FIXATION

Chemical fixation is the most common fixation method used to perform immunofluorescence on cells or tissue. Combined with expansion, chemical fixation choice depends on the type of structure observed. For instance, the observation of centrioles, highly stable microtubule-based organelles, does not require any pre-fixation step as the anchoring molecules used for the expansion are sufficient to preserve it during the process. However, other cellular elements need pre-fixation such as cytoskeleton elements (actin, microtubules) or membrane-based organelles (mitochondria, ER, Golgi…) (see Gambarotto et al., 2019; Laporte et al., 2022).

MATERIAL:

- Glutaraldehyde 25% solution (grade I) (Sigma Aldrich, G5882-10X1ML)
- Paraformaldehyde (formaldehyde) aqueous solution 8% (FisherScientific, 50-259-97)
- Methanol

EQUIPMENT:

- Tweezer Dumont Style 5
- 12 mm coverslips high precision No. 1.5H (Marienfeld Laboratory Glassware, lot number 47442)
- 12 well plate
- Pipettes and tips
- 1.5 mL Eppendorf tubes

SAMPLES:

All samples have been prepared by trainers prior to the course start: U2OS *tet3G EGFP-SEC61* adherent cells: 6 well plates, 4x12 mm coverslips/well, 2 mL medium, seeding density: 100.000 U2OS cells, 60 h before experiment and induced with 1 µg/mL doxycycline 6 h before fixation.

This protocol is optimized for 12 mm coverslips – All volumes should be scaled down/up according to the surface of the coverslip used. Note that you should optimize the cell number for the seeding step depending on the cell type. For example, we usually seed 200'000 RPE-1 cells or 400'000 HeLa cells 24 h prior fixation

WORKFLOW:

PFA/GA fixation (Slow fixation procedure known to best preserve membranous structures)

- Prepare a solution of PFA 3% / GA 0.1% diluted in PBS
- Transfer coverslip in 12 well plate (1 coverslip per well) filled with 1 ml PBS
- Remove PBS and add the mix PFA/GA (1 ml per well)
- Incubate RT for 20 min
- Wash cells in PBS for 5 min – Repeat 3x
- Proceed for expansion (see U-ExM and iU-ExM WORFLOW)

Methanol fixation (Good visualization of the microtubule network)

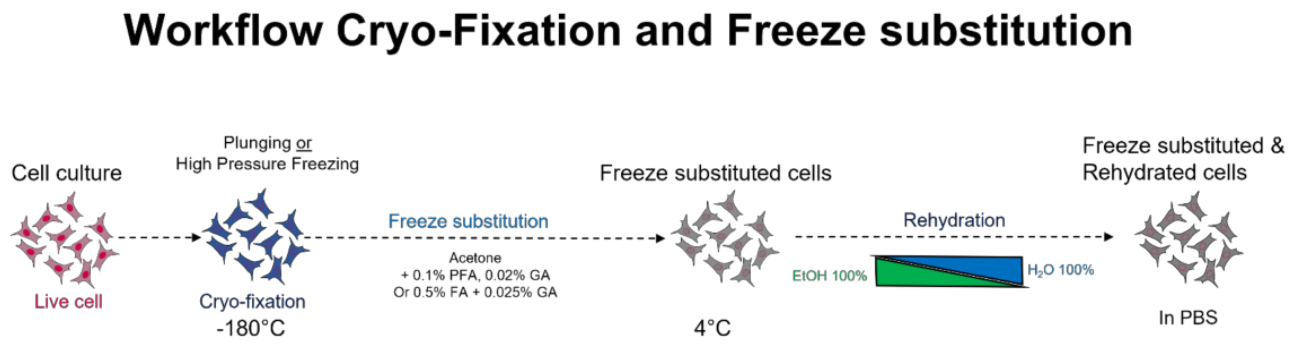
- Transfer coverslip in 12 well plate (1 coverslip per well) filled with 1 ml PBS
- Transfer coverslips into the fixation rack in PBS
- Transfer the fixation rack contaning the coverslips in ice-cold methanol
- Incubate -20°C for 7 min
- Transfer back the coverslips in the 12 well plate
- Wash cells in PBS for 5 min- Repeat 3x

- Proceed for expansion (see U-ExM and iU-ExM WORFLOW)

Practical: Cryo-fixation and Freeze Substitution

Cryo-fixation, also known as cryopreservation, is a method used to preserve the structural integrity of cells and tissues by rapidly freezing them at ultra-low temperatures, typically below -150°C. This technique originally used in electron microscopy is now adapted to techniques of cell biology such as immunofluorescence and expansion microscopy to best preserve the native cell architecture, as the rapid freezing prevents the formation of ice crystals that can damage cellular structures (Laporte et al, 2022). Importantly, cryo-fixation minimizes the artifacts that can be introduced during conventional chemical fixation, such as shrinkage, distortion, or loss of cellular components. In addition, note that cryo-fixed samples can be stored for long periods of time, allowing for future analysis and comparison with other samples. There are two ways to achieve cryo-fixation: cryo-plunging on glass coverslips and high pressure freezing (HPF) that should be chosen depending on the biological samples. This protocol can be used for a range of samples from human cells in culture to unicellular organisms or tissues (Laporte et al, 2022; Hinterndorfer et al, 2022, Mercey et al, 2020). In this practical we will focus on U2OS cells in culture to illustrate the protocol steps.

In the cryo-ExM protocol, the process of cryo-fixation is subsequently followed by a step of freeze substitution prior expansion. Freeze substitution is a technique used in electron microscopy to prepare biological samples for imaging. The principle of freeze substitution is to replace water in frozen biological samples with an electron microscopy resin, while avoiding ice crystal formation and preserving the structural and chemical integrity of the cells and tissues. In the case of the cryo-ExM protocol, the electron microscopy resin is replaced by the U-ExM anchoring solution (AA/FA). In practice, the frozen sample is first transferred to a frozen organic solvent (acetone) that will replace the water in the sample in the next steps. Next, the sample is slowly thawed while being kept in the organic solvent (acetone) to avoid the formation of ice crystals and to allow the acetone to penetrate the sample. After dehydratation, the sample is next infiltrated with the U-ExM anchoring solution prior to performing the next U-ExM steps.



