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Organoids

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Introduction (H.Y., Z.Z. and E.L.S.F.); Experimentation (Z.Z., K.B., E.L.S.F. and Q.W.); Results (X.C., A.M.D., A.Sl., L.L., Z.C., A.So., M.H. and Y.A.Z.); Applications (L.L. and A.So.); Reproducibility and data deposition (A.M.D., A.Sl. and M.H.); Limitations and optimizations (H.Y., A.M.D., A.Sl. and M.H.); Outlook (H.Y., Z.Z. and G.M.B.); Overview of the Primer (H.Y.).

Abstract (203/200 words)
Organoids have attracted increasing attention because they are simple tissue-engineered cell-based *in vitro* models that recapitulate many aspects of the complex structure and function of the corresponding *in vivo* tissue. They can be dissected and interrogated for fundamental mechanistic studies on development, regeneration, and repair in human tissues. Organoids can also be used in diagnostics, disease modeling, drug discovery, and personalized medicine. Organoids are derived from either pluripotent or tissue-resident stem (embryonic or adult) or progenitor or differentiated cells from healthy or diseased tissues, such as tumors. To date, numerous organoid engineering strategies that support organoid culture and growth, proliferation, differentiation and maturation have been reported. This Primer serves to highlight the rationale underlying the selection and development of these materials and methods to control the cellular/tissue niche; and therefore, structure and function of the engineered organoid. We also discuss key considerations for generating robust organoids, such as those related to cell isolation and seeding, matrix and soluble factor selection, physical cues and integration. The general standards for data quality, reproducibility and deposition within the organoid community is also outlined. Lastly, we conclude by elaborating on the limitations of organoids

109 in different applications, and key priorities in organoid engineering for the
110 coming years.

111
112 **Total (9251/9000)**

113
114 **[H1] Introduction (867/800 words)**
115 Stem cells are critical in maintaining organ size, structure and function
116 through cellular renewal, migration, differentiation and apoptosis[1]. Stem cells
117 reside in a defined microenvironment commonly referred to as the stem cell
118 niche, which provides the appropriate structural support, nutrients, and
119 mechano-chemical cues to regulate stem cell fate[2]. Given the importance of
120 these environmental cues, there have been numerous tissue engineering
121 attempts to mimic the stem cell niche *in vitro* to achieve high spatiotemporal
122 control over cell-cell and cell-matrix interactions and reproduce mechano-
123 chemical cues using engineered hydrogels and micro-devices[3,4]. In 1977,
124 Matrigel, a basement membrane extracellular matrix (ECM) containing a
125 unique mix of ECM components and growth factors, was extracted from
126 mouse sarcoma tumors and used to support *in vitro* cell culture[5]. Matrigel
127 was later shown to allow breast epithelial cells to grow in three-dimensions
128 (3D) and form lumens with milk protein secretion[6], and adult intestinal stem
129 cells embedded in Matrigel and in the presence of tissue-specific cocktail of
130 growth factors were also shown to self-organize into 3D crypt-villus
131 structures[7]. Organoid research interwined with 3D cell culture, stem cell and
132 tissue engineering for over a century, with various debates on the definition,
133 standard and scope.

134
135 In general, an organoid is a self-organized three-dimensional tissue that is
136 typically derived from stem cells (pluripotent, fetal or adult), and which
137 mimics the key functional, structural, and biological complexity of an organ[8-
138 12]. Cells comprising organoids can be derived from induced pluripotent stem
139 cells (iPSC), or tissue-derived cells (TDC), including normal stem/progenitor
140 cells, differentiated cells and cancer cells[13]. Compared to conventional two-
141 dimensional (2D) cultures and animal models, organoid cultures enable
142 patient specificity in the model while recapitulating *in vivo* tissue-like
143 structures and functions *in vitro*. Organoid cultures are more accessible for
144 manipulation and in-depth biological studies[14] than animal models. As such,
145 organoid cultures have been leveraged for a wide variety of applications
146 including drug discovery[15,16], personalized companion diagnostics[16] and cell
147 therapy[14].

148
149 However, organoid cultures exhibit significant heterogeneity, variable
150 complexity in cellular composition, can undergo poorly-controlled
151 morphogenesis in self-assembly process, and often lack stromal, vascular
152 and immunological components[4,13]. Hence, there is a great need to improve
153 organoid culture by leveraging our understanding of organogenesis as well

154 as how cells interact with their cellular and physical microenvirontment, i.e.,
155 the stem cell niche. Based on these insights, bioengineering strategies could
156 be developed to precisely control stem cell decisions during organoid
157 development. For example, from early embryogenesis studies, it is known
158 that morphogen gradients regulate tissue patterning and development[17,18].
159 Microfluidics systems can be used to create the required concentration
160 gradients of these by diffusing morphogens, giving rise to the desired cell
161 types with spatial patterning[17]. Beyond biochemical cues, it is now
162 increasingly appreciated that stem cells also experience active and passive
163 forces from their external microenvironment and convert these physical
164 stimuli into biochemical responses[19]. These physical cues could arise from
165 the matrix, external forces, and/or cell-cell interactions. Rather than relying
166 on natural or biologically-derived ECM such as Matrigel with limited stiffness
167 tunability, synthetic hydrogels or other ECM combinations can be leveraged
168 to control the physical properties of the matrix. Liquid friction against the cell
169 membrane can also exert shear stress on cells[20]. The dynamic biofluidic
170 environment has diverse effects on different cell types depending on
171 magnitude, direction, and frequency[20]. Hence, microfluidic systems and
172 bioreactors can be applied to provide perfusion at both the micro-scale and
173 macro-scales[21-23]. Lastly, it is now known that cells interact with their
174 neighbors and respond to external stimuli in a collective manner[24].
175 Topographical cues, such as curvature and shape of neighboring cells, can
176 affect stem cell decisions[25]. A recent neural tube model dissected the folding
177 process and demonstrated that geometry constraints by micropatterning can
178 control the final morphology of neural tube-like structures[26].
179
180 It is controversial whether engineered cell-based *in vitro* models such as
181 organoids need to faithfully recapitulate bulk of the structures and functions
182 of the *in vivo* organ-of-origin. One trend is to recapitulate as much *in vivo*
183 tissue architecture and functions as possible *in vitro* in order to demonstrate
184 the physiological relevance of the models of increasing complexity. For
185 engineers, the artificially created *in vitro* models only need to recapitulate
186 specific features of the *in vivo* tissue, relevant to the physiological or
187 diseased functions of interest (FOI). There is an optimism to create highly
188 complex models and expect them to accurately mimic the *in vivo* organ-of -
189 origin. For majority users, simpler ones are more robust for mechanistic
190 studies and applications[27-29].
191
192 In this Primer, we focus on the rationale underlying the establishment of
193 organoid cultures and provide guiding principles for the selection of suitable
194 materials and methods for different applications. We first discuss the
195 experimentation considerations for setting up organoid-based cultures,
196 categorized into four major components that make up organoid cultures –
197 cells, soluble factors, matrix, and physical cues and discuss approaches to
198 integrate these components (**FIG. 1**). We also discuss key considerations for

199 generating more complex yet robust organoids, such as those related to cell
200 isolation and seeding, matrix and soluble factor selection, physical cues and
201 integration. The general standards for data quality, reproducibility and
202 deposition within the organoid community is also outlined. Lastly, we
203 conclude by elaborating on the limitations of organoids in different
204 applications, and key priorities in organoid engineering for the coming years.

205

[H1] Experimentation (2575/2000 words)
[H2] Cell source

208 Under defined physicochemical conditions, tissues such as small intestine[7],
209 colon[30,31], stomach[32,33], esophagus[30], tongue[34], liver[35-38], lung[15], pancreas[39-41],
210 heart[42], ear[43], and skin[44] have been obtained from iPSCs, adult or fetal cells,
211 either stem/progenitor cells or differentiated cells. The starting cellular
212 population for any given organoid is of prime importance and not only can
213 affect the variability and heterogeneity in the structures obtained but the
214 function of the tissue they aim to model. Hence, to establish tissue-derived
215 organoids or cancer organoids, we obtain tissue resident
216 stem/progenitor/differentiated cells or tumour cells, respectively, through an
217 optimized tissue dissociation method. In contrast, for iPSC-derived organoids,
218 we establish and fully characterize iPSC lines as the starting cells.
219 Patient/tissue-derived stem cells will be obtained through an optimized tissue
220 dissociation method and then embedded into a 3D matrix mimic stem cell
221 niches. iPSC can be maintained and expanded as undifferentiated clonal
222 populations on feeder cells, defined 3D ECM substrates, or culture medium
223 suspensions. To exemplify the generation of tissue-derived organoids we will
224 use intestinal organoids as an example (**FIG. 2A**), as this was the first tissue-
225 derived-organoid type established[7]. The small intestine and colon are opened
226 longitudinally, washed, then cut into 2-4 mm fragments to increase surface
227 area for enzymatic digestion or further mechanical dissociation. EDTA
228 treatment is used to chelate calcium, disrupting cell-cell adhesion and tissue
229 integrity[45]. Larger tissue fragments and whole cells are remoed from
230 collected crypt fractions, and the harvested primary intestinal crypts are
231 used for seeding and generation of intestinal organoid cultures.

232

233 The starting cellular populations for organoid cultures can generally be
234 obtained from adult or fetal tissue biopsies. The most commonly used tissue
235 dissociation method is enzymatic digestion, which disolves the ECM[46]. The
236 composition of the enzymatic cocktail and efficacy of the enzymatic
237 dissociation process varies with tissue type[47], and in certain cases, DNase
238 can be added to remove excessive DNA released from necrotic cells[48].
239 Depending on the tissue type, the tissue fragments can be further incubated
240 with enzymes such as collagenase, elastase, or dispase to generate single-
241 cell suspensions and then seeded in Matrigel.The enzymatic dissociation
242 method may affect the cell state of retrieved cells as it may require

243 extended durations in the enzymatic mix to dissociate the majority of the
244 tissue-resident stem cells. Tissue dissociation can also be achieved
245 mechanically; although mechanical dissociation is much faster and less
246 expensive, cell yield and viability can be inconsistent[47]. Mechanical and
247 enzymatic dissociation can be combnined to generate better cell yield. After
248 tissue dissociation, TDCs for organoid development are identified and
249 collected using known biomarkers or physical characteristics[46]. Tissue-
250 specific stem cell markers are typically used to identify and isolate the
251 desired stem cells to generate organoids[1,2]. Fluorescence-activated or
252 magnetic-activated cell sorting (FACS or MACS) isolates cells based on
253 multiple parameters, including size, shape, and cell-surface marker
254 expression[46,47]. Other isolation techniques include laser capture
255 microdissection and manual cell picking[46].
256
257 iPSC can be maintained and expanded as undifferentiated clonal populations
258 over many generations. Undifferentiated human iPSCs are typically
259 maintained on feeder cells or defined ECM substrates. Since single iPSC does
260 not survive well *in vitro*, iPSC are typically harvested as cell aggregates
261 which preserve cell-cell contact, yielding cell populations with higher
262 viability. Physical scraping can also compensate for the lack of uniformity of
263 cell aggregates. The dissociation enzymatic mixture should be chosen based
264 on level of cell sensitivity[45] and whether the cultured cells secrete excessive
265 ECM, making it difficult to detach the cells from the cell culture plate (**FIG.
266 2B**).
267
268 Tumor tissue, derived from either biopsies or surgical resections, is also
269 typically processed akin to normal tissue to isolate tumour cells to grow as
270 organoids[16,49-51]. Tumor cells isolated from liquid samples such as peripheral
271 blood[52], ascites[50,53], and pleural effusions[54] can also be used as starting
272 material to generate organoids. Patient-derived tumor organoids can be
273 generated from samples obtained from minimally invasive Pap brush
274 material[55,56] . Due to their low numbers, tumor cells from biopsies or liquid
275 samples can be firstly expanded in animal models as xenografts in order to
276 obtain sufficient cells for organoid generation[57]. In the case of tumor tissue, it
277 can be preferable to limit tissue dissociation so that cell clusters rather than
278 single cells are isolated. A critical factor that can influence the generation of
279 tumour-derived organoids is the fact that isolated cells from tissue typically
280 contain both cancer and normal cells. While for some tumors it may be
281 possible to enrich for tumor-forming cells by sorting a priori[58], for the
282 majority there is currently no robust method to separate normal and tumor
283 cell populations prior to seeding into a matrix for culture. An approach to
284 overcome this issue is to take advantage of culture conditions by utilizing
285 selective media that omits certain factors required for growth of normal
286 organoids, as tumor cells gradually lose dependence on those factors during
287 malignant transformation[59]. Blood contamination, particularly erythrocytes,

