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TITLE:

Mucociliary Epithelial Organoids from Xenopus Embryonic Cells: Generation, Culture and High-Resolution Live Imaging

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KEYWORDS:

Spheroid, aggregate, mesenchymal-to-epithelial transition, goblet cell, multiciliated cell, tissue mechanics

SUMMARY:

We describe a simple protocol to develop mucociliary epithelial organoids from deep ectoderm cells isolated from *Xenopus laevis* embryos. The multipotent progenitors regenerate epithelial goblet cell precursors and allow live tracking of the initiation and progression of the cell transitions on the surface of organoids.

ABSTRACT:

Mucociliary epithelium provides the first line of defense by removing foreign particles through the action of mucus production and cilia-mediated clearance. Many clinically relevant defects in the mucociliary epithelium are inferred as they occur deep within the body. Here, we introduce a tractable 3D model for mucociliary epithelium generated from multipotent progenitors that were microsurgically isolated from *Xenopus laevis* embryos. The mucociliary epithelial organoids are covered with newly generated epithelium from deep ectoderm cells and later decorated with distinct patterned multiciliated cells, secretory cells, and mucus-producing goblet cells that are indistinguishable from the native epidermis within 24 h. The full sequences of dynamic cell transitions from mesenchymal to epithelial that emerge on the apical surface of organoids can be tracked by high-resolution live imaging. These in vitro cultured, self-organizing mucociliary epithelial organoids offer distinct advantages in studying the biology of mucociliary epithelium with high-efficiency in generation, defined culture conditions, control over number and size, and direct access for live imaging during the regeneration of the differentiated epithelium.

INTRODUCTION:

Injury, infection, and disease of mucociliary epithelium are associated with impaired production and clearance of mucus which is often found in pulmonary disorders such as chronic obstructive pulmonary disease, asthma, cystic fibrosis, bronchiectasis, and primary ciliary dyskinesia[1-4]. A recent advance in organoid technology, for instance, the basal cell derived lung organoid called

tracheosphere that recapitulates the regeneration of mucociliary epithelium arise as a promising model with therapeutic potential[1,5,6]. However, its use is currently limited, in part because of lack of the defined culture conditions and low efficiency in organoid productions. Mucociliary epithelium in the human airway and frog epidermis are remarkably similar in tissue morphology, cellular composition, and its function[7-12]. In both organisms, mucociliary epithelium provides first-line defense by secreting mucus and antimicrobial substances and clears harmful particles and pathogens through the synchronized action of cilia.

Here, we describe a simple protocol to generate mucociliary epithelial organoids using the multipotent progenitors of *Xenopus laevis* embryos[13,14]. Previously, we reported[14] that in the absence of exogenous growth factors and the extracellular matrix, the microsurgically isolated deep cells from the early gastrula stage ectoderm spontaneously assemble into aggregates, regenerate epithelium on its surface, and mature into mucociliary epithelium by intercalating multiciliated and other accessory cells within 24 h. In addition to the rapid development, this protocol offers a distinct opportunity for directly accessing the transitions of multipotent deep ectoderm cells into epithelial goblet cell progenitors that recapitulate the regeneration steps of a disrupted epithelium[14] which are not available from intact embryos and ectoderm (also known as the animal cap)[15-17]. The number and size of the organoids produced are scalable with high efficiency by controlling the starting materials from *Xenopus* embryos. Organoids in floating culture can be easily sorted and transferred at the desired stage for further analyses, including high-resolution imaging, mechanical testing, drug treatment, and genetic characterization[14]. This spontaneous, tissue mechanics-driven regeneration of the epithelium on the surface of embryonic cell aggregates results in mucociliary epithelial organoids and provide a novel three-dimensional (3D) model to study the biology of the mucociliary epithelium.

PROTOCOL:

Animal use and experimental protocols were approved by the institutional animal care and use committee (IACUC) of the Institute for Basic Science (IBS 18-01) and Korea Advanced Institute of Science and Technology (KA2017-22).

1. Embryos

1.1. Obtain *X. laevis* embryos using a standard procedure: manually collect eggs from stimulated female frogs and perform in vitro fertilization[18,19].

1.2. De-jelly the fertilized embryos with gentle agitation for about 5 min in 2% cysteine in 1/3x modified Barth's saline (MBS; see the recipe for 1X MBS below) at pH 8[19].

1.3. Optional step for live imaging: to fluorescently label specific proteins and observe their dynamics in the organoid, proceed to section 5.

1.4. (Optional) To monitor the contamination of superficial ectoderm cells within organoids, label the apical surface of the embryos with NHS-rhodamine at stage 9[14]. Incubate embryos in 1 mg/mL

NHS-Rhodamine in 1/3x MBS (pH 9.0) for 30 min with gentle nutation. Wash embryos three times by transferring to a Petri dish filled with 1/3x MBS for 15 min.

1.5. Culture the embryo in 1/3x MBS at the preferred temperature (14–26 °C) until the first signs of stage 10 are detected (i.e., the appearance of dark pigmented cells around the blastopore at the vegetal view).

2. Preparation of microsurgical tools, solutions, and culture vessels

2.1. Prepare the tools needed including a pair of surgical grade forceps and hair tools (hair loop and hair knife) for microsurgery[20].

2.2. Prepare the following culture media for embryos: 1/3X MBS, where 1X MBS is made with NaCl (88 mM), KCl (1 mM), $NaHCO_3$ (2.4 mM), $MgSO_4$ (0.82 mM), $Ca(NO_3)_2$ (0.33 mM), $CaCl_2$ (0.41 mM), and HEPES (10 mM). Adjust pH to 7.4 with 10 M NaOH.

NOTE: Optional: add drops of phenol red to indicate pH.

2.3. Prepare the following culture media for embryonic tissues and organoids: Danilchik's for Amy (DFA)[21] supplemented with fresh 1% antibiotic and antimycotic solution. Prepare DFA with NaCl (53 mM), Na_2CO_3 (5 mM), potassium gluconate (4.5 mM), sodium gluconate (32 mM), $CaCl_2$ (1 mM), and $MgSO_4$ (1 mM). Adjust pH to 8.3 with granular Bicine. Filter DFA (0.2 µm bottle-top filter), aliquot and store it at -20 °C.

2.4. Prepare calcium- and magnesium-free DFA for separating deep cells from ectoderm by using the recipe above and omitting $CaCl_2$ and $MgSO_4$. Aliquot and store at-20 °C.

2.5. Prepare non-adhesive PCR tubes for embryonic cell aggregation.

2.5.1. To induce spontaneous aggregation of the isolated embryonic cells, prepare non-adhesive PCR tubes by coating round-bottomed PCR tubes with 200 µL of 1% BSA (1 g of BSA in 100 mL of distilled water) overnight at 4 °C or for 2 h at room temperature. Each tube will be used to assemble one organoid.

