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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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1 **TITLE:**
2 2D and 3D Human Induced Pluripotent Stem Cell-Based Models to Dissect Primary Cilium
3 Involvement During Neocortical Development
4

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46 **KEYWORDS:**

47 primary cilium, human induced pluripotent stem cells (hiPSCs), neural stem and progenitor
48 cells, human cerebral cortical development, neural rosettes, dorsal forebrain organoids, 2D
49 and 3D hiPSC-based models of neocortical development, cerebral organoids, Sonic hedgehog,
50 whole-mount immunostaining, optical clearing, light sheet microscope

51

52 **SUMMARY:**

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

57

58 **ABSTRACT:**

59 Primary cilia (PC) are non-motile dynamic microtubule-based organelles that protrude from
60 the surface of most mammalian cells. They emerge from the older centriole during the G1/G0
61 phase of the cell cycle, while they disassemble as the cells re-enter the cell cycle at the G2/M
62 phase boundary. They function as signal hubs, by detecting and transducing extracellular
63 signals crucial for many cell processes. Like most cell types, all neocortical neural stem and
64 progenitor cells (NSPCs) have been shown harboring a PC allowing them to sense and
65 transduce specific signals required for the normal cerebral cortical development. Here, we
66 provide detailed protocols to generate and characterize two-dimensional (2D) and three-
67 dimensional (3D) cell-based models from human induced pluripotent stem cells (hiPSCs) to
68 further dissect the involvement of PC during neocortical development. In particular, we
69 present protocols to study the PC biogenesis and function in 2D neural rosette-derived NSPCs
70 including the transduction of the Sonic Hedgehog (SHH) pathway. To take advantage of the
71 three-dimensional (3D) organization of cerebral organoids, we describe a simple method for
72 3D imaging of *in toto* immunostained cerebral organoids. After optical clearing, rapid
73 acquisition of entire organoids allows detection of both centrosomes and PC on neocortical
74 progenitors and neurons of the whole organoid. Finally, we detail the procedure for
75 immunostaining and clearing of thick free-floating organoid sections preserving a significant
76 degree of 3D spatial information and allowing for the high-resolution acquisition required for
77 the detailed qualitative and quantitative analysis of PC biogenesis and function.

78

79 **INTRODUCTION:**

80 Primary cilia (PC) are microtubule-based organelles that sense and transduce a plethora of
81 chemical and mechanical cues from the extracellular environment. In particular, PC is the
82 central organelle for the transduction of the Hedgehog signaling pathway in vertebrates^{1,2}.
83 While most neural cells have long been shown harboring a PC, the contribution of this
84 organelle in shaping the central nervous system has long been undervalued. Studies on
85 neocortical development have led to the discovery of multiple neural stem and progenitor
86 cells (NSPCs), all harboring a PC which location has been proposed crucial for progenitor fate
87 determination³⁻⁷. PC has been shown crucial for cell mechanisms that are required for normal
88 cerebral cortical development including NSPC expansion and commitment⁸⁻¹² as well as
89 apicobasal polarity of radial glial scaffold supporting neuronal migration¹³. In addition, PC are
90 required during interneurons tangential migration to the cortical plate^{14,15}. Finally, a role for
91 the PC has been proposed in the establishment of synaptic connections of neurons in the
92 cerebral cortex^{16,17}. Altogether, these findings argue for a crucial role of PC at major steps of

93 cerebral cortical development^{18, 19} and raise the need to investigate their involvement in the
94 pathological mechanisms underlying anomalies of cerebral cortical development.

95

96 Recent studies have largely improved our understanding of important cellular and molecular
97 differences between cortical development in human and animal models, emphasizing the
98 need to develop human model systems. In this view, human induced pluripotent stem cells
99 (hiPSCs) represent a promising approach to study disease pathogenesis in a relevant genetic
100 and cellular context. Adherent two-dimensional (2D) cell-based models or neural rosettes
101 contain NSPCs similar to those seen in the developing cerebral cortex, which become
102 organized into rosette-shaped structures showing correct apicobasal polarity²⁰⁻²².
103 Furthermore, the three-dimensional (3D) culture system allows the generation of dorsal
104 forebrain organoids that recapitulate many features of human cerebral cortical
105 development²³⁻²⁶. Those two complementary cell-based modeling approaches offer exciting
106 perspectives to dissect the involvement of PC during normal and pathological development of
107 the cerebral cortex.

108

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

124

125 **PROTOCOL:**

126

127 **1. Generation of 2D hiPSC cell-based models of neocortical development**

128

129 **1.1. Neural rosette formation**

130

131 1.1.1. Start with hiPSC cultures harboring large regular colonies, exhibiting less than 10%
132 differentiation and no more than 80% confluency.

133

134 1.1.2. Rinse the hiPSCs with 2 mL of PBS.

135

136 1.1.3. Add 2 mL of NSPC induction medium supplemented with the Rock inhibitor (NIM + 10
137 μM Y-27632).

