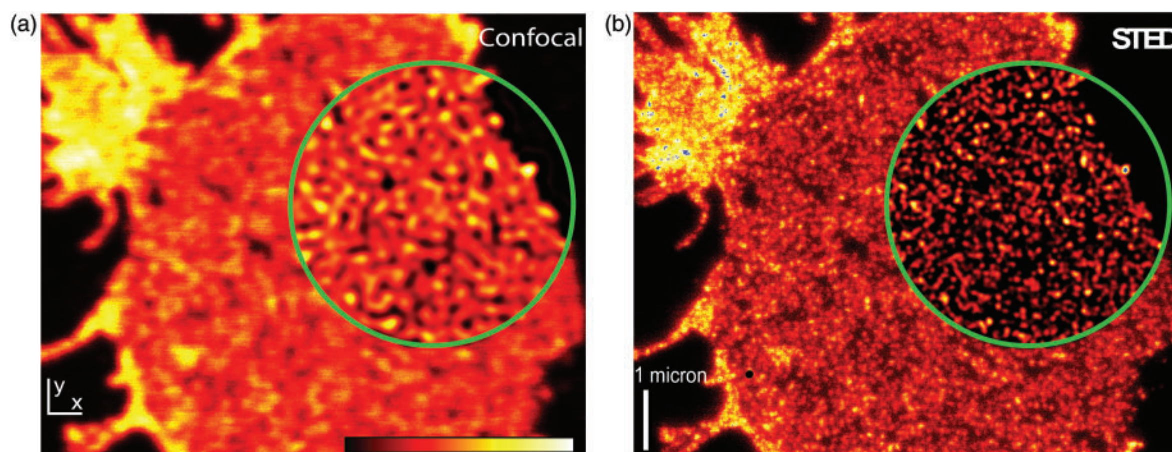

Figure 5: Stimulated emission depletion image. Revealing the nanopattern of the SNARE protein SNAP-25 on the plasma membrane of a mammalian cell. Confocal (a) vs. STED image (b) of the antibody-tagged proteins. The encircled areas show linearly deconvolved data. STED microscopy provides a substantial leap forward in the imaging of protein self-assembly; here it reveals for the first time that SNAP-25 is ordered in clusters of ‹60 nm average size. (reproduced with permission of the Institute of Physics from reference 39). The colour version of this figure can be found in www.bfgp.oxfordjournals.org

image deeper into tissue and the inherent confocality due to the squaring of the excitation intensity, obviating the need for a pinhole on the emission path. Another advantage of the square dependence is the reduction of sidelobe height, which is particularly useful in 4Pi microscopy.

However, other processes can achieve this nonlinear dependency with the help of visible light, and are also not limited to a maximal exponent of two in the polynomial expansion. The simplest case is saturation of the excited state of fluorophores by intense illumination [31]. Practically this can be realized as a microscopy method by combining the nonlinearity with the concept of structured illumination [31, 32], or by detecting high harmonics in the time domain under temporally sinusoidally modulated illumination intensity in a confocal system [33]. Also the spatial non-uniformity of a depletion, bleaching or light-induced inactivation process (dynamic saturation optical microscopy, [34]) can be used, which is again a nonlinear saturation effect. All of these methods are still in a developmental stage and some of them (nonlinear structured illumination, dynamic saturation optical microscopy) require a substantial amount of image processing, since the high-resolution information contained in the measured data has to be computationally extracted.

The most successful method so far is stimulated emission depletion (STED) microscopy [35–39]. The method itself is slightly more complex, but has the advantage of obtaining a directly scanned image without the need of image processing. The idea is to deactivate fluorophores surrounding the centre of the scanning spot by stimulated emission[5] by the help of a second beam (the doughnut-shaped STED beam), with a wavelength being within the emission spectrum of the fluorophore. If this was only done with a relatively dim STED beam this method would add another linear term, leading only to a moderate resolution improvement. However, driving this STED beam into saturation establishes the required nonlinearity of high order. In the centre of the spot to detect this STED beam is expected to have zero intensity, leaving the fluorophores unchanged in their excitation state. If a little bit of STED beam is present at a location only slightly away from its centre (zero intensity) position, its long enough interaction with the fluorophore pushes the latter into the ground-state. This will force emission of light at the wavelength of the STED beam, which can be specifically blocked by appropriate filters allowing most of the spontaneous fluorescence emission to pass. Also a temporal discrimination is possible. It has recently been demonstrated that STED can be applied to imaging of endogenous markers such as green fluorescent protein (GFP) [38]. By allowing the triplet states to relax between successive excitations, the fluorescence yield in STED microscopy could be dramatically improved [40].