2.5.2. Rinse BSA-coated PCR tubes with DFA three times to remove any residual BSA.

2.5.3. Fill PCR tubes with 200 µL of DFA.

3. Isolation of deep ectoderm cells

3.1. Select and gather embryos as they reach early stage 10 using hair tools under a stereoscope.

3.2. Using a disposable transfer pipette, transfer the selected embryos into a DFA-filled Petri dish.

3.3. Remove the vitelline membrane of the embryos using sharp forceps from the vegetal side without disrupting the animal side of the embryo.

NOTE: Be careful to avoid exposing embryos to the air. Introducing air bubbles into the solution or bringing embryos to the surface will cause the embryo to burst.

3.4. To isolate the animal cap, position the animal side of the embryo up.

3.5. Visually estimate the extent of the animal cap to be excised and make the first incision along the edge of the animal cap with a hair knife. Pull the hair knife outward to make a cut.

3.6. Repeat step 3.5 to create a chain of small cuts to excise the animal cap.

3.7. Trim the thick-layered edge of the animal cap using a hair knife to prevent the inclusion of mesoderm precursors.

NOTE: To prevent the healing and aggregation of isolated animal caps, proceed to the next step within 10 min. Typically, we isolate 5–10 animal caps at a time to assemble multiple organoids.

3.8. To separate deep ectoderm cells from the animal cap, transfer the excised animal caps to a Petri dish filled with calcium- and magnesium-free DFA with a disposable transfer pipette. Be careful not to introduce any air bubbles during the transfer.

3.9. To keep enough space for the next steps, using the hair tools, position the animal caps to face the animal-side up and maintain a generous distance from other explants.

3.10. Wait for 5–10 min and then monitor the explants under a stereoscope. Once loosened deep cells have been found from the edge of the dark-pigmented superficial layer, begin lifting the superficial layer away from the light-colored deep ectoderm cells using a hair knife under the stereoscope.

3.11. Carefully detach (peel-off) the superficial layer with a hair knife, starting at the edge.

3.12. Collect the deep ectoderm cells with minimum aspiration (10–15 µL) to limit the amount of calcium- and magnesium-free DFA that is transferred to the aggregation media in the next step.

NOTE: Detached superficial cells can be removed from the media to prevent contaminating the remaining deep ectoderm cells.

4. Generation of mucociliary epithelial organoids

4.1. Transfer collected deep ectoderm cells to a non-adhesive PCR tube containing 200 µL of DFA. Gently pipette the media (2–3 times) to disperse transferred cells in the PCR tube.

NOTE: The timing designated as hours post aggregation (hpa) starts at this step. The size of the resulting organoids is controlled by the number of deep ectoderm cells added to a PCR tube. Deep ectoderm cells from one or more animal caps can be used, depending on the desired size of the organoid.

4.2. Close the PCR tube. Keep PCR tubes upright to induce spontaneous aggregation at the bottom.

4.3. Monitor the aggregation process under a stereoscope. Cells typically gather at the bottom of the PCR tube within an hour and assemble into spherical aggregates within 2–3 h, depending on the size.

4.4. To conduct live imaging or drug testing during the development of the mucociliary epithelial organoids, collect aggregates at 2 hpa using a 200 µL pipette fitted with enlarged tips (cut with sterilized scissor) to avoid causing damage to the aggregates during collection.

4.5. To allow aggregates to develop into the mucociliary epithelial organoid in culture, collect spherical aggregates from the PCR tube at 5 hpa and transfer them to a DFA-filled Petri dish.

4.6. Position the aggregates far from others to prevent them from fusing. Within 24 h of culture at room temperature without any added factors, mature mucociliary epithelial organoids can be observed to be rotating with the action of beating cilia covering the surface of the differentiated epithelium

5. (Optional) High-resolution live imaging of developing organoids

5.1. Prepare mRNA for microinjection.

5.1.1. To visualize the epithelialization that occurs at the initial stage of organoid formation, prepare mRNA for the epithelial-specific zonula occludens protein-1 (ZO-1) and for outlining the cell membranes by amplifying pCS2-ZO1-RFP and pCS2-mem-GFP plasmids (a gift from Lance Davidson).

5.1.2. Extract and linearize the plasmid DNA, and then transcribe the capped mRNA using an SP6/T7 in vitro transcription kit.

5.1.3. Aliquot the transcribed mRNA and store it at -80 °C.

5.2. Microinject the mRNA into a fertilized embryo

5.2.1. Place fertilized embryos in 3% density gradient medium in 1x MBS.

5.2.2. Load 3–4 µL of mRNA using a micro-loader tip into a pulled glass needle (a long and fine tapered needle tip with inner diameter of 10–30 µm).

5.2.3. Attach the needle to a microinjector and adjust the time and pressure to deliver a constant volume of mRNA for microinjection.

5.2.4. Inject the mRNA just under the apical surface of the animal pole. A distinct pale-colored circular patch that is caused by the expansion of the cortex is visible at the time of microinjection.

5.2.5. Transfer the injected embryos to 1/3X MBS and culture them to stage 9.5.

5.2.6. Collect the fluorescently labeled embryos under a fluorescence stereoscope (excitation/emission settings for GFP (488/510) and RFP (532/588).

5.2.7. Proceed with step 1.3.

5.3. Assemble and culture the organoid (sections 3 and 4) until the desired stage of development.

5.4. Perform live imaging.

Prepare a glass-bottom imaging chamber by gluing a cover glass to a custom-milled acrylic chamber using silicon grease.

NOTE: Tightly seal the chamber to prevent leakage of the culture media.

5.4.2. Fill the imaging chamber with DFA.

5.4.3. Pick one hexagonal transmission electron microscopy (TEM) grid (75 mesh) from a container using forceps and apply a tiny amount of grease to the edge of the grid.

NOTE: The size of the mesh should be smaller than the diameter of the aggregate so that the aggregate sits on the grid.

5.4.4. Press down lightly to secure the TEM grid to the bottom of the imaging chamber.

5.4.5. Transfer the aggregates to the imaging chamber and position them within the grid.

NOTE: Avoid positioning the aggregates next to the grease. Throughout the experiment, the aggregates should sit on the TEM grid without contacting the bottom of the chamber to prevent physical compression.

5.4.5. Fill up the imaging chamber with DFA and seal it with a cover glass and grease.

NOTE: The chamber should be airtight with no air bubbles to prevent any turbulence or movement during imaging.

5.4.6. To follow the progression of mucociliary epithelial organoid formation, collect time-lapse z-stack images of aggregates (from 2 hpa) using a confocal microscope.

Note: We typically collect ~120 µm-thick z-stacks every 15 min using a 20X objective to follow the dynamic cell behaviors, but these specifications should be optimized for the goal of experiments.

