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2D and 3D Human Induced Pluripotent Stem Cell-based Models to Dissect Primary Cilium Involvement During Neocortical Development

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TITLE:

2D and 3D Human Induced Pluripotent Stem Cell-Based Models to Dissect Primary Cilium Involvement During Neocortical Development

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primary cilium, human induced pluripotent stem cells, hIPSCs, neural stem and progenitor cells, human cerebral cortical development, neural rosettes, dorsal forebrain organoids, 2D and 3D hIPSC-based models of neocortical development, cerebral organoids, Sonic hedgehog, whole-mount immunostaining, optical clearing, light sheet microscope

SUMMARY:
We present detailed protocols for the generation and characterization of 2D and 3D human induced pluripotent stem cell (hIPSC)-based models of neocortical development as well as complementary methodologies enabling qualitative and quantitative analysis of primary cilium (PC) biogenesis and function.

ABSTRACT:
Primary cilia (PC) are non-motile dynamic microtubule-based organelles that protrude from the surface of most mammalian cells. They emerge from the older centriole during the G1/G0 phase of the cell cycle, while they disassemble as the cells re-enter the cell cycle at the G2/M phase boundary. They function as signal hubs, by detecting and transducing extracellular signals crucial for many cell processes. Similar to most cell types, all neocortical neural stem and progenitor cells (NSPCs) have been shown harboring a PC allowing them to sense and transduce specific signals required for the normal cerebral cortical development. Here, we provide detailed protocols to generate and characterize two-dimensional (2D) and three-dimensional (3D) cell-based models from human induced pluripotent stem cells (hIPSCs) to further dissect the involvement of PC during neocortical development. In particular, we present protocols to study the PC biogenesis and function in 2D neural rosette-derived NSPCs including the transduction of the Sonic Hedgehog (SHH) pathway. To take advantage of the three-dimensional (3D) organization of cerebral organoids, we describe a simple method for 3D imaging of *in toto* immunostained cerebral organoids. After optical clearing, rapid acquisition of entire organoids allows detection of both centrosomes and PC on neocortical progenitors and neurons of the whole organoid. Finally, we detail the procedure for immunostaining and clearing of thick free-floating organoid sections preserving a significant degree of 3D spatial information and allowing for the high-resolution acquisition required for the detailed qualitative and quantitative analysis of PC biogenesis and function.

INTRODUCTION:
Primary cilia (PC) are microtubule-based organelles that sense and transduce a plethora of chemical and mechanical cues from the extracellular environment. In particular, PC is the central organelle for the transduction of the Hedgehog signaling pathway in vertebrates[1,2]. While most neural cells have long been shown harboring a PC, the contribution of this organelle in shaping the central nervous system has long been undervalued. Studies on neocortical development have led to the discovery of multiple neural stem and progenitor cells (NSPCs), all harboring a PC, the location of which has been proposed to be crucial for progenitor fate determination[3–7]. PC has been shown crucial for cell mechanisms that are required for normal cerebral cortical development, including NSPC expansion and commitment[8–12] as well as apicobasal polarity of radial glial scaffold supporting neuronal migration[13]. In addition, PC are required during interneurons tangential migration to the cortical plate[14,15]. Finally, a role for the PC has been proposed in the establishment of synaptic connections of neurons in the cerebral cortex[16,17]. Altogether, these findings argue for a crucial role of PC at major steps of cerebral cortical development[18,19] and raise the need to

investigate their involvement in the pathological mechanisms underlying anomalies of cerebral cortical development.

Recent studies have largely improved our understanding of important cellular and molecular differences between cortical development in human and animal models, emphasizing the need to develop human model systems. In this view, human induced pluripotent stem cells (hIPSCs) represent a promising approach to study disease pathogenesis in a relevant genetic and cellular context. Adherent two-dimensional (2D) cell-based models or neural rosettes contain NSPCs similar to those seen in the developing cerebral cortex, which become organized into rosette-shaped structures showing correct apicobasal polarity[20–22]. Furthermore, the three-dimensional (3D) culture system allows the generation of dorsal forebrain organoids that recapitulate many features of human cerebral cortical development[23–26]. Those two complementary cell-based modeling approaches offer exciting perspectives to dissect the involvement of PC during normal and pathological development of the cerebral cortex.

Here, we provide detailed protocols for the generation and characterization of neural rosettes and derived NSPCs as well as dorsal forebrain organoids. We also provide detailed protocols to analyze the biogenesis and function of PC present on NSPCs by testing the transduction of the Sonic Hedgehog pathway and analyzing the dynamics of crucial molecules involved in this pathway. To take advantage of the 3D organization of the cerebral organoids, we also set up a simple and cost-effective method for 3D imaging of *in toto* immunostained cerebral organoids allowing rapid acquisition, thanks to a light sheet microscope, of the entire organoid, with high resolution enabling to visualize PC on all types of neocortical progenitors and neurons of the whole organoid. Finally, we adapted immunohistochemistry on 150 µm free-floating sections with subsequent clearing and acquisition using resonant scanning confocal microscope allowing high-resolution image acquisition, which is required for the detailed analysis of PC biogenesis and function. Specifically, 3D-imaging software allows 3D-reconstruction of PC with subsequent analysis of morphological parameters including length, number, and orientation of PC as well as signal intensity measurement of ciliary components along the axoneme.

PROTOCOL:

1. Generation of 2D hIPS cell-based models of neocortical development

1.1. Neural rosette formation

1.1.1. Start with hIPSC cultures harboring large regular colonies, exhibiting less than 10% differentiation and no more than 80% confluency.

1.1.2. Rinse the hIPSCs with 2 mL of PBS.

1.1.3. Add 2 mL of NSPC induction medium supplemented with the Rock inhibitor (NIM + 10 µM of Y-27632).

1.1.4. Manually dissect each hIPSC colony from one 35 mm dish using a needle to cut each colony precisely in horizontal and vertical directions to create a checker-board pattern dividing each colony into equal clusters.

1.1.5. Detach the colony using a cell scraper and transfer them onto an ultra-low-attachment 35 mm dish.

1.1.6. Let them float overnight (ON) in a humidified incubator at 37 °C and 5% CO_2, so that they can form embryoid bodies (EBs).

NOTE: Embryoid bodies (EBs) are defined as floating spheroid clusters or aggregates of hIPSCs.

1.1.7. Transfer the EBs into poly-L-ornithine/laminin (PO/lam)-coated 35 mm dishes in 2 mL NIM + 10 µM of Y-27632.

1.1.8. Daily refresh NSPC induction medium (NIM without Y-27632) until the formation of neural rosettes that takes approximately 12 to 14 days. Check under the microscope.

NOTE: After this step neural rosettes can be expanded, differentiated, and processed for immunostaining analysis or dissociated to get isolated neural stem progenitor cells (NSPCs).

1.2. Neural rosette expansion and early differentiation

1.2.1. Cut neural rosettes in a grid-like pattern with a needle and dislodge the clusters using a cell scraper.

1.2.2. Transfer the rosettes into a 4-well plate containing PO/lam-coated glass coverslip (4–5 rosettes/well) in 0.5 mL of NSPC maintenance medium (NMM).

1.2.3. Incubate the plate in a humidified incubator at 37 °C and 5% CO_2.

1.2.4. Refresh NMM every other day until day 20.

1.2.5. From Day 20, culture neural rosettes in 0.5 mL of cytokine depleted NMM to allow differentiation. Refresh the medium every 2–3 days.

1.2.6. At Day 30 and Day 40 (10 or 20 days of differentiation), fix the rosettes with 0.5 mL of 4% PFA, 20 min, RT.

1.3. PC biogenesis and function analysis on isolated NSPCs

1.3.1. NSPC dissociation

1.3.1.1. Cut neural rosettes from step 1.1.8 in a grid-like pattern with a needle and dislodge the clusters using a cell scraper. Transfer the cells into PO/lam-coated 35 mm dishes in 2 mL of NMM and incubate in a humidified incubator at 37 °C and 5% CO_2.

1.3.1.2. Refresh NMM every other day until confluency (approximately 5–7 days).

1.3.1.3. After scraping away the large, clear cells surrounding neural rosettes, pick them manually and transfer them onto fresh PO/lam-coated 35 mm dishes to enrich for NSPCs and deplete non-neural cell types.

1.3.1.4. After one or two manual passages (repeat step 1.3.1.3 if needed) required to remove all contaminating cell types, remove the medium and rinse with PBS.

1.3.1.5. Add 300 µL of 0.05% trypsin and incubate for 5 min at 37 °C until most cells detach.

1.3.1.6. Add 2 mL of the medium containing 10% FBS (DMEM + 10% FBS) to inactivate the trypsin. Collect the cell suspension in a 15 mL conical tube. Gently pipette the cell suspension up and down three times to break up the cell clumps.

1.3.1.7. Centrifuge at 300 x *g* for 5 min.

1.3.1.8. Carefully aspirate the supernatant and resuspend the cells in NMN.

1.3.1.9. Seed the dissociated NSPCs as single cells (1 x 10^5 cells/cm^2) in NMM onto PO/lam-coated dishes and incubate them in a humidified incubator at 37 °C and 5% CO_2.

1.3.1.10. Expand NSPCs in NMM by changing the medium every other day.

NOTE: NSPCs are seeded at high density to avoid differentiation.

