

Breaking the Resolution Limit in Light Microscopy

22

Rainer Heintzmann^{*,1} and Gabriella Ficz^{†,2}

^{*}Randall Division of Cell and Molecular Biophysics, King's College London,
London, United Kingdom

[†]Laboratory of Developmental Genetics and Imprinting, The Babraham Institute,
Cambridge, United Kingdom

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Abstract

The advancement in fluorescence microscopy has dramatically enhanced the obtainable optical resolution enabling the users to inspect the structures of interest at finer and finer level of detail. This chapter describes some of these methods and how they break the classical resolution limit. The labeling of targets, such as individual genetic

¹Rainer Heintzmann (*1970) studied Physics in Osnabrück, Germany. After his Ph.D. in Heidelberg (Supervisor Professor 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 the Nanoimaging research group at King's College London, United Kingdom.

²Gabriella Ficz (*1978) studied Biology in Iasi, Romania. After her Ph.D. 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, United Kingdom.

loci, specific proteins, or organelles, is possible inside living cells, which led to the extensive use of fluorescence microscopy in life sciences. Other microscopic modes usually lack this high specificity but sometimes provide other useful information such as the orientation of molecular species in polarization microscopy. Modes, such as differential interference contrast, phase contrast, or dark field, are useful to discriminate and follow cells or structures within them without the need for specific labeling. However, classically the resolution of all of these light microscopic modes was far below that of the electron microscope, and only some recent approaches have made significant progress in resolution increase. 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.

INTRODUCTION

Fluorescence 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. Advances in fluorescence microscopy have dramatically enhanced the obtainable optical resolution ([Garini, Vermolen, & Young, 2005](#)) enabling the users to inspect structures of interest at finer and finer level of detail. The aim of our chapter is to describe some of these methods ([Table 22.1](#)) and how they break the classical resolution limit.

Usually, if the aim is to see the structure of a cell down to molecular detail one has to resort to electron microscopy or electron tomography ([Baumeister, 2005](#)). However, this requires sample fixation which by itself can alter the microstructure, 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 ([van den Pol, 1989](#)) help to some extent but usually serve as mere indicators that in the vicinity of these metal particles 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 labeling 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 ([Mahy, Perry, Gilchrist, Baldock, & Bickmore, 2002](#)). 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 ([Kawata, Inouye, & Ichimura, 2004](#)). Modes such as differential interference contrast (DIC), phase contrast, or dark field are useful to discriminate and follow cells or structures within them without the need for specific labeling.

Table 22.1 Fluorescence Microscopy Methods

Method	Common Abbreviations	Abbe Applies	Remarks	Best Resolution (nm)	References
Wide-field	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)	Pawley, 2006
Confocal	CLSM, LSM	Yes	In theory: twice the resolution of wide-field microscopy. Local dose of light is very high for a short time. Potentially more photodamage. Photons are blocked by the pinhole, wasting useful information. Fine with living cells.	~180 (xy), ~500 (z)	Pawley, 2006
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)	Bewersdorff et al., 2006; Pawley, 2006
I^5M	I^5M	Yes	Similar to the above except no scanning needed and an incoherent full wide-field illumination is used from both sides. Potentially faster.	~230 (xy), ~90 (z)	Gustafsson et al., 1999
Localization	PALM, STORM, PAINT	Yes	Optics within the Abbe limit, but the task (localizing single particles) is not restricted to it. PALM, STORM, and PAINT managed (for the first time) to reconstitute full images from many thousand localized molecules. Particle discrimination by color, fluorescence lifetime, blinking, bleaching, photo-activation followed by bleaching and transient binding. Not demonstrated in 3D, no living cells yet.	~20 (xy)	Betzig et al., 2006; Bystricky et al., 2004; Danuser & Waterman-Storer, 2006; Gordon et al., 2004; Heilemann et al., 2002; Hildenbrand et al., 2005; Kellermayer, 2005; Kubitscheck et al., 2005; Lacoste et al., 2000; Lidke et al., 2005; Mahy et al., 2002; Ober et al., 2004; Qu et al., 2004; Rust et al., 2006; Sharonov & Hochstrasser, 2007; Syed et al., 2006; Yildiz et al., 2003

