

A microwell platform for high-throughput longitudinal phenotyping and selective retrieval of organoids

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Summary

Organoids are powerful experimental models for studying the ontogeny and progression of diseases including cancer. Organoids are conventionally cultured in bulk using an extracellular matrix mimic. However, organoids in bulk culture physically overlap, making it impossible to track the growth of individual organoids over time in high throughput. Moreover, local spatial variations in bulk matrix properties make it difficult to assess whether observed phenotypic heterogeneity between organoids results from intrinsic cell differences or microenvironment variability. Here, we developed a microwell-based method that enables high-throughput quantification of image-based parameters for organoids grown from single cells, which can be retrieved from their microwells for sequencing and molecular profiling. Coupled with a deep-learning image processing pipeline, we characterized phenotypic traits including growth rates, cellular movement, and apical-basal polarity in two CRISPR-engineered human gastric organoid models, identifying genomic changes associated with increased growth rate and changes in accessibility and expression correlated with apical-basal polarity.

Keywords: organoids; high-throughput imaging; microwell arrays; quantitative phenotyping; deep learning; gastric tumorigenesis; single-organoid sequencing; genotype-to-phenotype linkage

Introduction

The advent of 3D organoid culture methods that recapitulate tissue structure, multi-lineage differentiation, pathology, and disease phenotypes while retaining the tractability of *in vitro* systems has revolutionized the study of various diseases, including cancer (1-6). Key strengths of organoids over conventional transformed 2D cell lines for studying cancer include the greater similarity of 3D versus 2D models to *in vivo* settings and the relatively clean genomic background of healthy primary organoids which can be engineered via CRISPR/Cas9 editing or other techniques to harbor alterations in oncogenes or tumor suppressors that promote malignant progression (7-9). Several studies have demonstrated the transformation of normal colon, stomach, and pancreas organoids into invasive carcinomas via the simultaneous introduction of multiple oncogenic 'hits' (7, 8, 10-12). These minimally transformed forward-

49 genetic models have yielded insights into the requirements for transformation and their tissue-
50 specific functional consequences.
51

52 For example, we recently developed individual and sequentially engineered organoid models to
53 study the earliest mutational events during gastric tumorigenesis (7, 13). Gastric cancer is the
54 third leading cause of cancer mortality worldwide and a major public health burden. Owing to
55 limited screening modalities and late presentation of clinical symptoms, gastric cancer is
56 commonly detected at an advanced stage, where treatment options are limited, emphasizing the
57 need for earlier detection and robust biomarkers (14). We sought to recapitulate molecularly
58 defined subgroups of disease via CRISPR/Cas9-mediated editing of genes commonly altered in
59 human gastric cancers, including *TP53*, which is altered in 70% of chromosomally unstable (CIN)
60 gastric cancer (15), and *ARID1A*, which is altered in 50% of all cases and enriched in the
61 microsatellite instable (MSI) and Epstein-Barr Virus (EBV) subgroups (15, 16). We first
62 biallelically inactivated *TP53* via CRISPR/Cas9 in normal human gastric organoids and
63 established clonally derived *TP53* knockout (KO) lines. In these *TP53*^{-/-} lines, we knocked out
64 *ARID1A*, yielding *TP53*/*ARID1A* double knockout (DKO) lines that exhibited morphologic
65 dysplasia, tumorigenicity, and mucinous differentiation, features that were not seen in *TP53*KO
66 organoids (7). Additionally, through *in vitro* evolution of *TP53*-deficient gastric organoids, we
67 demonstrated that this single initiating genetic insult is sufficient to recapitulate many molecular
68 features of the CIN subgroup of gastric cancer while remaining morphologically similar to normal
69 gastric organoids (13).

70 These single and double KO organoids thus represent powerful models of the earliest stages of
71 human cancer, corresponding to pre-malignant and malignant states, respectively, and are ripe
72 for further characterization. However, to date, the phenotypic characterization of single
73 organoids has remained challenging as most techniques suitable for quantifying the growth of
74 2D cell cultures generally do not extend to 3D and many methods were designed for bulk
75 populations (17-20). The most common strategy for culturing organoids in 3D involves
76 resuspending dissociated cells in Matrigel or Cultrex Basement Membrane Extract (BME),
77 commercially available extracellular matrix (ECM) mimics (21). Using this method, organoids are
78 formed from clusters of aggregated cells rather than from a single cell that expands
79 independently, making it difficult to determine whether an observed phenotypic trait reflects the
80 stochasticity of small deposited cell populations or is intrinsic to individual cells at the start of
81 organoid growth (22, 23). Distinguishing intrinsic from extrinsic heterogeneity is further
82 complicated by variations in the organoid's growth environment, as organoids seeded close to
83 other organoids may be affected by cell-cell paracrine or juxtacrine signaling (24, 25).
84 Additionally, the position relative to the margins of the ECM hemisphere can impact the diffusion
85 of growth factors and/or drugs (26, 27). Finally, the same observed bulk growth differences
86 could result from differences in the median organoid growth rate (28) or the fraction of cells that
87 grow, complicating the interpretation of such data.

88 Microfabricated microwells represent an alternative to bulk culture methods and have been used
89 in the materials science and tissue engineering fields for 3D cell culture (18, 22, 29-31).
90 However, most methods reported to date rely on cellular aggregation to generate spheroids (22,
91 29, 31-35) rather than growing organoids from a single cell, precluding investigations into
92 underlying cellular and phenotypic heterogeneity. In addition, unlike in 2D cell lines, image
93 analysis pipelines to quantitatively track the growth trajectories and other phenotypic traits of live
94 3D organoid cultures over time are less comprehensive (31, 36, 37). Finally, most platforms lack
95 the ability to selectively retrieve organoids of interest for downstream investigation or require

96 complex and expensive instrumentation, hindering efficient linkage of phenotype to genotype
97 (30).

98 Here, we present an open-source microwell platform and image-processing pipeline that
99 enables the characterization of a variety of phenotypes for thousands of single organoids in
100 parallel under near-identical conditions. These microwells are easy to fabricate and integrate
101 into traditional cell culture workflows and can be imaged using standard inverted microscopes.
102 Applying this new microwell platform to two established CRISPR-engineered organoid models of
103 gastric tumorigenesis, representing pre-malignant (TP53KO) and malignant (DKO) states, we
104 quantify cell size and position over time to determine single organoid growth rates and migration
105 patterns via time-lapse imaging and a neural network-based image analysis module (38, 39) for
106 nearly 100,000 cell trajectories and over 8,000,000 microwell images. By fluorescently labeling
107 nuclei and actin in both engineered organoid lines, we quantify the proportion of cells with
108 abnormal apical-basal polarity, a hallmark of malignant transformation (40, 41), and
109 demonstrate an enrichment in the DKO relative to the TP53KO model. We further implemented
110 a mechanism to selectively retrieve specific organoids of interest for downstream
111 characterization to facilitate efficient linkage of phenotype to genotype. With this, we
112 investigated the molecular features associated with this phenotype by retrieving individual DKO
113 organoids with normal versus disordered polarity and performing dual chromatin accessibility
114 and transcriptomic profiling. These analyses implicate changes in accessibility amongst cell
115 adhesion genes in apical-basal polarity. We anticipate that this microwell platform and
116 associated analysis pipeline can readily be integrated within existing culturing protocols to
117 interrogate the molecular and phenotypic features across a broad range of organoid and
118 spheroid models.

119 120 **Results**

121 *Organoid models of gastric tumorigenesis and growth rate measurements in bulk culture*

122 Here, we use two established organoid models of gastric tumorigenesis based on single and
123 combinatorial gene-editing via biallelic inactivation of the tumor suppressor, *TP53* (TP53KO) or
124 dual inactivation of *TP53* and *ARID1A* (DKO) (7, 13) (**Fig. 1A**). The *TP53/ARID1A* DKO
125 exhibited malignant phenotypes, including mucinous metaplasia and capacity for *in vivo* tumor
126 growth upon xenotransplantation in mice, implying this line has undergone malignant
127 transformation (7). In contrast, the TP53KO gastric organoids exhibit normal morphology and
128 are not tumorigenic even after long-term *in vitro* evolution despite harboring a constellation of
129 copy number variants associated with gastric cancer, implying this line mimics a pre-malignant
130 state (7, 13). These defining features render these excellent models in which to perform the
131 systematic and quantitative characterization of additional phenotypes within and between
132 models, including growth rates and the prevalence of apical-basal polarity.

133 As reported previously (7), images from bulk cultures grown in Cultrex BME revealed qualitative
134 phenotypic differences between these gastric organoid lines, with increasing cellular
135 disorganization for organoids lacking either *TP53* or both *TP53* and *ARID1A* (**Fig. 1A**). Changes
136 in cell growth, assessed by seeding bulk cultures with the same number of cells and then
137 quantifying the fold-change in the number of viable cells after 14 days of passaging in
138 conditioned media, also suggested that DKO organoids were more proliferative than TP53KO
139 organoids in bulk culture (**Fig. 1B**), consistent with previous observations (7). However, these
140 bulk culture methods could not determine: (1) if fold-change increases resulted from individual
141 cells growing at a faster rate or from differences in the fraction of cells that grow, (2) whether the
142 culture contained subpopulations with different growth behaviors, or (3) whether any observed

143 subpopulations resulted from intrinsic differences between cells or cellular microenvironment
144 (e.g. proximity to other organoids or to media surrounding the Cultrex BME boundary). Finally,
145 organoids in bulk culture physically overlap and can merge with one another (**Fig 1C**), making it
146 difficult to track the growth of individual organoids or isolate individual organoids of interest for
147 downstream analysis.

