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# Expansion Microscopy: Beyond limits

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The power of resolution of conventional light microscopy is physically limited by the wavelength of the visible light. This was well established by Abbe almost 150 years ago (1873). Resolution is the ability to recognise two structures as separate entities. The theoretical limit of light microscopy, i.e., the smallest distance (SD) separating two structures that can be recognised by conventional microscopes, can be determined by this mathematical equation:

SD = 1.22 x wavelength / (NA objective + NA condenser)

Where NA is the numerical aperture, a measure of the light-gathering ability of the lenses. It can be easily calculated that when we use good objectives and condensers of NA=1.4 and blue-green light (≈500 nm), the SD that can be detected between two separate structures is about 200-220 nm. Usually, the quality of the sample and other factors make difficult to reach even this limit in practice.

Thus, irrespective of the magnification provided by the objectives and oculars, the properties of the visible light establish a limit for the resolution and the quality of images that can be obtained by light microscopy. The invention of electron microscopy allowed for surpassing this limit, but the samples required a treatment that was for a long time incompatible with techniques such as fluorescence or 3D imaging, etc. However, some advances have led to a resurgence of interest for electron microscopy, including multicolor imaging (Gaietta et al., 2002), volume electron microscopy (reviewed in Peddie and Collinson, 2014), combined light and electron microscopy (Perkovic et al., 2014) and combined cryo-fluorescence microscopy and cryo-electron Microscopy (Schorb and Briggs, 2015).

During the last decade, the apparently absolute limit of light microscopy was broken by the work of a number of researchers that exploited the properties of fluorescence. Different approaches were considered, and most of them can be grouped in two categories:

- Deterministic super-resolution (also known as RESOLFT techniques for Reversible Saturable/Switchable Optical Fluorescence Transitions). For example, in the Stimulated Emission Depletion (STED) technique the fluorophores are excited by a laser beam, but switched off by another laser of a different wavelength. In this way, the emission of fluorescence can be concentrated in very small areas providing nanoscale resolution (Fig. 1). Other techniques such as Ground State Depletion (GSD) or Saturated Structured Illumination Microscopy (SSIM) exploit the nonlinear response of fluorophores to excitation, that can be used to enhance resolution.
- Stochastic super-resolution: Most of these techniques are based on single-molecule localization methods, for example Photoactivable Localization Microscopy (PALM) or Stochastical Optical Reconstruction Microscopy (STORM).

These techniques are reviewed, for example, in Heintzmann and Ficz (2013), Kusumi et al. (2014), Small and Parthasarathy (2014) and Long et al. (2014). They have allowed spectacular enhancements in resolution, giving rise to the new concept of “nanoscopy” or optical microscopy at nanomet-