138

139 1.1.4. Manually dissect each hiPSC colony from one 35 mm dish using a needle to cut each
140 colony precisely in horizontal and vertical directions to create a checker-board pattern
141 dividing each colony into equal clusters.
142

143 1.1.5. Detach the colony using a cell scraper and transfer them onto an ultra-low-attachment
144 35 mm dish.
145

146 1.1.6. Let them float overnight (ON) in a humidified incubator at 37 °C and 5% CO₂, so that
147 they can form embryoid bodies (EBs).
148

149 NOTE: Embryoid bodies (EBs) are defined as floating spheroid clusters or aggregates of hiPSCs.
150

151 1.1.7 Transfer the EBs into poly-L-ornithine/laminin (PO/lam)-coated 35 mm dishes in 2 mL
152 NIM + 10 μM Y-27632.
153

154 1.1.8 Daily refresh NSPC induction medium (NIM without Y-27632) until the formation of
155 neural rosettes that takes approximately 12 to 14 days. Check under the microscope.
156

157 NOTE: After this step neural rosettes can be expanded, differentiated, and processed for
158 immunostaining analysis or dissociated to get isolated neural stem progenitor cells (NSPCs).
159

160 **1.2. Neural rosette expansion and early differentiation:**
161

162 1.2.1. Cut neural rosettes in a grid-like pattern with a needle and dislodge the clusters using
163 a cell scraper.
164

165 1.2.2. Transfer the rosettes into a 4-well plate containing PO/lam-coated glass coverslip (4-5
166 rosettes/well) in 0.5 ml of NSPC maintenance medium (NMM).
167

168 1.2.3. Incubate the plate in a humidified incubator at 37 °C and 5% CO₂.
169

170 1.2.4. Refresh NMM every other day until day 20
171

172 1.2.5. From day 20, culture neural rosettes in 0.5 mL of cytokine depleted NMM to allow
173 differentiation. Refresh the medium every 2-3 days.
174

175 1.2.6. At Day 30 and Day 40 (10 or 20 days of differentiation), fix the rosettes with 0.5 mL of
176 4% PFA, 20 min, RT.
177

178 **1.3. PC biogenesis and function analysis on isolated NSPCs**
179

180 **1.3.1. NSPC dissociation**
181

182 1.3.1.1. Cut neural rosettes from step 1.1.8 in a grid-like pattern with a needle and dislodge
183 the clusters using a cell scraper. Transfer the cells into PO/lam-coated 35 mm dishes in 2 mL
184 of NMM and incubate in a humidified incubator at 37 °C and 5% CO₂.
185

186 1.3.1.2. Refresh NMM every other day until confluency (approx. 5-7 days).
187
188 1.3.1.3. After scraping away the large, clear cells surrounding neural rosettes, pick them
189 manually and transfer them onto fresh PO/lam-coated 35 mm dishes to enrich for NSPCs and
190 deplete non-neural cell types.
191
192 1.3.1.4. After one or two manual passages (repeat step 1.3.1.3 if needed) required to remove
193 all contaminating cell types, remove the medium and rinse with PBS.
194
195 1.3.1.5. Add 300 μ L of 0.05% trypsin and incubate for 5 min at 37 °C until most cells detach.
196
197 1.3.1.6. Add 2 mL of the medium containing 10% FBS (DMEM + 10% FBS) to inactivate the
198 trypsin. Collect the cell suspension in a 15 mL conical tube. Gently pipette the cell suspension
199 up and down 3 times to break up the cell clumps.
200
201 1.3.1.7. Centrifuge at 300 x g for 5 min.
202
203 1.3.1.8. Carefully aspirate the supernatant and resuspend the cells in NMM.
204
205 1.3.1.9. Seed the dissociated NSPCs as single cells (1×10^5 cells/cm²) in NMM onto PO/lam-
206 coated dishes and incubate them in a humidified incubator at 37 °C and 5% CO₂.
207
208 1.3.1.10. Expand NSPCs in NMM by changing the medium every other day.
209
210 NOTE: NSPCs are seeded at high density to avoid differentiation.
211
212 1.3.2. **PC biogenesis analysis**
213
214 1.3.2.1. Seed dissociated NSPCs at 100,000 cells /cm² and incubate them in a humidified
215 incubator at 37 °C and 5% CO₂ in NMM for 2 days.
216
217 1.3.2.2. Aspirate the medium and starve NSPCs in cytokine depleted medium (NSPC
218 starvation medium, Supplemental Table 1) for 48 h.
219
220 1.3.2.3. Fix NSPCs with 4% PFA for 20 min at RT.
221
222 1.3.2.4. Wash twice with PBS for 5 min at RT before immunostaining.
223
224 1.3.3. **PC function analysis: SHH signaling pathway**
225
226 1.3.3.1. Seed dissociated NSPCs at 100,000 cells /cm² on PO/lam-coated 8-well
227 Chamber Slides (300 μ L) or T25 dishes (5 mL) and incubate in a humidified incubator at 37 °C
228 and 5% CO₂ in NMM for 2 days.
229
230 1.3.3.2. Starve NSPCs in NSPC starvation medium (300 μ L per well of 8-well chamber slide and
231 5 mL in T25 flask) for 48 h.
232

Commenté [A1]: Is this 100 cells or 1×10^5 . Please check this throughout.

