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Version 1

U-ExM of diverse samples V.1

 Cell

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Wildlife disease



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Protocol status: Working

We use this protocol and it's working (depending on the species, see comments).

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Keywords: U-ExM, Expansion microscopy, expansion microscopy, staining in many specimen, physical enlargement of sample, specific antibody, cryo fixation, initial guideline for novel specimen type, exm, antibody, many specimen, list of antibody, novel specimen type, decrowding of the cellular environment, dey lab, cell wall, lab, efficient immuno

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Abstract

Expansion microscopy (ExM) allows for the physical enlargement of samples while maintaining relative positions. No only does this lead to an increase in resolution, but especially protocols utilising post-expansion staining approaches benefit of the removal of physical barriers (e.g. cell walls) and a decrowding of the cellular environment, enabling more efficient immuno-staining in many specimens previously refractory to labelling. The provided protocols offers a generic and versatile approach to ultrastructure expansion microscopy (U-ExM), expanding samples ~4-4.5 fold, which may be used as an initial guideline for novel specimen types. Different ways of chemical and cryo fixation are outlined as are tips on the handling of samples of distinct growth, size, and behaviour. A list of antibodies tested in U-ExM is included and will b updated to streamline processes for new users and act as a reference for experienced users looking for specific antibodies. For further questions, problems, and feedback please feel free to reach out to the Dudin Lab (UNIGE) or Dey Lab (EMBL Heidelberg).

Guidelines

This protocol is meant as a starting point when first trying expansion microscopy but is not a "one solution for all". In case of doubts and questions, feel free to reach out.

Materials

Monomer solution  250 µL

Note

This will make your gel. Prepare and store at -20°C for <2 weeks

	A	B	C	D
	Reagent	Stock Conc.	Amount (250 µL)	Final Conc.
	Acrylamid	40 %	62.5 µL	10 %
	Sodium acrylate	4 M / 38 %	125 µL	19 %
	N,N'-methylenbisacrylamide (BIS)	2 %	12.5 µL	0.1 %
	PBS	10 x	25 µL	1x

Prepare at least 4 hours in advance. Store at as aliquots at -20°C for no more than 2 weeks.

Denaturation Buffer  100 mL  9.0

	A	B	C	D
	Reagent	Stock Conc.	Amount (100 mL)	Final Conc.
	Tris pH 9.0	1 M	5 mL	50 mM
	NaCl	5 M	4 mL	200 mM
	SDS	20%	28.6 mL	200 mM

Adjust pH to 9.0 with HCl. Store at RT, warm if SDS precipitates.

 Acrylamide solution 40% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4058**

 N,N'-methylenbisacrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1533**

 Sodium Acrylate (purity note:*) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #408220** or home made



Protocol

NAME

Neutralising Acrylic Acid to make Sodium Acrylate

CREATED BY

Felix Mikus

[Preview](#) Ammonium Persulfate **Thermo Fisher Catalog #17874** TEMED **Thermo Fisher Scientific Catalog #17919** Sodium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #436143-25G** Tris HCl **P212121** Sodium chloride **P212121** Formaldehyde solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F8775-25ML*****Optional (depending on fixation)***

Poly-L-Lysine or

 Poly-D-Lysine solution 1.0 mg/mL **Merck Millipore (EMD Millipore) Catalog #A-003-E** or Cell-Tak **Corning Catalog #354240**

Liquid nitrogen

Ethane;Propane

 Acetone, glass distilled **Electron Microscopy Sciences Catalog #E10015** Glutaraldehyde EM Grade 25% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G5882-50ML** Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3641*****Optional (depending on sample)*** Zymolyase 20T (Arthrobacter luteus) **Carl Roth Catalog #9324.3** Collagenase Typ IV, Cls IV **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C4-28****Antibodies:****Anti-beta tubulin - ABCD antibodies** (Caution: Cross reacts with some chloroplast target)



Anti-alpha tubulin - ABCD antibodies

Anti-alpha tubulin - 12G10

Anti-gamma tubulin

Anti-Centrin 20H5

Anti-PsbA (PSII)