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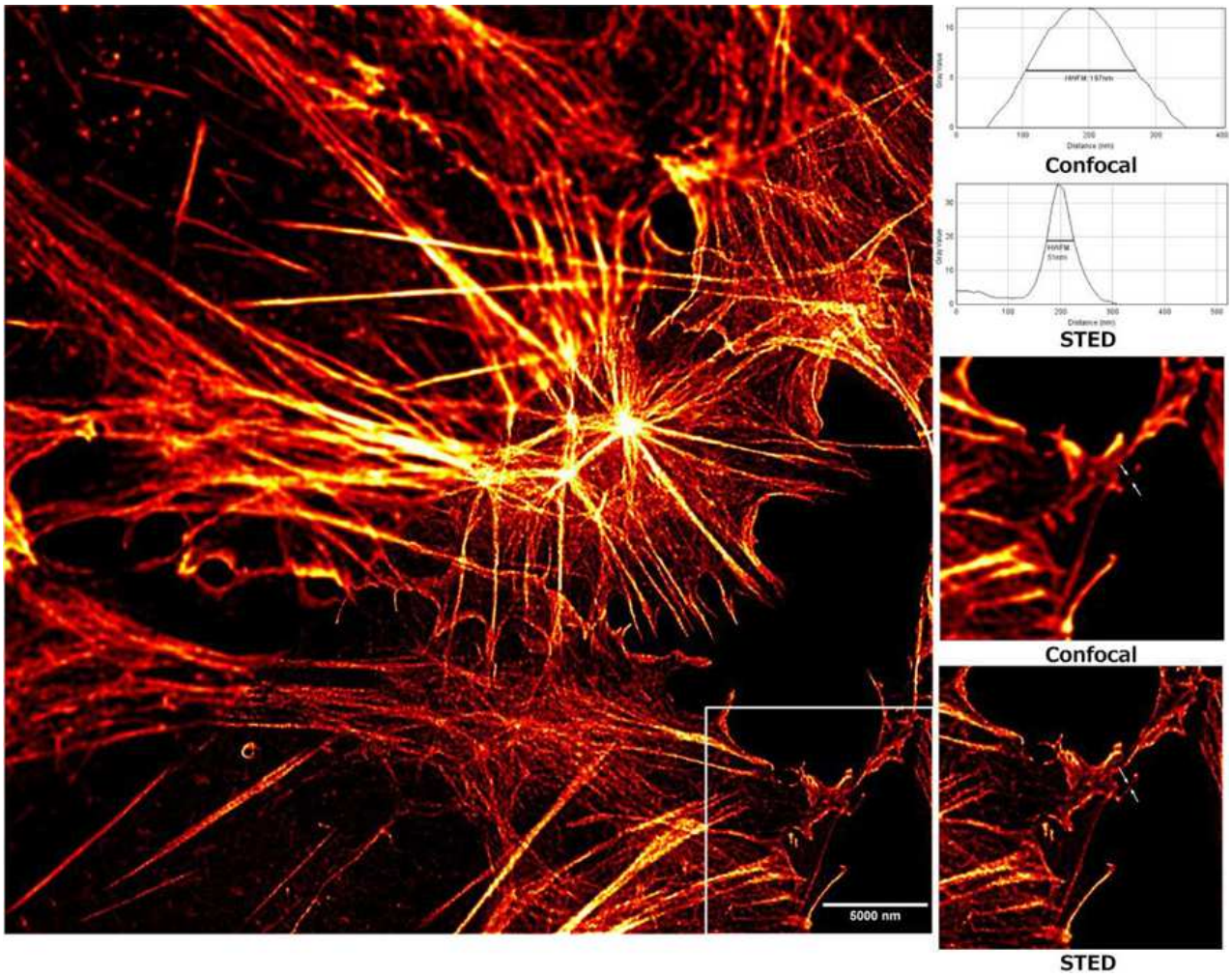


**Fig. 1.** Resolution improvement between traditional confocal microscopy and stimulated emission depletion (STED) microscopy. Cells were stained with 647-phalloidin to show actin filaments and the images were taken on a Leica SP5 2P STED microscope. FWHM - Full width at half maximum. By Howard Vindin - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=40722030

ric scale. Resolutions of 20-70 nm can be reached with these techniques. The extraordinary importance of these advances were acknowledged with the Nobel Prize of Chemistry 2014 awarded to Eric Betzig (Howard Hughes Medical Institute), William E. Moerner (Stanford University) who developed single molecule localization and PALM, and Stefan Hell (Max Planck Institute for Biophysical Chemistry) who had established the theoretical base of the STED technique in 1994 (Hell and Wichmann, 1994), and divulged the first STED super-resolution images in a paper published in *PNAS* after rejections by *Nature* and *Science* (Klar et al., 2000).

All these super-resolution techniques require expensive and complex microscopes, and specifically designed equipment are required to apply them, and for that reason nanoscopy is still out of reach of many laboratories.

A striking, alternative approach for nanoscopy consists in working with the sample, keeping unchanged the microscope technology. In other words, the idea is to keep intact the limit of the resolution, but to increase the size of the structures under examination. Obviously, if the sample volume increases four times, distances of 50 nm between structures become distances of 200 nm. Thus, cellular structures of sizes below the limit of resolution can be resolved with conventional microscopes (Fig. 2). The big issue is in that “if”. How can a sample be “inflated” without spatial distortion or structural damage?