288 can also affect organoid generation and matrix stability therefore standard
289 approaches to eliminate these through lysis are typically used[60].
290
291 **[H2] Matrix**
292 Following cell isolation, cells are typically seeded into biologically-derived
293 matrices such as Matrigel[7,61] or natural ECM such as collagen[62], or into
294 synthetic hydrogels[3,63,64]. Matrigel is mainly comprised of laminin, collagen IV,
295 entactin, perlecan and growth factors, and is similar in composition to the
296 basement membrane[62]. As a continuation from the above example using
297 intestinal organoids, we will now briefly describe how cells are encapsulated
298 into matrices. Isolated intestinal crypts are first re-suspended into cold
299 Matrigel and pipetted into a pre-warmed low-attachment well-plates for
300 culture. The resulting cell-matrix construct is typically a flat semi-sphere gel.
301 The complete composition of intestinal organoid culture medium have been
302 previously reported[30]. To passage organoids, the gel is first broken up
303 mechanically by pipetting culture medium to release the encapsulated
304 organoids, dissolved as much as possible using cold medium, PBS or special
305 dissolving solutions and then organoids are mechanically dissociated and
306 pieces are re-encapsulated in Matrigel for culture again. To measure the
307 proliferation rate of the cultured organoids, intestinal organoids are first
308 released from Matrigel using enzymatic digestion. The retrieved cell pellet is
309 then repeatedly washed to remove single cells, and crypts are counted. The
310 expansion rate is calculated as the number of organoids from each well
311 divided by the number initially crypts seeded in Matrigel in that well.
312
313 While Matrigel can support organoid culture, the inherently heterogeneous
314 and poorly defined composition of this biologically-derived matrix offers little
315 control over the biochemical and biophysical spatiotemporal cues that are
316 necessary for improving organoid culture. Therefore, other matrices with
317 defined compositions[64] have been explored as alternative matrices to
318 Matrigel, such as recombinant human collagen[62], fibrin[65], or synthetic
319 hydrogels[3,63]. Natural matrices can be recombinantly produced from proteins
320 or polysaccharides to address the batch-to-batch variability of Matrigel[61]. On
321 the other hand, synthetic hydrogels have emerged as powerful tools that
322 enable independent manipulation of biochemical and biophysical matrix
323 properties to control organoid features and enhance functionality. The ideal
324 organoid matrix should overall be stress-relaxing and highly dynamic in
325 biochemical and biophysical properties to accommodate or control changes
326 in organoid structure during culture. For example, dynamic hydrogels based
327 on poly (ethylene glycol) (PEG) were recently shown to enable reproducible
328 intestinal organoid formation and demonstrate how hydrogel properties
329 could be tuned to control stemness and differentiation in cultured organoids[3].
330 The viscoelastic profile of hydrogels has also been shown to define the
331 mechanical confinement of growing organoids[66]. In other examples, the
332 activity of adult stem cells can be controlled using PEG hydrogels with photo-
333 degradable moieties[67]; biomimetic polymers can be modified to incorporate

334 essential ECM signals to generate organoids with tailored features[3]. Tunable
335 PEG hydrogels can promote intestinal crypt budding[68]; while dextran-based
336 GMP-compatible hydrogels support expansion of cells for longer passages[69].
337 Lastly, microfabricated arrays were recently reported to enable the uniform
338 production of crypt-villi-shaped epithelium[70]. Even the recent advancements
339 using the synthetic matrix to grow organoids, organoid growth by the
340 synthetic matrix is still less efficient than Matrigel-cultured organoids. There
341 is an unmet demand to develop a better matrix.
342
343 Organoids can arise either from round colonies generated by single cells[7,35]
344 or, from initial multicellular structures such as intestinal crypts[30,63], cell
345 aggregates[24] or micropatterned cells[26]. The aim of the latter approach is to
346 establish a cellular niche which involves other cells of the same or different
347 type from the beginning. To form cell aggregates, the simplest method is to
348 use an ultra-low attachment dish coated with hydrophilic hydrogel[71] to
349 prevent cell attachment; subsequent centrifugation can promote aggregate
350 formation, enhancing cell-cell contacts[72]. The size and compaction of cell
351 aggregates can be tuned and controlled, such as through the use of
352 microwell arrays[72,73]. Another well-established method to form cell
353 aggregates is the hanging drop method, where aggregates form at the
354 bottom of the drop. In a recent example, droplet microfluidics was used to
355 aggregate murine cholangiocytes to form complex organoids with liver
356 mesenchymal cells[74]. Droplet microfluidics can print one organoid per well
357 and enable the rapid generation of intra-organoid heterogeneity[75]. Another
358 example of microfluidics use in organoid research comes from a recent
359 paper, where authors use droplet-based microfluidics to perform better
360 scRNA-seq analysis of intestinal organoid cell identities during various
361 developmental stages, revealing extensive population heterogeneity[76].
362 Microwell structures or microfabricated pillar arrays[70] have also been
363 developed to enable enhanced uniformity in cell aggregation[72,73,77]. In one
364 example, microfabricated patterns of Laminin-512 were shown to
365 reproducibly support human pluripotent stem cells to form lumen structures
366 in Matrigel and differentiate into human neural tube-like structures[26]. These
367 examples, amongst others emerging in the field, illustrate how our
368 knowledge of biomaterials and tissue engineering, can be extrapolated to
369 provide precise control over organoid structure and function.
370
371 **[H2] Soluble factors**
372 Organoid cultures are fundamentally based on our accumulated knowledge
373 of developmental biology[14], where soluble cues are presented to cells in a
374 spatiotemporally controlled manner (Soluble factors used to differentiate
375 TDC and iPSC into various tissue types are listed in **Supplementary Table
376 1**). Translated to organoid culture, these soluble cues are recapitulated *in
377 vitro* in the form of biologics, mainly as proteins such as growth factors[78], or
378 small-molecule drugs, which can activate or inhibit signaling pathways. While
379 growth factors could be costly and unstable, and many small-molecule drugs

380 can affect multiple pathways resulting in poor reproducibility[78], some
381 organoid protocols have combined the use of both biologics and small-
382 molecule drugs[79]. The use of conditioned medium from engineered cell lines
383 producing biologically active growth factors, e.g., L Wnt-3A, can replace
384 commonly used growth factors, such as Wnt3a ligands[80]. These conditioned
385 media face a similar issue of batch-to-batch variation and require stringent
386 tests to ensure reproducibility[81]. Thus, novel sorrugate molecules are starting
387 to arise as potential substitutes to condition medium[80,81].
388
389 It is critical to consider how and when soluble cues are are added to organoid
390 cultures because soluble cues *in vivo* are typically presented to cells by the
391 ECM or nearby cells, coordinated in time and space. This is the concept of
392 spatiotemporal presentation. For example, it is now known that fibroblast
393 growth factor (FGF) activity and specificity can be regulated by cell surface
394 heparan sulfate proteoglycans, suggesting how the addition of free FGF into
395 cell culture medium may not recapitulate the way FGF is presented *in vivo*[82].
396 The importance of presenting soluble cues in a spatiotemporally relevant
397 manner is especially important for growing human iPSC into complex
398 structures with multiple cell lineages such as in the case of kidney
399 organoids[83]. Based on previous studies[84], it is known that the ureteric
400 epithelium develops from early migrating presomitic mesoderm cells. To
401 recapitulate this process, the effect of different durations of initial Wnt
402 signaling before the addition of FGF was investigated[83]. The spatiotemporal
403 presentation of soluble factors can be achieved using different tissue
404 engineering approaches. In one strategy, these growth factors can be
405 encapsulated within nanoparticles, and conjugated onto cell surfaces for
406 controlled release[85-88]. To mimic how certain growth factors are bound to the
407 ECM *in vivo*, researchers conjugated polymers with heparin which can bind to
408 growth factors, or conjugated these growth factors to the polymer itself[89].
409 Surface tethering can also be achieved with nanotechnologies, such as nano-
410 imprint lithography, electron-beam, and electrospinning, can fabricate
411 substrates with nanopillars, nanopits, or nanochannels, mimicking the
412 basement membrane for 3D organoid culture[90]. Lastly, microfluidic systems
413 can be leveraged to create miniaturized niches with precise control over
414 mechano-chemical properties[91]. With directed flow and gradients of gas or
415 small molecules, these systems can finely control environmental parameters
416 within organoids[18]. In one example, a microfluidic neural tube device was
417 developed to present simultaneous opposing gradients of growth factors to
418 direct neural tube patterning, enabling recapitulation of the *in vivo*
419 structure[92].
420
421 **[H2] Physical cues**
422 Beside biochemical cues, it is also important to consider when and whether it
423 is necessary to provide appropriate physical cues to cultured organoids.
424 Nutrient supply and waste removal, which are diffusion-dependent, become
425 less efficient during organoid growth into larger tissue structures. This is the

426 reason why intestinal organoids need to be fragmented into smaller cell
427 clusters and reseeded regularly[7]. Inadequate nutrient and waste diffusion is
428 also problematic in brain organoid culture, where the resulting millimeter-
429 sized constructs often exhibit necrosis within the inner core due to nutrient
430 inaccessibility. This problem can be partially resolved using shaking
431 cultures[23 93], spinning bioreactors or suspension under continuous agitation[23],
432 or in continuously stirred bioreactors[93]. These bioreactors can monitor pH,
433 temperature, oxygen, and glucose levels to maximize mass transfer while
434 minimizing shear stress. In this regard, perfusable microfluidic chips have
435 also been developed to promote the long-term culture of organoids[21,22,94].
436 Lastly, it may be useful to consider providing topographical cues to control
437 organoid culture *in vitro*; the topography of the substrate is known to
438 modulate cell area, shape, and cell-cell interactions, resulting in biochemical
439 signals that can affect stem cell fate[25]. Topography-directed morphogenesis
440 has been demonstrated using intestinal organoids grown on soft hydrogels[95].
441
442 **[H2] Integrating cues**
443 In contrast to the original self-organizing organoid model, the above
444 described cues can be integrated to confer greater control over organoid
445 morphogenesis. Integration of cues is a commonly employed strategy in the
446 field of tissue engineering to construct tissues *in vitro* and *in vivo*[96]. Ideally,
447 specific physical or chemical cues should be presented in a spatiotemporally,
448 physiologically relevant manner using simple, reproducible and robust
449 methods (**Supplementary Table 2**). We will illustrate this using the
450 example on intestinal organoids[7]. Following the identification and isolation of
451 *LGR+* intestinal stem cells residing in crypts, biologically-derived Matrigel
452 was leveraged to mimic the laminin-enriched crypt environment,
453 supplemented with exogeneously added growth factors in the cell culture
454 medium. Most intestinal stem cells could survive, proliferate, and form
455 organoids[7]. However, given the stochastic nature of organoid development,
456 the resulting organoids vary in size, bud numbers and function. In one
457 example of how environmental cues (mechanical and biochemical) were
458 integrated to exert control over organoid morphogenesis, the organoid
459 maturation process was dissected into different stages, and biomaterials
460 engineering was used to identify the optimal mechano-chemical environment
461 at each stage[3]. It was shown that mechanically dynamic matrices that could
462 switch from high to low stiffness over time, enabled control over the
463 morphogenesis process[3]. In another example, human neural tube
464 morphogenesis[26] was simulated using micropatterning which created
465 mechano-chemical gradients to regulate cell-cell/matrix interactions, and
466 soluble factor presentation to orchestrate morphogenesis.
467
468 Besides these examples, other engineering methods have been used in
469 organoid culture to control cell proliferation, differentiation and
470 morphogenesis. A popular bioengineering approaches to reconstruct tissues
471 is bioprinting. Bioprinting is an additive manufacturing technique that

enables direct deposition of stem cells, organoids, and biomaterials to fabricate 3D organoid-based tissue structures with controlled cell-matrix structures[97]. This technology uses 'bioink', comprising living organoids encapsulated within a biomaterial, to precisely create 3D biological geometries that mimic that of the native tissue in a layer-by-layer approach. In one recent example of how bioprinting was used to generate large tissue constructs, human small intestinal organoids were used to form self-evolving tissue constructs by a concept called bioprinting-assisted tissue emergence (BATE), where centimeter-scale intestinal tissues were printed sequentially to mimic boundaries in the gastrointestinal tract[98]. Another bioengineering approach to reconstruct tissues, especially across different tissues and organs, is the use of microfluidic platforms. Microfluidic systems have been used to create miniaturized cellular models comprising organoids that recapitulate critical aspects of organ physiology, termed organ-on-a-chip[99] (OoC). OoC devices can potentially incorporate other bioengineering techniques to imitate the key physiological and structural features of organs and tissues. For example, physical constraints were engineered into the organoid environment using synthetic scaffolds to provide physical boundaries[100]. OoC devices also support co-culture or multi-organ systems. The two-organ system has been simulated using connected chambers containing different tissue constructs to mimic liver injury (with intestinal epithelial cells and liver cells)[101] and Type-2 diabetes (with human pancreatic islets and liver spheroids)[102]. A two-organoid microfluidic device was recently fabricated with multiple readouts using cardiac and hepatic organoids[103]. Moreover, OoC devices have improved the characteristics of hPSC-derived pancreatic islets[104], intestinal[105], stomach[106], and liver[77] organoids by supporting physiological flow rates. Kidney organoids have been cultured in an OoC device which enables fluid flow to simulate shear stress[107] and the generation of a vascular network[108].