233 1.3.3.3. To induce the SHH pathway, incubate the NSPCs with the starvation medium
234 supplemented with recombinant SHH (100 ng/mL) or SAG (500 nM); (150 µL per well of 8-well
235 chamber slide and 2 mL in T25 flask) for 24 h.
236
237 1.3.3.4. Fix NSPCs cultured on 8-well Chamber Slides in 250 µl 4% PFA per well for 20 min at
238 RT and wash them twice with 250 µL of PBS for 5 min at RT before immunostaining analysis.
239
240 1.3.3.5. Remove the medium and wash NSPCs cultured in T25 dishes with PBS.
241
242 1.3.3.6. Add 500 µL of 0.05% trypsin and incubate for 5 min at 37 °C until cells detach.
243
244 1.3.3.7. Add 2 mL of medium containing 10% FBS (DMEM + 10% FBS) to inactivate the trypsin
245 and collect the cell suspension to a 15 ml conical.
246
247 1.3.3.8. Centrifuge at 300 x *g* for 5 min and carefully aspirate the supernatant to obtain NSPC
248 pellets that can be cryopreserved at -80 °C before RNA extraction and RT-PCR analysis.
249
250 **2. Generation of dorsal forebrain organoids**
251
252 **2.1. Single-cell culture of hIPSCs**
253
254 2.1.1. Start with hIPSC cultures harboring large regular colonies exhibiting less than 10%
255 differentiation and that have been passaged almost once. Ensure that the cells are no more
256 than 80% confluent.
257
258 2.1.2. Wash the colonies with 2 mL of PBS.
259
260 2.1.3. Add 500 µL of Gentle Cell Dissociation Reagent (GCDR) and incubate for 5 to 7 min at
261 37 °C.
262
263 2.1.4. Aspirate the GCDR and add 2 ml of mTeRS1 medium supplemented with 5 µM Y-27632.
264
265 2.1.5. Pipette the cells gently up and down 10 times to dissociate all colonies into single cells.
266
267 2.1.6. Transfer the cells on vitronectin-coated dishes and incubate in a humidified incubator
268 at 37°C and 5% CO₂.
269
270 NOTE: Perform at least 1 passage of the hIPSCs using GCDR prior to the differentiation
271 experiment to adapt the hIPSCs to single-cell culture conditions.
272
273 2.2. On Day 0, allow hIPSCs to form embryonic bodies in EB medium containing a low
274 concentration of FGF2 and a high concentration of rock inhibitor required for hIPSC survival.
275 To do so follow the steps below.
276
277 2.2.1. Wash the colonies with 2 mL of PBS.
278

279 2.2.2. Add 500 μ L of Gentle Cell Dissociation Reagent (GCDR) and incubate for 5 to 7 minutes
280 at 37°C.
281
282 2.2.3. Aspirate the GCDR and add 1 mL of EB medium supplemented with 50 μ M Y-27632;
283 Use a 1 mL pipette to gently detach the cells and transfer them to a 15 mL conical tube.
284
285 2.2.4. Rinse once more with 2 ml EB medium supplemented with 50 μ M Y-27632, transfer
286 the remaining cells to the 15 ml conical tube. Immediately add 3 mL of EB medium
287 supplemented with 50 μ M Y-27632 to the conical tube and gently homogenize the solution
288 using a 5 mL pipette.
289
290 2.2.5. Centrifuge the dissociated cells at 100 x *g* for 5 min.
291
292 2.2.6. Gently aspirate the supernatant and resuspend the cells in 6 mL of EB medium
293 supplemented with 50 μ M Y-27632.
294
295 2.2.7. Centrifuge the cells at 100 x *g* for 5 min.
296
297 2.2.8. Aspirate the supernatant and resuspend the cells in 1 mL of EB medium supplemented
298 with 50 μ M Y-27632. Use a 1 mL pipette to gently dissociate the cells into a single-cell
299 suspension.
300
301 2.2.9. Immediately after resuspending the cells, dilute and count them.
302
303 2.2.10. Dilute the proper volume of cell suspension (containing 900,000 cells) into 30 mL of EB
304 medium supplemented with 50 μ M Y-27632 and 4 ng/ml bFGF and gently mix the solution.
305
306 2.2.11. Plate 9,000 cells/well into an ultra-low attachment 96U plate (300 μ l/well). To avoid
307 disturbing EB formation, incubate the plate in a humidified incubator at 37 °C and 5% CO₂ until
308 Day 3 without medium change.
309
310 **2.3. Neural induction (Dual SMAD inhibition)**
311
312 2.3.1. On Day 3, EBs should measure 300-400 μ m and show regular borders. If both criteria
313 are reached, replace half of the medium (150 μ L) with induction medium-1 containing Dual
314 SMAD inhibitors, leading to brightening of the contour of the EBs by day 6 to 7 indicating
315 neuroectodermal differentiation (**Supplemental Table 2**).
316
317 2.3.2. On day 4, replace 3/4 of the medium (225 μ L) with induction medium-1.
318
319 2.3.3. On day 6 & day 8: refresh half of the induction medium-1 (150 μ L).
320
321 **2.4. Organoid embedding into basement membrane matrix (Day 10):**
322
323
324 2.4.1. On Day 10, embed EBs in growth factor reduced basement membrane matrix (BMM).