Anti-RbsL (Ribosome)

Anti-AtpB

Anti-NPC - mab414

Anti-Nup98

Anti-H3

Protocol materials

⊗ Label-IT® Amine **Mirus Bio Catalog #MIR 3900**

⊗ BODIPY®; TR Ceramide **Thermo Fisher Catalog #D7540**

⊗ Poly-L-Lysine

⊗ Ethane;Propane

⊗ Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3641**

⊗ Sodium Acrylate (purity note:*) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #408220**

⊗ Sodium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #436143-25G**

⊗ Poly-D-Lysine solution 1.0 mg/mL **Merck Millipore (EMD Millipore) Catalog #A-003-E**

⊗ Acrylamide solution 40% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4058**

⊗ N,N'-methylenbisacrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1533**

⊗ TEMED **Thermo Fisher Scientific Catalog #17919**

⊗ Acetone, glass distilled **Electron Microscopy Sciences Catalog #E10015**

⊗ Glutaraldehyde EM Grade 25% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G5882-50ML**

⊗ Sodium chloride **P212121**

⊗ Liquid nitrogen

⊗ Collagenase Typ IV, Cls IV **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C4-28**

⊗ Cell-Tak **Corning Catalog #354240**

⊗ Zymolyase 20T (Arthrobacter luteus) **Carl Roth Catalog #9324.3**

⊗ Tris HCl **P212121**

⊗ Ammonium Persulfate **Thermo Fisher Catalog #17874**

⊗ Formaldehyde solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F8775-25ML**

Before start

Ensure required equipment and reagents are present, where needed prepare buffer in advance. Make yourself familiar with safety hazards presented and take steps to mitigate them.



Prior considerations and comments

- 1 This protocol outlines the steps recommended for U-ExM of various sample types, however further modification may be required depending on the species, structure, or protein of interest. For questions, feedback, or in case of problems please reach out to the Dudin Lab (UNIGE) or Dey Lab (EMBL HD)!
- 2 The first considerations should be focused on which type of fixation will yield the most accurate and reproducible results by minimising fixation-induced artifacts. Availability of infrastructure (e.g. access to vitrification equipment, abundance of the sample, limitations due to field settings) will dictate the best way forward.
- 3 Due to the denaturation of proteins, antibodies that work in unexpanded IF may not be applicable. **For a short list of antibodies used successfully by us, see in the Materials section.**
Note that many chemical dyes as well as most forms of autofluorescence will lose or drastically reduce their signals due to free radicals already in the gelation or later during the denaturation and expansion.
- 4 Depending on the sample type, structure of interest, and available equipment and expertise different fixation conditions may be suited.

STEP CASE

(Para) Formaldehyde fixation 30 steps

Often the easiest fixation to test on new samples yielding good results for a variety of structures of interest, but prone to fixation artifacts.

- 5
 - Grow samples to the preferred stage/density
 - When working with adherent samples on coverslips, move them into 4% FA in appropriate buffer/medium and incubate for 00:20:00 at Room temperature
 - Specimen in solution fix by transferring the required volume to a separate tube and add an equal volume of 8% FA (final concentration 4%). Mix by inverting a few times and incubate for 00:20:00 at Room temperature .

Note

If fixation protocols already exist that work well, start with durations outlined there.

Wash samples with PBS 3x 00:05:00 . When possible, avoid centrifugation and let samples settle. To stop fixation, add excess PBS to the tube and let specimen settle prior





to washes.

Sample pre-processing

6 Sample digestion



Additional steps may be required for some species - often repurposing protocols used for protoplasting.

- Zymolyase/Lyticase: beta-1,3-glucanase, good for yeasts and bacteria ([Hinterndorfer et al., 2022](#) and [Korovesi et al., 2022](#))
- Driselase: (Hemi)Cellulase, beta-glucanase, xylanase, pectinase, good for plants and some fungi ([Grison et al., 2025](#))
- Collagenase: Good for extracellular matrix like layers (multicellular)

Note

Concentrations, buffers, temperature, enzymes, and duration are very dependent on the specific sample used. It is advisable to do this step before anchoring of the samples, but in some cases digestion can be performed even after polymerising the gel by including 50 mM CaCl₂ in the buffer which prevents expansion of the gel and sample while undigested. Note that in some cases cryo fixation might help with cell wall cracking in some cases.