[H1] Results [1588/1500 words]

It is important to evaluate whether the cultured organoids recapitulate key aspects of the human tissue or organ. To achieve this, characterization of these organoids is typically performed by assessing organoid cellular composition, structure, functions, and robustness of phenotype. In this section, we discuss commonly employed analytical methods using representative examples.

[H2] Validation of organoid composition and structure

Organoid growth is a process of initial cell aggregation, proliferation, migration and differentiation. When assessing whether the organoids have been successfully established, it is critical to first determine whether the organoids contain the desired cell types, and the degree to which the organoids accurately mimic the functions of the corresponding tissue *in vivo*. To achieve this, both low-throughput gene expression validation and high-

518 throughput whole-genome transcriptome analyses are typically performed.
519 Real-time PCR is often first performed as an easy, fast, and quantitative
520 read-out on marker genes indicating cell identity, including key transcription
521 factors and differentiation markers. Western blotting provides further
522 quantitative information regarding protein abundance, protein degradation,
523 protein-protein interactions, and post-translational modifications, which
524 represent the activities of a specific signaling pathway in a committed cell
525 type. The most common tools used to evaluate organoid composition are
526 immunofluorescence and immunohistochemical imaging using sections or
527 whole-mount, and the specific cell markers antibody staining further
528 elucidates various cell types' spatial distribution and proportion. A high
529 throughput analysis of single cell RNA-sequencing (scRNA-seq) profiles all
530 the cell types in the organoids, undifferentiated and committed, at the
531 whole-genome transcriptome level. These cell types are then compared to
532 cells freshly isolated from the corresponding tissue or organ to evaluate the
533 degree of similarity for each cell population. Given how it is expected that
534 the *in vitro* cultured organoids contain different states of immature cells,
535 scRNA-seq can be helpful for determining the extent of organoid
536 heterogeneity in terms of cell differentiation status.

537

538 The assessment of organoid morphology is performed to determine whether
539 there is structural similarity between the cultured organoids and
540 corresponding *in vivo* tissue/organ[109]. For example, for secretory tissues such
541 as pancreatic islets, the islet-like globular structure should be present within
542 the organoids, and intra-cellular hormone vesicles[110] are also expected to be
543 observed. For branching epithelial organoids, such as breast organoids[111],
544 distinct branching structures are expected. For intestinal organoids, crypt-
545 like structures[100] should be observed. For more complex organoid culture
546 systems comprising incorporated supportive cells, like fibroblast[112] and/or
547 endothelial cells[107,110], it is expected that the incorporated endothelial cells
548 should form a vascular network[107] that recapitulates the endothelial-epithelial
549 interactions around the organoids.

550

551 **[H2] Validation of organoid functionality**
552 Evaluation of the functionality includes but is not limited to the generation of
553 mature cells, the formation of the vasculature or neuronal networks[26,44], the
554 accurate response to external stimuli, the effective secretion of cytokines or
555 hormones, etc. It should be noted that the organoids should be compared
556 with the freshly isolated tissues as a positive control. Discrete physiological
557 recapitulations were required for different tissues, such as acid secretion in
558 gastric organoids and mucus secretion in intestinal organoids. Various
559 methods of characterization to assess the structure and functions of
560 organoids are listed in **Table 1**.

561

562 *[H3] Pancreatic islet organoids*

563 We use the example of mouse pancreatic islet organoids to discuss various
564 characterization and validation methods (**FIG. 3**). The formation of four
565 endocrine differentiated cell types (β-cell, α-cell, δ-cell, PP-cell) is examined
566 by the expressions of the corresponding hormones, Insulin (Ins), Glucagon
567 (Gcg), Somatostatin (Sst), or Pancreatic polypeptide (Ppy), by
568 immunostaining[113,114]. To assess the extent of β-cell maturation, the
569 ultrastructural morphometric analysis of insulin secretory granules is
570 performed by immune-gold transmission electron microscopy (TEM)
571 image[110]. To evaluate the ability of the β-cells in the organoids'
572 responsiveness to glucose, one performs Glucose-stimulated insulin
573 secretion (GSIS) experiments, for example insulin or C-peptide ELISA assay
574 with cyclic high and low glucose incubation[115]. As the insulin release is
575 associated with the Ca^{2+} dynamics, the calcium signaling traces imaging
576 rapidly increased in response to glucose and returned to baseline[114,116]. To
577 test the full potential of the islet organoids, the *in vivo* functional evaluation
578 is to ameliorate the hyperglycemic phenotypes of streptozotocin (STZ, a
579 toxic to the insulin-producing β cells of the pancreas in mammals)-induced
580 type 1 diabetic mouse model upon organoid transplantation[110,114,117]. The
581 characterization parameters include normalized and stable blood glucose
582 level, normal plasma insulin level and maintained body weight.
583
584 *[H3] Intestinal organoids*
585 Intestinal organoids are among the first organoid types that were
586 successfully established *in vitro*[7,30]. The formation of classical crypt-villus-like
587 architecture is regarded as an important symbol of the successful organoid
588 establishment, exhibiting the proper branching morphology with robust
589 multicellular composition and location reflecting the organoids differentiation
590 and maturation level[118,119]. The maintenance of intestinal stem cells (ISCs)
591 can be analyzed by the expression of Lgr5 maker by real-time qPCR or by
592 imaging using a Lgr5- fluorescent reporter[7,120]. The differentiated cells are
593 assessed by the staining of their markers, including Lysozyme (Paneth cells),
594 Villin (enterocytes), Mucin 2 (goblet cells), and Chromogranin A
595 (enteroendocrine cells)[7,100,121]. These various cell types can also be
596 distinguished by TEM according to their characteristic subcellular
597 structure[7,121]. Furtherly, functional intestinal organoids exhibit a relatively
598 thick mucus layer due to mucus secretion from mature goblet cells[122], which
599 can be detected by Alcian blue (AB) and periodic acid-Schiff (PAS)
600 staining[123,124].
601
602
603 *[H3] Liver organoids*
604 In the case of the liver, both cholangiocyte (ductal cell of the bile duct)[35,36]
605 and hepatocyte organoids[125,126] have been established, both from mouse and

human tissue samples. To assess the organoids, passaging time and proliferation of the organoid cells are measured. Regarding marker expression, specific cholangiocyte (for example Keratin 19, Keratin 7, SOX9) or hepatocyte markers (such as Albumin, HNF4α, MRP4) are assessed both on the RNA (qRT-PCR, scRNA-seq) and protein level (immunofluorescence staining). Often also markers of liver progenitors (like LGR5) are used to assess the differentiation state of culture. For the assessment of organoid functionality, further markers such as CYP3A4, CYP3A11 for hepatocytes are analysed on the RNA level. Furthermore, Albumin secretion, LDL-uptake, presence of glycogen accumulation assessed with periodic acid-Shiff (PAS) staining, bile acid production and activities of liver enzymes such as CYP3A4 are assessed using chemical assays to confirm hepatocyte function. The ultra-structure of the organoids is also often assessed for typical hepatocyte or cholangiocyte morphology by transmission electron microscopy (TEM). Finally, for an ultimate test of functionality, organoids are often transplanted in FRG immune-deficient mice, to look at integration and functionality of the graft *in vivo*[35,125,126]. (**FIG. 4**)

[H3] Validation of tumor organoids

Patient-derived tumor organoids must maintain the genomic, transcriptomic, morphologic and functional profiles of the tissue of origin. As such, it is important to validate them by comparing to the tissue they are derived from in terms of histology and immunohistochemistry (IHC) profiles[16], transcriptomics as well as genomics[15,127]. Cellular organization, tissue structure and protein expression patterns of tumor organoids can be easily compared to the cancer of origin[16]. Similarly, organoids are expected to have gene expression profiles similar to the tumor they are established from. This has been shown by RNAseq on many tumor types including bladder cancer[128] and esophageal cancer[129] amongst others. Lastly, it is important to confirm whether tumor organoids have diverged from the patient tumor at the genomic level. Several studies have shown concordance of organoids and parent tissue in terms of mutations and copy number variations (CNV)[15,127]. A greater degree of divergence may be attributed to intra-tumor spatial heterogeneity and sampling bias: tumors can be spatially diverse, and organoids generated from a specific portion may lack the representativeness of distant regions[130,131]. In addition to this topographical issue, culture conditions can contribute to selecting specific clones, possibly altering or reducing clonality over time[129].

For this reason, validation of tumor organoids is even more critical when culturing over a long period of time. While some studies have reported that molecular characteristics can be maintained over long-term culture, others have observed tumor evolution after serial passaging[129,132]. For instance, exome sequencing of liver cancer organoids showed how mutation concordance decreased from 92% for organoids cultured for less than two months to 80% for those grown for over four months[132]. Colorectal tumor

652 organoids established from microsatellite instable tumors had higher *de*
653 *novo* mutations after long-term culture than stable ones[59]. Some of the
654 differences observed may be attributable to how different clones and
655 subclones adapt and expand in culture. Using deep targeted sequencing of
656 500 cancer-associated genes, truncal mutations were shown to be retained
657 while subclonal ones are gained or lost, with mutational changes at each
658 passage[127]. Most mutations and CNVs are are preserved in the late passage
659 of lung cancer organoids, while *de novo* mutations can be attributed to the
660 sub-clonal expansion of small subsets of cells present in the tumor of
661 origin[15]. Overall, these studies showed that genetic drift can occur in
662 organoids cultured for extended time, influencing their reliability as models
663 for functional and drug discovery studies[133]. This reiterates the importance of
664 validation in the context of tumor organoid establishment, characterization
665 and testing. As an example of analysis, crucial steps in characterisation of
666 liver cancer organoids are demonstrated (**FIG. 4**). Lastly, normal tissue
667 contamination is an issue for establishing tumor organoids and should be
668 addressed when validating patient-derived models. Presence of normal cells
669 can be estimated by histological comparison and IHC as well as
670 sequencing[55,134].
671
672 **[H2] Data analysis**
673 **[H3] Image processing and analyses**
674 ImageJ / Fiji[135], CellProfiler[136] and other software are used for image
675 processing. The number of organoids formed in a certain period, organoid
676 size (area or volume) and cell number (nuclei number) could measure the
677 cell proliferation rate. Cell proliferation could be quantified by the ratio of
678 EdU or Ki67 positive nuclei using the plugins in the software. Morphological
679 evaluation, for example, the bud formation ratio in the intestine organoid
680 system, is also necessary when quantifying the success rate considering
681 functional maturation. Mean/normalized fluorescence intensity of maturation
682 markers (either by live imaging with knock-in reporters or
683 immunofluorescence staining at the endpoint of the experiment) for different
684 tissue types could be recorded to measure their maturation level. As for
685 lineage tracing purposes, long-term live imaging could be analyzed using
686 software to track the cell behavior. Web resources could be used for pathway
687 analysis and visualization of multi-omics data to generate heatmaps for
688 understanding the differentiation stage of organoid[137,138]. Other software, like
689 e.g.: Profiler, GSEA, Cytoscape and EnrichmentMap, have been used for
690 biomolecular interaction networks, pathway enrichment analysis and
691 visualization[139-142].
692
693 **[H3] Machine learning and Artificial Intelligence data analytics**
694 **classification of phenotypes**
695 Miniaturization and automation could make it more consistent for high
696 throughput and multidimensional phenotyping of organoids; machine
697 learning and artificial Intelligence-based data analytics showed their

