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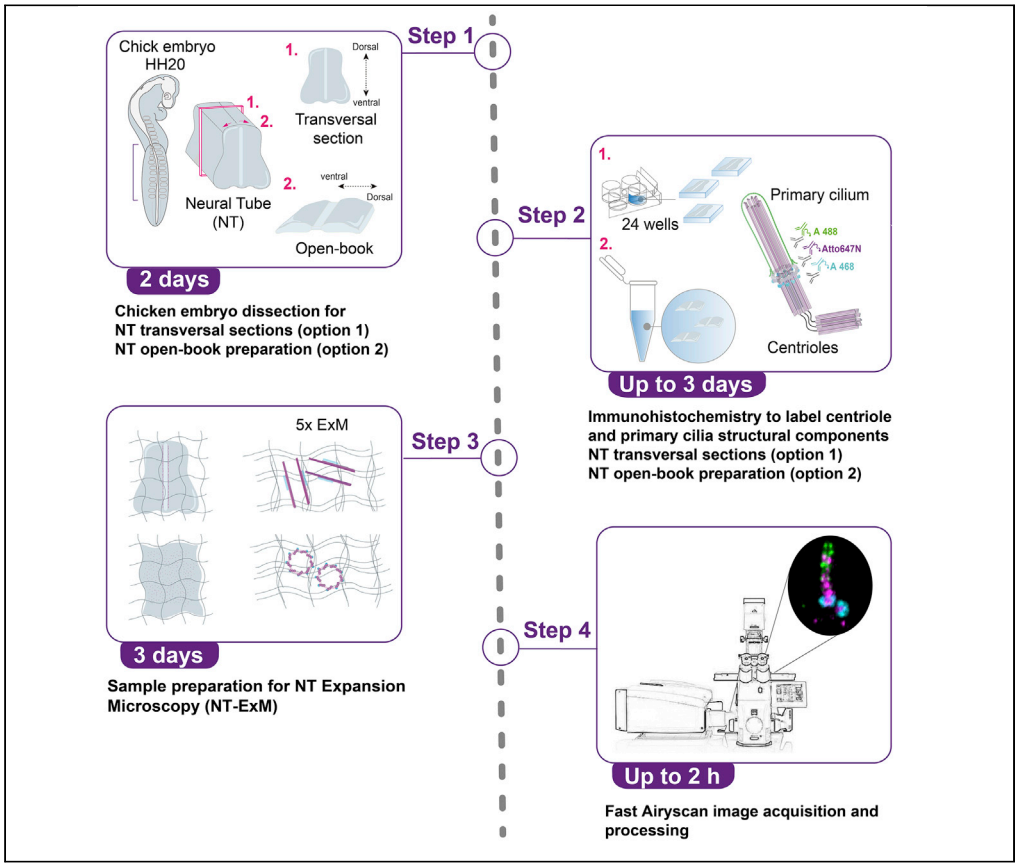
Expansion microscopy of the chick embryo neural tube to overcome molecular crowding at the centrosomes-cilia



We describe an optimized protocol for application of expansion microscopy (ExM) on chick neural tube (NT) which enables different oriented nanoscale resolution imaging of the centrosomes/cilia. We explain embryo NT transversal sections and open-book preparations, immunohistochemistry for labeling, and sample preparation for 5-fold tissue expansion. Further, we detail sample orientation and Fast Airyscan confocal acquisition and show that NT-ExM retains fluorescence signals and overcomes biomolecules crowding in structural features that to date were only imaged with electron microscopy on tissues.

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

Axelle Wilmerding, Paula Espana-Bonilla, Nikolaos-Nikiforos Giakoumakis, Murielle Saade

nikolaos.giakoumakis@irbbarcelona.org (N.-N.G.)
msabmc@ibmb.csic.es (M.S.)

Highlights

ExM optimized for chick neural tube (NT) slices and open-book preparation

NT-ExM allows nanoscale imaging of endogenous or electroporated cDNA encoding proteins

NT-ExM for quantitative lateral and axial nanoscale resolution of centriole composition

NT-ExM for nanoscale analyses of structural proteins during ciliogenesis

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Expansion microscopy of the chick embryo neural tube to overcome molecular crowding at the centrosomes-cilia

Axelle Wilmerding,[1,3] Paula Espana-Bonilla,[1] Nikolaos-Nikiforos Giakoumakis,[2,3,*] and Murielle Saade[1,4,*]

[1]Department of Cells and Tissues, Instituto de Biología Molecular de Barcelona, Consejo Superior de Investigaciones Científicas (IBMB-CSIC), Parc Científic de Barcelona, C/Baldiri i Reixac 20, 08028 Barcelona, Spain

[2]Advanced Digital Microscopy Facility Institute for Research in Biomedicine – IRB, Parc Científic de Barcelona, C/Baldiri Reixac 10, 08028 Barcelona, Spain

[3]Technical contact: awibmc@ibmb.csic.es; nikolaos.giakoumakis@irbbarcelona.org

[4]Lead contact

*Correspondence: nikolaos.giakoumakis@irbbarcelona.org (N.-N.G.), msabmc@ibmb.csic.es (M.S.)
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SUMMARY

We describe an optimized protocol for application of expansion microscopy (ExM) on chick neural tube (NT) which enables different oriented nanoscale resolution imaging of the centrosomes/cilia. We explain embryo NT transversal sections and open-book preparations, immunohistochemistry for labeling, and sample preparation for 5-fold tissue expansion. Further, we detail sample orientation and Fast Airyscan confocal acquisition and show that NT-ExM retains fluorescence signals and overcomes biomolecules crowding in structural features that to date were only imaged with electron microscopy on tissues.

BEFORE YOU BEGIN

The developing chick neural tube (NT) has served for decades to uncover the role of the centrosome/ciliary axis in normal and pathological neural development, and it is perfectly suited to be used as a fast, efficient and comparatively simple *in vivo* "test tube".[1–5] Early Neural Precursors Cells (NPCs) are easily accessible through *in ovo* electroporation for high resolution description at the organelle scale and functional assessment of cellular behaviors. Here we discuss super-resolution microscopy advances that propel deeper analyses of centrosome/cilia composition *in vivo* in the early developing chick embryo NT.

The presented protocol enables an axial and lateral visualization of centrosomes and primary cilia in transversal slices and open-book preparation of the embryo chick NT. Structural components of centrosomes and cilia are stained with immunofluorescence techniques or visualized with GFP- or RFP- tagged encoded proteins after cDNA electroporation.[1] For complete details on the use and execution of chick NT electroporation, transversal section, open-book preparation and immunohistochemistry, please refer to Saade et al.[1]

Further, all proteins within the sample are then covalently anchored to a swellable hydrogel. Through digestion with proteinase K, structural proteins are cleaved allowing isotropic swelling of the gel in the last step.[6] Immersion of the sample in water results in a 4–5-fold physical enlargement of the sample before imaging, rendering a potential resolution equivalent with a sub 50 nm image. Imaging was performed on an Elyra PS1 Zeiss LSM 880 confocal microscope equipped with an Airyscan Module. For the presented protocol the Fast Airyscan module with 16 detectors were used.

Thus, the resolution is further improved (1.5× according to the manufacturer) over a simple single-point scanning confocal microscope, by applying the established Fast AiryScan multichannel super-resolution imaging acquisition method.[7] We address key technical questions to successfully bridge the gap to the nanoscale spatial resolution of centrosomes and cilia structural components in the central nervous system.

Prior to initiating the protocol, eggs should be incubated to acquire the desired developmental stage of the NT and the features of electroporation and choices of fluorophores should be carefully considered as described below.

Institutional permissions

Although there is no need for ethical approval when working with chick in the first two thirds of embryonic developmental time, please consider revising the experimental procedures with the relevant Ethics Committee in each research institution.

Eggs incubation

Timing: 48–56 h

1. Incubate fertilized chick eggs in a stable horizontal position at 38.5°C in a humid incubator (~ 60% humidity).

   Note: Position the eggs horizontally allows moving the embryo inside the egg to an horizontal plane, avoiding its damage when cutting a window on the shell, later on.

