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EXPERT OPINION

Through the looking glass – the adventures of seeing beyond the diffraction limit

Cornelia Denz

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The dream of understanding the inner cell has initiated research since many centuries. Already the first inventor of a microscope, Antoni van Leuwenhoek, wanted to learn more about the inner structure and the dynamics of "dierkens", animalcules such as bacteria, protozoa and spermatozoa he observed in the 1650s and documented in fascinating drawings. His experiments, realized by fixing the spermatozoa into slender hollow glass pipes "not thicker than a man's hair", allowed him to "make the red globules of the blood appear" [1].

There was a significant progress in lens design in the following centuries, featured by introduction of corrected lenses and later achromatic lenses. By combining achromatic double lenses (achromatic doublet), the resolution limit reached the micrometer range. However, the configuration of these lenses was developed by trial and error because of the lack of a systematic mathematical description of light passing through the lenses. Driven by the founder of cell biology, Matthias Schleiden, a Jena-based entrepreneur from Germany, Carl Zeiss made an effort to improve and systematically craft reproducible high-resolution lenses. For this purpose he hired in 1866 young physicist Ernst Abbe as a freelancer and asked him to systematically improve lenses by introducing a mathematical basis. Abbe's results suddenly put an end to the euphoric hopes for future resolution improvements (Fig. 1): on the one hand, microscopes based on his calculations were worse than the ones fabricated by trial and error, and on the other hand, he developed a theory of image formation in a microscope that led to the discovery of the resolution limit due to the wave nature of light, namely diffraction.

Ernst Abbe found- in his looking glass book- that the interaction of a light wave with an optical system is essential, and that the aperture of the microscopic lens plays a decisive role because it defines the relative contribution of the diffracted light that enters the objective: for perpendicular incidence, half of the angle of the aperture is equal to the diffraction angle that the diffracted light

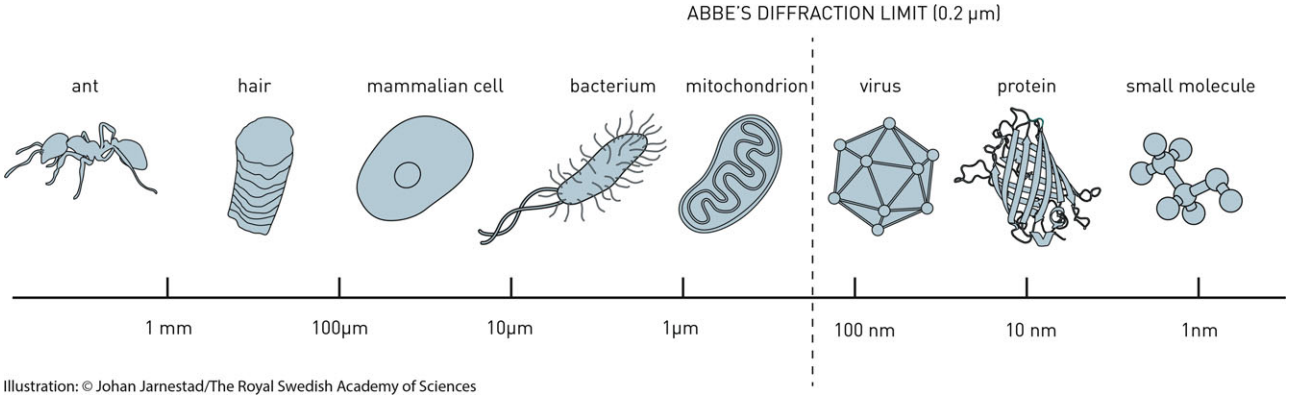

Figure 1 At the end of the 19th century, Ernst Abbe defined the limit for optical microscope resolution to roughly half the wavelength of light. Thus, scientists could distinguish whole cells, as well as some parts of the cell called organelles. However, they would not be able to discern something as small as virus or single proteins. (Image: http://www.kva.se/nobelchemistry2014).

can have in order to be still detected by the objective.

Since then, physicists have developed techniques to cope with that resolution limit by enhancing the contrast or by improving the depth of field. Among those techniques are phase-contrast microscopy developed by Frits Zernicke in 1934 [2], honored by the Nobel award in physics in 1953, which allowed low-refractive-index structures such as cells to be seen in a high-contrast mode, or confocal microscopy developed by Marvin Minski in 1955 [3]. Confocal microscopy introduced a paradigm shift in microscopy by exploiting spatial filtering to eliminate out-of-focus light in a specimen thicker than the focus depth. Confocal microscopy, being nowadays 'the workhorse' of biologists and medical researchers, has many advantages over conventional wide-field microscopy: it is able to control the depth of field, eliminates the image degradation due to out-of-focus contributions, and allows serial optical sections to be collected from thick specimens, however, at the expense of a scanning reconstruction of the image as compared to the "single-shot" wide-field imaging techniques.