1.3.2. **PC biogenesis analysis**

1.3.2.1. Seed dissociated NSPCs at 100,000 cells/cm^2 and incubate them in a humidified incubator at 37 °C and 5% CO_2 in NMM for 2 days.

1.3.2.2. Aspirate the medium and starve NSPCs in cytokine depleted medium (NSPC starvation medium, **Supplemental Table 1**) for 48 h.

1.3.2.3. Fix NSPCs with 4% PFA for 20 min at RT.

1.3.2.4. Wash twice with PBS for 5 min at RT before immunostaining.

1.3.3. **PC function analysis: SHH signaling pathway**

1.3.3.1. Seed dissociated NSPCs at 100,000 cells/cm^2 on PO/lam-coated 8-well Chamber Slides (300 µL) or T25 dishes (5 mL) and incubate in a humidified incubator at 37 °C and 5% CO_2 in NMM for 2 days.

1.3.3.2. Starve NSPCs in NSPC starvation medium (300 µL per well of 8-well chamber slide and 5 mL in T25 flask) for 48 h.

1.3.3.3. To induce the SHH pathway, incubate the NSPCs with the starvation medium supplemented with recombinant SHH (100 ng/mL) or SAG (500 nM); (150 µL per well of an 8-well chamber slide and 2 mL in T25 flask) for 24 h.

1.3.3.4. Fix NSPCs cultured on 8-well Chamber Slides in 250 µL of 4% PFA per well for 20 min at RT and wash them twice with 250 µL of PBS for 5 min at RT before immunostaining analysis.

1.3.3.5. Remove the medium and wash NSPCs cultured in T25 dishes with PBS.

1.3.3.6. Add 500 µL of 0.05% trypsin and incubate for 5 min at 37 °C until the cells detach.

1.3.3.7. Add 2 mL of medium containing 10% FBS (DMEM + 10% FBS) to inactivate the trypsin and collect the cell suspension in a 15 mL conical tube.

1.3.3.8. Centrifuge at 300 x *g* for 5 min and carefully aspirate the supernatant to obtain NSPC pellets that can be cryopreserved at -80 °C before RNA extraction and RT-PCR analysis.

2. Generation of dorsal forebrain organoids

2.1. Single-cell culture of hIPSCs

2.1.1. Start with hIPSC cultures harboring large regular colonies exhibiting less than 10% differentiation and that have been passaged almost once. Ensure that the cells are no more than 80% confluent.

2.1.2. Wash the colonies with 2 mL of PBS.

2.1.3. Add 500 µL of Gentle Cell Dissociation Reagent (GCDR) and incubate for 5 to 7 min at 37 °C.

2.1.4. Aspirate the GCDR and add 2 mL of mTeRS1 medium supplemented with 5 µM of Y-27632.

2.1.5. Pipette the cells gently up and down 10 times to dissociate all colonies into single cells.

2.1.6. Transfer the cells on vitronectin-coated dishes and incubate in a humidified incubator at 37 °C and 5% CO_2.

NOTE: Perform at least 1 passage of the hIPSCs using GCDR prior to the differentiation experiment to adapt the hIPSCs to single-cell culture conditions.

2.2. On Day 0, allow hIPSCs to form embryonic bodies in EB medium containing a low concentration of FGF2 and a high concentration of rock inhibitor required for hIPSC survival. To do so follow the steps below.

2.2.1. Wash the colonies with 2 mL of PBS.

2.2.2. Add 500 µL of Gentle Cell Dissociation Reagent (GCDR) and incubate for 5 to 7 min at 37 °C.

2.2.3. Aspirate the GCDR and add 1 mL of EB medium supplemented with 50 µM of Y-27632; Use a 1 mL pipette to gently detach the cells and transfer them to a 15 mL conical tube.

2.2.4. Rinse once more with 2 mL of EB medium supplemented with 50 µM of Y-27632, transfer the remaining cells to the 15 mL conical tube. Immediately add 3 mL of EB medium supplemented with 50 µM of Y-27632 to the conical tube and gently homogenize the solution using a 5 mL pipette.

2.2.5. Centrifuge the dissociated cells at 100 x *g* for 5 min.

2.2.6. Gently aspirate the supernatant and resuspend the cells in 6 mL of EB medium supplemented with 50 µM of Y-27632.

2.2.7. Centrifuge the cells at 100 x *g* for 5 min.

2.2.8. Aspirate the supernatant and resuspend the cells in 1 mL of EB medium supplemented with 50 µM of Y-27632. Use a 1 mL pipette to gently dissociate the cells into a single-cell suspension.

2.2.9. Immediately after resuspending the cells, dilute and count them.

2.2.10. Dilute the proper volume of cell suspension (containing 900,000 cells) into 30 mL of EB medium supplemented with 50 µM of Y-27632 and 4 ng/mL of bFGF and gently mix the solution.

2.2.11. Plate 9,000 cells/well into an ultra-low attachment 96U plate (300 µL/well). To avoid disturbing EB formation, incubate the plate in a humidified incubator at 37 °C and 5% CO_2 until Day 3 without medium change.

2.3. **Neural induction (Dual SMAD inhibition)**

2.3.1. On Day 3, ensure EBs measure 300–400 µm and show regular borders. If both criteria are reached, replace half of the medium (150 µL) with induction medium-1 containing Dual SMAD inhibitors, leading to brightening of the contour of the EBs by Day 6 to Day 7 indicating neuroectodermal differentiation (**Supplemental Table 2**).

2.3.2. On Day 4, replace 3/4 of the medium (225 µL) with induction medium-1.

2.3.3. On Day 6 and Day 8: Refresh half of the induction medium-1 (150 µL).

2.4. **Organoid embedding into basement membrane matrix (Day 10)**

2.4.1. On Day 10, embed EBs in growth factor reduced basement membrane matrix (BMM).

NOTE: From this step, always use sterile scissors to cut the opening of the pipette tips to avoid damaging the organoids by repeated pipetting.

2.4.2. First transfer around 15–17 organoids in conical tubes. Let the EBs settle and remove the medium. Add 50 µL of induction medium-2 and transfer them into a microcentrifuge tube containing 100 µL of BMM.

2.4.3. Spread the matrix-EB mixture onto the center of a well of an ultra-low-attachment plate and separate the EBs to prevent them from fusing.

2.4.4. Let the BMM solidify in the incubator at 37 °C for 45 min.

2.4.5. Add 3 mL of the induction medium-2 in each well and put the plate in a humidified incubator at 37 °C and 5% CO_2.

NOTE: Ensure that this procedure is done as quickly as possible as BMM solidifies at room temperature.

2.5. **Stationary culture of organoids embedded into basement membrane matrix**

2.5.1. Day 12, 14: Refresh induction medium-2.

2.5.2. Day 16: Refresh induction medium-2 and check under a microscope the expansion of neuroepithelial loops.

2.6. **Basement membrane matrix dissociation and culture on an orbital shaker**

2.6.1. On Day 17, dissociate organoids from BMM by pipetting up and down 10 times with a 5 mL pipette and transfer them into differentiation medium-1 (without vitamin A). Incubate onto an orbital shaker at 80 rpm in a humidified incubator at 37 °C and 5% CO_2 to improve nutritional absorption.

NOTE: Use a different incubator for stationary culture and culture onto an orbital shaker to avoid any vibrations detrimental to adherent hIPS cell growth.

2.6.2. Day 19: Refresh differentiation medium-1.

2.6.3. Day 21: Change differentiation medium-1 to differentiation medium-2 (with vitamin A).

2.6.4. From Day 23 to Day 35: Refresh medium with differentiation medium-2 every 2–3days.

2.6.5. From Day 35 to Day 70: Refresh differentiation medium-2 with 1% BMM and refresh every 2–3 days.

2.6.6. Collect the organoids after 28, 42, and 70 +/- 2 days of differentiation and fix them overnight at 4 °C, in 5 mL 4% PFA on a 15 mL conical tube.

2.6.7. For further *in toto* or free-floating immunostaining analysis, organoids are then rinsed twice in PBS and conserved at 4 °C in PBS + 0.05% sodium azide.

2.6.8. For immunostaining analysis on frozen sections, immerse 4% PFA fixed organoids in 10 mL 30% sucrose at +4 °C ON (or until organoids sink). Embed them into a frozen embedding matrix and store at -80°C until cryosectioning.

3. *In toto* immunolabeling, clearing, and lightsheet acquisition of dorsal forebrain organoids

3.1. Fixation

3.1.1. Collect the organoids in 6-well plates, remove the medium, and fix them with 2 mL of 4% PFA, at 4 °C, overnight.

3.1.2. Perform three washes in 2 mL of PBS at room temperature.

3.1.3. Transfer the organoids into 2 mL tubes and store at 4 °C in 2 mL of PBS + 0.05% sodium azide.

3.2. Permeabilization

3.2.1. Incubate organoids in 1 mL of PBS containing 0.2% Triton X100 at RT for 1 h. Do this twice.

3.2.2. Incubate in 1 mL of PBS containing 0.2% Triton X100 and 20% DMSO, at 37 °C, overnight.

3.2.3. Incubate in 1 mL of PBS containing 0.1% Tween20, 0.1% Triton X100, 0.1% Deoxycholate, 0.1% NP40 and 20% DMSO, at 37 °C, overnight.