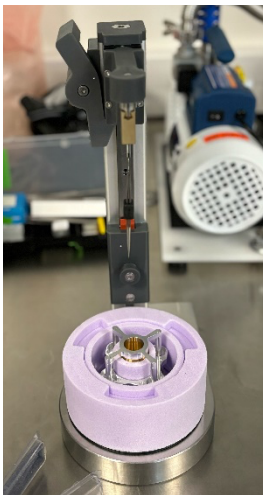
Cryo-fixation: Plunging

MATERIAL:

- Extra-Dry Acetone (Acros Organics/FisherScientific, 326801000)
- Ethanol (EtOH, Absolute) (ThermoFisher Scientific, 397691000)
- Dry Ice
- Glutaraldehyde 25% solution (grade I) (Sigma Aldrich, G5882-10X1ML)
- Paraformaldehyde (formaldehyde) aqueous solution 8% (FisherScientific, 50-259-97)
- Ethane:propane 37%:63% (PanGas)
- Liquid nitrogen

EQUIPMENT:

- Tweezer Dumont Style 5 with super thin tips or Negative-Action tweezer Style N5
- 12 mm coverslips high precision No. 1.5H (Marienfeld Laboratory Glassware, lot number 47442)
- Pipettes and tips
- 10 ml Syringe (used to pipet acetone)
- 1.5 mL Eppendorf tubes
- 5 mL Eppendorf tubes
- Whatman Filter Paper, 55 mm (GE Healthcare, catalogue number 1001-055)
- Polystyrene box (23 X 23 X 21 cm)
- Rocking machine
- Cryo-EM plunger (see picture below); in the practicals you will use a manual plunger home-made
- Light microscope (EVOS, Thermo)



Picture of cryo-EM plunger

SAMPLES:

All samples have been prepared by trainers prior to the course start: U2OS *tet3G EGFP-SEC61* adherent cells: 6 well plates, 4x12 mm coverslips/well, 2 mL medium, seeding density: 100.000 U2OS cells, 60 h before experiment and induced with 1 µg/mL doxycycline 6 h before fixation.

This protocol is optimized for 12 mm coverslips – All volumes should be scaled down/up according to the surface of the coverslip used. Note that you should optimize the cell number for the seeding step depending on the cell type. For example, we usually seed 200'000 RPE-1 cells or 400'000 HeLa cells 24 h prior fixation

WORKFLOW:

Before Plunging

- Fill 5 mL Eppendorf (1 per coverslip) with 1 mL of pure Extra-dry acetone using a syringe with needle (never open the bottle)
- Add 0.1% PFA and 0.02% Glutaraldehyde to the acetone
- Switch off the ventilation of the hood to limit evaporation of the liquid nitrogen and the liquid ethane/propane
- Freeze the Eppendorf tube by incubation in liquid nitrogen (keep the Eppendorf upright using a rack)
- Fill a polystyrene box with dry ice.
- Fill the cooling system for plunging entirely with liquid nitrogen. Refill as much as needed until it stops evaporate (bubbling stops). Wait 5 min for the system to be cooled.
- Fill the metallic plunging chamber with liquid ethane/propane. Be careful to always have the tip at the upper surface of the liquid to avoid freezing of the tip.
- Wait 10 min for the propane/ethane to be equilibrated. The system is ready for plunging.
- *Pure ethane works as well as the mix Ethane/Propane but has the tendency to freeze after a while. It creates a layer of "snow" that has to be removed with a tip before plunging. (On our current system, it's possible to fix the temperature at -177°C and avoid the freezing)*

Plunging and freeze substitution-Part 1

- Grab a 12 mm coverslip from the medium with thin tweezers.
- Soak up any excess medium with tissue paper.
- Hold the coverslip half-way with the tweezers.
- Place the tweezers holding the coverslip in the holder of the plunging apparatus.
- Blot any remaining excess of medium with Whatman paper.
- Activate the cryo-plunger to plunge the coverslip in the ethane/propane solution in the metal chamber.
- Transfer the coverslip into an Eppendorf containing frozen acetone. (*This is a tricky step! Practice this). During this step, it is possible to plunge the coverslip in the liquid nitrogen if it helps with the transfer into the Eppendorf.
- Incubate the Eppendorf in dry ice at an approximate angle of 45°.
- Agitate overnight (place the whole box on a shaker at room temperature).
- *Manual plunging is possible but has to be fast. More complicated to handle and less reproducible*
- *Freeze substitution can be shortened to 2h30 if needed*

Plunging and freeze substitution-Part 2

- Remove dry ice almost entirely (just keep a couple of pieces at the bottom of the box)
  CAUTION: Release the pressure inside the Eppendorf tubes by quickly opening and closing them under the hood, wear safety goggles
- Continue agitation for 45 min.
- Transfer coverslips from acetone to 100% ethanol pre-chilled at -20°C.
- *Use a 12-well plate during the following step for convenience*

Rehydration

- Rehydrate the samples by sequential incubations in a mixture of ethanol:water as follow :

EtOH 100% (5min) - EtOH 100% (5 min) - EtOH 95% (3 min) - EtOH 95% (3min) – EtOH 70% (3min) - EtOH 50% (3min) - EtOH 25% (3min) - H2O 100% - PBS

- *EtOH 100% and EtOH 95% should be stored at -20°C – EtOH 70% and EtOH 50% should be stored at 4°C.*
- *All rehydration solutions are complemented with 0.1% PFA and 0.02% glutaraldehyde except EtOH 25%.*
- Transfer the coverslips in PBS and orientate them. Scratch the coverslip surface with a tweezer under a light microscope to see if cells are up or down.

Continue with the respective protocol for U-ExM or iU-ExM.