A practical rule of thumb can be established as alteration to Abbe's equation [41], with the aim to

estimate a resolution criterion considering the limited signal to noise ratio available. However, such a rule of thumb should not be taken to be a new Abbe limit, as the Abbe limit sets a fixed limit to linear resolution methods, whereas the suggestion in [41] is an approximation to the width of the directly obtained PSF, which may or may not be a good indicator of the smallest periodical structure that can be discriminated for a given noise level in the system. In principle all of the saturation-based nonlinear methods have infinite resolution, but the high-resolution information becomes obscured by the noise.

A COMMENT ON NEAR FIELD METHODS AND PENDRY'S LENSE

Obviously the distribution of emitting fluorophores in the sample can have any structure, and is not constrained by the Abbe limit. The sample is just as it is with no limit to its periodical constituents, but the light intensity is known to fall off by an inverse square law (in the scalar approximation) with the distance to each emitting molecule. However, such a light distribution within the sample would also not be limited by the Abbe limit. The discrepancy to the light detection discussed so far arises from the fact that in the aforementioned theory, we only considered light waves that are able to propagate (so called far field waves). However, Maxwell's equations in a homogeneous medium allow also for a different kind of oscillating electro-magnetic fields called evanescent (or near field) waves. These solutions of the Maxwell equations in a homogeneous medium decay exponentially in a direction of space. Even worse; the finer the (diffracting) sample structure causing these evanescent waves is, the quicker their decay over distance. In the optical regime these evanescent waves become usually undetectable after about one micrometer.

Scanning near-field optical microscopy (SNOM) methods place a probe (a tip, a scatterer of particles that can fluoresce or resonantly be excited) close enough to the sample structure to measure these evanescent fields [42]. From the aforementioned it becomes clear that near-field methods are constrained to imaging surfaces. An additional problem is that at these small distances to the sample the interaction of the tip with the electromagnetic field can change the local field and the interpretation of the images may become difficult. Like with the described nonlinear methods, the Abbe limit does not apply to the near field. Practically SNOM achieves a resolution (expressed as half width of the direct PSF) of down to about 30 nm in a watery environment. An interesting perspective is the measurement of tip-enhanced Raman scattering [43].

Another particular example of a near-field effect is evanescent wave microscopy [44]. Here an exponentially decaying intensity distribution can be generated close to a surface by illumination above the critical angle of total internal reflection (TIRF) of a planar interface between an optically dense medium (e.g. glass, $n = 1.518$) and a less dense medium (e.g. water, $n = 1.33$). In contrast to SNOM, this evanescent wave just extends along Z and the resolution improvement gained is also just oriented along Z. However, the main advantage of TIRF is a relatively sharp discrimination between (usually membrane and vesicular) sample structures close to the optical interface (depending on the illumination angle closer than about 200nm) and structures further away from this interface. For cells which are adherently grown to this interface, this method is interesting as vesicles in the interior of the living cell can be suppressed, and only structures tethered to or fused with the plasma membrane are present in the image.

Recently the idea of the 'perfect lens' has arisen [45], which is also based on using non-propagating waves. Pendry realized that a medium of negative refractive index (e.g. a thin slab of silver) would lead to exponentially growing waves within this medium. This effect can, in theory, make up for the exponentially decreasing evanescent waves outside the medium such that they theoretically have similar levels at the focus position like in the sample space. This effect has recently been demonstrated experimentally for light at small distances [47], but it still remains to be shown how much the non-ideal media used for such lenses can re-amplify the 'lost' evanescent waves and to which maximal distance such an ideal lens could work.

Key Points

- Recently many microscopy methods have dramatically enhanced the resolution.
- Gradually these methods are now applied to solve biological problems.
- The most promising approaches are all based on fluorescence and use either nonlinear interaction of light with the sample (STED, nonlinear structured illumination, dynamic saturation optical microscopy or saturation in the time domain) or precise localization of individual particles or molecules with subsequent image generation.

Acknowledgement

We would like to thank Matthias Krause, Peter Fraser and Vassilios Sarafis for useful comments on the manuscript. Furthermore, we thank Ondrej Mandula, Liisa Hirvonen and Yannick Colpin for their contribution to the structured illumination setup and the image reconstruction (Figure 4). Darren Williams and Guy Tear are thanked for preparing the polytene squash sample and Elisabeth Ehler for the stained muscle support cells (Figure 3).