325 NOTE: From this step, always use sterile scissors to cut the opening of pipette tips to avoid
326 damaging the organoids by repeated pipetting.
327

328 2.4.2. First transfer around 15-17 organoids in conical tubes. Let the EBs settle and remove
329 the medium. Add 50 μ L of induction medium-2 and transfer them into a microcentrifuge tube
330 containing 100 μ L of BMM.
331

332 2.4.3. Spread the matrix-EB mixture onto the center of a well of an ultra-low-
333 attachment plate and separate the EBs to prevent them to fuse.
334

335 2.4.4. Let the BMM solidify in the incubator at 37 °C for 45 min.
336

337 2.4.5. Add 3 mL of the induction medium-2 in each well and put the plate in a humidified
338 incubator at 37 °C and 5% CO₂.
339

340 NOTE: Ensure that this procedure is done as quickly as possible as BMM solidifies at room
341 temperature.
342

343 2.5. **Stationary culture of organoids embedded into basement membrane matrix**
344

345 2.5.1. Day 12, 14: Refresh induction medium-2.
346

347 2.5.2. Day 16: Refresh induction medium-2 and check under a microscope the expansion of
348 neuroepithelial loops.
349

350 2.6. **Basement membrane matrix dissociation and culture on an orbital shaker**
351

352 2.6.1. On Day 17, dissociate organoids from BMM by pipetting up and down 10 times with a
353 5 ml pipette and transfer them into differentiation medium-1 (without vitamin A) and
354 incubate onto an orbital shaker at 80 rpm in a humidified incubator at 37 °C and 5% CO₂ to
355 improve nutritional absorption.
356

357 NOTE: Use a different incubator for stationary culture and culture onto an orbital shaker to
358 avoid any vibrations detrimental to adherent hIPS cell growth.
359

360 2.6.2. Day 19: refresh differentiation medium-1.
361

362 2.6.3. Day 21: change differentiation medium-1 to differentiation medium-2 (with vitamin
363 A).
364

365 2.6.4. From day 23 to day 35: refresh medium with differentiation medium-2 every 2-3days.
366

367 2.6.5. From day 35 to day 70: refresh differentiation medium-2 with 1% BMM and refresh
368 every 2-3 days.
369

370 2.6.6. Collect the organoids after 28, 42, and 70 +/- 2 days of differentiation and fix them
371 overnight at 4°C, in 5 ml 4% PFA on a 15 ml conical tube.

372
373 2.6.7. For further *in toto* or free-floating immunostaining analysis, organoids are then rinsed
374 twice in PBS and conserved at 4°C in PBS + 0.05% sodium azide.
375
376 2.6.8. For immunostaining analysis on frozen sections, immerse 4% PFA fixed organoids in 10
377 ml 30% sucrose at +4°C ON (or until organoids sink). Embed them into a frozen embedding
378 matrix and store at -80°C until cryosectioning.
379
380 **3. *In toto* immunolabeling, clearing and lightsheet acquisition of dorsal forebrain**
381 **organoids**
382
383 **3.1. Fixation:**
384
385 3.1.1. Collect the organoids in 6-well plates, remove the medium and fix them with 2 ml of
386 4% PFA, at 4 °C, overnight.
387
388 3.1.2. Perform 3 washes in 2 ml of PBS at room temperature.
389
390 3.1.3. Transfer the organoids into 2 ml tubes and store at 4 °C in 2 ml of PBS + 0.05% sodium
391 azide.
392
393 **3.2. Permeabilization:**
394
395 3.2.1. Incubate organoids in 1 mL of PBS - 0.2% Triton X100 at RT for 1 h. Do this twice
396
397 3.2.2. Incubate in 1 mL of PBS - 0.2% Triton X100 - 20% DMSO, at 37°C, overnight.
398
399 3.2.3. Incubate in 1 mL of PBS - 0.1% Tween20 - 0.1% Triton X100 - 0.1% Deoxycholate - 0.1%
400 NP40 - 20% DMSO, at 37°C, overnight.
401
402 3.2.4. Incubate in 1 mL of PBS - 0.2% Triton-X100, at RT, for 1 h. Do this twice
403
404 **3.3. Blocking and immunolabeling:**
405
406 3.3.1. Block in 1 mL of blocking solution (PBS - 0.2% Triton X100 - 6% Donkey Serum - 10%
407 DMSO), at 37°C, ON.
408
409 3.3.2. Incubate with primary antibodies diluted in 250 µL of PBS - 0.2% Tween20 - 0.1 µg/mL
410 Heparin - 5% DMSO – 3% Donkey Serum, at 37 °C, for 2 days.
411
412 3.3.3. Wash in 1 mL of PBS - 0.2% Tween20 - 0.1 µg/mL Heparin, for 1 h, at 37 °C. Do this four
413 times.
414
415 3.3.4. Wash in 1 mL of PBS - 0.2% Tween20 - 0.1 µg/mL Heparin, overnight, at 37 °C.
416
417 3.3.5. Incubate with secondary antibodies diluted in 250 µL of PBS - 0.2% Tween 20 - 0.1
418 µg/mL Heparin - 3% Donkey Serum, at 37 °C, for 2 days.

