An introduction to the methodology of expansion microscopy

Faulkner, Emma; Thomas, Steven; Neely, Robert

DOI:

[10.1016/j.biocel.2020.105764](https://doi.org/10.1016/j.biocel.2020.105764)

License:

Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Faulkner, E, Thomas, S & Neely, R 2020, 'An introduction to the methodology of expansion microscopy', *The International Journal of Biochemistry & Cell Biology*, vol. 124, 105764.
<https://doi.org/10.1016/j.biocel.2020.105764>

[Link to publication on Research at Birmingham portal](#)

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

An introduction to the methodology of expansion microscopy

Emma L. Faulkner¹, Steven G Thomas^{1,2} and Robert K. Neely^{2,3}

Affiliations

1 Institute of Cardiovascular Sciences, University of Birmingham, Edgbaston, Birmingham, UK.

2 Centre of Membrane Proteins and Receptors (COMPARE), University of Birmingham and University of Nottingham, Midlands, UK

3 School of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

For correspondence

r.k.neely@bham.ac.uk

ORCID numbers

Emma L Faulkner - 0000-0002-2728-7617

Steven G Thomas – 0000-0001-8733-7842

Robert K Neely - 0000-0002-5023-8960

Abstract

Expansion microscopy is a novel, fluorescence imaging technique, which allows three-dimensional nanoscale imaging of specimens on a conventional fluorescence microscope. This is achieved through an innovative sample treatment, which culminates in approximately 4.5-fold expansion of specimens in each dimension. This allows 70nm lateral and 200nm axial resolution. To further develop application of the technique, there has been considerable focus on improving the methodology by i) extending the efficacy of labelling, ii) enabling multi-colour labelling of different biomolecules simultaneously, iii) further improving resolving power through alterations to sample preparation and iv) by combination of expansion microscopy with other well-established super resolution techniques. This review will highlight some of these recent advances and suggest ways that the technique could be developed further in the future.

Key words

Expansion microscopy, super resolution, imaging.

Key facts

1. Expansion Microscopy (ExM) allows three-dimensional nanoscale visualisation of specimens on a conventional fluorescence microscope.
2. By embedding specimens into a polyelectrolyte gel, specimens can be physically expanded 4.5x in each dimension, resulting in decrowding of nanoscale information.
3. Broader application of ExM has been enabled by use of commercially available chemical cross-linkers to anchor labels to the gel meshwork.
4. Use of trifunctional labelling approaches enables retention of fluorescence intensity post-expansion and labelling of a range of biomolecules e.g. lipids, proteins.
5. Higher resolutions can be achieved by increasing expansion factor of gels, or by combining ExM with other super resolution approaches.

1. Introduction

Expansion microscopy (ExM) is a fluorescence imaging technique which enables three-dimensional nanoscale imaging of specimens on a conventional fluorescence microscope. This method relies on embedding samples into a polyelectrolyte gel which can be swollen by the addition of water. This results in a physical increase in specimen size by 4.5x in each dimension (Chen *et al*, 2015). The net effect of this is decrowding of features within the specimen, meaning super resolution imaging can be performed on a conventional microscope, thus eliminating the need for sophisticated software and expensive hardware as exists with other super resolution microscopy approaches (Gao *et al*, 2017 & Chozinski *et al*, 2016). ExM allows for rapid image acquisition, and samples are optically cleared during the process. Clearing eliminates scattering of light through the sample, meaning imaging depth and volumes accessible are orders of magnitude higher than those achievable by other super resolution techniques. As a result, ExM is suited for rapid, scalable super resolution imaging in cells and thicker specimens such as tissue sections.

A schematic summarising the steps required for ExM preparation of samples is given (Figure 1) (Tillberg *et al*, 2016 & Chozinski *et al*, 2016). The specimen (e.g. cells, tissue) is labelled through immunofluorescence or genetically encoded fluorescent proteins (Fig 1A & B). Labels are chemically equipped with anchors to allow their incorporation into the network of a polyelectrolyte gel which is formed evenly and densely throughout the specimen (Fig 1C). The polymer-embedded samples are homogenised (proteins are digested) to enable isotropic expansion of the gel (Fig 1D). Addition of water results in the physical expansion of the gel, which now carries anchored labels describing the features of interest in the cell, by up to 4.5x in all three dimensions. This allows visualisation of labelled structures with an effective resolution of ~70nm laterally, and ~200nm axially (Gao *et al*, 2017) (Fig 1E). In this review, we give an introduction to the expansion microscopy technique, and highlight its inherent limitations and recent efforts to address these. We will also give a brief outlook on the scope and future applications of this spectacular new approach for super-resolution imaging.