2. Wait until embryos reached the desired Hamburger & Hamilton (HH) stage of development for appropriate experiments.[8]

   Note: We decided to work with experimental stages HH12-16 (48–56 h post fertilization -hpf-, 16–28 somites) for *in ovo* electroporation (Figures 1A and 1B).

cDNAs preparation

Timing: 15 min

3. Plasmid preparation should be endotoxin free to ensure high electroporation efficiency and embryo survival. For this, we used the Macherey-Nagel™ NucleoBond™ Xtra Midi EF.
   a. Prepare cDNAs encoding for proteins at very low concentrations to avoid unspecific and ectopic distribution of the encoded proteins.[1,2]

   Note: For this experiment we prepared CEP152-GFP (component of proximal end of centrioles) and Arl13b-GFP (component of the ciliary membrane) at 100 ng/mL and 20 ng/µL, respectively.

   b. Since centrosomes and cilia cDNAs are electroporated at very low concentration, we recommend adding to the mix cDNA encoding for a nuclear marker. This will help locating under a dissecting microscope the electroporated region for further manipulation (Figures 1A and 1B).

   Note: For this experiment, we prepared H2B-RFP or H2B-GFP at 500 ng/mL.

   c. Mix Fast Green die at 50 ng/mL with cDNAs preparation to enable visualization during the injection of the cDNA preparation into the lumen of the NT.

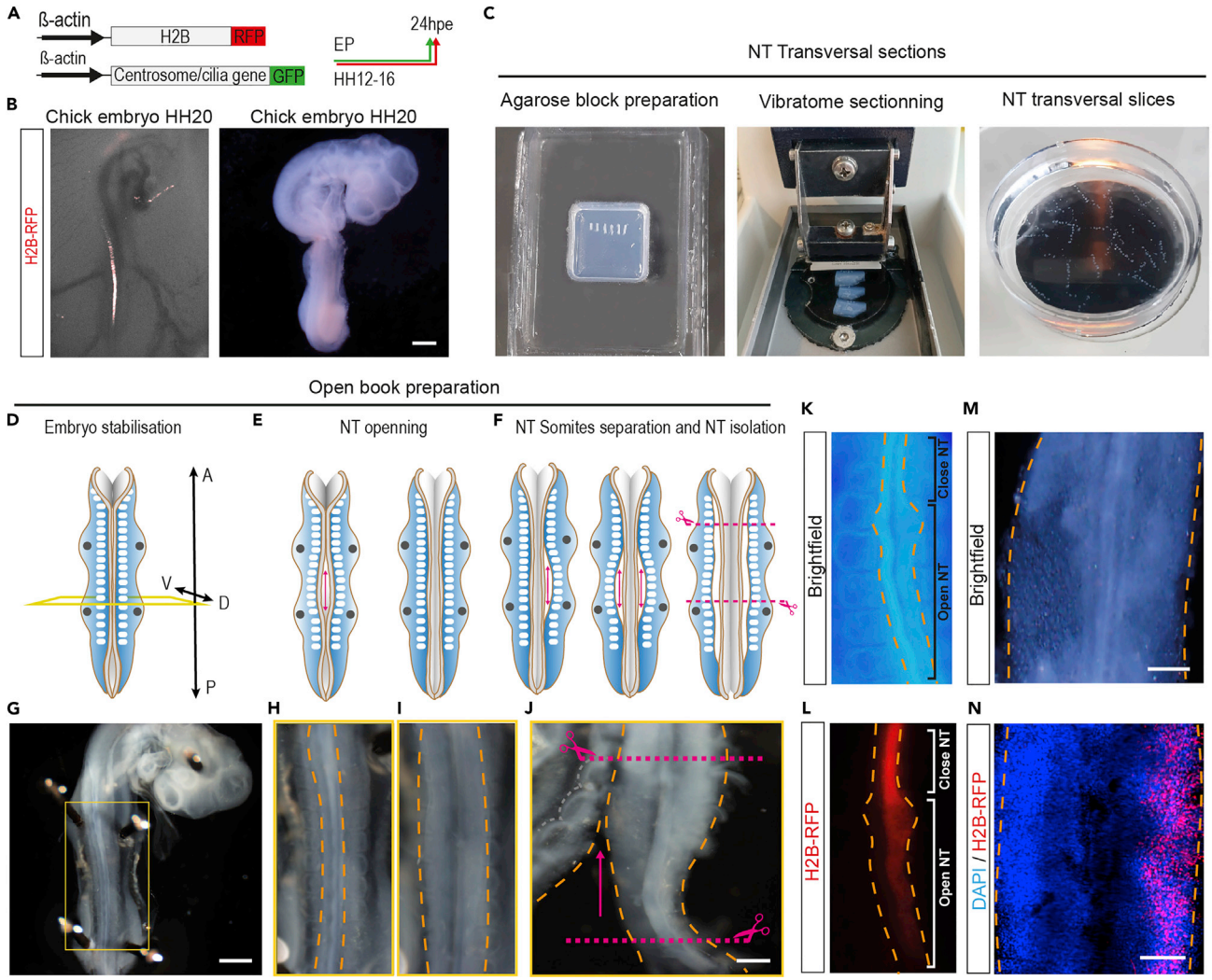

Figure 1. Transverse sections and open-book preparation of chick embryo electroporated NT

(A) Scheme showing the cDNAs co-electroporated (EP) at stages HH12-16. H2B-RFP is used as an electroporation marker in combination with centrosome/cilia genes fused to GFP.

(B) Dorsolateral view of a chick embryo 24 hpe (stage HH20) *in ovo* and after dissection in 1× PBS.

(C) Key steps for chick embryo NT free-floating transversal sections using a vibratome.

(D–J) Chick embryo NT open-book preparation. The key steps are represented as schematics (D–F) and as images (G–J) with the same embryo orientation highlighted in (D). (E) Pink arrows point the direction of dorsal NT opening. (F and J) Pink arrows point the direction of somites-NT separation. (H and I) Dashed line highlights the edge of the NT before and after NT opening.

(K and L) Dorsal view of a partially open electroporated NT in (K) brightfield and in (L) fluorescent light, with the same embryo orientation as in (D). The electroporated cells in L are expressing H2B-RFP. Dashed line highlights the edge of the NT.

(M and N) Electroporated open-book NT in (M) brightfield and in (N) fluorescent light, with the same embryo orientation as in (D). The electroporated cells in one side of the tube are expressing H2B-RFP and the nuclei are stained with DAPI (blue). Chick embryo in (D) is oriented as follows, A, Anterior; P, Posterior; D, Dorsal; V, Ventral. Scale bars, 1 mm (B and G), 0.5 mm (J), and 100 μm (M and N).

Chick embryo NT electroporation

Timing: 3 min per egg for electroporation

Timing: 16–24 h for incubation

4. Remove the eggs from the incubator and put them in a stable horizontal position.

5. Extract between 6–8 mL of albumin by inserting the 21G × 1″ needle in the smaller pole of the egg, in a 45° angle directed towards the bottom part and one of the sides, to avoid extracting the chalaza or any membrane surrounding the embryo.
6. Use the scissors to open a circular window in the upper part of the shell. Start from the small hole made previously to remove the albumin.
7. Inder a dissecting microscope, inject the cDNA-Fast Green mix into the NT lumen.
8. Add on top of the embryo 60 μL of 1× Penicillin/Streptomycin.
9. Place the electrodes on either side of the embryo at the level of the NT delivering five 50 ms pulses of 20–30V[9] using the Intracel Dual Pulse (TSS10) electroporator.
10. Tap the circulate window with parafilm and incubate again embryos at 38.5°C until reaching the desired stage (approximately 16 h–24 h).

Choice of fluorophores

Preservation of signal intensity in ExM is a challenge, due to the fluorophore decrowding that occurs in all 3 spatial dimensions during expansion (i.e., same signal spread across a greater area in the expanded gel) and to the potential damage/loss of fluorophores and dyes during the polymerization and digestion steps.[10] Most fluorescent dye-conjugated antibodies and β-barrel scaffolded fluorophores (i.e., GFP- and RFP-type) exhibit up to 50% signal retention in ExM.

11. For transiently expressed genetically-encoded fluorophore tags such as GFP or RFP, immunostain with a dye presenting similar spectral properties to boost the signal (Figures 3I and 3N–3P).