Expansion

7 Sample anchoring

This step adds anchor points to primary amines for their integration into the gel matrix. Concentrations are maintained low to reduce cross-linking. Alternative approaches tailored to e.g. anchoring nuclei acids use alternative reagents (e.g.

 Label-IT ® Amine **Mirus Bio Catalog #MIR 3900**)

7.1 Incubate samples in 1% acrylamide and 0.7% formaldehyde in PBS at  37 °C



 Overnight .

For cells in suspension, use tubes on a rotating wheel or shaker. Coverslips should be covered in appropriate volumes in e.g. multi well dishes.

8 Cell mounting (where required)



Cells, tissues, or large organisms fixed in suspension are mounted onto coverslips.

- 8.1 Coat coverslips with Poly-L-Lysine, Poly-D-Lysine, or Cell-Tak to facilitate cell attachment
- Add appropriate volumes to cover the entire surface and incubate at Room temperature for > 00:30:00 . Remove excess supernatant and rinse once with PBS.
 - Place (concentrated) sample onto coverslip and allow to settle at Room temperature for > 00:20:00 . Remove excess supernatant (non attached/excess cells may be collected and used for repeats) and rinse carefully once with PBS. Do not let them dry out.

9 Gelation

Samples are integrated into a swellable hydrogel.

9.1 Preparations

Recommended volumes to prepare by coverslip diameter

	A	B	C	D	E
		Custome volumes			
	Coverslip diameter (round) [mm]	defined by user	6	8	12
	number of samples	defined by user			
	Recommene d MS aliquot volume [μl]	Error: #VALUE!	18	36	72
	TEMED/APS each per aliquot [μl]		1	2	4
	Final volume per sample	Error: #VALUE!	8.5	15	34
	Required Aliquot number	Error: #VALUE!			

Table 1: Download to enter number of volumes and, if required, coverslip diameter. Aliquot volumes include a small buffer. Final volumes may be adjusted to suit needs.



Protocol

NAME

Neutralising Acrylic Acid to make Sodium Acrylate

CREATED BY

Felix Mikus

[Preview](#)MS recipe [ 250 μ L]:

	A	B	C	D
	Reagent	Stock Conc.	Amount (250 μ L)	Final Conc.
	Acrylamid	40 %	62.5 μ L	10 %
	Sodium acrylate	4 M / 38 %	125 μ L	19 %
	N,N'-methylenbisacrylamide (BIS)	2 %	12.5 μ L	0.1 %
	PBS	10 x	25 μ L	1x

Table 2: Store as aliquots (see table above for recommended aliquot volumes) at -20°C. Prepare >4h in advance to ensure proper temperature is reached, discard after ~2 weeks.

Denaturation Buffer  100 mL  9.0

	A	B	C	D
	Reagent	Stock Conc.	Amount (100 mL)	Final Conc.
	Tris pH 9.0	1 M	5 mL	50 mM
	NaCl	5 M	4 mL	200 mM
	SDS	20%	28.6 mL	200 mM

Table 3: Denaturation buffer can be stored at RT for several months - years. In case SDS precipitates, heat up the buffer before use. Clean spills, dried buffer makes quite a mess.

9.2 Fully thaw **Monomer solution (MS)**, **APS**, and **TEMED**

🌡 On ice

⌚ 00:20:00

20m

For aliquot volumes, see the table above.

Make sure samples on coverslips are ready to use. Have pipettes, tweezers, and a paper towel/filter paper ready at hand.

9.3 Build a gelation "platform" consisting of an ice bucket with ice, a solid and even and cold surface (e.g. petridish with moist paper tissue, metal block), with a Parafilm placed on top.