2. Expansion Microscopy: Techniques and applications

2.1 Ensuring robust, isotropic expansion of specimens

A key consideration when applying ExM is determining that expansion is isotropic on the macro- and nanoscale. Isotropy of expansion is a measure of the uniformity of the expansion at the nanoscale, where anisotropy in the expansion leads to distortion in the expanded image. The expansion isotropy is dictated by two key molecular factors: formation of a dense, homogeneous polymer network through the specimen, and efficient homogenisation of the sample. Expansion isotropy can also be influenced by the mechanical handling of the sample (Vanheusden *et al*, 2020). In developing ExM, Boyden and co-workers developed a gel composition which provided optimal expansion and mechanical stability (Chen *et al*, 2015). A key feature of these gels is the meshwork size is estimated to be 1-2nm; well below the size of biomolecules, meaning that biomolecules are pulled apart from each other evenly whilst retaining their relative spatial organisation, down to a precision of 5-10nm for measurements on the micron-scale (Wassie *et al*, 2019). The gel meshwork is thought to be critical to retention of nanoscale information in ExM prepared specimens (Tillberg & Chen, 2019).

In each variant of ExM, distortions introduced during expansion have been characterised (Chen *et al*, 2015, Chozinski *et al*, 2016, Tillberg *et al*, 2016, Chen *et al*, 2016, Chang *et al*, 2017, Pernal *et al*, 2020). This has been commonly achieved through a correlative imaging approach where images of highly conserved structures (e.g. microtubules) are acquired pre-expansion with a super resolution

technique (e.g. structured illumination microscopy (SIM)) and compared to post-expansion images of the same features in the same cells. Using this approach, and after a single round of expansion, Chen *et al.* measured 1 – 4% errors in length measurements on the scale of tens to hundreds of microns, a level similar to that seen in other studies listed above. Isotropy of expansion has also been determined by imaging of the nuclear pore complex protein, Nup153, which has a highly conserved molecular configuration meaning that structural comparisons can be made pre- and post-expansion without the need to image the same cells (Pesce *et al.*, 2019). ExM has now been meticulously validated across a range of cell and tissue types and even whole mouse organs (Wassie *et al.*, 2019).

Accurate quantification of the isotropy of expansion is challenging due to the potential for inducing heterogeneities and distortions during the sample preparation (Pesce *et al.*, 2019). Determining expansion factor is hampered by observations of ‘differential expansion’ (Pernal *et al.*, 2020). This is the result of varying ionic conditions in samples, which impact differentially on the expansion factor of those samples. Using particularly challenging samples that had been fixed for pathology, Zhao *et al.* report expansion factors between four- and five-fold, with an average expansion factor of 4.7-fold (Zhao *et al.*, 2017). Pernal *et al.* observe a similar effect across different samples with some wholesale variation in expansion factor, where the fixative (paraformaldehyde) concentration used prior to expansion is varied (Pernal *et al.*, 2020). This renders absolute distance measurements challenging.

Recent work by Martinez *et al.* has shown differential expansion of regions of the hydrogel that either carry or are devoid of cells. However, the same study also showed that nanoscale expansion of the gel around the cell is isotropic and, indeed, quantitative measurements in expanded samples can be made using a protein standard (microtubule) to calibrate the expansion factor (Martinez *et al.*, 2020). Works from Pesce *et al.* additionally identified Nup153 as an intrinsic reporter of expansion factor, with an accuracy of 5-10nm. This was determined by combining ExM with STED for nanoscale precision.

Users of ExM, should therefore be conscious of the sources of anisotropy in their experimental setups and, where measurements of distance are being recorded, that protein standards, or comparison to other techniques are used to corroborate them.