148

149 *Microwell arrays for high-throughput phenotyping of single organoids over time*

150 To address these challenges, we developed a microwell platform to perform time-resolved
151 phenotyping of thousands of organoids in parallel under near-identical conditions (**Fig. 1D**).
152 Each single-layer PDMS microwell device contains arrays of 2,500-10,000 microwells (either
153 100 x 100 x 80 μ m or 200 x 200 x 80 μ m, length x width x depth) placed directly at the bottom of
154 each well (“macrowell”) of a 12 well culture plate (**Fig. 1D; Supplementary Fig. S1**). Devices
155 are fabricated by spin coating polydimethylsiloxane (PDMS) onto master molds, thereby
156 ensuring uniform thickness and enhancing image quality (**Supplementary Fig. S2**). To facilitate
157 unique microwell indexing during downstream image processing, microwells are grouped within
158 subarrays of 20 x 20 (100 μ m) or 10 x 10 (200 μ m) microwells, with a pattern of rotated
159 microwells that uniquely identifies each subarray (**Supplementary Fig. S3**). The 100 μ m
160 diameter microwells are optimized for high-throughput imaging of thousands of organoids in the
161 same experiment, while 200 μ m diameter microwells are best suited for retrieval of organoids
162 with phenotypes of interest for single-organoid sequencing. All microwell devices were plasma-
163 treated and coated with 0.5% BSA to render them hydrophilic.

164 Initial measurements of single-cell occupancy as a function of starting cell concentration for
165 TP53KO cell lines established optimal concentrations of 6000 cells/mL for 100 μ m microwells
166 (26.15% of wells with a single cell) and 400 cells/mL for 200 μ m microwells (31.33% of wells
167 with a single cell), respectively; the fraction of microwells containing a given number of cells was
168 well-fit by a Poisson distribution, consistent with expectations for stochastic loading
169 (**Supplementary Fig. S4**).

170 For initial experiments, we seeded microwell arrays with single cells dissociated from TP53KO
171 organoids and then imaged microwells daily via tiled bright field imaging (**Fig. 1E**). To visualize
172 single organoid growth over time, we: (1) stitched tiled images into a single image per macrowell
173 per timepoint, (2) rotated stitched images to position microwell array edges parallel with image
174 edges, (3) manually determined corner locations for each macrowell, and then (4) extracted
175 individual microwell images from the rotated arrays by their position relative to the corner
176 locations (**Supplementary Fig. S3**). We then manually inspected a subset of 100 microwells for
177 each organoid line. Organoids grown in microwells appeared phenotypically similar to their bulk
178 culture counterparts, with circular and cystic structures (**Figs. 1E**). However, individual
179 organoids grown from single cells under identical experimental conditions often showed
180 dramatically different growth behavior: while some organoids showed rapid growth after
181 seeding, defined as growth to $\geq 25\%$ of the microwell area (25/100 TP53; 37/100 DKO) (**Fig. 1F**,
182 top), others either grew very little (<25% of the microwell area; 40/100 TP53; 31/100 DKO) (**Fig.**
183 **1F**, middle) or showed signs of cell death/apoptosis (35/100 TP53; 32/100 DKO) (**Fig. 1F**,
184 bottom). An approximately equal proportion of TP53KO and DKO organoids exhibited growth;
185 based on our manual classification, a greater proportion of DKO organoids showed larger and
186 more rapid growth ($\geq 25\%$ of the microwell area) compared to TP53 (37% versus 25% of
187 measured organoids, respectively) (**Fig. 1G**).

188

189 *High-throughput fluorescence imaging combined with deep learning tracks thousands of cells in*
190 *parallel*

191 Brightfield microscopy made it possible to qualitatively assess organoid growth, but manual
192 classification methods were both time-consuming and confounded by a high degree of
193 subjectivity. In addition, brightfield images lacked sufficient resolution to accurately determine
194 whether observed growth was a result of cell division or simply an increase in organoid volume
195 due to osmotic swelling of the lumen and the cells (42, 43). To distinguish between these
196 possibilities and simultaneously gain information about nuclear localization within organoids, we
197 engineered both TP53KO and DKO gastric organoid lines to express a nuclear fluorescent
198 reporter by lentivirally inserting a mCherry-tagged copy of histone 2B (mCherry-H2B) (**Fig. 2A**);
199 in parallel, we profiled the distribution of cytoskeletal proteins using live-cell labeling of actin or
200 tubulin (e.g. SiR Actin Kit from Cytoskeleton Inc.). To determine optimal conditions for
201 fluorescence imaging and pilot new analysis pipelines, we loaded TP53KO and DKO
202 engineered cells (at a concentration of 4000 cells/mL) into 100 μm microwells across three
203 separate replicate experiments. The first and second experiments took place after 5 and 7
204 months of continuous passaging in conditioned media; the third experiment took place after ~8
205 months of continuous passaging followed by a freeze-thaw cycle (required due to COVID
206 pandemic-related shutdowns) and an additional ~1 month of passaging post freeze-thaw (**Fig.**
207 **2A**). After loading, we mounted the entire plate assembly on an automated microscope with an
208 incubation chamber and collected tiled images across the device in the bright field, mCherry,
209 and Cy5 (for fluorophore-tagged actin molecules) channels at 2-hour intervals over 5 days for
210 each experiment (**Fig. 2A**). We then performed stitching, rotation, and microwell extraction for
211 each imaging channel, similarly to the initial brightfield tests (ref. Methods). Time-course image
212 processing for each well yielded 8,014,800 microwell images.

213 To efficiently extract information about the number, size, and relative positions of cells within this
214 large imaging dataset, we turned to a neural network originally designed for optical microscopy-
215 based image segmentation of cells grown in 2D monolayers (38, 39). To test whether and under
216 what conditions this deep learning model can recognize and track cells within 3D organoids, we
217 manually labeled individual mCherry-tagged nuclei positions in 2711 images from the first
218 experiment, used 2169 of these images and a transfer learning approach to train an organoid-
219 aware version of the model, and validated performance with the remaining 542 images
220 (Methods). To evaluate generalizability across experiments, we quantified model performance
221 based on the number of accurately identified cells within an additional 912 manually labeled
222 'test' images taken from all 3 experiments (306, 348 and 258 images from experiments #1-3
223 respectively) (**Fig. 2B**). The deep learning model counts showed strong concordance with
224 manual counts within the entire 5-day duration of the experiments ($R^2 = 0.83$ for the three
225 combined experiments; **Fig. 2C**; **Supplementary Fig S5A** for per-experiment correlation).
226 Precision and F1 scores were generally higher than recall for both validation and test sets, with
227 the validation set displaying higher scores across performance metrics, as expected (**Fig 2B**;
228 **Supplementary Fig. S5B**). Examination of error type revealed substantially more merges (in
229 which 2 labeled cells were predicted by the model to be a single cell) compared to splits (where
230 a single labeled cell was predicted to be 2 cells).

231 Further visual inspection of images showing regions identified as cells by the deep learning
232 model confirmed the accuracy of automated annotations over time (**Fig. 2D**). Identifying all cells
233 from microwells within a single macrowell required an average of 145 seconds for model
234 processing per time point. Fitting the model-annotated cell area and cell number over time for
235 each microwell to an exponential growth curve yielded quantitative growth rate estimates (**Fig.**

236 **2D**); linearly interpolating the centroid position of single cells over time returned a lower bound
237 for the distance traveled by a single cell prior to the first cell division (**Fig. 2E**).

238

239 *P53/ARID1A DKO cells grow at faster rates and higher occupancies decrease growth rates*

240 Using the trained deep learning pipeline for 3D cultures, we then quantified organoid growth
241 rates across all three initial fluorescence microscopy experiments (**Fig. 3A**). In total, the three
242 experiments profiled growth rates for 5812, 1328, and 3679 loaded microwells in experiments
243 #1-3, respectively, with the number of cells loaded per microwell following stochastic Poisson
244 distribution (**Supplementary Fig. S6A**). Somewhat surprisingly, per-organoid growth rates for
245 both organoid lines decreased as the number of cells initially seeded within the microwell
246 increased, suggesting that increased paracrine or juxtacrine signaling between cells in close
247 proximity does not enhance growth (**Fig. 3B**). We restricted analysis downstream to microwells
248 seeded with a single cell. Similar to growth rates observed in bulk cultures, the single-organoid
249 growth rates of the DKO line were higher than those of the TP53KO line (**Fig. 3C**). However, we
250 observed ~2-fold variability in growth rate across experiments, presumably due to differences in
251 the composition of conditioned media across batches (44). Despite this, DKO organoids
252 consistently grew faster than TP53KO organoids within the same experiment (**Fig. 3C**),
253 establishing that the higher fold-change in cell numbers for DKO organoids in bulk experiments
254 (**Fig. 1B**) is due to an enhanced growth rate in individual organoids rather than growth of a
255 larger proportion of cells. The single-organoid growth rates for the same organoid line across
256 different macrowells within the 12 well imaging plate varied only slightly, confirming lack of
257 macrowell-specific growth effects (**Fig. 3D**).

258 To calculate the time required for cells to complete a first division, we also determined the time
259 point at which 2 cells were first identified within microwells originally seeded with a single cell. In
260 all 3 experiments for both P53KO and DKO mutants, we observed a bimodal distribution in
261 which some cells divided soon after initial seeding (0-24 hours post-seeding) and the remainder
262 took longer to divide (24-120 hours post seeding) (**Fig. 3E**). We also observed experiment to
263 experiment variation in the time to first division. For example, most P53KO cells in experiment
264 #2 began dividing soon after seeding, and only a small subset of cells divided at later time
265 points, compared to experiment #1 in which the population was much more evenly distributed.
266 The total distance moved exhibited a similar modal pattern to the time to first cell division, as
267 cells that took longer to divide had more time for movement (**Supplementary Fig. S6B**). Total
268 distance moved was moderately positively correlated with the time to first division ($R^2 = 0.3209$;
269 **Supplementary Fig. S6C**), and weakly negatively correlated with growth rate ($R^2 = 0.0148$;
270 **Supplementary Fig. S6D**). Cells tended to move the furthest immediately after seeding, and
271 then settled down with decreasing movement before dividing (**Supplementary Fig. S6E**).