🌡 On ice

5m

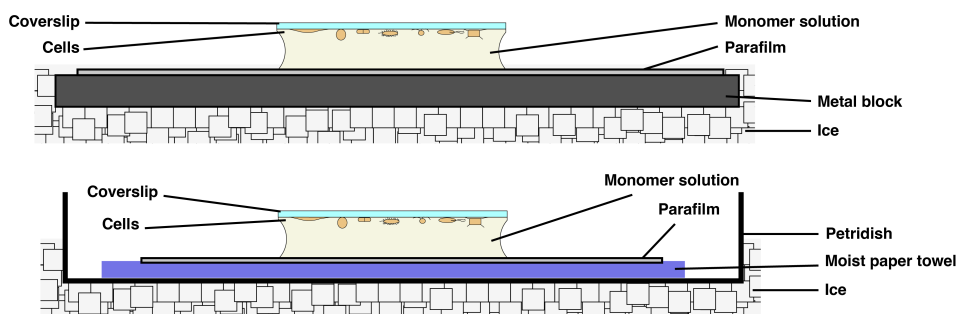


Figure 1: Sketch of commonly used gelation set-ups consisting of a cold platform and parafilm on which the MS and coverslip with the sample is added. Variations (including chamber setups for more consistent thickness and protection from condensation for longer incubations. See [Louvel et al., 2023](#) and [Klimas et al., 2023](#)) exist.

- Add TEMED and APS to aliquots of MS, briefly mix by vortexing and place droplets of $8\ \mu\text{L}$ (for 6 mm coverslips) or $35\ \mu\text{L}$ (for 12 mm coverslips) onto the ice cold parafilm (for different coverslip sizes, use the table above).
- Quickly, remove excess supernatant from coverslips with the samples, carefully blot off excess liquid with a paper towel or filter paper and place them onto the droplets with cells facing into the drop. Incubate 🌡 On ice for ⌚ 00:05:00

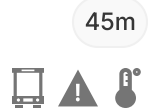
Note

Ensure cells are not lost by excessive blotting (only touch the very edge of the coverslip to the towel). If too much cell loss occurs, consider washing the coverslips with MS without added TEMED and APS to reduce dilution of the gelation solution. For samples not attaching well, consider inverting the setup by placing coverslips onto the parafilm, adding samples in MS without APS and TEMED and letting them settle for ⌚ 00:20:00 before adding TEMED and APS and a second coverslip to complete the sandwich.

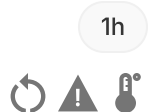
**Note**

Once APS and TEMED has been added, the polymerisation starts. Working too slow or not keeping everything cold will lead to the rapid formation of clumps and unequal gel formation.

- 9.4
- Incubate 37 °C for ≥ 00:45:00 in a humid chamber (e.g. plastic box, petridish with moist paper towels. Sealed if required) to allow the gel to fully polymerise.
 - Preheat heat denaturation buffer to 95 °C

**10 Denaturation**

Move coverslips with gels into preheated denaturation buffer. 6 mm coverslips can be dropped into 1.5 mL tubes with the gel attached. Larger coverslips require careful removal of the gel from the glass by briefly submerging them in denaturation buffer (hot if possible) until the gel edges visible ripple and carefully detaching the gel using a smooth edged spatula. Place the gel into denaturation buffer and incubate at 95 °C for 01:00:00 - 01:30:00 .

**Note**

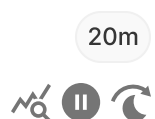
Gels will expand by ~2 fold in denaturation buffer and detach from the coverslips during. Ensure high enough temperatures to guarantee sufficient sample denaturation during this step to prevent artefacts introduced by the expansion of un-/partially denatured samples.

11 Expansion

Remove denaturation buffer and coverslips and wash the gels in PBS twice for 00:05:00 . Place the gels into ddH₂O for expansion using a sufficiently large dish. Incubate in water 3x for > 00:15:00 each.



- 11.1 Determine the gel expansion factor by measuring the diameter and comparing it to its original size.
- To guarantee complete expansion, the gel can be left in ddH₂O Overnight .
- Alternatively, gels can be shrunk in PBS for 3x 00:05:00 each and stored or used for staining.