6. (Optional) Imaging developing organoids by fixation and immunostaining

6.1. Fix organoids at the desired stage of development by transferring them to a glass vial filled with a fixative solution.

NOTE: Add a volume of fixative solution that is >20 times that of the samples to ensure complete fixation. Perform the following processes on a nutator unless otherwise noted. In general, organoids are fixed with 4% paraformaldehyde (PFA) in PBS. However, different fixatives may be required to detect specific proteins. For example, we used 4% PFA with 0.25% glutaraldehyde in PBS to detect F-actin and acetylated tubulin. To detect intelectin (ITLN) and ZO-1 organoids are fixed with ice-cold Dent's solution (4:1 methanol:dimethyl sulfoxide) for overnight at -20 °C. Dent's fixed organoids should be serially dehydrated before washing (step 6.3). Duration for antibody incubation and washing can be optimized for specific needs.

6.2. Fix organoids for 15 min at room temperature (RT) or overnight at 4 °C.

6.3. Wash 3 times with PBST (PBS with 0.1% Triton X-100) for 15 min at RT.

6.4. Block unspecific binding with 10% goat serum in PBST (PBSGT) for 1 h at RT.

6.5. Incubate with primary antibody (1:200) in PBSGT overnight at 4 °C.

6.6. Wash 3 times with PBST for 15 min at RT.

6.7. Incubate with secondary antibody (1:200) in PBSGT overnight at 4 °C.

6.8. Wash 3 times with PBST for 15 min at RT.

6.9. Transfer the fixed and immunostained organoids to an imaging chamber and proceed with confocal imaging.

REPRESENTATIVE RESULTS:

This standardized protocol generates a mucociliary epithelial organoid from multipotent progenitors isolated from the early gastrula stage *X. laevis* embryos within 24 h of cultivation[14]. Collected deep ectoderm cells self-assemble to form an aggregate in a non-adhesive PCR tube and undergo surface epithelialization and goblet cell differentiation. The newly epithelialized surface of aggregates provides a substrate similar to the native epithelium found in vivo for

intercalating inner cells (e.g., multiciliated and other accessory cells) and develops to form mucociliary epithelial organoids (**Figure 1A,B**). Within 24 h after aggregation, self-organized mucociliary epithelial organoids regenerate a mature epidermis that is indistinguishable from the epidermis of a tadpole. The organoids comprise fully differentiated epithelium (keratin), mucus-secreting goblet cells (ITLN), multiciliated cells (acetylated tubulin), and small secretory cells (peanut agglutinin, PNA) (**Figure 1C**).

In addition to confirming the development of different cell types with immunostaining, the dynamics of organoid development can be followed by live imaging (**Figure 2A**). To examine the epithelialization that emerges at the early stage of organoid formation (**Figure 1B**), we labeled embryos by expressing fluorescently tagged tight junction proteins (ZO-1-RFP) and membrane localizing proteins (mem-GFP). With dual-labeling, the sequential steps of ZO-1-positive tight junction formation can be marked and quantitatively analyzed during epithelialization (**Figure 2**). For example, for cells (**Figure 2B**, green-colored) at different stages of epithelialization (at 0 min), some regions of cell-cell adhesion have scattered puncta of ZO-1 (**Figure 2B**, green arrows). In contrast, other areas have fully assembled, contiguous ZO-1 expression (**Figure 2B**, yellow arrows). Over time, the puncta coalesce and connect to form contiguous tight junctions (**Figure 2B**, green arrows), and contiguous tight junctions maintain their morphology even during cell division (**Figure 2B**, yellow arrows). As the tight junctions mature, cells dynamically move in and out of the surface along the apical planes of the organoids (**Figures 2C,D**). Furthermore, by tracking cells spatiotemporally on the surface of differentiating organoids (**Figure 2B**, color-coded cells), multi-scale analysis is possible, ranging from individual puncta to contiguous tight junctions, cell-cell boundaries, and subsets of cell populations within organoids.

FIGURE AND LEGENDS:

Figure 1: Generation of mucociliary epithelial organoids. (**A**) A schematic showing the protocol to assemble deep ectoderm aggregates from *X. laevis* embryos. (**B**) A schematic for a model of mucociliary epithelial organoid formation originating from multipotent deep ectoderm cells (cross-sectional view). Surface-positioned cells transit into epithelial cells and differentiate into goblet cells. Differentiating ciliated cells, secretory cells, and ionocytes radially intercalate into the surface and regenerate a mature epidermis. (**C**) Maximum z-projection of mucociliary epithelium immunostained for ITLN (mucus-producing goblet cells), acetylated tubulin (ciliated cells), PNA (small secretory cells), and keratin (epithelial cells) in organoids at 24 hpa (upper panel) and tadpole epidermis (lower panel). Scale bar = 30 µm.

Figure 2: Live imaging of developing organoids. (**A**) A schematic of the imaging chamber for live organoids (not to scale). (**B**) Time-lapse sequences of confocal stacks collected from deep ectoderm cell aggregates expressing ZO-1-RFP and mem-GFP from 2.5 hpa. Scale bar = 20 µm. Cells are pseudo-colored for tracking over time. Green-colored cell have different cell adhesion statuses, including one progressively developing ZO-1 positive adhesion (green arrows) and one maintaining contiguous ZO-1 positive adhesion (yellow arrows) over time. (**C**, **D**) Time-lapse confocal images of ZO-1-RFP-expressing deep ectoderm cell aggregates show the radially

intercalating cells moving to the surface (C, yellow star) and moving inside the aggregates (D, blue star). Scale bars = 10 μm.

DISCUSSION:

Mucociliary epithelial organoids generated from deep ectoderm cells of *X. laevis* embryo are a powerful tool to study the epithelialization and differentiation of multipotent progenitors in vitro. In contrast to the widely adopted animal cap assay[16] used for in vitro organogenesis[13] and the development of mucociliary epithelia[15,17,22] that utilize the intact ectoderm, the deep ectoderm-derived organoids presented in this protocol offer a distinct opportunity to monitor the tissue mechanics-driven regeneration phases of the surface epithelium[14]. At around 2 hpa, the newly generated ZO-1 positive epithelial cells (**Figure 2**) begin to appear on the apical surface of organoids and increase their population to cover the entire organoid as the tissue solidify or reduces the compliance[14]. The regeneration of the epithelium and subsequent lineage specifications for mucus-producing goblet cells proceed spontaneously in a chemically defined culture media within a day. These rapidly developing mucociliary epithelial organoids provide a platform to examine dynamic cell behaviors in real-time, in high-resolution, during progressive steps of epithelial regeneration. They also enable investigation of fundamental questions that arise during mucociliary epithelium development, homeostasis, and associated diseases[2,9,23]. In particular, the mechanical sensitivity of the deep progenitor cells during the transition to epithelial goblet cell precursors identified in the organoids[14] may serve to link respiratory diseases associated with abnormal basal differentiation where mucus-secreting goblet cells are over- or under-produced[23].