272

273 *Microwell enabled examination of single organoids with disordered polarity*

274 Disordered apical-basal polarity is commonly seen in diverse epithelial cancers (41, 45) and is
275 believed to be an early hallmark of gastric cancer (45, 46). Intriguingly, in previous work, we
276 noted that TP53/ARID1A DKO gastric organoids exhibit a disordered polarity (7). To assess
277 whether apical-basal polarity could be visualized in organoids grown in microwells, we
278 performed confocal imaging of both TP53KO and DKO, which confirmed that both TP53KO and
279 DKO lines contained organoids with disordered polarity. To systematically quantify changes in
280 membrane organization, we visually inspected the final timepoint fluorescence images for 257
281 and 236 TP53KO and TP53/ARID1A DKO cells, respectively, from the first experiment and
282 classified organoids as having either 'normal' or 'abnormal' apicobasal polarity. Classification of

283 'abnormal' polarity was based on the requirement that organoids display two of the following
284 three characteristics: (1) disordered actin signal not restricted to organoid lumens, (2) lack of a
285 central lumen ringed with actin, and/or (3) presence of multiple small, disorganized lumens (**Fig.**
286 **3F-G**). A greater proportion of the DKO organoids exhibited an 'abnormal' phenotype (50.8% vs.
287 35.4% in the TP53KO organoids, $p = 0.03$, Fisher's exact test), suggesting that more complex
288 engineered genotypes drive greater cellular disorganization (**Fig. 3H**).

289

290 Chromosome alterations associated with enhanced organoid growth

291 The high-resolution phenotyping data from these initial three experiments (#1 - #3) provided
292 evidence that subtle differences in experimental conditions (*i.e.* different media batches)
293 between experiments can drive phenotypic differences large enough to obscure meaningful
294 intrinsic biological variation (**Fig. 3C**). To systematically characterize variability within and across
295 TP53KO versus DKO organoids, we performed three additional experiments under more tightly-
296 controlled conditions (ref. Methods) in which all cells were passaged using a standardized
297 chemically defined media (**Fig. 4A**); these experiments used arrays of 200 μm microwells to
298 enable retrieval of organoids of interest using tubing with an outer diameter of 250 μm . In total,
299 these experiments profiled 422, 147, and 1009 loaded microwell with growth in experiments #4-
300 6, respectively (**Fig. 4B**).

301 Similar to the previous experiments (**Fig. 3**), growth rates generally decreased as per-microwell
302 occupancy increased for both TP53KO and DKO organoids (**Fig. 4C**). Most other image-based
303 analyses such as distance moved and time to first division also recapitulated the observations
304 made in **Fig. 3** (**Supplementary Fig. S7**). The DKO organoid growth rates remained relatively
305 constant across experiments (**Fig. 4D**), highlighting the consistency of the growth media
306 composition. However, while TP53KO organoid growth rates were initially slower than those of
307 DKO organoids, consistent with prior experiments, TP53KO growth rates increased steadily
308 across experiments ($p=3.48\text{E-}7$, 2-tailed t test) until they eventually exceeded the DKO organoid
309 growth rates by a significant margin ($p = 0.025$, 2-tailed t test) (**Fig. 4D**).

310 Previously, we observed that TP53KO organoids rapidly and continuously accumulate copy
311 number variation (CNVs) and aneuploidy (13). To evaluate whether increased growth rates
312 might be attributable to additional CNVs, we performed shallow whole-genome sequencing
313 (sWGS) of both TP53KO and DKO organoid cells frozen during seeding of experiments #4 and
314 #6. The sWGS for the TP53KO organoids revealed additional large-scale copy number
315 alterations on chromosome 3 in experiment #6, as well as apparent chromosome shattering
316 encompassing all of chromosome 11, that were not present in experiment #4 (**Fig. 4E**). By
317 contrast, DKO cells showed no significant CNV changes across experiments (**Fig. 4E**),
318 corroborating the previous observation that DKO organoids did not exhibit molecular signatures
319 associated with the CIN subtype of gastric cancer, but instead displayed transcriptional profiles
320 characteristic of the MSI subtype (7).

321 Next, we investigated regions affected by CNVs in TP53KO cells to identify specific genetic
322 changes that could explain the observed increase in growth rate. While the TP53KO organoids
323 from both experiment #4 and experiment #6 (passage #9 and #13 post-thaw) harbored focal
324 deletion of FHIT locus, a common early alteration in this model and in gastric cancers (13), the
325 entire chromosome 3p arm was deleted in experiment #6 (**Fig. 4F**). FHIT is a secondary
326 regulator of DNA damage response commonly altered in TP53-null organoids (13), and in
327 gastric cancer (47, 48), and the data suggests that the loss of both FHIT and TP53 likely drives
328 rapid accumulation of additional CNVs. On chromosome 11, we observed loss of several
329 regions containing multiple mucin genes (MUC2, MUC5AC, MUC5B, MUC6, and MUC15) (**Fig.**

330 **4F)** that are often dysregulated during malignant progression (49-51). For instance, abrogation
331 of *MUC5AC* expression has been associated with increased cell proliferation and vascular
332 invasion in gastric tumor cells (52, 53). In addition, we observed amplification of *SPI1* which is a
333 proto-oncogene upregulated in various cancers and implicated in proliferation (54-56) (**Fig. 4F**).
334 These alterations likely explain the increased growth rate observed in the TP53KO organoids
335 over time.

336
337 *Sequencing of abnormal versus normal polarity organoids reveals changes in chromatin*
338 *accessibility associated with morphology and adhesion*

339 To explore the molecular changes driving differences in cell polarity, we extracted DKO
340 organoids of interest from the microwell arrays for downstream sequencing using a syringe
341 pump and 3D-printed microscope adapter (Methods; **Fig. 5A**). After staining organoids in
342 microwells at the end of experiment #6 with a live-cell actin dye (ref. Methods), we retrieved ten
343 organoids with normal polarity and ten with abnormal polarity (determined using the criteria
344 described in Methods) from the DKO line (**Fig. 5B; Supplementary Fig. S8**). We then prepared
345 libraries for single-organoid dual ATAC-seq and RNA-seq to identify differentially accessible
346 regions (DARs) or differentially expressed genes (DEGs), respectively, that might explain
347 differences in polarity.

348 Given the limiting biomass derived from single organoids, we used a dual library preparation
349 protocol developed for low-input specimens from Li *et al* (2021)(57). As the data quality was
350 generally higher for ATAC-seq libraries compared to RNA-seq libraries, we focused on the
351 ATAC-seq data initially. Following read deduplication and exclusion of six organoid samples with
352 <60,000 uniquely mapped reads or <500 accessible ATAC-seq peaks, we profiled seven normal
353 and seven abnormal polarity DKO gastric organoids with an average of 191,000 unique ATAC-
354 seq reads and 4,425 accessible peaks per sample (**Fig. 5C**). Clustering based on ATAC-seq
355 DARs clearly separated organoids with normal vs. abnormal polarity (**Fig. 5D**), and the DARs
356 were enriched for gene ontology (GO) terms related to cell adhesion and morphogenesis (**Fig.**
357 **5E**). Principle component analysis (PCA) also revealed a clear separation between normal and
358 abnormal polarity organoids (**Fig. 5F**). At a molecular level, transcription factor (TF) footprinting
359 analysis of the ATAC-seq data indicated that organoids with abnormal polarity had increased
360 accessibility in chromosomal regions bound by the SP family TFs (**Fig. 5G**), which are involved
361 in maintaining epithelial cell polarity (58, 59) and are dysregulated in a variety of cancer types
362 (60, 61). Consistent with this potential role, SP family TFs were more highly bound to upstream
363 promoter regions of several genes such as *PPP2R1B* and *LLGL1* involved in establishing
364 epithelial apical-basal polarity (**Fig. 5H**) (59, 62, 63). The RNA-seq data corroborated these
365 findings, as multiple upregulated genes with known SP family target sites were differentially
366 expressed in normal versus abnormal polarity organoids (**Fig. 5I; Supplementary Fig. S9**).
367 These data thus suggest a plausible molecular basis for abnormal polarity in TP53/ARID1A
368 DKO gastric organoids and demonstrate the feasibility and utility of molecularly characterizing
369 single organoids with distinct imaging-based phenotypes.

370
371 **Discussion**

372 Here, we describe an easy-to-use, open-source microwell platform for high-throughput, image-
373 based phenotyping of organoids with the ability to retrieve single organoids of interest for
374 additional downstream molecular profiling (e.g. single-organoid ATAC-seq or RNA-seq). As a
375 first demonstration of this platform, we used an organoid-optimized deep learning model to
376 identify and track nearly 100,000 individual cell trajectories from two engineering human gastric

377 organoid model lineages within >8,000,000 microwell images. Using these data, we then
378 quantified organoid growth rates and positions over time with high accuracy and granularity.
379 These measurements led to the identification of specific molecular features associated with
380 increased organoid growth and loss of apical-basal polarity.

381 This microwell platform offers multiple advantages over other existing organoid culture methods.
382 In comparison to bulk culture, the microwell method combined with our custom open-source 3D
383 image analysis module allows us to precisely measure quantitative phenotypes for individual
384 organoids, such as single organoid growth rates, division times, and migration distances, rather
385 than being restricted to bulk averages, such as fold change in cell number for an entire
386 population (64). Unlike commercially available methods or closed microfluidic systems (18, 65),
387 our microwell arrays do not require specialized instrumentation and are thus easy to adapt to
388 existing cell culture workflows (31). In addition, we were able to generate complete organoids
389 from single cells seeded in microwells (32-35), a particularly relevant advance for studying the
390 early stages of tumorigenesis, in which a single transformed cell develops into a tumor. We also
391 demonstrated that our microwell platform has comparatively higher throughput than other
392 existing methods, being able to profile thousands of organoids in parallel as opposed to
393 hundreds at a time (29, 34, 37). Finally, our microwell platform also facilitates retrieval of
394 individual organoids of interest, which can then be either directly sequenced to investigate the
395 molecular changes driving particular phenotypes or clonally expanded *in vitro* to generate
396 sufficient biomass for additional phenotypic or molecular profiling.