Notes

1 The spatial frequency is the number of cycles per meter of a periodic pattern.
2 Or by directly interfering laser beams.
3 'linear' means that the fluorescence is simply proportional to the amount of input light. A double input light intensity means double fluorescence emission intensity generated. The straight multiplication of input intensity with fluorescence concentration (brightness of the sample) leads to the addition of the illumination and the detection Abbe limit.
4 'nonlinear' means that the generated fluorescence is not any longer proportional to the illumination intensity.
5 Stimulated Emission is the physical effect that a fluorophore is more likely to emit a photon the higher the rate of photons with identical properties passing by.
6 A very common optical aberration which violates the linear shift invariance is field dependent astigmatism.
7 $NA = n \sin(\alpha)$, with the refractive index of the embedding medium n and the half opening angle of the objective being α
8 For high NA (Figure 1) the intensity does not reach zero at this minimum, and the minimum position is not accurately predicted by the commonly used paraxial approximation: $d = 0.61\ \lambda/NA$.
9 The surprising bit about interference is that depending on the mutual phase of the light, bright light (e.g. from the left part of the lens) plus bright light (e.g. from the right part of the lens) can yield darkness at a specific position in the image.
10 Incoherent light means light which cannot generate interference, whereas coherent light can interfere.

References

1. Garini Y, Vermolen BJ, Young IT. From micro to nano: recent advances in high-resolution microscopy. *Curr Opin Biotechnol* 2005;**16**:3–12.
2. Baumeister W. From proteomic inventory to architecture. *FEBS Lett* 2005;**579**:933–7.
3. van den Pol AN. Neuronal imaging with colloidal gold. *J Microsc* 1989;**155**:27–59.
4. Lippincott-Schwartz J, Patterson GH. Development and use of fluorescent protein markers in living cells. *Science* 2003; **300**:87–91.
5. Inoue S, Oldenbourg R. Microtubule dynamics in mitotic spindle displayed by polarized light microscopy. *Mol Biol Cell* 1998;**9**:1603–7.
6. Kubitscheck U, Grunwald D, Hoekstra A, *et al*. Nuclear transport of single molecules: dwell times at the nuclear pore complex. *J Cell Biol* 2005;**168**:233–43.
7. Kellermayer MS. Visualizing and manipulating individual protein molecules. *Physiol Meas* 2005;**26**:R119–53.
8. Betzig E, Patternson GH, Sougrat R, *et al*. Imaging intracellular fluorescent proteins at near-molecular resolution. Science, 2006. Express publication, doi: 10.1126/science. 1127344
9. Rust JM, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical resonctruction microscopy (STORM), Nature Methods, 2006. Advance online publication doi:10.1038/nmeth 929.
10. Heintzmann R. Band-limit and appropriate sampling in microscopy. In: Celis Julio E (ed). *Cell Biology: A Laboratory Handbook*, Vol. 3. Elsevier Academic Press, 2006:29–36, Chapter 3.
11. Heintzmann R, Sheppard CJR. The Sampling Limit in Fluorescence Microscopy, Micron, 2006, doi: 10.1016/ j.micron.2006.07.017 [Epub ahead of print].
12. Qu X, Wu D, Mets L, Scherer NF. Nanometer-localized multiple single-molecule fluorescence microscopy. *Proc Nat Acad Sci USA* 2004;**101**:11298–303.
13. Ober RJ, Ram S, Ward SE. Localization accuracy in single-molecule microscopy. *Biophys J* 2004;**86**:1185–200.
14. Lacoste TD, Michalet X, Pinaud F, Chemla DS, Alivisatos AP, Weiss S. Ultrahigh-resolution multicolor colocalization of single fluorescent probes. *Proc Natl Acad Sci USA* 2000;**97**: 9461–6.
15. Mahy NL, Perry PE, Gilchrist S, *et al*. Spatial organization of active and inactive genes and noncoding DNA within chromosome territories. *J Cell Biol* 2002;**157**:579–89.
16. Bystricky K, Heun P, Gehlen L, *et al*. Long-range compaction and flexibility of interphase chromatin in budding yeast analyzed by high-resolution imaging techniques. *Proc Natl Acad Sci USA* 2004;**101**:16495–500.
17. Heilemann M, Herten DP, Heintzmann R, *et al*. High-resolution colocalization of single dye molecules by fluorescence lifetime imaging microscopy. *Anal Chem* 2002;**74**:3511–7.
18. Gordon MP, Ha T, Selvin PR. Single-molecule high-resolution imaging with photobleaching. *Proc Natl Acad Sci USA* 2004;**101**:6462–5.
19. Lidke KA, Rieger B, Jovin TM, *et al*. Superresolution by localization of quantum dots using blinking statistics. *Opics Express* 2005;**13**:7052–62.
20. Yildiz A, Forkey JN, McKinney SA, *et al*. Myosin V walks hand-over-hand: Single fluorophore imaging with 1.5 nm localization. *Science* 2003;**300**:2061–6.
21. Syed S, Snyder GE, Franzini-Armstrong C, *et al*. Adaptability of myosin V studied by simultaneous detection of position and orientation. *EMBO J* 2006;**25**:1795–803.
22. Danuser G, Waterman-Storer CM. Quantitative fluorescent speckle Microscopy of cytoskeleton dynamics. *Annu Rev Biophys Biomol Struct* 2006;**35**:361–87.
23. Hildenbrand G, Rapp A, Spori U, *et al*. Nano-Sizing of Specific Gene Domains in Intact Human Cell Nuclei by Spatially Modulated Illumination Light Microscopy. *Biophys J* 2005;**88**:4312–18.
24. Akduman I, Brand U, Grochmalicki J, *et al*. Superresolving masks for incoherent high-numerical-aperture scanning