While this protocol offers a simple approach to generate these organoids, there are several critical steps for success in experiments. To prevent contamination of superficial epithelial cells during the isolation of deep ectoderm cells from the animal cap, one should monitor the animal cap placed in calcium- and magnesium-free DFA under a stereoscope and detect the right time to initiate the separation of the dark-pigmented superficial layer of the animal cap. If the tissue is kept in calcium- and magnesium-free DFA for too long, the entire tissue will dissociate and distinguishing between deep and superficial cells would then be impossible for deep ectoderm aggregates. To confirm the absence of superficial cells in deep ectoderm aggregates, we recommend fluorescently labeling the apical surface of the embryo with NHS-rhodamine (step 1.4[14]) prior to microsurgery; this would allow for easy identification of surface cells if they exist in the resultant organoids. Since epithelial regeneration is regulated by tissue mechanics[14], it is essential to avoid unintended force generation for self-organizing organoids. In particular, we suggest avoiding contact with the glass bottom of the imaging chamber during live imaging by placing aggregates at the edges of the TEM grids as this allows for free contact with the imaging window of live aggregates (step 5.1.2.). This in vitro-cultured, self-organized 3D model for mucociliary epithelium will serve as a tractable tool to answer the fundamental questions that arise during the regeneration of epithelium and the lineage specification of goblet cells.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

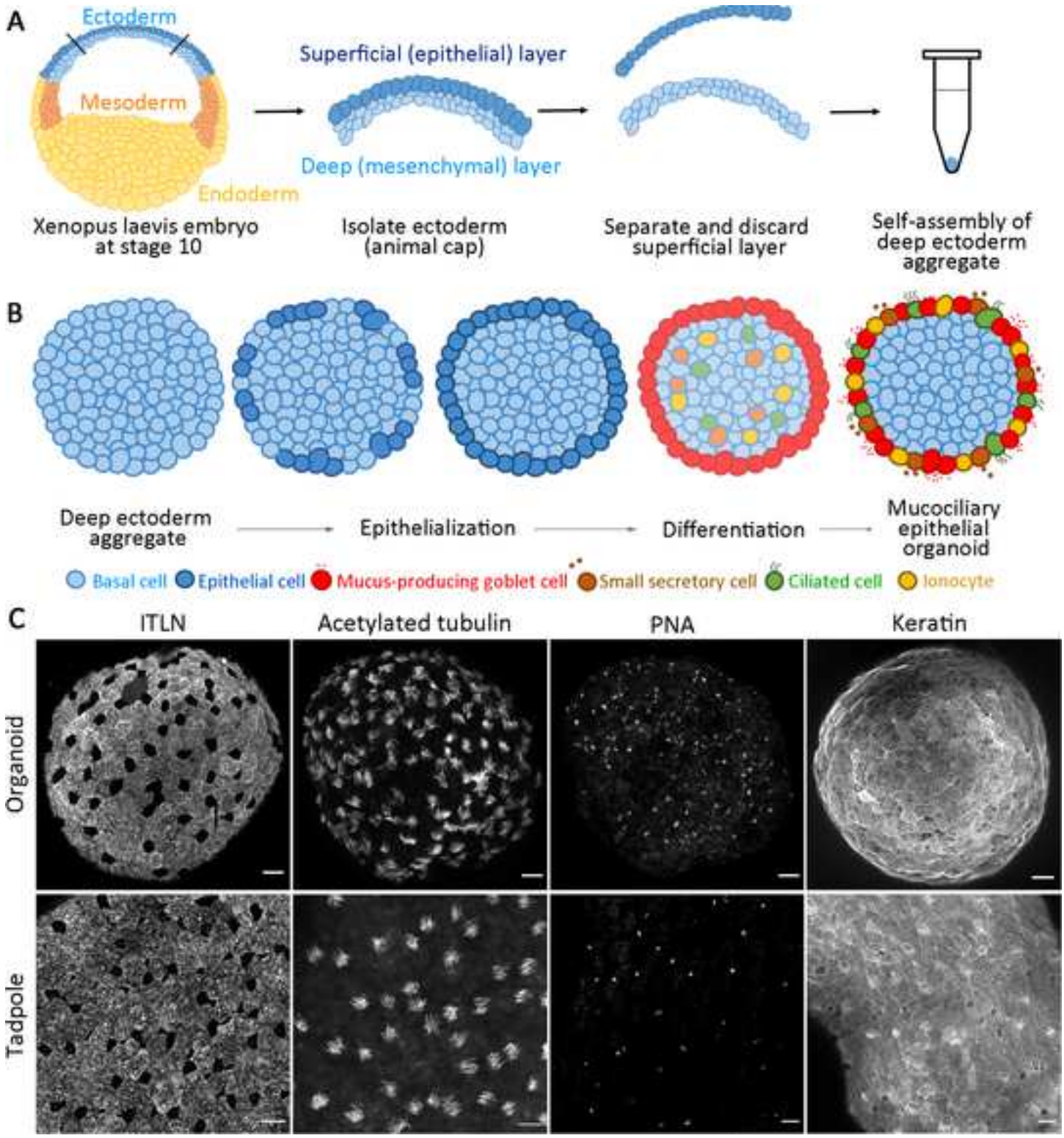
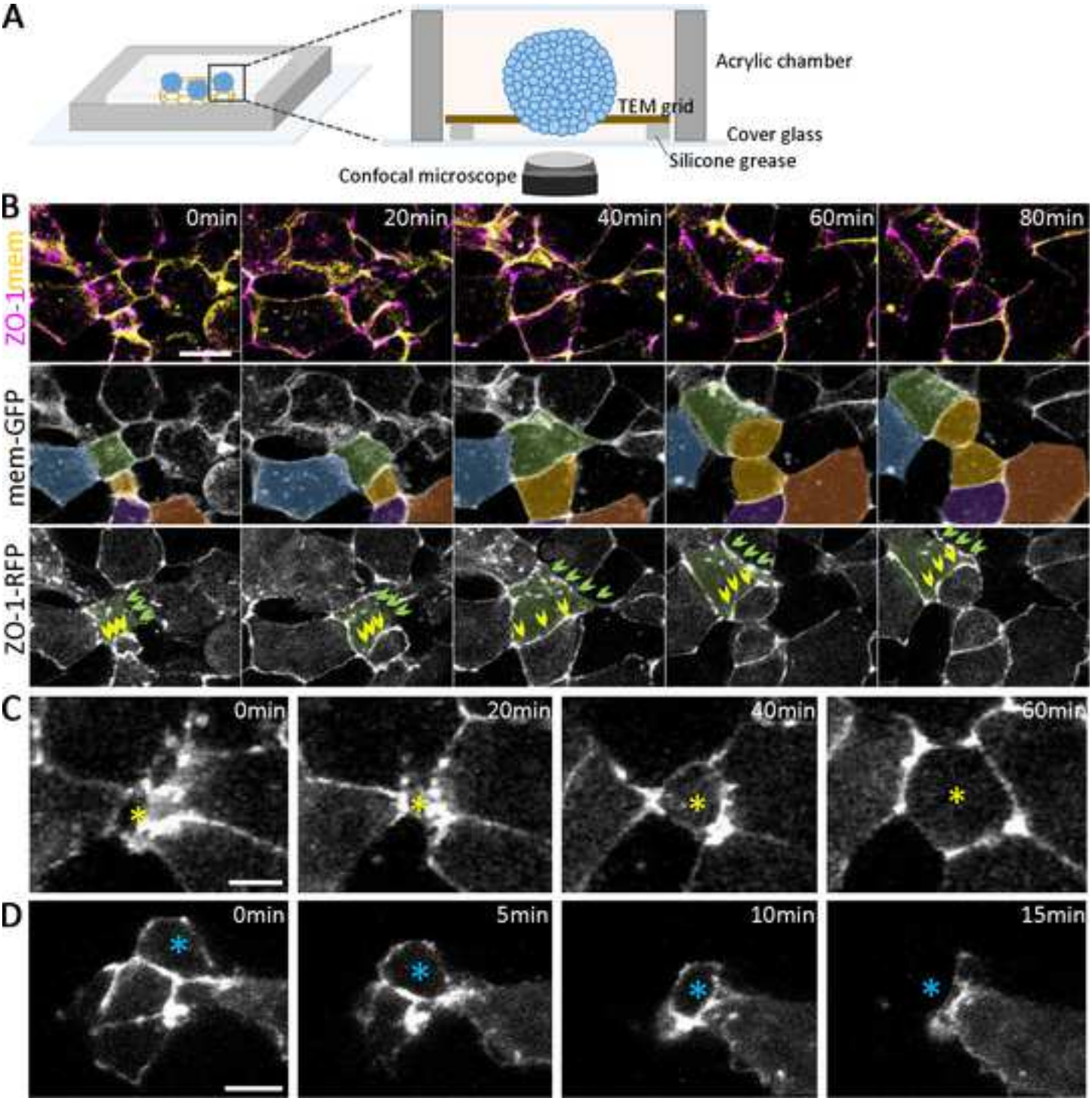