Measured gel diameter

Gels may be stored at this point in PBS at  4 °C for several days. If longer storage is expected, addition of 0.002% NaN₃ to PBS is recommended to prevent contamination.

Safety information

Sodium azide (NaN₃) is highly toxic and needs to be handled with caution and appropriate PPE.

- 11.2 Optional: cut gels into multiple pieces using a razor blade, glass slice, sharp spatula. Pieces can be used to test different antibodies. For larger specimens, trim the gel to fit the specimen and remove empty gel around it.



Note

When possible use straight cut to avoid nicks in the gel which are weakpoints and can lead to tearing during the downstream handling

Immuno & dye staining

9h 35m

- 12 All antibody stainings will be performed on shrunken gels (washed in PBS), this reduces the amount of antibody required and ensures proper binding to targets. For a list of tested antibodies **refer to this table**.

Note

Should you have more antibody recommendation, we would be happy to add more to the table as a resource!

To increase accessibility of samples, staining with the cell side towards the solution (e.g. facing inwards in tubes, facing up in well plates) is recommended if it can be determined before. Stainings will often still work when flipped due to antibodies flushing underneath the gel, however trapped air bubbles may introduce uneven signals.

- 12.1 Move gels/gel pieces into fresh tubes or multiwell dishes (depending on the size), wash once with 3% BSA-T (BSA in PBS, filter sterilised. 0.1% Tween-20 added after filtering)





and add antibody at appropriate concentration in 3% BSA-T. Ensure the gel will be evenly covered during the incubation.

Note

A good starting point is double the recommended concentration for IF and adjust from there.

Incubate  Overnight at  37 °C on an orbital shaker or rotating wheel.

Note

Like the concentration, this is a good starting point and can be adjusted from there (e.g. shorter incubation at 37°C, ON at 4°C....)

- 12.2 Remove staining solution (in some cases, it may be reused for testing further gels, for final analysis fresh antibody for each replicates is recommended). Wash 3x

 00:10:00 with PBS-T on an orbital shaker or rotating wheel at

 Room temperature .

30m



- 12.3 Add secondary antibody in 3% BSA-T and incubate at  37 °C for ~  04:00:00 on an orbital shaker or rotating wheel. Protect from direct light.

4h



Note

1:1000 dilutions of secondary antibodies are a good starting point.

- 12.4 Remove staining solution. Wash 3x  00:10:00 with PBS-T on an orbital shaker or rotating wheel at  Room temperature .

30m



13 **Dye staining**

Depending on what stainings are required, different points in the protocol may be best for dye labelling.

- Hoechst/DAPI (DNA) staining may be performed at any point throughout the staining protocol (e.g. together with secondary antibodies) or separately.





- NHS ester (whole proteome/pan labelling) staining may be performed prior to antibody staining to minimise the risk of "overlabeling" structures now coated with antibodies which can rarely occur. (before 9.1)
- Bodipy TR ceramid (membranes) labelling is best done at the end of the staining procedure to reduce loss during washes.

13.1 NHS ester staining:

Many different variants and providers now exist (coupled to a variety of dyes) which may show different affinities during staining (see [Sheard et al., 2023](#)) and might need distinct staining conditions.

Generally speaking, 2µg/mL NHS ester in PBS or bicarbonate buffer (

[M] 100 millimolar (mM) 8.3) for > 02:00:00 at 37 °C will yield good results.

If additional stainings or storage is planned, wash 3x 00:05:00 with PBS.

If no further stainings are needed, wash 3x 00:15:00 with water to expand fully and proceed to mounting.

13.2 Bodipy TR ceramid BODIPY®; TR Ceramide Thermo Fisher Catalog #D7540 staining:

To reduce dye loss, it is recommended to do this step last.

Incubation in PBS or bicarbonate buffer can be done in many cases for > 02:00:00 at 37 °C .

After staining, wash 3x 00:15:00 with water to expand fully and proceed to mounting.