Continued

Table 22.1 Fluorescence Microscopy Methods—cont'd

Method	Common Abbreviations	Abbe Applies	Remarks	Best Resolution (nm)	References
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)	Chung et al., 2006; Gustafsson, 2000; Heintzmann & Cremer, 1999; Pawley, 2006
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)	Gustafsson, 2005; Heintzmann et al., 2002
Two 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)	Pawley, 2006
Stimulated emission depletion	STED	No	The saturation of the stimulated emission circumvents the Abbe limit. Combination with 4Pi possible. Not yet demonstrated in 3D. No living cells demonstrated yet.	~16 (x), ~20 (xy), ~50 (z)	Donnert et al., 2006; Hell & Wichmann, 1994; Kittel et al., 2006; Willig et al., 2006a,b,c; Pawley, 2006
Evanescence 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)	Axelrod, 2003; Pawley, 2006
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)	Kawata et al., 2004; Wabuyele et al., 2005

Abbreviations: WF, wide-field; CLSM, confocal laser scanning microscopy; LSM, laser scanning microscopy; 4Pi, stands for the full solid angle of a sphere; Type A, coherent illumination; B, coherent detection; C, coherent illumination and detection; iFM, incoherent interference illumination image interference; PALM, photoactivated localization microscopy; STORM, stochastic optical reconstruction microscopy; PAINT, points accumulation for imaging in nanoscale topography; 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.

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

22.1 WHAT IS RESOLUTION?

The term “resolution” has to be distinguished from “sensitivity,” “sampling,” and “precision.” Below 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 ([Betzig et al., 2006](#); [Kittel et al., 2006](#); [Lacoste et al., 2000](#); [Sharonov & Hochstrasser, 2007](#)). 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 $0.5 \mu\text{m}$ 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 eye). 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 [Heintzmann, 2006](#); [Heintzmann & Ficz, 2006](#). 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. This means that the distance between the estimated and the true center position of the object has to be below the distance between the two objects. Localization precision can be far higher than the optical resolution ([Pawley, 2006](#); [Rust, Bates, & Zhuang, 2006](#)), 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 colors ([Bystricky, Heun, Gehlen, Langowski, & Gasser, 2004](#); [Lidke, Rieger, Jovin, & Heintzmann, 2005](#); [Ober, Ram, & Ward, 2004](#)), differences in fluorescence lifetime

(Heilemann et al., 2002), photobleaching (Gordon, Ha, & Selvin, 2004; Rust et al., 2006), the individuality of statistical blinking events (Lippincott-Schwartz & Patterson, 2003), photoactivation (Betzig et al., 2006; Sharonov & Hochstrasser, 2007), and transient binding (Heintzmann & Sheppard, 2007). 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 (Syed, Snyder, Franzini-Armstrong, Selvin, & Goldman, 2006; Yildiz et al., 2003). 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 (Lacoste et al., 2000). The method of speckle microscopy (Danuser & Waterman-Storer, 2006) uses precise localization for the tracking of molecular clusters to reveal their temporal behavior. Nanosizing (Inoue & Oldenbourg, 1998) 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 fibers) 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 below.

When we talk about high *resolution* of an optical instrument in this chapter, 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 (Boxes 22.1 and 22.2). The reader who is uninterested in the technical details of resolution definition may skip the boxed sections.

For classical treatments of the resolution proven see Chapter 1 by Sluder and Nordberg, this volume; and Chapter 2 by Wolf, this volume.

Although the definitions of resolution [FWHM (*Full Width of the PSF measured at Half its Maximum*) see Fig. 22.1, Rayleigh and Sparrow limit] mentioned in the boxed section “The point spread function” (Box 22.1) may be useful in some cases, we would like to use a different limit called the Abbe limit (see boxed section “The Abbe limit,” Box 22.2) within this chapter, 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” (Lippincott-Schwartz & Patterson, 2003). A very successful way of discriminating closely spaced molecules is using a repetitive cycle of faint photoactivation followed by bleaching of the activated molecules (Betzig et al., 2006; Sharonov & Hochstrasser, 2007). 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 a 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

Box 22.1 THE POINT SPREAD FUNCTION: THE SAMPLE AS A SUM OF POINTS

The point spread function (PSF) describes how a very small (in theory infinitesimally small) emitter gets imaged by an imaging system.

As a sample can be thought of consisting of many points (in the limit infinite) 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.³ This feature simplifies optical theory significantly as the imaging operation can then be described by a mathematical operation called “convolution.”