Note: We used primary antibodies paired with either Alexa Fluor 568- (data not shown) or AlexaFluor 488-(Figures 3I and 3N–3P) conjugated secondary antibodies to stain the encoded fluorescent proteins after cDNAelectroporation.

12. For endogenous proteins staining, use Alexa Fluor 488-, 568- and Atto647N-conjugated secondary antibodies since they have shown optimal fluorescent retention in NT-ExM (Figure 3).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse Centrin (1:500)	Sigma-Aldrich	20H5, cat#04-1624
Anti-rabbit FOP (1:500)	Dr. Olivier Rosnet (CRCM)	N/A
Anti-mouse Pericentrin (1:200)	Abcam	Cat#ab4448, RRID:AB_304461
Anti-Chick GFP (1:500)	AvesLabs	#GFP3717982
Anti-mouse Polyglutamylated-tubulin (1:500)	Enzo Life Sciences	Cat# AG-20B-0020, RRID: AB_2335608
Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat #: A-21206; RRID: AB_2535792
Alexa Fluor 488 donkey anti-mouse IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat #: A-21202; RRID: AB_141607
Alexa Fluor 555 donkey anti-rabbit IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat #: A-31572; RRID: AB_162543
Alexa Fluor 555 donkey anti-mouse IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat #: A-31570; RRID: AB_2536180
Alexa Fluor 568 donkey anti-mouse IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat #: A-11004; RRID: AB_2534072
Alexa Fluor Atto647N donkey anti-mouse IgG (H+L) (1:100)	Sigma-Aldrich	MFCD06798562
Recombinant DNA		
Arl13b-GFP (20 ng/μL)	Dr. Magdalena Götz (LMU)	N/A
Cep152-GFP (100 ng/μL)	OriGene	CAT#: RG211581
Chemicals, peptides, and recombinant proteins		
Penicillin and streptomycin	Sigma-Aldrich	Cat# P4333
Methanol	Supelco	MFCD00004595
DAPI	Sigma-Aldrich	Cat# D9542
Mowiol	Sigma-Aldrich	Cat# 81381
Sodium acrylate	Sigma-Aldrich	Cat# 408220

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium chloride	Sigma-Aldrich	S1679
N, N′-Methylenebisacrylamide	Sigma-Aldrich	Cat# M7279
Proteinase K, recombinant (fungal)	Invitrogen	Cat# 25530031
Acryloyl-X	Invitrogen	Cat# A20770
4-Hydroxy-TEMPO		Cat # 176141
TEMED ≥99% 50 mL	Santa Cruz Biotechnology. Inc.	Cat# sc-29111
Ammonium persulfate	Sigma-Aldrich	Cat # V800053
30% Acrylamide/Bis-Acrylamide Solution (37.5:1)	Laboratorios Conda, S.A.	Cat # A3626-0500
EDTA (0,5 M), pH 8,0	Thermo Fisher Scientific	Cat # R1021
Dimethyl sulfoxide	Sigma-Aldrich	D8148
Triton™ X-100	Sigma-Aldrich	X100
Hydrochloric acid	Sigma-Aldrich	258148
Trizma® base	Sigma-Aldrich	T1503
Calcium chloride	Sigma-Aldrich	C1016
Glycerol solution	Sigma-Aldrich	49781
Poly-L-lysine solution	Sigma-Aldrich	P8920
Polydimethylsiloxane (silicone) emulsion	Thermo Fisher Scientific	Cat #: 045088.A4
Experimental models: Organisms/strains		
white Leghorn chick embryos (Stages HH12-16)	Granja Gibert	N/A
Software and algorithms		
Fiji/Image J (2.0)	ImageJ; Schweizer et al.[11]	https://imagej.nih.gov/ij/ RRID: SCR_003070
Zen black (2.3) Zeiss	Zeiss	RRID: SCR_013672
Other		
NucleoBond™ Xtra Midi EF	Macherey-Nagel™	N/A
Parafilm® M Laboratory Sealing Film	SCT	sc-200311
Round coverslip dia. 13 mm #1	Epredia	10513234
Coverslip 24 × 60 mm #1.5	Epredia	15165452
Microscopy slides 26 × 76 mm	Knittel glass	N/A
Hypodermic needle 18G 1.2 × 50 mm	Sterican	4667123-02
20cc Luer Lock syringe w/o Needle	Terumo	SS-20L2
Insect pins 0.30 mm	F.S.T	26000-25
90 mm Petri dish	Sudelab	101200209
24 well plates	Cultek	88131020C

MATERIALS AND EQUIPMENT

- Anchoring Reagent Stock: Mix 5 mg of Acryloyl-X SE in 500 μL of Anhydrous DMSO for a final concentration of 35.4 nM.

Anchoring Reagent Stock should be stored at −20°C and can be kept for up to one year. We store it in 100 μL aliquots to avoid freeze-thawing cycles.

Monomer solution			
Reagent	Stock concentration	Final concentration	Amount
Sodium acrylate	380 g/l	86 g/l	2.25 mL
Acrylamide (30% solution)	292 g/l	25 g/l	0.857 mL
N,N′-Methylenebisacrylamide	20 g/l	1.5 g/l	0.75 mL
Sodium chloride (5 M)	292 g/l	117 g/l	4 mL
PBS pH 7.4	10×	1×	1 mL
ddH_2O	N/A	N/A	0.543 mL
10× Total	N/A	N/A	9.4 mL

The monomer solution can be divided into aliquots of 1 mL each and stored at −20°C for up to one year. Avoid exceeding freeze-thawing cycles.

- Sodium acrylate: dissolve 19 g in 50 mL of ddH2O to obtain a stock solution of 380 g/l.

The solution can be divided into aliquots of 5 mL and stored at −20°C for up to six months.

⚠ CRITICAL: Sodium acrylate in powder should be kept in a desiccated environment. Low-purity sodium acrylate may appear slightly yellow when dissolved in water which will lead to an incorrect polymerization of the gel. In this case, discard the solution and switch to a new batch or a new bottle of sodium acrylate.

- Acrylamide: The referenced product is already in solution at the desired stock concentration of 292 g/l.

Store at 4°C. We have used this reagent for one year without problems.

- N,N′-Methylenebisacrylamide: Dissolve 1 g in 50 mL of ddH2O to obtain a stock solution of 20 g/l.

The solution can be divided into aliquots of 5 mL and stored at −20°C for up to one year.

- Gelling solution.
  - 4-HT: dissolve 0.5 g in 100 mL of ddH2O.
  Store 0.5 mL–1 mL aliquots at −20°C. Freeze thaw cycles do not affect the solution. It is recommended to not store it for more than 6 months.
  - APS: 1 g of Ammonium persulphate was diluted in 10 mL of ddH2O.
  Store 50 µL aliquot at −20°C.

Digestion buffer

Reagent	Final concentration	Amount
Triton X-100	0.5% (v/v)	0.5 mL
EDTA, disodium (0.5 M, pH = 8)	1 mM	0.2 mL
TrisCl (1 M, pH = 8)	50 mM	5 mL
NaCl	800 mM	4.67 g
ddH_2O	N/A	up to 500 mL

Store the digestion buffer in 10 mL aliquots at −20°C for up to three months.

Proteinase K

Reagent	Final concentration	Amount
Proteinase K (40 U/mg)	8 U/µL	2 g
Tris HCL (pH = 7.5)	10 mM	1 mL
$CaCl_2$ (1 M)	20 mM	0.2 mL
Glycerol 87%	50% (v/v)	5,75 mL
ddH_2O	N/A	Up to 10 mL

The proteinase K solution can be divided into aliquots of 50–70 µL each and stored at −20°C.

⚠ CRITICAL: Methanol, DAPI and Triton X-100, Monomer solutions are hazardous: always wear proper protective equipment. Methanol waste needs to be collected and disposed of according to institutional regulations. Methanol, TEMED and acrylamide need to be handled inside a Chemical hood.