397 In future work, we anticipate that the ability to profile single organoids will enable personalized
398 drug screening in patient-derived tumor organoids. The microwell platform described here can
399 be used to characterize the drug response and associated phenotypic changes of each
400 organoid seeded in the microwells independently, offering the potential to screen patient-derived
401 tumor organoids against a wide variety of drugs using much lower input materials. Additionally,
402 the organoid retrieval mechanism described here confers the ability to retrieve resistant
403 organoids and either characterize them via 'omics' approaches or test them against additional
404 drug candidates.

405 Future modifications to our approach have the potential to unlock multiple additional
406 phenotyping capabilities. For example, the 3D-trained neural network could be optimized further
407 to track cell divisions and cell (39) lineages with more frequent imaging intervals (*e.g.* on the
408 order of minutes rather than hours) for fast-dividing cell types, extending our ability to measure
409 migration to multiple cells and enhancing resolution of cell division time measurements. In
410 addition, we found that microwells loaded with a greater number of cells tended to grow more
411 slowly, a phenomenon likely attributed to paracrine or juxtacrine signaling which could be
412 investigated with the loading of cells tagged with a different fluorescent protein. For example,
413 the addition of fluorescent tags on proteins such as MUC5AC and PGC, which are commonly
414 used as cell type markers in mucous cells and chief cells respectively, could reveal whether
415 certain cell types are likely to promote or suppress growth. Similarly, we could test whether
416 adjacent apoptotic cells slow the growth of nearby cells by fluorescently labeling Annexin V (66).
417 Finally, we could visualize chromosome mis-segregation errors during cell division using our
418 H2B-mCherry nuclear reporter with higher magnification imaging (67).

419 In summary, we present a microwell-based 3D culture platform that can be easily adapted for
420 the quantitative characterization of various image-based phenotypes. By applying this platform,
421 we quantify cell-to-cell variability in growth rate and apical-basal polarity in two engineered
422 gastric organoid models, and we discover potential molecular mechanisms underlying the
423 variability in both phenotypes. Through our findings, we demonstrate the importance of single
424 organoid measurements for characterizing morphological and molecular changes associated

425 with disease progression, which would be impossible to accomplish through traditional bulk
426 measurement approaches.

427

428 **Methods**

429 Culturing of CRISPR-engineered gastric organoids

430 P53KO and TP53/ARID1A DKO gastric organoids were generated as previously described (7,
431 13). Briefly, non-malignant gastric tissue from the corpus (stomach body) were obtained during
432 sleeve gastrectomy at Stanford University Hospital under an IRB approved protocol. Wild-type
433 gastric organoids were established followed by CRISPR/Cas9 mediated knockout of *TP53*
434 (P53KO) (13). CRISPR/Cas9-mediated *ARID1A* knockout (KO) in primary *TP53*^{-/-} human
435 gastric organoids, yielded double knockout lines (TP53/ARID1A DKO) (7, 13). These two
436 organoid lines, representing pre-malignant and malignant states were used in two separate sets
437 of experiments. In the first set (experiments #1 - #3), we conducted proof-of-principle tests to
438 determine various types of measurements that we could perform by culturing single-cell derived
439 gastric organoids in the microwells. Prior to the microwell experiments, organoids were cultured
440 in 24-well tissue culture plates. These organoids were maintained in growth media containing
441 50% Wnt3A/R-spondin1/Noggin conditioned media produced in house, 50% Advanced
442 DMEM/F12, 1X Penicillin/Streptomycin/Glutamine, 1X Normocin, 1X B-27 Supplement, 1X
443 GlutaMax, 1mM N-Acetyl-L-cysteine, 500nM A83-01, 10uM SB202190, 10mM Gastrin, and
444 50ng/mL EGF. The media was further supplemented with 10μM Y-27632 and 2.5μM CHIR-
445 99021 during passaging to promote stem cell survival. The culture media were refreshed every
446 7 days and the organoid cultures were passaged once every 12-14 days. During each passage,
447 old media was removed and 500ul of TrypLE was added to each well to dissolve the Cultrex
448 BME and dissociate organoids into single cells. After 30-40 minutes of incubation at 37°C, the
449 cells were pelleted down by centrifuging at 500g for 5 minutes. The supernatant was then
450 removed, and the cell pellet was resuspended in wash media (Advanced DMEM/F12, 1X
451 HEPES, 1X Penicillin/Streptomycin/Glutamine) for cell counting. Cell counting was performed by
452 combining 10ul of cell suspension with 10μl Trypan Blue, then loading the mixture into a
453 Countess chip for automated counting using the Countess II Cell Counter (Thermo Fisher). An
454 appropriate volume of cells was transferred to a new tube, spun down at 500g for 5 minutes,
455 and the pelleted cells were resuspended in fresh Cultrex BME. Cells were re-plated in a new 24-
456 well plate with 20000 cells per 40μl of Cultrex BME dome. The plate was then incubated at 37°C
457 for 20 minutes to allow Cultrex BME to solidify; after which, 500μl fresh growth media was
458 added. In the second set of experiments (#4 - #6), instead of using conditioned media as in the
459 first set of experiments, we opted to use chemically defined complete growth media to prevent
460 media batch effects from confounding our downstream analysis. The chemically defined media
461 was composed of Advanced DMEM/F12, 1X Penicillin/Streptomycin, 1X Normocin, 1X N21-Max
462 Supplement, 1X GlutaMax, 1mM N-Acetyl-L-cysteine, 500nM A83-01, 10uM SB202190, 10mM
463 Gastrin, and 50ng/mL EGF. During passaging, 10μM Y-27632, 2.5μM CHIR-99021 and
464 200ng/ml FGF10 were added. All other culture conditions remained the same as described in
465 the previous section.

466

467 Lentiviral transduction to fluorescently tag organoids with histone H2B protein

468 Addgene plasmid #89766 expressing H2B-mCherry fusion protein was packaged into lentiviral
469 particles (by the Gene Vector Virus Core at Stanford University). Prior to transduction, the
470 organoids were washed with PBS and incubated with TrypLE at 37°C for 40 minutes. FBS was
471 then added to quench the TrypLE reaction. After that, the dissociated cells were centrifuged at

472 500g for 5 minutes and resuspended in growth media supplemented with 10 μ M Y-27632. The
473 H2B-mCherry lentiviral particles were added at an MOI of 0.1 to aliquots of 500k cells to
474 maximize the number of successfully transduced cells harboring just one copy of the transgene.
475 This was done to reduce insertional mutagenesis and normalize the fluorescence intensity. The
476 cell/virus suspension was transferred to a single well of a 24-well plate, and a 1-hour
477 spinoculation at 600g at 32°C was performed. After spinoculation, cells were incubated 37°C for
478 four hours before being dissociated and pelleted down at 500g for 5 minutes. After which, the
479 pellet was resuspended in Cultrex BME followed by plating onto a 24-well plate. Organoids were
480 allowed to recover for 3-5 days until mCherry expression was visible, then the transduced cells
481 were FACS-sorted for cells expressing positive mCherry signal. Sorted cells were allowed to
482 recover and expand for several passages before being used for experiments.

483

484 Microwell design and fabrication

485 Design files for molds containing arrays of square microwells with a variety of different
486 dimensions (from 100 x 100 x 80 μ m to 1000 x 1000 x 80 μ m; breath x width x height) were
487 generated in AutoCAD and printed on standard transparencies at 30000 dpi. All design files are
488 available in Supplemental Information. Molds were created from SU-8 2100 photoresist
489 (Microchem, Inc.) on a 4" silicon test-grade wafer (University Wafer) according to the
490 manufacturer's instructions. After fabrication, molds were treated with vapor deposition of
491 1H,1H,2H,2H-perfluorooctyl-trichlorosilane (Sigma Aldrich) under vacuum for 10 minutes.
492 Microwell devices were made from the molds using standard soft-lithography (**Supplementary**
493 **Fig. S1**). Briefly, RTV 615 precursor solutions at a ratio of 10:1 (base elastomer:curing agent)
494 (R.S. Hughes) were mixed using a THINKY mixer with 3 minutes of mixing followed by 3
495 minutes of degassing, both at 2000 rpm, poured onto molds, and spun on a spin coater (Laurell
496 Technologies) at 200 rpm with an acceleration of 133 rpm/s for 30 seconds to spread the PDMS
497 and create a layer of PDMS approximately 0.5 mm thick (**Supplementary Fig. S2**); this
498 thickness could be easily peeled off but remained thin enough for high-quality imaging using a
499 long working distance objective. The PDMS was then degassed in a vacuum chamber for 15
500 minutes and baked at 80°C for 20 minutes. After baking, the PDMS was peeled from the mold
501 and cut into arrays of square devices (**Supplementary Fig. S1**). Prior to use, the devices were
502 treated with 20% oxygen plasma for 8 minutes, placed at the bottom of 12-well plates with
503 forceps, sterilized overnight by immersion in 70% ethanol, then treated with 0.5% PBS-BSA for
504 >1 hour to render the devices hydrophilic for cell growth.

505

506 Cell loading into microwells

507 Dissociated organoid obtained during bulk culture passage were resuspended in WENR media
508 to a concentration of 6000 cells/ml for 100 μ m microwells and between 600-2000 cells/ml for
509 200 μ m microwells. The cell suspension was pipetted directly onto BSA-treated microwell arrays,
510 and plates were centrifuged at 500g for 5 min to load cells into microwells. Following cell
511 loading, excess media was aspirated and Cultrex BME was pipetted dropwise onto the
512 microwell arrays containing cells. Plates were incubated at 37°C to polymerize the Cultrex BME,
513 then growth media supplemented with 10 μ M Y-27632 and 2.5 μ M CHIR-99021 and 200ng/ml
514 FGF10 was added.

515

516 High-resolution time-lapse image acquisition

517 For initial bright field imaging experiments (**Fig. 1**), organoids in microwells were grown in a
518 standard tissue culture incubator and imaged 1X per day over 7-10 days using a Keyence BX-
519 700 microscope. A grid acquisition with a 10X objective and 20X final magnification was
520 performed using the built-in brightfield capability of the Keyence microscope.