2.2 Optimising labelling and anchoring in ExM

Since its introduction, numerous variations of the technique have been reported which broadly simplify the process, and extend the scope of the approach. Successful application of ExM requires a labelling strategy which allows labels to retain their relative spatial organisation through the subsequent digestion and expansion steps (Chen *et al.*, 2015). Approaches to labelling and anchoring in ExM are summarised (Table 1). The choice of approach is dependent on sample type and biomolecules of interest, requiring careful design by users. Chen *et al.* originally achieved retention of labels by using a trifunctional fluorescent probe comprised of an antibody-conjugated DNA oligomer bearing an anchoring moiety for incorporation into the gel and a fluorophore for visualisation. Several trifunctional probes bearing different fluorophores and oligomers were synthesised which allowed multi-colour imaging in cells and tissue sections. However, the requirement for a custom-made labelling probe limited wider application of ExM. This hurdle was overcome by use of commercially available cross-linking monomers as the anchoring moiety (e.g. glutaraldehyde) (Chozinski *et al.*, 2016). The hypothesis was that by applying commercially available anchors following staining with conventional immunofluorescence, sufficient linkages would be formed between the labels and the polyelectrolyte gel to enable retention of fluorescence signal for

detection after expansion. The authors demonstrated good retention of fluorescence signal following digestion and expansion when applying glutaraldehyde or methacrylic acid N-hydroxysuccinimidyl ester (MA-NHS) in cultured cells and tissues. Interestingly, fluorescence intensity following expansion was observed to be higher in cells treated with DNA-labelled antibodies than in those treated with glutaraldehyde or MA-NHS (90% compared to 70%). However, the authors conclude the antibodies can conjugate more fluorophore than the DNA oligonucleotide based probes, which mitigates this effect. The retention of antibody labels by simple crosslinking moieties was similarly demonstrated in the development of protein retention ExM (ProExM) using 6-((acryloyl)amino)hexanoic acid (AcX) (Tillberg *et al*, 2016). These approaches were also shown to be compatible with retention of genetically encoded fluorescent proteins following ExM. GFP and GFP-like proteins have a high stability to proteases (e.g. proteinase K), meaning their fluorescence is retained through digestion. A range of fluorescent proteins were characterised and most tested proteins retained >50% of their fluorescence intensity following ExM preparation. Retention of fluorescent signal from both antibodies and fluorescent proteins was demonstrated in cells and tissues in a reproducible manner using these approaches.

A general limitation of ExM is reduced contrast in images due to a number of compounding factors; 100-fold volumetric dilution of fluorescence signal during expansion (Wassie *et al*, 2019), loss of fluorescently labelled antibody fragments due to digestion (Wen *et al*, 2020 & Shi *et al*, 2019), and destruction of fluorophores due to free radical generation during gel polymerisation (Min *et al*, 2020) (Figure 2). Whilst the dilution of fluorescence signal due to expansion may seem unavoidable, several approaches have been developed to mitigate this, as well as the loss of labels due to digestion and damage of fluorophores during polymerisation.

An approach to overcome volumetric dilution of fluorescence and destruction of fluorophores during polymerisation is to enable post-expansion labelling of specimens. This was attempted in the development of ProExM in which the non-specific proteinase digestion was replaced with milder digestion conditions (e.g. Endoproteinase LysC) to retain epitopes for post-expansion labelling (Tillberg *et al*, 2016). However, epitope preservation was variable and incomplete homogenisation resulted in anisotropic expansion of specimens. Post-expansion labelling has been demonstrated in magnified analysis of the proteome (MAP) and ultra-ExM (U-ExM) techniques (Ku *et al*, 2016 & Gambarotto *et al*, 2019). These approaches are based on combining tissue clearing methods and expansion, to retain endogenous proteins and allow for nanoscale imaging of specimens. Applying post-expansion labels following MAP and U-ExM preparation improves signal and contrast in images, but efficacy of labelling depends on antibody identity.

Whilst a portion of the antibody fragments are bound to the hydrogel through chemical anchors, the non-specific proteolytic digestion used for sample homogenisation results in fragmentation and subsequent loss of up to 50% of these fragments during the final expansion step. This issue has been addressed by direct grafting strategies (Wen *et al*, 2020), and label retention ExM (Shi *et al*, 2019). These approaches rely on use of trifunctional probes which are comprised of a targeting moiety, a reporter moiety and a polymerisation group. By conjugating the fluorophore directly to the anchoring moiety, any fluorescence loss during digestion and expansion is mitigated. Additionally, this simplifies the process as labelling and anchoring can be performed simultaneously. For example, Shi *et al* developed trifunctional probes to mitigate loss of fluorescence through antibody fragmentation and these probes were modified to enable labelling using enzymatic tags (e.g. SNAP-tag) in addition to conventional immunofluorescence labelling (Shi *et al*, 2019). The direct grafting

strategy has been exploited to use small molecules that target actin (e.g. phalloidin conjugates) or membrane lipids (e.g. trivalent fluorescent lipids carrying 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)) (Wen *et al*, 2020). During ExM, lipid membranes are lost, but by applying a covalently-tethered lipid probe, the signal derived from the membrane is preserved post-expansion.

