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Automated high speed 3D imaging of organoid cultures with efficient multi-scale phenotypic quantification.

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ABSTRACT

Current imaging approaches limit the ability to perform multiscale characterization of 3D organotypic cultures (organoids) in large numbers. Here, we present an automated multiscale 3D imaging platform synergizing high-density organoid cultures with 3D live single objective light-sheet imaging. It is composed of disposable microfabricated organoid culture chips embedding optical components and a custom laser beam steering unit coupled to a commercial inverted microscope. It streamlines organoid culture and high content 3D imaging on a single user-friendly instrument with minimal manipulations and unprecedented throughput of 300 organoids per hour in 3D. Collecting large number of 3D stacks allowed training deep learning-based algorithms to quantify the organoids morphogenetic organizations at multi-scales, ranging from the sub-cellular scale to the whole organoid level. We validated the versatility and robustness of our approach on intestine, hepatic, neuroectoderm organoids and oncospheres.

INTRODUCTION

In organotypic 3D cell cultures, referred hereafter as organoids, stem cells differentiate and self-organize into spatial structures that share strong morphological and functional similarities with real organs. Organoids offer valuable models to study human biology and development outside of the body^{1,2,3}. An increasing number of organoids are being developed

41 that mimic intestines, liver, brain, kidney, lung, and many other organs^{2,4,5}. Their
42 differentiation is directed by a precise sequence of exposure to soluble growth factors and
43 extracellular matrix during their development. However, in marked contrast to organs, the
44 morphologies of organoids could be quite heterogeneous. In organs, phenotypic alterations
45 can be used as a proxy to diagnose a diseased state. In contrast, organoids differentiate in
46 minimally reproducible micro-environments resulting in a large variability of their
47 development path and morphology at the individual organoid level. Characterizing and
48 controlling such a diversity results in several technological roadblocks^{6,7}. Fast, automated,
49 and multiscale imaging is one of them. In a recent study⁸ quantitative imaging of intestine
50 organoid shape coupled to the assessment of a few genetic markers allowed defining the
51 organoids phenotypic development landscapes. The different organization paths that the
52 organoids spontaneously adopted were quantified and causally linked to the biological
53 programs orchestrating the spatial induction of different cell fates. However, such studies,
54 like also in Renner et al.⁹, still required a methodological and technological ‘tour-de-force’¹⁰
55 using a multi-well plate for organoid growth, and commercially available high content imaging
56 techniques performed on fixed organoids at specific time point during their growth. Arguably,
57 the ability to relate the diversity of genomic expression in organoids with their phenotypic
58 behaviour is a major step to guide the establishment of their culture conditions as well as to
59 provide a system biology view of developmental pathways. Hence, it exists a real demand for
60 the development of dedicated high content organoid 3D imaging methods to characterize
61 organoid at sub-cellular, multi-cellular, and whole organoid scales both in 3D¹¹ and during
62 their growth^{11,12}.

63 Optical sectioning fluorescent microscopy techniques, such as confocal or
64 multiphoton laser scanning, hardly fit the needs of both high throughput and low
65 photobleaching/phototoxicity for 3D/live imaging. Commercially available high content
66 screening platforms (e.g. PerkinElmer’s Opera or Molecular Devices’ ImageXpress), are
67 usually well adapted for 2D cell cultures. However, they generally offer low speed,
68 penetration depth, high bleaching, and image analysis issues when applied to 3D cultures.
69 Light-sheet fluorescence microscopy (LSFM) is undoubtedly the most appropriate imaging
70 technique to visualize fine cellular details at the whole tissue level, with maximal speed and
71 minimal phototoxicity and photobleaching¹³. LSFM has thus been favored for the study of live
72 organoids, as it allows long-term monitoring in physiological conditions¹⁴. However, it
73 traditionally involves several objectives, and despite its superior imaging performances, it
74 results in low imaging throughput due to cumbersome sample mounting requirements.
75 Recent studies using dual objective SPIM have tried to engineer solutions to improve the
76 throughput¹⁵ using a liquid handling robot and complex dedicated imaging hardware.
77 However, this solution does not permit long term sterile culture (more than 24 hours), as
78 periodic medium or extracellular matrix exchange is mandatory for organoid development
79 and differentiation. To overcome these important limits, several approaches have been
80 developed to perform LSFM with a single objective. High speed, high content SCAPE¹⁶ was
81 used to obtain ultra-fast images of *in vivo* processes in individual *C. elegans* and *Zebra fish*.
82 Alleviating the multi-objective constraint, other approaches proved capable to image multiple
83 samples. Epi-illumination SPIM (ssOPM) enabled parallel 3D volume acquisition of single cell
84 down to the single molecule level in 96 well plates¹⁷. It also allowed the imaging of living
85 oncospheres in 3D seeded in agarose to detect glucose uptake¹⁸ but only during a short period

86 of time (3 hours). Moreover, the random positioning of the spheroids in the agarose limited
87 the study to 42 oncospheres.

88 It follows that existing technological solutions still offer important room for improvement for
89 3D high content imaging of live and fixed organoids.

90 In this context, we developed a versatile high content (i.e multi-scale) imaging platform to
91 streamline organoid culture (from isolated hESC, hiPSC or primary cells to 3D multicellular
92 differentiated organoids) with fast non-invasive 3D imaging. It integrates a new generation of
93 miniaturized 3D cell culture device, called the JeWell chip. They comprise thousands of well-
94 arrayed microwells (JeWells) flanked with 45° mirrors to enable large scale single objective
95 light-sheet microscopy (soSPIM)¹⁹. Compatible with standard commercial inverted
96 microscope after simple addition of a beam steering unit and a dedicated control software,
97 our system performs 3D imaging of up to 300 organoids in an hour with subcellular resolution
98 and minimum photobleaching. We tested it on various organoid models (hepatocytes,
99 neuroectoderm, intestinal) and 3D cell cultures (hESC, HCT116). We leverage the large
100 throughput of the approach to train several public-domain convolutional neural networks to
101 extract relevant biological features at different scales, ranging from the sub-cellular, cellular
102 and multi-cellular scales, up to the whole organoid. We demonstrate how the technique
103 enables correlative imaging at different magnifications and resolutions, or between live
104 development and endpoint imaging of immunolabeled proteins or gene expression profile of
105 organoids.

106 RESULTS

107 *Streamlining high density organoid culture with automated high content 3D imaging.*

108 **Figure 1** illustrates the dedicated disposable microfabricated JeWell chips used to create high
109 density arrays of organotypic cell aggregates for automated high content 3D soSPIM imaging.
110 JeWells are truncated pyramidal shaped micro-cavities moulded in a photocurable resin
111 (NOA73) with 45° gold coated faces (**Figure 1a**). The detailed protocol (Online Methods) for
112 their microfabrication is illustrated in the **Supplementary Figure 1**. The JeWell microcavities
113 are arrayed at a typical surface density of 150 JeWells/15 mm², depending on individual
114 JeWell sizes, corresponding to the equivalent of 96 organoids in a single well of a standard
115 384 well plate. This ensures an optimal scanning distance between JeWells for high-speed 3D
116 soSPIM imaging. We devised the JeWell chips with various sizes and shapes in the format of
117 single Petri dishes, 6 well format or 96 well plates (**Supplementary Figure 1-2**). A Lipidure®
118 coating was used as an enduring antifouling treatment to prevent any adhesion of the cell
119 culture on the JeWell chips over months allowing long term 3D cell cultures (detailed
120 protocols provided in **Online Methods**). Single cells or small cell aggregates were seeded
121 homogeneously over the chips (**Figure 1b**). Buoyancy mismatch leads cells to sink at the
122 bottom of the JeWells (**Figure 1b**). The JeWell chips compatibility with phase contrast imaging
123 allows monitoring the formation of cell aggregates and assess their homogeneity. Rapidly
124 after the seeding phase, it enabled counting the number of cells per individual JeWell as a
125 reference point (**Online Methods**). **Supplementary Figure 3** illustrates the reproducibility of
126 the seeding procedure, demonstrating how the initial cell concentration and the seeding time
127 allows to precisely control the number of cells per JeWell (eg: 0.5x10⁶ cells/ml for 10 min leads

128 to 96 ± 10 SD cells/JeWell (N=100 wells)). We then monitored by phase contrast microscopy
129 the projected area of the compact aggregates forming within the first few hours post-seeding
130 (**Online Methods**). We observed a batch-to-batch variability of 10% (3 batches) and 25 %
131 dispersion of inter-JeWells aggregate sizes (N=100 organoids). Phase contrast imaging was
132 also used over a prolonged period of time (up to 30 days) during the differentiation protocol
133 to monitor and demonstrate a fairly homogeneous proliferation rate of the organoids
134 (**Supplementary Figure 3**). This procedure proved to be robust for all the tested cell types. In
135 the rest of the paper, cells are subsequently differentiated and grown in the JeWell cavities,
136 except when specified otherwise.

137 We then positioned the chips on a commercial inverted Microscope (Nikon Eclipse) equipped
138 for single-objective Selective Plane Illumination Microscopy (soSPIM) as described in Galland
139 et al.¹⁹ The imaging system is composed of: *i*- interchangeable lenses (20x air (NA=0.75) and
140 60x WI (NA=1.27) used here but not limited to) (**Figure 1c**) *ii*- a custom laser scanning unit
141 and *iii*- an acquisition module operating inside MetaMorph commercial software. The **Online**
142 **Methods** and **Supplementary Figure 1** detail the acquisition set-up. In brief, the 45° gold
143 coated micromirrors of the JeWells enable to reflect a laser beam (**Figure 1d**) focused on the
144 sample by the same objective used for imaging. Scanning the laser beam along the mirror axis
145 (y-direction) creates a light-sheet perpendicular to the optical axis of the objective,
146 illuminating a thin section of the sample. The fluorescent signal is then collected through the
147 same and single objective used to create the light-sheet. Translating the laser beam
148 perpendicularly to the mirror (x-direction) translates of the light-sheet along the optical axis
149 of the objective (z-direction). Synchronizing the light-sheet position with the focal plane of
150 the objective ensures optimal 3D optical sectioning. The integration of the optical
151 components inside the chips eliminates the steric and alignment constraints of traditional
152 multi-objective light-sheet microscopes. We devised an automatic acquisition pipeline that
153 integrates *i*- an optional pre-screening step, *ii*- the automated identification and user-assisted
154 selection of the JeWells containing organoids to image, *iii*- the precise automatic positioning
155 of the light-sheet on the JeWell mirrors (**Online Methods** and **Supplementary Figure 4**). This
156 allows to rapidly setup the sequential 3D multi-colour imaging of hundreds of organoids in a
157 single acquisition workflow, with minimal user interaction. This approach builds on our
158 previous work¹⁹ devised for single molecule imaging in 3D using single objective SPIM
159 (soSPIM). With a 60x 1.27 NA objective, we could acquire images with a lateral resolution of
160 around 300 nm. The light-sheet thickness could be manually adjusted between 2 μm to 3.7
161 μm for a length of 12 μm to 60 μm respectively using a 60x magnification. A light-sheet of 5.7
162 μm thick for 135 μm long was obtained with a 20x magnification objective. We took benefit
163 of the JeWells as proxies for automatic alignment and calibration of the light-sheet as well as
164 for drift correction (**Supplementary Figure 4**).

165 This new integrated imaging platform provided a very efficient way to perform long term
166 automatic 3D imaging of various organoids with unprecedented throughput and limited
167 manipulation. **Figure 1e** illustrates a collection of 96 neuroectoderm organoids differentiated
168 from hESCs, fixed at day 8 and immunostained for Nuclei (405nm), Sox2 (488nm) and N-
169 cadherin (647nm) (**Material and Methods**). Setting-up the entire screening parameters and
170 selection of 96 organoids took around 10 min using a 60x WI 1.27NA objective, including the

171 high-resolution pre-screening of the JeWell chip. Each 3D stacks of immunostained fixed
172 organoids took about 7 sec per wavelength for 70 optical sections using one side light-sheet
173 illumination. The time between two adjacent JeWell acquisitions was 1.5 sec and the light-
174 sheet repositioning accuracy below 50 nm. It took 1h to acquire 96 organoids with 70 z-planes
175 for 3 wavelengths, representing a total of 173GB of raw data. The sectioning and the quality
176 of the images shown in **Figure 1e** insert illustrates the level of sub-cellular details that can be
177 resolved.

178 *Compatibility with various 3D cell culture types.*

179 We then demonstrated the robustness and the versatility of the approach by culturing and
180 imaging a wide variety of organotypic systems thanks to JeWell cavities that matched the final
181 size and shape of the targeted organoids (**Figure 2a, Supplementary Figure 2**). We grew a
182 variety of primary cells, embryonic cells, cancer cells in the JeWell chips according to
183 individual protocols described in **Online Methods**.