Cryo-fixation: High Pressure Freezing

MATERIAL:

- Planchettes (A carrier with 100 and 200 µm side & B carrier with 300 µm and flat side)
- Tweezers
- Membrane filter (1.2 µm pore size)
- Tubes
- Dry Acetone (Acros Organics/FisherScientific, 326801000)
- Glutaraldehyde 25% solution (grade I) (Sigma Aldrich, G5882-10X1ML)
- Paraformaldehyde (formaldehyde) aqueous solution 8% (Electron Microscopy Sciences 157-8)
- Vacuum pump
- Filtration system
- Liquid nitrogen

EQUIPMENT:

- Benchtop centrifuge for microfuge tubes (e.g. Eppendorf 5425)
- High pressure freezer (in this case a Leica EM ICE)

SAMPLES:

This protocol can also be used with different samples e.g. yeast, mammalian cells etc.

WORKFLOW:

<u>High pressure freezing-Part 1</u>

- Concentrate the sample on nitrocellulose membranes by applying vacuum
- Take care to not dry the samples - place the membrane onto agar dishes to keep them moist and apply more liquid to it when necessary OR
- Concentrate the sample by centrifugation

<u>High pressure freezing-Part 2</u>

- Prepare the carrier loading setup as demonstrated by the trainers
- Load ~1.5 -2 µL sample to the 200 µm side of the A carrier
- Place the B carrier on top smooth side facing down
- Freeze the sample by closing the lid
- *only 9 samples can be frozen until the storage dewar will need to be emptied*
- Store the carriers in liquid nitrogen until further use

<u>Freeze substitution using the AFS</u>

- Place the A carrier containing your sample into a cryotube with frozen (lN2) Acetone containing 0.5% formaldehyde and 0.025% glutaraldehyde
- Place the tube into a AFS precooled to -90°C
- Maintain the sample at -90°C for >24 hours
- Ramp the temperature to room temperature at 4 °C/h

Continue with the steps described above (Rehydration) the respective protocol for U-ExM.

U-ExM protocol

INTRODUCTION:

Expansion microscopy (ExM) is a technique developed by Ed Boyden in 2015 (MIT, USA) that allows for the enlargement of biological samples while retaining their molecular and cellular structures, which can next be imaged with high resolution using a standard fluorescence microscope (Chen et al., 2015). Several protocols have been subsequently developed based on 2 fundamental different approaches. The original ExM protocol is based on a proteinase K digestion of the biological sample and a pre-expansion labeling. The other, called MAP for magnified analysis of the proteome (Ku et al., 2016) relies on a heat protein denaturation to keep the proteome and a post-expansion labeling. Ultrastructure expansion microscopy (U-ExM) is an optimized variant of the MAP protocol, which enables the structural preservation of biological samples. Indeed, U-ExM uses temperature homogenization at 95°C to preserve the proteome while disrupting protein interactions prior to acrylamide/sodium acrylate gel expansion, thereby allowing for homogeneous isotropic expansion. Finally, U-ExM is a post-expansion labeling method.
This protocol can be used for a range of samples from human cells in culture to unicellular organisms or tissues (Gambarotto et al., 2019; Laporte et al., 2022; Hinterndorfer et al., 2022, Mercey et al., 2020). In this practical, we will focus on U2OS cells in culture to illustrate the protocol steps.

MATERIAL:

- Nuclease-free water (ThermoScientific, R0582) only for Sodium acrylate
- Poly-D-Lysine (Gibco, A3890401)
- Ammoniumpersulfat (APS, ThermoFisher, 17874)
- Tetramethylethylendiamine (TEMED, ThermoFisher, 17919)
- Formaldehyde (FA, 36.5-38%, Sigma, F8775)
- Acrylamide (AA, 40%, SIGMA, A4058)
- N,N'-methylenbisacrylamide (BIS, 2%,Sigma, M1533-100ML)
- Sodium Acrylate (97-99%, AK Scientific, R624-25g)
- SDS (sodium dodecyl sulfate) (Sigma, 75746)
- TWEEN-20 (Sigma, P9416)
- Bovine serum albumin (BSA) (Sigma, A2153)
- PBS (1× and 10×) (Gibco,14200-067)
- ddH_2O
- α-tubulin antibody guinea pig (ABCD antibodies, AA345)
- ß-tubulin antibody guinea pig (ABCD antibodies, AA344)
- anti-GFP antibody rabbit (Torrey Pines biolabs, TP401)
- goat anti-guinea pig 635 (Abberrior STAR 635P goat anti-guinea pig IgG, ST635P-1006-500UG)
- Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (Thermofisher, #A-11012)
- NHS-ester A488 (Alexa Fluor™ 488 NHS Ester, Succinimidyl Ester, Invitrogen, A20000)
- DAPI (Invitrogen, 28718-90-3)

WORKING SOLUTIONS:

All working solutions will be provided -ready to use- by your trainers. Please find some remarks that should be considered during this experimental protocol in blue.

- **Sodium Acrylate (SA):**
  Prepare a stock concentration of 38% (wt/wt) diluted in nuclease-free water. Add 19 g of SA, little by little, into 31 mL water while stirring. SA 38% is viscous; do not keep this solution longer than 6 months (4°C) or for longer at -20°C.
  Sodium acrylate can be ordered from SIGMA (408220), AK Scientific (R624-25g) or Combi-Blocks (QC-1489). Can appear slightly yellow but work as long as the resuspension is translucid. Be careful, each company has a lot of purity variability from lot to lot.
  Note: be careful of the presence of a viscous deposit at the bottom of the bottle. This could indicate a polymerization of the SA and then impact the amount of SA in the monomer solution. SA can display from a very light to visible yellow color. Both resulted in the same expansion factor, however if the SA solution displays an intense yellow color with an increased viscosity, trash it.
- **Monomer solution:**
  For 900 µL of solution mix:
  - 500 µL of Sodium acrylate (SA, 38%; 19% final)
  - 250 µL of Acrylamide (10% final)
  - 50 µL N,N'-methylenbisacrylamide (0.1% final)
  - 100 µL 10x PBS (Homemade)