In parallel, the quest on how to achieve an improved specificity in optical biomicroscopy was resolved by applying fluorescence into the standard microscopy. Although fluorescence has been a very well-known phenomenon since the early nineteenth century, it was Sir David Brewster who reported in 1833 on several observations of red radiation from green chlorophyll. The term fluorescence was defined by Sir George Stokes a couple of years later who described the luminescence in calcium fluoride excited by sunlight as fluorescence. The theory behind fluorescence as a phenomenon of absorption and subsequent emission of a light of longer wavelengths was formulated by Eugen von Lommel only in 1875.

It was, however, only in 1911–1913 that the well-known players in microscopy – Carl Zeiss in Jena and Carl Reichert in Vienna – developed a fluorescence microscope that was commercially successful. This became a reality after a so-called ultramicroscope that exploited the dark field to enhance the contrast and the resolution (Henry Siedentopf, 1903) as well as the Köhler illumination-based ultraviolet absorption microscope (August Köhler 1904) were designed, originally with the purpose to increase resolution. This allowed an observation of autofluorescence from a biological specimen and led to the construction of the first bright-field fluorescence microscope in 1910. They were commercialized almost in parallel in 1911 and 1912 in Jena and Vienna, respectively. In 1914 Stanislaus von Prowazek first introduced vital cellular staining into fluorescence microscopy using living protozoa. From there, the fluorescence microscopy started its triumphal development in biomedicine.

With the advent of modern physics, Peter Pringsheim described "fluorescence and phosphorescence in light of the newer atom theory" [4] in 1928, and Alexander Jablonski formulated his famous graphical representation of the process in 1935. After the possibility to use dyes to stain bacteria or cells had been fostered by the chemical dye industry, a number of demonstrations were made showing the use of this technique in biomedical applications. The combination of confocal microscopy with fluorescence microscopy in the 1950s marked a breakthrough in modern microscopy. These were the ingredients necessary to think of imaging schemes capable of ingeniously surpassing Abbé's diffraction limit and revolutionizing the optical microscopy, especially in the past twenty years.

In the late 1970s, Christoph and Thomas Cremer first came up with a path-breaking idea of using two oppositely placed objectives in a microscope to obtain interference in the focal plane, a so-called 4π technique in the confocal fluorescence microscopy [5] that improved resolution as well as inspection of the depth of field. Although it was not until 1994 that the first application of confocal microscopy was demonstrated, including the work of young Stefan Hell [6], the pioneering concept of Cremer has inspired many new developments.

Among others, the concept of two-photon quantum transitions in atoms that have been already theoretically studied by a Nobel awardee Maria Goeppert Mayer in 1931 in her doctoral dissertation [7] was translated into a real application in two-photon microscopy. With the invention of the laser, nonlinear optical second-harmonic generation was used in 1974 by Robert Helwarth and Paul Christensen [8] to investigate polycrystalline materials, and in 1978 J.N. Gannaway and Colin Sheppard [9] used a confocal scanning microscope to demonstrate second-harmonic microscopy. However, it was Winfried Denk together with James Strickler and Watt Webb [10] who demonstrated in 1990 the first application of two-photon absorption with fluorescence microscopy using a biomedical specimen, and only four years later it was applied to intravital microscopy that nowadays uses multiphoton absorption in living animals in vivo.

From then, challenging the diffraction limit in fluorescence microscopy became a red queen and exciting innovative inventions

followed at breathtaking speed. It now appears that there is no fundamental limit in achieving spatial resolution; using visible light, it is possible to resolve up to a few nanometers with these approaches, Therefore, it is straightforward that these techniques being the source of modern fluorescent materials, have been awarded together the Nobel price in 2014 in chemistry – and naturally have been known since many years under the name of nanoscopy.