3.2.4. Incubate in 1 mL of PBS containing 0.2% Triton-X100, at RT, for 1 h. Do this twice.

3.3. Blocking and immunolabeling

3.3.1. Block in 1 mL of blocking solution (PBS, 0.2% Triton X100, 6% Donkey Serum,10% DMSO), at 37 °C, ON.

3.3.2. Incubate with primary antibodies diluted in 250 µL of PBS, 0.2% Tween20, 0.1 µg/mL of Heparin, 5% DMSO and 3% Donkey Serum, at 37 °C, for 2 days.

3.3.3. Wash in 1 mL of PBS containing 0.2% Tween20 and 0.1 µg/mL of Heparin, for 1 h, at 37 °C. Do this four times.

3.3.4. Wash in 1 mL of PBS containing 0.2% Tween20 and 0.1 µg/mL of Heparin, overnight, at 37 °C.

3.3.5. Incubate with secondary antibodies diluted in 250 µL of PBS containing 0.2% Tween 20, 0.1 µg/mL of Heparin and 3% Donkey Serum, at 37 °C, for 2 days.

3.3.6. Wash in 1 mL of PBS containing 0.2% Tween 20 and 0.1 µg/mL of Heparin, for 1 h, on a wheel, at RT. Do this four times.

3.3.7. Wash in 1 mL of PBS containing 0.2% Tween 20 and 0.1 µg/mL of Heparin, overnight, on a wheel, at RT.

3.3.8. Wash in 1 mL of PBS, for 24 h, at RT.

3.3.9. Stock at +4 °C in 1 mL of PBS and 0.05% sodium azide until clearing.

3.4. **Clearing in TDE (2,2'-Thiodiethanol)**

3.4.1. Incubate the organoids in 1 mL of 30% TDE (3 mL of TDE + 7 mL of PBS), for 24 h, at RT.

3.4.2. Incubate in 1 mL of 60% TDE (6 mL of TDE + 4 mL of PBS), for 24 h, at RT.

3.4.3. Incubate in 1 mL of 80% TDE (8 mL of TDE + 2 mL of PBS), for 24 h, at RT.

3.5. **Organoid embedding prior to light sheet acquisition**

NOTE: Use a custom-made system; made with a 1 mL syringe with the tip cut with a scalpel.

3.5.1. Prepare 100 mL of 4% Low-Melting Agarose in 60% TDE solution and aliquot in 2 mL tubes. Conserve at +4 °C.

3.5.2. Prior to the organoid embedding, preheat one tube containing 1.5 mL of 4% low-melting agarose in 60% TDE in a water bath at 37 °C until it liquefies.

3.5.3. Meanwhile, prepare a custom-made molding system made from a 1 mL syringe the tip of which is cut off using a scalpel.

3.5.4. Pump in the syringe 600 µL of the gel solution using the plunger.

3.5.5. Position the sample using an enlarged pipette tip the opening of which has been cut using sterile scissors.

3.5.6. Fill in the syringe with 400 µL of gel solution so that the sample is positioned within the lower third of the syringe.

3.5.7. Let the gel polymerize and store in 80% TDE solution at 4 °C protected from light until acquisition.

3.5.8. For Light sheet acquisition, use a 20x objective immersed in 80% TDE solution added in the sample chamber to allow to accurately adjust to the refractive index of the clearing method.

3.5.9. Insert the syringe containing the agarose embedded organoid in the largest sample holder designed to accommodate a 1 mL syringe the plunger of which can be operated to position the organoid in front of the objective.

NOTE: Light sheet acquisition of an entire organoid takes approximately 5 to 10 min.

4. Immunostaining and clearing of free-floating sections of dorsal forebrain organoids

4.1. Fixation, agarose embedding, and sectioning of the organoids

4.1.1. Collect organoids after 28, 42, and 70 +/- 2 days of differentiation in a 6-well plate and fix overnight at 4 °C in 2 mL of 4% PFA.

4.1.2. Rinse twice in 2 mL of PBS and conserve at 4 °C in 2 mL of PBS + 0.05% sodium azide.

4.1.3. Embed the fixed organoids into 4% low-melting agarose in a plastic embedding mold (7 x 7 mm). Carefully remove the agarose block from the plastic mold and glue it to the vibratome stage.

4.1.4. Section the embedded organoids using a vibratome to obtain 150 µm free-floating sections transferred in a well of a 24-well plate containing 500 µL of PBS using a paintbrush to avoid damaging the sections.

NOTE: Low-melting agarose is recommended as its melting temperature is only approximately 60 °C. Prepare 4% low-melting agarose in PBS solution and leave it to cool just above 37 °C in a water bath prior to organoid embedding.

4.2. Permeabilization, blocking, and immunostaining

4.2.1. Incubate the sections in 500 µL of PBS containing 0.3% Triton X100, at RT, under agitation, for 20 min. Do this three times.

4.2.2. Incubate the sections in 500 µL of PBS containing 0.3% Triton X100 and 5% nonfat Milk, at RT, under agitation, for 2 h.

4.2.3. Incubate the sections in 250 µL of primary antibody solution (PBS + 0.3% Triton X100 + 1% nonfat Milk), under agitation, at +4 °C, overnight.

4.2.4. Wash the sections in 500 µL of PBS, at RT, under agitation, for 20 min. Do this four times.

4.2.5. Incubate the sections in 250 µL of secondary antibody solution (PBS + 0.3% Triton X100 + 1% nonfat milk), at RT, under agitation, for 2 h.

4.2.6. Wash the sections in 500 µL of PBS, at RT, under agitation, for 20 min. Do this four times.

4.2.7. Store the sections in 500 µL of PBS, at +4° C until clearing.

4.3. **Clearing in TDE (2,2'-Thiodiethanol)**

4.3.1. Incubate the sections in 500 µL of 30% and 60% TDE for 1 h each at RT, and then in 500 µL of 80% TDE overnight, at RT.

4.3.2. Store the cleared sections in 500 µL of 80% TDE until acquisition at +4 °C.

4.4. **Mounting of free-floating sections prior to acquisition with resonant scanning confocal**

4.4.1. Mount a free-floating section in a sealed chamber enabling to maintain the sample in 80% TDE solution and designed to adapt on a motorized XY stage of confocal microscopes.

4.4.2. Put one round coverslip in the chamber system.

4.4.3. Carefully transfer the cleared immunostained section using a paintbrush.

4.4.4. Fill the chamber with 80% TDE solution.

4.4.5. Add two standard coverslips plus one second round coverslip and a silicone seal.

4.4.6. Screw on the screwing ring of the chamber to perfectly seal the system.

5. Light sheet and resonant scanning confocal analysis

5.1. Process light sheet and resonant scanning acquisitions using software that enable 3D visualization and analysis of the entire immunostained sample.

NOTE: Such software allows to quickly open huge data to easily make snapshots and animations. It allows to move the sample in different orientations and to generate 2D views thanks to a 2D slicer tool in different orientations, XY and YZ for example.

5.2. For automatic detection of both centrosomes and primary cilia, use a spot wizard enabling to quantify their number in pathological versus control conditions.

5.3. For 3D reconstruction of PC enabling precise measurement of their length, use a filament wizard to manually fix the starting point of the PC and the software uses the fluorescence signal to reconstruct the PC precisely.

REPRESENTATIVE RESULTS:

2D hIPS cell-based models to study primary cilium biogenesis and function

The protocol detailed here has been adapted from previously published studies[20–22]. This protocol allows the generation of neural rosette structures that contain neocortical progenitors and neurons similar to those seen in the developing neocortex. Detailed validation can be performed by conventional immunostaining analysis using specific makers[3]. For instance, apical progenitors (AP) should be double-stained with SOX2 and PAX6, intermediate progenitors (IP) are revealed by TBR2/EOMES staining, and early-born neocortical neurons are revealed by CTIP2 staining (**Figure 1A–C**). Such neural rosette-like structures model interkinetic nuclear migration (INM) of AP that can be visualized by immunostaining with antibodies raised against phospho-vimentin, which stains mitotic nuclei and TPX2 that stains the mitotic spindle. These markers, therefore, allow the analysis of several characteristics of AP, including the INM with cell division to take place apically around the central lumen (**Figure 1D,D'**), as well as the division mode determined by measuring the angle between the division plane and the apical surface. Finally, ciliogenesis can be analyzed by immunostaining with antibodies raised against PCNT, which stains the basal body of PC, and ARL13B that stains the axoneme. On such rosette structures, PC extend from the apical pole of APs into the central lumen of each ventricle-like region (**Figure 1E,E'**), while they are also protruding from CTIP2+ neurons (**Figure 1E''**).

Dissociation of these rosette structures allows obtaining isolated NSPCs cultured on poly-L-ornithine/laminin-coated culture plates in NMM at a high density to allow maintenance of a stable and expandable population of NSPCs for at least 15 passages without accumulating karyotype abnormalities[21,27]. To analyze PC biogenesis, dissociated NSPCs are cultured to confluence and starved for 48 h. Immunostaining analyses using antibodies raised against PC markers show that those NSPCs harbor PC (**Figure 2A**). By combining two complementary open-source tools, Ilastik, a machine-learning-based image analysis tool useful for PC segmentation[28] and CiliaQ, a Fiji/ImageJ plugin package[29], that enable 3D reconstruction of PC from 3D confocal image stacks, several structural parameters can be easily evaluated, including the number of PC and their length.