521 High-resolution time-lapse images for experiments #1-6 (**Figs. 2-4**) were acquired on an
522 inverted fluorescence microscope (Nikon Ti) with a motorized xy-stage (ASI MS-2000) and a
523 camera (Andor Zyla 4.2+) set to acquire images at 2x2 binning for a final resolution of
524 1024x1024. Broad spectrum illumination was provided by a solid-state light source (Lumencor
525 Sola). Images were acquired with a 10X objective at 20x final magnification. For multi-day
526 acquisitions, organoids were kept in a stage-top incubator to maintain 37°C, 5% CO₂, and 95%
527 relative humidity. Imaging was controlled with a Jupyter notebook that used a custom Python
528 library (AcqPak) to manage experimental acquisitions and the Micro-Manager API for hardware
529 control; all AcqPak software is freely available for download from Github
530 (<https://github.com/FordyceLab/AcqPak>). Each position in a rastered acquisition was imaged
531 with the following filter cubes and exposures: brightfield (Semrock BRFD-A-NTE-ZERO) at 1
532 ms, Cy5 (Semrock 49002) at 15 ms, and mCherry at 50ms. Rasters were acquired every 2
533 hours.

534

535 Initial image processing

536 All image processing was performed using custom Python libraries which are openly available
537 for download from Github. To extract per-microwell information, we first stitched raw tiled
538 images are first stitched into a single reconstructed image of the microwell array
539 (MicrowellStitcher; <https://github.com/FordyceLab/ImageStitcher>). The pixel locations of the
540 array corners are used to rotate the image so the array edges are parallel with the image edges.
541 Subarrays containing either 400 microwells (100um microwells) or 100 microwells (200um
542 microwells) are extracted from the stitched array image using the corner pixel locations
543 (MicrowellProcessor; <https://github.com/FordyceLab/MicrowellProcessor>), and image prediction
544 for nuclear segmentation is performed with a trained deep learning model on the subarrays
545 (Wellception; <https://github.com/juliaschaepe/wellception>).

546

547 Deep learning model training and testing

548 To enable automated quantification of organoid growth from microwell images, we first trained a
549 deep learning model (38, 39) to predict fluorescent organoid nuclei. To construct the dataset
550 used to train the model, individual microwell images of 114x114 pixels from experiment #1 were
551 saved as .npz files, and each image was randomly assigned to either training data (80%),
552 validation data (20%). Additional images from experiments #1 - #3 were used as test data (306,
553 348, 258 images respectively). We used automated quality control to remove microwell images
554 containing >8 cells after initial testing revealed images with higher cell numbers were
555 challenging for annotators to label manually (data not shown), leaving a final dataset of 2711
556 training images, 542 validation images and 912 test images. The Caliban desktop module (39)
557 was used to manually label individual nuclei in each image, and the annotations were saved to
558 the .npz files. We then used deepcell-tf (<https://github.com/vanvalenlab/deepcell-tf>) to train a
559 deep learning model, with model weights stored in a hdf5 file, and performance was measured
560 on the validation and test sets using the deepcell-toolbox
561 (<https://github.com/vanvalenlab/deepcell-toolbox>)(38, 39).

562 To predict nuclei in microwell images, the organoid segmenter was initialized with trained model
563 weights stored in an hdf5 file. The segmenter took in input images and preprocessed them with
564 histogram normalization using a kernel size of 32 x 32. Each macrowell was imaged in 9-15
565 subarrays, with each 2280 x 2280 pixel subarray containing 400 microwells of 114x114 pixels
566 each (100 μ m microwells) or 100 microwells of 224 x 224 pixels each (200 μ m microwells). Each
567 subsection was first rescaled by a factor of two before being passed into the model. Model
568 outputs were postprocessed with a watershed filter with a detection threshold of 0.25, a distance
569 threshold of 0.1 and a minimum distance of 2.5. The model was run on a NVIDIA GPU. Nuclear
570 predictions were saved as an hdf5 file containing all subarrays coming from a single microwell
571 array. A .csv file containing summary statistics of the predictions for each timepoint and
572 microwell (indexed by pixel location), including the predicted number of nuclei, area of each
573 nucleus, the total area of the organoid, and centroid locations for each organoid, alongside
574 pertinent metadata such as timepoint, microwell ID, mutant, and microwell pixel location was
575 generated.

576

577 Quantification of organoid growth rates using deep learning

578 To quantify organoid growth rates, we first used the extracted deep learning predictions to
579 identify microwells initially loaded with a ≥ 1 cells for which the model identified $\geq n+1$ cells at the
580 final timepoint in the timecourse. Total predicted organoid nuclear area was plotted as a function
581 of time for up to 60 timepoints to include only timepoints with >8 cells. A model of exponential
582 growth was then fit to the data, and parameters corresponding to initial area and growth rate as
583 the percent change in area per day were extracted from the exponential model. To compare
584 growth rate distributions across mutants and experiment, a one-way ANOVA with three
585 variables was performed, and Bonferroni correction was used to account for multiple hypothesis
586 testing.

587

588 Polarity measurements with confocal imaging

589 Initial confocal imaging to assess apical-basal polarity was performed in microwells. Growth
590 media was aspirated from plates, arrays were washed with 1X phosphate-buffered saline (PBS),
591 the arrays were incubated in BD Cytfix/Cytoperm Fixation/Permeabilization solution for 30min
592 at 4°C, then cells were washed 3x with 1X BD Perm/Wash buffer, with 10 minute incubations at
593 room temperature between washes. Fixed cells were then stained with DAPI at 300 nM
594 concentration diluted in PBS solution and Alexa Fluor 647 phalloidin at 165 nM concentration
595 diluted in PBS solution, then incubated at room temperature in the dark for 1 hr. Cells were
596 washed with PBS solution before imaging. Imaging was performed with a Leica SP5 upright
597 multi-photon confocal microscope with a HCX APO L20x/1.00 water immersion objective.
598 Organoid images were acquired in a z-stack with images taken every 10 μ m. Representative
599 single-plane images were extracted using Volocity software.

600

601 Polarity measurements with live-cell fluorescence imaging

602 For experiments #1 - #3, 1X SiR-actin dye (Cytoskeleton Inc.) and 1X verapamil were added to
603 growth media before addition to macrowells at the start of the timecourse. For experiments #4 -
604 #6, growth media was aspirated from microwells at the final timepoint of the timecourse, and
605 growth media supplemented with 1X SiR-actin dye and 1X verapamil was added to macrowells.
606 This was done in order to reduce the extrinsic effects introduced by the addition of the actin dye
607 on the growth measurements of the organoids. Imaging, image processing, and individual

608 microwell extraction were performed as described above. Blinded manual polarity classification
609 was performed on a subset of images from the final timepoint of experiment #1, with 408
610 microwells classified for TP53KO and 403 microwells for DKO. Organoids were classified as
611 “normal”, “abnormal”, or “unknown”. To be classified as “abnormal”, organoids were required to
612 display 2 of the following 3 characteristics: (1) disorganized actin signal not restricted to
613 organoid lumens, (2) lack of a central lumen ringed with actin, and/or (3) the presence of
614 multiple small, disorganized lumens. Aspiration of organoids with varying polarity was performed
615 at the end of experiment #6, using the same classification requirements.

616

617 Shallow WGS of bulk organoids

618 The TP53KO and DKO organoids were harvested using TrypLE solution in a fashion similar to
619 organoid passaging, prior to the start of experiments #4 and #6. The cells were then lysed and
620 the nucleic acid was extracted using Qiagen Allprep DNA/RNA Mini Kit (Qiagen). Aliquots of
621 DNA were sent to Novogene Co. for the construction of sequencing library and shallow WGS at
622 1X coverage. Sequencing reads were aligned to the hg38 human reference genome using BWA
623 (68). Samtools (69) was used to convert the alignment files into bam format with indexes. The
624 bam files were subsequently analyzed with QDNAseq (70) to infer copy-number variations using
625 50kb read bin size and median normalization. The output was log2 transformed and visualized
626 using a custom R script.

627

628 Single organoid retrieval from microwell

629 A syringe pump (Harvard Apparatus Pump 11 Elite) was used to drive a 1 ml syringe fitted with
630 a blunt Luer probe tip. The syringe was attached to a 255µm ID/510µm OD PEEK tubing via a
631 short 1 cm Tygon splint. The PEEK tubing was then threaded through a tightly-fitting 20µl
632 pipette tip such that approximately 0.5cm extended past the tip. The splint and tip connections
633 were secured with super glue. The tip was affixed to the microscope condenser z-stage using a
634 3D-printed liquid light guide holder (**Supplementary Fig. S8**). The syringe and tubing were
635 primed with phosphate-buffered saline (PBS). The touch-down position of the tip was adjusted
636 to the center of a live field of view under 4X magnification using thumbscrews on the condenser
637 slot; this position was noted by drawing a reference box around the tip location in the Micro-
638 Manager GUI. To pick an organoid of interest, the xy-stage was moved until the organoid was
639 centered in the reference box. The tip was then lowered to the microwell surface using the
640 condenser z-stage. The syringe pump was then used to withdraw the organoid into the tip; if
641 necessary, adherent organoids were loosened by toggling withdraw/infuse on the syringe pump
642 and/or by incubation with TrypLE for 5 minutes. The tip containing the aspirated organoid was
643 then positioned just above an empty container (e.g. a well of a multiwell plate) and dispensed
644 using the infuse button on the syringe pump into a 1.5ml centrifuge tube. A total of 20 organoids
645 were retrieved from the DKO culture from experiment #6 in microwells. Ten organoids had
646 normal apical basal polarity as evidenced by the actin ring with a large lumen (**Fig. 4B**). Ten
647 organoids had abnormal apical basal polarity as defined throughout based on the presence of
648 two of the three criteria: (1) disorganized actin signal not restricted to organoid lumens, (2) lack
649 of a central lumen ringed with actin, and/or (3) the presence of multiple small, disorganized
650 lumens.