Figure 2



Name of Material/ Equipment	Company
Equipment	
Dual-stage Glass Micropipette Puller	Narishige
Picoliter microinjector	Warner Instruments
Confocal Laser Microscope	
Stereoscope	
Tools	
Forcep	Dumont
Hair knife	
Hair loop	
hCG injection	
human chorionic gonadotropin	Sigma
MBS solution	
10M Sodium hydroxide	Sigma
Calcium chloride	Sigma
Calcium nitrate	Sigma
HEPES	Sigma
Magnesium sulfate	Sigma
Phenol-red	Sigma
Potassium chloride	Sigma
Sodium bicarbonate	Sigma
Sodium chloride	Sigma
Sodium hydroxide reagent grade, 97%, powder-25g	Sigma
dejellying solution	
L-Cysteine hydrochloride monohydrate	Sigma
Sodium hydroxide 10M	Sigma
Ficoll solution	

Ficoll	Sigma
DFA solution	
Sodium chloride	Sigma
0.22μm Filter	Millipore
Antibiotic Antimycotic Solution	Sigma
Bicine	Sigma
Calcium chloride	Sigma
Magnesium sulfate	Sigma
Potassium gluconate	Sigma
Sodium carbonate	Sigma
Sodium gluconate	Sigma
mRNA in vitro transcription	
SP6/T7 in vitro transcription kit	Invitrogen
mRNA microinjection	
Borosilicate glass capillary tubes	Harvard Apparatus
Eppendorf microloader pipette tips	ThermoFisher
Mineral oil	Sigma
PCR tube coating	
BSA	Thermofisher
PCR tubes	SSI
Imaging	
Custom-milled acrylic chamber	
Coverglass 24mmX50mm	Duran
SPI Hexagonal TEM Grids, Gilded Nickel (50mesh)	SPI
SPI Hexagonal TEM Grids, Gilded Nickel (75mesh)	SPI
Silicone grease	Shinetsu

Fixation

20ml screw top-cap vial	Wheaton
2ml screw top-cap vial	
Benzyl alcohol	Sigma
Benzyl benzoate	Sigma
Dimethyl sulfoxide (DMSO)	Sgima
Glutaraldehyde 10% EM GRADE	Electron Microscopy Sciences
Goat serum	Jackson
Methanol	Sigma
Paraforlamdehyde	Sigma
Phosphate-buffered saline (PBS)	LPS Solution
Triton X-100	Sigma

Primary antibody (1:200)

acetylated tubulin	Sigma
Itln1	Proteintech
Keratin	Developmental Studies Hybridoma Bank
ZO1	Invitrogen

Vectors

pCS2-mem-GFP
pCS2-ZO1-RFP

Catalog Number	Comments/Description
PC-100	
PLI-100A	
Dumont #5	
Reference (Kay, B.K.; Peng, H.B., 1991)	
Reference (Kay, B.K.; Peng, H.B., 1991)	
cg10-10vl	
72068	
C3881	
C1396	
H4034	
230391	
P0290	
7447-40-7	
S6014	
S9625	
655104	
C7880	
72068	

F4375

S9625
S2GPT05RE
A5955
B3876
C3881
230391
G4500
222321
G9005

AM1340

GC100-10
A25547
M5904

26140079
SSI-3245-00

B01_001650
275HGN-XA
2775GN-XA
HIVAC-G

WH.986580

305197
B6630
D4540
16120
005-000-121
322415
P6148
CBP007B
T8787

clone 6-11B-1
11770-1-AP
1h5
402200

Gift from Dr. Lance Davidson
Gift from Dr. Lance Davidson

We thank the reviewers and editorial committees for their comments. We have revised the manuscript to address each of their comments. A line-by-line response to reviewers' points is offered below and shown in blue text.

Editorial Comments:

We have revised the manuscript to adopt the editorial comments listed below.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

• If your figures and tables are original and not published previously or you have already

obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
This is a description of the production of mucociliary organdies using Xenopus embryos. The authors do a great job of describing their protocol for isolating the inner epithelial layer, specifically, and allowing these cells to aggregate in to a spheroid that will quickly develop into a ciliated epithelium indistinct from the ciliated epithelium found on the embryo.