PC function of NSPCs can also be evaluated by testing the transduction of the Hedgehog signaling pathway. To assess the transduction of the SHH signaling pathway, NSPCs are starved for 48 h and treated with recombinant SHH (rSHH) or a smoothened agonist (SAG) for 24 h. Using antibodies raised against GPR161, SMO, and GLI2, immunofluorescent (IF) analysis allows testing the trafficking of these key SHH signaling actors along the PC, with GPR161 normally exiting the PC while GLI2 and SMO accumulate within the PC in response to SHH pathway activation (**Figure 2C–D**). Analysis of 3D confocal image stacks using CiliaQ tools allows quantifying GPR161 exit from the PC as well as GLI2 and SMO accumulation within the PC after SHH pathway activation (**Figure 2F–G**). In addition, semi-quantitative RT-PCR analysis on mRNA extracted from rSHH- or SAG-treated NSPCs shows the induction of two SHH target genes, *GLI1* and *PTCH1*, attesting for normal SHH signaling transduction in NSPCs derived from control hIPSCs (**Figure 2B**).

Overall, 2D cell-based models of developing dorsal forebrain clearly reproduce several aspects of normal cerebral cortex development and represent promising tools to dissect PC-associated mechanisms underlying anomalies of neocortical development using control versus patient hIPSCs harboring mutations in genes responsible for human developmental anomalies of the cerebral cortex.

3D hIPS cell-based models to study primary cilium involvement during neocortical development

The protocol described here (**Figure 3A**) has been adapted from previously published protocols[23–26,30] and successfully tested on five distinct control hIPSC lines. It allows the generation of hIPSC-derived dorsal forebrain organoids that increased in size over time while forming large neuroepithelial loops (**Figure 3B**). Immunolabeling analysis either on organoids cryosections (cryostat, 20 µm), free-floating sections (vibratome, 150 µm), or *in toto*, can be performed for quality controls. At day 28 ± 2, organoids consist of stratified neuroepithelial loops co-expressing the neural progenitor marker SOX2 and the forebrain marker PAX6, attesting for dorsal forebrain identity. At day 42 ± 2 (6 weeks of differentiation), those loops should display a more complex stratified organization. From the apical to the basal surface of these loop structures, a ventricular zone (VZ)-like region with SOX2/PAX6-positive apical radial glial progenitors (aRG) can be delineated as well as a subventricular zone (SVZ)-like region with TBR2-positive intermediate progenitors (IPs) and a cortical plate (CP)-like region containing CTIP2-positive early-born neocortical neurons (**Figure 3C,E**). The apical molecular polarity of aRG can be evaluated by the apical enrichment at the ventricular surface of ZO-1 and N-CADH (**Figure 4A, Video 1**). The aRG progenitor division properties can be evaluated using TP2X and P-VIM markers to analyze INM that normally leads to the alignment of aRG mitotic nuclei at the ventricular surface of the cortical loops (**Figure 4B, Video 2**). Such immunolabeling also allows measurement of the division angle to evaluate symmetric versus asymmetric division mode of aRG. P-Vim positive progenitors can also be observed in the SVZ-like region, harboring a unique basal process extending to the basal surface reminiscent of outer radial glia (oRG or basal radial glia, **Figure 4C, Video 2**). While they are absent from day 42 organoids, SATB2 positive late-born neurons should be detected from day 70 (10 weeks of differentiation) (**Figure 3D,F**), attesting for *in vivo*-like timing of neocortical neuronal differentiation.

Immunolabeling experiments using the basal body (gamma Tubulin) and axoneme (ARL13B) markers allow visualization of PC on 20 µm cryosections (**Figure 3G,H**). To take advantage of the 3D organization of dorsal forebrain organoids, whole-mount immunostaining can be performed. Light sheet acquisition of such *in toto* immunostained and cleared organoids allows detection of both centrosomes and PC on all NSPC types as well as on neocortical neurons (**Video 3**). In addition, to perform more accurate analyses of PC biogenesis, immunohistochemistry analysis on free-floating thick sections (150 µm) of dorsal forebrain organoids may be performed. After clearing, such sections may be acquired using a 40x (NA 1.3) or 63x (NA 1.4) oil objective of an inverted resonant white light confocal laser microscope, allowing to improve resolution while preserving the 3D spatial information of organoids. Such laser scanning confocal microscope enables rapid acquisition of thick sections, with a precise and flexible excitation and detection without any interference (**Video 4**). Several structural parameters of PC can be evaluated, including PC number, length, and orientation, using 3D-imaging software. Dedicated open-source, freely available tools such as Ilastik[28], a machine-learning-based image analysis tool as well as the CiliaQ plugin package on Fiji/ImageJ[29] are highly useful for automatic PC segmentation allowing subsequent qualitative and quantitative analysis of PC biogenesis and function in control versus pathological conditions.

FIGURE LEGENDS:

Figure 1: Generation and characterization of 2D neural rosettes. Immunohistochemical characterization of neural rosette structures using (**A**) SOX2 and PAX6 antibodies to detect AP, (**B**) TBR2/EOMES to reveal IP, and (**C**) CTIP2 to stain early-born neocortical neurons. Proliferating AP are detected with phospho-vimentin and TPX2 antibodies and are located at the apical surface (**D,D'**). PC are revealed by double immunostaining with PCNT and ARL13B antibodies. PC extend from each AP into the central lumen of each rosette, while they are also detected on IP and early-born neurons (**E,E',E''**).

Figure 2: Isolated NSPCs derived from 2D neural rosettes harbor functional PC. Dissociation of 2D-neural rosettes gives rise to isolated NSPCs harboring PC after starvation for 48 h as revealed by ARL13B and PCNT immunostaining (**A**). NSPCs harbor functional PC as revealed by RT-PCR quantification of two SHH target genes *GLI1* and *PTCH1* after starvation for 48 h and subsequent activation of the pathway by treatment with recombinant SHH (rSHH) for 24 h. *GLI1* and *PTCH1* expression data were performed in triplicate and normalized to *ACTB* expression data. Data were analyzed with the $2^{-\Delta\Delta Ct}$ method and presented as relative expression ± SEM (Mann Whitney test). (**B**). IF analysis on starved NSPCs with (**D**) or without (**C**) rSHH treatment to test the dynamics of two crucial actors of the SHH signaling pathway, GLI2, and GPR161, which respectively accumulate or exit the PC after SHH pathway activation. By combining Ilastik and CiliaQ tools for the analysis of confocal images, GPR161 (**F**) and GLI2 (**G**) signal intensity within PC was compared in +/- rSHH treated NSPCs. ARL13B staining was used for PC segmentation, and as expected PC length was unchanged in +/- rSHH treated NSPCs (**E**). Data are summarized in box and whisker plots (mean values ± SEM). ****$p < 0.0001$ (Mann Whitney test).

Figure 3: Generation and characterization of dorsal forebrain organoids. (**A**) Schematic overview of the protocol to generate dorsal forebrain organoids. (**B**) Representative bright-field images of organoids at days 1, 3, 7, 24, and 42 of differentiation. Confocal images of 20 µm cryosections of organoids at day 42 (**C**) and 70 (**D**) after immunostaining with SOX2, TBR2, and CTIP2 antibodies delineating, respectively, the ventricular zone (VZ), the subventricular zone (SVZ) containing TBR2 positive IP, and the preplate-like region (CP) containing CTIP2 positive early-born neocortical neurons. Confocal images of 20 µm cryosections of organoids at day 42 (**E**) and 70 (**F**) after immunostaining with PAX6, CTIP2, and SATB2 antibodies showing the appearance of SATB2 positive late-born neurons at Day 70. Confocal images of 20 µm cryosections of an organoid at day 42 after immunostaining with ARL13B and PCNT antibodies revealing PC at the apical pole of radial glial progenitors (**G**) while they are also present on CTIP2 positive neurons (**H**).

Figure 4: 3D imaging of dorsal forebrain organoids. (**A**) Light sheet acquisition of an entire dorsal forebrain organoid (Day 42) stained with PAX6, N-CADH, and CTIP2 antibodies showing the multiple neuroepithelial loops with PAX6 positive radial glial progenitors exhibiting correct apicobasal polarity as revealed by the accumulation of N-Cadherin at their apical side and CTIP2 positive early-born neocortical neurons delineating a preplate-like region. (**B**) Light sheet acquisition of an entire dorsal forebrain organoid (Day 42) stained with TPX2, P-Vim, and CTIP2 antibodies illustrating interkinetic nuclear migration of ventricular radial glial progenitors. (**C**) Zoom on the light sheet acquisition of an entire dorsal forebrain organoid (Day 42) stained with TPX2 and P-Vim antibodies showing a progenitor extending a unique

basal process and localized in the subventricular zone, reminiscent of an outer radial glial progenitor. (**D**) Resonant Scanner acquisition of a 150 µm section of a dorsal forebrain organoid (Day 42) stained with PCNT and ARL13B antibodies showing PC in NSPCs and neocortical neurons.