651

652 Dual ATAC/mRNA library preparations and sequencing of single retrieved organoids

653 Each single organoid retrieved from microwell was subjected to dual ATAC/mRNA sequencing
654 library preparation following a recently published protocol that was designed for low input (57).
655 Briefly, the organoid was spun down at 500g for 5 minutes using a benchtop centrifuge. After
656 removing supernatant, the organoid was then resuspended in a direct
657 permeabilization/tagmentation mastermix (25µl TD buffer (from Illumina Nextera XT DNA Library
658 Prep Kit), 2.5µl Tn5, 16.5µl DPBS, 0.5µl 1% digitonin, 0.5µl 10% Tween-20, 2.5µl RNase
659 inhibitor and 2.5µl nuclease-free water) without prior nuclei isolation. The reaction mixture was
660 incubated in a 37°C water bath for 30 minutes with occasional hand-pipetting. At the end of
661 incubation, 2.5µl of stop buffer containing 10mM EDTA and 0.5M lithium chloride was added to
662 neutralize the permeabilization/tagmentation reaction. The tagmented cells were then lysed
663 using 100µl of Lysis/Binding Buffer from the Dynabeads mRNA Direct Micro Kit (Invitrogen).
664 After lysis, 20µl of pre-washed Dynabeads Oligo-dT beads were added to the lysate which was
665 thereupon incubated at room temperature for 5 minutes to allow mRNA to anneal to the Oligo-
666 dT. The beads with annealed mRNAs were separated from the supernatant using a magnetic
667 rack. The supernatant which contained genomic DNA was transferred to a new tube for
668 subsequent genomic DNA extraction using Qiagen MinElute PCR Purification Kit. Meanwhile,
669 the beads-mRNA complex was resuspended in 20µl of reverse transcription mastermix (without
670 any primer) from Superscript IV First Strand cDNA Synthesis Kit (Invitrogen). The Oligo-dT on
671 beads served as primers for the reverse transcription reaction. The reaction mix was incubated
672 at 50°C for 5 minutes then at 55°C for an additional 10 minutes. The resulting cDNA/mRNA
673 hybrid was thus covalently bound to the magnetic beads. The beads were washed twice with
674 100µl ice-cold 10mM Tris-HCl (pH 7) and resuspended in 5µl of Tris-HCl. Sequencing library
675 preparation of the cDNA was performed on beads using Nextera XT DNA Library Prep Kit where
676 the reagent volumes were halved (keeping the reagent ratio consistent). Meanwhile, the
677 previously tagmented ATAC-DNA was amplified using Q5 High-Fidelity 2X Mastermix (NEB)
678 with universal i5 and indexed i7 adapters. PCR was performed with 18 cycles due to the low
679 input nature of the samples. Both ATAC and cDNA libraries were cleaned using AMPure XP
680 beads according to manufacturer's recommendations. The libraries were then sent to Novogene
681 Co. for sequencing on the Illumina Novaseq platform with 75bp paired-end reads.

682

683 *Bioinformatic analysis of ATAC-sequencing libraries*

684 The raw sequencing reads were first processed with Trim-Galore to remove Illumina adapter
685 sequences. The Nextflow atacseq pipeline (nf-core/atacseq) was used to process the
686 sequencing trimmed reads with default parameters. Briefly, reads were aligned to hg38 human
687 reference genome using BWA. The alignment files were further processed with Picard and
688 Samtools to mark duplicate reads and to create bigWig files for downstream analysis and data
689 visualization. Open chromatin peaks were called using MACS2 and annotated with HOMER.
690 Due to low sample input, the number of PCR cycles used to generate the library was more than
691 the recommended number, and this resulted in higher read duplication rate. As a result, the data
692 was subjected to additional filters: 1) samples with less than 60000 deduplicated, uniquely
693 mapped reads, and 2) samples with less than 500 total peaks were removed. The final dataset
694 consisted of seven organoids each with normal and abnormal apical-basal polarity (**Fig. 5C**).
695 The peak regions were then analyzed in DESeq2 to determine differential accessibility regions
696 (DARs) (71). The transcription factor footprinting analysis was performed using TOBIAS with
697 default parameters (72). The DARs were subjected to gene ontology (GO) analysis using default
698 parameters (73-75). The GO terms with a p-value <0.01 were retained for Revigo analysis to
699 remove redundant GO terms (76).

700

701 *Bioinformatic analysis of mRNA-sequencing libraries*

702 The raw sequencing reads were first processed with Trim-Galore (77) to remove Illumina
703 adapter sequences. The trimmed reads were then aligned to hg38 human reference
704 transcriptome using STAR (78), and processed using RSEM to calculate gene expressions in
705 each sample (79). The data was further examined with PCA analysis to remove outlier samples.
706 In addition, the genes with zero readcount in more than or equal to 30% of the samples were
707 removed from the differential analysis to ensure that the analysis was not skewed by missing
708 data. DESeq2 was used to determine differential gene expression between the normal and
709 abnormal groups. Gene ontology analysis was performed in similar manner as described for
710 ATAC-sequencing libraries with one modification: GO terms with a p-value <0.05 were retained
711 for Revigo analysis due to a low number of differentially expressed genes (**Supplementary Fig.**
712 **S9**).

713

714 **Data Availability**

715 Raw sequencing data for shallow WGS and dual ATAC/RNA sequencing is available on SRA
716 with accession number PRJNA858865 at the NCBI Short Read Archive
717 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA858865/>). Other data, including image files for
718 experiments #1-6, labeled training, validation, and test set data for deep learning model training,
719 processed data files, and per-experiment and per-macrowell summary reports are available
720 through OSF at <https://osf.io/r4s3m/>.

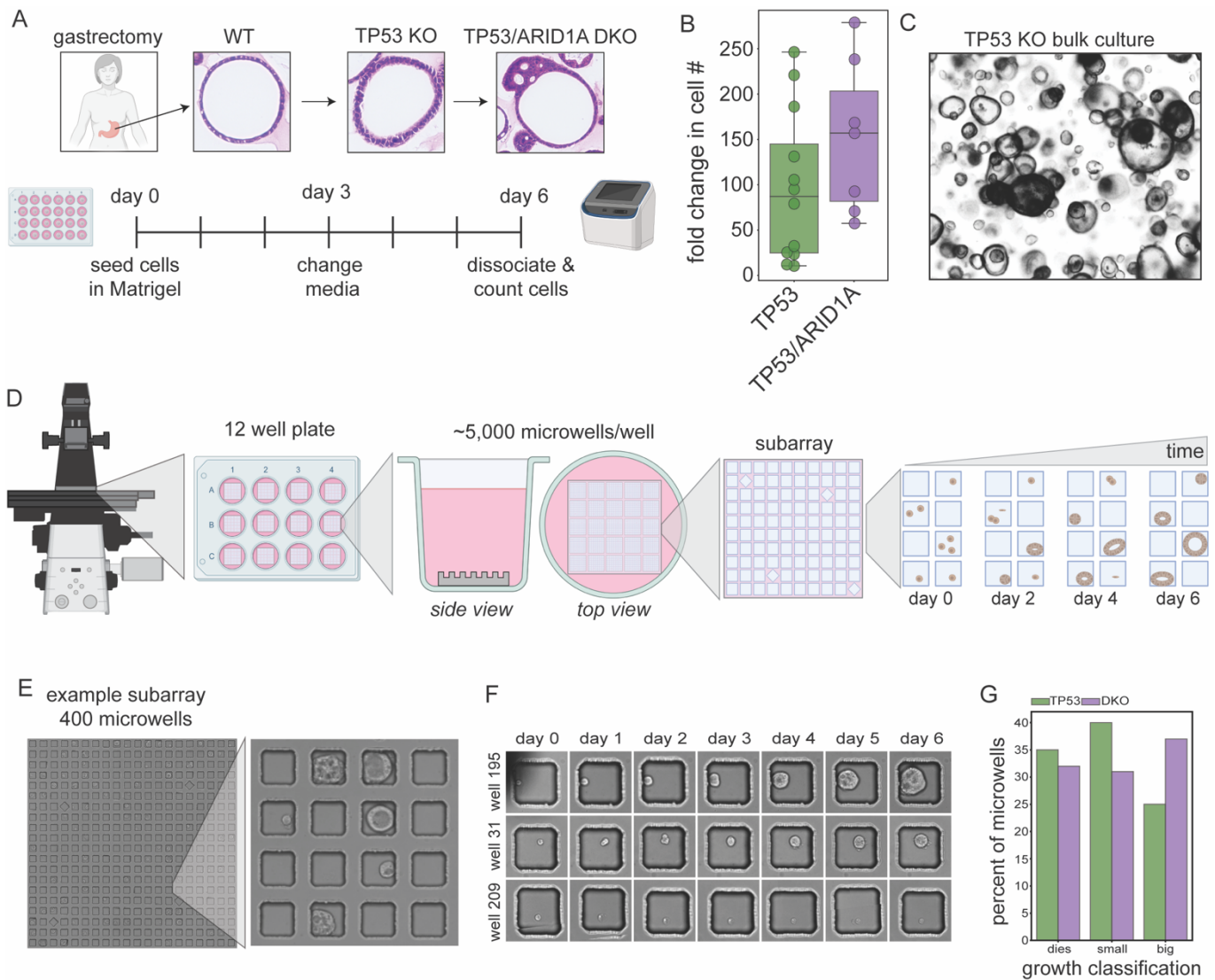
721 **Acknowledgments**

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727 Curtis and Fordyce lab for helpful feedback on this manuscript.