Many of the labelling approaches described focus on imaging of proteins post-expansion. It has also been demonstrated that ExM approaches are compatible with imaging nucleic acids (Chen *et al*, 2016). This was demonstrated in the development of expansion fluorescence in situ hybridisation (ExFISH) in which they developed a small molecule linker, LabelX, to allow retention of RNA transcripts through ExM sample preparation. These transcripts were subsequently detected using FISH probes. It was also shown this small molecule linker allowed for expansion of the nuclear compartment. Therefore, by combining ExM methodologies, imaging of DNA, RNA and proteins within the same specimen can be achieved.

The polymerisation reaction is thought to lead to chemical damage of some fluorophores rendering them non-fluorescent, meaning fluorophore choice is crucial. Retention of fluorescence depends on the identity of the fluorophore with some (e.g. Alexa Fluor (AF) 647) being completely destroyed following gelation, whilst others (e.g. AF488) retain approximately 50% of their brightness post-expansion (Min *et al*, 2020). The best-performing fluorophores for ExM were identified as AF488, Tamra, Atto565 and Atto647N (Chen *et al*, 2015). This list has since been further extended to include AF405, AF546, AF568 and GFP (Chozinski *et al*, 2018). In addition, Min *et al* methodically assessed brightness of cyanine-based (CF) and Alexa Fluor dyes to achieve four colour imaging with maximal signal-to-noise ratio during standard ExM preparation. Fluorophores and fluorescent proteins characterised for use in ExM are summarised (Table 2). The values in this table are based on the ability of fluorescent labels to survive the polymerisation step required for ExM preparation. Labels may also be applied post-gelation allowing for staining with labels which would not survive the gelation process (Chozinski *et al*, 2016).

These approaches to ExM have extended the range of labelling strategies and fluorophores compatible with the method, and have largely overcome limitations surrounding fluorescence intensity following expansion.

2.3 Improving resolution of ExM

ExM protocols result in a 4.5-fold expansion of specimens in each dimension, offering a resolution of 4.5-fold smaller than the diffraction limit ($250\text{-}300\text{nm}/4.5 = 55\text{-}66\text{nm}$ lateral resolution). Two approaches have been developed which further increase expansion factor and therefore the effective resolution: iterative ExM (iExM) (Chang *et al*, 2017) and X10 ExM (Truckenbrodt *et al*, 2018). iExM enables 25nm resolution, facilitated by performing a preliminary gelation and expansion, followed by formation of a second polymer mesh in the spaces generated by the first expansion. All information is transferred from the first to the second gel by chemical means (Wassie *et al*, 2019). Expansion is then performed on the second gel resulting in a 4.5 x 4.5, or 20x physical expansion. X10 ExM similarly achieves 25-30nm resolution and this is achieved by modifying the gel composition to incorporate N,N-dimethylacrylamide (DMAA) and sodium acrylate. DMAA is a self-crosslinking monomer meaning the gel can be formed without additional multifunctional monomers. These gels have excellent mechanical stability and are superabsorbent, absorbing up to 3000x their weight in water (Cipriano *et al*, 2014). More uniform distribution of crosslinks in the gel networks reduced local stress and distortions during the expansion process.

ExM can be successfully combined with other super resolution techniques including structured illumination microscopy (SIM) (Halpern *et al*, 2019), stimulated emission depletion (STED) microscopy (Gao *et al*, 2018) and stochastic optical reconstruction microscopy (STORM) (Xu *et al*, 2019). These combined approaches result in lateral resolutions of ~30nm, ~10nm and ~10-20nm, respectively. Such resolutions match or exceed the resolving power of existing imaging techniques when applied individually. When combining ExM with these other techniques, care must be taken to minimise trade-offs in the imaging experiment. In all cases, imaging depth is increased 4.5-fold, necessitating use of water-based instead of oil-based immersion objectives to prevent spherical aberrations. Sample preparation and data acquisition parameters may need to be altered to mitigate lower fluorescence intensity and labelling density caused by ExM and for each combined imaging experiment, specific parameters may need optimisation. For example, ExM gels must be completely immobilised to prevent severe reconstruction artefacts derived from gel movement when combining ExM with SIM. When applying STED to ExM prepared samples, high fidelity dense labelling of epitopes is critical to achieving the best resolution.