184 **Figure 2b** shows that aggregates of primary rat hepatocytes developed secreting bile
185 canaliculi (2 μm diameter intercellular lumens). Using MRP2 (bile transporter) as a marker of
186 functional canaliculi, we could reconstitute the canalicular network in 3D (**Movie 1**).

187 **Figure 2c** exemplifies the culture of neuroectoderm organoids successfully differentiated
188 from human embryonic stem cells (hESCs). They displayed the characteristic transcription
189 factors for early ectoderms and developed *bona fide* rosette multicellular organisation. 3D
190 soSPIM imaging with a 60x 1.27NA WI objective allowed to resolve single actin protrusions
191 within the rosette core with a lateral resolution of about 300 nm.

192 **Figure 2d** exemplifies the culture of HCT116 human colon carcinoma cells aggregated in
193 oncospheres that displayed a clear hypoxic core when cultured under low oxygen conditions.
194 Staining with Ki67 revealed the proliferation stages of the cells inside each oncosphere.

195 **Figure 2e** displays the images of human stem cells cultured in Matrigel for 7 days within
196 alginate micro-capsules (TreeFrog Therapeutics technology), removed from their capsules,
197 seeded and imaged in large number in JeWell cavities with large top opening (270 μm). It
198 revealed the multilayer cyst that formed in each alginate capsule.

199 **Figure 2f**, finally, shows early (8 days) and late (20 days) intestinal organoids cultured and
200 imaged in elongated JeWell grooves. At day 8, the organoids displayed sparse regions of CDX2
201 positive cells. At day 20, CDX2 was ubiquitously expressed with a strong Villin signal lining the
202 apical pole of the elongated lumen within each of the organoids. The format of the elongated
203 JeWell grooves provided a scaffold to orient the organoids' growth.

204 Altogether, we successfully grew and imaged functional organoids and oncospheres in the
205 JeWell chips. It illustrates the great potential of our approach to collect large collections of
206 high resolution 3D images of organoids that are either directly grown inside the JeWell chips,
207 or produced outside and seeded after. The image quality is in agreement with state-of-the-
208 art single side Gaussian light-sheet illumination expectation.

209 *Automatic quantification of cell localization and proliferation states*

210 Single side illumination scheme proved to be an excellent compromise between acquisition
211 speed, phototoxicity, and image quality to perform reliable automated quantitative analysis.

212 The simple and fast 3D acquisition of a large number of organoids, makes the system
213 particularly suited to implement deep learning-based quantitative analysis. As an illustration,
214 we first analysed HCT116 aggregates (human colon cells) grown in the JeWell chips. The
215 aggregates originated from 80 cells ± 20 , which reached different sizes after 10 days
216 proliferation. We then fixed and stained them for Nuclei (DAPI) and Ki67 as a marker for
217 chromosome contours, and acquired 3D stacks with the soSPIM using the high content 3D
218 imaging protocol described above. We trained the convolutional neural network (CNN)
219 StarDist over 471 planes (5274 nuclei) manually annotated in 3D from the DAPI images
220 (**Online Methods**). The quality of the 3D segmentation of the nuclei (**Online Methods**) is
221 exemplified in **Figure 3a**. We manually compared the ground truths to the predictions in 3D
222 on 10 organoids, assessing a detection accuracy superior to 85% (**Movie 2**). Importantly the
223 detection accuracy was equivalent on both sides of the oncosphere, demonstrating that the
224 Stardis CNN was insensitive to the inhomogeneities originating from the single sided Gaussian
225 illumination (**Supplementary Figure 5**).

226 We then used the Ki67 channel to detect and classify proliferating cells²⁰. We used the Yolov5
227 convolutional neural network to detect and classify dividing cells (**Online methods**) into three
228 proliferation phases: G1/S/G2 (large dense clusters of Ki67), Mitosis (condensed chromatide
229 marked by Ki67) and Early G1 (Nuclei with scattered Ki67 aggregates), with an accuracy of
230 90% (**Figure 3b, Movie 3**). G0 cells were Ki67 negative. We found that the number of nuclei
231 scaled logically with the volume of the organoids (**Figure 3c**). However, the fraction of Ki67
232 positive nuclei decreased with the total number of nuclei per organoids, indicative of a
233 reduced proliferation rate with the organoids size. Further analysis showed that cells were
234 arrested more specifically in G0 phase as the organoids grew. Indeed, amongst the
235 proliferative cells, the proportion of early G1 and Mitosis was insensitive to the organoid
236 volume, indicating that the division cycle time was independent of the organoid size with no
237 arrest at any other proliferation check point.

238 *3D multiscale analysis of organoid morphologies*

239 Beyond nuclei segmentation and proliferation analysis, we further demonstrated that our
240 method enables a multiscale analysis of the organoids morphology through the analysis of
241 neuroectoderm organoids²¹ at day 8. We used YoloV5 CNN to localize in 3D mitotic and cell
242 death events directly from the DAPI images. Using 28 annotated organoids, we reached an
243 accuracy of 89% (4% false positive, 7% false negative, benchmarked on 20 test organoids by
244 a trained human). **Figure 4a** shows the 3D organization of the mitotic and cell death events.
245 They appeared homogeneously distributed within the organoid volume, as expected for
246 healthy proliferating organoids. This spatial homogeneity was confirmed by the equal average
247 density of mitotic and cell death computed in 3D regions of interest located in the central vs
248 peripheral regions of the organoids (**Online Methods**). It demonstrates that the classification
249 of proliferation events can be performed on different types of organoids.

250 We then characterized the spatial distribution of cells expressing differentiation markers.
251 Neuroectoderm organoids organized locally in multicellular rosette structures (**Figure 4b**)
252 characterized by layers of aligned cells surrounding a central streak that develops into a
253 lumen^{22,23,24,25}. We used the 3D StarDist CNN to segment individual nuclei from the DAPI

254 channel, and computed the average expression of Sox2 in each segmented nucleus. The
255 distribution of expression levels proved to be bimodal. We thus binarized the distribution
256 between Sox2 positive and Sox2 negative cells by setting a threshold value at the bimodal
257 intersection point (680 au). It allowed us to reconstitute a 3D map of the Sox2 expressing cells
258 in each organoid (**Figure 4b**), clearly revealing the rosette organisation with Sox2 positive cells
259 surrounding the central streak. We then endeavoured to segment the rosettes based on the
260 actin organization, an ubiquitous marker for any cell type that could be used generically in
261 live cell imaging. In that perspective, we trained a U-Net CNN (**Online Methods**) to segment
262 the rosettes in 3D (**Figure 4c, Movies 4-5**). It enabled to extract the number of rosettes per
263 organoids as well as their morphological features for further classification (**Supplementary**
264 **Table 3, Supplementary Figure 7**) (N=415 streaks longer than two cells (20µm)).

265 Finally, we assessed the neuroectoderm variability of the organoid whole shapes using the
266 shape recognition CNN DenseNet121²⁶ (**Online Methods**) to classify automatically the
267 organoids in two morpho-classes: bean shaped (B-shaped) or spherical shape (O-shaped)
268 (**Figure 4e**) with 99 % accuracy (**Supplementary Figure 6b**). Interestingly, B-shape organoids
269 systematically displayed fewer nuclei and internal rosettes than O-shape ones.

270 Altogether, we demonstrated that our integrated densely arrayed culturing JeWell chips
271 combined with our high content 3D imaging platform allows training efficiently different CNN
272 to quantitatively assess neuroectoderm organoids at several scales, ranging from the single
273 cell level, up to the whole organoid level.

274 *Multi-magnification capabilities and image analysis allow efficient extraction of rare events*

275 We then demonstrated that the organoids can also be imaged at different magnifications to
276 facilitate the localization of rare cellular clusters composed of a few cells expressing specific
277 markers. We used intestinal organoids that have been grown and differentiated for 8 days
278 (early stage) (**Figure 5**). We fixed and stained them for nuclei (DAPI), actin, CDX2 (a major
279 regulator of intestine-specific genes) and E-cadherin (E-cad). As expected, at these early
280 stages of differentiation, only a fraction of the cells was CDX2 positive. We leveraged on the
281 arrayed organization of the organoids grown in JeWell chips to perform correlative multi-
282 magnification 3D imaging. First, we rapidly scanned 100 organoids in 3D (10 min for 100
283 organoids) at 20x magnification (air objective, 10 Z plans, 7 microns Z-steps) only for two
284 channels (CDX2 and actin) (**Figure 5a**). The CDX2 channel was then automatically analyzed
285 and we identified the CDX2 positive organoids (CDX2⁺) as representing 20% of the whole
286 library. We next performed a second scanning of the CDX2⁺ organoids for the 2 other channels
287 (E-cad and DAPI) to screen the E-cad expression profile at this early stage, still at low
288 magnification (20x) but with higher axial sampling (**Figure 5b**, 50 plans, 1 micron z-steps, 10
289 min for 20 organoids). An automated segmentation (**Online Methods**) within the E-cad
290 channel (**Figure 5b**) allowed identifying E-cad expressing cells clusters (Ecad⁺) present in 25%
291 of the CDX2⁺ organoids (5% of the whole library of 100 organoids). Finally, a third round of 3D
292 acquisitions at high resolution using a 60x magnification (WI 1.27NA) objective was performed
293 to image exclusively the regions with E-cad⁺ cells (4 channels, 50 Z plans, 1 micron z-steps,
294 **Figure 5d**). This high-resolution 3D imaging enabled to reconstruct in 3D the E-cad⁺ junctions
295 of CDX2 positive cells, which was not possible at 20x magnification. **Figure 5e** displays one

296 representative example of 3D reconstruction of E-cad⁺ cellular cluster where one central cell
297 was surrounded and linked to 4 cells (only five E-Cad⁺ cells among thousands constituting one
298 single organoid). These 5 cells, localized at the border of the lumen, showed also a very high
299 level of CDX2 compared to the adjacent cells, confirming the link between CDX2 and E-cad
300 expression profiles. Altogether, the acquisition and the analysis took less than 1h. Finding rare
301 cellular clusters within the organoids population was greatly facilitated by the capability of
302 multi-magnification imaging of the soSPIM imaging system.

303 *Live imaging modalities*

304 We finally demonstrated that our approach performs correlative imaging between the
305 morphology of live developing organoids and the end-point analysis of their transcription
306 profiles. We followed in live the 3D development of neuroectoderm organoids. We first
307 stained the intercellular clefts in the organoids (**Figure 6a**) with extracellular dyes (Calcein,
308 AlexaFluor 647). **Supplementary Figure 8** demonstrates that the dyes infiltrated within 15
309 minutes the entire organoid intercellular volume without effect of the JeWell cavities. It
310 allowed us to monitor in 3D cellular movements during the development of rosettes and
311 streaks (**Movie 6**). To establish the maturation stage of the cells around the rosettes, we fixed
312 the organoids at day 7 and stained them for Sox2 (early development marker) and Pax6 (later
313 development marker). The quality of the repositioning method of individual JeWell enabled
314 to overlap the last images of the live sequence with the immunostained images of the same
315 organoid (**Figure 6b**) by a simple registration in z. It revealed the presence of Pax6 in some
316 rosettes and Sox2 in others. We then used the 3D time lapse to back track the rosette
317 formation process (**Figure 6a**), which revealed that the Sox2⁺/Pax6⁺ rosettes (white arrow)
318 appeared 3 days before the Sox2⁺/Pax6⁻ rosettes. However, immunostaining only provided
319 access to the spatial distribution of a limited number of transcription factors or protein of
320 interests.

321 To identify the expression level of a higher number of expression factors and proteins of
322 interest, we thus performed RTqPCR on libraries of organoids after imaging. We first tracked
323 the development of neuroectoderm organoids using stem cells expressing life-act GFP and
324 Histone 2B-mCherry²⁷ (**Movie 7**). We monitored 25 organoids, capturing one z-stack of 70
325 optical planes every 15 minutes for 8 days, representing a total of 500 3D image stacks per
326 organoid acquired in 125h (**Online Methods**). During these 8 days, we did not notice any
327 obvious effect of photodamaging as mitosis and proliferation continued and no cluster of
328 death cells were observed. Furthermore, at day 8, we did not notice any differences in the
329 organoids size or shape of cellular patterns between the imaged vs non-imaged organoids in
330 the JeWell chips. Backtracking the rosettes from day 8, we could follow the time course of the
331 first development steps based on local cell arrangements (**Figure 6c, Movie 8**). It revealed
332 that rosette-like structures started to appear in the organoids after 4 to 5 days (consistently
333 with **Figure 6a**). We then collected the organoids alive by peeling-off the JeWell membrane
334 from the glass slide with a sterilized tweezer (**Figure 6c**), and subsequently performed RTqPCR
335 on eleven specific markers (**Online Methods**). Results in **Figure 6c** unambiguously
336 demonstrated the differentiation into neuroectoderm lineage after 8 days of culture in the
337 JeWells as compared to day 2 (undifferentiated stem cell aggregates), characterized by a 10-

338 fold reduction in Ecad and NANOG, and 500-fold increase in Pax6 for example. This
339 demonstrates that organoids cultured in JeWell chips can be collected for further omics
340 analysis or also be re-seeded in other devices or even injected *in vivo* as the sterility and
341 viability has been conserved.

