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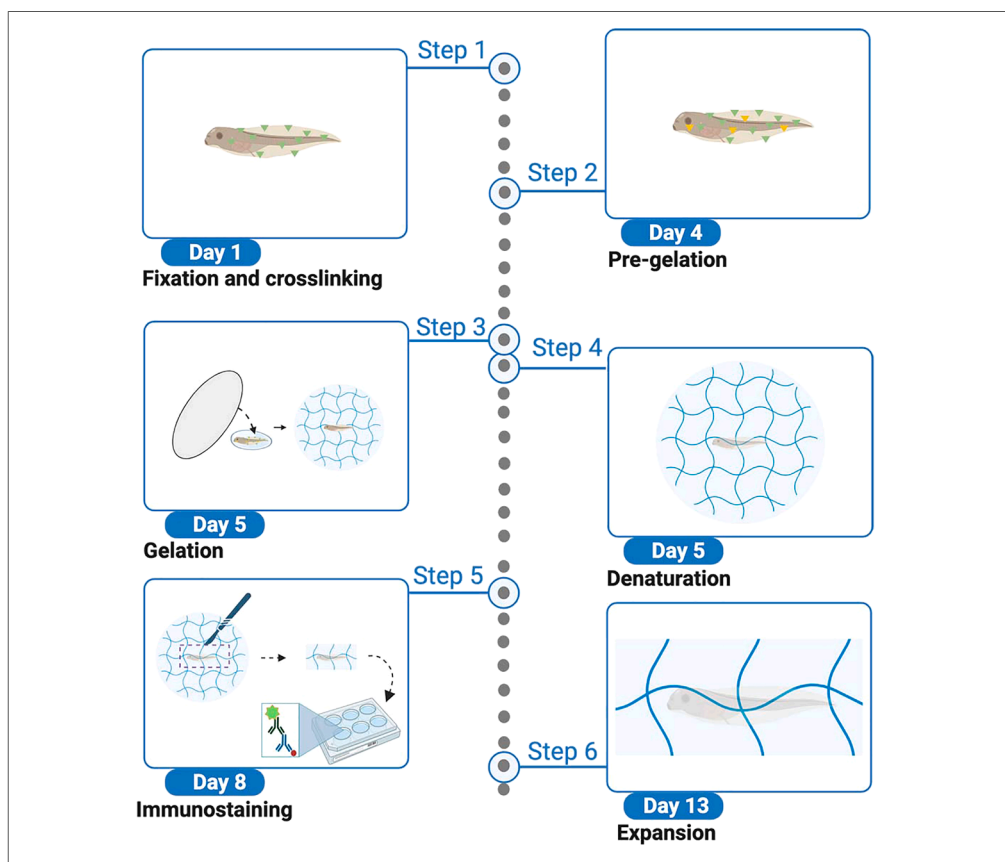
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## Protocol

# Protocol for tissue expansion microscopy for ultrastructure expansion of *Xenopus* embryos



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**Highlights**  
Instructions for 4-fold  
expansion of whole  
*Xenopus* embryos  
using TissUExM

Protocol for fixation,  
cross-linking,  
gelation, expansion,  
and immunolabeling

Steps for analyzing  
subcellular structures  
in epidermal  
multiciliated cells

Tissue expansion microscopy (TissUExM) allows super-resolution imaging by physically expanding biological samples. Here, we present a protocol for ultrastructure expansion microscopy of *Xenopus* embryos using TissUExM. We describe steps for tissue fixation, embedding, fluorescence labeling, and expansion. We demonstrate that our protocol provides enhanced resolution for studying subcellular structures, such as centrioles and cilia. This protocol has the potential to offer a broad range of applications to decipher sub-cellular organization in *Xenopus* embryos.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

Protocol for tissue expansion microscopy for ultrastructure expansion of *Xenopus* embryosSalomé Dussert,<sup>1,2</sup> Virginie Thomé,<sup>1,2,3</sup> Laurent Kodjabachian,<sup>1</sup> and Camille Boutin<sup>1,4,\*</sup><sup>1</sup>Aix Marseille University, CNRS, IBDM, Turing Centre for Living Systems, Marseille, France<sup>2</sup>These authors contributed equally<sup>3</sup>Technical contact<sup>4</sup>Lead contact\*Correspondence: [camille.boutin@univ-amu.fr](mailto:camille.boutin@univ-amu.fr)  
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## SUMMARY

Tissue expansion microscopy (TissUExM) allows super-resolution imaging by physically expanding biological samples. Here, we present a protocol for ultrastructure expansion microscopy of *Xenopus* embryos using TissUExM. We describe steps for tissue fixation, embedding, fluorescence labeling, and expansion. We demonstrate that our protocol provides enhanced resolution for studying subcellular structures, such as centrioles and cilia. This protocol has the potential to offer a broad range of applications to decipher sub-cellular organization in *Xenopus* embryos.

## BEFORE YOU BEGIN

Inspired by previously published articles,<sup>1–3</sup> this protocol provides a comprehensive and detailed description of TissuExM on *Xenopus laevis* embryos, for observation under fluorescent confocal microscope. Specifically, we report how to implement TissuExM to visualize the fine organization of multiciliated cells at the surface of NF stage 25–30 *X. laevis* embryos.