3. Conclusions and future directions

Expansion microscopy is a unique approach to super resolution imaging, enabling rapid and easy nanoscale imaging of specimens in 3D. The nature of the preparation offers several technical advantages. Samples are optically clear which reduces the effects of diffraction and scatter (Gao *et al*, 2017). This enables greater imaging depth with minimal introduction of optical aberrations. Multi-colour applications of ExM are possible with minimal constraints on fluorophore choice (Min *et al*, 2020), and recent innovation of this technique has extended the range of biomolecules and labelling approaches compatible with it (Wen *et al*, 2020 & Shi *et al*, 2020). However, with all of these advances, the key caveat of ExM i.e. its incompatibility with live cell imaging, remains.

At present, ExM allows resolutions comparable to the best-performing super resolution techniques. It has been suggested that higher expansion factors and therefore resolution may be achieved and would supersede other super resolution techniques (Chang *et al*, 2017 & Truckenbrodt *et al*, 2018). It has also been demonstrated that ExM approaches may be combined (e.g. ExFISH and ProExM) (Chen *et al*, 2016). If a unified protocol could be developed, imaging of DNA, RNA, proteins and lipids may be combined to reveal organisation of heterogeneous complexes. The aqueous nature of specimens and the decrowding effect of ExM has been postulated to allow multiplexed readout of molecular information with nanoscale precision. This has been demonstrated using fluorescence in situ hybridisation approaches, and may be extended to being compatible with DNA-PAINT style probes (Wassie *et al*, 2019). In theory, any biomolecule could be labelled with an oligonucleotide barcode which can be identified post-expansion meaning that nanoscale mapping of biomolecules in a highly multiplexed fashion may be possible.

References

- Alon, S., Huynh, G. H., & Boyden, E. S. (2019). Expansion microscopy: enabling single cell analysis in intact biological systems. *FEBS Journal*, 286(8), 1482–1494.
- Chang, J., Chen, F., Yoon, Y., Jung E. E., Babcock H., Kang J. S., Asano, S., Suk, H., Pak, N., Tillberg, P. W., Wassie, A. T., Cai, D., Boyden, E. S. (2017). Iterative Expansion Microscopy. *Nature Methods*, 14(6), 593-604.

1 Chen F., Wassie A. T., Cote A. J., Sinha A., Shahar A., Shoh A., Daugharthy E. R., Chang J., Marblestone
2 A., Church G. M., Raj A., Boyden E.S. (2016). Nanoscale imaging of RNA with expansion microscopy.
3 *Nature Methods*, 13(8), 679–84.

4 Chen, F., Tillberg, P. W., & Boyden, E. S. (2015). Expansion microscopy. *Science*, 347(6221), 543-548.

5 Chozinski T. J., Halpern, A. R., Okawa, H., Tremel, G. J., Wong, R. O. L., Vaughan, J. C. (2016)
6 Expansion microscopy with conventional antibodies and fluorescent proteins. *Nature methods* 13:
7 485-488.

8 Cipriano B.H., Banik S.J., Sharma R., Rumore D., Hwang W., Briber R.M., Raghavan S.R. (2014)
9 Superabsorbent hydrogels that are robust and highly stretchable. *Macromolecules*, 47: 4445 – 4452.

10 Gambarotto, D., Zwettler, F. U., Le Guennec, M., Schmidt-Cernohorska, M., Fortun, D., Borgers, S.,
11 Heine, J., Schloetel, J., Reuss, M., Unser, M., Boyden, E. S., Sauer, M., Hamel, V., Guichard, P. (2019).
12 Imaging beyond the super-resolution limits using ultrastructure expansion microscopy (UltraExM).
13 *Nature methods*, 16: 71-74.