Major Concerns:
My biggest concern for this is their use of the term mesenchymal for the cells they are isolating and the claim that they are multipoint stem cells. That terminology might be a bit strong. These cells are likely already committed to an ectodermal fate. None the less the ability to regenerate multiple ectodermal cell types is remarkable and a fantastic system. I just think some of the terminology could be more conservative.

We revised to use the term multipotent progenitors throughout manuscript.

The two-layered embryonic ectoderm of Xenopus does not fall into any of the mammalian classifications of simple, pseudostratified, stratified, or transitional epithelia. A static picture of a stratified epithelia includes a deep layer of cells associated with a basement membrane and a superficial layer of apical-basally polarized cells, stratified epithelia are dynamic tissues where the deep layer alone is mitotically active and replaces cells lost from the surface(Elias and Friend 1975). The animal cap ectoderm appears superficially like this static image, however, in Xenopus, both the superficial and deep layers are mitotically active and there is no transfer of cells, outside of intercalating cells, from the deep layer to the superficial. Furthermore, the superficial population is not derived from the deep, as goblet cell precursors can be tracked from the superficial layer from blastula stages (Chalmers, Pambos et al. 2005) to mid-to late-larval stages. A stratified epidermis does emerge from the ectoderm, however, this occurs many days later when the embryonic skin transforms into adult skin (Yoshizato 2007). While there are only limited parallels between mammalian and Xenopus embryonic epithelia we propose the Xenopus animal cap and derivatives in the early tadpole skin are best described as a simple epithelium. We argue against an epitheloid characterization of the bulk of cells in the deep layer (Shook and Keller 2003) since they do

not assemble keratin filaments and exhibit no signs of apical-basal polarity indicative of epithelial cells.

A number of groups have utilized ectodermal spheroids from Xenopus caps. In the intro it might be useful to add a broader description of those spheroids and how the ones generated here are distinct. While both might be good for certain things this protocol is better for others and I think that distinction is really important and makes this protocol really nice.

We have revised to include this point in the introduction and discussion.

Minor Concerns:
The description of DFA media leaves out the detail that the Ph should be brought to 8.3 using 1M Bicine. If the authors are using Bicine this detail should be added and if not then the description should change.

We have revised the manuscript (Step 2.3).

I would recommend adding a sentence to the portion about antibody staining that details of incubation and wash time will alter based on particular antibodies.

We have revised the manuscript (Step 6.1).

Reviewer #2:
Manuscript Summary:
Kim and Kang present a protocol where animal caps of early gastrula embryos where processed to „organoids", resembling the mucociliary epidermis of Xenopus embryos. Technically the authors use basal cells which after dispersion and reaggregation develop to spheres which differentiate into the 4 epidermal cell types. The protocol is widely in agreement with my understanding of the epidermal differentiation processes. However, I do have major doubts on its practicability and further questions.

Major Concerns:
1. Animal caps (ACs) excised at blastula stage have been long used for analysis and it has been shown that without any treatments ACs differentiate into a mucociliary epidermis. In addition, the term "organoids" has been used for this setting (Haas et al., 2019). I wonder what are advantages of the proposed protocol by Kim and Kang over the classical way. In other words why should researchers use this rather sophisticated protocol?

We have revised the manuscript to emphasize the distinction between mucociliary organoids generate from animal caps and our protocol (generate from deep ectoderm cells) in introduction and discussion.

2. I am quite surprised that goblet cells (GC) seemed to develop de novo from deep cells, which is in stark contrast to the embryo. GCs have been shown to originate directly from

superficial cells of the animal cap i. e. the cells that have been removed in the protocol by Kim and Kang. How do the authors explain this apparent discrepancy?

Thank you for raise the question. In contrast to goblet cell differentiation originate from superficial epithelial cells *in vivo* and animal caps, surface positioned newly epithelialized cells in deep ectoderm aggregates differentiate into goblet cells (Kim, Jackson et al. 2020). This new finding is published (Kim et al. 2020, Nat. Commun.) and is a key difference between mucociliary organoids generated from animal caps.

I would suggest that remnants of or even single superficial cells in the explant could be sufficient to provide enough mitotic GCs which finally cover the organoid.

We have thoroughly tested the possibility of contamination from superficial cells in deep ectoderm aggregates by labeling the apical surface of embryos (see Figure 1c and d, Kim et al 2020), and confirmed that epithelialization emerges from deep ectoderm cells. We have also recommended to confirm possible contamination of superficial cells by NHS-Rhodamine labeling in the current protocol (Step.1.5) for deep ectoderm aggregates.

Minor Concerns:
3. Are ACs of early gastrulas specifically required for the protocol or does it work with ACs of blastulae as well?

We have been generated the deep ectoderm aggregates from animal caps of 10 and 10+ stage embryos and did not test with animal caps of blastula stage embryos. While we suspect our protocol would work with blastula stage animal caps we have not tested them.

4. Dispersion of animal cap cells using Ca2+ and Mg2+ free media has been used to induce neural cell fate. Why this is not an issue in the presented protocol?

Neural induction of single isolated animal cap cells occurs after more than 6 hours of incubation in calcium and magnesium-free media (Grunz and Tacke 1989, Kuroda, Fuentealba et al. 2005). In our protocol, isolated animal caps were briefly exposed to calcium and magnesium- free media (maximum 10 minutes) to loosen the cell adhesions between the deep and superficial layers of animal caps. Separated deep cells were immediately transferred to DFA for aggregation. Limited exposure to calcium and magnesium-free media do not induce neural cells.

Reviewer #3:
Manuscript Summary:
The manuscript "Mucociliated epithelial organoids from Xenopus embryonic cells: generation, culture, and high-resolution live imaging" by Kim and Kang describes the derivation of mucociliated epithelial organoids from deep ectoderm cells in Xenopus Laevis embryos. The protocol is detailed and clearly written. A few clarifications are necessary.

Minor Concerns:
1) I suggest the authors provide academic email addresses instead of Gmail addresses

We have corrected this.

2) Lines 62-65: A more recent reference (e.g. (Sachs et al., 2019)) can be added.

We have updated references.

3) Line 73: What do the authors mean by "pluripotent stem cells"? Can the cells they isolate give rise to cells from all embryonic germ layers?

Ectoderm (animal cap) cells been used to induce for mesodermal, endodermal, and neural tissues and organs *in vitro* (Asashima, Ito et al. 2009, Buitrago-Delgado, Nordin et al. 2015). We have adopted the term 'multipotent progenitors' for deep ectoderm cells in the revised manuscript.

4) Line 110, Line 114: Are there preferential buffers for pH regulation? If so, please add the names.

We have revised the manuscript.

5) Line 111: Give the original reference for Danilchik's for Amy (DFA) medium

We have updated the reference.