342 **DISCUSSION**

343 Our automatized high content 3D imaging platform streamlines high-density organoid culture
344 with light-sheet-based 3D fluorescence microscopy. It enables monitoring hundreds of live or
345 fixed organoids in 3D, with an acquisition rate of about 300 organoids per hour, without the
346 need for complex and time-consuming sample manipulation between culturing vessels and
347 imaging supports. It is a versatile platform allowing investigating diverse types of biological
348 systems thanks to the new JeWell culturing device, which dimensions can be scaled according
349 to the expected size and shape of the organoids. In the variety of cells we cultured, we did
350 not see any specific impact of the JeWells on the organoid growth. Organoids can also be
351 grown outside, in dedicated culturing devices, and deposited later inside JeWells for 3D
352 imaging. Our approach leverages on the soSPIM technique developed earlier¹⁹ to perform in
353 depth 3D single molecule localization microscopy in isolated cells. We redesigned the beam
354 steering unit and the acquisition workflow to allow, in combination with the JeWell device, a
355 fully automated high content 3D live imaging of a large number of cell aggregates. It can be
356 easily mounted on any standard inverted microscope, making multi-modal correlative
357 imaging straightforward. We demonstrated that its high-speed acquisition capacity fulfills the
358 requirements of advanced deep learning analysis tools, which require large training data
359 dataset, and provided examples of multiscale quantitative analysis of organoid development.
360 While we performed all the analysis off-line, after the acquisition, the high-speed of the
361 prediction algorithms once trained, will likely allow real time predictive analysis of the
362 organoids while they develop, opening new applications for drug screening and regenerative
363 medicine.

364 All the soSPIM images we presented were intentionally raw data, with single-side
365 illumination. Their quality proved a good compromise between speed, image quality and
366 processing time. The native quality of our imaging platform was good enough to extract
367 cellular details with good accuracy. However, there are several ways to improve the quality
368 of the images. Multi-side illumination, up to 4, is obviously possible using the different mirrors
369 composing the JeWell cavities. But it comes with a cost of instrumentation complexity,
370 imaging time, photobleaching and image processing time, and should thus be used only if
371 necessary. Line confocal and structured illumination for contrast and sectioning enhancement
372 could also be implemented like for any other traditional SPIM, at not instrumental cost. A
373 significant advantage of our technology over most existing light-sheet approaches, including
374 single-objective ones, is its simplicity to change the objective lens to probe various types of
375 3D cultures with optimal spatial resolution and field of view, and perform multimodal
376 imaging. Changing magnification also alleviates the requirement of soSPIM to fit both the
377 mirror and the samples within the field of view of the objective. Therefore, with a 60x
378 magnification it is limited to 200 μm width organoids, and with a 20x magnification to 600 μm
379 width organoids. This is not really an issue since most reported organoids are within this size

380 range: cancer pancreatic²⁸: 100 μm , intestinal²⁹: 50-200 μm , gastruloids³⁰: 100 μm diameter
381 and 400 μm length, liver³¹: 300 μm , micro kidney³²: 150-200 μm , cancer spheroids³³: 250-750
382 μm

383 A natural future extension of our method is to provide high content screening capacities to
384 allow multi-condition testing in a single workflow. As a proof of concept, we developed a
385 prototype of a 96 well plate integrating arrays of 96 JeWells in each compartment
386 (**Supplementary Figure 1**). Even with the demonstrated unprecedented acquisition speed,
387 imaging the potential 9,216 organoids in 3D with 70 optical planes would take 0.6 TB (resp
388 1.7 TB) and 1.3 day (resp 4 days) of imaging for a single color (resp 3 colors). The typical
389 analysis time we measured per organoid for the longest convolutional neural network we
390 used is 40 sec per organoids on a standard workstation. Therefore, we estimate that the
391 analysis of the full 96 well plate could be done in 4 days. It could hence be performed almost
392 in parallel as the organoids are acquired. This demonstrates that the imaging scheme we
393 describe here is a good candidate to develop a functional high content screening platform
394 dedicated to organoids.

395 Our high content organoid 3D imaging approach provides a unique alternative to existing high
396 content screening techniques, offering unsurpassed culturing and imaging throughput,
397 opening new perspectives in the field of organoid research.

398 **DATA AND MATERIAL AVAILABILITY**

399 The raw images of the organoids acquired in this study as well as the training datasets for the
400 convolutional neural networks will be available upon request. We will provide free access to
401 JeWell chips for testing based on Material Transfer Agreement.

402 **CONFLICT OF INTEREST.**

403 The authors declare no conflict of interests.

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414 **ONLINE METHODS**

415 **Fabrication methods for the JeWell chips**

416 JeWell pyramidal microwells were produced by casting a textured polymeric thin film using
417 capillarity filling. The textured film was then coated with metal to enhance the reflectivity of
418 the 45° slanted surfaces, flipped, and finally glued to a coverslip to form the final JeWell chips.
419 The detailed fabrication procedure is as follow. First, a silicon primary mold featuring array of

420 45° truncated pyramidal microwells was made with an optimized process based on the
421 protocol previously published in Galland et al.¹⁹. The JeWell design required to at least double
422 the previously achieved etch depth, while keeping the same optical finishing. To achieve this,
423 we have revised and modified the overall procedure in order to: (1) Reduce the number of
424 steps required to minimize the introduction of defects during handling of the wafer, (2)
425 Increase the thickness of the oxide layer used to 500 nm, which allowed for longer etching
426 times before the deformation or breaking of the masking layer could introduce defects, and
427 (3) modify the lithographic process and the RIE pattern transfer in order to comply with the
428 higher thickness of the oxide. The fabrication of the JeWell devices required also to modify
429 the fabrication process previously published. First, the silicon primary mold is replicated in
430 PDMS (Supplementary Figure 1a). PDMS Sylgard 184 (Dow Corning) base resin and
431 reticulation agents are mixed in 10:1 ratio and out-gassed (20-30 min in a vacuum jar
432 evacuated to 5-10 mbar). The liquid PDMS is poured on the silicon mold and further out-
433 gassed to ensure filling of the cavities. (vacuum jar at 5-10 mbar for 10 min). After thermal
434 curing (2h at 75 °C on a hot plate) the PDMS mold is peeled-off and cut in 1 x 1 cm² pieces.
435 Each 1 x 1 cm² cut can be used multiple times to produce JeWell devices.
436 The PDMS mold features need then to be reproduced onto a glass coverslip. To do so, a PDMS
437 1x1 cm² piece is first flipped face-down on a flat PDMS substrate (Supplementary Figure 1a),
438 then a small amount of liquid UV-curable optical resin (NOA73, Norland Optics, USA) is
439 dropped at one edge of the mold. To be noted that differently from the soSPIM devices, using
440 of an index matched polymer is not required, since the laser sheet is never going to pass
441 through the textured film along its path to reach the organoids inside the pyramidal wells.
442 Thus, cheaper and biocompatible curable resins can be used, such as NOA73. Driven by
443 capillarity, the liquid fills the cavity between the PDMS mold and the flat substrate. Once the
444 whole structured cavity is filled, the NOA is cured by flood exposure to UV light (UV LED KUB2,
445 Kloe France, 365 nm with power density of 25 mW/cm², 1 min exposure). Then, the PDMS
446 mold is peeled-off leaving the cured NOA featuring inverted JeWells on the flat PDMS
447 substrate that will have to be flipped 180° around onto a coverslip to end with correctly
448 oriented pyramidal wells. Excess material is trimmed on all 4 edges and a film of Au is
449 deposited by sputter-coating (Jeol Auto fine-coater, Jeol Japan). A sputtering process of 45 s
450 with setting at 20 mA was repeated 4 times to achieve a final thickness of the coating of about
451 40 nm, with 1 min waiting between each step to allow for sample cooling. Meanwhile, a glass
452 coverslip (such as #1.5H) is prepared as a final substrate for the JeWell device by coating it
453 with a thin layer of NOA73. A layer of NOA of about 20-30 μm thick is deposited on the
454 coverslip by spin-coating and pre-cured under UV-light (30s in the same UV LED KUB2) so to
455 become solid but still retaining adhesive properties. The Au-coated NOA structured film (still
456 on the flat PDMS substrate) is flipped and placed in contact with the NOA-coated coverslip
457 and gently pressed to ensure good contact. The NOA adhesive layer is then fully cured with a
458 final step of UV flood exposure (1 min in the same UV LED KUB2 as previously) and the flat
459 PDMS substrate can finally be peeled-off removing at the same time the portion of the metal
460 film which is not coated on the NOA73 film.

461 **Long term passivation of JeWell chips**

462 This step is mandatory to avoid any adhesion of cells to the chips during the time of the
463 experiments (>25 days) and to allow 3D culture of the cells. A solution of Lipidure® (CM5206,
464 NOF America) at 0.5% (w/v) in pure ethanol is poured to completely cover the JeWell chips.
465 Optionally, a O₂ plasma cleaning of the coverslip (5min) has be done before. The JeWell chips
466 covered with Lipidure solution is out-gassed (5-10 min in a vacuum jar evacuated to 5-10

467 mbar. Then, the ethanol-Lipidure solution is allowed to completely be evaporated under a
468 sterile hood (>2 hours) in order to form a coating with same molecular structure as polar
469 group of phospholipids. Before cells seeding, JeWell chips are rinsed with PBS, out-gassed as
470 previously, and finally filled with cell culture medium.

471 **Stem cell lines and their maintenance.**

472 The human embryonic stem cell lines (hESCs) used for the experiments were H1 (WiCell
473 Research institute, Inc. Madison, WI, USA) and K21 (lifact-GFP, H2B-mCherry) (gift from Orly
474 Reiner's lab, Weizmann Institute, Israel). Both cell lines used were on early passage number
475 and confirmed to be contamination free.

476 We used hPSC-qualified Matrigel™ (354277, BD Biosciences) as the basement membrane
477 matrix to culture and maintain the cells in the maintenance medium mTeSR™1 medium
478 (05850, StemCell™ Technologies) for H1 and RSeT™ medium for K21 (05978, StemCell™
479 Technologies). The 80% confluent H1 cultures were passaged using Dispase (07923, StemCell™
480 Technologies). We used TrypLE™ Express Enzyme (12605028, Thermofisher scientific) for K21
481 cultures. Gentle mechanical scrapping detached the cells. We resuspended them in fresh
482 medium before to be re-plated on Matrigel™ coated wells.

483 **Embryoid body formation in JeWells**

484 For formation of embryoid bodies (EB) in JeWells, we dissociated the hESCs into single cell
485 suspension by enzymatic with ReLeSR™ (05872, StemCell™ Technologies) for about 9 minutes.
486 The hPSC cells were suspended at a concentration of 0.5 million cells/ml in EB formation
487 medium supplied in STEMdiff™ Cerebral Organoid Kit (08570, StemCell™ Technologies)
488 supplemented with 10 μM Y27632 (72304, Calbiochem, Merck Millipore) and seeded onto
489 the JeWells. The cells were incubated for 10 minutes to allow adequate number of cells to
490 enter per JeWell. Then we washed the chips with DMEM/F12 medium to remove excess cells.
491 We incubated in EB formation medium supplemented with 10μM Y27632 for 24 hours. The
492 neuroectoderm differentiation of EBs in JeWells was then initiated after 24 hours of cell
493 seeding and/or when the EBs where $\geq 120\mu\text{m}$ in size.

494 **Induction of neuroectoderm from hESCs in JeWells**

495 To induce neuroectoderm differentiation, the embryoid bodies were cultured in STEMdiff™
496 Cerebral Organoid Kit (08570, StemCell™ Technologies) according to the manufacturer's
497 instructions with slight modifications: when the EBs in JeWells reached $\geq 120\mu\text{m}$ in size we
498 induced them to neuroectoderm using the induction medium from Cerebral Organoid Kit for
499 4 days. On day 5, the induction medium was removed and the induced EBs were embedded
500 in 100% hPSC-qualified Matrigel™ (354277, BD Biosciences) for 30 mins. They were then
501 allowed to undergo neural expansion using expansion medium from Cerebral Organoid Kit for
502 3 days.