14 Gao, M., Maraschini, R., Beutel, O., Zehtabian, A., Eickholt, B., Honigmann, A., & Ewers, H. (2018).
15 Expansion Stimulated Emission Depletion Microscopy (ExSTED). *ACS Nano*, 12(5), 4178–4185.

16 Gao, R., Asano, S. M., & Boyden, E. S. (2017). Q&A: Expansion microscopy. *BMC Biology*, 15(1), 50.

17 Halpern, A. R., Alas, G. C. M., Chozinski, T. J., Paredez, A. R., & Vaughan, J. C. (2017). Hybrid
18 Structured Illumination Expansion Microscopy Reveals Microbial Cytoskeleton Organization. *ACS*
19 *Nano*, 11(12), 12677–12686.

20 Ku, T., Swaney, J., Park, J.-Y., Albanese, A., Murray, E., Cho, J. H., Park, Y., Mangena V., Chen, J.,
21 Chung, K. (2016). Multiplexed and scalable super-resolution imaging of three-dimensional protein
22 localization in size-adjustable tissues. *Nature Biotechnology*, 34(9), 973-981.

23 Martínez, G. F., Gazal, N. G., Quassollo, G., Szalai, A. M., Cid-Pellitero, E. Del, Durcan, T. M., Fon E. A.,
24 Bisbal M., Stefani F. D., & Unsain, N. (2020). Quantitative expansion microscopy for the
25 characterization of the spectrin periodic skeleton of axons using fluorescence microscopy. *Scientific*
26 *Reports*, 10(1), 1–11.

27 Min, K., Cho, I., Choi, M., & Chang, J. B. (2020). Multiplexed expansion microscopy of the brain
28 through fluorophore screening. *Methods*, 174(February 2019), 3–10.

29 Pernal, S. P., Liyanaarachchi, A., Gatti, D. L., Formosa, B., Pulvender, R., Kuhn, E. R., Ramos, R., Naik,
30 A. R., George, K., Arslanturk, S., Taatjes, D. J., & Jena, B. P. (2020). Nanoscale imaging using
31 differential expansion microscopy. *Histochemistry and Cell Biology*, doi: 10.1007/s00418-020-01869-
32 7. [(Epub ahead of print)]

33 Pesce, L., Cozzolino, M., Lanzaò, L., Diaspro, A., & Bianchini, P. (2019). Measuring expansion from
34 macro- to nanoscale using NPC as intrinsic reporter. *Journal of Biophotonics*, 12(8), e201900018.

35 Shi, X., Li, Q., Dai, Z., Tran, A., Feng, S., Ramirez, A. D., Lin, Z., Wang, X., Chow, T. T., Seiple, I. B.,
36 Huang, B. (2019). Label-retention expansion microscopy. *BioRxiv*, 687954.

37 Tillberg P.W., Chen F., Piatkevich K.D., Zhao Y., Yu C. English B.P., Gao L., Martorell A., Suk H., Yoshida
38 F., DeGennaro E.M., Roosen D.H., Gong G., Senevirtene U., Tannenbaum S.R., Desimone R., Cai D.,
39 Boyden E.S. (2016). Protein-retention expansion microscopy of cells and tissues labeled using
40 standard fluorescent proteins and antibodies. *Nature Biotechnology* , 34(9): 987-992.

- 1 Tillberg, P. W., & Chen, F. (2019). Expansion Microscopy: Scalable and Convenient Super-Resolution
2 Microscopy. *Annual Review of Cell and Developmental Biology*, 35(1), 683–701.
- 3 Truckenbrodt, S., Maidorn, M., Crzan, D., Wildhagen, H., Kabatas, S., & Rizzoli, S. O. (2018). X10
4 expansion microscopy enables 25-nm resolution on conventional microscopes. *EMBO Reports*, 19(9),
5 1–12.
- 6 Wassie, A. T., Zhao, Y., & Boyden, E. S. (2019). Expansion microscopy: principles and uses in
7 biological research. *Nature Methods*, 16(1), 33–41.
- 8 Wen, G., Vanheusden, M., Acke, A., Valli, D., Neely, R. K., Leen, V., & Hofkens, J. (2020). Evaluation of
9 Direct Grafting Strategies via Trivalent Anchoring for Enabling Lipid Membrane and Cytoskeleton
10 Staining in Expansion Microscopy. *ACS Nano*.
- 11 Vanheusden, M., Vitale, R., Camacho, R., Janssen, K. P. F., Acke, A., Rocha, S., & Hofkens, J. (2020).
12 Fluorescence photobleaching as an intrinsic tool to quantify the 3D expansion factor of biological
13 samples in expansion microscopy. *ACS Omega*, 0–7.
- 14 Xu, H., Tong, Z., Ye, Q., Sun, T., Hong, Z., Zhang, L., Bortnick, A., Cho, S., Beuzer, P., Axelrod, J., Hu, Q.,
15 Wang, M., Evans, S. M., Murre, C., Lu, L., Sun, S., Corbett K. D., Cang, H. (2019). Molecular
16 organization of mammalian meiotic chromosome axis revealed by expansion STORM microscopy.
17 *Proceedings of the National Academy of Sciences*, 116(37), 18423–18428.
- 18 Zhao Y, Bucur O, Irshad H, Chen F, Weins A, Stancu AL, Oh E-Y, DiStasio M, Torous V, Glass B et al.
19 (2017) Nanoscale imaging of clinical specimens using pathology-optimized expansion microscopy.
20 *Nature Biotechnology* 35, 757–764.