6) Line 181: Who is the supplier for the plasmids?

We have updated the manuscript.

7) Pages 23-24: There is text whose relation to the protocol is not clear.

Those texts (p23; comments/Description, p24; Shinetsu frequent use silicone grease) might be from the Table of Material, and should be corrected.

8) Have the authors attempted to stain for ionocytes on their organoids and tadpole epidermis? If so, these data need to be included in Figure 1C.

We have not stained ionocytes yet in our organoids and tadpole.

9) In vitro tracheospheres originate from highly enriched basal cells, isolated from adult mouse/human tracheas. Also to derive functional, differentiated, pseudostratified epithelium, culture of basal cells in ALI is necessary. The amphibian system presented here originates from embryonic ectodermal progenitors. Could the authors add a couple of sentences in the Discussion to elaborate on how their system can be used by lung biologists studying progenitor dynamics and regeneration following various types of injury in the mammalian trachea/large airways?

We have revised the discussion.

Reviewer #4:

Manuscript Summary:

The protocol will be very useful for the community and offers some important advantages as compared to the standard animal cap explant organoids. There are a few things that should be improved in the text and the protocol steps and the omitted literature should be cited to provide adequate context and reference for the interested reader with less experience in the field that this form of protocol aims at attracting. After those improvements were made, I would highly recommend this manuscript for video production and publication.

Major Concerns:

It should not be referred to as regeneration model, as this systems merely recapitulates early development of the epidermis rather than regeneration of a mature mucociliary epithelium, such as in ALI cultures. Also, it should be pointed out what specific advantages/novelties this system has as compared to the widely used animal cap explants (I think that there is indeed a benefit to this new type of culture, but it would be incorrect to claim that drug screening and genetic characterization is not possible in the animal cap organoid model, which has been used for exactly that very successfully over the years by many groups).

A key distinction of our deep ectoderm derived organoids from intact animal cap organoids is that the deep ectoderm aggregates newly generate a layer of epithelium on the surface of organoids by transitioning to epithelial goblet cell precursors under the control of tissue mechanics (Kim et al.2020) that we refer to regeneration. We have revised the manuscript to add those points in the introduction and discussion.

Protocol:

Step 1.2: spell the time required until dejelly procedure and how long that procedure should last.

We have revised the manuscript.

Step 1.4: stage 10 is actually a bit too late, the explantation of animal cells should happen at st. 8-9, and before organizer signals start to induce neuroectodermal fate in a subset of those cells.

We isolate ectoderm explants from the animal pole of early stage 10 embryo distance from thick layered marginal cells of the embryo. We note the cells expressing neural induction signals move ventrally at gastrula (Kuroda, Wessely et al. 2004) while a population of ectoderm cells maintains their blastula pluripotency until neurula stage (Buitrago-Delgado, Nordin et al. 2015).

Step2.1: name the grade of the forceps and which kind of hair tool you are referring to (hairloop or eyebrow knife?)

We have revised the manuscript.

Step2.3: indicate that DFA can be made and stored at -20 as aliquoted 2x solution, but without the CaCl2, which should be supplemented fresh.

We have added details for DFA and calcium-magnesium-free DFA (Step.2.3 - 2.4).

Section 3: say in which buffer the explant should be cut. 1x MBS would be standard.

Explants were cut and cultured in DFA that matches the interstitial fluid of the gastrula stage embryo (from Section 3-2).

Step3.3: As note, one could add that the safest way is to turn the embryo vegetal up and poke into the membrane on that side.

We have added it to note.

Where are steps 3.4-3.6?

We have corrected typos.

Step3.7: Add as a note that the explant should not be too big and one should avoid taking the thicker-layered areas of transition into mesoderm to prevent inclusion of mesodermal precursors that generate signals which would then prevent the induction of epidermal fate.

We have revised the manuscript.

Step3.10: Lower Calcium levels might be used to increase time until healing of tissue occurs.

Exposure to calcium- and magnesium-free media were limited to prevent alteration of native cell behaviors (e.g., cell-cell adhesions)

Step3.11: No protocol is given above for Ca/Mg-free DFA.

Recipe for calcium- and magnesium-free DFA is added to the manuscript (Step.2.4).

Also transfer pipettes should be BSA coated for this step, that helps a lot!

3.16: Here as well, pipettes should be BSA coated for this step, that helps a lot!

We limited the use of BSA coating to prevent excessive residual BSA maintained in culture media as it may alter cell behaviors during aggregation and subsequent differentiation steps. However, we agree that BSA coating with sufficient washing could help the transferring procedures.

Step4.1: Say that this is DFA with Ca/Mg and don't the deep cells should be dissociated beforehand?

Step4.1 is for the beginning of aggregation in normal DFA. Loosened deep cells in calcium-magnesium-free DFA were dissociated during transfer (step3.16-4.1).

Step4.6: One can use 0.5x MBS or 1/3xMR here as well, might be beneficial, because the high BSA content of DFA makes it prone to contamination and bacterial overgrowth.

The aggregate could develop in either 1/3 MBS or DFA (Kim et al. 2020) for mucociliary epithelial organoids. We do not add BSA for DFA and supplemented with antibiotic and antimycotic.

Step4.8: The epithelium is not fully mature after 24h. SSCs are still missing, alignment of MCC cilia is not finished, etc. (see papers from Wallingford, Kintner/Keller, Walentek, Mitchell).

The stage of organoid at 24 hours post aggregation match to embryonic stage of 27-28 when small secretory cells appear on the epithelium *in vivo* (Dubaissi, Rousseau et al. 2014, Walentek, Bogusch et al. 2014) and our organoids (Figure 1C -PNA). Alignment of cilia may not be possible with organoids lacking Anterior-Posterior polarity (Chien, Keller et al. 2015).

Step4.6: I would really not use DFA for that…

Figure1A: This is not a st. 10 blastula, but st.8-9.

We have revised the cartoon.

Quite a few relevant citations are missing:
e.g. Quigley & Kintner 2017 (transcriptomics), Stubbs et al. 2006 & Sedzinski et al. 2016 (radial intercalation and apical expansion), Chien et al. 2015 (mechanical strain and PCP), Walentek 2018 (manipulation, cell types and imaging protocol), Huang & Niehrs 2014 & Haas et al. 2019 (deep cell characterization), Walentek et al. 2014 (SSCs in laevis, Dubaissi only described them in tropicalis).

We have added citations.

Minor Concerns:
1. I would suggest to use mucociliary instead of mucociliated as this is the usually used term for these epithelia.

We have adopted ‘mucociliary’ throughout manuscript.

2. I would indicate that those organoids are related to animal cap explants.

We have revised manuscript in introduction and discussion.