503 **Formation and growth of intestinal organoids in JeWells**

504 The hESCs H1cell line was used to generate intestinal organoids. The induction of cells was
505 done using STEMdiff™ Intestinal Organoid Kit (05140, StemCell™ Technologies) according to
506 the manufacturer's instructions with slight modifications: H1 cells were seeded as cell
507 aggregates of $>200\mu\text{m}$ on Matrigel™ coated 24-well plate and allowed to reach ~90%
508 confluence. At confluence, the cells were induced to definite endoderm for three days
509 followed by mid/hindgut differentiation for next 6 days. Once the mid/hindgut differentiation
510 was achieved the cells were dissociated as clumps of around 50-75μm by physical pipetting
511 and seeded at a high density to cover all the JeWells. The aggregates were incubated for 15

512 minutes to allow adequate number of aggregates to enter the JeWells. Then the JeWells were
513 washed with DMEM/F12 medium to remove excess cells and coated with Corning® Matrigel™
514 Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free (356231,
515 Corning) for 30 minutes. Once the Matrigel™ has solidified, the cell aggregates were supplied
516 with intestinal organoid growth medium to generate intestinal organoids for 8-9 days. The
517 medium was change every other day to keep the culture healthy.
518 For long-term culture of intestinal organoids in JeWells (20 days), pre-formed intestinal
519 organoids in conventional culture system were passaged and broken into small clumps and
520 seeded into JeWells in a similar way as described above. The JeWells were supplemented with
521 fresh intestinal organoid growth every alternate day till required.

522 **Primary rat hepatocytes isolation and seeding in JeWells**

523 Hepatocytes were harvested from male Wistar rats by the two-step collagenase perfusion
524 method as described by Seglen et al.³⁴. Animals were handled according to the protocol
525 established by Institutional Animal Care and Use Committee (IACUC) approved by the IACUC
526 committee of National University of Singapore. Each isolation yielded >10⁸ cells, with the
527 viability ranging from 75-90% as tested by Trypan Blue exclusion assay. 0.15x10⁶ cells/ml
528 suspended in cold hepatocytes William's E culture media (A1217601, Thermofisher scientific)
529 supplemented with 1 mg/ml BSA (A7906, Sigma-Aldrich), 100nM Dexamethasone
530 (D4902, Sigma-Aldrich), 2mM L-Glutamine (25030081, Thermofisher scientific), 0.3µg/ml of
531 insulin (I9278, Sigma-Aldrich), 50µg/ml linoleic acid (L1376, Sigma-Aldrich), 100 units/ml
532 penicillin and 100µg/ml streptomycin (15140122, Thermofisher scientific) were seeded as
533 previously described per JeWell chip and the chips placed in the incubator for 5-10min to
534 allow for the cells to settle. The dishes were then swirled, and the supernatant was collected
535 to remove the floating cells. 1ml of fresh hepatocytes culture medium was added to the
536 JeWell chip and were left to incubate during 2 to 4 days before fixation and immunostaining.

537 **Cancer cell lines seeding in JeWells and formation of spheroids**

538 HCT116 cells, colorectal cancer cells, were maintained in RPMI 1640 media (Invitrogen,
539 11875093) supplemented with 10% FBS (10082147, Invitrogen), 100 U/mL Penicillin-
540 Streptomycin (15070063, Invitrogen) and 10mM / mL of Hepes (15630080, Invitrogen) at 37°C
541 and 5% CO₂. After trypsinization, the cells were suspended in complete 20% FBS
542 DMEM (11965092, Invitrogen) media and the cell suspension was adjusted to
543 0.5X10⁶ cells/ml. 1ml of cell suspension was placed in 35mm JeWell plates for 10 mins to get
544 approximately 80 cells per JeWell. The cell suspension was removed, washed once with DPBS
545 (14190250, Invitrogen) and 2ml complete 20% FCS DMEM media was added to the plate. The
546 media was changed on day 3 and day 6. The cells were fixed at day 10 for immunostaining.

547 **Encapsulated cysts of stem cells culture and seeding in JeWells**

548 Encapsulated cysts of hiPSC line IMAGINEi004 stem cells, were produced according to the
549 protocol described in³⁵. They were kindly provided by TreeFrog Therapeutics
550 <https://treefrog.fr/> in alginate capsules produced according to the system described in³⁶. The
551 cell/matrix suspension was entrapped inside a closed and permeable micro-sphere. They
552 were cultured in mTeSR1 culture medium within T-flasks and maintain in a cell culture
553 incubator at 37°C at 5% CO₂. The medium (mTeSR1) was supplemented with 10 µM Y-27632
554 for ROCK inhibition during the first 24h of cultures. Medium culture were change every day
555 until day 6. The encapsulated cyst were provided fixed in PBS. The capsules were removed by
556 incubating them in EDTA solution for 2 min (1 µM) and then stored in PBS + 0.1 tween20

557 before staining according to the protocol detailed below and seeding into JeWell chips. The
558 stained cysts were seeded in square JeWells with 250 micron top opening at 1000 cyst/ml.

559 **Organoids immunofluorescence staining for imaging (all organoids)**

560 Organoids/spheroids in JeWell chips were fixed for 20 minutes in 4% paraformaldehyde
561 (28906, Thermofisher scientific) at room temperature. IN the case of the capsules, we
562 dissolved the alginate membrane using EDTA (10 mM) for 1 min at RT. The 3D cultures
563 permeabilized for 24 hours in 1% Triton X-100 (T9284, Sigma Aldrich) solution in sterile PBS
564 at 4°C on an orbital shaker, followed by 24 hours incubation in blocking buffer (2% bovine
565 serum albumin BSA (37525, Thermofisher scientific) and 1% Triton X-100 in sterile PBS) at 4°C
566 on an orbital shaker. Samples were then incubated with primary antibodies (Suppl Table1 for
567 references and dilutions) diluted in antibody dilution buffer (2% BSA, 0.2% Triton-X 100 in
568 sterile PBS) at 4°C for 48 hours. The samples were then rinsed 3 times with washing buffer on
569 an orbital shaker (3% NaCl and 0.2% Triton-X 100 in sterile PBS) and incubated with secondary
570 antibodies, donkey anti-mouse IgG-Alexa Fluor® 488 (A21202, Invitrogen, 1:500), donkey anti-
571 goat IgG-Alexa Fluor® 633 (A21245, Invitrogen, 1:500), 0.5µg/ml DAPI (62248, Thermofisher
572 scientific) and Alexa Fluor® 488 phalloidin (A12379, Thermofisher scientific, 1:200) at 4°C for
573 24 hours on an orbital shaker followed by 5 rinsing steps with washing buffer. The samples
574 were finally washed following similar washing steps as after primary antibody incubation and
575 mounted using RapiClear® 1.52 (RC 152001, Sunjin lab) pre-warmed at 37°C.

576 **Automated cell counting in JeWells after seeding**

577 We imaged the JeWell Chip in transmitted bright field mode using the Metamorph ScanSlide
578 module at 20X magnification just after the seeding and rinsing steps. The positions of the
579 individual wells were automatically identified on the stitched image. We then cropped the
580 stitched image into sub images containing a single well by threshold detection of the bright
581 top aperture of each well. All the detection was performed using OpenCV-Python. We then
582 trained a convolutional neural network for biomedical image segmentation published by the
583 computer science department of the University of Freiburg U-Net using 200 raw images that
584 we manually annotated. The training dataset was artificially augmented (3 times) applying
585 transformations to the original images. We then estimated the area A_c per cells by using a
586 linear regression between the segmented area predicted by the U-net per well and the ground
587 truth number cell in the training set. We computed the number of cells per wells dividing the
588 total predicted segmentation area by A_c . We found an error of around 6% as compared to the
589 ground truth (Supplementary Figure 5). (N=200 wells). Once trained, the cell counting process
590 by the U-net network is almost immediate.

591 **Automated acquisition process**

592 The microscope, the motorized stages, and the Multi-Dimensional Acquisition (MDA) process
593 were controlled by MetaMorph software (Molecular Device). A dedicated home-made plugin
594 (Visual basic) integrated into Metamorph allowed to control the soSPIM beam steering unit
595 and therefore the sample illumination. It especially enabled to synchronize the 3D position of
596 the light-sheet (after reflection onto the 45° mirror) with the objective's axial movement and
597 the acquisition process to ensure a proper superposition of the excited and imaged plane for
598 optimal 3D imaging as described in Galland et al.¹⁹. The light-sheet calibration and
599 repositioning during multi-position acquisition are performed using brightfield images of the
600 JeWells. The calibration is performed semi-automatically, by simply user-drawing a line in the
601 direction of the mirror, at the in-focus position, which is clearly visible from a bright-field
602 image of a JeWell. Repeating this process for two different Z positions (eg. at the top and at

603 the bottom of the JeWell), allows calibrating the position of the light-sheet on the mirror with
604 respect to the Z position, and guarantees a perfect alignment of the light sheet with the focal
605 plane of the objective. The precise arrayed structure of the JeWells ensures the mirrors
606 orientations to remain the same for all the JeWells contained in a JeWell chip, facilitating
607 multi-position acquisition as long as JeWells are repositioned exactly at the same position in
608 the camera field of view. Indeed, the soSPIM architecture couple the lateral (XY) JeWell
609 position with the axial (Z) position of the light-sheet (**Supplementary Figure 4a**). To control
610 the exact positioning of the JeWell in the camera field of view for both automatic multi-
611 position acquisition and drift correction during time-lapse acquisition we developed a fully
612 automatic positioning tool. It relied on the cross-correlation of the JeWells high-contrasted
613 brightfield images in between a reference position on which the soSPIM imaging
614 synchronization has been performed and the JeWell that will be imaged with a subpixel
615 resolution (**Supplementary Figure 4b**). The positioning accuracy was estimated to be less than
616 45.8 nm for a computational time of 188 ± 13 ms (with an image binning factor of 4) ensuring
617 optimal 3D soSPIM imaging of all positions (and time-point) defined with neglectable impact
618 on the acquisition duration. To ease and fasten multi-dimensional acquisition set-up
619 leveraging the arrayed structures of JeWells, we developed a 4 steps process as described in
620 **Supplementary figure 4c**. It relies on (*step 1*) the acquisition of a brightfield preview of the
621 whole JeWell chip, (*step 2*) the automatic JeWells positions detection and (*step 3*) the
622 interactive selection of the positions to be imaged, before (*step 4*) the screen of those position
623 according to the acquisition's parameters preliminary defined (frequency, excitation
624 wavelengths, Z series, ...). The integration of the repositioning tool within this acquisition
625 pipeline ensures optimal 3D soSPIM imaging of all the positions and time-points of the screen.
626 Overall, it took 25 min to set-up a screen on 321 positions (*steps 1 to 3*) including 20 min for
627 the chip acquisition preview.

628 **Nuclei segmentation by StarDist**

629 The 3D segmentation of nuclei was performed using StarDist³⁷ convolutional neural network
630 (CNN). All the image stacks were resized from 2048*2048 to 614*614, keeping the number of
631 planes constant, to allow StarDist training on a standard desktop computer equipped with a
632 Geforce RTX 2080 Ti NVIDIA GPU with 11Go of graphical memory. We manually annotated 7
633 organoids using Napari³⁸, corresponding to 471 frames and 5,274 3D nuclei. After resizing,
634 the median size of our annotated nuclei was 28*27*7 voxels. We used a patch size of
635 96*96*24 voxels to guarantee that the network's field of view encapsulates at least one
636 nucleus. Data augmentation was performed with random flips, rotations, and intensity
637 modifications, and we used 85% of the data set for training and 15% for validation of the
638 network predictions. Training used 400 epochs and required approximately 4 hours.
639 Prediction of a complete 3D stack required 40 seconds (4 seconds for resizing and 36 ± 21
640 seconds for the StarDist prediction **Supplementary Movie 2**). Segmentation applied to 96
641 organoids resulted in 55,976 nuclei and has been used to measure intensities of transcription
642 factors for all organoids. Distributions of the nuclei volume were computed using the
643 MorphoLibJ³⁹ plugin.