698 superiority in this case. Various software have been developed in recent
699 years, like ilastik[143], OrganoidTracer[144] and Phindr3D[145]. Most of them have
700 Fiji or CellProfiler plugins, which makes it possible for users without
701 substantial computational expertise to handle larger data set in a much
702 shorter time. Those software usually contain functions for image
703 segmentation, object classification, morphological characterization,
704 fluorescence intensity quantification, cell counting and tracking for hundreds
705 of images. Good image quality (homogeneous) is a prerequisite for a
706 trustable result, so optimizing the image acquisition process is crucial. At
707 different stages of the data processing and different parts of the data,
708 validation of the accuracy of the trained algorithm is recommended.
709
[H1] Applications (1406/1500 words)

Organoids as engineered cell-based models to recapitulate relevant
physiological structures and functions of interest have shown great potential
in both basic research and clinical applications. (Supplementary **FIG. 1**)

[H2] Tissue regeneration

Tissue-derived organoids can be a potential source of transplantable material
for regenerative medicine. Organoids of murine intestines[31], livers[35,125,126] and
pancreas[69,146] were successfully transplanted into mice, and the transplanted
organoids could restore organ dysfunction. For instance, liver organoids have
been generated from hepatocytes isolated from murine livers[126]. The
organoids were injected into mice with a deficiency in fumarylacetoacetate
hydrolase (*Fah*), a defect causing liver injury resulting in short survival (40
days) after treatment with the hepato-protective nitisinone drug is
withdrawn[35]. Organoid engraftment rates were as high as 80%, and the
engrafted mice survived more than 100 days after suspension of nitisinone
therapy[126]. Similarly, pancreatic islets cultured in an artificial ECM could be
successfully engrafted into either streptozocin-induced or autoimmune-
driven diabetic mouse models, rescuing insulin production and reversing
hyperglycemia[147]. Beyond murine organoids, Huch and colleagues
successfully engrafted human liver organoids derived from EpCAM+ ductal
cells from liver samples into immunodeficient mice with acute liver damage
induced[35]. Human pancreas organoids can be generated from ALDH[high] stem
cells and have the potential to produce insulin[146] without *in vivo* evidence yet
to confirm engraftment. Sachs and colleagues showed that intestinal
organoids could fuse into tubular structures resembling *in* vivo
intestines[148,149]. A more complicated intestinal organoid can also be
generated in an artificial ECM with modulable biodegradability[3]. While there
is a long way for organoids to be used in regenerative medicine, improved
protocols to generate normal tissue organoids, good manufacturing practices
assuring high reproducibility, grafting, and function can be established[150].

[H2] Drug discovery and development studies

Technical challenges to rapid and high-throughput screening of 3D organoids have been addressed in recent years and surpassed by different means, including alternative plate design or seeding geometries[16,151,152]. Phan and colleagues screened 240 targeted agents on organoids derived from patients with ovarian or peritoneal tumors, with results available within a week from surgery. The study included a carcinosarcoma of the ovary, a sporadic ovarian cancer for which no -line therapy was established[151]. Tumor organoids can be screened to select effective drugs from structurally similar candidates[153] and investigate the synergic effects of different drugs for combination therapy[154]. Non-tumor cells from adjacent normal tissue in biopsies or surgically resected samples can be used to develop control healthy organoids to confirm the tumor-specific efficacy of the tested drugs[155]. Organoids can be screened with libraries of immune cells engineered to express chimeric antigen receptors (CAR) against different neo-antigens[156]. Checkpoint inhibitors and other immuno-oncology (IO) drugs can also be screened in tumor organoid models that include immune cells[157].

[H2] Biomarker research
Since organoids can maintain the genomic profile of the parent tissue[158], drug screening can be used to provide connections between genetic mutations and drug responses[37,154,159]. By screening gastric cancer organoids from seven patients against 37 drugs, Yan and colleagues showed that tumors with ARID1A mutations were sensitive to ATR inhibitors[159], supporting a previous study[160] and functionally identifying an intervention for a class of mutations that were considered undruggable.

[H2] Precision medicine applications
There is growing interest in tailoring cancer therapy to each patient. Precision medicine is often synonymous with genomics[161]. However, as shown in several clinical trials[161-165], only a small proportion of patients (approx. 10-20%) have an actionable alteration that can be coupled to a specific intervention[161]. In addition, the SHIVA trial has shown how this strategies based on tumor sequencing do not outperform better than the conventional physician's choice[161,162]. While the clinical efficacy of genomics precision medicine interventions can be debated, it is unquestionable that most cancer patients have no targetable alteration to guide therapy. Functional precision medicine (FPM) has been proposed as a more direct alternative[161,166]. FPM is based on testing drug responses on patient tumors to identify effective regimens and does not require any knowledge of a tumor molecular profile or characteristics a priori. Organoids are ideally suited for FPM due to their ease of establishment, similarity with the tissue of origin and tractability[158,167] and have been deployed to identify therapeutic leads in a number of tumors[168], including rare cancers that tend to be understudied and poorly characterized at the molecular level[16,151,169,170]. A recent paper by Guillen et al leveraged organoids established from patient-derived xenografts to identify eribulin as therapy for a recurrent triple negative

789 breast cancer patient. Progression-free survival was 3.5 times longer on the
790 eribulin regimen than the previous therapy the patient received[171]. This case
791 study is indicative of the potential of organoids to impact clinical care.
792
793 **[H2] Source material to establish xenografts**
794 An application of organoids that is becoming more popular involves using
795 them as starting seeding material to generate xenografts with high take
796 rates. These involve using intact organoids[15,59], or individual cells after
797 digestion[55,172]. Matano and colleagues seeded human intestinal organoids
798 transformed by CRISPR-Cas9 to carry mutations in APC, SMAD4, TP53, KRAS,
799 and/or PIK3CA in subcapsular kidney space of immunocompromised mice.
800 They found that benign organoids carrying mutations in 1-2 genes could not
801 form tumors while more malignant ones with mutations in 4-5 genes were
802 tumorigenic[173].
803
804 Patient-derived organoids can also generate xenografts, confirm their
805 tumorigenic capacity and recapitulate the histology of parental tumors[15,55]. In
806 a study of colorectal cancer, benign tumor organoids showed no or minimal
807 engraftment in mice. In contrast, colorectal cancer organoids derived from
808 metastases were more invasive than those from primary tumors[59]. Hubert
809 and colleagues directly compared the morphology of xenografts formed by
810 glioblastoma multiforme cells cultured *in vitro* either as tumor spheres or
811 organoids. They showed that while cells from tumorspheres displayed a solid
812 growth pattern, organoids were more diffusive, similar to the tumor of
813 origin[172]. Lee and colleagues showed how organoid lines derived from
814 bladder tumors of lumenal origin transplanted in mice gave rise to tumors
815 with the same lumenal phenotype[127].
816
817 **[H2] Infectious diseases**
818 Organoid models are now widely used in research on host-pathogen
819 interactions. For instance, organoids derived from tissues and organs such as
820 intestine[174,175], livers[176], lungs[175,177], oral mucosa[178], stomach[33,179], have been
821 cocultured with pathogens such as bacteria[33,179,180], viruses[174,176-178,181] and
822 parasites[175]. In human intestinal organoids, differentiated intestinal cells are
823 more susceptible to human rotavirus (HRV) infection than undifferentiated
824 ones. Organoids infected with HRV or rotavirus enterotoxin showed lumenal
825 swelling, a characteristic of rotavirus-induced diarrhea[181]. Human liver
826 organoid was used as a drug screening platform to test anti-HRV drugs and
827 antibodies, identifying mycophenolic acid, IFN-γ and anti-rotavirus-VP7
828 antibodies capable of blocking rotavirus replication *ex vivo*[176]. Organoids can
829 also be used in studies of oncogenic pathogens. Infection of murine gastric
830 organoids by H. pylori showed increased cytosolic β-catenin levels induced
831 by bacterially originated CagA, which led to increased proliferation in
832 infected organoids[179].
833

Since the start of the Covid-19 pandemic, several laboratories have investigated the effects of the SARS-Cov-2 virus on organoids. Salahudeen and colleagues generated SARS-Cov-2 infectable distal lung organoids with ACE2-expressing lumenal cells[182]. SARS-Cov-2 targets goblet cells rather than ciliate ones, in contrast to a previous study in a 2D air-liquid interface model[183]. Studies in intestinal organoids show that SARS-Cov-2 can also infect enterocytes[183,184]. Zang and colleagues used CRISPR-Cas9 and drug screenings to show that knock-outs of either TMPRSS2 or TMPRSS4, or treatment with Camostat can reduce viral infection[184].

[H2] Biology of common and rare diseases

Organoids have demonstrated significant utility in modelling and investigating both common as well as rare diseases arising from various different organs. Cystic fibrosis (CF) is a genetic disorder caused by mutations in the gene CFTR, which expresses a transmembrane chloride and bicarbonate transporter. Over 2,000 mutations have been reported, yet small molecule therapeutics deployed clinically only target a subset of these[185,186]. Mutations in CFTR cause multi-organ dysfunctions affecting lungs, pancreas, liver and intestine[187]. CF organoid models of colon[188], liver and bile ducts[189], rectum[190,191], the respiratory system[177,192], and the small intestine[188,193], have been derived from adult stem cells of relevant organs of either mouse models or patients with applications from investigating the biology of disease to mirroring patients' drug responses[190]. Berkers and colleagues used forskolin, a CFTR activator, to induce organoid swelling due to chloride and fluid flux into the lumen[191]. They demonstrated how reduced swelling of patient-derived rectal organoids correlated with clinical response parameters of patients and could mimic individualized clinical responses to specific therapeutic regimens[191]. Schwank and colleagues have used CF organoids to investigate gene therapy interventions and were able to rescue the effect of CFTR mutations in intestinal organoids using CRISPR-Cas9[193].

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease have also been studied with organoid models[194]. For instance, organoids derived from intestinal epithelial cells of pediatric IBD patients had IBD-specific DNA methylation signatures despite retaining similar morphologies[195]. Liver organoids were generated to model liver diseases like α-1-antitrypsin deficiency[36], Alagille syndrome[36] and primary sclerosing cholangitis[196]. Lung organoids have been established to investigate diseases such as goblet cell metaplasia[197].

[H1] Reproducibility and data deposition [981/800 words]

[H2] Heterogeneity and reproducibility

Organoids exhibit heterogeneity and variability between cells forming the organoid (intra-organoid heterogeneity), between organoids in the same dish

and between individual patients (inter-organoid heterogeneity). It is valuable to recapitulate the individual-to-individual differences, especially in the context of human diseases. It is also exploited in cancer research to mimic cancer development in a patient-specific manner for personalized medicine[127-129,132,155,159,167,198-200]. Similarly, the intra-organoid variation reflects the complexity of cellular composition of the tissues. This is advantageous for modeling tissue development or regeneration, with cells forming an organoid encompassing different cellular states, from stem/progenitor to more differentiated cells[36,125,201]. While these levels of variation and heterogeneity reflect the complexity of biological systems, left uncontrolled, they can jeopardize the reproducibility and robustness of the system.