In Fig. 22.1, two examples of typical PSFs of a wide-field fluorescence microscope are shown. As can be seen, the PSF sizes are quite different for the numerical apertures⁴ 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 taken at the full width of the PSF measured at half its maximum (FWHM) (Fig. 22.1). Thinking about the situation of three-dimensional (3D) samples to “paint,” one will have to 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 Fig. 22.1). 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 (Fig. 22.1) where the image of the other object has its first intensity minimum.⁵

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 labeled “zero intensity” points to in Fig. 22.1), which are unwanted. Due to their nonabsolute nature, these limits could be called “soft,” whereas the Abbe limit as discussed in the other boxed section (Box 22.1) 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.

resolution of about 20 nm (Betzig et al., 2006). Currently, a problem is the rather long imaging time well above an hour for a single 2D image, but further development should obviate this. An alternative is to exploit the transient binding of fluorophores, yielding rare bursts of signal which can be localized (Heintzmann & Sheppard, 2007).

³A very common optical aberration which violates the linear shift invariance is field-dependent astigmatism.

⁴NA = $n \sin(\alpha)$, with the refractive index of the embedding medium n and the half-opening angle of the objective being α .

⁵For high NA (Fig. 22.1), the intensity does not reach zero at this minimum and the minimum position is not accurately predicted by the commonly used paraxial approximation: $d = 0.61\lambda/NA$.

Box 22.2 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 abundances), 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 of 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 lower 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 interference⁶ (Fig. 22.2A). 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 (Fig. 22.2B, see discussion in Chapter 14 by Wolf et al., this volume). 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 $NA_{\text{illumination}}$) 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:

⁶The 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.

$$d_{\min} = \frac{\lambda}{NA_{\text{illumination}} + NA_{\text{detection}}}. \quad (22.1)$$

In *fluorescence*, the situation is slightly different, as the light from different fluorescent molecules has to be incoherently⁷ 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 (Fig. 22.2C). 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 wide-field fluorescence microscopy, the corresponding Abbe limit becomes:

$$d_{\min} = \frac{\lambda}{2NA_{\text{detection}}}. \quad (22.2)$$

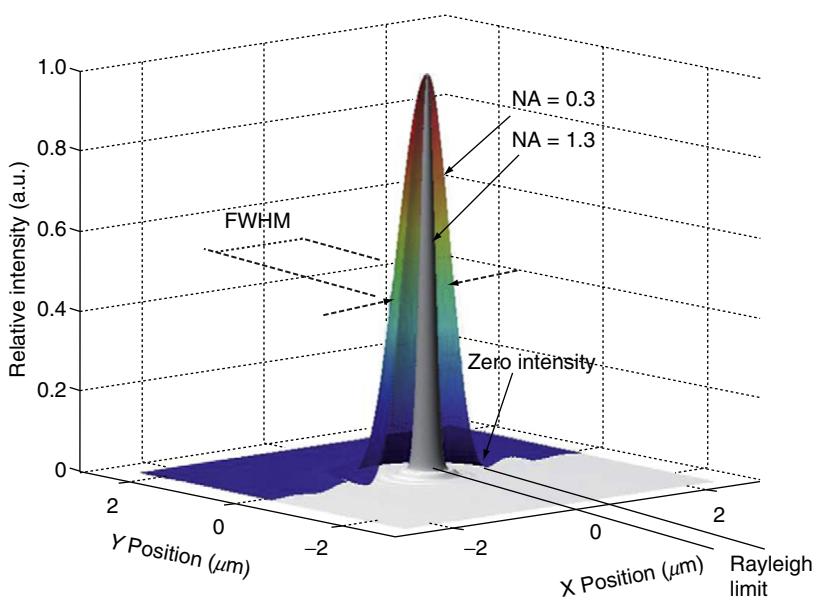
This factor of 2 in Eq. (22.2), when compared with $NA_{\text{detection}}$ in Eq. (22.1), stems from the fact that in a incoherent fluorescence PSF the finest detail stems from mutual interference of the highest angled rays (e.g., left with right side), whereas in transmission the finest scattering structure would be defined by the difference of the incident illuminating ray (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. Even finer structures would only be imaged as a homogeneous level of brightness with no modulation whatsoever.

One may now wonder, whether changing the fluorescence 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 nonuniform illumination will be discussed.