644 **Detection of Ki67 classes, mitosis, and apoptosis in 3D by YOLOv5**

645 The detection of Ki67 classes²⁰ (Early G1, G1/S/G2, Mitosis) and apoptosis and mitosis (DAPI)
646 events was performed using the YOLOv5 CNN published by ZeroCostDL4Mic, run on our GPU
647 resources (NVIDIA Quadro RTX6000 24GB) (**Supplementary Figure 6a**). For the Ki67 classes, a
648 custom-made image analysis algorithm based on local thresholding and morphology

649 mathematics has been implemented under ImageJ to help for the detection of the Ki67
650 positive region (surrounding boxes, **Supplementary Movie 3**) following by a manual step of
651 classification. This has been done on 13 images stacks of 71 z-planes of the Ki67 signal only.
652 For the mitosis/apoptosis detection on DAPI signal, 28 image stacks of 71 z-planes were
653 manually annotated by selecting 30x30 pixels bounding boxes around the central plane of the
654 apoptosis and 50x50 pixels bounding boxes around the central plane of the mitosis using
655 ImageJ. For both type of detection, these annotated stacks constituted the ground truth of
656 the training dataset, representing a total of 35000 positive region in 2D for Ki67 signal, and
657 for DAPI signal a total of 344 mitosis and 1,123 apoptosis 2D region. For mitosis/apoptosis
658 only, an additional data augmentation was performed using image flipping and rotation,
659 representing 4,128 2D region of mitosis and 13,476 apoptosis. We used 90% of the data set
660 for training and 10% for validation of the network prediction. After training, the prediction of
661 a single plan image took 20msec and around 1sec for a full 3D stack. The AI network identified
662 bounding boxes around individual events in each plan independently (2D). 3D detection was
663 achieved using a custom MATLAB routine identifying and fusing the same event detected in
664 consecutive planes. The centre coordinates of each 3D box were plotted as a localization map.
665 We measured the confusion matrix by comparing the overlap between the predicted
666 bounding box with the ground truth (**Supplementary Figure 6a**).

667 **Classification of organoid shapes by DenseNet121**

668 Raw images of global organization of organoids based exclusively on the actin (Phalloidin) and
669 nuclei (DAPI) staining were analyzed using deep learning algorithms developed for image
670 classification. We manually distinguished 2 classes of organoids: 'O-shape' organoids
671 displaying a spherical global shape, and 'B-shape' ones for which an external streak
672 surrounding by 2 cellular lobes tending to join can be clearly distinguished. We analyzed 132
673 (2048*2048*64 planes) 3D images using DenseNet121²⁶ CNN for classify the 3D images into
674 those two classes (see **Supplemental Figure 6b**). To accommodate the memory constraint of
675 the graphics card (Geforce RTX 2080 Ti NVIDIA GPU with 11Go of graphical memory), we
676 resized the whole dataset to 128*128*64, a size where the structures are still roughly
677 distinguishable. A 5-fold cross-validation was performed with a 60/20/20 split for training,
678 validation and test set definition. Each fold was launched with a DenseNet121
679 implementation with Adam optimizer⁴⁰. To counterbalance large imbalance between the two
680 classes, each training iteration was launched with a batch consisting of as many 'O-shape' as
681 'B-shape'. After training, the prediction of the classes among a set of 130 organoids took less
682 than 1s in total, with 99% of accuracy. Subsequently, each 'O-shape' organoids were analyzed
683 for internal streaks segmentation.

684 **Segmentation of internal streaks by 3D U-Net**

685 Classified 'O-shape' organoids were proceeded for the segmentation of internal streak based
686 only on morphological staining (DAPI and Phalloidin) to permit the detection of morphological
687 patterns independently of gene expression level (**Supplementary Figure 6c**). The 3D dataset
688 was first resized to 1024 * 1024 * 71, and each image plane was manually segmented using
689 3D Slicer software⁴¹, giving a ground truth mask. Original image and corresponding masks
690 were again resized to 256*256*71 for faster testing of deep learning algorithms. The same 5-
691 fold cross-validation procedure described previously were applied using 3D U-Net⁴² CNN. The
692 prediction produced probability maps of size 256 * 256 * 71 for each image. An arbitrary
693 threshold of 0.5 was applied to each map, resulting in a binary streak segmentation mask.
694 Once the network trained, the streak detection with the generation of the corresponding

695 mask took approximately 5 seconds for each organoid. A 3D connected components
696 algorithm was used to label each voxel of the binary masks. Most of segmented objects
697 seemed relevant for internal streaks as showed in **Figure 6** and **Supplementary Figure 6**.

698 **Image analysis of intestinal organoids for CDX2 and E-cadherin signals**

699 The 20x resolution (air objective, N.A 0.75) image stacks of 100 intestinal organoids were
700 automatically analysis using a custom-made ImageJ routine based on the maximum intensity
701 projection followed by thresholding and morphological mathematic operations to first classify
702 organoids positive for CDX2 transcription factor (CDX2⁺) and then, after the second round of
703 acquisition, to delineate the region of positive E-cadherin (E-cad⁺) clusters. These 2 steps of
704 analysis have been launched just after each round of acquisitions and took less than 10
705 minutes each. The positive organoids and region of interest have been registered to allow the
706 following acquisition using a higher resolution objective (60X, WI, N.A 1.27).

707 **JeWells peeling and real-time PCR**

708 Organoids were released from the JeWells by peeling off the top layer. Organoids were then
709 flushed twice with DMEM/F-12 1:1 (Gibco) and collected within a microcentrifuge tube. Total
710 RNA was extracted from these organoids using RNeasy Plus Micro Kit (74034, QIAGEN)
711 according to the manufacturer's protocol. 450ng of total RNA was used to form the cDNA
712 using cDNA synthesis kit (SensiFAST™, BIO-65054, Biotline). qPCR was performed using
713 FastStart Universal SYBR Green Master (ROX) mix on a CFX96 Touch Real-Time PCR detection
714 system (Bio-Rad). Primers sequences for the 12 genes tested are listed on the **Table 1**. GAPDH
715 was used as a housekeeping gene.

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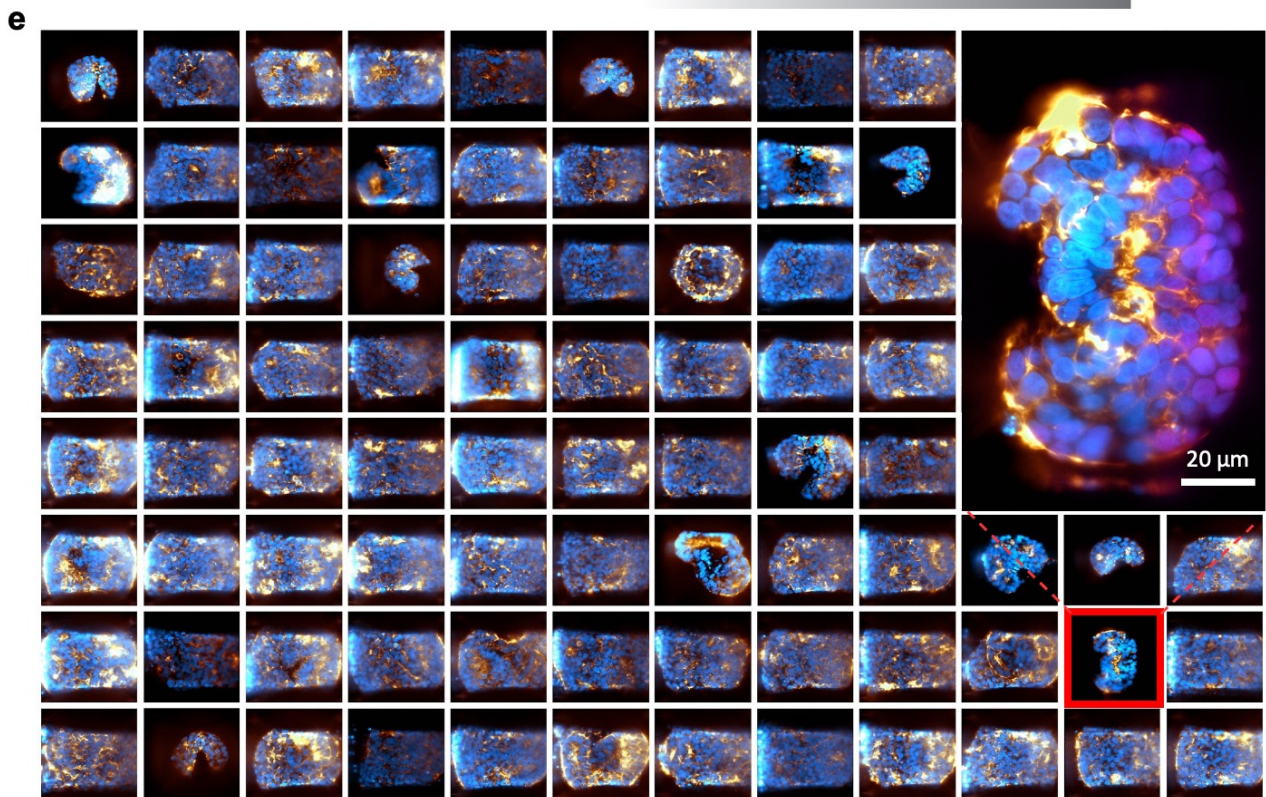
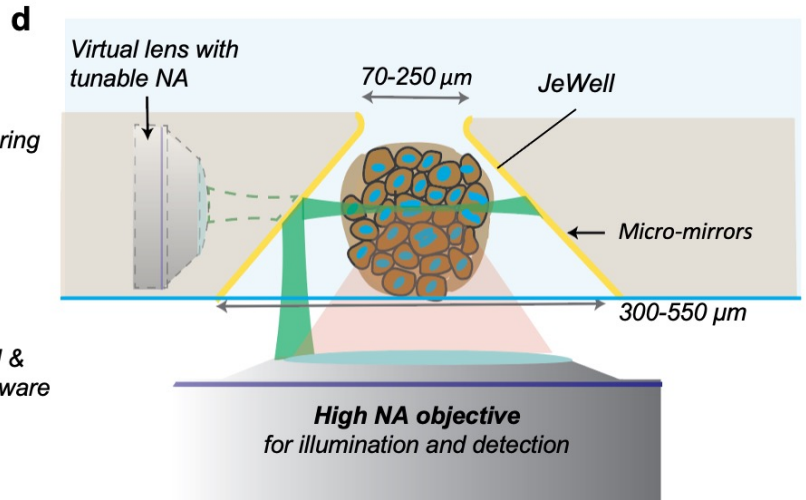
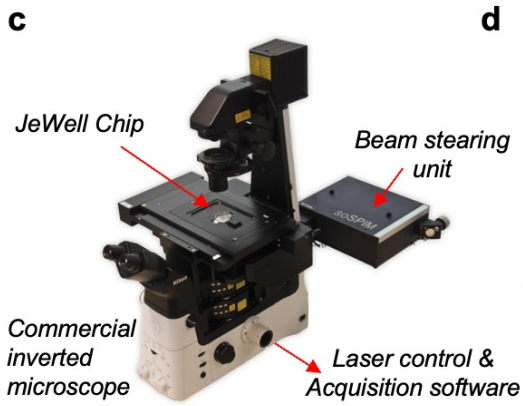
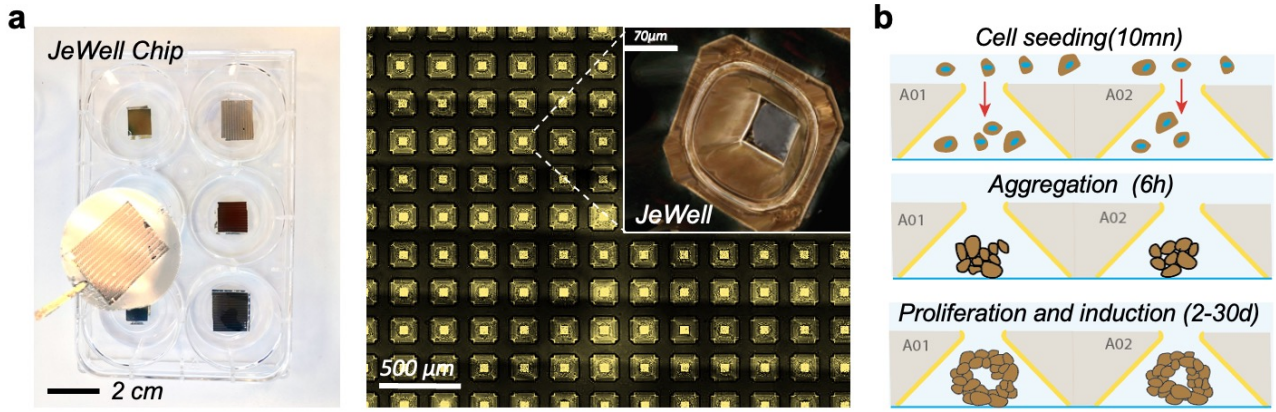
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820



96 organoids, 70 z plans each, 3 wavelengths, 173GB of (2048x2048x70) stacks, 1h acquisition time

Figure 1:

Working principles. **a.** JeWell chips in a 6 well dish (left) and close-up image of the JeWells array with a density of 16 JeWells/mm² (right). Inset shows a zoom on a JeWell inverted pyramidal microcavities flanked with four 45° mirroring surfaces. **b.** Schematic of the seeding procedure. Dissociated cells are homogeneously seeded above the chip. They sediment inside the pyramids for 10 mins, then aggregate for the next hours and finally proliferate and differentiate for up to 30 days in the JeWell cavities. **c.** Photography the imaging set-up comprising a commercial inverted microscope, combined with JeWell chips, a laser scanning unit and its custom-made control software. **d.** Principles of the single objective selective plane illumination microscopy (soSPIM). The light-sheet perpendicular to the optical axis is created by reflection of the laser beam (green) onto the 45° mirrored surfaces of a JeWell, acting as a virtual second objective. The fluorescence emission (red) is then collected through the same objective. **e.** Representative gallery of 96 neuroectoderm organoids (median plane of the 3D stacks) labelled with Actin (gold), Sox2 (magenta) and DAPI (blue) acquired in an automated workflow in less than 1 hour.