Significant differences in morphology and functionality or even organoid formation efficiency depend on the different cells-of-origin of the culture or different cells in the organoid as well as different medium composition[120,202-204]. Regarding the cell of origin, the batch and quality of the iPSC/PSC used to generate the starting culture can influence the variability of the organoid line obtained. Similarly, the different primary cells isolated from different animals or patients also impact the subsequent cultures[13]. Also, the media is usually optimized for cell proliferation whereas differentiation toward certain cell fates can be largely influenced by the culture conditions, thus increasing variability and heterogeneity. In that regard, a nice example was described by Fujii M et al. who demonstrated how refining of culture conditions could increase the diversity of differentiated cell fates in human small intestinal organoids[120]. Similarly, undefined ECMs (e.g. Matrigel) suffer from their unknown composition and batch to batch variability, making reproducibility a significant concern[13,205]. Another important factor becomes apparent when looking into transcriptomic changes during organoid development[206] or studying gene expression changes during organoid passages[35,202]. When organoids mimic development, these changes can lead to confounding effects – the temporal heterogeneity. Therefore, recording the passage and the time in culture is essential to identify sources of variation that affect the results.

To better understand and control heterogeneity, the field has been moving from bulk analysis towards employing single cell approaches, such as scRNAseq, to help delineate the different populations of organoid-forming cells. Many single-cell-resolved datasets are emerging, with several notable examples in more homogeneous organoid production[3,70,100,207] and resolving organoid heterogeneity during growth progression[204,208]. Pooling together different cells and/or organoids for bulk analysis can lead to inaccurate results[3,70,100,204,207,208]. Therefore, good record-keeping about what was pooled in experiment deems crucial to drawing robust conclusions.

Additionally, it is critical to reduce the sources of variability and heterogeneity as the field grows in complexity. Our efforts to generate more

923 complete organoid structures containing different cellular populations
924 (reviewed in[209,210]), such as the recently published endothelial and healthy
925 human colon and human colorectal cancer organoids[211], murine
926 cholangiocyte and mesenchymal organoids[74], and murine pancreatic islet
927 and endothelial organoids[110], will require controlling sources of variation
928 derived from incorporating additional cells to an already heterogeneous
929 system. Exerting control over the variation while generating more
930 functionally relevant structures represents a significant challenge for the
931 field.
932
933 Finally, significant heterogeneity is also observed following organoid
934 treatments. While this represents a drawback when trying to use organoids
935 as a tool for investigating molecular mechanism, it is beneficial when aiming
936 to model patient responses. In particular, for cancer treatment, several
937 different patient-derived organoid models tested, including but not limited
938 to: pancreas, colorectal and liver cancer all exhibit heterogeneous responses
939 to known chemotherapeutic and anticancer agents[49,132,212]. This parallels the
940 variability in patient outcomes observed in the clinic and have positioned
941 cancer organoids as potential predictive tool for drug testing (Extensively
942 reviewed in[213]). This heterogeneity in treatment responses is now being
943 exploited for the treatment of cystic fibrosis patients[191], with a large ongoing
944 European multicentre clinical trial study, led by the HIT-Cystic Fibrosis
945 consortium, to identify potential responders[214]. Similar initiatives are lagging
946 behind for cancer patients, though mostly because of disparities in
947 establishment rates and lengthy timelines (extensively reviewed in[215]). In
948 that regard, while this manuscript was under revision, the group of Markus
949 Heim reported the use of organoids to inform the treatment of a patient
950 suffering from a rare form of liver cancer. Unfortunately, the treatment was
951 discontinued due to deterioration of the patient's general condition,
952 preventing the authors to draw conclusions regarding the predictive value of
953 the organoid system for the drug treatment of that patient[216].
954
955 **[H2] Minimum reporting standards**
956 To improve the reproducibility and reliability of the results, it is essential to
957 account for the variability between different cell isolations and intra-organoid
958 heterogeneity. While one could consider that at least 3 independent
959 experiments with at least 3 different biological replicates are a minimum
960 requirement, they might not always be sufficient. In fact, the number of
961 organoids to study per biological replicate as well as the number of biological
962 replicates will very much depend on the question asked, the specific
963 experiment and the variability of the phenotype observed. Whenever
964 possible, to calculate the number of independent organoids per biological
965 replicate, sample size calculations similar to the ones used for mouse
966 experiments, that take into account statistical power and variance of the
967 experiment, would be highly recommended. The data should be robust,
968 meaning repeated in at least three independent biological replicates, which

969 refers to independent isolations from different animals or patients. However,
970 replicates and the question asked in each experiment should be determined
971 in advance to avoid hypothesis fishing and increasing replicates to get a
972 significance between conditions[217,218]. Data should be reported correctly as a
973 mean of each replicate, with each replicates being an average of a similar
974 number of organoids. Recently, several publications have provided
975 guidelines to improve reproducibility, including general biological reporting
976 and accurate statistics[219-221]. Especially recommended is the use of
977 SuperPlots for data visualization, which show both the mean of each
978 independent biological replicate and the spread of the individual data
979 points[222,223]. This type of plot is ideal for organoid reporting, as it allows
980 immediate visualization of any potential artifact concerning batch variation.
981 Journal collections that deepen all aspects of statistics used in biological
982 sciences are valuable resources to facilitate accurate reporting[224].

983

984 Organoids should be characterized in full and not only checked for the
985 expression of markers, but also benchmarking to the tissue of origin. Beside
986 determining if the cells have similar morphology and expression patterns as
987 the tissue-of-origin, showing a similar function (for eample, ELISA for
988 Albumin, bile acid production by liver/hepatocyte organoids) is a must.
989 Whenever possible, results should be normalized against DNA content or
990 structural proteins such as actin, while functional data should be normalised
991 to the total number of cells or area of structures. Improving reporting
992 standards is the first step toward increasing reproducibility, thus enabling
993 the field to move forward.

994

[H2] Data Deposition

996 Standardized organoid data repositories are currently missing. Only a
997 patient-derived repository[225] led by the National Cancer Institute (NCI in the
998 USA) and the Human Cancer Models Initiative (HCMI), a combined
999 international effort of several institutions (Cancer Research UK; NCI;
1000 Hubrecht Organoid Technology's the HUB; and the US Office of Cancer
1001 Genomics's OCG), have started to generate organoid data repositories, but
1002 only for cancer organoids (**Supplementary Table 3**). However, healthy
1003 patient-derived organoids and organoid models from non-cancer diseases,
1004 both human and murine, are yet to be documented in one database,
1005 evidencing the apparent need to fully document, report and deposit all
1006 organoid lines generated. The recent launch of the Human Cell Atlas-
1007 Organoid initiative, documenting all human-derived organoids[226], is the first
1008 step in that direction.

1009

1010 On another note, when depositing data from human organoids, important
1011 aspects to consider are all ethical regulations and the necessity of obtaining
1012 patient consent and keeping patient anonymity. Critical points have been
1013 extensively reviewed in [227,228].

1014

1015 A consensus in organoid nomenclature is also missing. Aside from a couple
1016 of notable examples, where leaders in the intestine, liver and pancreas
1017 addressed the nomenclature of gut, hepatic, pancreatic and biliary
1018 organoids[229,230], the field is waiting for a more clearly-defined nomenclature
1019 system. Similarly, there is no repository where to submit organoid data. For
1020 transcriptomics-related data, such as gene expression, non-coding RNA,
1021 ChIP, genome methylation, high-throughput RT-PCR, SNP arrays, SAGE,
1022 protein arrays, Gene Expression Omnibus (GEO) is generally
1023 recommended[231,232]. For code used to analyze organoid datasets, GitHub is
1024 recommended (https://github.com). We envision that data and organoid
1025 depositories will arise shortly to fill in the presently missing gap in data
1026 repositories for organoid work.
1027
1028
1029 **[H1] Limitations and optimisations (814/1,000 words)**
1030
1031 The reproducibility, both morphological and functional, of the obtained 3D
1032 organoid systems, remains a major bottleneck. In this section, we will
1033 elaborate on the limitations and the recent developments in organoid
1034 research, providing a path towards a more optimal pipeline for developing,
1035 characterizing, and benchmarking organoid systems.

1036 **[H2] Limited level of maturity and function**

1037 None of the present organoid model systems reproduce the entire
1038 physiological repertoire of cell types, maturation level and/or functions of
1039 their respective organ; they rather exhibit certain functions of the tissue they
1040 predominantly form. The vast majority of tissue-derived organoid models are
1041 missing tissue-specific cell types, including niche-specific mesenchyme,
1042 immune cells, vascularization, innervation or microbiome. Recently, ductal
1043 cell-liver mesenchymal cell co-cultures have been shown to recapitulate part
1044 of the liver portal tract architecture[74]. Specifically challenging is that not all
1045 cell types have the same proliferation rate, growth factor requirements, or
1046 even requirements for oxygen exposure (hypoxia for vasculature).
1047 Pluripotent-stem cell derived organoids are better in recapitulating different
1048 cell types and cellular interactions of the developing organ but fail on
1049 exhibiting adult-tissue structures and functions, as well as cell maturation.
1050 One strategy that helps is *in vivo* transplantation[233]. However, this is at
1051 expenses of giving up control over the formed tissue constructs. Meanwhile,
1052 optimizations on differentiation protocols is to enrich maturation and specific
1053 functions of interest.

1054 Another factor contributing to limited maturity and function is the nutrient
1055 (in)accessibility and accumulation of dead cells in hollow lumens. This is
1056 particularly important for iPSC-derived organoids. As organoids grow in size,
1057 the nutrient supply to cells localized in the center of the organoid gets

1058 restricted, resulting in cellular death. It is common with organoids that form
1059 a more compact structure, such as brain organoids. For tissue-derived
1060 organoids forming a hollow cyst (cholangiocyte, pancreas), dead cells will
1061 eventually start accumulating in their lumens which cannot be avoided, but
1062 can be resolved by mechanical fragmentation of organoids. Constant
1063 fragmentation of formed structures prevents carrying out the long-term
1064 studies. However, PSC-derived organoids cannot be fragmented and
1065 passaged; and new strategies to solve the nutrient accessibility problem are
1066 being developed, including the long-term maintenance of brain slices *in*
1067 *vitro*[234].

1068

[H2] Limited control of organoid heterogeneity

1070 Once cells form an organoid, we have minimal input in cellular behavior
1071 within the organoid. Even in the same experimental settings, the result is
1072 often a plethora of phenotypic traits (shape, size, cell composition) rather
1073 than a stereotypic culture. Optimizing morphogenic gradients, tissue-specific
1074 cell-ECM interaction, and local biochemical and biophysical properties are
1075 essential for minimizing batch-to-batch heterogeneity[235].

1076

1077 To generate more complex multicellular mature and functional structures,
1078 the organoid field has started to create assembloids as demonstrated for
1079 human cortico-motor assembloids[236]. Such effort allows the creation of more
1080 complex structures, connecting multiple types of tissues with defined
1081 interface such as connecting cerebral cotex, spine and skeletal muscle with
1082 neuro-muscular junctions, but at the cost of reproducibility. As recently
1083 discussed in another review focused on hepatic, biliary and pancreatic (HBP)
1084 organoids[230], reproducibility in multi-cellular and multi-tissue organoid
1085 systems decreases as it is challenging to coordinate proliferation and
1086 differentiation of multiple cell types.

1087

1088 The limited control of intra-organoid heterogeneity is detrimental for high
1089 throughput screening applications and makes it difficult for studies requiring
1090 high spatiotemporal resolution imaging. Instead of creating more complex
1091 organoid systems, simpler models of reduced dimensions to recapitulate the
1092 essential tissue structures and functions of interest are gaining momentum.
1093 Variants of ECM combinations, micropatterned 2D mono-culture or co-
1094 culture[237,238], cell sheets[239], stacked 3D structures[26], and micro-positioned
1095 ECM substrates[70,240] allow the formation of reproducible tissue structures and
1096 functions with a high degree of spatiotemporal control such as stretching[241]
1097 and osmotic forces[242] (**FIG. 5**).