STEP-BY-STEP METHOD DETAILS

Chicken embryo dissection for NT transversal sections and open-book preparation

Timing: 2 days (for step 1)

Timing: 5 min per embryo for NT dissection (for step 1a)

Timing: 16 h for NT fixation (for step 1b)

Timing: 10 min per block for block preparation (for step 2a to 2g)

Timing: 10 min per block for block sectioning (for step 2h to 2j)

Timing: 15 min per embryo (for step 3)

This section describes the procedure for chick embryo dissection and fixation as well as transversal sections (option 1, Figure 1C) and open-book preparation (option 2, Figures 1D–1N). Transversal sectioning of the chick NT will lead to a visualization of the centrosomes forming the basal body of the protruding primary cilia localized at the NPCs apical endfeet lining the entire NT lumen.[1,12] Open-book NT will lead to an en-face view of the NPCs apical endfeet with a better visualization of centrosomes duplication in coordination with cell-cycle progression before the entry into mitosis.[1,13]

1. NT isolation from chick embryos and tissue fixation.
   a. Embryos dissection:
      i. Remove the eggs form the incubator.
      ii. With long and thin tip scissors, cut around the vascular area and remove the embryo from the egg using forceps or a small spoon.
      iii. Place it in 1× PBS in a Petri dish under a dissecting microscope.
      iv. Remove the extraembryonic membranes by gently scraping using fine forceps.
   b. Whole embryos can be fixed here, by replacing the 1× PBS with ice cold methanol and incubating for 16 h at −20°C.

⚠ CRITICAL: Methanol is highly toxic. Make sure to work under the hood and use appropriate personal protective equipment, particularly nitrile gloves, safety goggles and lab coat. Gloves are changed as soon as they are contaminated.

   c. After 16 h incubation, carefully pour off cold methanol and replace it with 1× PBS.

Pause point: Embryos can be stored at 4°C up to one week after fixation.

2. NT transversal sections (Option 1).

Note: All the following steps should be performed under a dissecting microscope.

   a. Prepare the agarose/saccharose solution (0.5% low-melt agarose 1% saccharose in H_2O) and keep it molten in a warm bath for block preparation.
   b. Place the embryos in 1× PBS in a Petri dish and remove the head and the heart by gently scraping with fine forceps.
   c. With fine forceps, cut through the NT at the level of the wing bud caudal to the limb bud and transfer the embryo trunk into the plastic sectioning mold.

Note: In case of electroporation, use fine forceps under a fluorescent dissecting microscope to isolate the electroporated part between the wing and the limb bud.

   d. Place molten agarose/saccharose solution into plastic sectioning mold.

Note: Avoid embedding the tissue in overheated agarose /saccharose solution. This could damage tissue integrity, denaturate endogenous proteins and thus affect the efficiency of the immunostaining.

Note: The agarose/saccharose solution remains fluid at 37°C and will set rapidly at temperatures below 25°C.

e. Use fine forceps to lift and orient all NTs in parallel and flat on their ventral side in the agarose (Figure 1C, left panel).
f. Once the agarose is hardened, use a flat surface tool, such as a spatula, to remove the agarose block from the mold.
g. Carve with a razor blade the block to remove the excess of agarose and orient it to obtain NTs in the desired transversal section plane.
h. Add a drop of super glue to the vibratome stage, place the agarose block on it and fill the vibratome stage with water (Figure 1C, middle panel).
i. Set the parameters of the vibratome to: speed-6; frequency: 65 Hz (if applicable) thickness-50 μm and section.
j. With a thin paint brush, collect sections carefully trying to not break tissue slices and place into a 1× PBS-filled 24 multiwell plate (Figure 1C, right panel).

Pause point: If necessary, tissue slices can be stored in the dark at 4°C in 1× PBS for up to one week until ready for immunostaining.

3. NT open-book preparation (option 2).
   a. Embryo stabilization:
      i. Remove the head, the heart and limbs using spring scissors and gently scraping with fine forceps.

Note: The somites and NT should be visible if all surrounding tissues have been removed completely.

      ii. Lay the embryo on its ventral side.
      iii. Stabilize it on a petri dish coated with PDMS by pinning at the level of the wing and the limb buds, from one side and another of the NT (using 0.20 mm insect pins), with gentle stretching (Figures 1D and 1G).

Note: Make sure that the pins are inserted at an angle away from the embryo so that they do not interfere with the dissection.

   b. NT opening and dissection:
      i. Use a tungsten needle to gently scrap and open the dorsal part of the NT. Gently scrap longitudinally by making caudo-rostral movement along the dorsal NT (Figures 1E, 1H, 1I, 1K, and 1L).
      ii. Use a tungsten needle to separate the somites from the NT. Gently scrap longitudinally along this line on each side of the NT (Figures 1F and 1J).

Note: Look for a dark line between the NT and the somites to be able to separate them. Adjust the illumination angle, if necessary.

Note: The somites should separate from the NT due to the gentle stretching of the pinned embryo.

      iii. Cut the NT at the level of the wing bud and caudal to the limb bud (Figures 1F and 1J).

Table 1. Tested antibodies to visualize centrosome and ciliary structures

Primary antibody	Dilution	Localization
FOP/CEP43	1:500	Distal end of the 2 centrioles
Centrin 1	1:500	Distal lumen of the 2 centrioles
Pericentrin	1:200	Pericentriolar material
Poly E	1:500	Centriole microtubule and ciliary axoneme
GFP	1:500	CEP152-GFP centriole proximal end and Arl13b-GFP ciliary membrane

iv. Lift the whole NT out of the embryo in one smooth rostral to caudal motion, using forceps.

c. With a glass pipette, transfer the open-book NT to a 200 µL PCR tube containing 1× PBS.

Note: In case of electroporation, under a fluorescent dissecting microscope, make sure to select the H2B-RFP electroporated region (Figures 1K–1N).

Pause point: If necessary, open-book NT can be stored in the dark at 4°C in 1× PBS for up to one week until ready for immunostaining.

Immunohistochemistry to label centriole and primary cilia structural components

Timing: 40 min for permeabilization and saturation (for step 4a to 4b)

Timing: 16 h for primary antibody incubation (for step 4c)

Timing: 2 h for secondary antibody incubation (for step 4e)

Timing: 18 h for permeabilization and saturation (for step 5a to 5b)

Timing: 16 h for primary antibody incubation (for step 5c)

Timing: 16 h for secondary antibody incubation (for step 5e)

In this section we describe standard immunocytochemistry workflow for labeling structural proteins at the two centrioles that form one centrosome and at primary cilia. Immunohistochemistry has been performed in both, fixed NT transversal sections (option 1) and open-book preparation (option 2).

4. Immunochemistry on NT transversal sections (option 1).

Note: All the following steps are performed in a 24 multiwell plate in the dark, with a buffer volume of 300 µL.

a. Incubate tissue slices in permeabilization buffer (0.1% Triton X-100 in 1× PBS) for 10 min on an orbital shaker at low speed at 20°C.
b. Incubate tissue slices in the saturation buffer (1% BSA/0.1% Triton X-100 in 1× PBS) for 30 min on an orbital shaker at 20°C.
c. Incubate for 16 h on an orbital shaker at 4°C tissue slices with primary antibodies diluted in the blocking solution (1% BSA/0.1% Triton X-100 in 1× PBS [Table 1]).

Alternatives: Incubation with primary antibody could remain over weekend on an orbital shaker at 4°C in the dark.

d. The next day, wash three times in the permeabilization buffer (0.1% Triton X-100 in 1× PBS) for 15 min each wash.

e. Incubate the tissue slices with the secondary antibodies diluted at 1:500 in the blocking solution (1% BSA/0.1% Triton X-100 in 1× PBS) for 2 h on an orbital shaker at 20°C.
f. Wash three times with the permeabilization buffer (0.1% Triton X-100 in 1× PBS) for 15 min each wash.

Pause point: If necessary, tissue slices can be stored in the dark at 4°C in 1× PBS for up to two weeks before the next steps of the protocol.

5. Immunochemistry on NT open-book preparation (option 2).

Note: NT should be kept in the dark and requires a minimum volume of 250 µL solution for each step.

a. Incubate open-book NT in permeabilization buffer (0.5% Triton X-100 in 1× PBS) for 2 h on a rotary shaker at 20°C.

Note: As open-book preparation is 4 time thicker than a transversal section, it is required to boost permeablization by increasing the percentage of Triton X-100 which in turn will allow a better entry of the antibodies through the cell membrane deep within the tissue.

b. Incubate open-book NT in saturation buffer (1% BSA/0.5% Triton X-100 in 1× PBS) for 16 h on an orbital shaker at 4°C.
c. The next day, incubate open-book NT with primary antibodies diluted at the working concentrations in the blocking solution (1% BSA/0.5% Triton X-100 in 1× PBS, Table 1) and incubate them 16 h, on a rotary shaker, at 4°C.