Figure 5: Generation and characterization of 2D and 3D hIPS cell-based models of dorsal forebrain development to dissect PC involvement to the pathophysiology of cerebral cortical anomalies. hIPS cells are allowed to form embryoid bodies (EBs) into low adhesion culture plates, and are then incubated into an induction medium containing dual SMAD inhibitors to induce neuroectodermal differentiation. Adhesion of EBs into PO/lam-coated dishes allows the generation of 2D neural rosette structures while the free-floating culture of EBs with subsequent embedding into BMM allows the generation of dorsal forebrain organoids. Withdrawal of mitogenic factors from adherent neural rosette medium promotes the onset of neurogenesis that can be tested by IF analysis. Dissociation of adherent neural rosettes allows the generation of isolated NSPC cultures useful for PC biogenesis and function analysis. Dorsal forebrain organoids are collected after 4, 6, or 10 weeks of differentiation and fixed for subsequent immunostaining analysis either *in toto*, on 150 µm thick sections, or 20 µm cryosections.

Video 1: Light sheet acquisition of an entire dorsal forebrain organoid (Day 42) immunostained with PAX6, CTIP2, and NCADH antibodies.

Video 2: Light sheet acquisition of an entire dorsal forebrain organoid (Day 42) immunostained with TPX2, P-VIM, and CTIP2 antibodies.

Video 3: Light sheet acquisition of an entire dorsal forebrain organoid (Day 42) immunostained with PCNT and ARL13B antibodies.

Video 4: Resonant scanning confocal acquisition of a 150 µm section of a dorsal forebrain organoid (Day 42) immunostained with ARL13B and PCNT antibodies.

SUPPLEMENTARY FILES:

Supplementary Table 1: Recipe of the media used for the generation of 2D-neural rosettes and NSPCs.

Supplementary Table 2: Recipe of the media used for the generation of dorsal forebrain organoids.

DISCUSSION:

PC are now regarded as key organelles regulating crucial steps during normal cerebral cortical development[18,19,31] including NSPC expansion and commitment[8–12] as well as neuronal migration[13,14] and synaptogenesis. In addition to analysis in animal models or human fetal cerebral tissues, the generation of highly innovative and relevant patient-derived hIPSC-based models of neocortical development is essential to dissect the role of PC during both normal and pathological cerebral cortical development.

The 2D hIPSC-based modeling protocol detailed here was adapted from three major publications[20–22]. Neuroepithelial differentiation is induced by dual SMAD inhibition using small molecule inhibitors of the activin/nodal (SB-431542) and BMP (LDN-193189) pathways[22]. Cells are organized in rosette-shaped structures containing NSPCs as well as neocortical deep layer neurons after 20 days of differentiation. In addition, these rosette-like structures show correct apicobasal polarity, interkinetic nuclear migration of apical progenitors as well as PC extending from all NSPCs and neurons. After dissociation of these rosette structures, a homogeneous and stable population of cortical progenitors is obtained[21]. When cultured to confluence and starved for 48 h, those cortical progenitors harbor PC for which the morphology, number, and length can be easily analyzed by combining Ilastik[28] and CiliaQ[28,29] plugin package on Fiji/ImageJ. Furthermore, by using cytokines to induce the SHH signaling pathway, PC function can also be used by IF to test the dynamics of crucial signaling pathway actors along the PC such as GLI2, SMO, and GPR161. In addition, semi-quantitative RT-PCR assays enable testing of the induction of SHH target gene expression, including *GLI1* and *PTCH1*, in response to SHH pathway activation. Other signaling pathways dependent upon PC function should also be tested, including WNT and IGF pathways[2,32,33]. To conclude on this 2D modeling approach, which clearly reproduces several aspects of normal cerebral cortex development, it represents a useful and relevant tool for testing PC biogenesis and function in normal versus pathological conditions and should contribute to gain further insight into the involvement of PC during neocortical development.

Complementary to 2D hIPSC-based modeling approaches, dorsal forebrain organoids offer unprecedented opportunities to investigate normal and pathological cerebral development *in vitro* as they recapitulate many features and characteristics of the early developing human cerebral cortex. Two main types of protocols are currently used: the intrinsic and the guided methods. The intrinsic protocol developed by Lancaster and colleagues[23] relies on the intrinsic ability of IPSCs to self-organized with minimal external factors and gives rise to cerebral organoids containing rudiments of distinct brain regions offering a unique opportunity to model the interactions between different brain regions. However, the high variability and heterogeneity inherent to such modeling strategy present significant challenges of reproducibility. Guided organoid differentiation protocols allow the generation of brain region-specific organoids with minimal heterogeneity[24–26]. This approach allowed us to successfully generate dorsal forebrain organoids with ventricle-like structures that recapitulate major processes of early human cerebral cortical development. The first critical issue is to start with high-quality hIPSC cultures harboring large regular colonies exhibiting less than 10% differentiation and which have been passaged almost once as a monolayer to adapt the hIPCs to single-cell culture conditions. Indeed, to limit organoid heterogeneity, one of the major challenges in the field, the formation of EBs with a homogeneous size is a prerequisite that implies the need for dissociation of hIPSC colonies into single-cell suspension allowing seeding at the defined cell density. Another critical step is BMM inclusion of EBs, needed to support 3D structure and neuroepithelial expansion. We favored the group inclusion of about fifteen organoids over individual inclusion even if it involves an additional step, the BMM dissociation. BMM dissociation prior to shaking culture has been shown to reduce variability within and between organoid batches, thus resulting in higher reproducibility[34]. In addition, it allows getting rid of cell processes extending into the BMM which are not detrimental for the following differentiation steps but which make it difficult to observe organoids for quality inspection during subsequent steps of the procedure. To improve nutritional absorption and

oxygen exchange, we compared organoid maturation on orbital shakers and spinning bioreactors that led to similar results. We, therefore, chose the orbital shaker option, as it allows to significantly reduce the medium volumes and therefore the total cost of the experiments. Importantly, use a different incubator for stationary and shaking culture on an orbital shaker to avoid any vibrations detrimental to adherent hIPSC growth. We successfully applied this protocol on five distinct control hIPSC lines[35] that give rise to homogeneous results ensuring the robustness of this procedure.

Characterization of such organoids can be achieved using several methods. To preserve the 3D spatial information within organoids, we set up a protocol allowing whole-mount immunostaining and clearing of entire organoids with subsequent light sheet acquisition. Different clearing methods have emerged with efficiency depending upon sample origin and thickness[36,37]. Here, we set up a simple, fast, and cost-effective clearing method that relies on TDE (2,2'-thiodiethanol), a glycol derivative previously used to clear mouse brain and intestinal organoids[38,39]. Acquisition of immunostained and cleared organoids was performed on a light sheet microscope using a 20x objective immersed in 80% TDE. In comparison to other 3D imaging acquisition methods, light sheet microscopy is of interest for several reasons: fast acquisition, good penetration, and reduced photobleaching. Optimization of the permeabilization step enables to reach efficient and homogeneous antibody penetration allowing to visualize basal bodies and axonemes of PC extending from all progenitor and neuronal cell types of the whole organoid. Furthermore, acquisition of free-floating 150 µm thick sections using an inverted resonant scanning confocal microscope with 40x (NA 1.3) or 63x (NA 1.4) oil objectives enables to gain further into resolution, while preserving a significant degree of 3D spatial information, and allowing qualitative and quantitative analysis of PC biogenesis and function.

Combining such 2D and 3D cell-based models and 3D imaging analysis (**Figure 5**) on hIPSCs generated either by reprogramming ciliopathy patient cells or by using CRISPR/CAS9 technology to specifically edit centrosomal or ciliary genes should allow significant progress in the understanding of the contribution of the PC during normal and pathological development of the cerebral cortex. Importantly, genome editing technology also allows to specifically rescue patient mutations to obtain isogenic controlled hIPSCs to overcome genetic heterogeneity that challenges the detection of disease mechanisms. In addition, single-cell genomic approaches are now widely used throughout the field and represent relevant and complementary approaches to immunostaining analysis. Thus, despite some limitations and difficulties inherent in all emerging technologies, and which are extensively being addressed, such 2D and 3D hIPSC-based models and the characterization methods we presented here offer powerful and relevant tools to dissect PC involvement into the pathological mechanisms underlying human developmental neocortical anomalies.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

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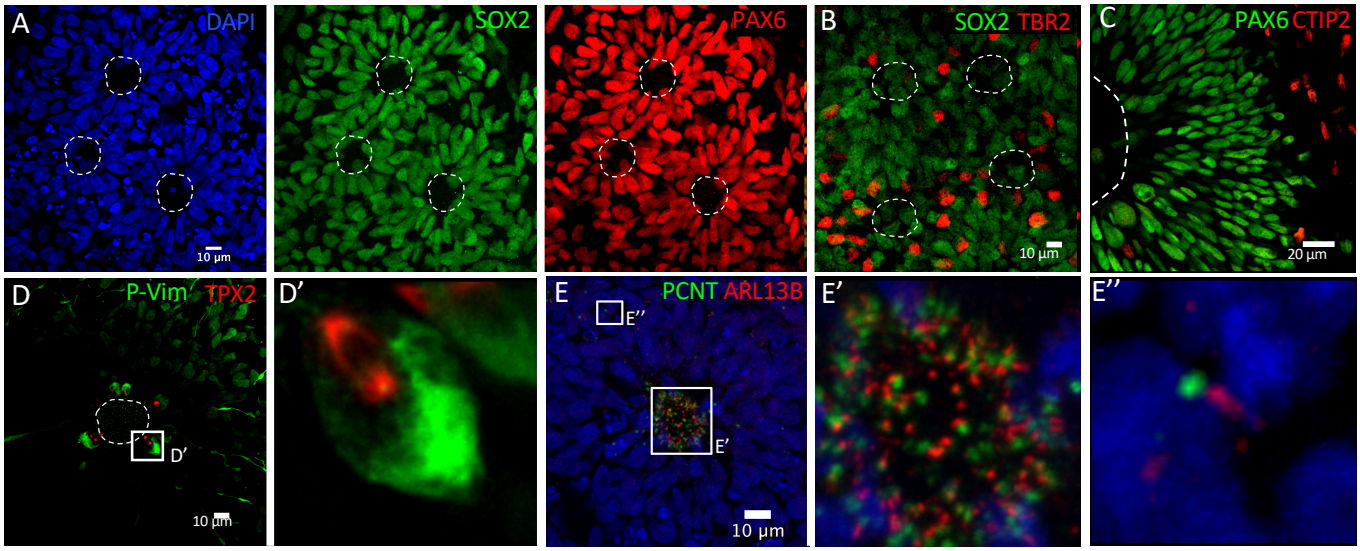