## Institutional permissions

All experiments were performed following the Directive 2010/63/ EU of the European parliament and of the council of September 22, 2010 on the protection of animals used for scientific purposes and approved by the “Direction Départementale de la Protection des Populations, Pôle Alimentation, Santé Animale, Environnement”, des Bouches du Rhône (agreement number F1305521).

## Number of embryos per experiment

For beginners, we recommend preparing 6 embryos per condition. More experienced handlers can limit themselves to 2 embryos per condition. To calculate sample size, users must integrate that confocal imaging should be done within a maximum of two days after completion of the protocol.

## Solution preparation

Given the number of solutions needed for this protocol, we recommend to prepare them in advance and store them according to the guideline provided below.

All solutions are prepared similarly to Gambarotto et al.,<sup>4</sup>; Steib et al.<sup>3</sup> and Steib et al.<sup>2</sup> An optional solution containing 4-hydroxy-TEMPO (4-OH) can be used to slow down the gelation kinetic. Most solutions can be stored over several weeks or months. Note that the monomer solution containing sodium acrylate should be prepared at least 24 h prior to gelation.



## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Paraformaldehyde (PFA, 32%)	EMS-Euromedex	Cat# EM-15714
DMSO	Fisher Chemical	Cat# D/4121/PB08
Methanol	VWR	Cat# 20847.295
Triton X-100	Euromedex	Cat# 2000-B
Formaldehyde (FA, 36.5%–38%)	Merck	Cat# F8775
Acrylamide (AA, 40%)	Merck	Cat# 1003584583
N,N'-Methylbisacrylamide (bis-AA, 2%)	Merck	Cat# M1533
Sodium acrylate (SA, 97%–99%)	AK Scientific	Cat# R624
4-hydroxy-TEMPO	Sigma	Cat# 176141
Ammonium persulfate (APS)	Thermo Fisher Scientific	Cat# 17874
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific	Cat# 17919
Sodium dodecyl sulfate (SDS)	Carl Roth	Cat# 1057.1
Tris	VWR	Cat# 28811.295
Tween 20	Euromedex	Cat# 2001-A
Phosphate-buffered saline	Eurobio	Cat# CSOPBS01-08
Poly-D-lysine (PDL, 1 mg/mL)	Gibco	Cat# A38904-01
12 mm diameter, 1.5 mm thick coverslips	Eprédia	Cat# CB00120RAC20MNZO
35 mm glass bottom dish	Cellvis	Cat# D35-20-1,5-N
<b>Antibodies</b>		
Ac- $\alpha$ -tubulin (1:500)	Sigma	Cat#T7451
Centrin (1:250)	Millipore	Cat#04-1624
Cep164 (1:250)	Kind gift from Pr Rhee	N/A
Goat anti-mouse IgG2b Alexa Fluor A488 (1:400)	Thermo Fisher Scientific	Cat# A-21141
Goat anti-mouse IgG2b Alexa Fluor A568 (1:400)	Thermo Fisher Scientific	Cat# A-21144
Goat anti-mouse IgG2a Alexa Fluor A488 (1:400)	Thermo Fisher Scientific	Cat# A-21131
Goat anti-mouse IgG2a Alexa Fluor A568 (1:400)	Thermo Fisher Scientific	Cat# A-21134
<b>Experimental models: Organisms/strains</b>		
<i>X. laevis</i> WT stage 29–30	Nasco	N/A
<b>Other</b>		
Confocal microscope	Zeiss	LSM880

## MATERIALS AND EQUIPMENT

### Gelation chamber

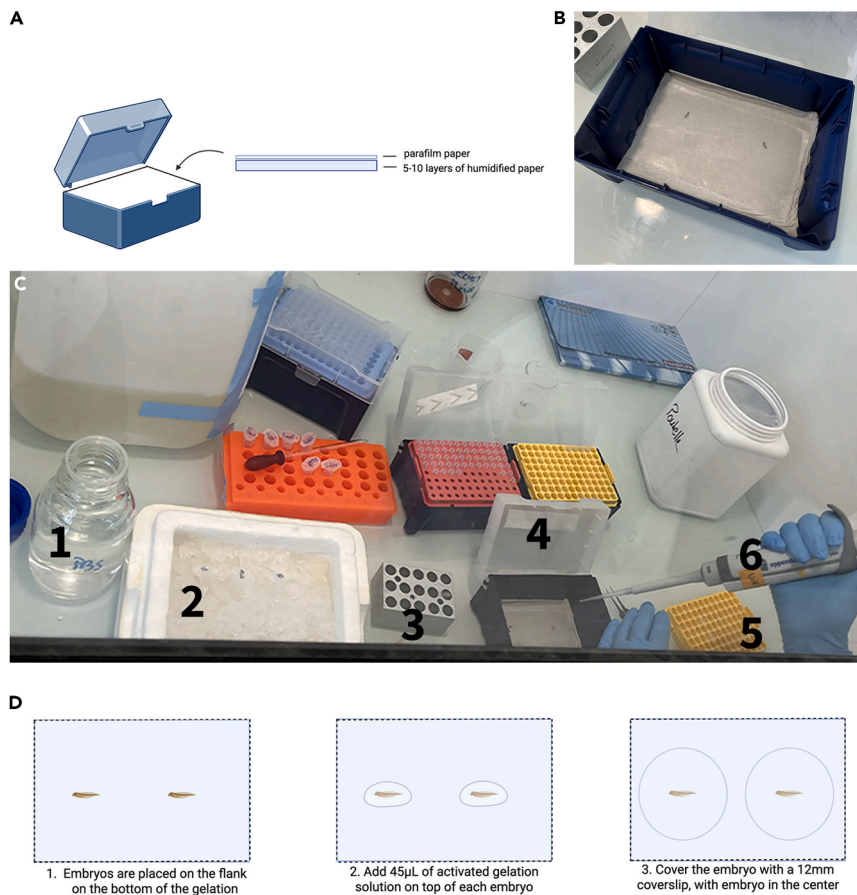
To prepare the gelation chamber, fill an empty tip box with 5–10 layers of tissue paper. Lightly wet the paper with water before placing a parafilm paper on top (Figure 1A). The surface of the parafilm should be as flat as possible. A small western blot roller can be used to remove bubbles between the paper and parafilm. Store at  $-20^{\circ}\text{C}$  until use. We recommend preparing one gelation chamber for two embryos. Gelation chambers can be re-used several times, ensuring that the surface of the parafilm remains flat.