Note: Incubation with primary antibodies could last to up to one week.

d. The following day, perform three 15 min washes in the permeabilization buffer (0.5% Triton X-100 in 1× PBS).
e. Incubate open-book NT with the secondary antibodies in the blocking solution (1% BSA/0.5% Triton X-100 in 1× PBS) and incubate them 16 h on an orbital shaker, at 4°C.
f. After the 16 h incubation, wash three times with the permeabilization buffer (0.5% Triton X-100 in 1× PBS), 15 min each wash.

Pause point: If necessary, open-book preparation can be stored in the dark at 4°C in 1× PBS for up to two weeks before the next steps of the protocol.

Sample preparation for NT expansion microscopy (NT-ExM)

Timing: 3 days

Timing: 20 min setup, 16 h incubation (for step 6)

Timing: 30 min setup, 2–2,5 h incubation (for step 7)

Timing: 10 min setup, 4–4,5 h incubation (for step 8)

Timing: 10 min setup, 1 h incubation (for step 9)

To analyze centrosomes/cilia protein distribution in tissue at the super-resolution level, we took advantage of ExM, a recently developed technique that improves spatial resolution by physically

increasing sample size.[14] The tissue is embedded in a polymer-based gel where polymer is cross-linked with the proteins. The tissue is then digested, leaving only the anchored proteins within the gel, which is then expanded by osmosis in multiple water baths. Several variants of the initial protocol have been published, with optimizations for analyzing centrosomes and cilia structures in cell culture.[11,15–17] Recently, ExM has started to be used to visualize centrosomes/cilia structural proteins in various tissues such as sectioned mouse olfactory epithelium and mouse retina.[18,19] The present protocol is adapted from the Basic Protocol 2 from Asano and colleagues,[6] with practical twists optimized for NT transversal sections and open-book preparation (summary timetable in Figure 2A).

6. Chamber preparation and molecular anchoring.
   a. Prepare the gelation chamber by using a suitable large container with a lid. For up to four 13 mm coverslips, a 100 mm petri dish is adequate (Figure 2D). Cover it externally with aluminum foil and the internal bottom with parafilm.
   b. Add on top of the parafilm a round coverslip of 13 mm diameter (Figure 2D).
   c. Add 60 μL of 1× PBS on the coverslip.
   d. Maintain humidity by applying along the circular periphery of the chamber tissue paper soaked in sterile water (Figures 2D and 2E).
   e. Transfer NT open-book or slices on a petri dish.
   f. Isolate tissue slices by using tungsten needles to gently separate the tissue from the surrounding gel, under the dissection microscope (Figure 2B).
   g. For open-book NT expansion, use fine forceps to gently isolate the H2B-RFP electroporated side under a fluorescent dissecting microscope.
   h. Use spoon micro-spatula or handmade spoon glass micropipette to transfer the tissue to the 60 μL of 1× PBS on top of the coverslip in the gelation chamber (Figure 2C).

   Note: Before dissection, it is important to soak the tungsten needles and spoons in horse serum to avoid tissue slices sticking and breaking.

   i. Prepare the anchoring solution by diluting the anchoring Reagent Stock (10 mg/mL Acryloyl-X in anhydrous DMSO) to 0.1 mg/mL in 1× PBS pH7.4.
   j. After tissue transfer to coverslip, remove the 1× PBS with a 10 μL pipette and add 60 μL of anchoring solution, ensuring that the entire surface coverslip is covered.
   k. Place the lid on the humidified chamber and leave the tissue in the AcX solution 16 h in the dark at 20°C.

   Note: Make sure that the tissue is soaked in the Acryloyl-X and localized close to the coverslip.

   ⚠ CRITICAL: Exceeding tissue number could affect gel expansion. Up to 2 electroporated sides of open-book NT cut in 2–3 pieces or 6 transversal tissue slices (50 μm) could be loaded on the same coverslip for gelation.

   ⚠ CRITICAL: The size of the gelation chamber and the amount of anchoring and gelling solution should be scaled up or down according to the size of the open-book or tissue slice and should be at least 100-fold excess in volume.

7. Sample gelation.
   a. Start by thawing the components for the gelling solution (Monomer solution, 4HT, Ammonium Persulfate -APS and TEMED) on ice.
   b. Meanwhile, remove the Acryloyl-X from the coverslip and wash the tissues with 60 μL of 1× PBS twice, each time for 15 min.

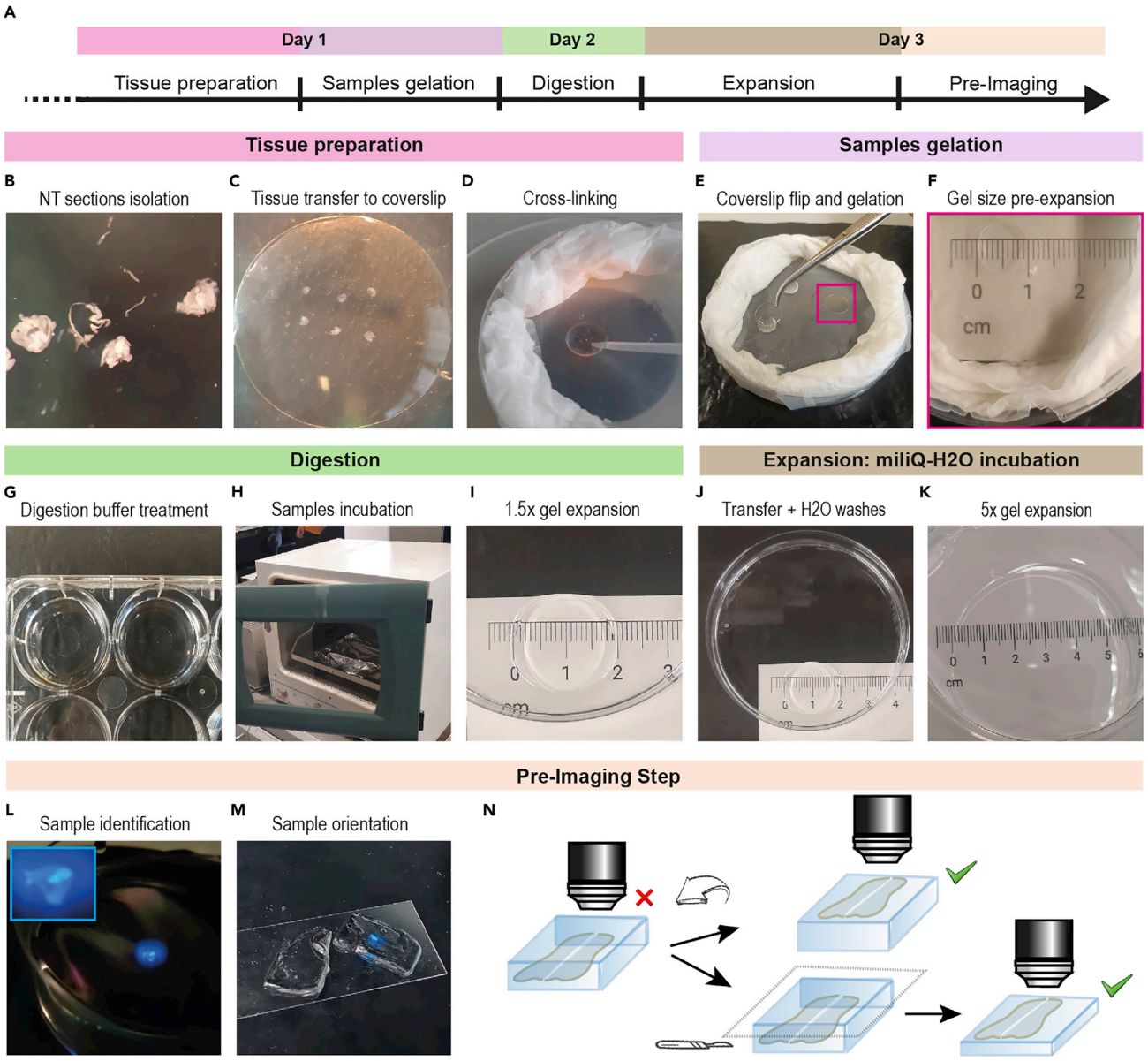

Figure 2. Sample preparation for NT-ExM

(A) Timeline of NT-ExM.