[H2] Optimizing ECM composition

1099 Engineering methods have been implemented to optimize these limitations
1100 (**FIG. 6**). There are two main paths to overcome the use of non-specific ECM
1101 such as Matrigel: one is the use of synthetic matrices with more complete

1102 control over composition and stiffness, and the other is to take decellularized
1103 tissue and create tissue-specific matrices[205,243]. There are significant efforts
1104 to identify chemically-defined, GMP-compatible ECMs that enable the growth
1105 and the long-term expansion of human organoids. In that regard, some
1106 advances were shown with human pancreas organoids, intestinal organoids
1107 and colorectal cancer organoids, which could grow in a dextran-based fully
1108 defined ECM; however, they would not expand long term[3,69].
1109
1110 **[H2] Organoids meet organs-on-a-chip**
1111 It has been shown that by maximizing the mass transfer and minimizing the
1112 shear stress in the perfusive soluble microenvironment, cells grown in organ-
1113 on-a-chip set-ups upregulate their functions, getting a step closer to a native
1114 tissue[94,244,245]. A more recent example shows how the presence of fluid flow
1115 enhances kidney organoids' maturation and favors their vascularization *in*
1116 *vitro*[107]. Nikolaev et al. engineered physical constraints into the organoid
1117 environment and intestinal cells when provided with boundaries through
1118 engineered scaffolds self-organized in crypts of the same size. At the same
1119 time, they overcame the inaccessibility of cystic organoids and clearance of
1120 cell debris by creating a perfusable culture of mini-intestines where cells are
1121 arranged to form tube-shaped epithelia and similar spatial arrangement as
1122 the *in vivo* tissue[100].
1123
1124 **[H1] Outlook (989/800 words)**
1125 Moving forward, the trend is to develop more complex models that
1126 recapitulate *in vivo* structure and function as faithfully as possible, in terms
1127 of the recapitulating cell types over time, tissue architecture, measurable
1128 molecular events and phenotypic functions. Rather than focusing exclusively
1129 on the most prominent markers or functional assays, architectural
1130 benchmarking of native tissue should also be performed. In the example of
1131 hepatocyte organoids[125], hepatocyte functions are preserved, yet the liver
1132 tissue architecture does not match the native tissue where hepatocytes are
1133 arranged in cords. Similarly, organoids like pancreatic or colon cancer
1134 organoids grow isotropically, forming a cyst instead of the tubular structure
1135 they would form in their native tissue. To derive more complex functions,
1136 organoids with multi-cellular and multi-tissue structures will be important,
1137 especially in the context of studying cell-cell interactions[246]. Along this vein,
1138 assembloids and organs-on-chips are also becoming increasingly complex
1139 and more broadly adopted.
1140
1141 On the other hand, the engineer's (cell as a machine) approach[28,247] is to
1142 pursue simpler reductionist models defined by the minimal functional
1143 modules (MFMs) that drive a complex cellular or tissue FOI, to study the
1144 mechanobiological causation in development or repair, or to develop a
1145 robust system for high throughput screening. The basic premise is that a
1146 complex biological function is executed by coordinated operation of a limited
1147 number of functional modules, each described by a small set of molecules,

1148 and chemical reactions driving physical attribute changes of mesoscale (sub-
1149 cellular or inter-cellular tissue/multi-cellular) structures associated with FOI in
1150 the specific spatiotemporal stage/phase/step. For example bile canaliculi in
1151 liver exhibit hourly cycles of expansion and contraction. To study the
1152 causative contraction events with high resolution, only regions of adjacent
1153 hepatocytes forming the bile canaliculi are directly studied in the context of
1154 the entire regulatory machinery of adjacent hepatocytes[27,248]. One can
1155 choose to create a much larger structure involving cholangiocytes than the
1156 one operated by the MFMs but the model will be noisy and costly. Each
1157 functional module is coupled to another and can be modeled together or
1158 independently at different length scale. Simple reductionist models have
1159 been useful for high resolution mechanistic understanding of tissue
1160 morphogenetic events such as defects[29,240,249].
1161
1162 Geometrically constraining the size of the initial 2D seeding pattern and 3D
1163 formation by micropatterning and supporting 3D cell growth using Matrigel,
1164 Karzburn et al., induced tissue-like neural tube morphogenesis and produced
1165 highly reproducible neural tubes. They identified the mechanisms of neural
1166 tube folding and modeled neural tube defects[26]. They focused on neural tube
1167 formation and characterizing a selected subset of relevant molecular events.
1168 In another example, symmetry breaking in a uniform sphere of cells and the
1169 emergence of a Paneth cell is a critical event in the early stage of intestinal
1170 organoid formation. The mechanism was unknown until Serra et al. showed
1171 that it is caused by transient activation of mechanotransducer YAP1, which
1172 induces NOTCH-DLL1 lateral inhibition events[204]. Gjorevski et al. translated
1173 this knowledge to control YAP1 activation by applying geometrical
1174 constraints in hydrogel scaffolds and producing a uniform and reproducible
1175 intestinal microtissues[3].
1176
1177 Organoids can be constrained by reducing the 3rd dimension in a 2.5D
1178 culture. 2.5D is 3D culture with restricted 3rd dimension. Typical examples
1179 are culturing cells on curved or patterned surfaces, flattened or constrained
1180 cellular construct[250], and overlaying ECM on a flat cell monolayer at high
1181 confluency which would pull the cells upward, forcing more cell-cell
1182 interactions to adopt 3D cell morphology. For example, hepatocytes in a
1183 collagen sandwich have sufficient contact area to attain polarity and form a
1184 bile canalicular lumen that contracts in the same periodic cycles as *in vivo*,
1185 although missing the 3D network, and lunes are wider and cholestatic
1186 compared to the native tissue. This cell-based model enables high-resolution
1187 dissection of the mechanism of bile canaliculi contraction into steps and an
1188 understanding of the molecular machinery regulating the phase
1189 transitions[27,251]. 2.5D culture reduces the depth-driven variabilities of a
1190 typical organoid: diffusional constraints in the hypoxic core, limited
1191 accessibility for drugs/transfection agents, and impeded imaging
1192 transparency[250]. We would also envision more engineered organoid models

based on CRISPR-edited cells for disease modeling, even though these cells and models are synthetic.

Beside these technological advances on creating more physiologically relevant, robust and easier to use organoid models, we shall see greater impact in applications. In the last two decades, while there have been discussions to replace animal testing, these efforts have not panned out in the form of concrete actions. However, this is rapidly changing with regulatory hardstops now established on multiple fronts[252]. Organoids that can recapitulate the complex physiological functions *in vivo* has also boosted confidence that the new alternative methods are now viable options. There will be more extrapolation of animal research findings in human organoids to better understand human biology and pathophysiology. We envision widespread adoption of organoids as cell sources for cell therapy, regenerative medicine, *in vitro* diagnostics, and drug discovery.

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This review disccused about the current stage of precision cancer and suggest the future applications.

Declaration of competing interests:

MH is inventor in several patents on organoid technology. A.So and LL are inventors on a patent on organoid technology. A.So is a founder and owner of Icona BioDx. HY is inventor in several patents on cell-based models. The remaining authors declare no competing interests.

Tables
Methods used in organoid research to assess/characterise organoid structure and function

	Result characterization methods	Function	Example Applications	Ref.
Organoid structure	Bright field imaging	Qualitative assessment of morphology and viability. Quantitative assessment of organize size and number.	Crypt-like structures for intestinal organoids, islet-like globular structure for pancreatic islet organoids, branching structure for breast or lung organoids, hollow cystic structures for cholangiocyte and cholangiocarcinoma organoids, grape-like structures for hepatocyte and hepatocellular carcinoma organoids	35-37,42,100,110,111,125,126,132,155,201,203
	Immunofluorescent staining	Quantitation viable cells. Edu and Ki67 staining to evaluate the survival and proliferation of the cells in the organoids. Staining of differentially expressed proteins in different layer or geometry. Qualitative and quantitative assessment of cellular composition, spatial distribution, and proportion of different cell types and maturation state of the different subpopulations.	Bile canaliculi can be shown to form between the hepatocytes (identified by markers, such as such as BSEP or MRP2 for bile canaliculi, Albumin or HNF4alpha for hepatocytes)) and in some cases bile ducts can also be formed between the cholangiocytes (identified by markers such as Keratin19 and SOX9)	36,100,107,110,111,125,126,132,155,201,203
	Transmission and scanning electron microscopy	Characterizing the cellular interaction and ultrastructure of the cells.	Measuring the size and density of mitochondria and cellular secretion, or for hepatocyte organoids glycogen accumulation, Golgi and mitochondria morphology, bile canaluciuli presence.	7,36,41,42,100,107,109,117,125
Organoid function	qPCR and single and bulk cell RNA-sequencing	Quantitation of the expression of marker genes, including key transcription factors and differentiation markers, to indicate the cell identity and cellular composition of the organoids.	Single cell RNA-sequencing can profile all cell types in the organoids. Also useful for screening differentially expressed markers under different stimuli.	35,37,38,41,42,100,107,110,117,125,126,132,201,203
	Immunofluorescent imaging	Use of fluorescently labelled dyes to determine specific functions of certain tissues. Transport of Rhodamine123 (substrate of MDR1 on cholangiocytes) or fluorescein diacetate (in the bile canaliculi of hepatocytes) to verify correct cell functionality of organoids	Swelling assays to determine functionality of ion transporters (e.g for CFTR functionality in gut, lung and pancreas organoids), CMFDA (5-chloromethylfluorescein diacetate) dyes to determine bile transporter functionality, or dextran-based fluorescent dyes	36-38,41,100,107,111,114,125,126,132,155,201,203

			to investigate permeability of epithelium or endothelium.	
	Alcian blue (AB) and periodic acid-Schiff (PAS) staining	Detection of neutral and acidic glycoproteins produced by specific cells in organoids	Measurement of mucus secretion ability of the differentiated goblet cells in intestinal organoids	**123,124**
	ELISA and colorimetric assays	Secretome quantification in response to external stimuli	Determine c-peptide secretion in pancreas organoids or endogenous production of secretome molecules by organoids as assessment of their maturity.	35,36,38,41, 110,114-116,125,126, 132
	Luciferase assays	Luminescent assays for measuring enzyme activity	Various Cytochrome activity for hepatocyte organoids (CYP3A4, CYP1A2, P450).	35,36,125,126
	Calcium signaling	Characterizing the electrophysiology property of the organoid	Used for organs like the heart, neuron, retina, skeletal muscle and pacncreatic islet.	27,114,116,125,126
	Implantation	Testing the in vivo function of organoids for cell therapy.	The full potential of the islet organoids in mouse transplantation can be tested by characterization of normalized and stable blood glucose level, normal plasma insulin level and maintained body weight	35,41,110,114,116,117,125,126,132,155,203

2125 *TEM – transmission electron microscopy, SEM– scanning electron microscopy, qPCR
2126 – quantitative polymerase chain reaction, ELISA – enzyme-linkedimmunosorbent
2127 assay
2128

2129 **Figure legends:**
2130
2131 **Fig. 1 Components to engineer organoids**
2132 The set up of organoid-based culture requires considerations about four
2133 major components that make up organoid cultures – cells, soluble factors,
2134 matrix, and physical cues and how to integrate these components
2135
2136 **Fig. 2 Flowchart of the procedures**
2137 Organoids can be generated from TDC or iPSC.
2138
2139

Fig. 3 Representative results of pancreatic islet organoids validation analysis

1. Representative view of pancreatic islet organoids.
2. Cell types and hormones secretion level validation by immunofluorescence or immunohistochemical staining.
3. Real-time qPCR analysis for some key transcription factors and differentiation markers.
4. The maturation of the organoids can be induced through prolonged culture for a total of 30 days at any passage.
5. Schematic of the pancreatic islet organoids function validation *in vitro*
6. Measurement of the secreted C-peptide by ELISA
7. Intensity of calcium signalling traces imaging indicating the capability of responding acutely to glucose
8. Schematic of the islet organoids function validation *in vivo*

Figure 4. typical characterization of cancer organoids: liver cancer subtype[132]

1. Isolation of cells from patient samples and organoid culture; schematic of tissue isolation and processing;
   HCC, hepatocellular carcinoma; CC, cholangiocarcinoma; CHC, combined HCC/CC tumors.
2. Histological analysis of liver cancer samples: top, tissue; middle, organoid brightfield images; bottom, histological H&E staining of organoids; scale bar, middle row, 100 µm; top and bottom rows, 50 µm.
3. Analysis of specific marker gene expression: immunofluorescene staining for AFP (hepatocyte/HCC marker; red) and EpCAM (ductal/CC marker; green); blue - DAPI, scale bar, 30 µm.
4. Organoid formation efficiency: growth and splitting curves; dot, splitting time point, arrow, continuous expansion.
5. Transplantation into immunodeficient mice: xenograft and histopathology analysis, matching to the patient origianal tissue sample; scale bars, left, 125 µm; right, 62.5 µm
6. Analysis of genetic changes in the cancer organoids and their concordance to the mutations in the original tumor sample.
7. Organoid sensitivity to drugs: IC50 curves for gemcitabine treatment.