### Solutions

#### PFA 4%

Reagent	Final concentration	Amount
PFA 32%	4%	8 ml
PBS 10X	1X	8 ml
ddH <sub>2</sub> O	NA	64 ml
TOTAL	NA	80 ml

Aliquot and store at  $-20^{\circ}\text{C}$  for 12 months.



**Figure 1. Gelation chamber preparation, gelation set up and gelation**

(A) Schematic representation of the gelation chamber.

(B) Gelation chamber with embryos positioned just before gelation.

(C) The gelation set up is installed under a fume hood and includes: PBS (1); APS and SDS (*optional 4OH-TEMPO*) on ice (2); cooling block for iMS solution (3); gelation chamber (4); coverslips placed on a support to be easily accessible (5) and pipettes preset to the volumes required for preparation of the activated gelation solution (6).

(D) Schematic representation of embryo positioning in the gelation chamber and gelation procedure.

△ **CRITICAL:** Work with personal protective equipment (PPE) including gloves and mask. This work should be performed under the fume hood.

Triton 10%		
Reagent	Final concentration	Amount
Triton (100%)	10%	5 ml
PBS1X	NA	45 ml
TOTAL		50 ml

Store at 4°C for several months.

Sodium Acrylate (SA) 38%		
Reagent	Final concentration	Amount
Sodium Acrylate (99%)	38%	3 g
ddH <sub>2</sub> O	NA	4.89 ml
TOTAL	NA	~8 ml

Progressively dissolve SA in water while stirring on ice. Filter 0.45 µm. Store at 4°C for 6 months.

△ **CRITICAL:** Work with PPE including gloves and mask. This work should be performed under the fume hood. The solution should be beige and not display a strong yellow color associated with batch impurity. Contact provider if needed.

<b>PBS-T</b>		
Reagent	Final concentration	Amount
PBS 10 X	1X	50 ml
Triton (10%)	0,1%	5 ml
ddH2O	NA	445 ml
<b>TOTAL</b>	<b>NA</b>	<b>500 ml</b>

Store at RT for 2–3 months.

<b>Inactivated Monomer Solution (iMS)</b>		
Reagent	Final concentration	Amount
Sodium Acrylate (38%)	21%	50 µl
Acrylamide (40%)	10%	25 µl
Bis-Acrylamide (2%)	0,1%	5 µl
Triton 10%	0,1%	1 µl
PBS 10X	1X	9 µl
<b>TOTAL</b>	<b>NA</b>	<b>90 µl</b>

Work on ice or at 4°C. Aliquot in tubes containing exactly 90 µl. Store at –20°C for up to 2 weeks. The solution should not freeze.

△ **CRITICAL:** Work with PPE including gloves and mask. This work should be performed under the fume hood. The iMS is used on day 4 and should be prepared at least 24 h in advance.

<b>4-hydroxy-TEMPO (4OH-TEMPO)</b>		
Reagent	Final concentration	Amount
4-hydroxy-TEMPO	0,5%	6 mg
ddH2O	NA	1.2 ml
<b>TOTAL</b>	<b>NA</b>	<b>1.2 ml</b>

Dissolve. Filter 0.45 µm. Aliquot and store at –20°C. Do not re-use aliquots.

△ **CRITICAL:** Work with PPE including gloves and mask. This work should be performed under the fume hood.

<b>TEMED</b>		
Reagent	Final concentration	Amount
TEMED	10%	10 µl
ddH2O	NA	90 µl
<b>TOTAL</b>	<b>NA</b>	<b>100 µl</b>

Vortex. Aliquot and store at –20°C. Do not re-use aliquots.

△ **CRITICAL:** Work with PPE including gloves and mask. This work should be performed under the fume hood.

<b>APS</b>		
Reagent	Final concentration	Amount
APS (99%)	10%	0.01 g
ddH <sub>2</sub> O	NA	100 µl
TOTAL	NA	100 µl

Vortex. Aliquot and store at -20°C. Do not re-use aliquots.

△ **CRITICAL:** Work with PPE including gloves and mask. This work should be performed under the fume hood.