In analogy to the above discussion on the Abbe limit, 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.

⁷Incoherent light means light which cannot generate interference, whereas coherent light can interfere.

**FIGURE 22.1**

The point spread function (PSF). Two PSFs are shown, one for high numerical aperture ($NA = 1.3$ in gray) 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 gray peak in the middle) is much finer but does not have the rings of zero intensity.

22.2 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 Box 22.2 “The Abbe limit”) by a spatially nonuniform illumination (Table 22.1). Nonuniform illumination implies that adjacent positions in the sample can receive different illumination intensities. Below we will detail on how the resolution enhancement is achieved for these methods.

In confocal microscopy, 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 wide-field fluorescence microscope. If the pinhole is very small, the light distribution used for illumination is very similar to the spatial sensitivity map of light detection from the sample. For light particles (photons) to reach the detector, a

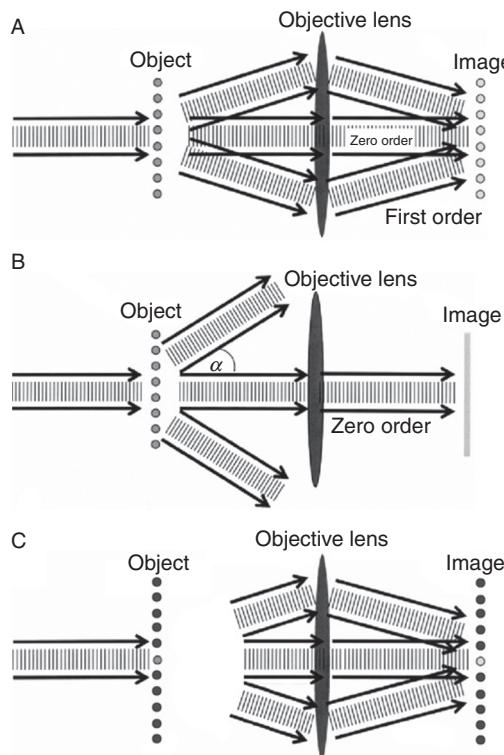


FIGURE 22.2

The Abbe limit. (A) Diffraction of a parallel light beam from a periodic grating structure (only the zero and first diffraction orders 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.

particular sample point needs to be illuminated (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 $1/10 \times 1/10 = 1/100$) 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 twofold finer periodicity.

However, in practice this is almost never achieved as the fine details (high spatial frequency⁸) 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

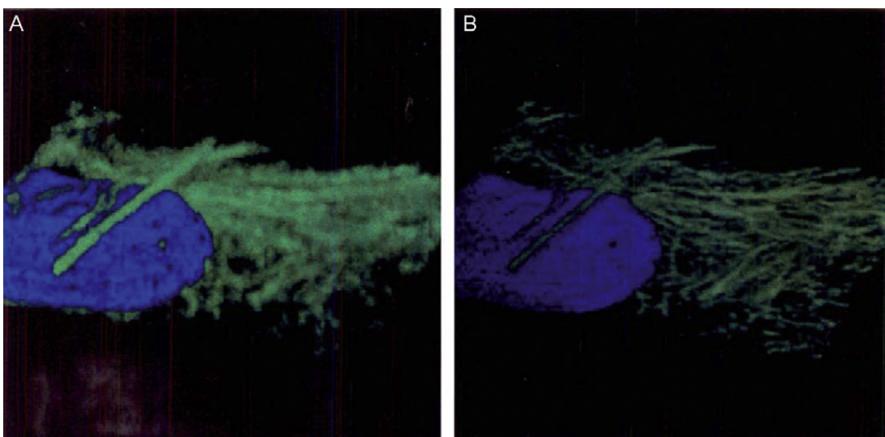
⁸The spatial frequency is the number of cycles per meter of a periodic pattern.

in the range of the diameter of an Airy disc (where the first theoretical minimum occurs 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 led 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 limit of twice the wide-field resolution with uniform illumination (Akduman et al., 1998; Cichocki, Martinez-Corral, Kowalczyk, & Andres, 1998). 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 (Bewersdorf, Schmidt, & Hell, 2006; Gustafsson, Agard, & Sedat, 1999). 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 from laser and detector to the sample (through the top and through 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 (becoming identical to the refractive index) is obtained along the optical axis (z) connecting the two opposing objectives, 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 ~ 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 ~ 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 (Fig. 22.3B). 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 dataset. Similar arguments apply to the I^5M method, where incoherent illumination is used (Gustafsson et al., 1999).