  Aliquot (90ul) and freeze (a minimum of 24h of freezing is necessary to ensure agood quality of the gel) – Keep up to 2 weeks.
  Note that the stock solution is indicated for 900 µL only. The "missing" 100 µL are for the 5 µL TEMED and 5 µL APS which are added last minute in each 90 µL aliquot
- **Denaturation buffer:**
  - 200 mM SDS
  - 200 mM NaCl
  - 50 mM TrisBase in water, pH 9
- **TEMED (10%):** mix 100 µL of TEMED to 900 µL of nuclease free water
- **APS (10%):** dilute 0.1 g into nuclease-free water and fill up to 1 mL. Aliquot (100ul) and freeze or use fresh – Keep up to 1 month
- **Poly-D-Lysine:** Ready to use (0.1 mg/ml) stored at 4°C

EQUIPMENT:

- Tweezer Dumont Style 5 with super thin tips or Negative-Action tweezer Style N5
- 12 mm coverslips high precision No. 1.5H (Marienfeld Laboratory Glassware, lot number 47442)
- 24 mm coverslips, high precision No. 1.5H (Marienfeld Laboratory Glassware, lot number 48639)
- 12-well plates (ThermoFisher Scientific, catalog number 150200)
- 6-well plates ((ThermoFisher Scientific, catalog number 150239)
- Pipettes and pipette tips
- Spatula
- Spoon
- Razor blade
- 250 mL beaker

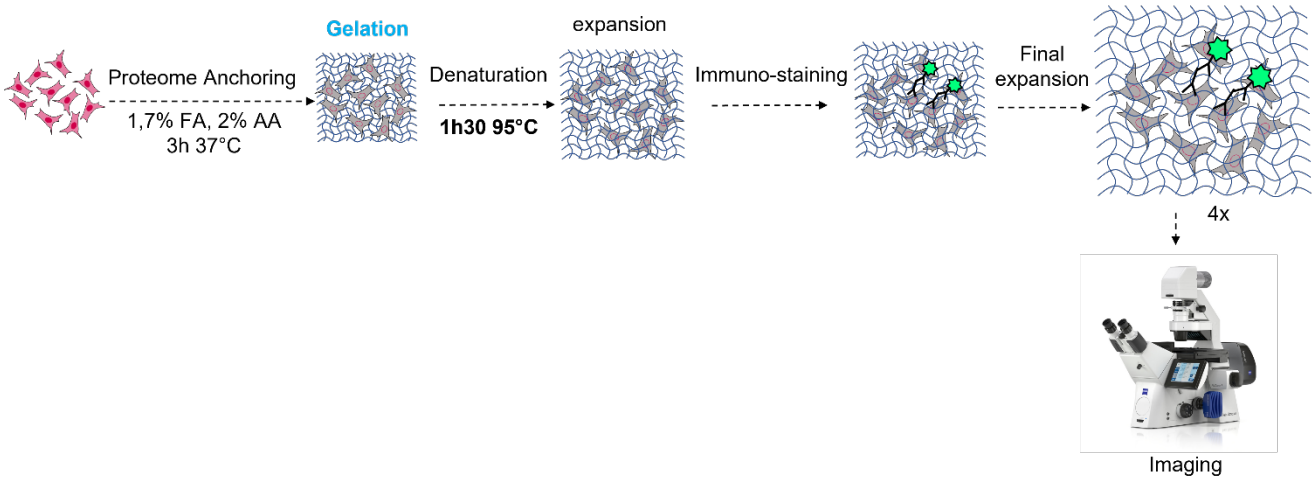
- 500 mL beaker
- Caliper
- Parafilm
- Scissors
- 1.5 mL Eppendorf tubes
- 35 mm imaging chamber (metallic O-ring 35mm, Okolab, RA-35-18 2000–06).
- 37 °C incubator or climate-controlled room
- Heat block

SAMPLES:

U2OS *tet3G EGFP-SEC61* adherent cells: 6 well plates, 4x12 mm coverslips/well, 2 mL medium, seeding density: 100.000 U2OS cells, 60 h before experiment, and induced with 1 µg/mL doxycycline 6 h before fixation.

This protocol is optimized for 12 mm coverslips – All volumes should be scaled down/up according to the surface of the coverslip used. Note that you should optimize the cell number for the seeding step depending on the cell type. For example, we usually seed 200'000 RPE-1 cells or 400'000 HeLa cells 24 h prior fixation

U-ExM workflow



WORKFLOW:

Step 1: Fixation

In this course you will be introduced to a manual cryo-fixation method. In addition, we will use chemical fixation methods including Methanol and Formaldehyde/Glutaraldehyde.

- *Note that the type of fixation will affect the expansion results. For instance, no fixation leads to cytoplasmic microtubule depolymerisation and is ideal for the study of centrioles (Gambarotto et al, 2019).*

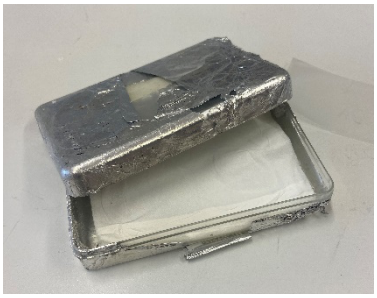
Glutaraldehyde fixation (GA) is not optimal with U-ExM since it can impede the expansion. Methanol, PFA/GA and cryo-fixation are possible ways to fix the sample prior to U-ExM if needed.