(B–D) Key steps of tissue preparation for NT transversal sections.

(E) Gelation of the sample.

(F) Size of the polymerized gel before expansion.

(G–I) Proteinase K digestion results in a 1.5× expansion of the gel.

(J and K) MilliQ water incubation results in the final 5× gel expansion.

(L) Prior to imaging, expanded NT (stained with DAPI) is found and oriented using UV light.

(M) Cut gel containing the NT and placed onto a polylysine-coated coverslip.

(N) Illustration shows incorrect (cross) and correct (tick) orientation of the gel for fast airyscan acquisition. In case your region of interest is still located out of working distance, the gel should be cut horizontally and carefully with a scalpel.

c. During the last wash, use a tungsten needle to gently reposition tissues in the center and close to the coverslip, reorient open-book NT apically facing the coverslip and drain the excess of 1× PBS.

d. Prepare 60 μL of gelling buffer respecting the following order: First, add in the tube the monomer solution, 4HT, TEMED and lastly add the APS in a 47:1:1:1 ratio and vortex for a few seconds. Prepare the gelling solution immediately before use as it solidifies fast (troubleshooting problem 1).

⚠ CRITICAL: To prevent premature polymerization of the gelling solution, TEMED and APS should be added to the solution last before vortexing.

⚠ CRITICAL: The mixed gelling solution should be kept always on ice to prevent premature gelation.

e. Remove the 1× PBS from the coverslip and place the 60 μL drop of gelation buffer on the parafilm and carefully flip over the coverslip where the tissues are attached (Figure 2E).
f. Incubate tissues for 2–2.5 h at 37°C.

⚠ CRITICAL: Be careful not to create bubbles or air-pockets inside the gelling solution drop.

8. Digestion.
   a. Prepare the digestion buffer with a final concentration of Proteinase K at 8 U/mL.

⚠ CRITICAL: The volume of the digestion buffer should be adjusted according to the thickness of the gel, the size of the coverslip and the size of the container (troubleshooting, problem 5).

⚠ CRITICAL: We recommend preparing enough digestion buffer to have at least a 10-fold excess in volume compared with that of the gel.

   b. Take out the gelation chamber from the incubator. The gel has polymerized underneath the coverslip (Figure 2F).
   c. Carefully insert flat tweezers from the side between the parafilm and the gels and gently flip the gel that remain attached to the coverslip.
   d. Transfer the gel and the remain attached coverslip to a 35 mm diameter well plate to proceed with the digestion step (Figure 2G).

⚠ CRITICAL: Since the gel will expand to about 1.5× during digestion, it is important to choose a suitably large container, such as a 35 mm diameter well plate.

   e. Add 3 mL of digestion buffer for 60–70 μL gel attached to a 13 mm diameter coverslip placed inside a 35 mm diameter well plate.
   f. Leave the gel immersed in the digestion buffer for 4–4.5 h at 37°C in the dark (Figure 2H).
   g. After digestion, the gel is detached from the coverslip and expanded 1.5× (Figure 2I).
   h. After digestion, wash three times the gel with 1× PBS, 10 min each to remove the excess of proteinase K (troubleshooting, problem 5).

Note: Changing the incident angle of light might help to locate it inside the solution.

   i. Add to the gel DAPI at 1:1000 dilution in 1× PBS for 16 h at 4°C.

Note: Since samples become transparent during expansion, DNA staining facilitates the localization of the tissue after expansion (Figures 2L and 2M).

Pause point: if later expansion is desired, remove the digestion buffer, add enough 1× PBS to avoid dehydration, and store the gel at 4°C 16 h up to one week in the dark.

9. Expansion.
   a. Transfer the gel to a 100 mm petri dish by inversion (Figure 2J).
   b. To start the expansion step, remove the 1× PBS with a 15 mL pipette and immerse the gel in milliQ-H_2O for 20 min at 20°C.
   c. Repeat the milliQ-H_2O washes 2 more times. The sample should be fully expanded and optically cleared prior to imaging (Figure 2K).

Note: The speed and extent of the expansion depends on the dish volume, wash time, number, and volume.

Note: More washes and/or longer incubation with a higher volume of water in a larger dish will result in greater expansion. We found that optimal expansion was reached after 3 × 20 min washes in petri dishes.

⚠ CRITICAL: If the gel is not fully expanded, continue washing the gel with milliQ-H_2O at 20°C for several rounds of 20 min, until no further expansion is observed.

Note: Expansion can be assessed by measuring sample size before digestion and after every bath (Figures 2I and 2K). In our hands, we routinely reached a 4–5× expansion.

Fast Airyscan image acquisition and processing

Timing: Up to 2 h per expanded tissue

Timing: 10 min setup (for step 10)

Timing: 30 min setup, 1 h acquisition (for step 11)

Super resolution Fast Airyscan imaging acquisition was performed on fully expanded tissues using an inverted microscope. Fast Airyscan improves the resolution imaging by a factor of 1.5× and a signal to noise ration by 4× compared to standard confocal imaging.[20] Alternatively, any ISM capable microscope can produce fast images with high signal to noise ratio.[21] In this section, we describe how we prepared and imaged the expanded samples using this technique.

10. Sample preparation for imaging.
    a. Under a dissecting microscope locate the tissue inside the gel with the use of UV light (Figure 2L).
    b. Use a sharp scalpel blade to cut and select the gel containing the tissue (Figures 2M and 2N).

Note: Make sure to cut a reasonable size in order to easily handle it. It is important to consider the shrinkage of the gel when it will be stored again back in 1× PBS.

    c. Place the gel directly onto a polylysine-coated coverslip and proceed by removing carefully the excess of water with a paper tissue.
    d. Transfer the gel onto the microscope stage.

Note: Alternatively, you can place the gel within a glass bottom dish coated with polylysine. This can decrease the gel drift however it is much more challenging to flip the gel in case the tissue is out of working distance for higher magnification lenses (troubleshooting, problem 3).

Note: To avoid breaking the thin cover glass, you could use a common metal coverslip stage adaptor.