The methods of structured illumination (Chung, Kim, & So, 2006; Gustafsson, 2000; Heintzmann & Cremer, 1999) exploit the fact that the excitation and emission Abbe limit can be added. The highest possible spatial frequency is intentionally

**FIGURE 22.3**

Confocal and 4Pi images. 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) Two-photon confocal view of the sample and (B) two-photon 4Pi (type A) view (after three-point deconvolution) of the sample. The improved resolution and better discrimination of biological structures is visible. This dataset corresponds to a width of $23.4\ \mu\text{m}$, a height of $2.9\ \mu\text{m}$, and a depth of $30.0\ \mu\text{m}$.

introduced during excitation via a fine sinusoidal illumination pattern in the sample volume. This is achieved by projecting a fine grating⁹ 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 (Fig. 22.4) 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 (Gustafsson, 2000), albeit at the expense of requiring extensive computational reconstruction.

22.3 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. Below, the importance of the nonlinearity is motivated and exemplified with two-photon microscopy, nonlinear structured illumination, and stimulated emission depletion (STED) microscopy.

As mentioned in the boxed section “The Abbe Limit,” the linear¹⁰ response of the sample led us to define the combined illumination and detection Abbe limit as a

⁹Or by directly interfering laser beams.

¹⁰“Linear” means that the fluorescence is simply proportional to the amount of illumination intensity. A double input light intensity means double fluorescence emission intensity generated. The straight multiplication of input intensity with fluorophore concentration (brightness of the sample) leads to the addition of the illumination and the detection Abbe limit.

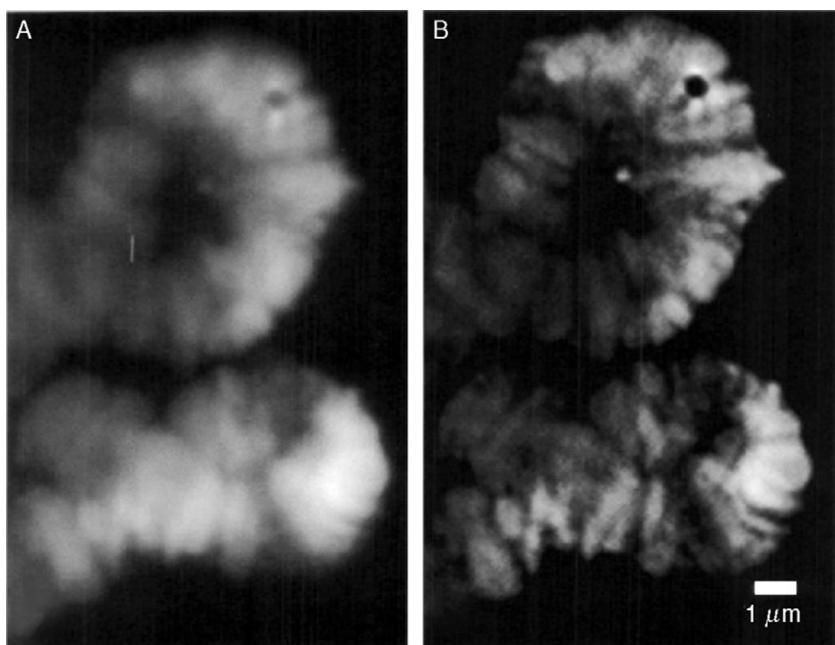


FIGURE 22.4

Linear structured illumination of *Drosophila* polytene chromosomes stained with Sytox Green. (A) Wide-field image obtained by summing all the individual images under structured illumination. (B) Reconstruction of the sample from the structured illumination data (five phases, two angles).

