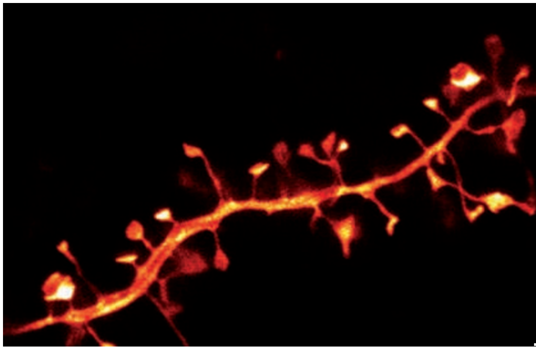
# Light microscopy at the limit



Stimulated-emission-depletion image recorded from a living neuron of an organotypical hippocampal slice, showing dendritic spines in unprecedented detail. The neuron is expressing yellow fluorescent protein. Image courtesy of K. Willig, V. Nägerl, N. Urban, T. Bonhoeffer & S. W. Hell, MPI Biophysical Chemistry, Göttingen, Germany

For most of the twentieth century, it was held that far-field light microscopy could not resolve objects closer than 150–200 nanometres (see Milestone 3). This is because light diffracts as it passes through different media, so that the light emanating from a point is detected as emerging from a larger volume. Light microscopy was therefore said to be 'diffraction-limited'.

Yet many cellular processes take place within these diffraction-limited distances, at length scales of tens to hundreds of nanometres (nm). To be able to visualize these processes, therefore, microscopy with substantially improved resolution was needed. It became necessary to 'break' the diffraction barrier.

After early work that achieved the maximum theoretical resolution — including the standing-wave illumination reported by Fred Lanni and Jans Taylor and the two-photon 4Pi microscope of Stefan Hell and Ernst Stelzer — Hell showed, first theoretically and then experimentally, that a stimulated-emission-depletion (STED) microscope can break the diffraction barrier. To do this, the excitation focal spot is shrunk to a very small size by depleting the fluorophores at its rim through stimulated emission with a doughnut-shaped STED beam of red-shifted light. The tiny spot is then scanned over the sample to generate a sub-diffraction image. In the first experimental implementation of STED, which included imaging of live yeast and bacteria, resolution was improved both axially and laterally and reached about 100 nm. The resolution has since been improved further, allowing the technique to be used to show that the synaptic vesicle-associated protein synaptotagmin remains clustered on the neuronal plasma membrane after exocytosis.

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While STED was being demonstrated, Mats Gustafsson was developing an approach called structured illumination microscopy (SIM). In SIM, the excitation light is structured in a controlled pattern, which renders normally unresolvable information accessible after image processing. Although still diffraction-limited, SIM improved lateral resolution by about twofold and produced clearer images of biological structures. The technique has since been extended to three dimensions using 3D structured light; moreover, it enables the diffraction barrier to be broken, using nonlinear processes.

Finally, by exploiting the known ability to localize single molecules with nanometre precision, several single-molecule approaches to achieving sub-diffraction resolution were developed. The pioneers of these methods — Eric Betzig and Harald Hess for photoactivated localization microscopy (PALM), Xiaowei Zhuang for stochastic optical reconstruction microscopy (STORM) and Samuel Hess for fluorescence photoactivation localization microscopy (FPALM) — used the combination of photoactivatable or photoswitchable fluorophores and high-accuracy single-molecule localization to break the diffraction barrier. Although the details differ, all three methods are based on a single principle: a sparse subset of fluorophores is switched on at any one time, and each molecule is localized with high accuracy. These fluorophores are then switched off and the process is repeated with another subset until enough information has been collected to generate a sub-diffraction image.

The hope for super-resolution microscopy is that visualizing cells at this unprecedented scale will yield unprecedented insights. Although the application of these methods to biological questions has begun, the full realization of their potential is still to come. Their extension to imaging in all three dimensions and within living cells — capabilities that are developing rapidly — will be a crucial part of this process.

*Natalie de Souza, Associate Editor,*
Nature Methods

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