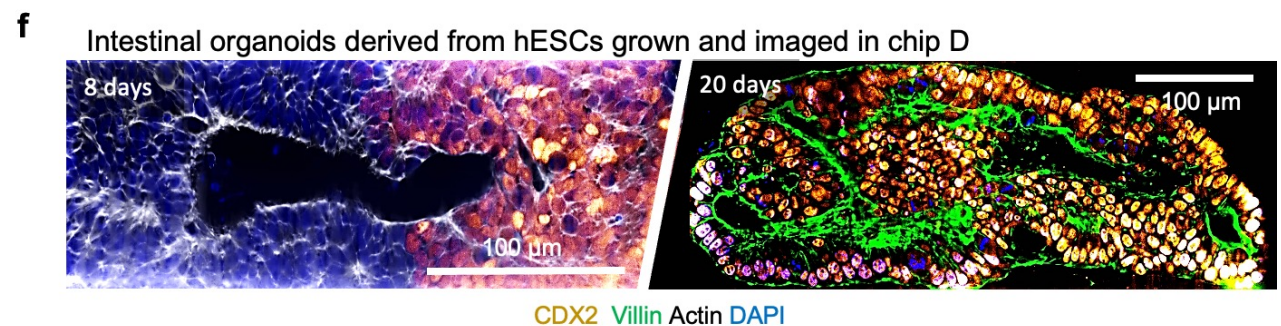
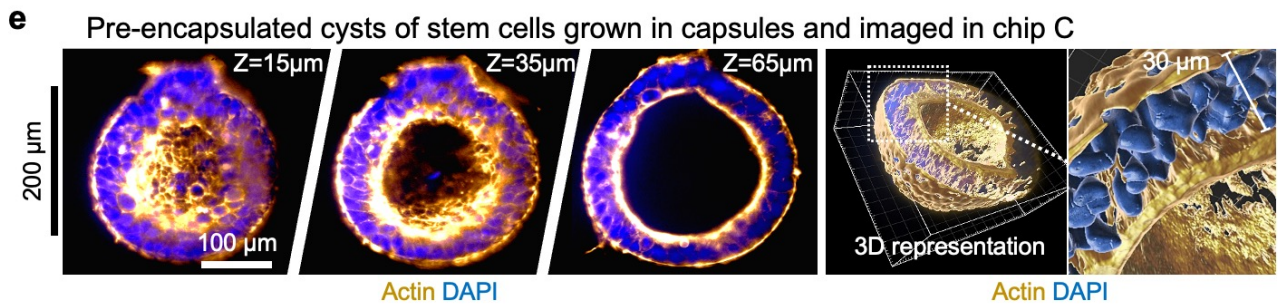
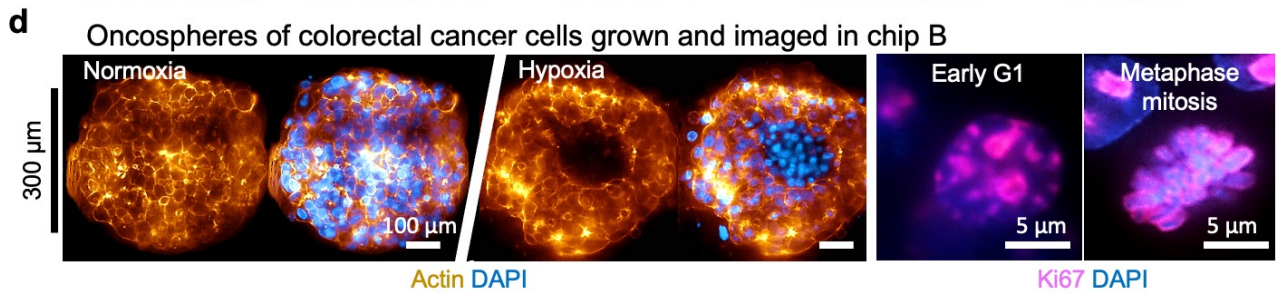
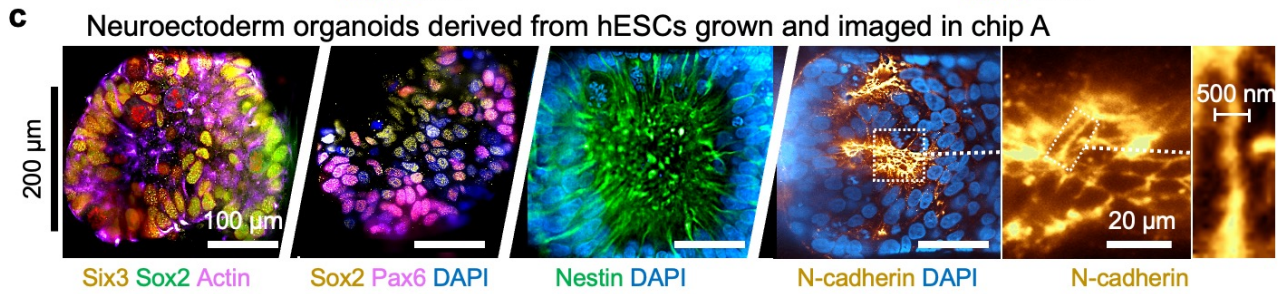
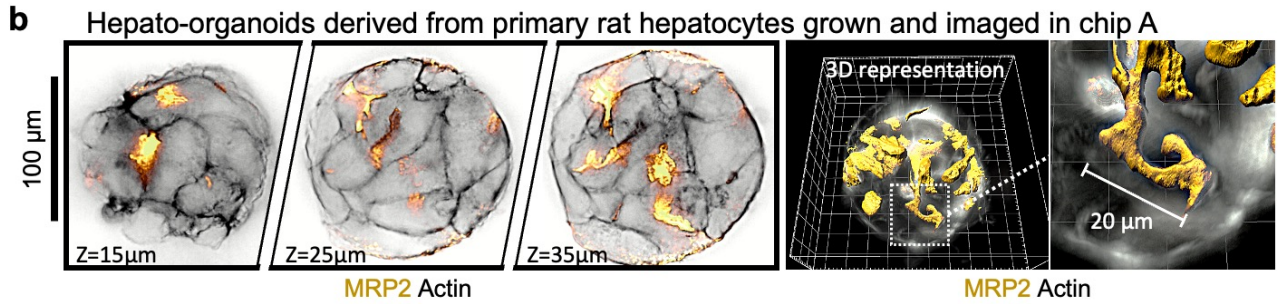
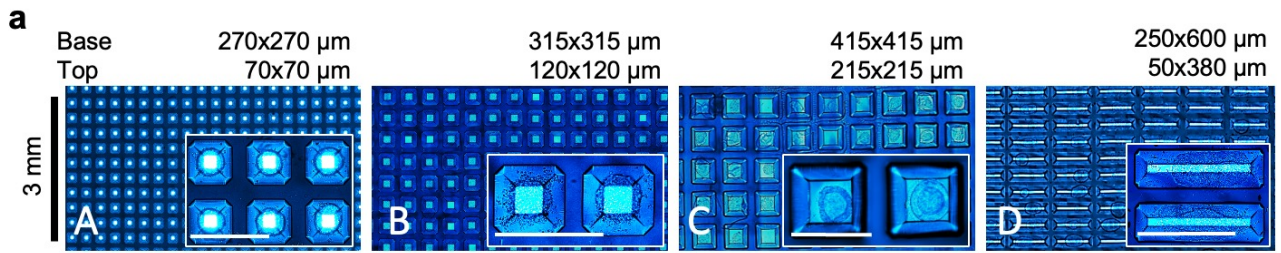
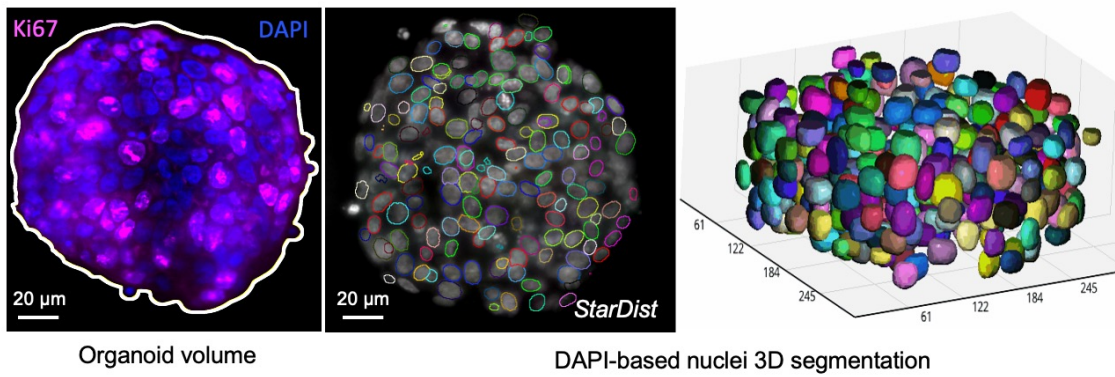


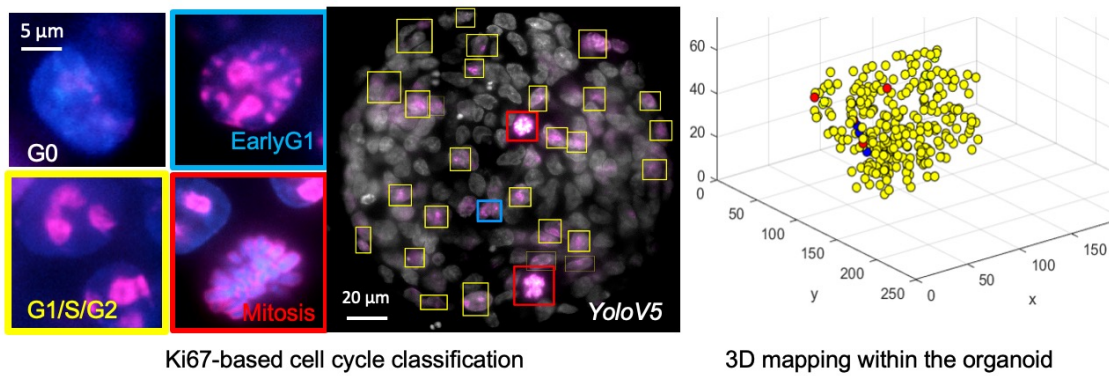
Figure 2:

Matching JeWell chips to different sorts of organoids. **a.** Examples of JeWell cavities dimensioned to fit the size of the organoids (scale bars 500 μ m). It maximized the image quality, protected the organoids for external flux, allowed embedding in Extra-cellular matrix (Matrigel) and provided scaffolds for the organoid growth. **b** Representative examples of 3D stacks (60X, WI) and 3D reconstruction of primary rat hepatocyte aggregates stained for actin (grey) and MRP2 (canalicular marker) (gold) after 4 days of culture (N=96). **c** Representative median plane images (60X, WI) of hESCs derived Neuroectoderm organoids (8 days old) immunolabeled for various transcription factors (as indicated). 3D rosettes could easily be identified with the N-cadherin rich core. Intra-rosette N-cadherin structures (<300nm) were visible (N=400). **d** Typical images (20X) of oncospheres (HCT116) (~ 200 μ m in diameter) grown in hypoxic conditions displayed a hypoxic center as compared to the normoxic conditions. The imaging resolution was fully capable of distinguishing proliferation stages (G0, G1, S, mitosis) based on Ki67 staining (N=400). **e.** Representative 3D stack (20X) and 3D reconstructions of hESCs cells pre-encapsulated in alginate capsules. The encapsulated cells were grown outside the JeWells and the capsules were seeded in pyramids with large top openings (215 μ m). **f** Representative median plane (20x) of intestinal organoids derived from hESCs at day 8 (left) and day 20 (right). The intestines grew in rectangle grooves. They elongated along the long axis. Staining at early stage (D08) showed clustered expression of CDX2 that became ubiquitous at later stage organoids (D20) presenting Villin lining the central lumen (N=96). All images in the figure are raw images from soSPIM set up without any post-processing .

