

# An introduction to the methodology of expansion microscopy

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DOI:

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

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*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>

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# 1 An introduction to the methodology of expansion microscopy

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15

## 16 Abstract

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

## 27 Key words

28 Expansion microscopy, super resolution, imaging.

## 29 Key facts

- 30 1. Expansion Microscopy (ExM) allows three-dimensional nanoscale visualisation of specimens  
31 on a conventional fluorescence microscope.
- 32 2. By embedding specimens into a polyelectrolyte gel, specimens can be physically expanded  
33 4.5x in each dimension, resulting in decrowding of nanoscale information.
- 34 3. Broader application of ExM has been enabled by use of commercially available chemical  
35 cross-linkers to anchor labels to the gel meshwork.
- 36 4. Use of trifunctional labelling approaches enables retention of fluorescence intensity post-  
37 expansion and labelling of a range of biomolecules e.g. lipids, proteins.
- 38 5. Higher resolutions can be achieved by increasing expansion factor of gels, or by combining  
39 ExM with other super resolution approaches.

## 1 1. Introduction

2 Expansion microscopy (ExM) is a fluorescence imaging technique which enables three-dimensional  
3 nanoscale imaging of specimens on a conventional fluorescence microscope. This method relies on  
4 embedding samples into a polyelectrolyte gel which can be swollen by the addition of water. This  
5 results in a physical increase in specimen size by 4.5x in each dimension (Chen *et al*, 2015). The net  
6 effect of this is decrowding of features within the specimen, meaning super resolution imaging can  
7 be performed on a conventional microscope, thus eliminating the need for sophisticated software  
8 and expensive hardware as exists with other super resolution microscopy approaches (Gao *et al*,  
9 2017 & Chozinski *et al*, 2016). ExM allows for rapid image acquisition, and samples are optically  
10 cleared during the process. Clearing eliminates scattering of light through the sample, meaning  
11 imaging depth and volumes accessible are orders of magnitude higher than those achievable by  
12 other super resolution techniques. As a result, ExM is suited for rapid, scalable super resolution  
13 imaging in cells and thicker specimens such as tissue sections.

14 A schematic summarising the steps required for ExM preparation of samples is given (Figure 1)  
15 (Tillberg *et al*, 2016 & Chozinski *et al*, 2016). The specimen (e.g. cells, tissue) is labelled through  
16 immunofluorescence or genetically encoded fluorescent proteins (Fig 1A & B). Labels are chemically  
17 equipped with anchors to allow their incorporation into the network of a polyelectrolyte gel which is  
18 formed evenly and densely throughout the specimen (Fig 1C). The polymer-embedded samples are  
19 homogenised (proteins are digested) to enable isotropic expansion of the gel (Fig 1D). Addition of  
20 water results in the physical expansion of the gel, which now carries anchored labels describing the  
21 features of interest in the cell, by up to 4.5x in all three dimensions. This allows visualisation of  
22 labelled structures with an effective resolution of ~70nm laterally, and ~200nm axially (Gao *et al*,  
23 2017) (Fig 1E). In this review, we give an introduction to the expansion microscopy technique, and  
24 highlight its inherent limitations and recent efforts to address these. We will also give a brief outlook  
25 on the scope and future applications of this spectacular new approach for super-resolution imaging.

## 26 2. Expansion Microscopy: Techniques and applications

### 27 2.1 Ensuring robust, isotropic expansion of specimens

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

41 In each variant of ExM, distortions introduced during expansion have been characterised (Chen *et al*,  
42 2015, Chozinski *et al*, 2016, Tillberg *et al*, 2016, Chen *et al*, 2016, Chang *et al*, 2017, Pernal *et al*,  
43 2020). This has been commonly achieved through a correlative imaging approach where images of  
44 highly conserved structures (e.g. microtubules) are acquired pre-expansion with a super resolution

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

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

20 Recent work by Martinez *et al.* has shown differential expansion of regions of the hydrogel that  
21 either carry or are devoid of cells. However, the same study also showed that nanoscale expansion  
22 of the gel around the cell is isotropic and, indeed, quantitative measurements in expanded samples  
23 can be made using a protein standard (microtubule) to calibrate the expansion factor (Martinez *et al.*,  
24 2020). Works from Pesce *et al.* additionally identified Nup153 as an intrinsic reporter of expansion  
25 factor, with an accuracy of 5-10nm. This was determined by combining ExM with STED for nanoscale  
26 precision.