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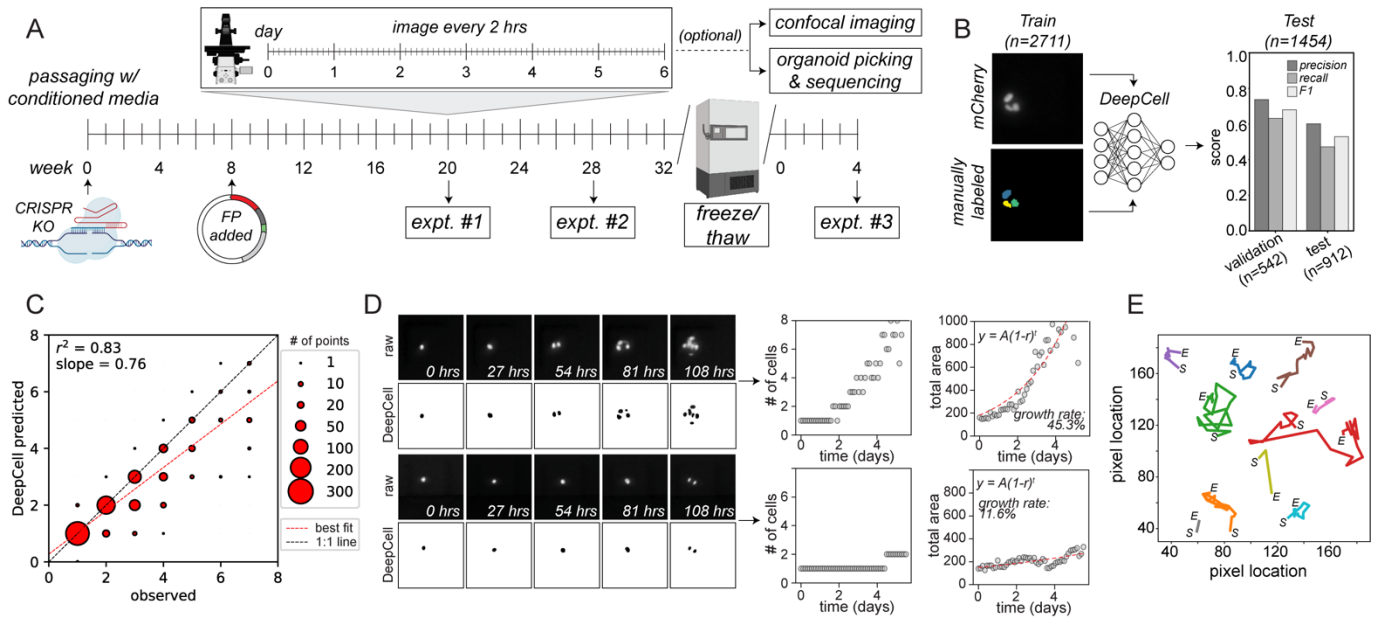
729 **Author Contributions**

730 A.S., W.W., K.K., C.C., and P.M.F. conceptualized the initial research idea. A.S. and W.W.
731 performed the microwell experiments. A.S. tested and made the microwell devices and
732 performed image processing bioinformatic analysis. W.W. performed organoid picking
733 experiments, made single-organoid sequencing libraries and performed bioinformatic analysis.
734 S.L. and D.M. set up the microscope, performed initial testing, and implemented the organoid-
735 picking platform. T.V. performed manual labeling of deep learning training and test data. V.C.
736 performed initial growth and cell seeding experiments. D.V.V., J.S. and A.S. set up the deep
737 learning pipeline. K.K. and Y.L. performed the organoid gene-editing. A.S., W.W., C.C., and
738 P.M.F. wrote the manuscript.



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Figure 1. A microwell platform for high-throughput phenotyping of gastric cancer organoids. (A) Sequential genome editing of TP53 and ARID1A in human gastric organoids revealed progressive phenotypic changes. Loss of epithelial apical-basal polarity was associated with TP53/ARID1A DKO organoids. Organoid cultures were seeded in Cultrex BME, and allowed to grow for 6 days before bulk measurements of growth and microscopy examination. (B) DKO organoids appeared faster than TP53KO organoids in bulk measurement when comparing fold change in cell number from start to end of passage but the difference was not significant (Wilcoxon rank-sum test). (C) Example image of TP53 organoids grown in bulk culture, illustrating challenges with isolating individual organoids. (D) Schematic of the initial microwell experiments using brightfield microscopy. Each microwell array was placed onto a well of a 12-well tissue culture plate. Single cells dissociated from organoids were then seeded into the microwells and allowed to expand. (E) An example image of 100 μ m microwell subarrays with organoids at the end of a 6-day timecourse. (F) Growth trajectories of single cells seeded in microwells, demonstrating heterogeneity in growth patterns. (G) Manual classification of growth trajectories for single cells by organoid size at end of time course (small = <25% of well occupied, big = \geq 25% of microwell occupied). A greater proportion of DKO organoids were classified as “big” compared to TP53KO.



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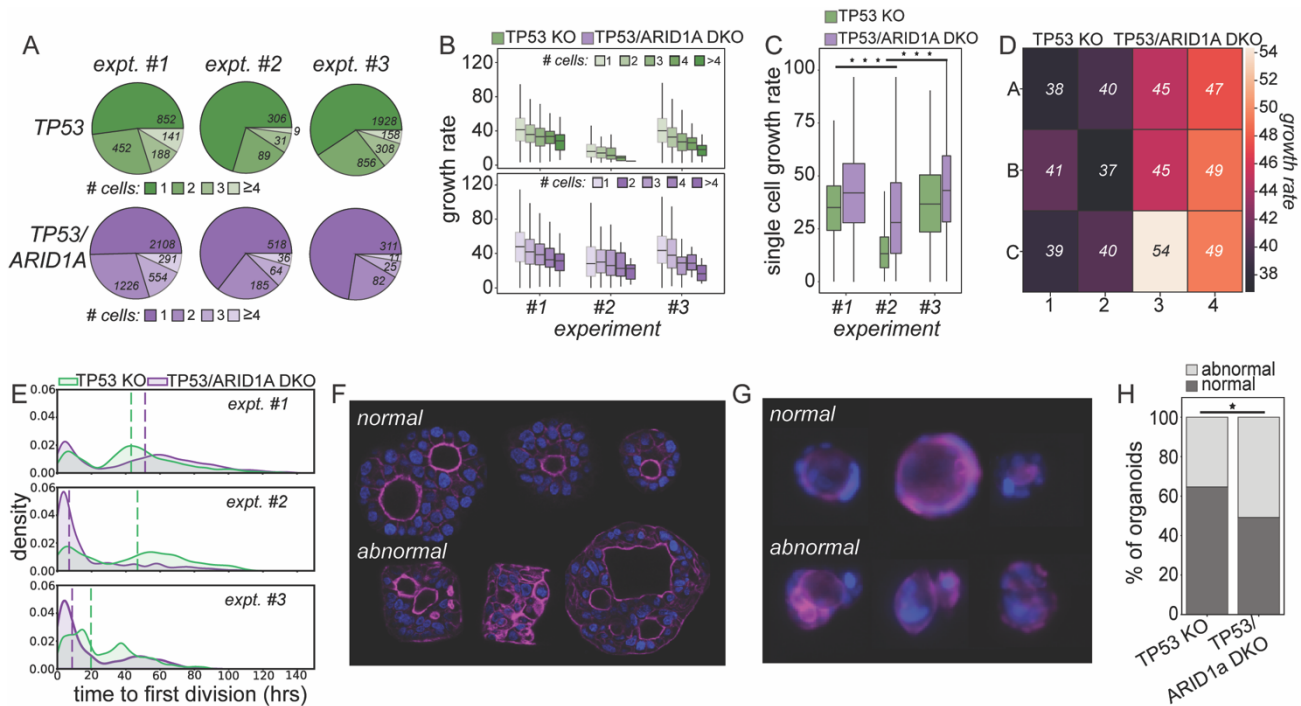
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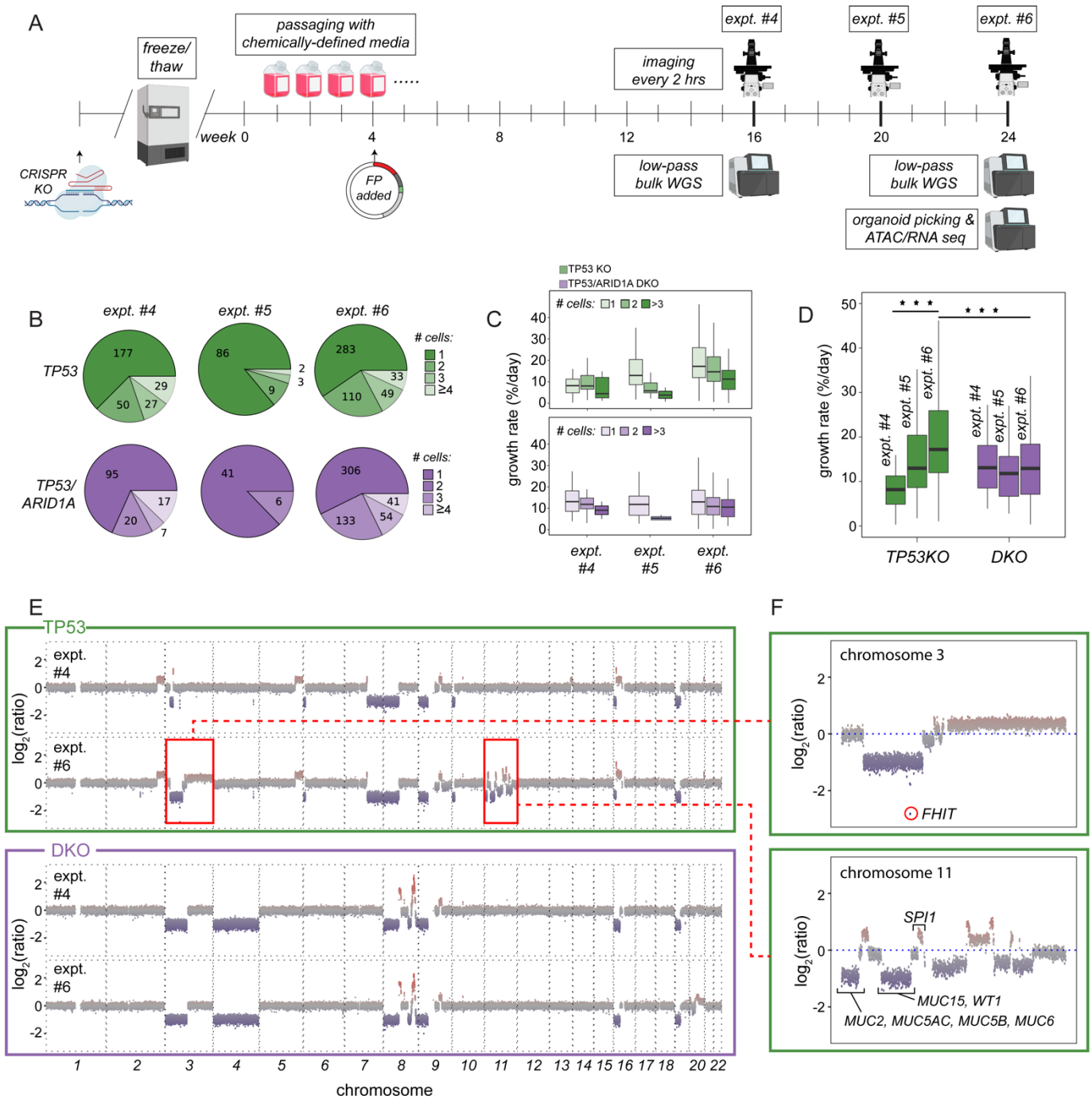
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Figure 2. Neural net accurately identifies cells within fluorescence images to enable high-throughput image processing and organoid growth quantification. (A) Experimental timeline for cell engineering and imaging experiments. **(B)** Pipeline for model training and validation (left) and performance metrics (right). **(C)** Scatter plot comparing the number of model-predicted cells and manually annotated cells per microwell (N = 912 total microwells). Marker size indicates the number of events, dashed red line indicates linear regression, and dashed black line indicates 1:1 line. **(D)** Example raw and model-predicted images (left) and model-annotated cell numbers and areas over time (right) for 2 microwells over 108 hours of imaging (left). Red line shows the exponential growth equation using the indicated best fit parameters; growth rate is reported based on the % daily change in area). **(E)** Example single cell position traces over time.