<b>SDS</b>		
Reagent	Final concentration	Amount
SDS (99%)	350 mM	10 g
ddH <sub>2</sub> O	NA	~95 ml
TOTAL	NA	100 ml

Progressively dissolve SDS in 80 ml ddH<sub>2</sub>O while stirring. Once dissolved, fill up to 100 ml. Keep at RT for 6 months. Place for 10 min at 37°C if crystals form.

△ **CRITICAL:** Work with PPE including gloves and mask. This work should be performed under the fume hood.

<b>NaCl</b>		
Reagent	Final concentration	Amount
Sodium Chloride (99%)	5 M	29.2 g
ddH <sub>2</sub> O	NA	~95 ml
TOTAL	NA	100 ml

Filter 0.45 µm. Keep at RT for 12 months.

<b>Denaturation buffer</b>		
Reagent	Final concentration	Amount
Tris (99%)	50 mM	1.2 g
ddH <sub>2</sub> O	NA	10 ml
SDS (350 mM)	200 mM	114.8 ml
NaCl 5 M	200 mM	8 ml
Dilute Tris while stirring using water pre-warmed at 37°C before adding SDS and NaCl. Adjust pH to 9 with HCl		
ddH <sub>2</sub> O	NA	~70 ml
TOTAL	NA	200 ml

Filter 0.45 µm. Keep at RT for 6 months.

△ **CRITICAL:** Work with PPE including gloves and mask.

#### Denaturation like buffer

Reagent	Final concentration	Amount
Tris (99%)	50 mM	0.6 g
ddH <sub>2</sub> O	NA	10 ml
Dilute Tris while stirring using water pre-warmed at 37°C		
NaCl 5 M	200 mM	4 ml
ddH <sub>2</sub> O	NA	~85 ml
TOTAL	NA	100 ml

Adjust pH to 9 with HCl. Filter 0.45 µm. Keep at RT for 6 months.

#### Blocking buffer

Reagent	Final concentration	Amount
DMSO	1%	500 µl
Triton 10%	0,5%	250 µl
FCS	5%	2.5 ml
BSA	2%	1 g
PBS 1X	NA	~45 ml
TOTAL	NA	50 ml

Filter 0.45 µm. Keep at 4°C for 12 months.

#### Tween 20

Reagent	Final concentration	Amount
Tween 20 (99%)	10%	10 ml
ddH <sub>2</sub> O	NA	90 ml
TOTAL	NA	100 ml

Keep at RT for 12 months.

#### PBS – T

Reagent	Final concentration	Amount
Tween 20 (10%)	0,1%	5 ml
PBS10X	1X	50 ml
ddH <sub>2</sub> O	NA	445 ml
TOTAL	NA	500 ml

Keep at 4°C for 12 months.

#### PBS - BSA

Reagent	Final concentration	Amount
BSA	3%	3 g
PBS1X	NA	~95 ml
TOTAL	NA	100 ml

Dissolve while stirring. Aliquot and keep at –20°C for 24 months.

### STEP-BY-STEP METHOD DETAILS

#### Day 1—Embryo fixation and crosslinking

⌚ Timing: 30 min manipulation, 6 + 72 h incubation

In this step, embryos are fixed before crosslinking AA and FA that will functionalize cell components, enabling subsequent binding of Sodium Acrylate swellable gel.

**Note:** This work should be performed under the fume hood.

1. Fixation of embryos.
  - a. Remove vitelline membrane from the embryos.
  - b. Immerse embryos in glass vials containing 2 ml PFA 4%.
  - c. Incubate for 6 h at 23°C–25°C under 100 rpm agitation.
2. Preparation of cross-linking solution.
  - a. Prepare 500 µl of crosslinking solution per embryo.

#### Crosslinking solution mix for 1 ml

Reagent	Final concentration	Amount
PBS 1X	0,45X	453 µl
Triton (10%)	0,1%	10 µl
AA (40%)	20%	500 µl
FA (38%)	1,4%	37 µl
TOTAL	NA	1 ml

- b. For each embryo distribute 500 µL of crosslinking solution in 1,5 ml Eppendorf tube.
- c. Transfer one embryo per Eppendorf tube.
- d. Seal Eppendorf tube with parafilm.
- e. Incubate for 72 h at 37°C.

#### Day 4—Pre-gelation

⌚ Timing: 30 min preparation, overnight incubation

This step allows the penetration of gelation components into the embryo.

**Note:** This work should be performed under the fume hood.

3. Incubation with inactivated Monomer Solution (iMS).
  - a. Remove the cross-linking solution.
  - b. Rinse 3x with 500 µL PBS-T 5 min.
  - c. Add 45 µL of iMS per embryo.
  - d. Incubate 15–20 h at 4°C.

#### Day 5—Gelation of embryos

⌚ Timing: 10 min for manipulation of 2 embryos, 3 h incubation

This step requires careful handling to ensure the integrity of embryos and their proper positioning into the gel. Optional addition of 4OH-TEMPO may be used to slow down gelation kinetics to ensure proper positioning if large series of embryos are proceeded. We did not observe differences in expansion efficiency comparing samples expanded with or without 4OH-TEMPO.