419
420 3.3.6. Wash in 1 mL of PBS - 0.2% Tween 20 - 0.1 µg/mL Heparin, for 1 h, on a wheel, at RT.
421 Do this four times.
422
423
424 3.3.7. Wash in 1 mL of PBS - 0.2% Tween 20 - 0.1 µg/ml Heparin, overnight, on a wheel, at RT
425
426 3.3.8. Wash in 1 mL of PBS, for 24 h, at RT
427
428 3.3.9. Stock at +4 °C in 1 mL of PBS + 0.05% sodium azide until clearing
429
430 **3.4. Clearing in TDE (2,2'-Thiodiethanol):**
431
432 3.4.1. Incubate the organoids in 1 mL of 30% TDE (3 ml TDE + 7 ml PBS), for 24 h, at RT
433
434 3.4.2. Incubate in 1 mL of 60% TDE (6 ml TDE + 4 ml PBS), for 24 h, at RT
435
436 3.4.3. Incubate in 1 mL of 80% TDE (8 ml TDE + 2 ml PBS), for 24 h, at RT
437
438 **3.5. Organoid embedding prior to light sheet acquisition:**
439
440 NOTE: Use a custom-made system; made with a 1 mL syringe with the tip cut with a scalpel.
441
442 3.5.1. Prepare 100 mL of 4% Low-Melting Agarose in 60% TDE solution and aliquot in 2 mL
443 tubes. Conserve at +4 °C.
444
445 3.5.2. Prior to organoid embedding, preheat 1 tube containing 1.5 ml of 4% low-melting
446 agarose in 60% TDE in a water bath at 37 °C until it liquefies.
447
448 3.5.3. Meanwhile, prepare a custom-made molding system made from a 1 ml syringe which
449 tip is cut off using a scalpel.
450
451 3.5.4. Pump in the syringe 600 µL of the gel solution using the plunger.
452
453 3.5.5. Position the sample using an enlarged pipette tip which opening has been cut using
454 sterile scissors.
455
456 3.5.6. Fill in the syringe with 400 µL of gel solution so that the sample is positioned within the
457 lower third of the syringe.
458
459 3.5.7. Let the gel polymerize and store in 80% TDE solution at 4°C protected from light until
460 acquisition.
461
462 3.5.8. For Light sheet acquisition, use a 20x objective immersed in 80% TDE solution added
463 in the sample chamber to allow to accurately adjust to the refractive index of the clearing
464 method.
465

466 3.5.9. Insert the syringe containing the agarose embedded organoid in the largest sample
467 holder designed to accommodate a 1 mL syringe which plunger can be operated to position
468 the organoid in front of the objective.
469

470 NOTE: Light sheet acquisition of an entire organoid takes approximately 5 to 10 minutes.
471

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

473 **4.1. Fixation, agarose embedding, and sectioning of the organoids:**

474 4.1.1. Collect organoids after 28, 42, and 70 +/- 2 days of differentiation in a 6 well plate and
475 fix overnight at 4 °C, in 2 mL of 4% PFA.

476 4.1.2. Rinse twice in 2 mL of PBS and conserve at 4°C in 2 ml PBS + 0.05% sodium azide.
477

478 4.1.3. Embed the fixed organoids into 4% low-melting agarose in a plastic embedding mold
479 (7 x 7 mm). Carefully remove the agarose block from the plastic mold and glue it to the
480 vibratome stage.
481

482 4.1.4. Section the embedded organoids using a vibratome to obtain 150 µm free-floating
483 sections transferred in a well of a 24-well plate containing 500 µl PBS using a paintbrush to
484 avoid damaging the sections.
485

486 NOTE: Low-melting agarose is recommended as its melting temperature is only approximately
487 60 °C. Prepare 4% low-melting agarose in PBS solution and left it to cool just above 37°C in a
488 water bath prior to organoid embedding.
489

490 **4.2. Permeabilization, blocking, and immunostaining:**

491 4.2.1. Incubate the sections in 500 µL of PBS - 0.3% Triton X100, at RT, under agitation, for
492 20 min. Do this three times.
493

494 4.2.2. Incubate the sections in 500 µl of PBS - 0.3% Triton X100 - 5% nonfat Milk, at RT, under
495 agitation, for 2 h.
496

497 4.2.3. Incubate the sections in 250 µl of primary antibody solution (PBS - 0.3% Triton X100 -
498 1% nonfat Milk), under agitation, at +4°C, overnight.
499

500 4.2.4. Wash the sections in 500 µl of PBS, at RT, under agitation, for 20 minutes. Do this four
501 times.
502

503 4.2.5. Incubate the sections in 250 µl of secondary antibody solution (PBS - 0.3% Triton X100
504 - 1% nonfat milk), at RT, under agitation, for 2 h.
505

506 4.2.6. Wash the sections in 500 µl of PBS, at RT, under agitation, for 20 minutes. Do this four
507 times.
508

