Super-resolution microscopy writ large

Peter Engerer, Caroline Fecher & Thomas Misgeld

Improved expansion microscopy provides a super-resolution method that is accessible to any laboratory with a fluorescence microscope.

Seeing is believing, but many subcellular structures are simply too small and densely packed to be resolved with light microscopes. Over the past two decades, ever-more sophisticated techniques for 'super-resolution' microscopy have succeeded in surpassing the capabilities of conventional microscopy. And last year, a new technology called expansion microscopy[1] achieved the precision of super-resolution imaging not by optical tricks but by a radically different approach in which the biological specimen is physically enlarged through chemical treatments. In this issue and in a recent issue of *Nature Methods*, three groups, Tillberg *et al.*[2], Ku *et al.*[3], and Chozinski *et al.*[4], improve on this method with simplified protocols that use off-the-shelf chemicals and are compatible with standard immunofluorescence techniques and genetically encoded fluorophores. The new protocols should broaden the applications of expansion microscopy and bring it within reach of almost any laboratory.

Light microscopy has contributed tremendously to our understanding of cell biology. But the physics of light diffraction limits the spatial resolution of conventional microscopes to ~200 nm in the lateral dimension and ~500 nm in the axial dimension, which means that distinct structures in closer proximity cannot be resolved. Many subcellular organelles are smaller than 200 nm and are densely packed, eluding visualization as individual structures by light microscopes. For many years, only electron microscopy could circumnavigate the diffraction limit of light, but conventional

Peter Engerer, Caroline Fecher and Thomas Misgeld are at the Institute of Neuronal Cell Biology, Munich, Germany, the Center of Integrated Protein Science (CIPSM), Munich, Germany, the German Center for Neurodegenerative Diseases (DZNE), Munich, Germany, and the Munich Cluster of Systems Neurology (SyNergy), Munich, Germany.
e-mail: thomas.misgeld@tum.de