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*¹¹ 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 (Hell, 2004). 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 nonlinear dependence is encountered in two-photon microscopy. Due to the probabilistic nature of the two-photon absorption process (both photons need to interact with the fluorophore 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

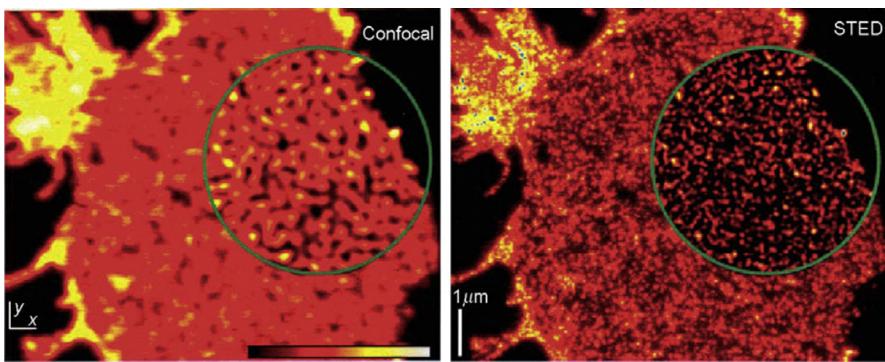
¹¹“Nonlinear” means that the generated fluorescence is not any longer proportional to the illumination intensity.

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 twofold 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 two-photon microscopy is the reduced absorption and scattering, allowing to 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 side-lobe 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 (Hell, 2004). Practically, this can be realized as a microscopy method by combining the nonlinearity with the concept of structured illumination (Gustafsson, 2005; Hell, 2004) or by detecting high harmonics in the time domain under temporally sinusoidally modulated illumination intensity in a confocal system (Fujita, Kobayashi, Nakamura, & Kawata, 2006). Also, the spatial nonuniformity of a depletion, bleaching, or light-induced inactivation process (dynamic saturation optical microscopy, Enderlein, 2005) 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 STED microscopy (Hell & Wichmann, 1994; Kubitscheck et al., 2005; Willig, Keller, Bossi, & Hell, 2006; Willig et al., 2006; Willig, Rizzoli, Westphal, Jahn, & Hell, 2006; Fig. 22.5). 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 center of the scanning spot by stimulated emission¹² caused by 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. The center of 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 center (zero intensity) position, its interaction with the fluorophore is long enough to push 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 been demonstrated that STED can be applied to imaging of

¹²Stimulated emission is the physical effect that a fluorophore is more likely to emit a photon higher than the rate of photons with identical properties passing by.

**FIGURE 22.5**

Stimulated emission depletion image. Revealing the nanopattern of the SNARE protein SNAP-25 on the plasma membrane of a mammalian cell. Confocal (A) versus 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 Willig et al. (2006a).

endogenous markers such as GFP (Willig et al., 2006). By allowing the triplet states to relax between successive excitations, the fluorescence yield in STED microscopy could be dramatically improved (Donnert et al., 2006).

A practical rule of thumb can be established as alteration to Abbe's equation (Hildenbrand et al., 2005), 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 Hildenbrand et al., 2005 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.

22.4 A COMMENT ON NEAR FIELD METHODS AND PENDRY'S LENS

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 with distance to each emitting molecule. 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 theory above 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 electromagnetic fields called evanescent (or near field) waves. These solutions of the Maxwell's 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 1 μm .

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 ([Wabuyele, Culha, Griffin, Viallet, & Vo-Dinh, 2005](#)). From the above, 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. As with the nonlinear methods described, the Abbe limit does not apply to the near field. Practically, SNOM achieves a resolution down to about 30 nm in a watery environment. An interesting perspective is the measurement of tip-enhanced Raman scattering ([Kellermayer, 2005](#)).

Another particular example of a near field effect is evanescent wave microscopy ([Axelrod, 2003](#)). 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 200 nm) 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.

The idea of the “perfect lens” has arisen ([Qu, Wu, Mets, & Scherer, 2004](#)), which is also based on using nonpropagating 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 as in the sample space. This effect has been demonstrated experimentally for light at small distances ([Fang, Lee, Sun, & Zhang, 2005](#)) but it still remains to be shown how much the nonideal media used for such lenses can reamplify the “lost” evanescent waves and to which maximal distance such an ideal lens could work.

22.5 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.

Acknowledgment

This chapter is reprinted with modification from [Heintzmann & Ficz, 2006](#).

We would like to thank Klaus Suhling, Matthias Krause, Peter Fraser, and Vassilios Sarafis for useful comments on the chapter. Furthermore, we thank Ondrej Mandula, Liisa Hirvonen, and Yannick Colpin for their contribution to the structured illumination setup and the image reconstruction ([Fig. 22.4](#)). Darren Williams and Guy Tear are thanked for preparing the polytene squash sample.

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