21 Acknowledgements

22 Work in the lab of Steve Thomas is supported by the British Heart Foundation (PG/15/114/31945;
23 IG/18/2/33544; NH/18/3/33913) and Rob Neely by the EPSRC (EP/N020901/1).

Figure Legends

Figure 1: Schematic overview of the expansion microscopy method.

A) Cells are fixed and permeabilised and (B) immunolabelled using antibodies, tethered to a DNA oligonucleotide functionalised with a fluorophore (red star) and an anchoring acryloyl group (anchor). C) The labelled sample is infused with acrylamide monomers, sodium acrylate and bis-acrylamide crosslinking reagent and the polyelectrolyte gel is formed in situ, around the fixed sample. The anchoring acryloyl groups are bound into the polymer network. D) The sample is treated with a non-specific protease, which removes the cell and antibodies, leaving the tethered fluorophores in place. E) The sample is immersed in water and expands by approximately 4.5-fold in one dimension, resulting in an enlarged copy of the labelled cell.

Figure 2: Schematic showing possible mechanisms for loss of fluorescent signal from a sample in ExM.

A) Heterogeneity inherent in the labelling of antibodies means only a fraction of labels are retained for imaging. B) Digestion can lead to cleavage of an antibody between the fluorophore and the anchoring group. C) The polymerisation reaction can lead to chemical damage of fluorophores.

1 Figures
2 Figure 1
3

4
5
6
7
8
9
10
11
12
13
14
15
16

Figure 2

- 1 Table 1. Table summarising approaches to labelling and anchoring in ExM approaches. Advantages,
- 2 limitations and validated uses of each approach are provided.

Cross-linking/ labelling strategy	Advantages	Limitations	Uses	Reference
Trifunctional fluorescent probe	Excellent retention of labelling probes throughout digestion and expansion steps as probes directly incorporated into polyelectrolyte gel.	Custom-made probes requiring expensive reagents and 1-2 day multi-step preparation.	Validated for use in cells and tissues with a range of fluorophores attached.	Chen <i>et al</i> , 2015.
Glutaraldehyde / MA-NHS approach	Commercially available reagents which are cheap and no complex preparation required. Good retention of fluorescence following digestion and expansion. MA-NHS incorporation into gels highly efficient as structurally similar to the methacryloyl group used previous.	Glutaraldehyde preferred for cells and not tissues as induces higher background than MA-NHS. Incorporation of glutaraldehyde-anchored probes into the polyelectrolyte gel may be affected by glutaraldehyde becoming topologically entangled in the polymer network. Relies on sufficient linkages being formed between proteins and gel so could be variable.	Validated for use in tissues and cells for a range of fluorescent antibodies and fluorescent proteins.	Chozinski <i>et al</i> , 2016.
Acryloyl-X (AcX)	Commercially available reagent. No complex preparation required.	Relies on sufficient linkages being formed between proteins and gel so could be variable.	Validated for use in tissues and cells for a range of fluorescent antibodies and fluorescent proteins.	Tillberg <i>et al</i> , 2016.
Direct grafting strategies	Trifunctional probe for targeting, labelling and anchoring simultaneously. Fluorescence loss mitigated. Range of biomolecules which can be labelled extended.	Custom-made probes.	Small molecule targeting labels to label cytoskeletal components. First example of lipid membrane ExM. Compatibility with in situ	Wen <i>et al</i> , 2020.