a Nuclei 3D segmentation



b Proliferation stage quantification



c Quantification of a library of hundreds oncospheres

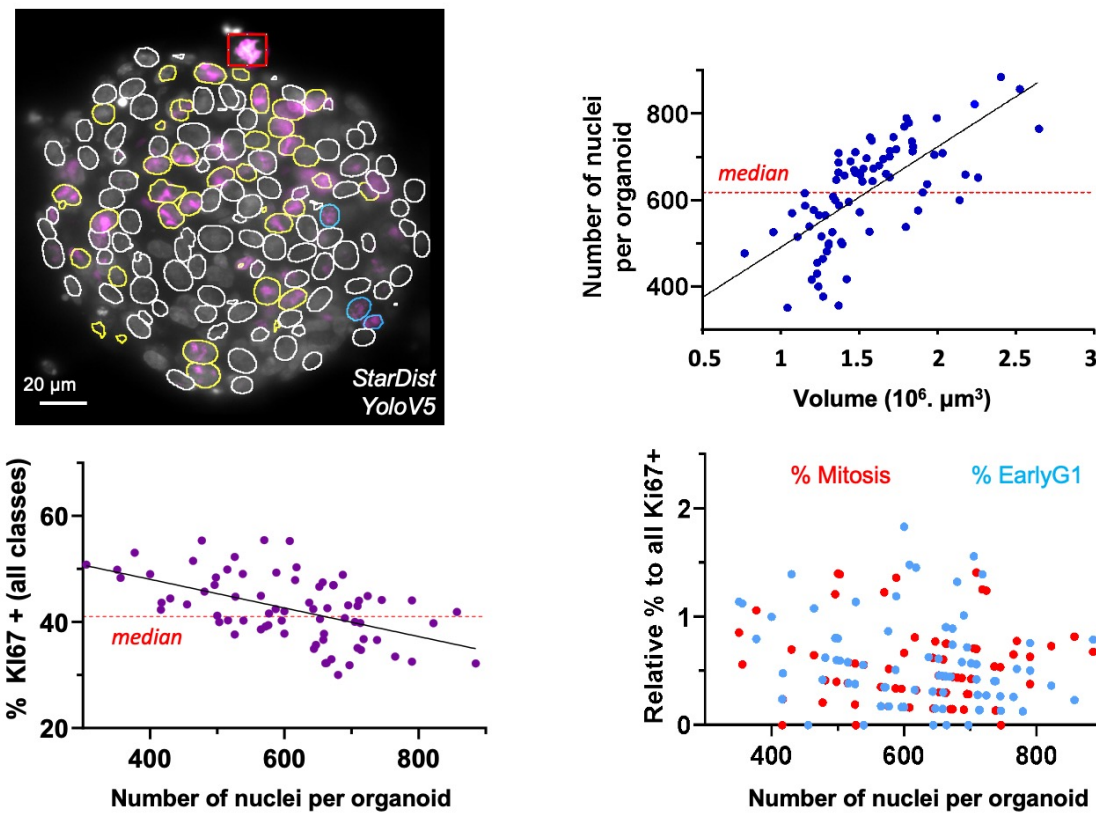
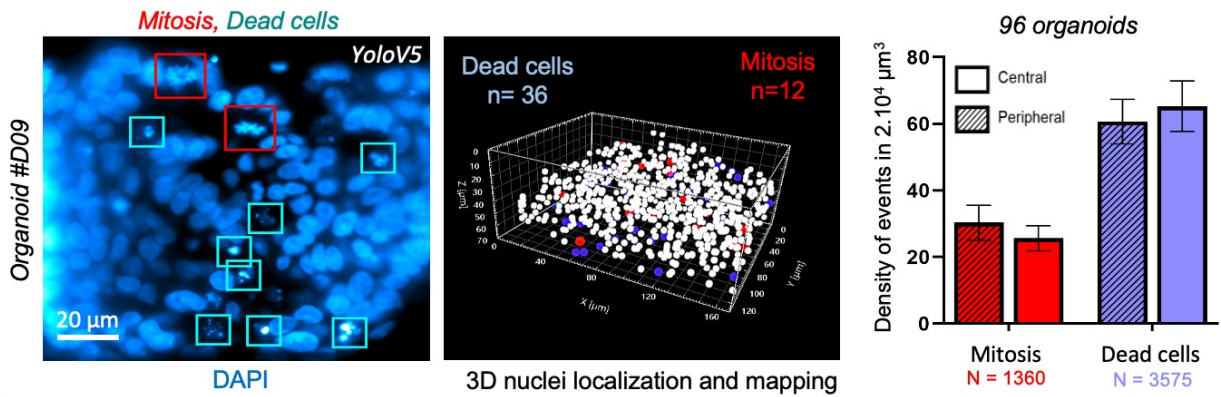


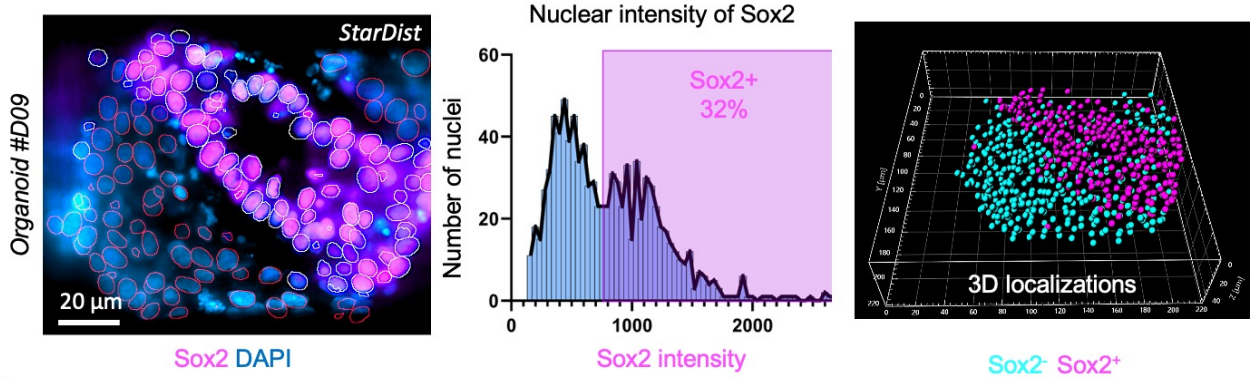
Figure 3:**Sub-cellular quantification of cell proliferation in oncospheres.**

a. Image of the median plane of an oncosphere (HCT116) stained for nuclei (DAPI) and ki67 in normoxy conditions. The volume of the oncosphere is measured by thresholding the aggregate contour in 3D. Individual nucleus positions are obtained by segmenting the DAPI channel using the 3D StarDist neural network. 3D reconstruction of segmented nuclei by surface rendering. **b.** AI detection of three different stages of cell division based on the ki67 signal using YoloV5. The 3D localization of individual proliferating nucleus is represented in 3D by a sphere. The colors code for the proliferation stages (blue for Early G1, yellow for G1/S/G2, red for mitosis). **c.** Correlation between the predictions of both AI networks. The color code of the nuclei contours corresponds to the class of proliferation stages. The total number of nuclei linearly scaled with the approximated volume of the oncosphere. The fraction of cells in each proliferative state decreased proportionally to the total number of cells. However, there are no sign of arrest at any proliferation checkpoint as demonstrated by the constant proportion of each phase relative to the total number of proliferative cells. Each point represents one organoid.

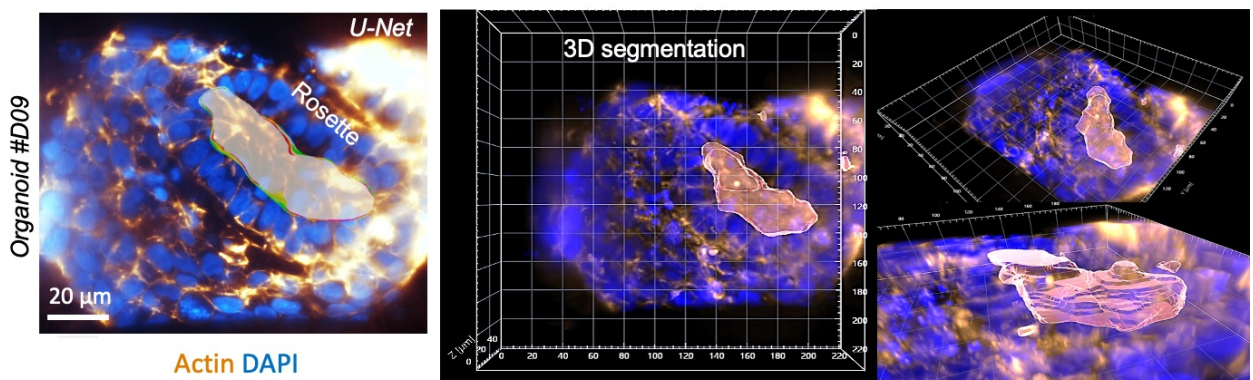
a Division and mortality detection



b Cellular phenotyping



c 3D segmentation of multicellular patterns



e Classification of the organoid morphologies

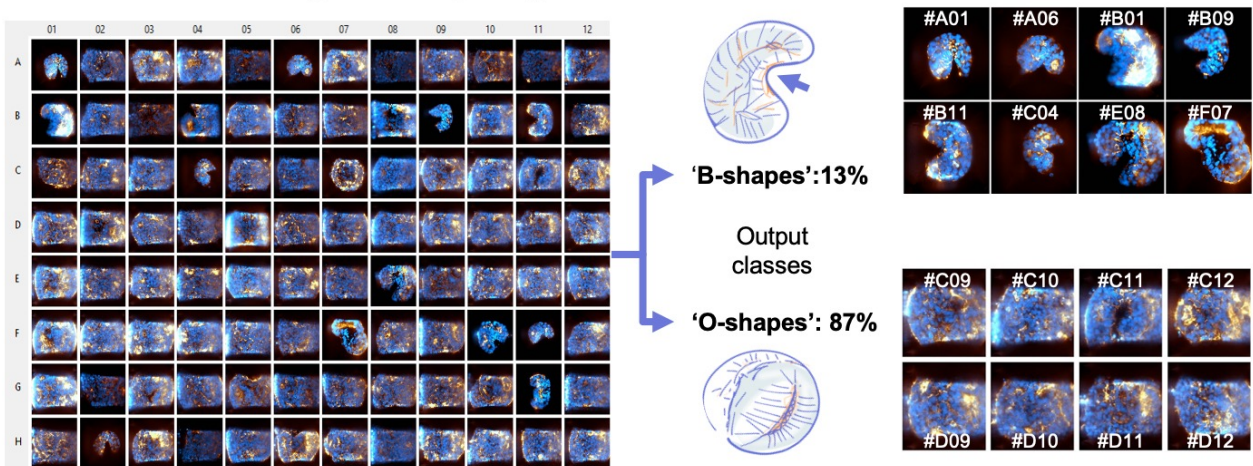


Figure 4: Multiscale analysis of neuroectoderm organoids.

a. Example of detection of mitosis and cell death based on DAPI signal using YoloV5 convolutional neuronal network (CNN). 3D localization of mitosis and cell death events within one full organoid. White: all nuclei (N=680) (detected by StarDist), blue: cell death (N=36) and red: mitosis (N=12). Comparison of the density of mitosis and cell death in the core vs the periphery of organoids (N=96) for the entire organoid collection. The density of events (out of 1,360 mitosis and 3,575 cell death) showed no significant differences between sub-volumes ($2 \cdot 10^4 \mu\text{m}^3$) in the central and the peripheral regions. It suggests that the proliferation and cell death were homogeneously distributed within the organoids. **b.** 3D segmentation of the nuclei in neuroectoderm organoid using 3D StarDist CNN on DAPI signal. Bimodal distribution of the average intensity levels of Sox2 signals within each nuclei. Green contours correspond to Sox2⁻ cells, red contours to Sox2⁺ cells. 3D representation of the Sox2⁻ (cyan) and Sox2⁺ (purple) nuclei based on the cut-off derived from the histogram. Sox2⁺ cells localized around the rosette. **c.** 3D segmentation of the streak regions of the rosettes based on actin structure (gold) were segmented using 3D U-net CNN. **d.** DenseNet121 CNN classification of the neuroectoderm organoid morphologies in 2 classes: B-shape or O-shape (N= 96). The classification of each organoid was achieved with 100% accuracy.