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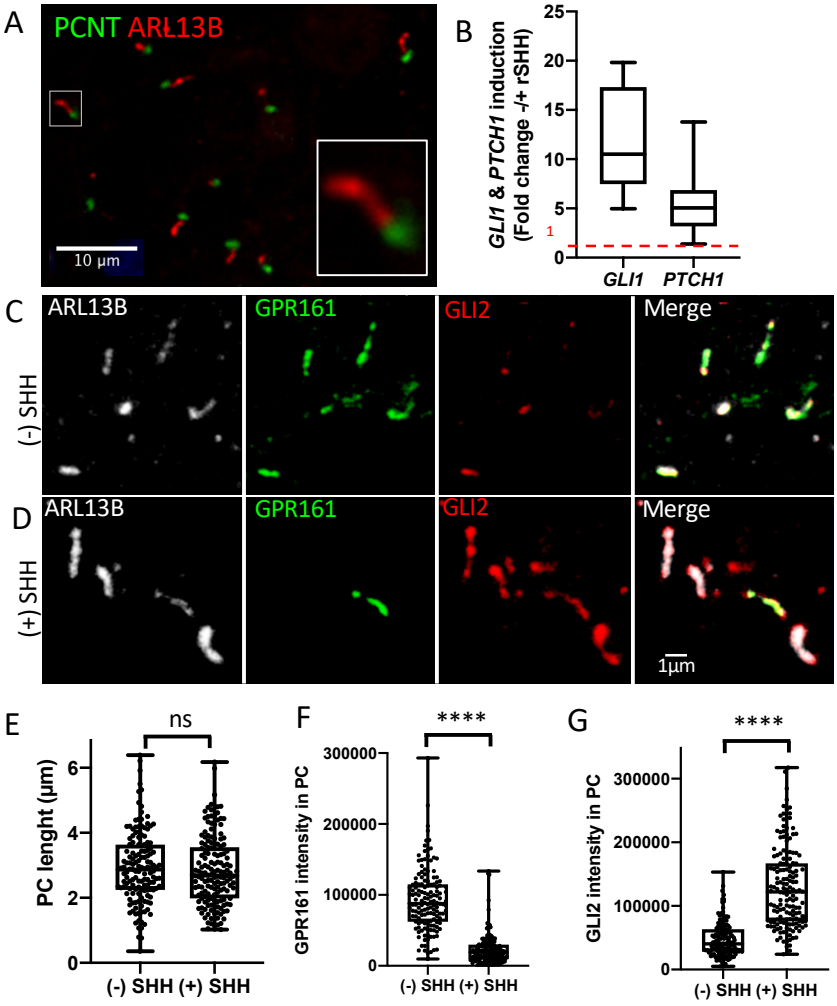

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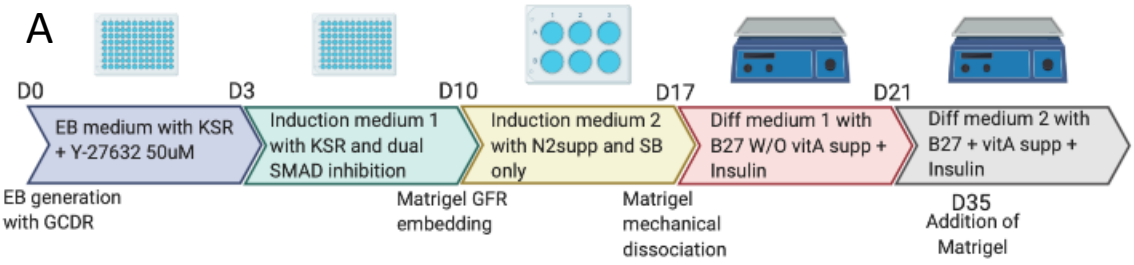

