Improving the spatial resolution of optical microscopes is important for a vast number of applications in the life sciences. Optical microscopy allows intact samples and living cells to be studied in their natural environment, tasks that are not possible with other microscopy methods (e.g., electron microscopy). Major advances in the past two decades have significantly improved microscope resolution. By using interference and structured light methods microscope resolution has been improved to ~100 nm, and with non-linear methods a ten times improvement has been demonstrated to a current resolution limit of ~30 nm. These methods bring together old theoretical concepts such as interference with novel non-linear methods that improve spatial resolution beyond the limits that were previously assumed to be unreachable.

Abbreviations
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CCD charge-coupled device
HELM harmonic excitation light microscopy
MPM multiphoton microscopy
NSOM near-field scanning optical microscopy
PSF point spread function
SHG second harmonic generation
SPIM selective plane illumination microscopy
SPR surface plasmon resonance
SSIM saturated structured illumination microscopy
STED stimulated emission depletion
TIRF total internal reflection fluorescence

Resolution
What is resolution anyway? Even when a very small object (say one nanometer in diameter) is observed with a microscope, its image is significantly broadened compared with the original object. First described by Abbe [1], this phenomena is a result of the diffraction of light and depends on the wavelength and the finite size of the objective lenses of the microscope. The intensity distribution of the image of a very small object is called the point spread function (PSF) (Figure 1). The PSF usually has a lateral (in-plane) radial symmetry (Figure 1a) and a larger axial broadening (along the optical axis). Figure 2 shows the intensity distribution as observed from a 'side-view' relative to the plane of the object. Based on the commonly used Rayleigh criterion, the minimal distance of two points that can still be resolved is approximately equal to the width of the PSF (Figure 1).

According to the Rayleigh criteria, the resolution limit of a conventional microscope is given by

\[ \text{Lateral (in-plane): } d_{x,y} = \frac{0.61 \lambda}{NA} \]
\[ \text{Axial (along the optical axis): } d_z = \frac{2\lambda}{NA^2} \]

where \( \lambda \) is the wavelength of light and \( NA \) is the numerical aperture of the lens, which is the sine of half of the focal length of the objective lens.
The intensity of a small object (diameter much smaller than \( \lambda \)) as viewed through an optical microscope, also called the point spread function (PSF). (a) The intensity of a small object as observed in the plane (solid line). To distinguish two small objects from one another, the distance between them should be approximately as plotted (see the distance between the solid and dashed blue lines). The total intensity is the sum (red line) that has been artificially raised a little. It is common to use the Rayleigh criteria for the resolution. With this criterion, the sum intensity of two close objects (red line) should have an intensity minimum that is 20–27% lower than the peak intensity. (b) The same graphs shown along the optical axis \( z \). Note that the resolution along the optical \( z \) axis is worse than the resolution in the lateral \( x,y \) plane. Two axes are shown for each graph. The upper one shows the distance for a numerical aperture (NA) of 1.0 and a wavelength of 500 nm. The lower axis is drawn for a general case and the resolution can be calculated with it for any given value of NA and \( \lambda \).
observation angle of the lens multiplied by the index of refraction, $n$, of the material that is in between the lens and the sample. These limits, known as the diffraction limit, form the starting point for the struggle to improve the resolution; the narrower the PSF of a system, the better the resolution that can be achieved.

**High-resolution three-dimensional methods**

It is fascinating to see how spatial resolution has gone through evolution and revolution. We divide the existing high-resolution microscopy methods into four categories: conventional and confocal microscopy; interference methods; non-linear methods; and surface methods. The classification of a method into a category is not always well defined, but we found it easier to conceptually understand the similarities and differences between the methods using this approach. The ‘methods map’ (Figure 3) provides a general overview and the categories and methods are described below.