Alternatively, staining in water with 0.2% propyl gallate Overnight might yield superior results (see [Liffner et al., 2024](#)). Fully expand the gel before adding staining solution and mount directly from the staining without washing.

2h 20m



2h 15m



Microscopy

20m

14 Finally the time has come to see if it has all been worth it!

Of course, steps depends on what types of microscope you have access to. Some ideal circumstances:

- A confocal is great, but wide field will do a fantastic job depending on what is studied. Especially optimisation and testing steps will be most convenient on a wide field setup. The same holds true when screening multiple conditions (e.g. antibodies)
- Water immersion objectives (40x/60x) are fantastic since they increase the working distance in comparison to oil/silicon objectives and have a higher NA than air objectives. Further, the refractive index is more closely matched up with the water based sample.

- Inverted microscopes make mounting a lot easier

14.1 Mounting

20m

For higher magnification imaging, having the samples as close to the objective as possible is critical due to the short working distances. The aim is therefore to get the side of the gel with the specimen (which used to be the coverslip surface) mounted onto objective slides or glass bottom dishes.

- Coat a coverslip or glass bottom dish with Poly-L-Lysine for ~  00:20:00 . Rinse one with water to remove residual salts. This will reduce drift of the gel during imaging.
- Determine which side of the gel the cells are facing (this can be done on any microscope, either by trying to find specimen in brightfield - they are Godin to be very transparent when expanded - or using one of the stainings). For checking, use uncoated coverslips!
- Carefully blot excess water from the gel but do not dry it completely. Place the gel onto the coated coverslip in its final position ensuring it sits flat and without air bubbles. Do not try to adjust the position afterwards since the gel might stick to the Poly-L-Lysine and tear apart!

14.2 For imaging at inverted microscopes, different options exist:

*

- Glass bottom chambers (e.g. Ibidi chambers, MatTer chambers, Cellvis plates) (Benefits: ease of handling, easy to store after in case of reimaging, range of different sizes. Con: Expensive)
- Magnetic or screw chambers and coverslips (e.g. <https://www.warneronline.com/quick-release-magnetic-imaging-chambers-gr-series>) (Benefits: one time purchase, coverslips are cheap. Con: limited in size, storage of coverslips with gels not straightforward)
- Coverslips with iSpacer (Benefits: Cheapest option, sealed system prevents drying, allows for imaging of both sides (to some degree), can be stored. Con: handling a little tricky when using sliding sample holders, can bend slightly without supports)

I have no experience with upright microscopes, but I assume an iSpacer setup will work the best.

15 At the microscope:



- Try finding samples first in low magnification and determine the depth in the gel to prevent running higher magnification objectives into the sample
- Required laser powers tend to be quite high
- Image low energy/long wavelength channels first to reduce bleaching
- If any drift can be noticed, poly-L-lysine coating might not have been sufficient. In chambers, try removing as much water as possible from the bottom with a paper towel and only add a small drop on top of the gel to prevent drying.
- Expect increased file sizes (4 times larger sample > 4 times larger image)

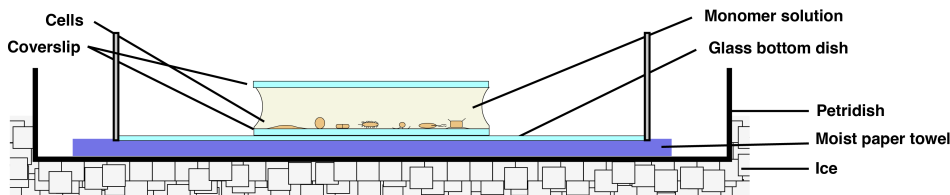
16 Image analysis

- Represent scale bars and measurements as accurately as possible. Make clear if you are indicating real sizes or distances adjusted for the expansion factor (and which expansion factor you used - gel expansion, population level estimates, direct comparison)
- Be cautious of unisotropic expansion of e.g. organelles and fixation artefacts
- Cavities in the gel resulting of e.g. incomplete sample expansion, gaps remaining from cell walls fragmented during expansion, or dirt particles will often trap unbound antibody and lead to increased background