3. The overall language quality could be improved a bit.

Reviewer #5:

Manuscript Summary:

Overall, I think this is a very useful protocol that is suitable for JoVE given that some aspects of the method are quite technical and would best be described through visual demonstration. The authors describe each step very well in the main and the protocol appears to be easy to follow. The premise of the manuscript is that stem cells can be extracted from inner layers of the early ectoderm through micromanipulation. These cells can then be cultured in a simple buffer and subsequent steps of mucociliary development can be imaged live. The authors explain how the accessibility of the organoid allows for mechanical manipulation, drug treatments and imaging that may not be possible with other systems. This protocol is particularly important for understanding the process of re-epithelialization and differentiation of outer layer goblet cells after the stem cells have aggregated. The protocol takes the reader through the process of isolating tissue from correctly staged embryos, separating the deep cells from superficial cells and culturing these deep cells so they form aggregates. The protocol also describes how mRNA can be injected into early stage embryos so as to visualise membranes and the processes of epithelialization and intercalation by expression of fluorescently-tagged proteins (e.g. membrane GFP and ZO-1-RFP). A description of how to fix and immunostain aggregates at different stages of development is also provided. The authors also describe how to set up an imaging platform to visualise the aggregates in real-time (see minor concerns below). The two figures provided give a good overview of the process and show some real examples of imaging these aggregates at various time points.

I have some minor concerns and suggestions for improvement.

Major Concerns:

None

Minor Concerns:

*The authors use the term 'mesenchymal stem cells'. Do we know that these deep layer cells are mesenchymal stem cells - do they express specific markers that would allow us to describe them as such? I prefer the term multi-potent progenitors, which they have used several times. They also used the term pluripotent at some point. Can they be more consistent?

We have revised to multipotent progenitors throughout the manuscript. Please see the discussion about ‘mesenchymal’ in our response to Reviewer #1-1.

*The authors make no reference or comparison with the animal cap assay, which has been used for many years and gives rise to a mucociliary epithelium too. They of course use the cap assay to isolate the original tissue and then they dissect out the deep layer cells in order to form aggregates. The standard animal cap assay is in fact simpler because you don't have to separate layers. The benefit of the protocol described is that the process of re-epithelialization and differentiation of the outer layer goblet cells can be visualised, whereas in the standard animal cap assay the outer layer is already an epithelium. What I don't understand is how the protocol in this paper adds anything to visualising the transition of

inner layer cells (e.g. ciliated cells, ionocytes and SSCs) into the outer layer that the standard animal cap assay cannot already do? Is it just an alternative method in this regard or am I missing something? I think the protocol described is still valuable, but perhaps the authors should consider a comparison with the standard cap assay as well as to other models of mucociliary epithelia to really describe the value of the new protocol. Some references to the animal cap assay include Green J (1999), The animal cap assay, Methods Mol. Biol, 127:1-13 and Dingwell and Smith (2018), Dissecting and culturing animal cap explants, Cold Spring Harb. Protoc. These references, or similar, should be included anyway for more detail of the animal cap technique that is the start point for this protocol.

We have revised the manuscript to add distinctions to animal cap assay in introduction and discussion. Our protocol that using deep cells of ectoderm (animal cap) covers the regeneration of epithelial goblet cells that has not been accomplished in other protocols before (Kim et al.2020). Once surface cells are epithelialized in deep ectoderm aggregates, radial intercalation of inner cells to the newly epithelialized surface might be indistinct from the radial intercalation in the animal cap. Although we have not thoroughly tested differences of newly epithelized surface in aggregates and the native epithelium of the animal cap during radial intercalations, it will be interesting to investigate how the newly formed epithelium act as a substrate for intercalating cells.

Specific points for each section:
*Introduction - reference paper in line 75

We have revised the manuscript.

*Protocol - line 104: mention blastopore: dark pigmented cells around the blastopore

We have revised the manuscript.

-Line 107: might be worth showing the viewer how to make an eyebrow knife in the video

We will consider to add it for video production.

-Line 128: take vitelline membrane from vegetal side (where blastopore is)

We have revised the manuscript.

-Line 144: this ensures animal caps do not aggregate I presume

We have revised the manuscript for clarification.

-Line 146: do you use the eye brow knife to lift?

Yes, we have revised manuscript for clarification.

-Line 150-153: I guess 200P is same as P200? Also, you say use 10-15 uL to collect, so why do you use a P200? Minor detail but could you clarify and rephrase

We have revised the manuscript for clarification.

-Line 162-164: Is this just done at room temp.? Can this be slowed down or speeded up at different temperatures?

It could be done at different temperatures similar to explant culture, but we have not tested yet.

-Line 198-216: This is the most difficult part to visualise for me. How to make the imaging chamber and position the aggregate. I know this will be in the video, but could you also not put a simple diagram of the positions of everything (e.g. hard to understand how the cover glass goes on to the acrylic chamber and then how the TEM grid goes down. It also says to fill the imaging chamber with DFA twice - once in 4.2 and then in 4.6). This is the most confusing part of the protocol for me.

A schematic of the imaging chamber is added in Figure 2 to better introduce the setup. The imaging chamber first filled enough to set grids and aggregates (step5.4.2). Once organoids were ready for positioning, the chamber was filled up to the top (step 5.4.6.) to seal it without air bubbles inside. We have revised the manuscript to clarify this point.

-Line 226: Intelectin not Interlectin

We corrected the typo.

*Representative Results - line 244-246: what about the other cell type, the ionocyte? You mention ionocytes in Figure 1B, but no marker in Fig. 1C or in the text - is this because you don't have one or can't get one. Could you provide an example marker that could be used

We currently have not stained for ionocytes. We have revised the manuscript to clarity the point.

-Line 261: Colour-coded cells in Figure 2. Its not exactly clear what these represent. Do they represent specific cell types (e.g. ciliated cells, goblet cells etc.) or is it just saying that individual cells can be traced - could you clarify. Would also be useful to put this in the Figure 2 legend. In the legend you should say what the coloured cells represent and also what the coloured arrows represent so the figure stands alone.

We have revised the manuscript.

*Discussion - could add more about its potential uses, why it is superior to other techniques etc.

We have revised the discussion.

-Line 325: use of mesenchymal stem cells. Are they mesenchymal-like or actually mesenchymal stem cells?

We have revised the manuscript for clarification. Please see the discussion about 'mesenchymal' in our response to Reviewer #1-1.

-Line 338: it's good that specific steps to succeed are highlighted, but I feel like this part about NHS-rhodamine might be best to put as an optional step in the protocol rather than tucked away in the discussion. Most users will be simply following the protocol, so might be good to see this part there

We have updated the protocol (Step.1.3).

-Line 341-343: Again information on positioning of the aggregate might be better served within the protocol rather than at the end, when it might be too late

We have updated the protocol (Step.5.4.5).

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