509
510
511
512

513 4.2.7. Store the sections in 500 µl of PBS, at +4°C until clearing.
514
515 **4.3. Clearing in TDE (2,2'-Thiodiethanol)**
516
517 4.3.1. Incubate the sections in 500 µl of 30% and 60% TDE for 1 hour each at RT and then in
518 500 µl 80% TDE overnight, at RT.
519
520 4.3.2. Store the cleared sections in 500 µl of 80% TDE until acquisition at +4°C.
521
522 **4.4. Mounting of free-floating sections prior to acquisition with resonant scanning**
523 **confocal**
524
525 4.4.1. Mount free-floating section in a sealed chamber enabling to maintain the sample in
526 80% TDE solution and designed to adapt on a motorized XY stage of confocal microscopes.
527
528 4.4.2. Put one round coverslip in the chamber system.
529
530 4.4.3. Transfer carefully the cleared immunostained section using a paintbrush.
531
532 4.4.4. Fill the chamber with 80% TDE solution.
533
534 4.4.5. Add two standard coverslips plus one second round coverslip and a silicone seal.
535
536 4.4.6. Screw on the screwing ring of the chamber to perfectly seal the system.
537
538 **5. Light sheet and resonant scanning confocal analysis**
539
540 5.1. Process light sheet and resonant scanning acquisitions thanks to software
541 enabling 3D visualization and analysis of the entire immunostained sample.
542
543 NOTE: Such software allows to quickly open huge data to easily make snapshots and
544 animations. It allows to move the sample in different orientations and to generate 2D views
545 thanks to a 2D slicer tool in different orientations, XY and YZ for example.
546
547 5.2. For automatic detection of both centrosomes and primary cilia, use a spot wizard
548 enabling to quantify their number in pathological versus control conditions.
549
550 5.3. For 3D reconstruction of PC enabling precise measurement of their length, use a
551 filament wizard to manually fix the starting point of the PC and the software uses the
552 fluorescence signal to reconstruct the PC precisely.
553
554 **REPRESENTATIVE RESULTS**
555
556 **2D hIPS cell-based models to study primary cilium biogenesis and function**
557 The protocol detailed here has been adapted from previously published studies²⁰⁻²². This
558 protocol allows the generation of neural rosette structures that contain neocortical
559 progenitors and neurons similar to those seen in the developing neocortex. Detailed validation

560 can be performed by conventional immunostaining analysis using specific makers³. For
561 instance, apical progenitors (AP) should be double-stained with SOX2 and PAX6, intermediate
562 progenitors (IP) are revealed by TBR2/EOMES staining, and early-born neocortical neurons are
563 revealed by CTIP2 staining (**Figure 1A-C**). Such neural rosette-like structures model interkinetic
564 nuclear migration (INM) of AP that can be visualized by immunostaining with antibodies raised
565 against phospho-vimentin, which stains mitotic nuclei and TPX2 that stains the mitotic spindle.
566 These markers, therefore, allow the analysis of several characteristics of AP, including the INM
567 with cell division to take place apically around the central lumen (Figure 1D, D'), as well as the
568 division mode determined by measuring the angle between the division plane and the apical
569 surface. Finally, ciliogenesis can be analyzed by immunostaining with antibodies raised against
570 PCNT, which stains the basal body of PC, and ARL13B that stains the axoneme. On such rosette
571 structures, PC extend from the apical pole of APs into the central lumen of each ventricle-like
572 region (**Figure 1E, E'**), while they are also protruding from CTIP2+ neurons (**Figure 1E''**).

573
574 Dissociation of these rosette structures allows obtaining isolated NSPCs cultured on poly-L-
575 ornithine/laminin-coated culture plates in NMM at a high density to allow maintenance of a
576 stable and expandable population of NSPCs for at least 15 passages without accumulating
577 karyotype abnormalities^{21, 27}. To analyze PC biogenesis, dissociated NSPCs are cultured to
578 confluence and starved for 48 hours. Immunostaining analyses using antibodies raised against
579 PC markers show that those NSPCs harbor PC (**Figure 2A**). By combining two complementary
580 open-source tools, Ilastik, a machine-learning-based image analysis tool useful for PC
581 segmentation²⁸ and CiliaQ, a Fiji/ImageJ plugin package²⁹, that enable 3D reconstruction of PC
582 from 3D confocal image stacks, several structural parameters can be easily evaluated including
583 the number of PC and their length.

584
585 PC function of NSPCs can also be evaluated by testing the transduction of the Hedgehog
586 signaling pathway. To assess the transduction of the SHH signaling pathway, NSPCs are starved
587 for 48 hours and treated with recombinant SHH (rSHH) or a smoothened agonist (SAG) for 24
588 hours. Using antibodies raised against GPR161, SMO, and GLI2, immunofluorescent (IF)
589 analysis allows testing the trafficking of these key SHH signaling actors along the PC, with
590 GPR161 normally exiting the PC while GLI2 and SMO accumulate within the PC in response to
591 SHH pathway activation (**Figure 2C-D**). Analysis of 3D confocal image stacks by using CiliaQ
592 tools allows quantifying GPR161 exit from the PC as well as GLI2 and SMO accumulation within
593 the PC after SHH pathway activation (**Figure 2F-G**). In addition, semi-quantitative RT-PCR
594 analysis on mRNA extracted from rSHH- or SAG-treated NSPCs shows the induction of two
595 SHH target genes, *GLI1* and *PTCH1*, attesting for normal SHH signaling transduction in NSPCs
596 derived from control hIPSCs (**Figure 2B**).

597
598 Overall, 2D cell-based models of developing dorsal forebrain clearly reproduce several aspects
599 of normal cerebral cortex development and represent promising tools to dissect PC-associated
600 mechanisms underlying anomalies of neocortical development by using control versus patient
601 hIPSCs harboring mutations in genes responsible for human developmental anomalies of the
602 cerebral cortex.