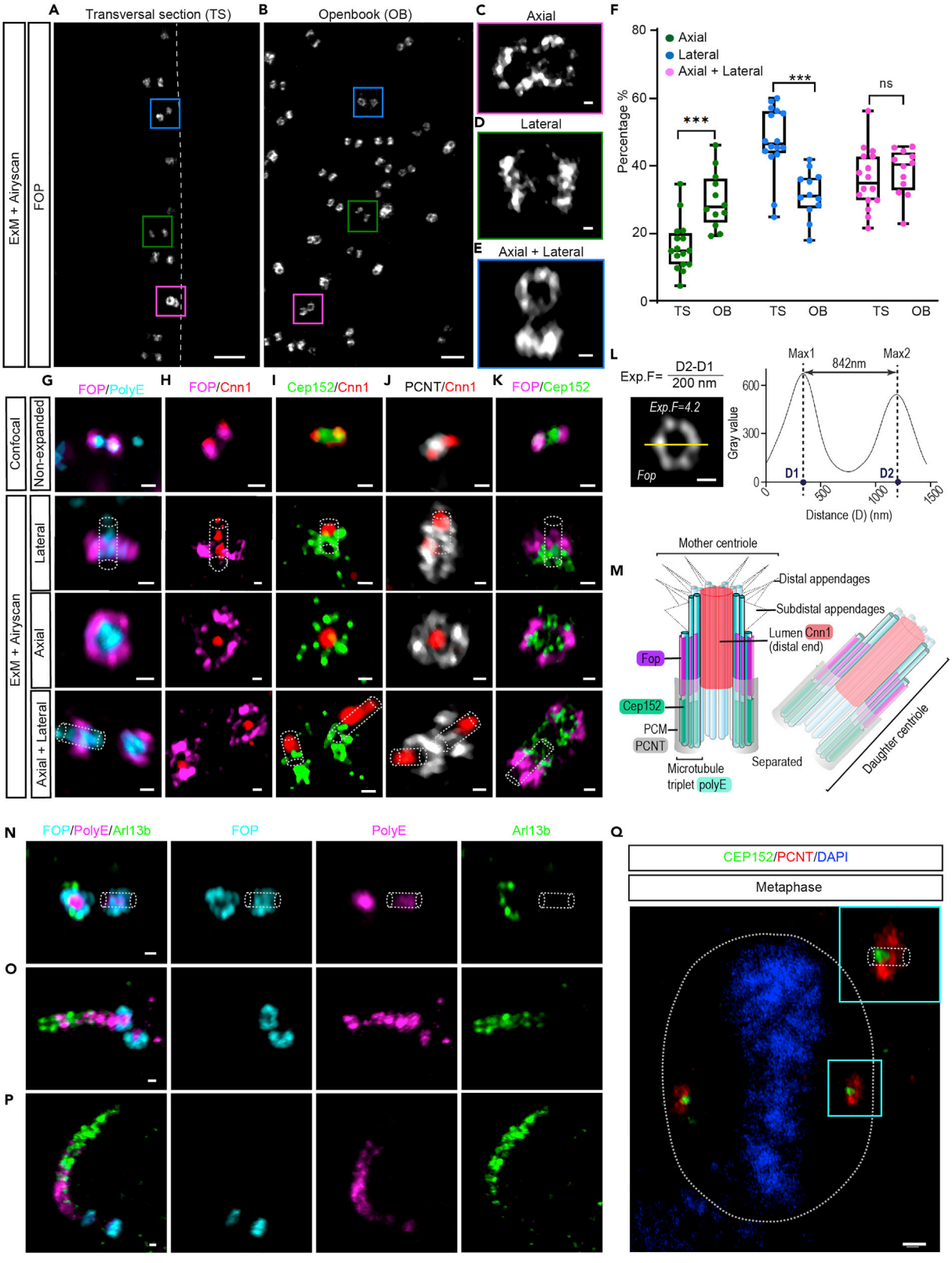
A
Transversal section (TS)
B
Openbook (OB)
C
Axial
D
Lateral
E
Axial + Lateral
ExM + Airyscan
FOP
F
Axial
Lateral
Axial + Lateral
Percentage %
***
***
ns
TS
OB
TS
OB
TS
OB
G
FOP/PolyE
H
FOP/Cnn1
I
Cep152/Cnn1
J
PCNT/Cnn1
K
FOP/Cep152
Confocal
Non-expanded
ExM + Airyscan
Lateral
Axial
Axial + Lateral
L
Exp.F= D2-D1 / 200 nm
Exp.F=4.2
Fop
Max1
842nm
Max2
Gray value
D1
D2
Distance (D) (nm)
M
Mother centriole
Distal appendages
Subdistal appendages
Lumen Cnn1
(distal end)
Fop
Cep152
PCM
PCNT
Microtubule
triplet polyE
Separated
Daughter centriole
N
FOP/PolyE/Arl13b
FOP
PolyE
Arl13b
O
P
Q
CEP152/PCNT/DAPI
Metaphase

Figure 3. ExM+LSM880 Fast Airyscan images of centrosome/ciliary components in the chick NT

(A–F) Comparison of centrioles pairs orientation located at the apical side of the NT in (A) transversal section, and (B) open-book preparation using the distal end centriolar marker FOP. The color square boxes highlight the 3 types of centrioles pairs' orientation that are magnified in (C–E): (C) pink, axial view; (D) green, lateral view; (E) blue, axial and lateral view. (F) Quantification of the percentage of the 3 types of orientation in transversal sections (TS n=16 images) vs open-book (OB n=12 images) NTs. The upper and lower lines indicate the interquartile range and the middle line indicates the median. Unpaired two-tailed *t-test*, ***p < 0.001; ns, not significant.

(G–K) Images of centrioles structural proteins in non-expanded NT acquired with an LSM780 vs expanded samples acquired with an LSM880 Fast Airyscan with centriole pairs oriented in lateral, axial, or axial and lateral views.

(L) Expansion factor (Exp.F) formula. Axially oriented centrosome stained with anti-FOP was selected for Exp. F. measurement. The plot profiling across the yellow line allows us to calculate the width of the expanded centriole, and divide it by 200 nm to calculate the Exp. F.

(M) Scheme presenting the localization of the different structural proteins at the centrioles imaged in (G–K).

(N–P) Images of cilia/centrosome structural proteins at different stages of ciliogenesis according to cell cycle progression in expanded NT transversal sections acquired with Fast Airyscan.

(Q) Image of lateral oriented centrioles (inset) in mitotic NPC after NT expansion and acquisition with the LSM880 Fast Airyscan. Scale bars 5 μm (A, B), 0.5 μm (C-E, G-L, N-P), 2 μm (Q).

11. Images acquisition.
    a. First locate and image the whole NT using a 10× dry lens with a 0.3 NA. With this lens the electroporated side of the NT is detected.

Note: Be sure to be within the working distance as you move to higher magnification lenses (troubleshooting, problem 2, problem 3).

    b. To identify centrosomes and cilia within the cells, switch to a 63× oil-immersion lens with a 1.4 NA and a 190 μm working distance (Plan-Apochromat 63×/1.4 Oil DIC M27) or a 100× oil- immersion lens with a 1.46 NA and a 110 μm working distance for superresolution imaging.
    c. Determine a rectangular cropped region of interest to speed up the imaging at least 1.5× zoom with optimal XY acquisition settings.
    d. Acquire images with a 40 nm pixel size (Superresolution mode of FastAiryscan).
    e. Aquire Z-slices with a step size of 0.5 μm and with the piezoelectric stage of the microscope to avoid drift during acquisition (troubleshooting, problem 4).
    f. Take the images with a frame by frame acquisition. In the current experiment, up to 4 channels (405 nm, 488 nm, 561 nm and 633 nm) were acquired with the proper filter settings, for each fluorophore (troubleshooting, problem 5).
    g. Reconstruct Raw Fast Airyscan data automatically with the Zen black software.

Note: Once the gel is on the coverslip, imaging should be performed as soon as possible. The heat from the laser will cause the gel to begin to deposit water and shrink. In our hands, this tended to happen in 1 h.

12. Use ImageJ to calculate the distances between local maxima from the intensity plot profiles on the expanded sample images (Figure 3L).

EXPECTED OUTCOMES

We present an adaptation of expansion microscopy approach, specifically developed for a robust and routine analysis of centrioles and cilia in tissues. Our protocol can be used for the analysis of centriole number, duplication status, length, localization of various structural components of centrosomes and cilia during cell-cycle progression of NPCs in tissues. We validate our approach, by comparing centriolar and ciliary features obtained by expansion of 50 μm transversal sections and 200 μm open-book NTs (Figure 3). We explored whether changing the orientation of the tissue may change the orientation of the centrioles (Figures 3A–3F). Indeed, a higher percentage of lateral oriented pair centrioles are observed in NT transversal sections while a higher percentage of axial oriented pair centrioles are observed in open-book NT. We tested several antibodies commonly used to detect centrosomes and primary cilia structural components, for their retention after expansion (Figure 3). We found that FOP is suitable for detection of the distal end of the pair centrioles