**Conventional and confocal microscopy**

Conventional microscopy refers to the compound microscope, principally composed of an objective lens, an eyepiece lens, and (for infinity-corrected microscopes) a tube lens. This setup has been used for 350 years since the development of the compound microscope by Hook and his outstanding 1664 publication of Micrographia [2]. Main improvements in the optical resolution over the course of the past centuries have resulted from the improved quality of the elements, most importantly the correction of aberrations in the objective lens. There are also works that have demonstrated the correction of aberrations by using adaptive optics [3,4]. These developments are important in light of the fact that almost all high-resolution methods are based on a conventional microscope.

**Conventional microscopy and deconvolution**

With the development of digital imaging and image processing, including deconvolution methods [5], it has become possible to process two- and three-dimensional images that have been acquired with a conventional microscope and to improve the spatial resolution [6]. Most of the digital images that are measured through a microscope use a charged-coupled device (CCD). These detectors have high efficiency, collect the light from the whole image simultaneously, have a large dynamic range and long possible exposure times. This allows for the measurement...
of high signal-to-noise ratio images, which are essential for the acquisition of high-resolution data. In some cases (e.g. thin samples with a small number of fluorescing objects), this method provides results that are as good as those obtained with a confocal microscope (Figure 4).

Confocal microscopy

Confocal fluorescence microscopy [7,8] was one of the earliest methods developed for improved resolution and is the most well-established and wide-spread method in use at present.

The PSF for a confocal microscope is approximately the square of the PSF of a conventional microscope and the resolution improves by $\sim \sqrt{2}$. Even more importantly, it provides good isolation of out-of-focus fluorescence. This aspect is not obvious from observing the PSF but is described, as an example, by Wilson [8].
Confocal imaging is achieved by a single point illumination with a laser and detection through a pinhole, followed by a raster scan of the entire image. This indicates the weakness of confocal microscopy, namely the long time it takes to measure an image with a good signal-to-noise ratio. The scanning time can be reduced, for example, by using a spinning-disk that has many holes [9], but the resolution is somewhat reduced.

Selective plane illumination microscopy
Selective plane illumination microscopy (SPIM) is a newly developed method that allows one to measure large-size specimens (up to few millimeters) with an improved semiconfocal illumination [10]. The high-efficiency of this method, relative to confocal microscopy, results from the illumination of a whole image plane instead of a single point. SPIM permits the rapid capture of three-dimensional images so that transient biological processes in a large specimen with a resolution of \(\approx 1 \mu m\) is of major importance (Figure 5).

Interference and structured illumination methods
Light waves can interfere, as was shown for the first time in 1801 in Young's wave experiments. Interference of two or more light sources can result in a periodic pattern of light on the sample (object) plane. When these patterns are used to excite fluorescence, they interact with the sample structure and the recorded emission carries higher resolution information than can be achieved by conventional microscopy. Usually, two objective lenses are used at the front and rear of the sample. This also increases the numerical aperture of the setup and results in an improved resolution. A similar interference effect can be achieved when the emitted fluorescence is collected from both objectives and combined to interfere on the CCD plane. Interference methods are usually limited to thin samples, because the quality of the interference pattern is reduced when the light passes through the sample.

Interference methods were initiated a decade ago by Lanni and colleagues [11,12] and development has continued with the introduction of the I3M, HELM and 4Pi microscopy methods [13]. A method based upon the work of Wilson and colleagues [14] that uses structured light illumination has also recently been introduced to the market (Apotome from Carl Zeiss, Gottingen, Germany). It provides the same axial resolution as a confocal microscope, while providing a better signal-to-noise ratio using ‘only’ a conventional microscope.

4Pi microscopy
4Pi microscopy [15] is based on the interference principle. It uses laser light for illumination in a confocal mode. When image restoration is added to the three-dimensional scanned data [16], the resolution can be improved to the 100 nm range.

Through further improvements, the acquisition time has been shortened by using many beams in parallel and a two-photon configuration [17]. This method — termed by Hell and colleagues multifocal, multiphoton 4Pi confocal microscopy (MMM-4Pi) — overcomes the long acquisition time of a single point system and allows one to measure very fine details of organelles such as mitochondria [17] and the Golgi apparatus [18] in living cells with a resolution of \(\approx 100 \text{ nm}\) (Figure 6).