603
604 **3D hIPS cell-based models to study primary cilium involvement during neocortical**
605 **development**

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

630
631 Immunolabeling experiments using the basal body (gamma Tubulin) and axoneme (ARL13B)
632 markers allow visualization of PC on 20 µm cryosections (**Figure 3G, H**). To take advantage of
633 the 3D organization of dorsal forebrain organoids, whole-mount immunostaining can be
634 performed. Light sheet acquisition of such *in toto* immunostained and cleared organoids
635 allows detection of both centrosomes and PC on all NSPC types as well as on neocortical
636 neurons (**Video 3**). In addition, to perform more accurate analyses of PC biogenesis,
637 immunohistochemistry analysis on free-floating thick sections (150µm) of dorsal forebrain
638 organoids may be performed. After clearing, such sections may be acquired using a 40x (NA
639 1.3) or 63x (NA 1.4) oil objective of an inverted resonant white light confocal laser microscope,
640 allowing to improve resolution while preserving the 3D spatial information of organoids. Such
641 laser scanning confocal microscope enables rapid acquisition of thick sections, with a precise
642 and flexible excitation and detection without any interference (**Video 4**). Several structural
643 parameters of PC can be evaluated, including PC number, length, and orientation, by using
644 3D-imaging software. Dedicated open-source, freely available tools such as Ilastik²⁸,
645 a machine-learning-based image analysis tool as well as the CiliaQ plugin package on
646 Fiji/ImageJ²⁹ are highly useful for automatic PC segmentation allowing subsequent qualitative
647 and quantitative analysis of PC biogenesis and function in control versus pathological
648 conditions.

649 **FIGURE LEGENDS**

650
651

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

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

674 (Mann Whitney test).
675 **Figure 3: Generation and characterization of dorsal forebrain organoids.** (A) Schematic
676 overview of the protocol to generate dorsal forebrain organoids. (B) Representative bright-
677 field images of organoids at days 1, 3, 7, 24, and 42 of differentiation. Confocal images of
678 20 μ m cryosections of organoids at day 42 (C) and 70 (D) after immunostaining with SOX2,
679 TBR2, and CTIP2 antibodies delineating respectively the ventricular zone (VZ), the
680 subventricular zone (SVZ) containing TBR2 positive IP, and the preplate like region (CP)
681 containing CTIP2 positive early-born neocortical neurons. Confocal images of 20 μ m
682 cryosections of organoids at day 42 (E) and 70 (F) after immunostaining with PAX6, CTIP2, and
683 SATB2 antibodies showing the appearance of SATB2 positive late-born neurons at Day 70.
684 Confocal images of 20 μ m cryosections of an organoid at day 42 after immunostaining with
685 ARL13B and PCNT antibodies revealing PC at the apical pole of radial glial progenitors (G) while
686 they are also present on CTIP2 positive neurons (H).

687 **Figure 4: 3D imaging of dorsal forebrain organoids.** (A) Light sheet acquisition of an entire
688 dorsal forebrain organoid (Day 42) stained with PAX6, N-CADH, and CTIP2 antibodies showing
689 the multiple neuroepithelial loops with PAX6 positive radial glial progenitors exhibiting correct
690 apicobasal polarity as revealed by the accumulation of N-Cadherin at their apical side and
691 CTIP2 positive early-born neocortical neurons delineating a preplate-like region. (B) Light
692 sheet acquisition of an entire dorsal forebrain organoid (Day 42) stained with TPX2, P-Vim,
693 and CTIP2 antibodies illustrating interkinetic nuclear migration of ventricular radial glial
694 progenitors. (C) Zoom on the light sheet acquisition of an entire dorsal forebrain organoid
695 (Day 42) stained with TPX2 and P-Vim antibodies showing a progenitor extending a unique
696 basal process and localized in the subventricular zone, reminiscent of an outer radial glial
697
698

699 progenitor. (D) Resonant Scanner acquisition of a 150 µm section of a dorsal forebrain
700 organoid (Day 42) stained with PCNT and ARL13B antibodies showing PC in NSPCs and
701 neocortical neurons.

702

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

716

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

719

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

722

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

725

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

728

729 **SUPPLEMENTARY FILES**

730 **Supplementary table 1:** Recipe of the media used for the generation of 2D-neural rosettes
731 and NSPCs

732 **Supplementary table 2:** Recipe of the media used for the generation of dorsal forebrain
733 organoids.

734

735 **DISCUSSION**

736 PC are now regarded as key organelles regulating crucial steps during normal cerebral cortical
737 development^{18, 19, 31} including NSPC expansion and commitment⁸⁻¹² as well as neuronal
738 migration^{13, 14} and synaptogenesis. In addition to analysis in animal models or human fetal
739 cerebral tissues, the generation of highly innovative and relevant patient-derived hiPSC-based
740 models of neocortical development is essential to dissect the role of PC during both normal
741 and pathological cerebral cortical development.