The first technique to substantially achieve the subdiffraction resolution was a stimulated emission depletion (STED) microscopy that is now known as Stefan Hell's path-breaking noble invention, first proposed in 1994 [11, 12]. As instructively described in the Nobel lecture article by Stefan Hell in this issue, the invention is based on an innovative idea to saturate fluorescence transitions by pushing them into a state where they can no longer send a fluorescence signal. The ingenious trick to considerably enhance the resolution is the de-excitation of the outer part of an illuminated region by a red-shifted ring-shaped beam, a so-called donut beam. The excited molecules in this state experience stimulated emission by this beam and relax into the previous state. Thus, the fluorescent volume is considerably shrunk, and thereby the microscopy resolution is enhanced.

This concept is different from the previous ones because it does not use a shorter wavelength or nonlinear optics to create better resolution and it does not require confocal imaging but can be applied to a wide field. Thus, it is a White Queen among super resolution techniques: it operates in anticipating and changing future events in fluorescent molecules, having two distinguishable states where reversible switching is possible and at least one such transition state can be optically controlled. It can be tailored in such a way to achieve resolution far below the diffraction limit, almost unlimited down to the molecular scale. Today, a number of techniques based on this reversible saturable optical fluorescence transition principle (RESOLFT) have been invented, including STED as well as ground-state depletion microscopy, also first suggested by Stefan Hell [13].

The 1990s also witnessed another approach to superresolution microscopy based on wide-field microscopy to image cellular nanostructures stained with fluorescent markers. It is based on the statistical fluorescence behavior of single fluorescence emitters known as "blinking" that can be exploited for single-molecule detection. However, in order to improve statistical evidence and make these methods more applicable, single elements are "optically isolated" from their surroundings. Thus, only a single nanoparticle or molecule is present in the field of view at a certain time, thus allowing the resolution of its size to be achieved, mostly in the range of a few hundred nanometers. These techniques are known as spectral precision distance microscopy (SPDM).

One way is to use the structured illumination or spatially modulated illumination (SMI [14]) to optically isolate single elements in the transverse space. Nowadays, spatially nonuniform illumination is considered as the most attractive way to achieve superresolution [15].

Another way is to controllably turn fluorescent proteins on and off in time by using different wavelengths. William Moerner was the first to discover that the "workhorse", green fluorescent protein GFP could be excited with a blue light with a wavelength of 488 nm, then faded, and could be again returned to life with a light of slightly smaller wavelength of 405 nm [16]. This idea served as a base for photoactivated localization microscopy (PALM) that exploits blinking by optically isolating single fluorescent molecules in time, just stochastically switching single molecules on and off for the time of a wide-field image acquisition with a weak laser pulse. In this way, only a fraction of the fluorescence tags light up, enough to take a first picture. The next light shot then activates a different fraction of tags, and by repeating the process many hundreds of times, and processing them on a computer, the highest resolution images can be obtained. This technique was awarded the Nobel prize as well and was developed on the basis of William Moerners' concept by Eric Betzig and Harald Hess at the Howard Hughes Medical Institutes [17]. The same principle was independently applied by Sam Hess from the University of Maine to a nanoscopic tool [18] and by Xiaowei Zhuang from Harvard University to stochastic optical reconstruction microscopy (STORM) [19]. Nowadays these techniques allow imaging of cell interiors to unprecedented levels of detail in the range of a few tens of nanometers. Even three-dimensional stochastic optical reconstruction of living biological species and imaging of molecular structures of the cell are possible at present.

Thus superresolution imaging does not only allow stepping from microscopy to nanoscopy but it also narrows the gap between the optical microscopy and the imaging schemes relying on other electromagnetic waves of smaller wavelengths, such as X-ray and electron microscopy.

The impact and future potential of these complementary techniques are thus tremendous. On the one

hand, these techniques allow non-invasive investigation of the interior of biological cells and observe the dynamics of molecules, which is fascinating by itself and opens up a completely new insight for biologists. Peek inside nerve cells, track proteins that cause such disastrous diseases as Alzheimer or cancer, or watch how stem cells divide in living embryos have been the visions of medical researchers for years, and they are now coming true – just as going through a new looking glass. On the other hand, they promise nanoimaging of semiconductor devices in the information industry, where new generations of computer chips in the range of a few tens of nanometers are now produced.

The advent of superresolution optical microscopes will make it possible to observe the world around us with a new level of clarity – as being Lewis Carroll´s Alice seeing clearly through the looking glass. This will for certain lead to a very exciting new science, particularly within but not limited to the field of biomedicine.

Cornelia Denz
Institute of Applied Physics, University of Muenster, Germany
E-mail: denz@uni-muenster.de

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