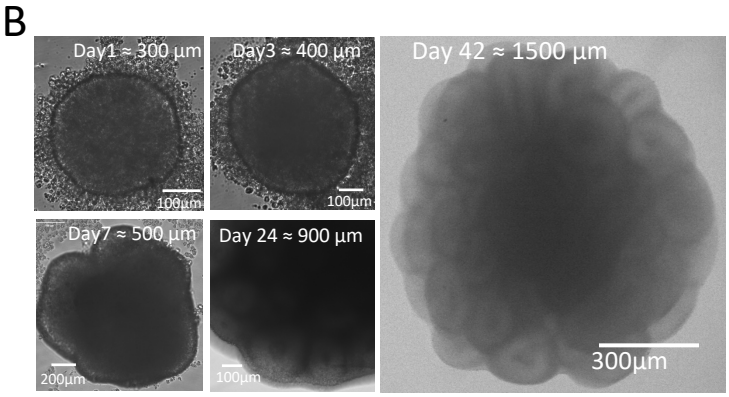

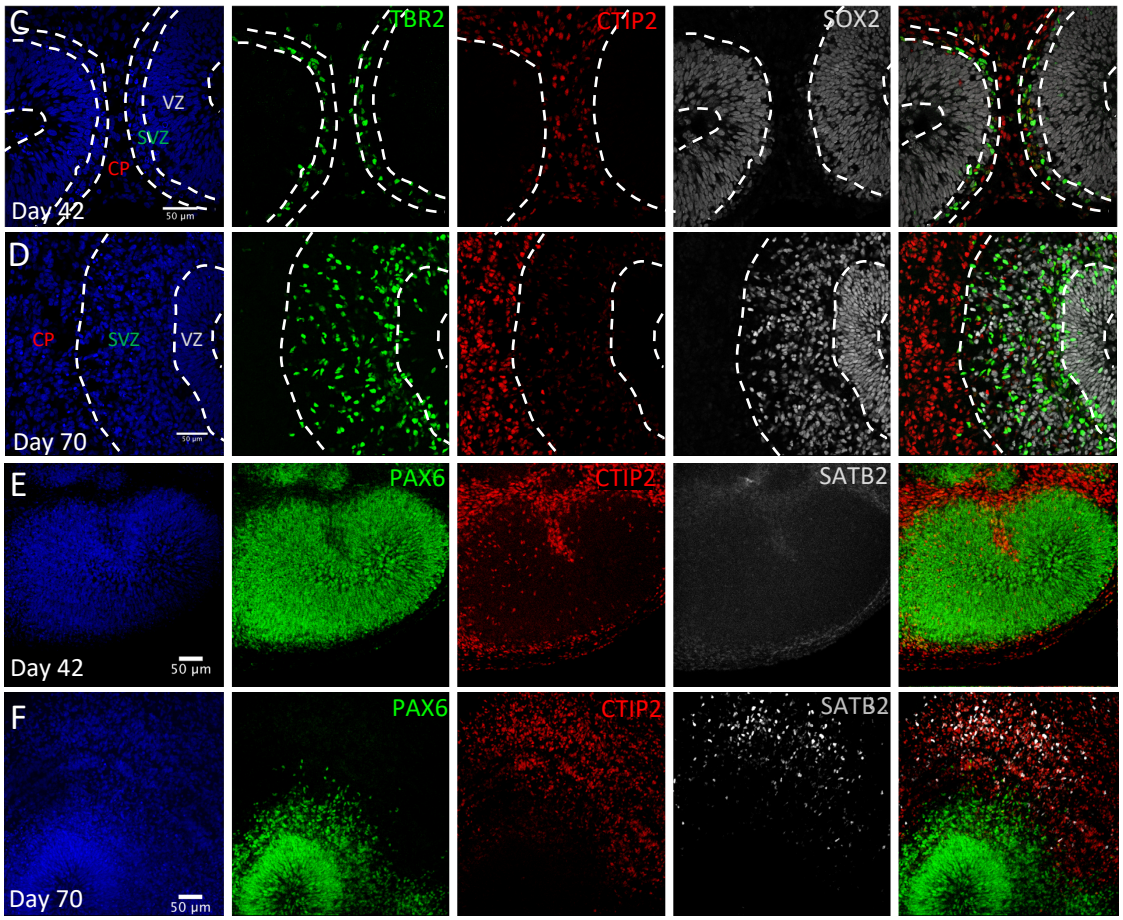

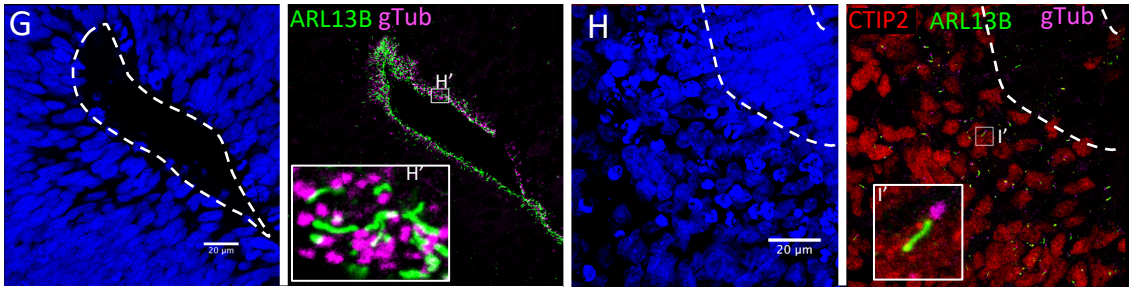

Figure 4

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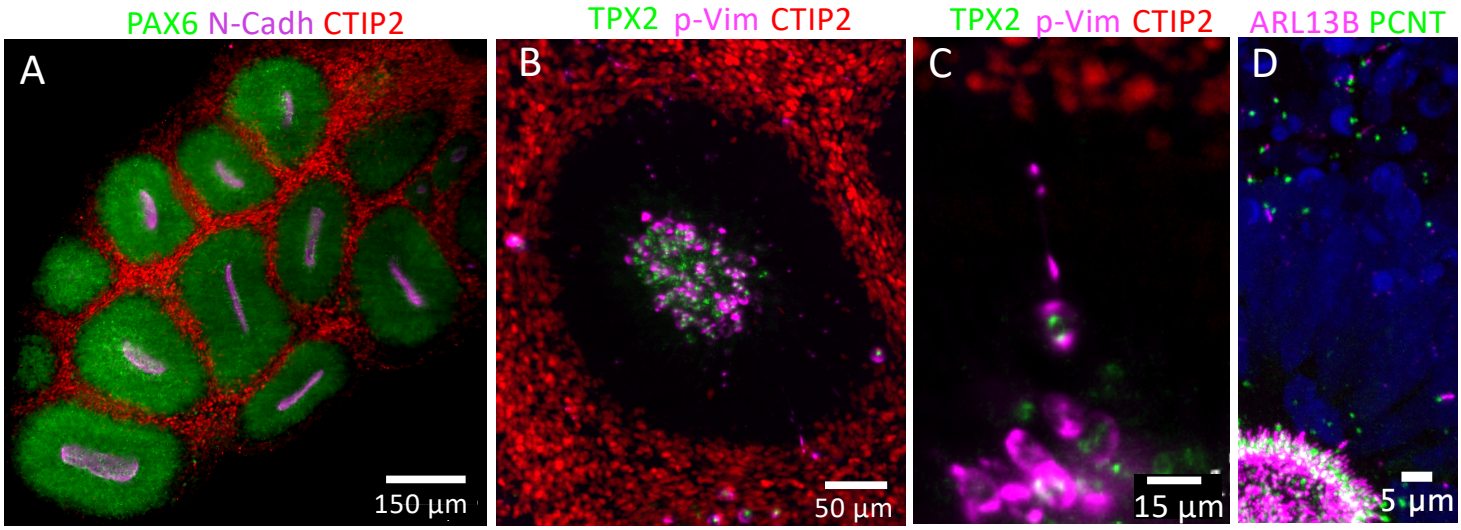
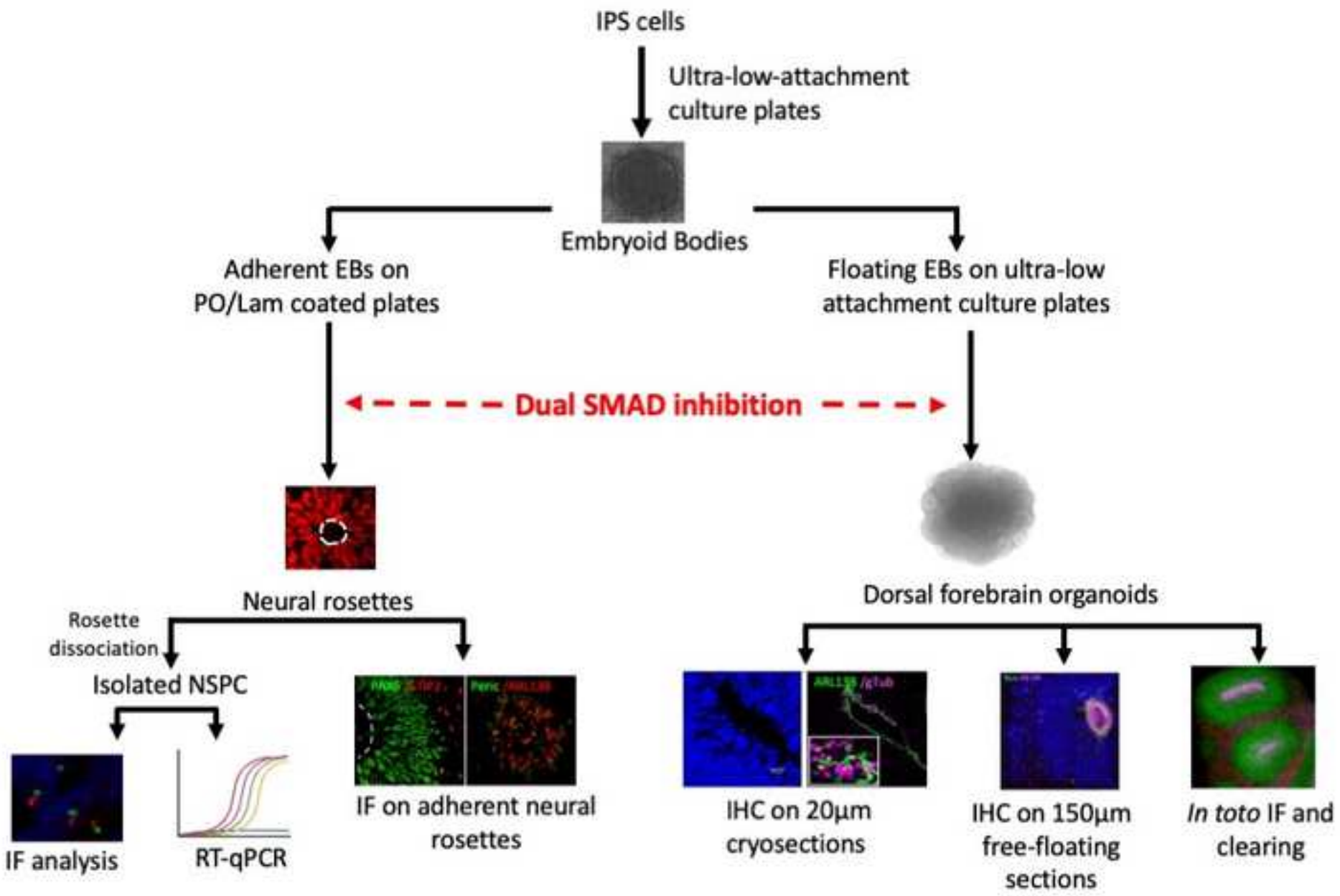


Figure 5



Click here to access/download

Video or Animated Figure

Video 1 Lightsheet N-CADH PAX6 CTIP2 Boutaud et al.mp4

Click here to access/download

Video or Animated Figure

Video 2 Lightsheet TPX2 P-VIM short.mp4

Click here to access/download

Video or Animated Figure

video 3 Lightsheet ARL13B PCNT Boutaud et al.mp4

Click here to access/download

Video or Animated Figure

video 4 Resonant Scanning Confocal PCNT ARL13B Boutaud et al.mp4

Click here to access/download

Table of Materials

Table of materials Boutaud et al..xls

We thank the reviewers and the editorial board for their useful comments helping us to improve our manuscript and video.
This rebuttal document addresses each of the peer review and editorial comments individually.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors describe with details methods to culture and then perform immunostainings of 2D neural rosettes and 3D cerebral organoids. Moreover, they suggest and, in the case of 2D rosettes, also show possible manipulations and analyses to assess the biogenesis and functionality of primary cilia and the effect on neocortical development.

Major Concerns:
The title points to the use of patient iPS cells to study the importance of primary cilia in neocortical development. The specific mention of patient-derived iPS cells sets the expectation to see 2D and 3D culture derived from patients (possibly affected by primary cilium-related defects). Indeed, the authors mention using iPS cells but never clarify from which type of patient the cells are derived and if they carry any relevant mutation. Moreover, it would be appreciated if the authors could show that the protocol works when primary cilia function is affected in iPS cells (e.g., iPS cells with a specific mutation in primary cilia genes). Finally, to assess that the suggested methods are helpful to dissect the involvement of primary cilia in neocortical malformation, the authors need to show that 2D and 3D culture can recapitulate some of the known effects of ciliopathies-related genes (e.g., number of mitotic neural progenitors).