742

743 The 2D hiPSC-based modeling protocol detailed here was adapted from three major
744 publications²⁰⁻²². Neuroepithelial differentiation is induced by dual SMAD inhibition by using
745 small molecule inhibitors of the activin/nodal (SB-431542) and BMP (LDN-193189)

746 pathways²². Cells organized in rosette-shaped structures containing NSPCs as well as
747 neocortical deep layer neurons after 20 days of differentiation. In addition, these rosette-like
748 structures show correct apicobasal polarity, interkinetic nuclear migration of apical
749 progenitors as well as PC extending from all NSPCs and neurons. After dissociation of these
750 rosette structures, a homogeneous and stable population of cortical progenitors is obtained
751 ²¹. When cultured to confluence and starved for 48 hours, those cortical progenitors harbor
752 PC which morphology, number, and length can be easily analyzed by combining Ilastik²⁸ and
753 CiliaQ^{28, 29} plugin package on Fiji/ImageJ. Furthermore, by using cytokines to induce the SHH
754 signaling pathway, PC function can also be tested by IF to test the dynamics of crucial signaling
755 pathway actors along the PC such as GLI2, SMO, and GPR161. In addition, semi-quantitative
756 RT-PCR assays enable testing of the induction of SHH target gene expression, including *GLI1*
757 and *PTCH1*, in response to SHH pathway activation. Other signaling pathways dependent upon
758 PC function should also be tested including WNT and IGF pathways^{2, 32, 33}. To conclude on this
759 2D modeling approach which clearly reproduces several aspects of normal cerebral cortex
760 development, it represents a useful and relevant tool for testing PC biogenesis and function
761 in normal versus pathological conditions and should contribute to gain further insight into the
762 involvement of PC during neocortical development.

763 Complementary to 2D hiPSC-based modeling approaches, dorsal forebrain organoids offer
764 unprecedented opportunities to investigate normal and pathological cerebral development *in*
765 *vitro* as they recapitulate many features and characteristics of the early developing human
766 cerebral cortex. Two main types of protocols are currently used: the intrinsic and the guided
767 methods. The intrinsic protocol developed by Lancaster and colleagues²³ relies on the intrinsic
768 ability of iPSCs to self-organized with minimal external factors and gives rise to cerebral
769 organoids containing rudiments of distinct brain regions offering a unique opportunity to
770 model the interactions between different brain regions. However, the high variability and
771 heterogeneity inherent to such modeling strategy present significant challenges of
772 reproducibility. Guided organoid differentiation protocols allow the generation of brain
773 region-specific organoids with minimal heterogeneity²⁴⁻²⁶. This approach allowed us to
774 successfully generate dorsal forebrain organoids with ventricle-like structures that
775 recapitulate major processes of early human cerebral cortical development. The first critical
776 issue is to start with high-quality hiPSC cultures harboring large regular colonies exhibiting less
777 than 10% differentiation and which have been passaged almost once as a monolayer to adapt
778 the hiPCs to single-cell culture conditions. Indeed, to limit organoid heterogeneity, one of the
779 major challenges in the field, the formation of EBs with a homogeneous size is a prerequisite
780 which implies the need for dissociation of hiPSC colonies into single-cell suspension allowing
781 seeding at the defined cell density. Another critical step is BMM inclusion of EBs, needed to
782 support 3D structure and neuroepithelial expansion. We favored the group inclusion of about
783 fifteen organoids over individual inclusion even if it involves an additional step, the BMM
784 dissociation. BMM dissociation prior to shaking culture has been shown to reduce variability
785 within and between organoid batches, thus resulting in higher reproducibility³⁴. In addition, it
786 allows getting rid of cell processes extending into the BMM which are not detrimental for the
787 following differentiation steps but which make it difficult to observe organoids for quality
788 inspection during subsequent steps of the procedure. To improve nutritional absorption and
789 oxygen exchange, we compared organoid maturation on orbital shakers and spinning
790 bioreactors that led to similar results. We, therefore, chose the orbital shaker option, as it
791 allows to significantly reduce the medium volumes and therefore the total cost of the
792

793 experiments. Importantly, use a different incubator for stationary and shaking culture on an
794 orbital shaker to avoid any vibrations detrimental to adherent hiPSC growth. We successfully
795 applied this protocol on 5 distinct control hiPSC lines³⁵ that give rise to homogeneous results
796 ensuring the robustness of this procedure.

797

798 Characterization of such organoids can be achieved by using several methods. To preserve the
799 3D spatial information within organoids, we set up a protocol allowing whole-mount
800 immunostaining and clearing of entire organoids with subsequent light sheet acquisition.
801 Different clearing methods have emerged with efficiency depending upon sample origin and
802 thickness^{36,37}. Here, we set up a simple, fast, and cost-effective clearing method that relies on
803 TDE (2,2'-thiodiethanol), a glycol derivative previously used to clear mouse brain and intestinal
804 organoids^{38,39}. Acquisition of immunostained and cleared organoids was performed on a light
805 sheet microscope using a 20x objective immersed in 80% TDE. In comparison to other 3D
806 imaging acquisition methods, light sheet microscopy is of interest for several reasons: fast
807 acquisition, good penetration, and reduced photobleaching. Optimization of the
808 permeabilization step enables to reach efficient and homogeneous antibody penetration
809 allowing to visualize basal bodies and axonemes of PC extending from all progenitor and
810 neuronal cell types of the whole organoid. Furthermore, acquisition of free-floating 150 µm
811 thick sections using an inverted resonant scanning confocal microscope with 40x (NA 1.3) or
812 63x (NA 1.4) oil objectives enables to gain further into resolution, while preserving a significant
813 degree of 3D spatial information, and allowing qualitative and quantitative analysis of PC
814 biogenesis and function.

815

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

829

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838

839 **DISCLOSURES**

840 The authors declare no conflicts of interest.

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