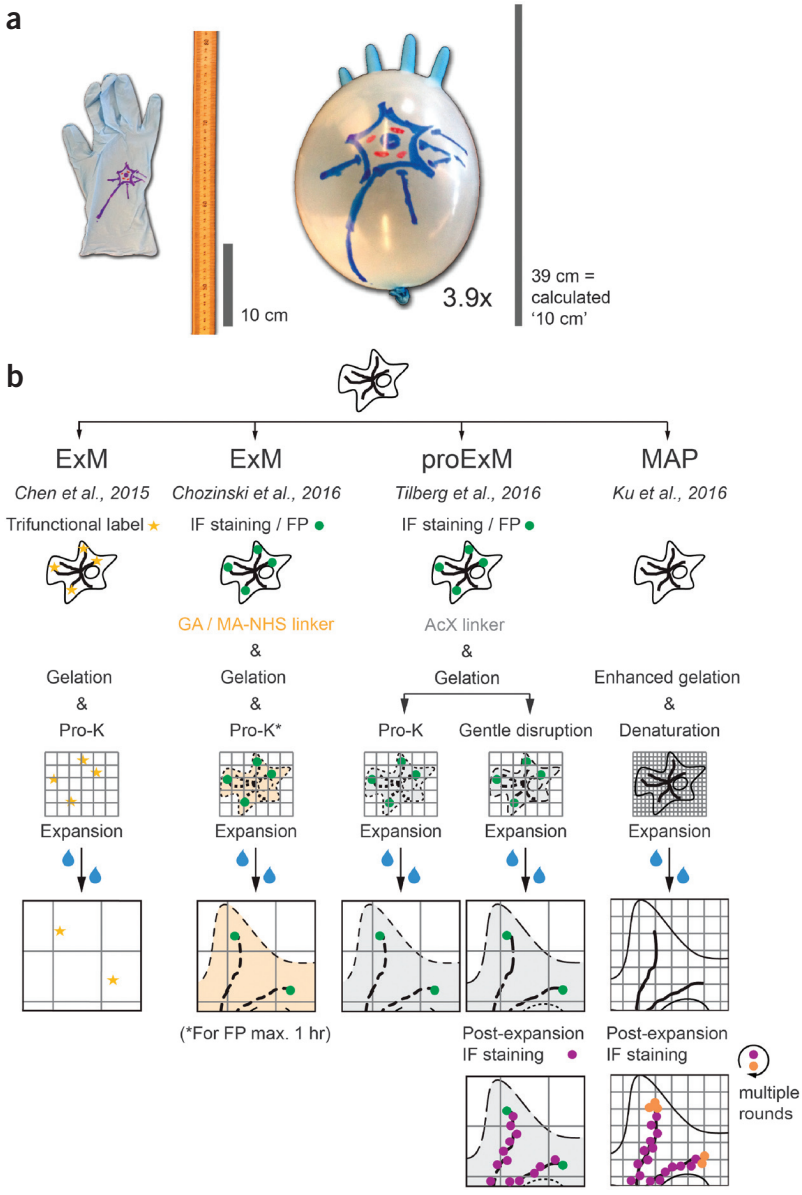

Figure 1 Concept and workflow of refined expansion microscopy techniques. (**a**) Expansion microscopy expands tissue embedded in a hydrogel, similar to how the expansion of a balloon reveals details of a sketch drawn on its surface (i.e., in 2D). (**b**) Simplified comparative workflow of different expansion microscopy protocols.

Table 1| Comparison of demonstrated expansion microscopy techniques as reported

	ExM[1] Boyden laboratory	ExM[4] Vaughan laboratory	proExM[2]	MAP[3]	ExFISH[9]
Target	Proteins	Proteins and DNA (dye)	Proteins	Proteins and sugar residues	RNA and DNA (dye)
Matrix	2.5% acrylamide sodium acrylate and MBAA	2.5% acrylamide sodium acrylate and MBAA	2.5% acrylamide sodium acrylate and MBAA	30% acrylamide, sodium acrylate and BA	2.5% acrylamide sodium acrylate and MBAA
Linking agent	Trifunctional DNA-oligomer	MA-NHS, GA	AcX	None	LabelX, (AcX plus Label-IT amine)
Sample disruption	Digest proteinase K, >12 h, RT	Digest proteinase K, 30 min to >12 h, 37 °C	Digest proteinase K, >12 h, RT; 4 h, 60 °C	Denaturation with SDS, 37 °C and dissociation at 70/95 °C, 6 h	Digest proteinase K, >12 h, 37 °C
Expansion factor (resolution)	4.5 (~70 nm)	4.0–4.2 (65 nm)	~4.0 (~70 nm)	4.0 (~60 nm)	3.3 (not reported)
Time (brain slice)	~6 d (100 µm)	~4.5 d (100 µm)	~3.0 d (100 µm)	~7 d (100–500 µm)	~4.5 d (50–200 µm)
FP preservation	No	Yes, max. digest 30 min to 1 h	Yes, ~50% intensity	No	Not applicable (combination with proExM[2])
IF staining	No	Yes, modified mAb	Yes	Yes	No
Sample	Cells, tissue: brain	Cells, tissue: brain	Cells, tissue: brain, pancreas, lung, spleen	Cells, tissue: brain, lung, heart, spinal cord, liver, kidney, intestine	Cells, tissue: brain
Comment	First report of the concept, customized reagents needed	Pre-expansion staining	Pre-expansion staining; post-expansion staining possible	Post-expansion staining of preserved epitopes; multiplexing	Post-expansion FISH with multiplexing and HCR amplification

AcX, Acryloyle-X; mAb, antibody; BA, bisacrylamide; FISH, fluorescence *in situ* hybridization; FP, fluorescent protein; GA, glutaraldehyde; HCR, hybridization chain reaction; MA-NHS, methacrylic acid-*N*-hydroxysuccinimidyl ester; MBAA, *N,N'*-methylenebisacrylamide; RT, room temperature.

electron microscopy is extremely laborious and restricted to minuscule tissue volumes, and offers only limited options for labeling specific antigens or other molecular targets. More recently, super-resolution fluorescence microscopy has improved resolution of light microscopes by about an order of magnitude (in optimal cases as low as ~20 nm lateral resolution) through techniques for spatially shaping the illumination and exploiting stochastic variations in fluorescence emission[5]. However, super-resolution techniques are not in wide use owing to the high cost of the microscope, the need for specialized expertise, or other inherent shortcomings (e.g., the difficulty of multicolor experiments, the limited imaging depth, the high power of excitation light, and the slow acquisition speed).

Expansion microscopy promises to overcome a number of these practical and scientific limitations. In essence, the approach amounts to taking an organ, fixing it, and blowing it up like a balloon (**Fig. 1a**). Could it really be this simple to bypass the resolution limit of light microscopy? Although many might have had initial doubts, the new studies[2–4] provide evidence that expansion microscopy is a viable and robust technology.