**Note:** This work should be performed under the fume hood.

4. Embryo wash.
  - a. Remove the 45  $\mu\text{L}$  of iMS from the embryos.
  - b. Wash 3 x 5 min with 500  $\mu\text{L}$  PBS-T under 100 rpm agitation.
5. Preparation of the working station ([Figure 1C](#)).
  - a. Take out one aliquot of TEMED and APS (*Optional: 4OH-TEMPO*), vortex well and keep on ice.
  - b. Prepare a 12 mm coverslip for each embryo that will be rapidly accessible.
  - c. Pre-set the pipettes to the volume to be used for activated gelation solution preparation.
  - d. Take out iMS aliquots, vortex well as crystals may have formed, keep on a cooling block.
6. Embryo orientation ([Figures 1B and 1D](#)).
  - a. Take the gelation chamber out from  $-20^{\circ}\text{C}$ .
  - b. Use a plastic pipette to place the embryo on the parafilm paper.
  - c. Carefully aspirate the excess liquid.
7. Activated gelation solution preparation (45  $\mu\text{L}$  per embryo).
  - a. For 100  $\mu\text{L}$  solution add to 90  $\mu\text{L}$  iMS aliquot rapidly, in this order and vortexing between each reagent:
    - 5  $\mu\text{L}$  PBS1X.
    - 2.5  $\mu\text{L}$  TEMED.
    - 2.5  $\mu\text{L}$  APS.

**Optional:** For 100  $\mu\text{L}$  solution add to a 90  $\mu\text{L}$  iMS aliquot rapidly, in this order and vortexing between each reagent:

- 3  $\mu\text{L}$  PBS1X.
- 2  $\mu\text{L}$  4OH-TEMPO.
- 2.5  $\mu\text{L}$  TEMED.
- 2.5  $\mu\text{L}$  APS.

8. Embryo gelation ([Figure 1D](#)).
  - a. Gently add 45  $\mu\text{L}$  of activated gelation solution on top of each embryo, making sure to avoid bubbles.
  - b. Flip a 12 mm coverslip on top of the embryo making sure the embryo stays right in the middle of the coverslips.
  - c. When two embryos have been positioned, close the chamber, carefully transfer at  $4^{\circ}\text{C}$  and incubate 1 h.
  - d. Incubate 2 h at  $38^{\circ}\text{C}$ .

**Optional:** Slow down gelation kinetics.

**Note:** In case more time is needed to install the embryos, 4OH-TEMPO can be used to slow down gelation kinetics. In that case use option b. for activated gelation solution preparation.

[Troubleshooting 1.](#)

[Troubleshooting 2.](#)

### Day 5—Gel denaturation

⌚ Timing: 3 min manipulation per embryo, 72 h incubation

**Note:** This work should be performed under the fume hood.

In this step, the sample is denatured by heat and SDS to ensure a proper swelling in the subsequent steps.

9. Gel denaturation.
  - a. Using forceps carefully detach the gel and coverslips from the parafilm.
  - b. Transfer one gel and coverslip per well of 6 wells plate filled with 2 ml denaturation buffer. For an easy manipulation the coverslip should be on top.
  - c. Incubate 10 min at 23°C–25°C under 100 rpm agitation.
  - d. Remove the coverslip with forceps.
  - e. Using a spatula, transfer each gel to a 1.5 ml Eppendorf tube filled with 1 ml denaturation buffer.
  - f. Incubate for 72 h at 70°C.

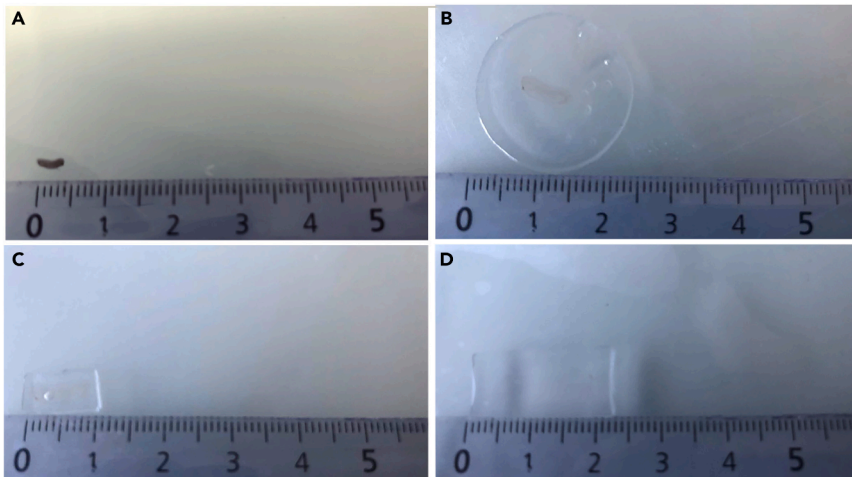
### Day 8—Gel equilibration, control, and trimming; immunostaining part 1

⌚ Timing: 30 min manipulation, 72 h incubation

This step is the final preparation of the gel. The gel that has already partially expanded is trimmed around the embryo to reduce the quantity of reagents required in the subsequent steps. The gel is then processed for the first steps of immunolabeling process.