16.1 Controls and the ideal experiment

To test for faithful and isotropic expansion some comparison of expanded samples to nativ, unexpanded samples is required

1. Measure metrics across populations (e.g. length, width, nuclear size, shape descriptors) in both conditions and compare. Note that expansion may work unequally well across different structures!
 2. In the ideal case, compare the same specimen before and after expansion. Many samples may shift and move during the processing (adherent cells are an exception in many cases) so imaging in the gel prior to denaturation is the safest option.
- Stain samples with e.g. NHS ester & Hoechst/DAPI when required. Note: some chemical dyes are (partially) destroyed during gelation due to free radicals. AlexaFluor647 worked decently for me
 - Polymerise the gel onto glass bottom chambers as shown below (Fig. 2). This will allow the use of immersion objectives while reducing the risk of losing the sample during imaging (at inverted microscopes). The drawback is the requirement for longer working distance objectives (two coverslip[s plus sample) and loss of resolution due to refractions. If mounting of samples directly onto glass bottom chambers is possible - perfect!
 - Take an overview of the coverslip/central area. Pick areas that are easily recognised - clumps of samples, gaps, scratches made on purpose. Take closeups with the best objective that works (e.g. 40x)
 - Denaturation, expansion, staining of samples followed by the same imaging workflow. Overview, refine areas used as landmarks, re image closeup areas with the same objective as before



Gelation set-up that allows for imaging of the unexpanded gel at inverted microscopes. The entire petridish is transferred to 37°C to initiate polymerisation, the glass bottom chamber can be mounted directly at the microscope. Add some moist tissue to the glass bottom chamber to prevent the gel from drying during longer imaging sessions, ensure no condensation accumulates).

Note

While more tedious, time intensive and certainly with drawbacks to the imaging this has been proven to be the best way of controlling for isotropic sample expansion in addition to population measurements (especially when re-finding several specimen) and the best way of visualising the protocol with all benefits and drawbacks.

When working with suspension cells (not fixed on coverslips e.g. plunge freezing) try mounting them onto small poly-L-lysine coated areas on the glass bottom chamber instead - take steps to prevent MS from spilling over when placing the droplet by using for example hydrophobic marker barriers.

Storage

17 Storage before expansion:

- after chemical fixation in PBS or PBS 0.002% NaN₃ 4 °C
- after cryo fixation/vitrification in liquid nitrogen storage
- after freeze substitution and rehydration of vitrified samples, in PBS or PBS 0.002% NaN₃ 4 °C
- after anchoring in PBS or PBS 0.002% NaN₃ 4 °C (fresh anchoring may be required for expansion)

Storage after expansion:

- after denaturation, wash min 2x in PBS and store in PBS or PBS 0.002% NaN₃ 4 °C
- after expansion in water 4 °C for few days, or wash min 2x in PBS and store in PBS or PBS 0.002% NaN₃ 4 °C
- after antibody staining in PBS 0.002% NaN₃ 4 °C



- after NHS ester and/or DNA staining in PBS or PBS 0.002% NaN₃  4 °C
- after imaging in water  4 °C to prevent tearing it from the coated coverslip

Long term storage: Freezing in 50% glycerol of gels before or after staining (Louvel et al., 2023 <https://doi.org/10.1038/s41467-023-43582-8>). Wash fully expanded gels 3x  01:00:00 in 50% glycerol and freeze at  -20 °C . Thaw by placing them in PBS 2x  00:30:00 and washing them excessively.

Protocol references

The backbone of this protocol has been developed by [Gambarotto et al., 2019](#), based on [Chen et al., 2015](#) and [Tillberg et al., 2016](#). Further refinements were introduced by [Laporte et al., 2022](#), [Damstra et al., 2022](#), [M'Saad & Bewersdorf, 2020](#), [Louvel et al., 2023](#).

For alternative protocols refer to [Klimas et al., 2023](#) and their available kits and services, [Steib et al., 2022](#), [Damstra et al., 2023](#) .