Expansion microscopy relies on a flaw of some tissue clearing protocols whereby the size of specimens is inadvertently altered[6]. It takes advantage of this effect by increasing the extent and isotropy of the expansion to enlarge the specimen about fourfold and achieve an effective lateral resolution of ~70 nm on conventional light microscopes[1]. First-generation expansion microscopy was carried out by labeling protein antigens, embedding the sample in a hydrogel, tethering the label to the hydrogel, digesting the original tissue, and expanding the hydrogel 'blue print' (**Fig. 1b**). But because any fluorescent protein or antibody used for labeling would be degraded, it was necessary to use specialized, non-protein reagents.

The new studies[2–4] address this limitation in different ways (**Fig. 1b** and **Table 1**). Tillberg *et al.*[2] and Chozinski *et al.*[4] introduce expansion microscopy variants, which preserve proteins in the sample during the expansion process to a degree that allows standard fluorescent protein fusions and off-the-shelf secondary antibodies to remain detectable. This is achieved by anchoring proteins to the hydrogel and minimizing proteolytic homogenization by proteinase. Ku *et al.*[3] present magnified analysis of the proteome (MAP), which allows for multiple rounds of immunostaining in an expanded specimen. Repetitive rounds of immunostaining have previously been reported for ultrathin sections[7] and for cleared tissue[8]. Now, the intersection of repetitive immunostaining and tissue expansion makes it possible to study at high resolution a wide array of antigens (although, currently, nothing close to a full proteome)[3]. MAP achieves expansion through preventing intra- and interprotein crosslinking during the hydrogel–sample hybridization. By applying a high concentration of acrylamide the interaction of sample and hydrogel is maximized and isotropic expansion is achieved after high-temperature denaturation without using proteinase K.

Expansion microscopy has already been applied successfully to several tissues and species, and it is likely to be adaptable to a wide range of specimens. As these applications are developed, the isotropy of the expansion will remain a key concern, especially for tissues with special mechanical properties, such as muscles and tendons. Thus, similar to previous new fixation and microscopy techniques, it will be important to validate results and distinguish facts from artifacts by painstaking comparative analysis across different imaging modalities.

Properly controlled, however, refined expansion microscopy techniques[1–4,9] (**Table 1**) offer several advantages over 'conventional' super-resolution microscopy, including low cost and the ease of performing multicolor experiments. Another notable advantage is that thick

specimens can be imaged with great detail in all three spatial dimensions. Because refractive index heterogeneities of cells and tissues cause optical aberrations, the spatial resolution of super-resolution microscopy quickly deteriorates as the imaging plane is moved deeper into the specimen. This typically restricts the usefulness of super-resolution microscopy to a few micrometers, which is often not enough to capture the depth of one cell, let alone the architecture of complex tissues. In contrast, specimens in expansion microscopy consist largely of water, so that optical aberrations are negligible and deep imaging is relatively straight forward.

Of course, expansion microscopy faces several challenges of its own. The samples can become rather bulky, although objectives with extremely long working distances have already been designed for cleared tissue, and re-slicing approaches (e.g., using an on-stage vibratome) are conceivable. In addition, image brightness diminishes as fluorophore density is diluted with expansion (i.e., by the third power of the expansion factor, as in all super-resolution approaches) and also from the necessary chemical treatments (up to a factor of two in the present reports[2,4]). Thus, depending on the degree of desired resolution gain and the strength of chemical treatment needed for tissue homogenization, expansion microscopy techniques might be restricted to relatively bright samples or might require additional amplification steps. Ameliorating this concern is the near-transparency of specimens, which will facilitate imaging modalities geared toward dim samples and fast imaging, such as light-sheet microscopy[10]. Moreover, the limits of expansion microscopy in improving resolution remain to be determined. It is unclear whether specimens can be isotropically expanded tenfold (or more) to reach the equivalent to 20-nm lateral resolution, now achievable with super-resolution microscopy. Finally, as expansion microscopy is a fixed-tissue method, it cannot be used on live samples, an area in which super-resolution microscopy is making steady progress.

All of this suggests that super-resolution microscopes and 'ballooned' samples will be allies, not competitors, in the search for finer cellular detail —yet another example of how physics and chemistry together shape biological inquiries.