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

## 30 2.2 Optimising labelling and anchoring in ExM

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

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

16 A general limitation of ExM is reduced contrast in images due to a number of compounding factors;  
17 100-fold volumetric dilution of fluorescence signal during expansion (Wassie *et al*, 2019), loss of  
18 fluorescently labelled antibody fragments due to digestion (Wen *et al*, 2020 & Shi *et al*, 2019), and  
19 destruction of fluorophores due to free radical generation during gel polymerisation (Min *et al*,  
20 2020) (Figure 2). Whilst the dilution of fluorescence signal due to expansion may seem unavoidable,  
21 several approaches have been developed to mitigate this, as well as the loss of labels due to  
22 digestion and damage of fluorophores during polymerisation.

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

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

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

6 Many of the labelling approaches described focus on imaging of proteins post-expansion. It has also  
7 been demonstrated that ExM approaches are compatible with imaging nucleic acids (Chen *et al*,  
8 2016). This was demonstrated in the development of expansion fluorescence in situ hybridisation  
9 (ExFISH) in which they developed a small molecule linker, LabelX, to allow retention of RNA  
10 transcripts through ExM sample preparation. These transcripts were subsequently detected using  
11 FISH probes. It was also shown this small molecule linker allowed for expansion of the nuclear  
12 compartment. Therefore, by combining ExM methodologies, imaging of DNA, RNA and proteins  
13 within the same specimen can be achieved.

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

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

### 30 2.3 Improving resolution of ExM

31 ExM protocols result in a 4.5-fold expansion of specimens in each dimension, offering a resolution of  
32 4.5-fold smaller than the diffraction limit ( $250\text{-}300\text{nm}/4.5 = 55\text{-}66\text{nm}$  lateral resolution). Two  
33 approaches have been developed which further increase expansion factor and therefore the  
34 effective resolution: iterative ExM (iExM) (Chang *et al*, 2017) and X10 ExM (Truckenbrodt *et al*,  
35 2018). iExM enables 25nm resolution, facilitated by performing a preliminary gelation and  
36 expansion, followed by formation of a second polymer mesh in the spaces generated by the first  
37 expansion. All information is transferred from the first to the second gel by chemical means (Wassie  
38 *et al*, 2019). Expansion is then performed on the second gel resulting in a 4.5 x 4.5, or 20x physical  
39 expansion. X10 ExM similarly achieves 25-30nm resolution and this is achieved by modifying the gel  
40 composition to incorporate N,N-dimethylacrylamide (DMAA) and sodium acrylate. DMAA is a self-  
41 crosslinking monomer meaning the gel can be formed without additional multifunctional monomers.  
42 These gels have excellent mechanical stability and are superabsorbent, absorbing up to 3000x their  
43 weight in water (Cipriano *et al*, 2014). More uniform distribution of crosslinks in the gel networks  
44 reduced local stress and distortions during the expansion process.

1 ExM can be successfully combined with other super resolution techniques including structured  
2 illumination microscopy (SIM) (Halpern *et al*, 2019), stimulated emission depletion (STED)  
3 microscopy (Gao *et al*, 2018) and stochastic optical reconstruction microscopy (STORM) (Xu *et al*,  
4 2019). These combined approaches result in lateral resolutions of ~30nm, ~10nm and ~10-20nm,  
5 respectively. Such resolutions match or exceed the resolving power of existing imaging techniques  
6 when applied individually. When combining ExM with these other techniques, care must be taken to  
7 minimise trade-offs in the imaging experiment. In all cases, imaging depth is increased 4.5-fold,  
8 necessitating use of water-based instead of oil-based immersion objectives to prevent spherical  
9 aberrations. Sample preparation and data acquisition parameters may need to be altered to mitigate  
10 lower fluorescence intensity and labelling density caused by ExM and for each combined imaging  
11 experiment, specific parameters may need optimisation. For example, ExM gels must be completely  
12 immobilised to prevent severe reconstruction artefacts derived from gel movement when combining  
13 ExM with SIM. When applying STED to ExM prepared samples, high fidelity dense labelling of  
14 epitopes is critical to achieving the best resolution.