10. Gel equilibration.
  - a. Remove as much as possible denaturation buffer from the Eppendorf tube.
  - b. Transfer one gel/well in a 6 wells plate in 2 mL of denaturation-like buffer.
  - c. Incubate for 15 min at 23°C–25°C, under 100 rpm agitation.
  - d. Remove 2 ml of denaturation like-buffer.
  - e. Add 2 ml of PBS 1X.
  - f. Incubate for 15 min at 23°C–25°C, under 100 rpm agitation.
11. Gel control.
  - a. Transfer the gel on a parafilm.
  - b. Take a picture with a ruler to appreciate the expansion coefficient (Figure 2B).
12. Gel trimming.
  - a. Use a scalpel to trim the gel at 1 mm around the embryo.
  - b. Take a picture with a ruler to evaluate the expansion coefficient (Figure 2C).
13. Immunostaining part 1.
  - a. Transfer the trimmed gel in 6 wells plate filled with 2 ml blocking buffer.
  - b. Incubate for 2 h at 23°C–25°C under 100 rpm agitation.
  - c. Replace blocking buffer by 2 mL of primary antibodies diluted in blocking buffer.
  - d. Fill empty wells with water to limit evaporation.
  - e. Seal the plate with parafilm.
  - f. Incubate for 72 h at 37°C.

**Note:** Working concentration must be adjusted for each antibody. We recommend to start using antibodies 2 times more concentrated than for a classical immunofluorescence experiment.



**Figure 2. Measurements to evaluate expansion factor and gel trimming**

(A) Embryo after fixation.

(B) Gel on day 8 after equilibration. At this stage the 12 mm initial gel was expanded by a factor 2.

(C) Gel on day 8 after trimming at 1 mm around the embryo. In this example the length of the trimmed rectangle is 12 mm.

(D) Gel on day 14 before imaging. The trimmed gel measured 24 mm and was thus expanded by a factor 2. The total expansion factor is calculated by adding the factors for the two expansion stages, giving a final value of 4X.

**Note:** For immunostaining up to 2 trimmed gels can be incubated in the same well. Negative controls to be included—especially when a new antibody is used with this protocol—are incubation with the secondary antibody alone and isotype controls (i.e. primary antibodies that lack specificity to the target, but match the class and type of the primary antibody used in the application).

### Day 11—Immunostaining part 2

⌚ **Timing:** 10 min manipulation, 48 h incubation

This step describes the second part of the immunolabeling process.

#### 14. Immunostaining part 2.

- a. Remove primary antibodies solution.
- b. Wash with 1 mL PBS-T 3 x 45 min at 23°C–25°C under 100 rpm agitation.
- c. Add 2 ml per well of secondary antibodies diluted in PBS1X-3%BSA.
- d. Incubate for 48 h at 37°C.

**Note:** We recommend to use antibodies 2X more concentrated than for conventional microscopy.

⏸ **Pause point:** Gels can be incubated into secondary antibodies up to 72 h.

### Day 13—Expansion

⌚ **Timing:** 20 min manipulation, overnight incubation

This step describes the final expansion of the gel.

15. Wash.
  - a. Remove secondary antibodies solution.
  - b. Wash with 1 ml PBS-T, 3 x 45 min at RT under 100 rpm agitation.
  - c. Wash with 1 ml PBS 10 min at 23°C–25°C under 100 rpm agitation.
16. Water expansion.
  - a. Transfer each gel in a 90 mm Petri dish filled with 20 ml ddH<sub>2</sub>O.
  - b. Incubate for 30 min at 23°C–25°C.
  - c. Replace ddH<sub>2</sub>O with 20 ml new ddH<sub>2</sub>O.
  - d. Incubate 15–20 h at 23°C–25°C.

**Note:** Gels are transparent at this stage, be careful not losing them while replacing water.

▣▣ **Pause point:** Gels can be kept in water at RT for an additional 24 h before observation.

### Day 13—Preparation of glass bottom dish for observation

⌚ **Timing:** 5 min manipulation, 2 h incubation

This step describes the preparation of glass bottom dishes that are used for observation of expanded gels.

17. Coating of glass bottom dishes with poly-D-lysine.
  - a. Add 1 mL of 100 µg/ml poly-D-Lysine on a glass bottom dish.
  - b. Incubate for 1 h at 37°C.
  - c. Remove the poly-D-lysine.
  - d. Wash 3x with 1 mL ddH<sub>2</sub>O.
  - e. Remove the ddH<sub>2</sub>O.
  - f. Air-dry for 1 h at 23°C–25°C.
  - g. Store at 4°C.

**Note:** Prepare one dish per gel. Prepare additional dishes that may be required if the gel cannot be positioned in the correct orientation under the microscope. Dishes must be prepared at least 24 h before observation and can be stored at 4°C for one month.

### Day 14—Microscope observation

⌚ **Timing:** 10 min preparation, 1–3 h observation

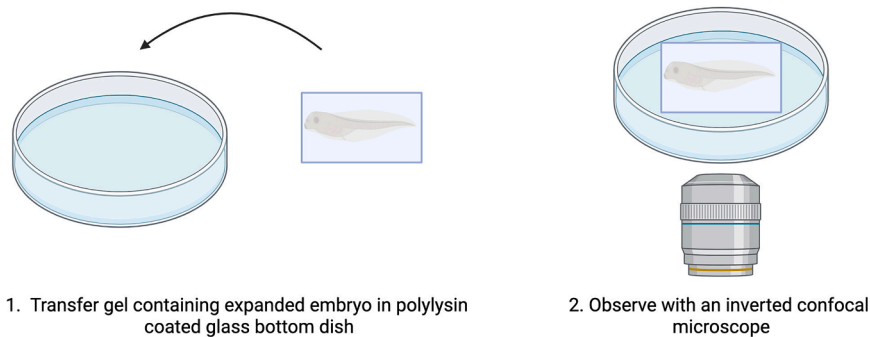
This step describes how to prepare expanded sample before observation at the microscope.