→ Reviewer 1 is right; our original title was confusing and suggested the use of cells carrying ciliary gene mutations. This is not the case since we only present data obtained through the use of IPS cells generated by reprogramming cells from control individuals. This was not the purpose of this article which aims to describe the tools we have developed but which we hope will be very useful to study the consequences of the invalidation of ciliary genes. To overcome the confusion, we have modified the title of the manuscript: "**2D and 3D IPS Cell-based Models to dissect Primary Cilium involvement during Neocortical Development.**"

Minor Concerns:
Aside from the analysis of primary cilia function in 2D rosettes, the authors only suggest possible other possible analyses. It would have been good to show in more detail a few of them.

→ We added in the manuscript and video some possible analysis that can be performed on cerebral organoids, especially PC and centrosome count as well as 3D-reconstruction of PC to measure precisely their size.

Reviewer #2:
The manuscript by Boutaud et al. describes a detailed procedure for the analysis of primary

cilia in IPS cell-based 2D/3D models.
The presented methods would be certainly important for dissecting the functional role of primary cilia during both pathological and normal cerebral cortex development and for enabling functional analysis of SHH signaling pathway components.
The manuscript is well presented and protocols are clearly described in the text and associated videos.
I have no major concerns.
Authors could consider eliminating video 3, as possibly redundant.

→ Many thanks to reviewer 2 for positive comments on our manuscript. To us, video 3 is not redundant, as it illustrates that PC as well as centrosomes can be visualized by using the method we set up for whole-mount immunostaining and clearing of entire cerebral organoids and that can be highly useful.

Reviewer #3:
Manuscript Summary:
This manuscript details protocols to analyze primary cilia in the context of SHH signaling. Specifically, immunofluorescence is used with state of the art 3D human models and light sheet or confocal imaging to look at changes in cilia along with cell fate markers or mitotic cells. RT-PCR analysis is included as well. These are technically challenging assays and the images/movies are excellent in the quality. Only minor issues should be addressed.

→ Many thanks to reviewer 3 for interesting comments on our manuscript

Minor Concerns:
1) How are the organoids anchored to the stage for vibratome sectioning? Is the agarose block glued to the stage or is the organoid nicked and directly glued to the stage with the agarose block.

→ The organoids are embedded into 4% low-melting agarose and the agarose block is glued to the vibratome stage. This step has been clarified in the manuscript and video

2) Can the details from issue #1 be added to the text and/or movie

→ More details on the protocol used for 2D modeling have been added to both the manuscript and video

3) Why is blocking continued in the secondary solution. Once the tissue is blocked, continued addition of such reagents can often just lead to precipitation.
https://www.jacksonimmuno.com/technical/products/protocols/multiple-labeling

→ In our protocols, as well as in many published protocols, blocking solutions contain a high concentration of blocking agent, while primary and secondary antibody solutions contain

low concentration of blocking agent. And fortunately, we have never observed any precipitation.

4) Milk is not often an advisable blocking agent
https://www.jacksonimmuno.com/secondary-antibody-resource/technical-tips/controls-diluents-blocking/ Have other blocking agents been tested?

→ We usually use dry milk as first line for immunostaining analysis as it is a cheap blocking agent and works well for the reported experiments so we have not had to test any other agents. Importantly, we only use nonfat dry milk. This has been clarified in the manuscript.

Editorial and production comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

→ done

2. Please make the title concise.

→ The title has been changed: **2D and 3D IPS Cell-based Models to dissect Primary Cilium involvement during Neocortical Development.**

3. Please provide an email address for each author.
lucile.boutaud@institutimagine.org
marie.michael@institutimagine.org
celine.banal@institutimagine.org
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tania.attie@inserm.fr
nadia.bahi-buisson@aphp.fr
nathalie.lefort@institutimagine.org
sophie.thomas@inserm.fr

4. Please expand all abbreviations during the first time use.
→ Done

5. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

→ Done

6. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “This protocol presents…”
→ The summary has been rephrased and contains 34 words

7. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
→ The long abstract has been modified and contains 242 words

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.
→ Done

9. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
→ Done

10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
→ Done

11. The Protocol should contain only action items that direct the reader to do something. Please describe the action in complete sentences.
→ Done

12. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
→ Done

13. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
→ We revised the protocol section and add more details to some important steps

14. How do you identify embryonic bodies?

→ Embryoid bodies (EBs) are floating spheroid clusters/aggregates of IPS cells.
This has been added in the manuscript.

15. Please include all the volumes and concentration throughout the protocol.
→ Done

16. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
→ Done

17. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.
→ Done

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
→ No figures have been reused in the present manuscript

19. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

20. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Please remove commercial terms from the figure legend as well. For example: Imaris, etc.
→ "Imaris" and "matrigel" have been removed from the manuscript and the video and replaced by generic terms.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally,

all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
→ The homogeneity between the manuscript and the video has been increased.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol and is in imperative tense.
→ The narration has been revised to be more homogeneous with the written manuscript

3. Please make the title concise.
→ The title has been modified and is now more concise: "**2D and 3D IPS Cell-based Models to dissect Primary Cilium involvement during Neocortical Development.**"

4. For the last two interviews the audio and video doesn't match.
→ Those videos have been reshot

5. Please ensure that the protocol subheadings are the same both in the text and the video.
→ Done

6. Please ensure that the video and the narration match each other.
→ The narration and the video match more each other now.

7. Please do not use commercial terms in the video e.g., Imaris. Please use a generic term instead.
→ Done: Imaris and Matrigel have been replaced by generic terms

8. Filming of the microscopy part is a bit shaky. Please use a still camera.
→ The video has been modified

9. Please include more video shots to show how the protocol actions are performed.
→ We added more video shots

10. Please ensure that the results are described with respect to the protocol being presented. Essentially you performed your experiment, how did it help you to conclude what you wanted to and how is it in line with the title.
→ Done

11. Please do not have interviews in the representative result section.
→ Interviews in the representative result section have been removed

12. Suggested Changes

• Please Reduce the timing of Every "Name Caption'
→ Done

• Please remove the period from the title
→ Done

• Please capitalize the first letter of every important word in your title.
→ Done

• Please add corresponding institution(s) to your title card.
→ Done

• Missing institution(s) in the title card which is absolutely required.
→ Institutions have been added in the title card

13. Audio:

00:14 The audio here seems garbled and over-processed, as though a noise reduction filter has been applied. Consider reprocessing the audio with a weaker setting.
→ The video has been reshot

07:11 The audio here seems garbled and over-processed, as though a noise reduction filter has been applied. Consider reprocessing the audio with a weaker setting.
→ The video and audio have been reshot

14. Video:

00:14, 13:36 Consider Reshooting the interview shot, Because the lighting is a little bit dark and dull. Beware of the Audio Noise. Try to shoot in a Quiet and Bright Location. This shot seems very blurry and noisy due to the heavy scale/zooming, consider zooming out a little more to reduce the noise but not too much that we don't notice the detail in the shot.
→ The videos have been reshot

* 01:42 to 02:00 The video-audio synchronization is not correct and noticeably out of sync. Consider tightening the synchronization at these parts.
→ The video and audio have been reshot

Once done please ensure that the video is no more than 15 min and upload it to https://www.dropbox.com/request/Zad8oUlehXy6wD5BdHaj?oref=e

NSPC maintenance Medium (NMM)	**NSPC induction medium (NIM)**	**NSPC starvation medium**
Neural Basal Medium (50%)	Neural Basal Medium (50%)	Neural Basal Medium (50%)
DMEM/F-12, GlutaMAX (50%)	DMEM/F-12, GlutaMAX (50%)	DMEM/F-12, GlutaMAX (50%)
B27 supplement w/o Vit A (1X)	B27 supplement w/o Vit A (1X)	B27 supplement w/o Vit A (1X)
N2 Supplement-B (1X)	N2 Supplement-B (1X)	N2 Supplement-B (1X)
P/S (1X)	P/S (1X)	P/S (1X)
FGF2 (10 ng/ml)	FGF2 : 5 ng/ml	
EGF (10 ng/ml)	LDN193189 500 nM	
BDNF (20 ng/ml)	SB431542 20 µM	
	+/- Y-27632 10 µM	

Table 1: Recipe of the media used for the generation of 2D-neural rosettes and NSPC

EB medium	Induction medium-1	Induction medium-2
DMEM-F12/Glutamax	DMEM-F12/Glutamax	DMEM-F12/Glutamax
20% (v/v) KoSR	20% (v/v) KoSR	1x N2
1× MEM-NEAA	1× MEM-NEAA	1× MEM-NEAA
100 µM b-ME (55mM)	100 µM b-ME (55mM)	5 µM SB431542
P/S 0,1%	P/S 0,1%	P/S 0,1%
Y-27632 50µM	200 nM LDN-193189/Dorso 5µM	
+/- bFGF 4ng/ml	5 µM SB431542	

Differentiation medium-1	Differentiation medium-2
DMEM-F12/ Glutamax	DMEM-F12/ Glutamax
1x MEM NEAA	1x MEM NEAA
1x B27 w/o Vit A	1x B27 with Vit A
1x N2	1x N2
50 µM b-ME (55mM)	50 µM b-ME (55mM)
Insulin 2,5µg/ml	Insulin 2,5µg/ml
P/S 0,5%	P/S 0,5%
	Matrigel 1% (from Day 35)

Supplemental table 2: Recipe of the media used for the generation of dorsal forebrain organoids