microscopy in three dimensions. *J Opt Soc Am A* 1998;**15**: 2275–87.

25. Cichocki T, Martinez-Corral M, Kowalczyk M, *et al*. Annular binary filters for controlling the axial behaviour of optical systems. *J Mod Opt* 1998;**45**:227–37.
26. Gustafsson MGL, Agard DA, Sedat JW. I^5M: 3D widefield light microscopy with better than 100 nm axial resolution. *J Microsc* 1999;**195**:10–16.
27. Bewersdorf J, Schmidt R, Hell SW. Comparison of I^5M and 4Pi-microscopy. *J Microsc* 2006;**222**:105–17.
28. Heintzmann R, Cremer C. Lateral modulated excitation microscopy: improvement of resolution by using a diffraction grating. *Proceedings of SPIE* 1999;**3568**: 185–96.
29. Gustafsson MGL. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J Microsc* 2000;**198**:82–7.
30. Chung E, Kim D, So PTC. Extended resolution wide-field optical imaging: objective-launched standing-wave total internal reflection fluorescence microscopy. *Opt Lett* 2006; **31**:945–7.
31. Heintzmann R, Jovin TM, Cremer C. Saturated patterned excitation microscopy (SPEM) - a novel concept for optical resolution improvement. *J Opt Soc Am A* 2002;**19**: 1599–609.
32. Gustafsson MGL. Nonlinear structured-illumination microscopy: Wide-field fluorescence imaging with theoretically unlimited resolution. *Proc Natl Acad Sci USA* 2005;**102**: 13081–6.
33. Fujita K, Kobayashi M, Nakamura O, *et al*. Fluorescence microscope and observation method, Patent WO/2006/ 061947.
34. Enderlein J. Breaking the diffraction limit with dynamic saturation optical microscopy. *App Phys Lett* 2005;**87**, Art. No. 097105.
35. Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated emission – Stimulated-emission-depletion fluorescence microscopy. *Opt Lett* 1994;**19**:780–2.
36. Willig KI, Rizzoli SO, Westphal V, *et al*. STED microscoy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. *Nature* 2006;**440**:935–9.
37. Kittel RJ, Wichmann C, Rasse TM, *et al*. Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release. *Science* 2006;**312**:1051–4.
38. Willig KI, Kellner R, Medda R, *et al*. Nanoscale resolution in GFP-based microscopy. *Nat Methods* 2006. doi:10.1038/nmeth922.
39. Willig KI, Keller J, Bossi M, *et al*. STED microscopy resolves nanoparticle assemblies. *New J Phys* 2006;**8**, Art. No. 106.
40. Donnert G, Keller J, Medda R, *et al*. Macromolecular-scale resolution in biological fluorescence microscopy. *Proc Nat Am Soc* 2006;**103**:11440–5.
41. Hell SW. Strategy for far-field optical imaging and writing without diffraction limit. *Phys Lett A* 2004;**326**:140–5.
42. Wabuyele MB, Culha M, Griffin GD, *et al*. Near-field scanning optical microscopy for bioanalysis at nanometer resolution. *Methods Mol Biol* 2005;**300**:437–52.
43. Kawata S, Inouye Y, Ichimura T. Near-field optics and spectroscopy for molecular nano-imaging. *Sci Prog* 2004;**87**: 25–49.
44. Axelrod D. Total internal reflection fluorescence microscopy in cell biology. *Methods Enzymol* 2003;**361**:1–33.
45. Pendry JB. Negative refraction makes a perfect lens. *Phys Rev Lett* 2000;**85**:3966–9.
46. Pawley JB (ed), Handbook of Biological Confocal Microscopy, 3rd edn, Springer USA, 2006, 265–79.
47. Fang N, Lee H, Sun C, Zhang X. Sub-diffraction-limited optical imaging with a silver superlens. *Science* 2005;**308**: 534–7.