(Figures 3A–3E, 3G, 3H, 3K, 3L, 3M–3P), Poly E for the detection of the centriole microtubule (Figures 3G and 3M) and ciliary axoneme (Figures 3N–3P), Cnn1 for the distal lumen of the pair centrioles (Figures 3H–3J and 3M), and PCNT for pericentriolar material (Figures 3J, 3M, and 3Q). NT-ExM results in a decrease in signal strength as the fluorophores are stretched over a larger area and possibly lost due to anchoring and digestion; we recommend to always use fresh primary and secondary antibodies. Alternatively, we tested several cDNAs commonly used to detect centrosomal and ciliary structural components (Figures 3I, 3K, and 3M–3P). We found that CEP152-GFP is suitable for detection of the proximal end of the pair centrioles (Figures 3I, 3K, 3M, and 3Q) and Arl13b-GFP is suitable for detection of the ciliary membrane (Figures 3N–3P). We recommend to not exceeding 200 ng/μL of cDNA concentration to avoid structural abnormalities or ectopic distribution. In addition, using antibodies against the encoding fluorescent proteins such as GFP and RFP is needed to increase the signal and maintain their retention after expansion. To study ciliogenesis, we recommend ExM on NT transversal sections (Figures 3N–3P). To analyze centrioles at spindle poles, both tissue preparations should be suitable (Figure 3Q). The modified protocol of Tillberg et al.,[10] routinely provided us with a successful expansion factor ranging between 4–5 × for both open-book NT preparations and transversal sections. Fast airyscan further improves the resolution by a factor of 1.5×, combined the spatial resolution is improved by a factor of 7.5× and in parallel enables very fast multi-channel scanning, with very low bleaching during the image acquisition. Also, Fast Airyscan is not point scanning making it ideal for fast imaging over larger volumes of data. In detail Fast Airyscan is scanning in a 4px vertical line,[20] thus while inducing much less photobleaching during acquisition than traditional confocal systems or SIM systems that use high powered lasers. SIM acquisition is possible in case the fluorescence retention is sufficient and there is no recording bleaching during the SIM image acquisition. In optimal conditions this will further increase the resolution, although reconstruction of the images might require a more careful design to detect and reduce image artefacts.[15,22] dSTORM is not easily used in combination with Expansion microscopy as it has limitation in the channels that can be used, usually need oxygen scavenging buffers, that are not compatible with the hydrogels and also demands long acquisitions times per reconstructed image, with the risk of extensive gel drift and shrinkage during acquisitions. However, recent advances on this field permit using 3× Expansion microscopy in combination of dSTORM for specific applications.[23] Finally, we demonstrate that ExM goes beyond centrioles analysis in flat cell monolayers and that it can be applied for the studies of centrioles and cilia even in complex and hard to image samples such as the chick NT and that it can be used to detect a variety of centrosomes and cilia components. Overall, NT-ExM should prove invaluable to study pathological centrosome and ciliary features associated to neurodevelopmental disorders (NNDs) in vertebrate central nervous system.

QUANTIFICATION AND STATISTICAL ANALYSIS

The expansion factor can be calculated by measuring the size of the centrioles, since they are rigid structures that can act as molecular rulers. There are several possible quantification methods for the determination of expansion factors; the most accurate way to measure the expansion factor is to measure centriole diameter with axial view before and after expansion. To proceed with the measurement, we used FOP staining as a molecular ruler. Tissue from the same embryo (few sections or half of the electroporated open-book NT) should be directly mounted after immunostaining and used to measure the average value of centriole axial diameters in pre-expansion images (Figures 3G, 3H, and 3K upper panel). These measurements should be therefore compared with the average value of central diameters obtained from the distance between the inner parts of the FOP axial rings in ExM images (Figure 3C). Another alternative is to compare the average value of centriole axial diameters obtained in ExM with centriole diameters obtained from electron micrographs (~200 nm, Figure 3L).[15] Using the two type of measurements mentioned before, we calculated the expansion factor in 10 individual experiments and obtained an expansion factor ranging between 4× and 5× in both transversal sections and open-book NTs showing an optimal expansion regarding the thickness of the tissue.

LIMITATIONS

The loss of fluorescence intensity encountered in ExM, due to a combination of loss of fluorophores and a physical "dilution" of the signal during expansion of the sample, poses a major challenge. Several advices could be suggested to increase the signal in the pre-expansion sample in order to attain a sufficient labeling density in the expanded gel. For ExM on tissues, always use fresh primary antibody since loss of fluorescence intensity has been observed by using recycled primary antibody (data not shown). Although the technique is compatible with a wide range of commercial fluorescently-conjugated secondary antibodies and genetically encoded fluorophores, certain caveats apply.[10] Examples of fluorescent dyes that are not suitable for ExM include cyanine family dyes such as Cy5 and Alexa Fluor 647 which are degraded during the polymerization step and the bacteriophytochrome IR protein iRFP that is degraded during the proteinase K digestion. For alternative far red dye choices, Atto647N has shown optimal fluorescent retention after NT-ExM (Figures 3N–3P). For genetically encoded –GFP and –RFP fluorophores, the labeling density in the expanded gel was very low. The best alternative was to proceed with signal amplification by immunostaining with a dye with similar spectral properties (Figures 3I and 3N–3P). We validated ExM on centrioles and cilia in sectioned and not sectioned chick NT which is relatively a soft tissue with low mechanical resistance to expansion. We do not rule out that other ExM methods give better results for specific organelles or antibodies of interest.[11,24] Researchers may need to compare protocols for their field of expertise.

TROUBLESHOOTING

Problem 1

Polymerization of the gel starts prematurely (see: step 7-Sample gelation).

Potential solution

Make sure that you work on ice. All the ingredients of the gel should be chilled or freshly thawed, except the TEMED that is at 20°C. Use small volumes and minimize the time needed for this step. Try to hold the Eppendorf from the top and not from the bottom close to the solution to avoid heating.

Problem 2

Having trouble finding the tissue in the gel under the microscope (see: step 11-Images acquisition).

Potential solution

Once the coverslip is placed on the inverted microscope, it is advisable to start focusing on the sample with the 10× lens. Increase the magnification up to 63× or 100×, if the working distance allows it.

Problem 3

Tissue is out of lens working distance in high magnification lenses (see: step 11-Images acquisition and Figures 2M and 2N).

Potential solution

In case, that your region of interest is located in a low magnification lens, but it is out of working distance for the higher magnification lenses, try first flipping the gel (Figure 2N upper panel). This can be easily done by placing another 24 × 60 mm glass coverslip on top of the gel and flip the coverslips. However, if the tissue is still at the limits of the working distance, cut the gel horizontally and carefully (Figures 2M and 2N lower panel). This can be done with the help of a scalpel pushed on two coverslips. For open-book NTs, the apical side of the opened NT should be positioned closer to the objective.

Problem 4

Gel is drifting during image acquisition (see: step 11-Images acquisition).

Potential solution

Drift of expanded samples during imaging is a recurring problem and the most difficult of all to solve. Gel drift during image acquisition can gravely affect the imaging by either creating misalignments, artifacts, or even losing the field of view under study. There are various strategies that you can employ to address this issue while mounting and during the image acquisition. As it was aforementioned, it is important to prepare the mounting of the gel on the slide by mitigating sliding. Be sure to aspirate and take away any excess water around the gel or on the coverslip to prevent the gel from sliding. Polylysine coated coverslips can decrease sliding. In addition, two component dental silicon can be put around the gel as a barrier in order to impede sliding. Moreover, glass bottom petri dishes, like the 35 mm ibidi high glass bottom can be used since the gel will be safely secured inside the well that is in the middle of the plate. Set fast scanning settings, without compromising the lateral and axial resolution. Frame by frame acquisition is advised. Image processing and registration algorithms can be applied to correct, to an extent, the drift. By using the MultistackReg plugin in ImageJ and a rigid body transformation on a centriole marker channel you can save the transformation matrix and reapply it on the different channels.[25] This protocol can be found at https://github.com/miura/MultiStackRegistration.

Problem 5

Low fluorescence retention (see: step 8- Digestion and step 11-Images acquisition).

Potential solution

This is a quite common issue throughout the different protocols of ExM and for different fluorophores. Optimizing the digestion step and washing thoroughly the Proteinase K after digestion can improve the fluorescence retention. Also boosting the endogenous fluorescence with antibody staining can also help. Using more photostable fluorophores is also important. Post expansion staining is another solution, provided that the epitopes are preserved through the gelation and digestions steps. Scanning of the sample should be done fast and with a high signal to noise ratio, making the Fast Airyscan mode an ideal solution that combines both speed and high signal-to-noise Ratios (SNR) in a superresolution manner. Scanning fast with an image scanning microscopy systems (ISM) or, at least using, spinning disk confocal systems to enhance the signal to noise ratio or without using slow point scanning systems is highly recommended.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Murielle Saade (msabmc@ibmb.csic.es).

Materials availability

This study did not generate new reagents.

Data and code availability

The datasets supporting this study are available from the corresponding authors upon request.

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AUTHOR CONTRIBUTIONS

A.W. designed, performed, and supervised experiments; P.E.-B. performed experiments; N.-N.G. performed Fast Airyscan acquisition and analyses and supervised microscopy manipulation; M.S. designed and supervised all stages of the project; and all authors wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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