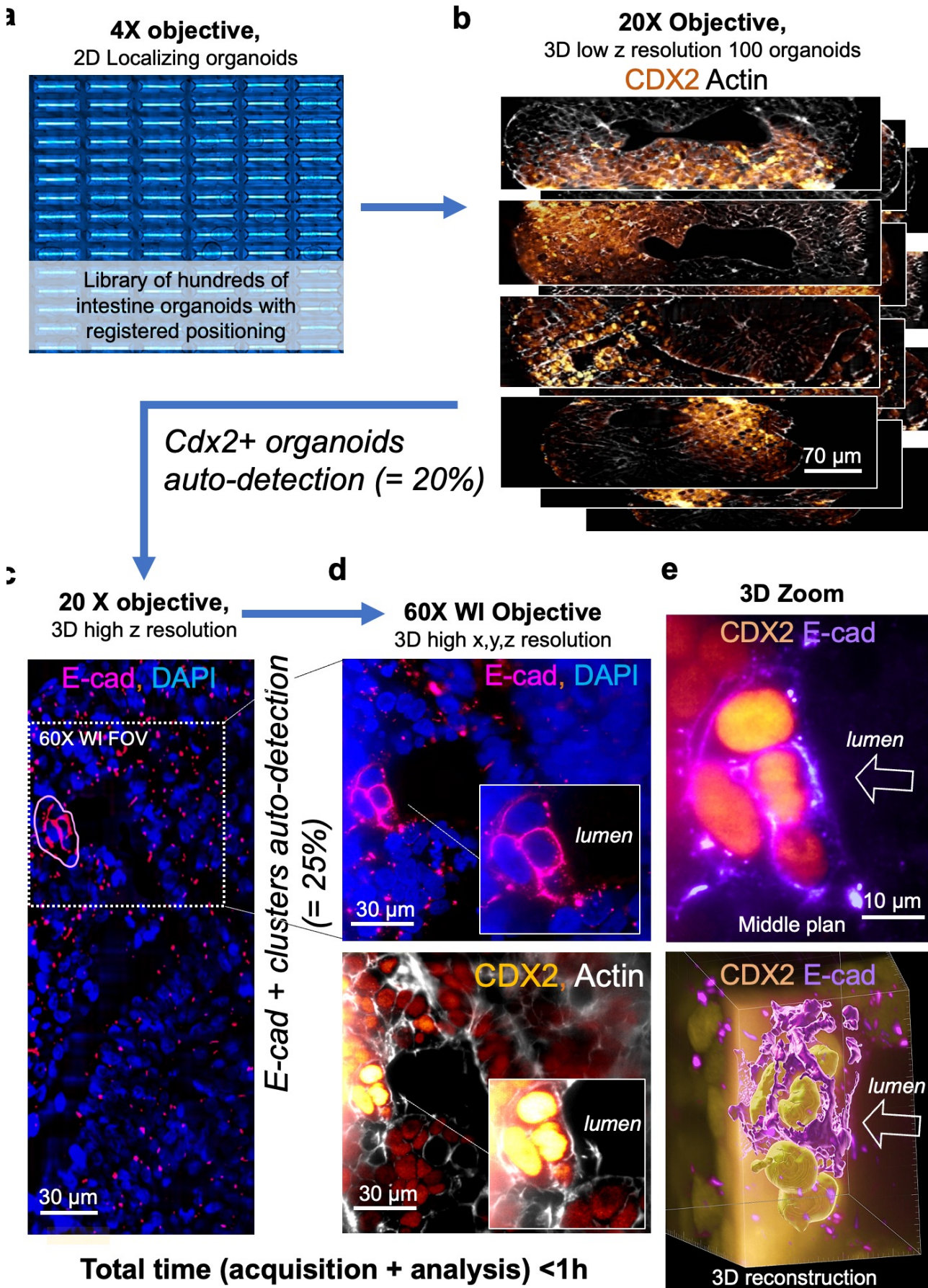


Figure 5: Efficient rare events detection by multi-magnification imaging pipeline.

a. A representative brightfield image using a 4x objective of a rectangular JeWell chip with intestinal organoids (Day 8). **b.** A collection of 100 intestine organoids with registered positions were imaged in less than 10 min using a 20X objective (air) in soSPIM (7 plans per organoid, step size of 10 microns, 2 channels : CDX2 and Actin). We automatically selected the CDX2 positive organoids (~ 20% of the whole batch, N=100). **c.** Rare clusters of E-cad⁺ cells (pink contour) were automatically detected by a second round of imaging of the selected CDX2⁺ organoids (20X with a higher Z resolution of 1 micron, 50 plans, 10 min) (= 25% of the CDX2⁺ organoids, =m5% of the whole batch). **d.** Optical zoom using a 60X, WI, 1.27 N.A objective on the selected field of views containing E-Cad⁺ clusters. The total time of the process was less than 1hour. **e.** The final high-resolution imaging enabled to reconstruct and to localize in 3D the E-cad⁺ junctions of the CDX2⁺ cells.

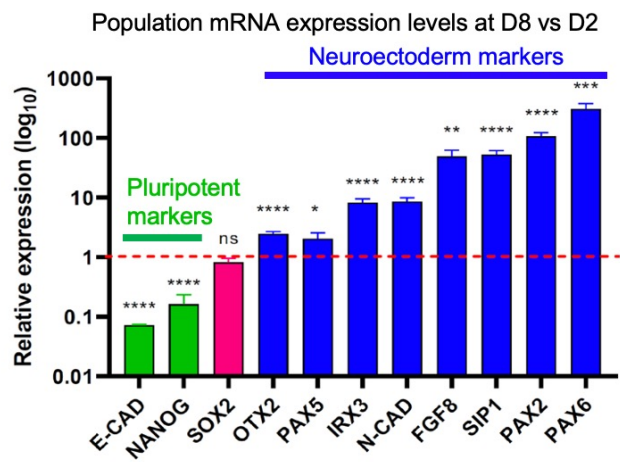
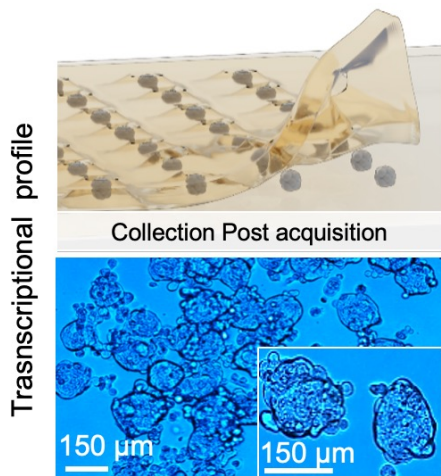
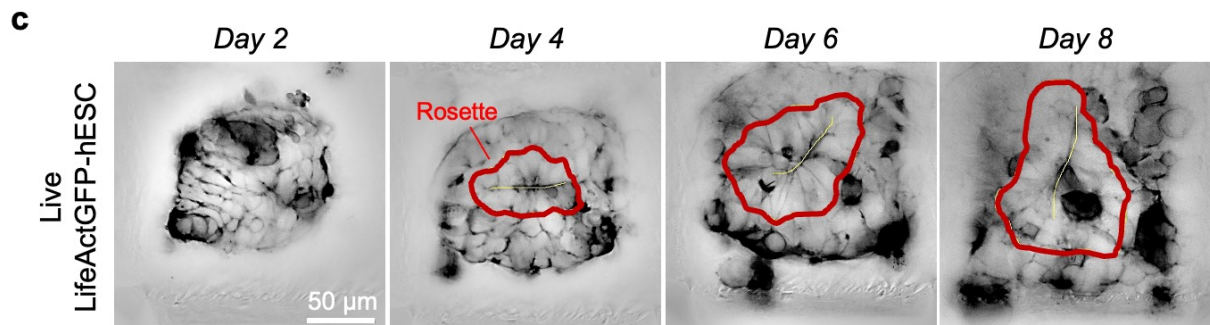
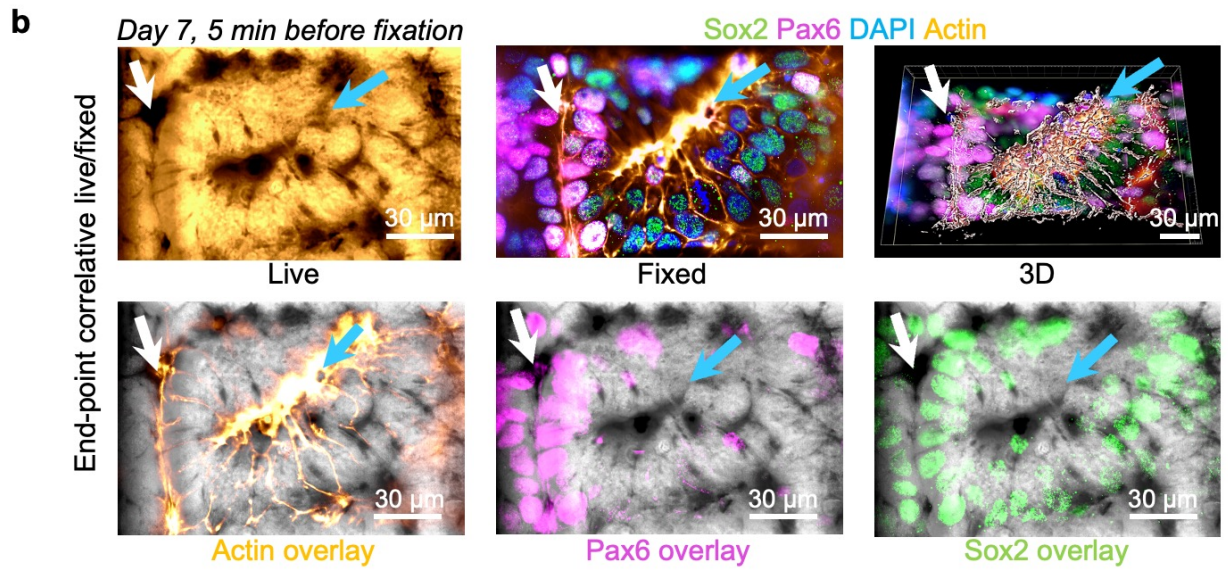
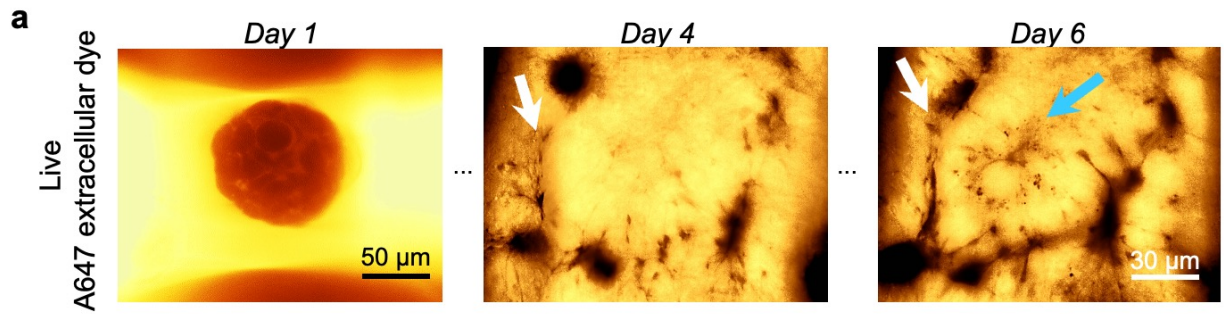


Figure 6. Correlative imaging of live vs transcription factors expression.

a. Live imaging performed on neuroectoderm organoid during 7 days of development. Alexa647 staining of the intercellular clefts. 3D time lapse imaging (1 frame every 15 min for 8 days) revealed the sequential formation of several rosettes (white and blue arrow) in the organoids. **b.** Immunostaining images of the organoids at day 7 (actin (gold), DAPI (blue), Pax6 (magenta) and Sox 2 (green)) could be overlaid with the last image of the live imaging (5 min before fixation) by manual registration of the focus offset. It revealed the presence of Pax6⁺ cells exclusively around the older rosettes (white arrow). **c.** Representative time lapse imaging of live neuroectoderm (lifact GFP) (N=100) imaged sequentially in 3D (70 optical sections separated by 1 μ m), every 15 minutes during 125 hours (spread over 8 consecutive days). The rosettes were manually tracked (red contour) from the day of induction (day 2). Schematic of the peeling of the Jewell chip and collection of the organoids after 3D live monitoring to enable mRNA extraction. RT-qPCR performed on 11 genes, demonstrating the expected reduction in pluripotent reporters (green) and the increase of the neuroectoderm markers (blue) (N=3 replicates, > 1000 organoids/replicate). P-values: ****<0.0001, ***<0.001, **<0.01, *<0.05, ns: non-significative.