	Multiplexed approaches enabled.		hybridisation (ISH) probes and amplification through hybridisation chain reaction (HCR).	
Label retention probes	Trifunctional probe for targeting, labelling and anchoring simultaneously. Fluorescence loss mitigated. Compatible with SNAP- and CLIP-tags in addition to immunofluorescence approaches.	Custom-made probes.	Validated for use in cells.	Shi <i>et al</i> , 2019.
ExFISH (LabelX anchoring)	Anchoring approach for retention of nucleic acids (RNA, DNA). Commercially available reagents, simple preparation.	Retention of nuclear architecture is questionable.	Validated for use in cells and tissues. Compatible with FISH approaches.	Chen <i>et al</i> , 2016.
MAP/ U-ExM approach	Preservation of epitopes for post-expansion labelling facilitated by using a custom fixation approach. Commercially available chemicals.	Preservation of epitopes is variable. Efficacy depends on antibody identity.	MAP - validated in cultured cells and mouse brain. U-ExM – validated in isolated centrioles and cultured cells.	Ku <i>et al</i> , 2016. Gambarotto <i>et al</i> , 2019.

1
2
3
4
5
6
7
8
9
10
11

- 1 Table 2. A summary of fluorophores and fluorescent proteins assessed for use in ExM preparations is
 2 provided.

Tested Fluorophores and fluorescent proteins	Retention of fluorescence following polymerisation	Method of determining fluorescence retention	Reference
AF488 Atto565 Atto 647N AF647	57.2% 76.2% 58.5% -	Fluorescence intensity compared pre-expansion and post-gelation with no expansion.	Chen <i>et al</i> , 2015.
AF405 Atto488 AF532 AF546 AF568 GFP YFP DsRed Hoechst 33342 SYBR gold.	All fluorophores and fluorescent proteins listed reported to survive the gelation process.	Quantification is not reported for each individual fluorophore or fluorescent protein. Fluorescence retention assessed for antibody (Atto 488) in presence of glutaraldehyde and MA-NHS (>60% retention). GFP assessed and reported >80% retention.	Chozinski <i>et al</i> , 2016.
Dylight 405 CF405M AF488 AF546 AF594 CF633 AF647 Atto647N EBFP2 mTagBFP2 mTurquoise2 mCerulean3 ECFP mTRP1 mEmerald EGFP mClover EYFP mVenus mCitrine mOrange2 LSSmOrange tdTomato mRuby2 mCherry mKate2 mCardinal	28±5% 51±4% 48±2% 68±3% 46±2% 51±10% 7±3% 55±2% 62±4% 65±9% 68±8% 69±4% 51±2% 70±7% 53±4% 65±5% 61±4% 64±7% 44±5% 54±7% 32±2% 42±3% 67±4% 90±7% 72±3% 37±3% 36±3%	% fluorescence intensity compared between live cells and those which have undergone gelation and digestion steps of ExM protocol.	Tillberg <i>et al</i> , 2016.

iRFP	14±1%			
CF405S CF405M CF430 CF440	CF405S yielded highest fluorescence*.	CF405S**	Measured % fluorescence retention by comparing pre-expansion images with images acquired following ExM preparation. The authors shrunk the gels for the post-ExM image acquisition.	Min <i>et al</i> , 2020
CF450 CF488A CF514 CF532	CF488 and CF514 yielded highest fluorescence*. CF488 higher than CF514.	CF488A and CF514**. CF488A yielded highest fluorescence retention.		
CF543 CF555 CF568 CF583 CF594	CF568 and CF594 yielded highest fluorescence*. CF568 higher than CF594.	CF568 and CF594**. CF568 yielded highest fluorescence retention.		
CF633 CF640R CF647 CF660C CF660R CF680 CF680R AF 405 AF 488 AF 568	CF640R, CF660R and CF680R yielded highest fluorescence*. CF640R higher than CF660R and CF680R.	CF640R, CF660R and CF680R**. CF660R yielded highest fluorescence retention.		

1 AF = Alexa Fluor

2 * Fluorescence intensity quantified pre-expansion

3 ** Relative fluorescence retention calculated by comparing post-expansion specimens with pre-
4 expansion

5