ACKNOWLEDGMENTS

The authors thank the Neurobiology Course at the Marine Biological Laboratory for being their summer home for many years. T.M.'s laboratory is supported by the Deutsche Forschungsgemeinschaft (DFG) through the Munich Center for Systems Neurology (SyNergy; EXC 1010), the Center for Integrated Protein Science Munich (CIPSM, EXC 114), Collaborative Research Center 870, Priority Program 1710 and DFG Research Grant (MI 674/7-1). Further support came from the European Research Council under the European Union's Seventh Framework Program (FP/2007-2013; ERC Grant Agreement n. 616791) and the German-Israeli Foundation. T.M. is also associated with the German Center for Neurodegenerative Diseases (DZNE Munich). P.E. was supported by the DFG Research Training Group 1373 and the Graduate School of the Technische Universität München (TUM-GS); C.F. is supported by the Graduate School of Systemic Neuroscience (GSN, GSC 82).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Chen, F., Tillberg, P.W. & Boyden, E.S. *Science* **347**, 543–548 (2015).
2. Tillberg, P.W. *et al. Nat. Biotechnol.* **34**, 987–992 (2016).
3. Ku, T. *et al. Nat. Biotechnol.* **34**, 973–981 (2016).
4. Chozinski, T.J. *et al. Nat. Methods* **13**, 485–488 (2016).
5. Galbraith, C.G. & Galbraith, J.A. *J. Cell Sci.* **124**, 1607–1611 (2011).
6. Richardson, D.S. & Lichtman, J.W. *Cell* **162**, 246–257 (2015).
7. Micheva, K.D. & Smith, S.J. *Neuron* **55**, 25–36 (2007).
8. Murray, E. *et al. Cell* **163**, 1500–1514 (2015).
9. Chen, F. *et al. Nat. Methods* **13**, 679–684 (2016).
10. Dodt, H.U. *et al. Nat. Methods* **4**, 331–336 (2007).

Precision medicine for autoimmune disease

Lucienne Chatenoud

An antigen-specific cell therapy for autoimmune disease avoids compromising immunity as a whole.

The field of oncology is abuzz over recent clinical trial results showing that patient T cells engineered in the laboratory are remarkably effective against a few intractable cancers. Chimeric antigen receptor (CAR) T cells express a recombinant receptor that binds a specific tumor antigen, inducing the cell to kill target tumor cells. Writing in *Science*, Ellebrecht *et al.*[1] have now adapted the approach to autoimmune disease. They developed chimeric autoantibody receptor (CAAR) T cells as an antigen-specific therapy for the autoimmune disease pemphigus vulgaris and showed, both *in vitro* and in mice, the capacity of the cells to selectively eliminate B lymphocytes that produce autoantibodies to desmoglein (Dsg) 3, the pathogenic mediators of the disease. If these results can be translated to human autoimmune disease, this would represent a major leap toward achieving durable remissions or cures for certain severe and disabling conditions.

Autoimmune diseases can affect almost any organ, and stem from a breakdown in immune tolerance to host-derived or 'self' antigens. Their frequency has risen steadily over the past four decades in industrialized countries[2], and together they represent the third leading cause of morbidity and mortality after cardiovascular disease and cancer. Although both autoreactive T and B lymphocytes contribute to some degree to the development of all autoimmune diseases, injury of a given tissue usually results from the predominant action of either one cell type or the other.

Current treatments for autoimmune diseases are based on anti-inflammatory and immunosuppressive agents—including engineered biologics; human or humanized monoclonal antibodies; and fusion proteins selective for certain immune cell subsets or signaling pathways[3]—but their effect is transient and not antigen-specific. Chronic administration of these agents leads to the common side effects of general immunosuppression, such as an increased incidence of infections.

Major efforts have been devoted to selectively targeting the autoantigen-specific response in various autoimmune diseases (multiple sclerosis, type 1 diabetes, and uveitis) by administering the autoantigen(s) through different routes (subcutaneous, oral, and parenteral). However, the efficacy seen in induced or spontaneous mouse models of autoimmunity has never been successfully translated to the clinic. Among biological agents, CD3 antibodies have shown particular promise for reversing established autoimmunity and durably restoring self-tolerance in animal models. Clinical development of this approach is still in progress, but present data suggest that combination therapies may be needed to achieve a sustained effect in humans[3].

Ellebrecht *et al.*[1] have approached the challenge of antigen-specific therapy by designing a modified form of a CAR that leads T cells to kill autoreactive B cells. A conventional CAR fusion protein consists of an extracellular antibody moiety specific to an antigen of interest

Lucienne Chatenoud is at the Université Paris Descartes, Sorbonne Paris Cité, Paris, France, and INSERM U1151, CNRS UMR 8253, INEM Hôpital Necker-Enfants Malades, Paris, France.
e-mail: lucienne.chatenoud@inserm.fr