This issue has been solved by the group of Edward Boyden at the Massachusetts Institute of Technology (Chen et al., 2015). The method used is related with CLARITY, a technique that we already described in a former article (Muñoz-Chápuli, 2013). CLARITY consisted in the infusion of an acrylamide solution in the sample together with a detergent (SDS) and a cross-linking fixative (formaldehyde). Acrylamide polymerization originates a stiff, nanoporous hydrogel that preserves the 3D structure of the sample. Proteins and nucleic acids remain crosslinked to the polyacrylamide

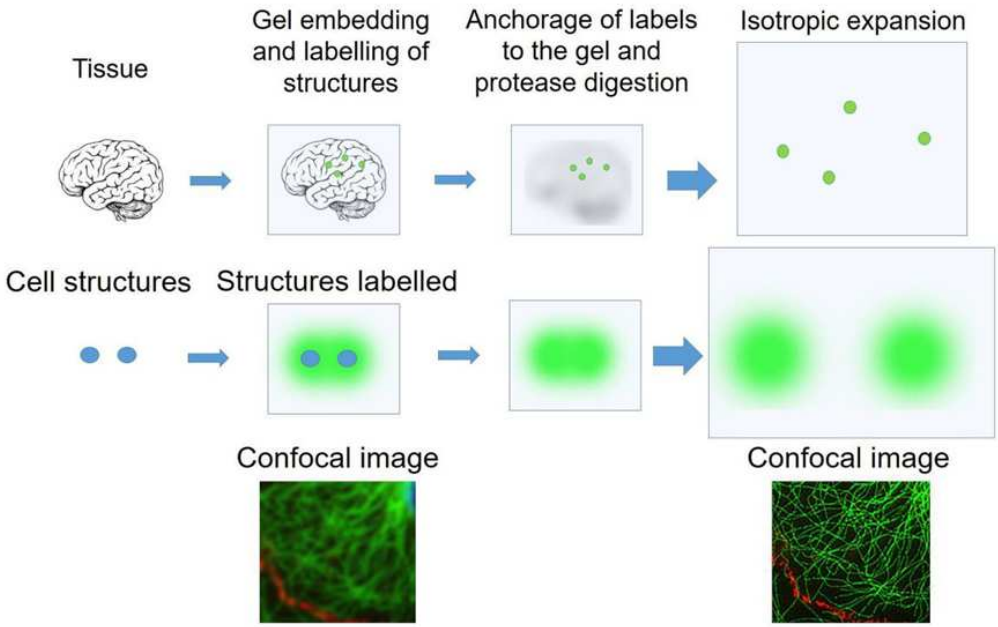


**Fig. 2.** Scheme of the expansion microscopy technique as originally described by Chen et al. (2015). The tissue is embedded in a hydrogel, the structures of interest are labelled with fluorochrome-conjugated antibodies, and the fluorochromes become anchored to the gel before protease treatment. Then, isotropic expansion of the gel increases the distance between labels and allows for a much improved resolution of the image. In this way the limit of resolution of conventional light microscopy can be surpassed. Further refinements of this technique are described in the text.

chains. Finally, the detergent extracts all the lipids of the tissue rendering the sample optically transparent. In this way, whole brains from adult mice can be studied *in toto* with long working distance optical microscopy. The retention of proteins and nucleic acids also allow for immunohistochemistry and *in situ* hybridization techniques.