Step 2: Anchoring

- Prepare 1.4% formaldehyde/2% acrylamide (FA/AA) solution in PBS
- Per coverslip, dilute 38 µL of FA and 50 µL of AA in 912 µL of 1X PBS (=1 mL solution per coverslip)
- Place coverslips into a 12 well plate and fill with 1mL AA/FA
- Fill the empty wells with water and seal the plate with parafilm to reduce evaporation
- Incubate for 3 h, at 37°C
- *Prepare the AA/FA solution fresh*
- *Incubation time can be modified depending on the samples (i.e. overnight for tissue)*
- *AA/FA concentration is also a critical step to play with depending on the sample*

Step 3. Gelation

- Thaw TEMED, APS and MS aliquots on ice and place an Eppendorf rack at least 10 min before gelation
- Put a humid chamber (with parafilm and wet paper; **see picture below**) at 4°C for 10 min.
- Take out the plate with the coverslips from the 37°C incubator
- Place the humid chamber on ice
- Take out the maximum two coverslips at once, since TEMED and APS induced polymerisation is a fast process
- Add first 5 µL of TEMED and 5 µL APS into the monomer solution, vortex 2-3 seconds, make 2 drops of 35 µL on the parafilm of the humid chamber
- Rapidly cover each drop with a coverslip; **cells facing the solution**
- Incubate 5 min on ice for a better gel penetration
- Incubate at 37°C for 30 min



Always work on ice – or in the cold room if necessary to prevent gel polymerization; prepare everything before adding TEMED and APS

Step 4 and Step 5. Denaturation and Expansion

- Transfer the coverslips + gels into a 6 well plate filled with 1 mL of denaturation buffer
- Incubate for 10-15 min RT with agitation (to allow the gel to detach from the coverslip)
- Transfer the gel into a 1.5 mL Eppendorf tube filled with fresh denaturation buffer
- Incubate for 1h30min at 95°C
- *Gel starts to expand a bit during the denaturation – Transfer the gel with a spatula into the 1.5 mL Eppendorf tube (tricky step)*
- *Cells are "absorbed" into the gel very close to its surface*
- Washes of the gel: Put the gel in 250 mL beaker (or similar container) + ddH20 for 10 min.
- Repeat, several washes in ddH20

- Transfer the gel in PBS overnight or proceed for immunostaining directly
- *Be gentle with the gel after the first wash it becomes fragile.*

Step 6. Staining

- Wash the gel with PBS for 5 min (repeat if needed – The gel must be small enough to hold into a 6 well plate)
- Cut the gel in quarters and transfer in 12 well plate
- Incubate with 500 µL of antibody diluted in PBS-BSA 2% for 2h30min at 37°C with agitation
  Here: primary antibodies are: anti-alpha and -beta tubulin (1:250 each) and rabbit anti-GFP (1:500)
- Wash gel 3xwith PBS+Tween 0.1% for 10 min at RT with agitation
- Incubate the gel into 500 µl of secondary ab in PBS-BSA 2% for 2h at 37°C with agitation
  Here: secondary antibodies are: anti-guinea pig STAR 635P (1:1000), anti rabbit Alexa 594 (1:1000) and Hoechst or DAPI (1:1000)
- Wash gel 3x with PBS+Tween 0.1% for 10 min at RT with agitation
- Incubate the gel with 2 µg/ml NHS ester in PBS ON at 4°C with agitation (alternatively, incubation can be performed for 1h30 min at RT)
- Wash gel 3x with PBS+Tween 0.1% for 10 min RT with agitation
- *Use 2x concentrated ab compared to regular IF*
- *After this procedure, the entire gel has been stained in 1 mL of antibody solution (total: Primary and secondary ab) in a 6 well plate*

Step 7. Final expansion

- Put the gel in 100 mL beaker (or equivalent) + ddH2O for 3x 15 min or 2x 15 min and overnight.
- *The important point is to have at least 3x water bath before imaging. Leave it overnight for convenience*
- *Do not keep the gel at 4°C for more than 1 week.*
- *Gels can be frozen in 50% glycerol either before or after staining (Wash the gel in water - add 50% glycerol into a piece of gel in the desired container – incubated for 30/60 min with agitation – Freeze at -20°C in the container sealed with parafilm). Thawing the gel by washing the glycerol in water or PBS according to the following step (immunostaining or imaging). Wash 3x 10 min with agitation.*

Imaging

- Before imaging prepare coated coverslips:
  For example, with 24 mm coverslips: Add 200 µL of 100 µg/ml poly-D-Lysine on a coverslip and incubate for 15 min (37°C) or 1h (RT).
  Note that the coating can be done up to overnight for convenience
- wash 3x with ddH2O, dry completely (store in the fridge for 1 week)
- Orientate the gel: Place the gel on a non-coated coverslips and try to focus on samples. If this is not possible, the gel is very likely upside down
- *The presence of the cells bends the surface of the gel. Therefore, the cells are on the concave part of the gel*
- *The part of the gel containing the cell is smoother than the other part which contains asperities/ruggedness/impurities*
- Dry the gel on a tissue paper; **be careful that cells never face the tissue**. Putting the cell directly in contact of the tissue will trigger cell loss (aspirated into the tissue).
- Flip the gel on a coated coverslip
- Image

iU-ExM protocol

INTRODUCTION:

Iterative ultrastructure expansion microscopy (iU-ExM) is an advanced technique that combines ultrastructure expansion microscopy (U-ExM) with the principle of iterative expansion (iExM: Chang et al., 2017, or Pan ExM: M'Saad et al., 2020) to achieve even higher resolution imaging of biological samples. The underlying principle of iU-ExM is to repeat the expansion multiple times, with each iteration resulting in a higher resolution (Louvel et al., 2023).

The steps involved in iU-ExM are similar to those in U-ExM albeit with some specific modifications, with the addition of iterative cycles of expansion and imaging. The biological sample is first fixed according to the user's preferences and subsequently embedded in a cleavable, swellable polymer, which contains N,N'-(1,2-Dihydroxy-1,2-ethanediyl)bis(acrylamide) (DHEBA) instead of BIS that is used in regular ExM protocols (including U-ExM). The sample is next homogenized through heat denaturation at 85 °C since DHEBA melts at 95°C (the original U-ExM denaturation condition) and expanded first. iU-ExM introduces an intermediate staining after the first gel, enabling good labeling efficiency. Next, the first gel is embedded in a second DHEBA gel to stabilize the first one, itself subsequently embedded in a third BIS-acrylamide-based gel. Note that the first and second gels are dissolved prior the second expansion and imaging. This protocol can be used for a range of samples from human cells, isolated organelles, such as basal bodies, or tissues. In this practical, we will illustrate the power of iU-ExM on EGFP-SEC61 U2OS cells in culture, labelling the tubulin network and the endoplasmic reticulum.