### 15 3. Conclusions and future directions

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

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

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## 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).

24

1 [Figure Legends](#)

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3 **Figure 1: Schematic overview of the expansion microscopy method.**

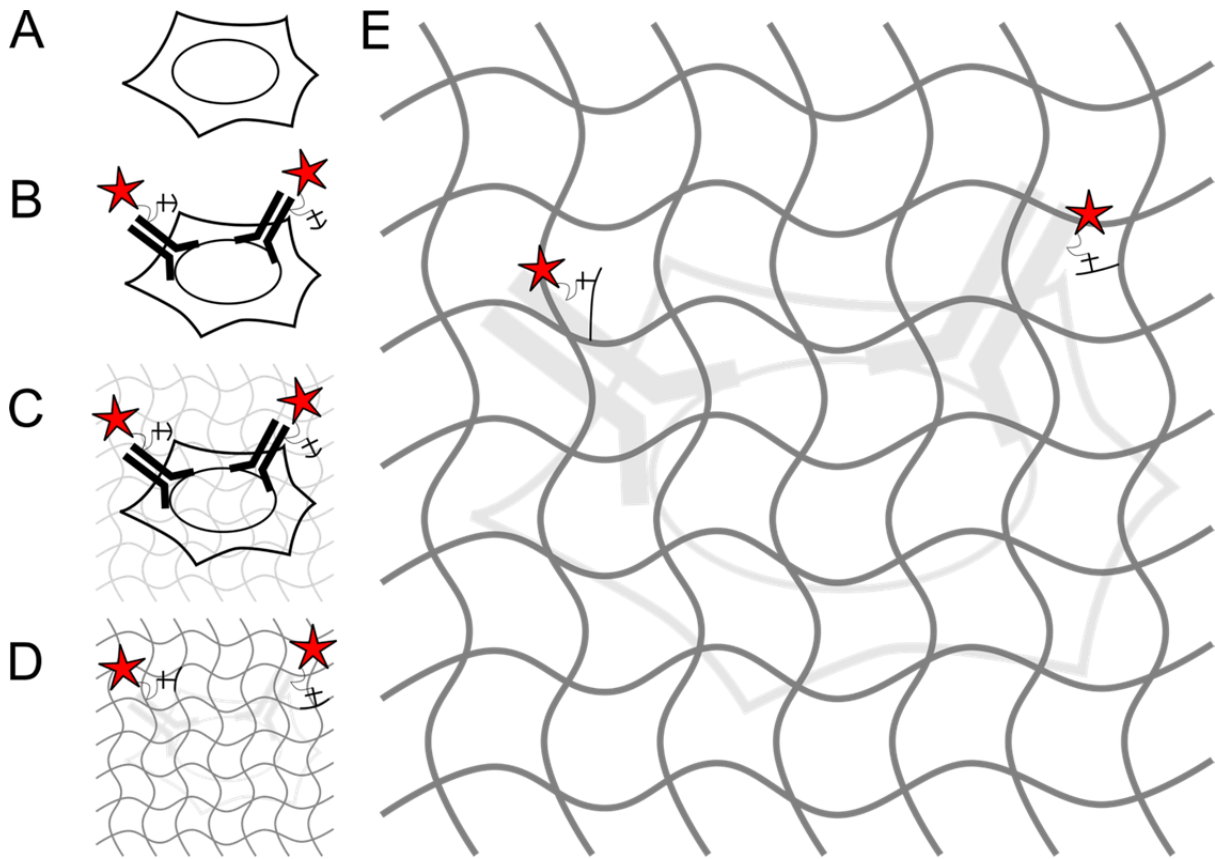
4 A) Cells are fixed and permeabilised and (B) immunolabelled using antibodies, tethered to a DNA  
5 oligonucleotide functionalised with a fluorophore (red star) and an anchoring acryloyl group  
6 (anchor). C) The labelled sample is infused with acrylamide monomers, sodium acrylate and bis-  
7 acrylamide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

