Would it not be great to observe the cell organelles and the myriad of proteins in a living cell in 3D with nanoscale resolution and with a non-invasive method? Until recently, devising or even building such a microscope had been a fundamental scientific problem. The reason for this is that a non-invasive, high-resolution microscope needs to rely on focused visible light. Fluorescently labeled proteins, nucleic acids, and lipids can be mapped in a living cell with unmatched sensitivity. However, the established light microscopy methods suffer from a critical disadvantage, namely their diffraction-imposed resolution, which is limited at best to ~500 nm in the direction of light propagation (z-axis) and ~200 nm in the transverse (xy) direction. The diffraction barrier was discovered by Abbe in 1873 and has been paradigmatic ever since.

We have recently succeeded in introducing physical concepts that break Abbe’s barrier. In fact, we intend to explore all the possibilities of making the microscope sharper. Our primary scientific aim is to push fluorescence microscopy resolution down to the nanometer range and to eventually apply it to the field of biology. This challenging endeavour requires a joint effort of physicists, chemists, engineers, and biologists alike.

Perhaps the most intuitive method for sharpening the spot is to selectively inhibit the fluorescence at the circumference by stimulated emission. Technically, the Stimulated Emission Depletion (STED-) microscope relies on pairs of synchronized laser pulses. The primary excitation pulse produces a 3D spot of excited molecules of regular diffraction size, and the immediately following red-shifted STED pulse quenches the excited molecules down to the ground state. By arranging the STED pulse in a doughnut mode, only the fluorescence at the periphery of the spot is inhibited, whereas the very centre ideally remains untouched. To date, an improvement of factor 3 in the transverse direction and up to 6 along the optic axis has been demonstrated (Fig. 1). In a similar manner, it is possible to deplete the ground state of the fluorophore. Both concepts rely on the nonlinear optical phenomenon of saturation and have the potential to provide resolution down to the molecular scale.

A powerful way of sharpening the axial resolution of a microscope is the coherent superposition of focal wavefronts. This idea has been realized in our 4Pi-microscope for which we use two opposing objective lenses, adding up their wavefronts to form a 3- to 7-fold improvement in spatial resolution along the optic axis. The resolution is further enhanced by the application of (non-linear) image
Fig. 1. Stimulated emission depletion microscope. The diffraction-limited distribution of excited molecules generated by the green beam is depleted at the periphery through stimulated emission with the red beam (a). The resulting fluorescence spot (b) is fundamentally reduced as compared to its standard counterpart (c).

Fig. 2. Optical section-image through the microtubules of a fixed mouse-fibroblast cell, labeled by immunofluorescence. In conjunction with image restoration techniques (Fig. 2). The superior 3D-resolution of a 4Pi-confocal microscope is illustrated in Fig. 3, which depicts a 3D-rendered image of the mitochondrial network of a live budding yeast cell. The combination of both microscopes, namely the STED-4Pi-microscope, was the first to demonstrate a spatial resolution in the double-digit nanometer range (30-40 nm) with visible light and regular objective lenses.

Fig. 3. 3D-rendered image of the mitochondrial network of a live yeast cell. The mitochondrial matrix is labeled with green fluorescent protein, whereas the cell wall is counterstained with the dye calcofluor white and displayed by ray tracing. Live cell 4Pi-imaging with ~100 nm 3D-resolution allowed us to study the influence of selected mitochondrial proteins on mitochondrial morphology.