Fig. 5 Reducing the heterogeneity with complexity reduction
Simpler models of reduced dimensions to recapitulate the essential tissue structures and functions of interest are gaining momentum. Micropatterned 2D mono- or co-culture allow the formation of reproducible initial 2D condition which can further form the initial 3D structure[26,70]. Then a high degree of spatiotemporal

2181 control, such as stretching[241] and osmotic forces[242] can be applied to direct certain
2182 tissue morphogenesis.
2183
2184 F**ig. 6 Side-by-side comparison of the current limitations for**
2185 **organoid culture and approaches to overcome them**
2186 **Top panel:** Accummulation of dead cells and cell debris inside of cystic organoid
2187 lumina (left) has been overcome by (right) designing a perfusable open-end
2188 structures that use inducible flow to wash out cell debris, which are compatible with
2189 long-term experiments.
2190 **Middle panel**: Organoids grown in Matrigel domes display high-variability of cell
2191 heterogeneity and morphology (left) which can be overcome by utilising (right)
2192 grids with patterned synthetic ECM which provide cues for cell differentiation. These
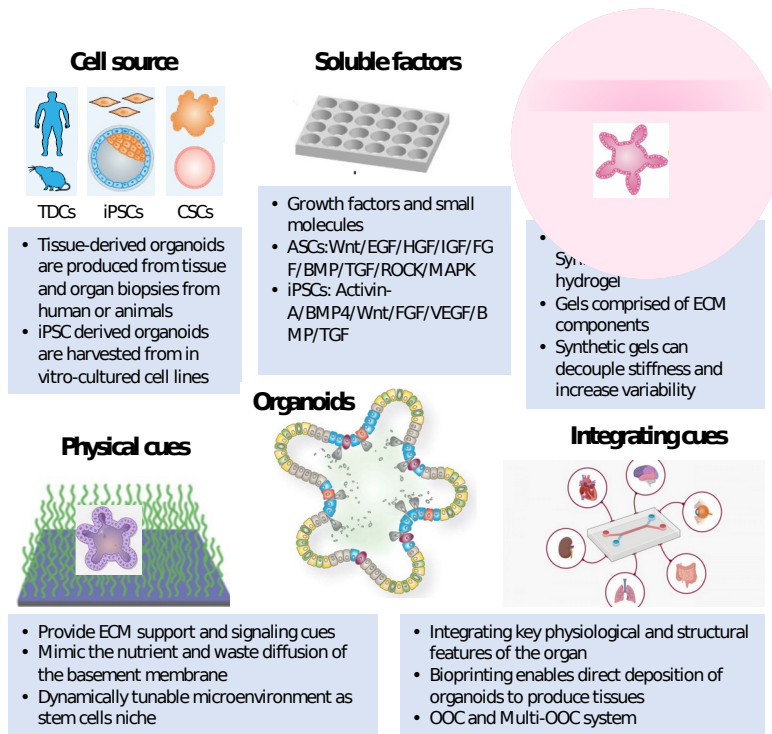2193 platforms are additionally compatible with high-throughput screenings.
2194 **Bottom panel**: Single-cell type derived organoids do not recapitulate the cellular
2195 and physiological complexity of native tissue (left), but (right) combining organoids
2196 with organ-on-chip (OoC) as novel technology would enable creating controlled
2197 micro-envirorment, suitable for multiple cell types
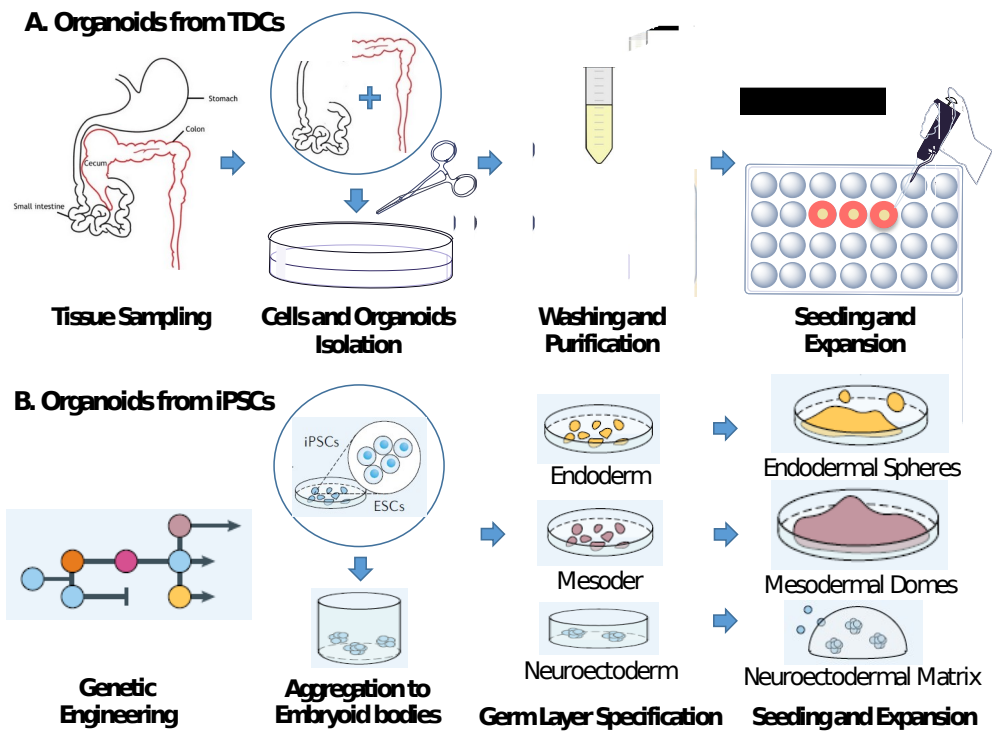2198
2199 **Figure legends:**
2200
2201 **Fig. 1 Components to engineer organoids**

Cell source
TDCs   iPSCs   CSCs
- Tissue-derived organoids are produced from tissue and organ biopsies from human or animals
- iPSC derived organoids are harvested from in vitro-cultured cell lines

Soluble factors
- Growth factors and small molecules
- ASCs:Wnt/EGF/HGF/IGF/FGF/BMP/TGF/ROCK/MAPK
- iPSCs: Activin-A/BMP4/Wnt/FGF/VEGF/BMP/TGF

- Syn... hydrogel
- Gels comprised of ECM components
- Synthetic gels can decouple stiffness and increase variability

Organoids

Physical cues
- Provide ECM support and signaling cues
- Mimic the nutrient and waste diffusion of the basement membrane
- Dynamically tunable microenvironment as stem cells niche

Integrating cues
- Integrating key physiological and structural features of the organ
- Bioprinting enables direct deposition of organoids to produce tissues
- OOC and Multi-OOC system

2202
2203 **Fig. 2 Flowchart of the procedures**

A. Organoids from TDCs

Tissue Sampling   Cells and Organoids Isolation   Washing and Purification   Seeding and Expansion

B. Organoids from iPSCs

iPSCs   ESCs

Endoderm   Endodermal Spheres

Mesoder   Mesodermal Domes

Neuroectoderm   Neuroectodermal Matrix

Genetic Engineering   Aggregation to Embryoid bodies   Germ Layer Specification   Seeding and Expansion

2204
2205
2206 **Fig. 3 Representative results of pancreatic islet organoids validation**
2207 **analysis**
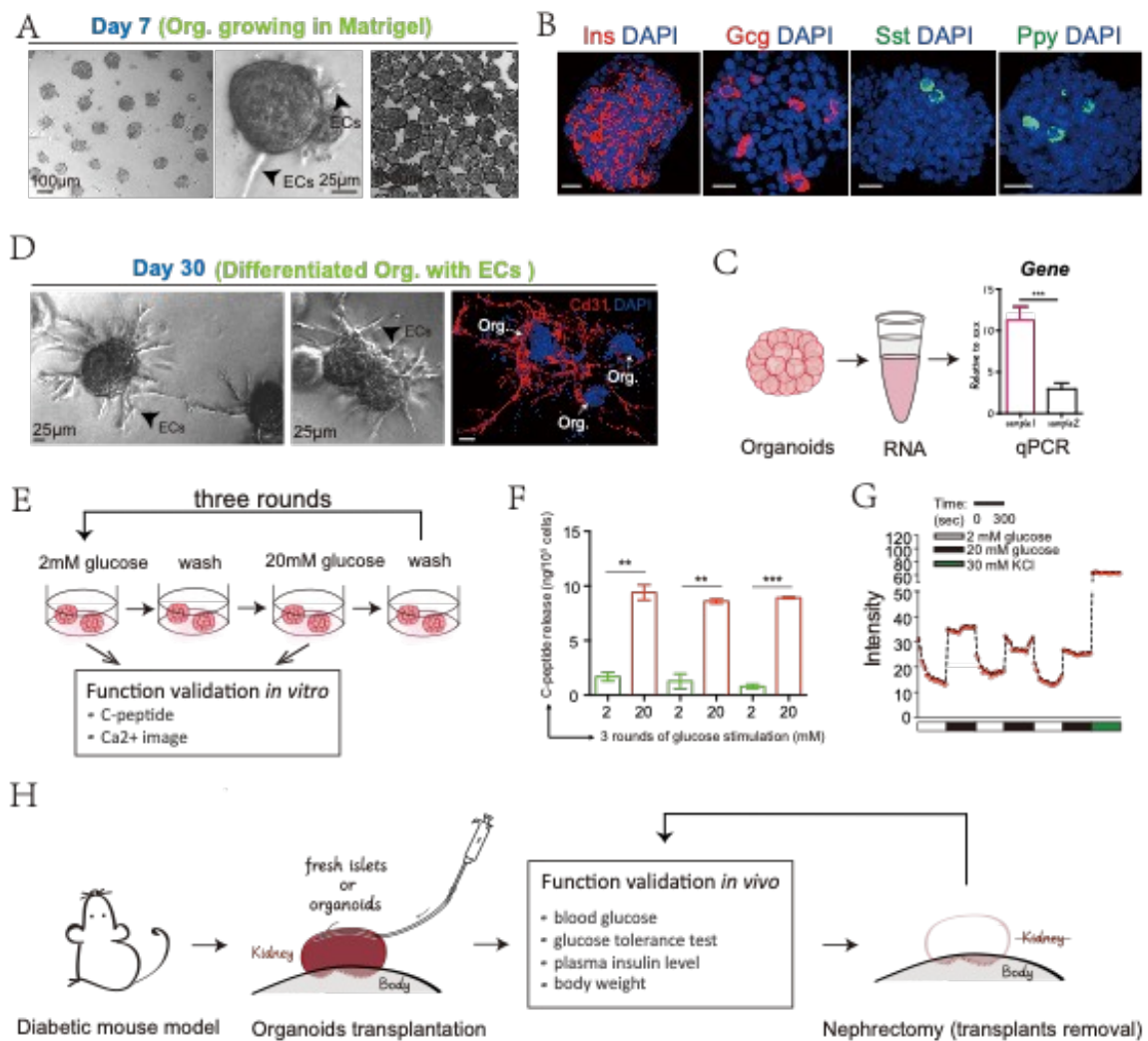
Figure 1. Representative results of pancreatic islet organoids validation analysis

A, Representative view of pancreatic islet organoids. **B**, Cell types and hormones secretion level validation by immunofluorescence or immunohistochemical staining. **C**, Real-time qPCR analysis for some key transcription factors and differentiation markers. **D**, The maturation of the organoids can be induced through prolonged culture for a total of 30 days at any passage. **E**, Schematic of the pancreatic islet organoids function validation *in vitro*, including measure the secreted C-peptide by ELISA (**F**) and calcium signaling traces imaging (**G**), indicating the capability of responding acutely to glucose. **H**, Schematic of the islet organoids function validation *in vivo*.