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

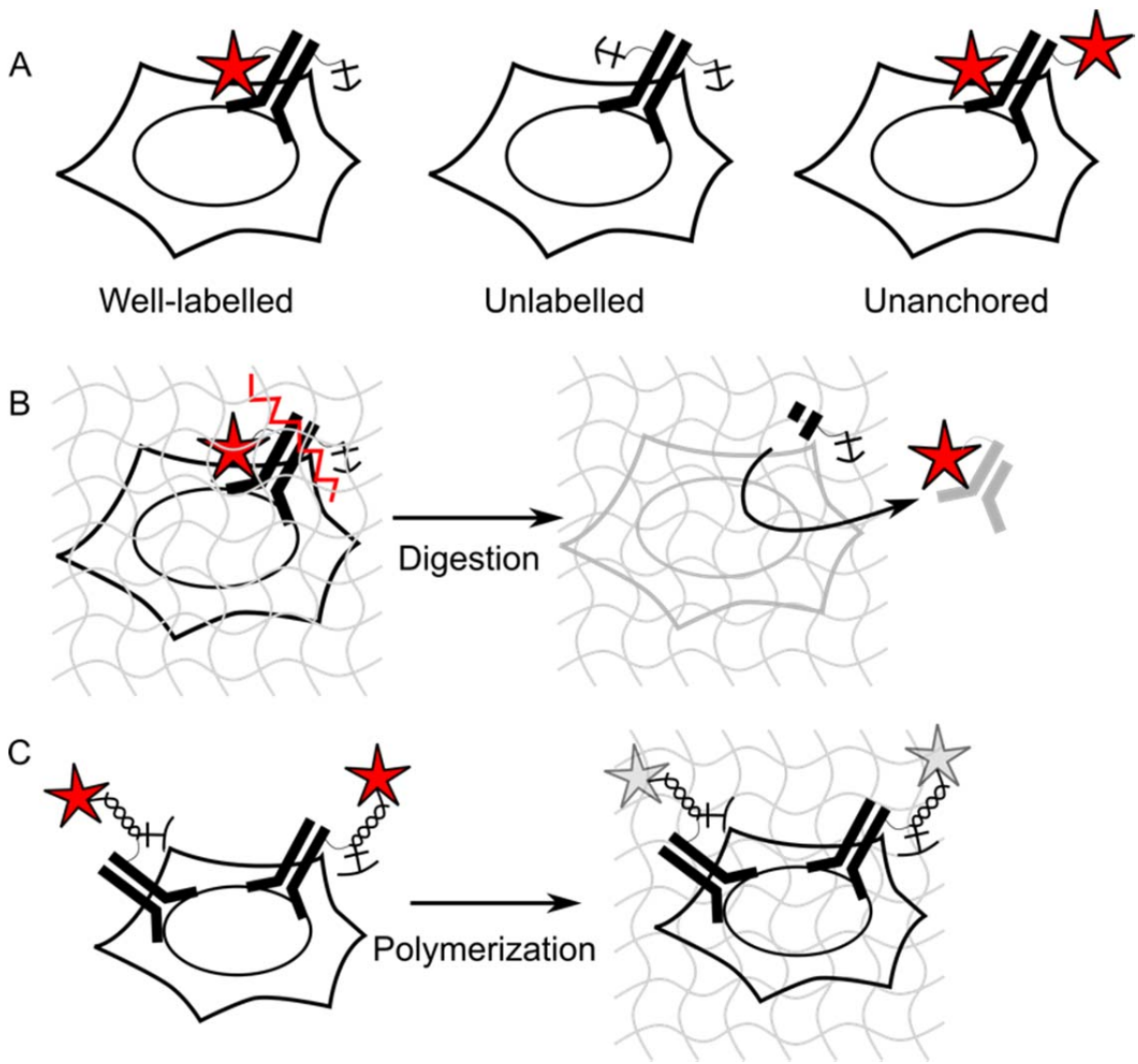
15 A) Heterogeneity inherent in the labelling of antibodies means only a fraction of labels are retained  
16 for imaging. B) Digestion can lead to cleavage of an antibody between the fluorophore and the  
17 anchoring group. C) The polymerisation reaction can lead to chemical damage of fluorophores.

- 1 Figures
- 2 Figure 1
- 3



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Figure 2



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- 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.

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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

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