18. Gel control.
  - a. Transfer the gel on a parafilm.
  - b. Take a picture with a ruler to evaluate the expansion coefficient (Figure 2D).
19. Gel observation (Figure 3).
  - a. Transfer the expanded gel in poly-D-Lysine coated glass bottom dish.
  - b. Observe using an inverted confocal microscope.

**Note:** Sample must be observed within 2–3 h.

[Troubleshooting 3.](#)

[Troubleshooting 4.](#)

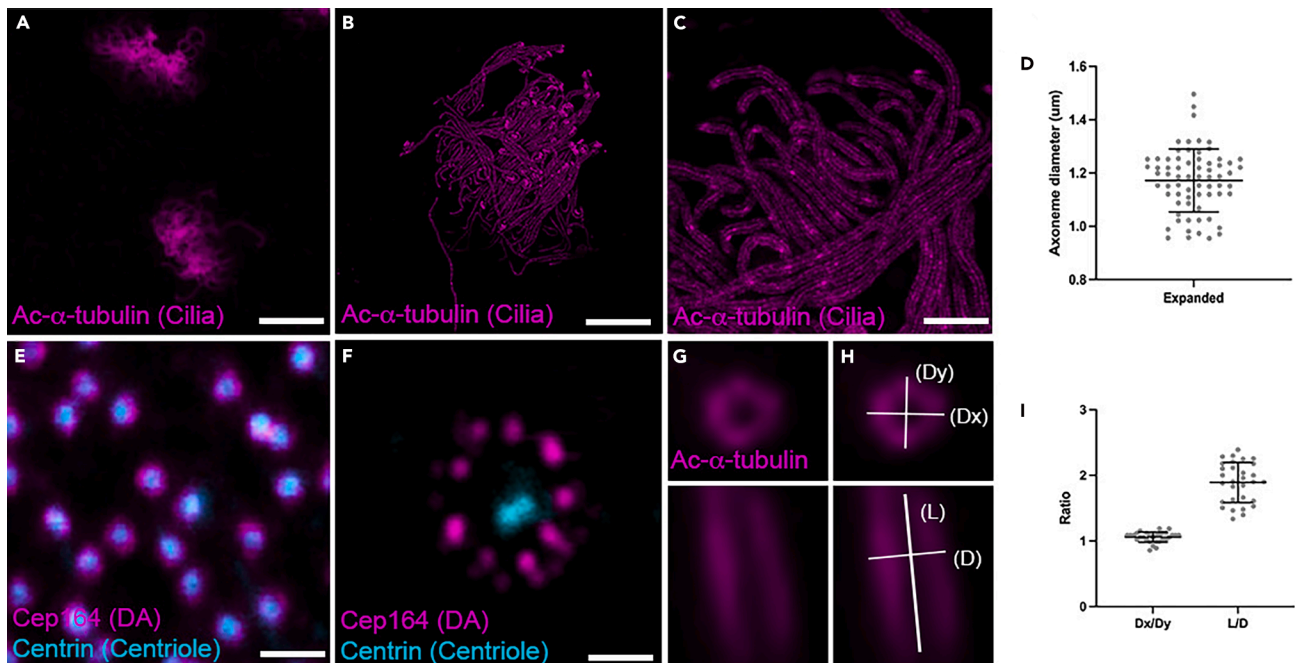


**Figure 3. Schematic representation of embryo mounting before observation**

### Troubleshooting 5.

## EXPECTED OUTCOMES

The expected outcome of this protocol is the capacity to visualize cellular structures in whole *Xenopus* embryos, that would be difficult or impossible to discern with conventional fluorescent confocal microscopy. At the end of the protocol, embryos should appear transparent and should have been expanded 4-fold. To illustrate the gain in resolution obtained with this method, we analyzed the multiciliated cells (MCCs) of the *Xenopus* epidermis that generate a flow of liquid at the surface of the embryo.<sup>5</sup> MCCs feature hundreds of cilia on their apical surface, each of them built from a modified centriole known as basal body. We used antibody against Acetylated- $\alpha$ -Tubulin, a major component of cilia. On non-expanded samples observed with a conventional confocal, this antibody reveals cilia as long continuous filaments at the surface of the MCCs, but their density is such that it is impossible to distinguish each individual cilium (Figure 4A). On expanded samples, individual intertwined cilia could be readily observed (Figure 4B). Observation at higher magnification even reveals longitudinal lines corresponding to axonemal microtubules making up the cilia (Figure 4C). Next, we analyzed the organization of basal bodies (BBs) at the base of cilia. In conventional microscopy, Cep164, a marker for distal appendages (DAs),<sup>6</sup> appears as a continuous circle around the Centrin signal, which stains the core of the BB (Figure 4E). In contrast, the TissUEXM protocol revealed nine dots corresponding to individual DAs (Figures 4F and 4G). Once the expansion factor is taken into account those dots appear at an average distance of 278 nm from the Centrin-positive BB core, which is consistent with its reported location on the outer part of the DAs.<sup>6</sup> We performed multiple measurements to evaluate the isotropy of expansion at the ultrastructural scale. First, we took advantage of the fact that cilia are oriented in multiple directions upon fixation, to measure and compare their diameters. We observed minimal variation of cilium diameter, suggesting that regardless of their orientation, cilia expand in a similar manner (Figure 4D). Next, we analyzed basal bodies labeled with Acetylated- $\alpha$ -Tubulin (Figure 4G). First, we calculated the ratio of the diameters measured along the X and Y axes in "front" views (Figure 4G, top). This ratio was around 1, as expected for isotropic X-Y expansion of a circular organelle (Figure 4I). We also calculated the length/diameter ratio from lateral views of basal bodies (Figure 4G, bottom). This ratio was 1.8 on average (Figure 4I), demonstrating isotropic expansion comparable to that of previously published protocols.<sup>1</sup> Finally, we calculated the ratio of basal body diameter to cilium diameter. We found a value of 1.06, very close to the 0.95 ratio observed in Transmission Electron microscopy,<sup>7</sup> demonstrating a correct preservation of relative sizes across the entire ciliary structure. Altogether, these results show that our TissUEXM protocol for *Xenopus laevis* embryos is highly effective in revealing nanoscale cellular structures. It should be easily transposable to *Xenopus tropicalis* and other amphibian embryos, promising to reveal cellular features with unprecedented precision in these organisms.