The 4Pi microscopy method has recently been commercialized as an extension to a confocal/two-photon microscope (Leica, Mannheim, Germany).

Image interference microscopy
Image interference microscopy (I3M) uses two, high numerical aperture objective lenses and beam splitters to collect fluorescence images from the same focal plane and to let them interfere on a CCD plane [19,20]. These objective lenses can also be used to illuminate the sample from both sides with an incoherent light source (such as a mercury lamp). When used in this way, the method is called incoherent, interference, illumination microscopy (I3M) and results in an interference pattern on the sample. Gustafsson and colleagues, who first developed I3M, also combined this approach with I3M to form I3M [21]. During the measurement, a set of images is collected by scanning the sample through the system focal plane and the data are appropriately deconvolved to provide
high-resolution three-dimensional information. I\(\text{M}\) provides a resolution in the range of 100 nm.

**Harmonic excitation light microscopy**

In harmonic excitation light microscopy (HELM), four beams are combined to interfere in the sample creating high-frequency interference lines along the two main axes of the sample [22]. The system uses a laser beam coupled to the sample through a set of beam splitters and a glass block. The fluorescence is measured with a high numerical aperture objective lens on the other side of the sample. Five images are recorded with a CCD camera for different positions of the pattern on the sample, and processed to provide high-resolution images. HELM provides lateral resolution of \(\approx 100\) nm; it has also been shown that both axial and lateral resolution of \(\approx 100\) nm can be achieved [23].

**Non-linear methods**

The methods described above try to reach high resolution by improving the PSF either directly (e.g. by increasing the numerical aperture) or indirectly by using interference phenomena followed by image processing.

Non-linear methods are based on a different approach. In one non-linear method (multiphoton microscopy), the fluorochromes are excited only when absorbing more than one photon. In another method (reversible saturation), the reaction of the fluorochromes to light is used to manipulate the volume of sample that actually fluoresces. Non-linear phenomena can reach very small fluorescing volumes, which results in improved spatial resolution. We believe that these methods carry the highest potential for the future, especially when combined with some of the other methods mentioned above.

**Multiphoton microscopy**

A major improvement for studying both living cells and thick samples was achieved with the development of multiphoton microscopy (MPM) [24]. In this non-linear method, each fluorochrome is usually excited by two photons (sometimes three) such that the total energy is equal to the excited fluorochrome energy. This process is only effective at the center of the focused beam in the sample where the photon density is high, but the resolution is subsequently reduced because photons with a wavelength of \(2\lambda\) (or \(3\lambda\)) are required. This can be improved to a certain extent by using a confocal pinhole. MPM has become a powerful method for imaging thick samples, as it causes minimal harm to the surrounding cells or tissues through the unwanted absorption of short wavelength excitation energy [25]. This important advantage is achieved while still maintaining a good spatial resolution owing to the long wavelength used in the excitation (which is less damaging and penetrates deeper into tissues) and the efficient fluorescence detection that is spectrally far from the excitation. Typically excitation is in the infrared spectrum, whereas detection is in the visible spectrum.
Other non-linear methods include second harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS). SHG was demonstrated to have advantages in thick samples and for the fast recording of small intensity changes, such as the detection of action potentials [26]. CARS provides the advantage that no labels are required, because the Raman-based method is sensitive to the specific chemical in the tissue itself [27,28].

**Stimulated emission depletion**

Stimulated emission depletion (STED) microscopy was conceptually introduced a decade ago by Hell and colleagues [29] and has been demonstrated recently [30**]. The principle of the method is to ensure that the volume that emits fluorescence in the sample is extremely small. This is accomplished by using two pulsed lasers. The first laser has a wavelength that excites the fluorescent molecules and the second illuminates the sample with a donut-like pattern in a wavelength that drives (depletes) the excited states of the fluorescent molecules back to the ground state. The only volume that is left with fluorescent molecules in the excited state is in the hole of the donut, from where fluorescence is actually detected. Because pulsed lasers are used, the depleting laser is very powerful and the only molecules that are not fully depleted are very close to the donut hole. Images with a resolution of \( \sim 30 \) nm have been measured using this technique [31], and given that the method is still in its infancy its potential is very high. This is a major step towards improving resolution: going from \( \sim 100 \) nm (with 4Pi, F\(^2\)M and HELM) to \( \sim 30 \) nm (Figure 7) [32**].