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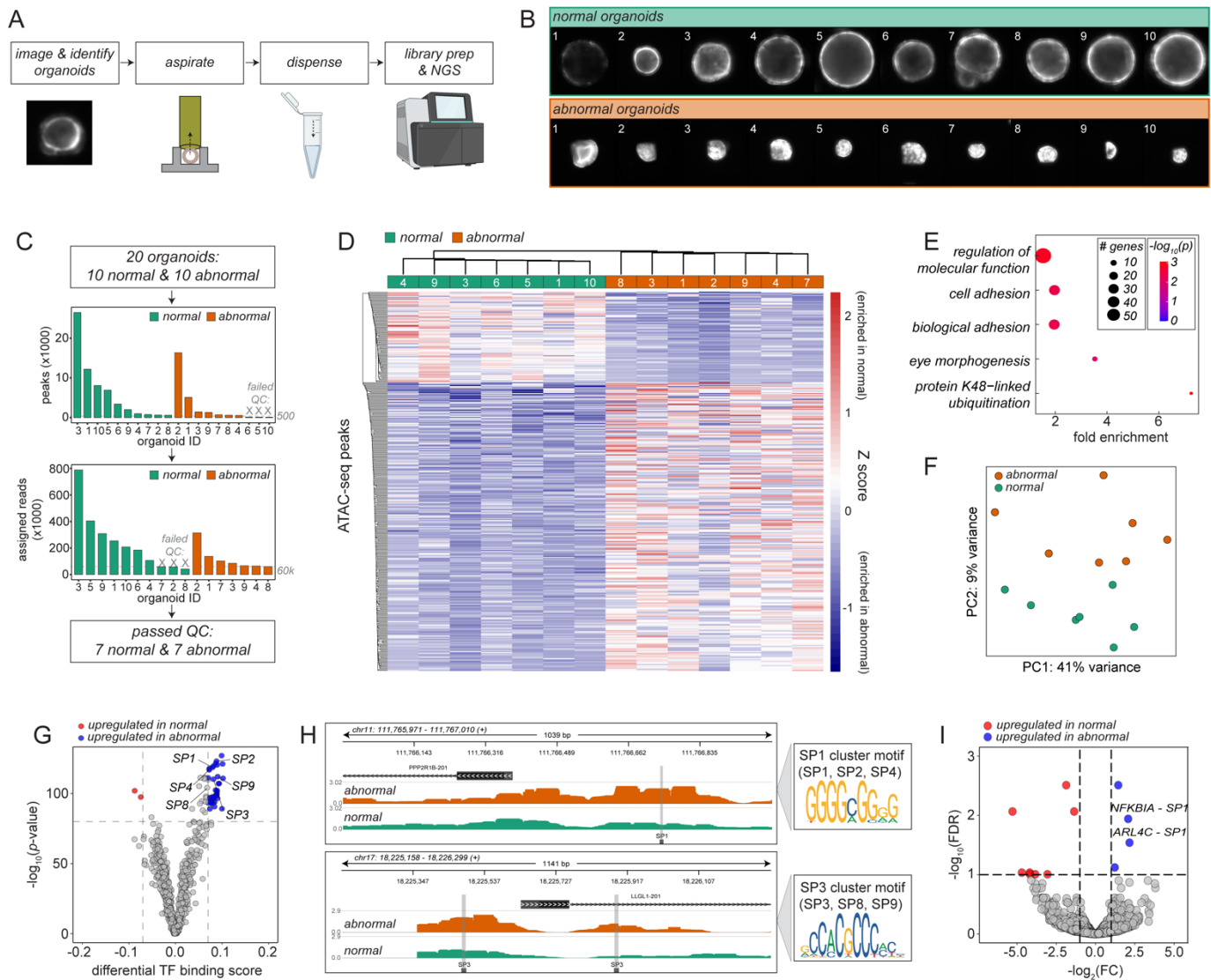
Figure 3. Microwell experiments and analysis reveal differences in growth rates and epithelial apical/basal polarization. (A) Pie charts indicating the number of TP53KO and TP53/ARID1A DKO cells loaded per microwell that exhibit growth across each experiment. **(B)** Growth rates as a function of the number of cells per microwell across each experiment for TP53KO (top, green) and TP53/ARID1A DKO (bottom, purple) organoid lines. **(C)** Growth rates for all single cells across each experiment for TP53KO (green) and TP53/ARID1A DKO (purple) organoid lines. Wilcoxon rank-sum tests yielded significant differences between experiment #2 and either experiment #1 or #3 for both within-TP53KO and within-DKO comparisons (***) denotes Bonferroni-adjusted p-value <0.001). **(D)** Heat map showing median growth rates (% daily change in cell area) for all microwells within macrowell A1 for experiment #1; median growth rates are indicated within each well. **(E)** Kernel density plots showing the time to first cell division for microwells containing a single cell from TP53KO or TP53/ARID1A DKO organoid lines; dashed lines indicate median time to first cell division. **(F)** Example confocal images of normal and abnormal polarity organoids. **(G)** Example fluorescent images of normal and abnormal polarity organoids. **(H)** Percentage of organoids with normal vs. abnormal polarity TP53KO vs. DKO lines. Fisher's exact test yielded a significant difference between the two groups (p-value=0.03).



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Figure 4. Microwell experiments under controlled experimental conditions reveal genomic changes associated with increased growth rates for TP53KO organoids. (A) Schematic of experiments #4 - #6 using chemically-defined growth media. TP53KO and DKO organoid lines were revived from cryopreservation and lentivirally transduced with H2B-mCherry at a defined MOI of 0.1 in 200µm microwells; shallow whole-genome sequencing was performed on bulk cultures at the start of experiments #4 and #6. (B) Pie charts indicating the number of TP53KO and TP53/ARID1A DKO cells loaded per microwell across each experiment. (C) Growth rates as a function of the number of cells per microwell across each experiment for TP53KO (top, green) and TP53/ARID1A DKO (bottom, purple) organoid lines. (D) Growth rates for all single cells across each experiment for TP53KO (green) and TP53/ARID1A DKO (purple) organoid lines. TP53KO organoids grew increasingly faster with each experiment while the growth rates of DKO organoids remained relatively constant (***) denotes Bonferroni-adjusted p-value < 0.001;

798 Wilcoxon ranked-sum tests). (E) Whole-genome copy number plot for TP53KO (green box) and
799 DKO organoids (purple box) from experiments #4 and #6. (F) Zoomed-in view showing copy
800 number variations across chromosomes 3 and 11 for TP53 organoids at the start of experiment
801 #6.



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 803 **Figure 5. Single organoid retrieval and sequencing reveals changes in chromatin**
 804 **accessibility associated with changes in apical/basal polarity. (A)** Schematic of experimental
 805 pipeline for single organoid retrieval. Single organoids were retrieved using a syringe pump
 806 connected to a 3D-printed microscope adapter via PEEK tubing and dispensed into a clean vial
 807 prior to preparing ATAC-seq and mRNA-seq libraries. **(B)** Images of all retrieved organoids. Top:
 808 organoids with normal polarity (*i.e.* a robust actin ring around the lumen). Bottom: organoids with
 809 abnormal polarity (*i.e.* disorganized actin signals). **(C)** ATAC-seq quality control pipeline
 810 eliminating samples with insufficient read depths or peaks. **(D)** Heatmap showing differentially-
 811 accessible peaks between normal and abnormal organoids. **(E)** Gene ontology analysis of
 812 differentially-accessible peaks showing enrichment of the peaks in pathways associated with
 813 cellular adhesion and morphology. **(F)** Principal component analysis of ATAC-seq data showing
 814 clear separation between normal and abnormal organoids. **(G)** Transcription factor footprinting
 815 analysis of ATAC-seq data showing enriched binding of SP-family transcription factors in
 816 abnormal organoids. **(H)** Representative plots showing increased binding of SP-family
 817 transcription factors in PPP2R1B and LLGL2 regulatory regions involved in regulating apical-basal
 818 polarity in epithelial cells. **(I)** Volcano plot showing differentially expressed genes in RNAseq data.
 819 Two of the four upregulated genes in abnormal organoids are known to harbor binding sites for
 820 SP-family transcription factors.

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