**Figure 4. Multiciliated cell nanoscale organization revealed by TissUExM on *Xenopus* embryos**

(A) Confocal field of view of *Xenopus* embryo epidermis stained for Acetylated- $\alpha$ -Tubulin.

(B) Expanded *Xenopus* embryo epidermis stained for Acetylated- $\alpha$ -Tubulin imaged with the same confocal parameters as in A.

(C) Zoom on expanded cilia stained with Acetylated- $\alpha$ -Tubulin.

(D) Quantification show minimal deviation in cilia diameters (Mean= 293.12 nm, SD= 29.51 (10%), n=68 cilia from two independent experiments).

(E) Confocal field of view of *Xenopus* embryo epidermis stained for distal appendages (DA, Cep164, green) and centrioles (Centrin, red).

(F) Expanded *Xenopus* embryo epidermis stained for distal appendages (DA, Cep164, green) and centrioles (Centrin, red) imaged with the same confocal parameters as in E.

(G) Top (top) and lateral (bottom) views of basal bodies from expanded *Xenopus* embryo epidermis stained with Acetylated- $\alpha$ -Tubulin.

(H) Same pictures as in G indicating where the diameter x (Dx), diameter y (Dy), diameter (D), and length (L) were measured.

(I) Dx/Dy and L/D ratio quantification. Average is 1.06 for Dx/Dy and 1.89 for L/D. Data are represented as mean  $\pm$  SD. (n=30 centrioles from two independent experiments).

Scale bar A, B: 10  $\mu$ m; C: 2.5  $\mu$ m, E, F, G, G': 1  $\mu$ m.

## LIMITATIONS

The protocol has been tested only on stage 25–30 *Xenopus* embryos. Optimization might be required when expanding earlier or later stage embryos. It should be noted that the shape of the embryo sometimes differs before and after expansion, suggesting that the conditions described here may result in heterogeneous expansion factors between tissues, probably due to variation in their physical properties. Collagenase treatment, such as that used on whole zebrafish embryos,<sup>2,3</sup> may be necessary to ensure homogeneous expansion in particular for late embryos.

## TROUBLESHOOTING

### Problem 1 (Day 5—Gelation of embryos)

Gelation is not completed after 2 h incubation at 38°C.

### Potential solution (Day 5—Gelation of embryos)

Incubate for longer time monitoring gelation every 30 min.

### Problem 2

When managing large series of embryos, gelation may be too fast to correctly position and orientate embryos in the gel.

### Potential solution

Use the 4OH-TEMPO option to slow down gelation.

### Problem 3 (Day 14—Microscope observation)

Impossible to visualize structures of interest under the microscope.

### Potential solution

Turn the gel over in a new observation dish, as the embryo may be too far from the objective. Another possibility is to work with an objective with a longer working distance.

### Problem 4 (Day 14—Microscope observation)

During acquisition at the microscope, the gel moves or shrinks.

### Potential solution

During mounting, it is necessary to remove as much PBS as possible so that the gel does not slide on the PDL-coated dish. If the gel tends to move during acquisition, remove it from the microscope and dry as much PBS as possible. However, for long observations, it may be necessary to re-humidify the gel with PBS to prevent shrinkage.

### Problem 5 (Day 14—Microscope observation)

Labeling of interest is not detected on expanded samples.

### Potential solution

TissUEXM physically enlarges the samples, with the result that antibodies are distributed over a greater distance, potentially preventing good confocal detection. One solution may be to increase the concentration of primary and secondary antibodies. In addition, different antibody references can be tested.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Camille Boutin ([camille.boutin@univ-amu.fr](mailto:camille.boutin@univ-amu.fr)).

### Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Virginie Thomé ([virginie.thome@univ-amu.fr](mailto:virginie.thome@univ-amu.fr)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

Data will be made available on request.

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## AUTHOR CONTRIBUTIONS

Conceptualization: L.K. and C.B.; investigation: S.D. and V.T.; formal analysis: S.D., V.T., and C.B.; writing: C.B.; editing: S.D., V.T., L.K., and C.B.; funding acquisition: L.K. and C.B.; supervision: C.B.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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