Base on Wang et al. 2022. *Nature Protocols*

Figure 4. typical characterization of cancer organoids: liver cancer

subtype[132]

Figure 4 - Typical characterisation of cancer organoids; liver cancer subtypes [109]
A) Isolation of cells from patient samples and organoid culture; schematic of tissue isolation and processing;
HCC, hepatocellular carcinoma; CC, cholangiocarcinoma; CHC, combined H CC/CC tumors.
B) Histological analysis of liver cancer samples: top, tissue; middle, organoid brightfield images; bottom, histological H&E staining of organoids;
scale bar, middle row, 100 μm; top and bottom rows, 50 μm.
C) Analysis of specific marker gene expression: immunofluorescene staining for AFP (hepatocyte/HCC marker; red)
and EpCAM (ductal/CC marker; green); blue - DAPI, scale bar, 30 μm.
D) Organoid formation efficiency: growth and splitting curves; dot, splitting time point, arrow, continuous expansion.
E) Transplantation into immunodeficient mice: xenograft and histopathology analysis, matching to the patient origianal tissue sample; scale
bars, left, 125 μm; right, 62.5 μm.
F) Analysis of genetic changes in the cancer organoids and their concordanc e to the mutations in the original tumor sample.
G) Organoid sensitivity to drugs: IC50 curves for gemcitabine treatment.
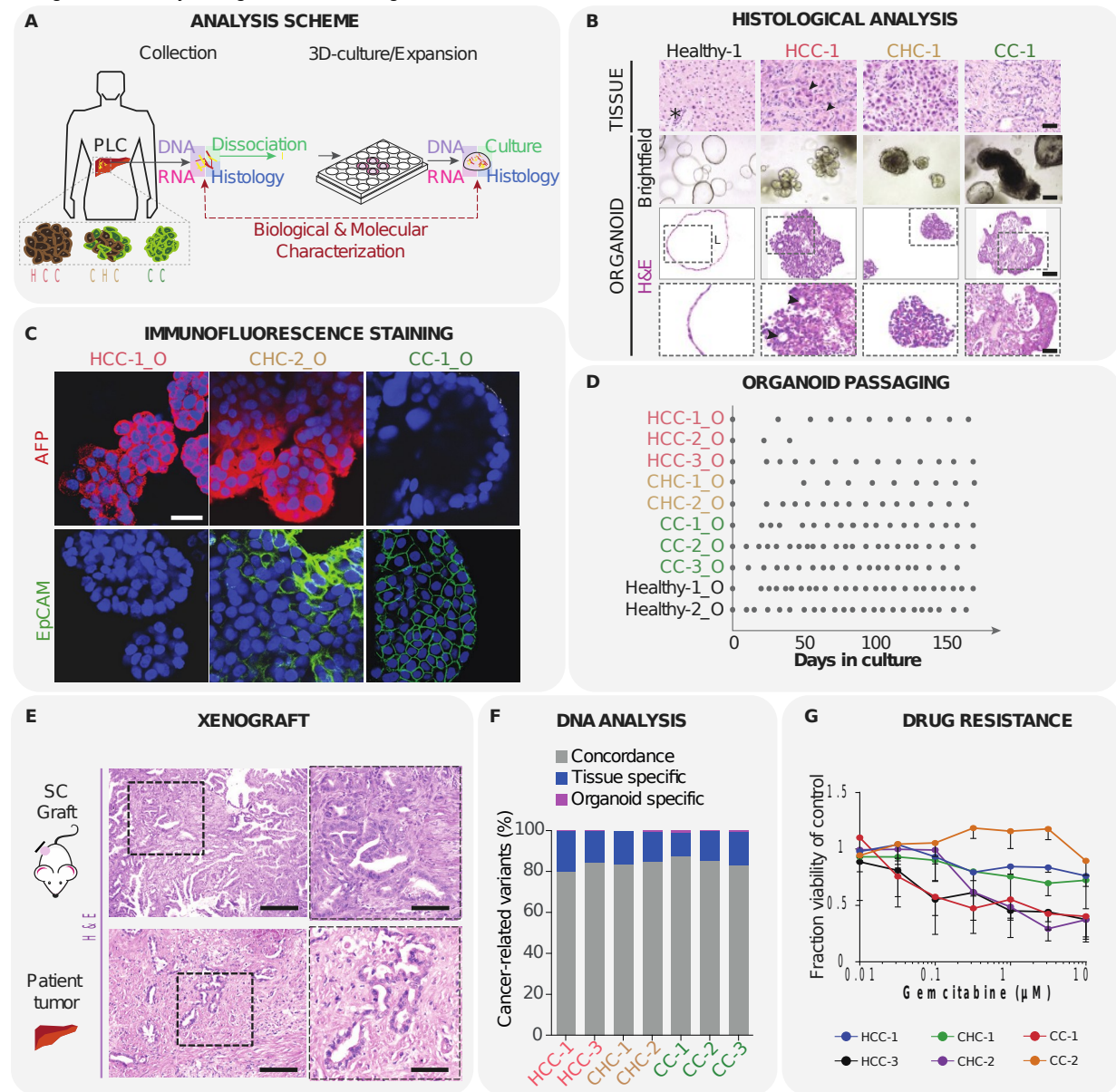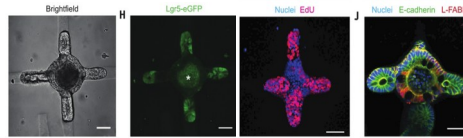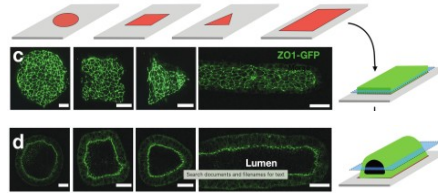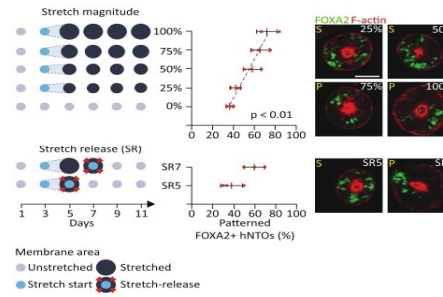

2211
2212
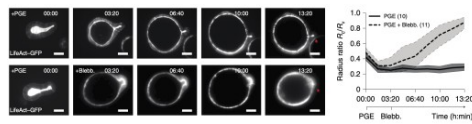## Fig. 5 Reducing the heterogeneity with complexity reduction

initial 2D condition

Micropattern

3D structure formation
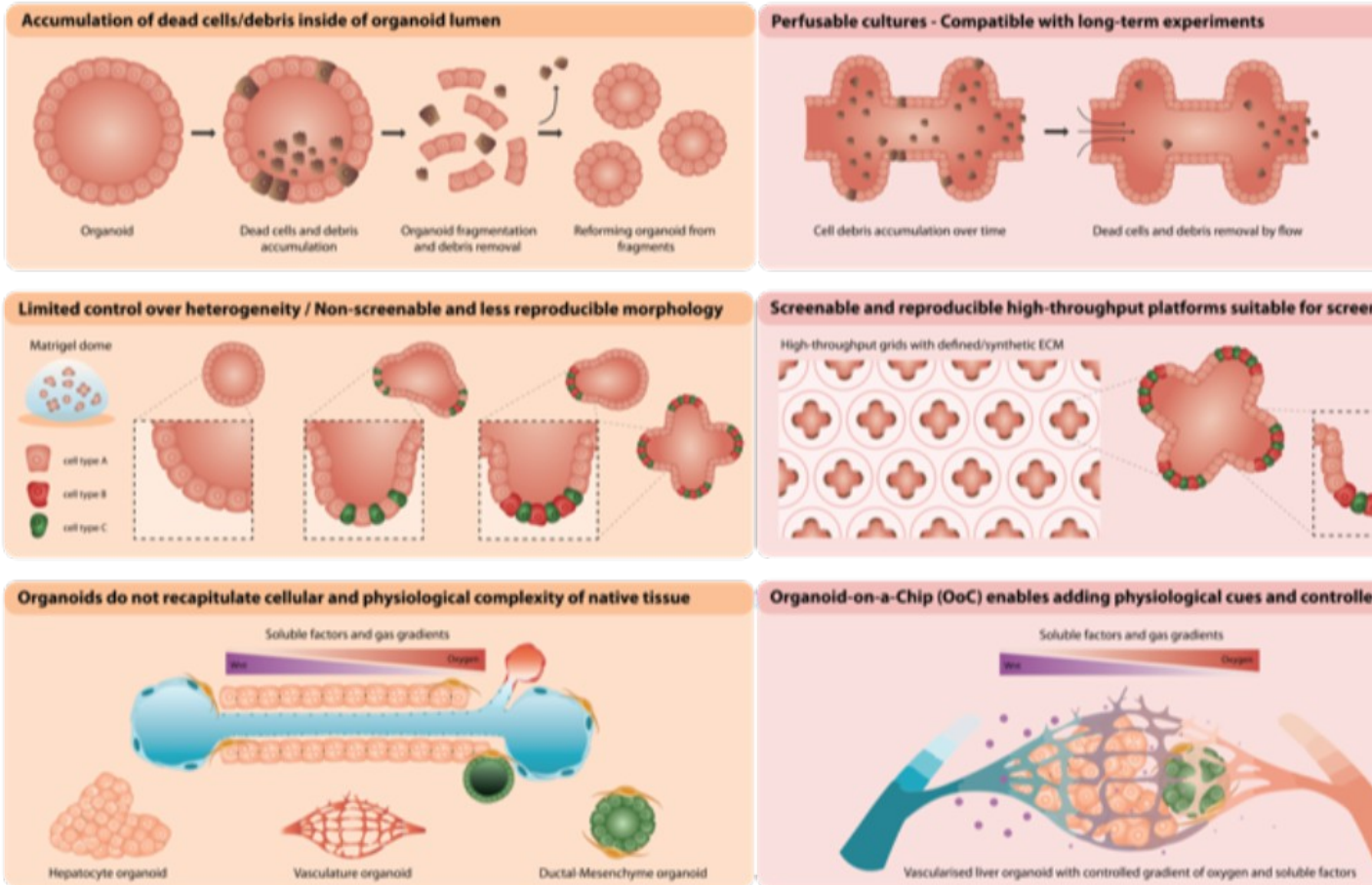
Stretching

tissue morphogenesis

Osmotic force

2214
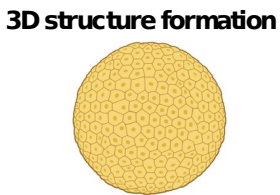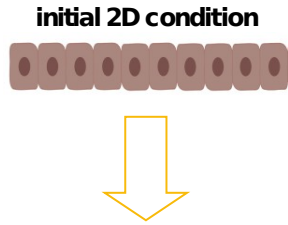
2215 **Fig. 6 Side-by-side comparison of the current limitations for**
2216 **organoid culture and approaches to overcome them**

Limitation and Optimisation

2218
2219 **Glossary terms:**
2220   1. Stem cell niche: A specific tissue microenvironment where stem cells
2221   both reside and receive stimuli that regulate cell fate
2222   2. Extracellular matrix (ECM): A large network composed of an array of
2223   (glycol)proteins and other macromolecules that provides structural and
2224   mechano-chemical support to cells and tissues.

3. Induced pluripotent stem cell (iPSC): Immature cells that are generated from an adult (mature) cell and that have regained the capacity to differentiate into any type of cell in the body
4. Tissue-derived cells (TDC): Adult or fetal cells derived from tissues, either stem/progenitor cells or differentiated cells
5. Organoid: A self-organized three-dimensional tissue that is typically derived from stem cells (pluripotent, fetal, or adult), or even differentiated cells and which mimics the key functional, structural, and biological complexity of an organ
6. Perfusion culture: A perfusion cell culture process involves the constant feeding of fresh media and removal of spent media and product
7. Organ-on-a-chip (also known as micro-physiological systems) (OoC/MPS): Systems containing engineered or natural miniature tissue constracts grown inside microfluidic chip
8. Passage (also known as splitting): It represents the subculture of organoids by either creating smaller organoid fragments or single cells using mechanical dissociation or enzymatic digestion
9. Engraftment rate: Degree of organoid retention in a host tissue after transplantation
10. Assembloids: Complex 3-dimensional structures combining several separately pre-generated cellular compartments/entities
11. Functional module: A structured design part of a system with inputs, processing, and outputs.
12. Functional precision medicine: A strategy whereby live tumor cells from individuals affected from a specific disease are directly perturbed with drugs to provide immediately translatable, personalized information to guide therapy
13. Copy number variations: A phenomenon in which sections of the genome are repeated and the number of repeats in the genome varies between individuals