MATERIAL:

- See materials for U-ExM
- N,N'-(1,2-Dihydroxy-1,2-ethanediyl)bis(acrylamide) DHEBA (Merck- sigma aldrich, 294381)
- Sodium hydroxide (Merck, 1.06498.1000)
- Rabbit anti-GFP (Torrey Pine-TP401; working dilution 1:250)
- Guinea pig anti-alpha Tubulin and anti-beta Tubulin (ABCD Antibodies, aa345 and aa344; working dilution 1:250)
- Alexa Fluor 594 Goat anti-Rabbit (Thermo A-11012; working dilution 1:250)
- Abberior 635p Goat anti-Guinea pig (Abberior ST635P; working dilution 1:250)
- Gelation chamber
- Humid chamber
- 10 cm Petri dish

WORKING SOLUTIONS:

All working solutions will be provided -ready to use- by your trainers. Please find some remarks that should be considered during this experimental protocol in blue.

- **Anchoring solution (AS):** 2% Acrylamide, 1.4% Formaldehyde, in 1x PBS.
- **DHEBA Denaturation buffer (DDB):**
  - 200 mM SDS
  - 200 mM NaCl
  - 50 mM TrisBase in water, **pH=6.8**

The pH is critical here, be careful to have a well calibrated pH-meter and to regularly monitor the pH of the denaturation buffer, it should not exceed pH=7. A pH exceeding 7 would result in either dissolved gels or overly expanded gels that will be highly breakable. In contrast, a pH below 6.8 would result in a lower expansion of the 1st gel. 1x difference on the 1st gel will impact the final expansion factor of about 3-4x)

- **Sodium Acrylate (SA): see U-ExM section**
- **40% acrylamide, 0.4% DHEBA stock:**
  Weigh 0.02 g of DHEBA powder and dissolve it in 5 mL of 40% acrylamide. Agitate until the DHEBA powder completely dissolves. Prepare aliquots of 250 µL in 1.5 mL Eppendorf tube. Those tubes can be directly used to prepare 1MS. Freeze and keep up to 2 years.
  Note that the DHEBA powder dissolvability shows a very important lot to lot variability. Some powder cannot be dissolved at all. Do not hesitate to buy different lot at the same time.
- **1st Monomer solution (1MS):**
  For 950 µL of solution mix:
  - 500 µL of 38 % Sodium acrylate (SA, 19% final)
  - 250 µL of 40% Acrylamide, 0.4% DHEBA (10% AAm=acrylamide monomer, 0.1% DHEBA final)
  - 100 µL 10x PBS
  - 100 µL ddH_2O.

  Aliquot (95 µL) and freeze (a minimum of 24h of freezing is necessary to ensure a good quality of the gel) – Keep up to one month.
  Note that the stock solution is indicated for 950 µL only. The "missing" 50 µL are for the TEMED and APS which are added last minute in each 95 µL aliquot
- **Neutral Gel Solution (NGS):**
  For 50 mL of solution mix:
  - 12.5 mL of 40% Acrylamide (10% final)
  - 0.025 g of DHEBA powder (0.05% final)
  - 37.5 mL ddH_2O.

  Mix the DHEBA powder with the 12.5 mL of 40% acrylamide before adding the water. Put on a shaker until a complete dissolution of the DHEBA powder. Prepare 10 mL aliquots and keep in the freezer up to 2 weeks.
- **2nd Monomer solution (2MS):**
  For 50 mL of solution mix:
  - 12.5 mL of 40% Acrylamide (10% final)
  - 25 mL of 38% SA (19% final)
  - 2.5 mL of 2% N,N'-methylenbisacrylamide (0.1% final)
  - 10 mL of ddH_2O.

  Vortex strongly the solution until complete homogenisation. If your sodium acrylate solution as a yellow color, it is possible that the monomer solution presents a foggy aspect. This is expected and we never experienced any variations in expansion factor. Also, vortex strongly the solution before use as the SA can form a pellet at the bottom of the tube. With a very clear (not yellow) SA, the solution should remain clear. We have noticed a reduced expansion factor with clear SA.
  Prepare 10 mL aliquots and store them at -20°C for several months.
- **TEMED (10%)**-see U-ExM
- **APS (10%)** - see U-ExM section
- **Dissolution-Solution:** 200 mM NaOH in water
- **Poly-D-Lysine** - see U-ExM section

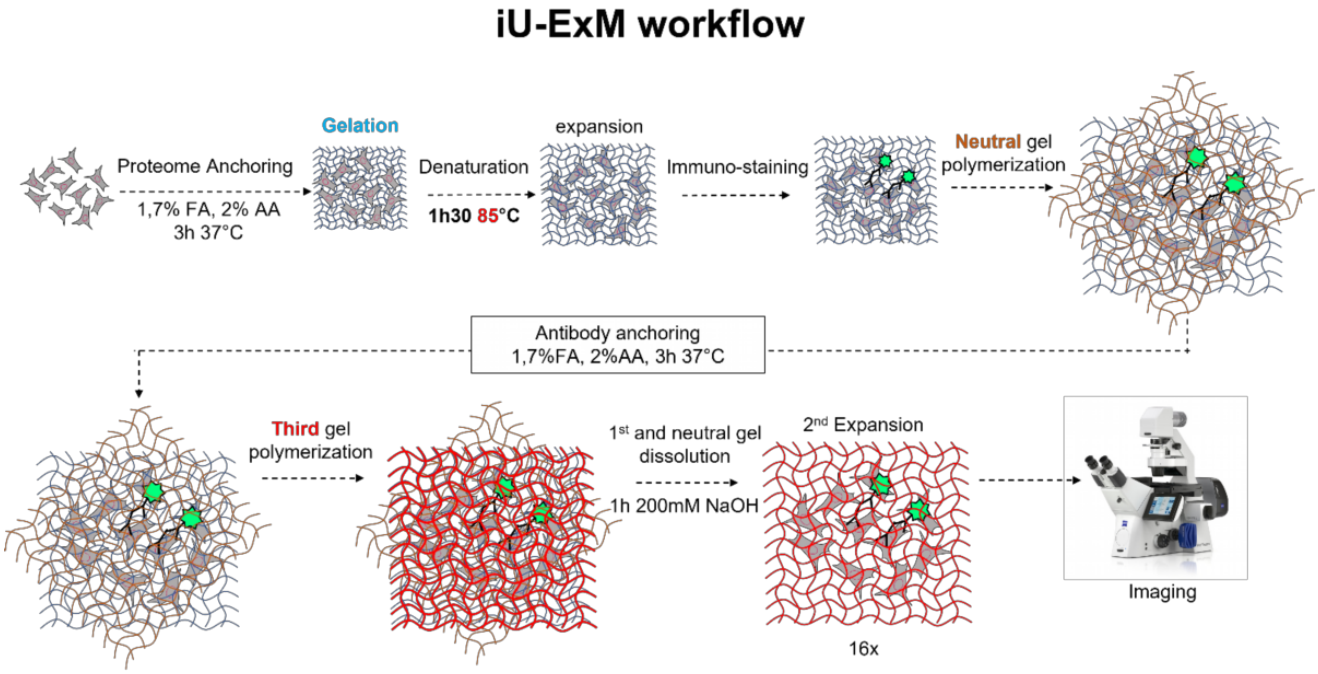
EQUIPMENT:

- **See Equipment list in U-ExM section**
- 22*22mm squared coverslips (DIVERS DUTSCHER, 100032N)
- Thermometer
- 10 cm petri dish

SAMPLES:

U2OS *tet3G EGFP-SEC61* adherent cells: 6 well plates, 4x12 mm coverslips/well, 2 mL medium, seeding density: 100.000 U2OS cells, 60 h before experiment, and induced with 1 µg/mL doxycycline 6 h before fixation.

This protocol is optimized for 12 mm coverslips – All volumes should be scaled down/up according to the surface of the coverslip used. Note that you should optimize the cell number for the seeding step depending on the cell type. For example, we usually seed 200'000 RPE-1 cells or 400'000 HeLa cells 24 h prior fixation



WORKFLOW:

Step 1: Fixation of choice

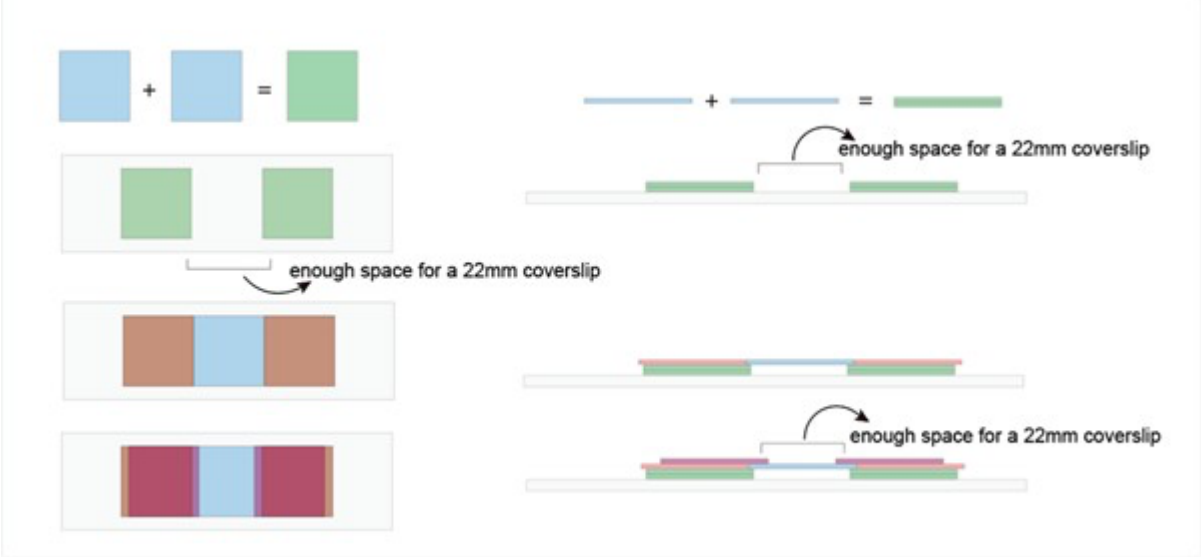
- According to the type of structures to visualize, different fixations can be used. For this course, we will use cryo-fixation to better preserve the ER and the microtubule network.

Step 2: Anchoring

- Fill one row of a 12 well plate with water.
- Incubate the coverslips in 1 mL of Anchoring solution (AS, 2% AA; 1.4% FA) in the remaining wells, one coverslip per well. (Dry them slightly with a tissue before adding them in the Anchoring solution, to remove excess medium. *Be careful not to dry the cells completely*)
- Seal the plate with parafilm and incubate for 3h at 37°C.

Construction of the gelation chamber (see scheme)

a. Build the gelation chamber according to the scheme below
b. Glue two stacks of two 22*22mm glass coverslips together
   Do not add a lot of glue, otherwise you will increase the height of the stack.
c. On a classic glass microscope slide, glue the stacks of 22*22mm coverslips at 14 mm one from each other, taking care to not space them too much so that it's still possible to add another 22*22mm on top to cap the chamber
d. On each 22*22mm stacks, glue another 22*22mm coverslip on top of it, leaving enough space to add other coverslips between the two stacks to cover the chamber.



Step 3: First Gelation

- Thaw APS/TEMED/1MS solutions on ice 30 min before.
- Put a humid chamber at -20°C at least 20 min before. Add the gelation chamber on it before gelation
- Fixation of the cell coated coverslip on the gelation chamber:

- Remove the Anchoring solution from the 12 well plate well.
- Open gelation chamber.
- Take out the coverslip with tweezers.
- Gently dry the coverslip by briefly touching a facial tissue.
- Put a **little** drop of glue on the gelation chamber.
- Dry the cell-free side of the coverslip with a facial tissue.
- Put the cell free side on the drop of glue.
- Gently press with the head of tweezers making little rounds to spread the glue.
- The glue should fix the coverslip within seconds.
- Close the gelation chamber.