**Saturated structured illumination microscopy**

Saturated structured illumination (SSIM) is conceptually the opposite of STED. The theory was established by Heintzmann and colleagues [33,34] and was recently successfully tested by Gustafsson and colleagues [35]. By using a structured light illumination from two powerful interfering beams, most of the fluorescent molecules in the illuminated beams saturate, leaving only small volumes unsaturated at the shadows of the interference pattern. These volumes become very small when increasing the light intensity, smaller than any PSF width. The sample has to be scanned and the images have to be processed to extract the high-resolution data [35]. Resolutions smaller than 50 nm have already been demonstrated using this method (Figure 8).

**High-resolution measurements of surfaces**

A few methods are suitable for high-resolution measurements of surfaces, but not for three-dimensional measurements. These include near-field scanning optical microscopy (NSOM) and methods that create entangled fields on the surface, including surface plasmon resonance (SPR) and total internal reflection fluorescence (TIRF).

**Near-field scanning optical microscopy**

NSOM improves optical resolution by circumventing the use of lenses [36]. This is achieved by shining laser light through a fiber with a small tip aperture (diameter of 20–200 nm). The tip scans the surface at a close distance (\( \sim 10–50 \) nm) and the effective illuminated area has the tip diameter with a shallow penetration depth (less than...
NSOM has gone through major improvements such as fast measurement (100 images per second) and the detection of single molecules on surfaces, such as the membrane of dendritic cells.

Total internal reflection fluorescence and surface plasmon resonance

TIRF and SPR provide intrinsic high axial resolution owing to the unique optical setup that is used to illuminate the sample. A sample is attached to a substrate such as a glass cover slip adjacent to a prism that is illuminated at an angle that does not allow the light to propagate directly through the substrate to the sample. It is, however, enough to create an evanescent light field in a thin layer close to the substrate (~100 nm) and can excite fluorescent molecules that are within this shallow layer (see also the review by Schneckenburger in this issue). The fluorescence emission is collected with an objective lens and thus provides a high axial resolution (~100 nm or less). TIRF has been recently applied to the dynamic study of molecular motor steps by combining it with an interference pattern illumination; a spatial resolution of 8 nm has been achieved with a time resolution of 100 μs [42]. In SPR, an evanescent field is also created, but here a glass surface is coated with a metal layer. Subtle changes in the refractive index of the surface can be detected by measuring changes in the intensity of the illumination beam as a function of the impinging angle [43]. This method is highly effective for detecting interactions at the surface without the need for a label. An attempt is currently underway to extend the method so that full imaging on a surface can also be achieved [44]. We believe that this method will have a number of advantages for monitoring the physical properties of biomacromolecules and protein–protein interactions. It has also been shown that SPR can be combined with nanostructured devices to provide an extraordinary behavior of light when it is transmitted through nanometer-size holes [45]. This has the potential to evolve into a new type of microscopy with a resolution that is yet to be explored [46].

Conclusions

For many years, it was believed that optical microscopy measurements had to be limited to a spatial resolution of ~200 nm in the plane and ~400 nm along the optical axis. Major developments, especially in the past few years, have demonstrated that this can be improved up to tenfold. Among the novel methods that have enabled these improvements, we believe that the non-linear methods combined with high numerical aperture measurements (e.g. using STED and SSIM) have the potential to evolve into systems that will be widely used, although the process may still take years.

Furthermore, sophisticated methods are still being developed to improve the optical resolution for situations that involve thick samples (SPIM), fast measurements (SHG) and living cells (MPM).

These methods will significantly improve the efficiency of research on biological samples and will provide new and necessary tools for subcellular and molecular level studies.

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References and recommended reading

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

- of special interest
- of outstanding interest