Expansion microscopy (ExM) utilizes a similar principle, but expanding the gel through dialysis. The original study of Chen et al. (2015) demonstrates that this expansion is isotropic, and the spatial relationship among the structures remains constant (Figure 2). ExM starts with a fixation step with formaldehyde, followed by permeabilization and infusion of an acrylamide solution together with a heat-activated cross-linker agent. After acrylamide polymerization, the sample is treated with proteases. Protein digestion homogenizes the mechanical properties of the sample. Then, dialysis of the gel isotropically increases its volume until a factor of 4-5, keeping constant the spatial relationships within the sample. It is obvious that the protease treatment provokes the loss of most cellular structures (however, this is not completely true, as we will see below). For that reason a previous step of labelling is required. The labelling is performed before acrylamide gelification, and involves specific reactives. In the original paper of the Boyden's group, primary antibodies against the epitopes of interest are used, followed by incubation with special secondary antibodies conjugated with oligonucleotides. Complementary oligos are designed conjugated with fluorophores and metacryloil groups. Thus, before gelification, primary and secondary antibodies are localized on the epitopes of interest. During gelification the metacryloil groups become covalently linked to the acrylamide chains. After protease digestion the antibodies disappear, but the fluorochromes remain *in situ* due to their anchorage to the gel. Interestingly, what can be observed by confocal or conventional fluorescence microscopy are not the structures themselves, but a "ghost" image of the precise places where the epitopes were located in the structures. Due to the isotropic expansion of the gel, the resolution attained with conventional microscopes is beyond the 200 nm limit and it can reach 70 nm. Furthermore, the transparence of the gel to visible light adds to ExM the advantages of the CLARITY technique. Thus, the original paper of Chen et al. (2015) shows high resolution, three-colour images of a mouse hippocampus ($10^7$ $\mu m^3$ in volume) and also images of cultured cells.

Further refinement of ExM has been performed more recently by the Boyden's group and by other research teams (Tillberg et al., 2016; Chozinski et al., 2016). Chen et al. (2016) combined fluorescent *in situ* hybridization with ExM (Ex-FISH). In this particular technique, the cellular RNA is covalently linked to the gel, and then conventional FISH is

performed. In this way, individual RNA molecules can be visualized by confocal microscopy, and precise subcellular localization of these molecules can be investigated. The anchorage of the RNA to the acrylamide chains is performed using custom molecules (called LabelX) synthetized from commercial precursors. Ex-FISH allowed to nanoscale imaging of large non-coding RNA (lncRNA). Xist (X -inactive specific transcript), for example, is an lncRNA involved in the inactivation of the X chromosome by binding to specific areas of the chromatin. Images of Xist RNA were obtained by Chen et al. (2016). Also the ring-like shape of Neat1 lncRNA (nuclear enriched abundant transcript 1), another regulator of gene expression at chromatin level, was demonstrated by Ex-FISH.

It was assumed that the protease treatment required for ExM degradated all the proteins precluding for example the observation of native fluorescent proteins. However, two recent papers (Tillberg et al., 2016; Chozinski et al., 2016) have demonstrated that proteins can also be covalently anchored to the acrylamide gel in such a way that after protease digestion enough protein fragments remain to use conventional antibodies for immunofluorescence.

The original paper by the Boyden's group required the use of customized, non-commercial molecules (secondary antibodies linked to oligonucleotides and fluorochrome/methacryloyl-conjugated oligos). The technique described in Tillberg et al. (2016) involves the use of Acryloyl-X, a reactive that modifies the amino groups of proteins with an acrylamide group. After polymerization, the proteins become covalently linked to the polyacrylamide gel. The tissue is then subjected to a soft digestion, using for example an alkaline detergent in autoclave or the endoproteinase Lys-C that only cut proteins at lysil groups. The resistance of protein fragments to this treatment allowed for immunofluorescence after tissue expansion. Even proteinase-resistant proteins such as GFP keep their native fluorescence and they can be detected at nanoscale with this method.

Chozinski et al. (2016) describe two alternative treatments, methacrylic acid N-hydroxysuccinimidyl ester and glutaraldehyde postfixation. In both cases, the linkage of proteins to the polyacrylamide gel is improved, and the results are similar to those shown by Tillberg et al. (2016).

In summary, ExM is a very promising and attractive set of techniques that allows super-resolution and nanoscale microscopy to the laboratories equipped with conventional confocal microscopes. No specific reactives, antibodies or probes are required, multicolor and 3D imaging is possible and the clearing of the tissue makes possible to work with thick samples.

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