- Add 2.5 µL of each APS/TEMED to the 1MS 95 µL aliquots and vortex for 2-3 seconds
- Fill the space between the coverslip and the capping coverslip with monomer solution MS.
- Put the gelation chamber on the humid chamber and let it incubate for 15 min on ice.
- Transfer the humid chamber at 37°C for 45 min.

Step 4 and Step 5: Denaturation/Expansion

- Turn on the heater at 85°C. Check with an external thermometer to be sure of the temperature.
  A few °C below might create non-homogeneous expansion and few °C upper can cause gel over-expansion or disintegration.
- Take out the gelation chamber from 37°C.
- Disassemble gently the gelation chamber. The coverslip lid should detach with the gel in between the 12 mm coverslip and the lid. If the gel detaches from the 12 mm coverslip and sticks to the coverslip lid, put the lid on a 6 well plate with the gel up. If the lid detaches and the gel with the 12 mm coverslip stays glued to the gelation chamber, push gently with a spatula on the coverslip, it should detach from the gelation chamber. If not (which might be due to a too large amount of glue), put the gelation chamber on a petri dish.
- Add 1mL of DDB on the gel and put the plate on a rocking platform for 15 min.
  If the coverslip does not detach from the gelation chamber, do not shake and only add the DDB on it.
- Add 1 mL of fresh DDB in a 1.5 mL Eppendorf tube and gently transfer the gels on it with a spatula.
- Put the Eppendorf tube in the heater for 1h30 at 85°C.
- Remove the DDB solution and put the gel in a 12 cm petri dish filled with ddH_2O, then wash 3 times for 30 min then let it for an overnight expansion.

Step 6: Intermediate Staining

- Immunolabelling is performed on the first expanded gel (see UExM protocol). However, NHS-Ester staining can be performed at the final step post-expansion.

Step 7 and Step 8: Neutral gel and 2nd anchoring

- Thaw on ice APS/TEMED/NGS at least 1h before. 5 mL per sample of activated NGS is needed per sample
  (can be reduced and optimized according to the size of the sample of gel. The most important thing is that the gel is totally covered with NGS)
- With a razor blade, cut a 1.5*1.5 cm sample *(the size can be adjusted, but a too large sample can be tricky to process)* in the first expanded gel and transfer it in a 6 well plate.
- Put the 6 well plate on ice and on a rocking platform.

- Still on ice, add 25 µL of TEMED/APS solution in 5 mL of NGS and let the tube open to prevent gelation. Vortex for 2-3 seconds.
- Add 5mL of NGS with TEMED/APS per well on ice under agitation keeping the lid open to prevent gelation for 25 minutes.
- Remove the NGS, and with a spatula gently take the gel and put it on a classic microscope slide.
- With a facial tissue, gently touch the gel to remove the excess of NGS.
  Be careful, the gel can stick to the tissue. Do not over dry, the gel would not polymerize well.
- Add a 22*22mm coverslip on top of the gel making sure of not making any bubbles. If there are some bubbles, try to gently push them out and/or move the coverslip to remove them. If it doesn't succeed, do not insist.
- Put the microscope slide with the gel on a humid chamber and transfer the chamber at 37°C for 1h.
- Take out the gel from the microscope slide and put it directly in a 6 well plate.
- Add 2 mL of Anchoring solution and incubate the plate at 37°C for 3h under agitation.

Step 9: 2nd Monomer solution embedding and 3rd Gelation

- After the 3 hours, remove the Anchoring solution and wash the gel 2 times with PBS for 10 minutes.
- Repeat step 7 but with 2nd monomer solution, and the gelation time is reduced to 45 minutes.
  Be careful, the third monomer solution polymerise faster than the second monomer solution. Once the coverslip is added on top of the gel, it is tricky to remove bubbles. If there is, do not insist and pursue the protocol.

Step 10: Dissolution of neutral and first gels

- In a glass beaker, add 25 mL of dissolution solution (DS).
  Make sure that the beaker is big enough to permit the gel to move with the agitation.
- Take the gel out of the microscopic slide and put it in the beaker. Gently take it off from the slide, without deforming it.
  A blue blur visible with dark background will form in the gel due to the very high concentration of monomers in the gel. This is expected. If you do not have this blur, the polymerization might have gone wrong.
- Agitate for 1h at RT; stir several times.
  This step is very critical to ensure proper complete expansion of the gel. If the gel looks bended/deformed, the dissolution might not be complete. In this case, change the dissolution solution and pursue another 30 min of dissolution.
- Remove the DS solution and rinse once with PBS quickly to remove the excess of DS.
- Wash with PBS for 15 min.
- *If an NHS-Ester staining is planned after, follow these steps:*
  - *Test the pH with pH paper.*
  - *Wash again until the pH drops to 7-8.*
- The sample is ready to be labelled or expanded.

Step 11: Final Expansion

- Be careful, as the gel is very thick, you might think that it is totally expanded but it might not be. Let the gel expand with several water baths for at least 15h.
  If you see that the gel is bending when you slice it, it is either not completely expanded or not well dissolved.

Imaging

- Before imaging prepare coated coverslips:
  For example, with 24 mm coverslips: Add 200 µL of 100 µg/ml poly-D-Lysine on a coverslip and incubate for 15 min (37°C) or overnight (RT).
- Wash 3x with ddH2O, dry completely (store in the fridge for 1 week).
- Orientate the gel: Place the gel on a non-coated coverslips and try to focus on samples. If this is not possible, the gel is very likely upside down.
- Flip the gel on a coated coverslip.
- Dry the gel by putting the cell face on a kimwipe. Gently press on it to remove water and then put it on the coated coverslip. It might create bubbles. If so, gently press on the gel with a spatula or brush. If it doesn't remove the bubbles, change the coverslip or focus on a bubble-free area.
- Image
- *With a 15-20X expansion the fluorescence signal is extremely diluted and therefore the intensity of the staining will be much lower dans IF or U-ExM gels. Do not hesitate to push the laser and really focus while looking for focal plan and change location as it is possible that there are no cells in the area you are looking at.*
- *With the high expansion factor, the working distance of the objective might be limited. Not being able to see the entire cell from top to bottom is expected. Be careful of not pushing to high.*