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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

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

Total (9251/9000)

[H1] Introduction (867/800 words)

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

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

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

as how cells interact with their cellular and physical microenvirontment, i.e., the stem cell niche. Based on these insights, bioengineering strategies could be developed to precisely control stem cell decisions during organoid development. For example, from early embryogenesis studies, it is known that morphogen gradients regulate tissue patterning and development[17,18]. Microfluidics systems can be used to create the required concentration gradients of these by diffusing morphogens, giving rise to the desired cell types with spatial patterning[17]. Beyond biochemical cues, it is now increasingly appreciated that stem cells also experience active and passive forces from their external microenvironment and convert these physical stimuli into biochemical responses[19]. These physical cues could arise from the matrix, external forces, and/or cell-cell interactions. Rather than relying on natural or biologically-derived ECM such as Matrigel with limited stiffness tunability, synthetic hydrogels or other ECM combinations can be leveraged to control the physical properties of the matrix. Liquid friction against the cell membrane can also exert shear stress on cells[20]. The dynamic biofluidic environment has diverse effects on different cell types depending on magnitude, direction, and frequency[20]. Hence, microfluidic systems and bioreactors can be applied to provide perfusion at both the micro-scale and macro-scales[21-23]. Lastly, it is now known that cells interact with their neighbors and respond to external stimuli in a collective manner[24]. Topographical cues, such as curvature and shape of neighboring cells, can affect stem cell decisions[25]. A recent neural tube model dissected the folding process and demonstrated that geometry constraints by micropatterning can control the final morphology of neural tube-like structures[26].

It is controversial whether engineered cell-based *in vitro* models such as organoids need to faithfully recapitulate bulk of the structures and functions of the *in vivo* organ-of-origin. One trend is to recapitulate as much *in vivo* tissue architecture and functions as possible *in vitro* in order to demonstrate the physiological relevance of the models of increasing complexity. For engineers, the artificially created *in vitro* models only need to recapitulate specific features of the *in vivo* tissue, relevant to the physiological or diseased functions of interest (FOI). There is an optimism to create highly complex models and expect them to accurately mimic the *in vivo* organ-of -origin. For majority users, simpler ones are more robust for mechanistic studies and applications[27-29].

In this Primer, we focus on the rationale underlying the establishment of organoid cultures and provide guiding principles for the selection of suitable materials and methods for different applications. We first discuss the experimentation considerations for setting up organoid-based cultures, categorized into four major components that make up organoid cultures – cells, soluble factors, matrix, and physical cues and discuss approaches to integrate these components (**FIG. 1**). We also discuss key considerations for

generating more complex yet 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 in different applications, and key priorities in organoid engineering for the coming years.

[H1] Experimentation (2575/2000 words)

[H2] Cell source

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

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

extended durations in the enzymatic mix to dissociate the majority of the tissue-resident stem cells. Tissue dissociation can also be achieved mechanically; although mechanical dissociation is much faster and less expensive, cell yield and viability can be inconsistent[47]. Mechanical and enzymatic dissociation can be combnined to generate better cell yield. After tissue dissociation, TDCs for organoid development are identified and collected using known biomarkers or physical characteristics[46]. Tissue-specific stem cell markers are typically used to identify and isolate the desired stem cells to generate organoids[1,2]. Fluorescence-activated or magnetic-activated cell sorting (FACS or MACS) isolates cells based on multiple parameters, including size, shape, and cell-surface marker expression[46,47]. Other isolation techniques include laser capture microdissection and manual cell picking[46].

iPSC can be maintained and expanded as undifferentiated clonal populations over many generations. Undifferentiated human iPSCs are typically maintained on feeder cells or defined ECM substrates. Since single iPSC does not survive well *in vitro*, iPSC are typically harvested as cell aggregates which preserve cell-cell contact, yielding cell populations with higher viability. Physical scraping can also compensate for the lack of uniformity of cell aggregates. The dissociation enzymatic mixture should be chosen based on level of cell sensitivity[45] and whether the cultured cells secrete excessive ECM, making it difficult to detach the cells from the cell culture plate (**FIG. 2B**).

Tumor tissue, derived from either biopsies or surgical resections, is also typically processed akin to normal tissue to isolate tumour cells to grow as organoids[16,49-51]. Tumor cells isolated from liquid samples such as peripheral blood[52], ascites[50,53], and pleural effusions[54] can also be used as starting material to generate organoids. Patient-derived tumor organoids can be generated from samples obtained from minimally invasive Pap brush material[55,56] . Due to their low numbers, tumor cells from biopsies or liquid samples can be firstly expanded in animal models as xenografts in order to obtain sufficient cells for organoid generation[57]. In the case of tumor tissue, it can be preferable to limit tissue dissociation so that cell clusters rather than single cells are isolated. A critical factor that can influence the generation of tumour-derived organoids is the fact that isolated cells from tissue typically contain both cancer and normal cells. While for some tumors it may be possible to enrich for tumor-forming cells by sorting a priori[58], for the majority there is currently no robust method to separate normal and tumor cell populations prior to seeding into a matrix for culture. An approach to overcome this issue is to take advantage of culture conditions by utilizing selective media that omits certain factors required for growth of normal organoids, as tumor cells gradually lose dependence on those factors during malignant transformation[59]. Blood contamination, particularly erythrocytes,

can also affect organoid generation and matrix stability therefore standard approaches to eliminate these through lysis are typically used[60].

[H2] Matrix

Following cell isolation, cells are typically seeded into biologically-derived matrices such as Matrigel[7,61] or natural ECM such as collagen[62], or into synthetic hydrogels[3,63,64]. Matrigel is mainly comprised of laminin, collagen IV, entactin, perlecan and growth factors, and is similar in composition to the basement membrane[62]. As a continuation from the above example using intestinal organoids, we will now briefly describe how cells are encapsulated into matrices. Isolated intestinal crypts are first re-suspended into cold Matrigel and pipetted into a pre-warmed low-attachment well-plates for culture. The resulting cell-matrix construct is typically a flat semi-sphere gel. The complete composition of intestinal organoid culture medium have been previously reported[30]. To passage organoids, the gel is first broken up mechanically by pipetting culture medium to release the encapsulated organoids, dissolved as much as possible using cold medium, PBS or special dissolving solutions and then organoids are mechanically dissociated and pieces are re-encapsulated in Matrigel for culture again. To measure the proliferation rate of the cultured organoids, intestinal organoids are first released from Matrigel using enzymatic digestion. The retrieved cell pellet is then repeatedly washed to remove single cells, and crypts are counted. The expansion rate is calculated as the number of organoids from each well divided by the number initially crypts seeded in Matrigel in that well.

While Matrigel can support organoid culture, the inherently heterogeneous and poorly defined composition of this biologically-derived matrix offers little control over the biochemical and biophysical spatiotemporal cues that are necessary for improving organoid culture. Therefore, other matrices with defined compositions[64] have been explored as alternative matrices to Matrigel, such as recombinant human collagen[62], fibrin[65], or synthetic hydrogels[3,63]. Natural matrices can be recombinantly produced from proteins or polysaccharides to address the batch-to-batch variability of Matrigel[61]. On the other hand, synthetic hydrogels have emerged as powerful tools that enable independent manipulation of biochemical and biophysical matrix properties to control organoid features and enhance functionality. The ideal organoid matrix should overall be stress-relaxing and highly dynamic in biochemical and biophysical properties to accommodate or control changes in organoid structure during culture. For example, dynamic hydrogels based on poly (ethylene glycol) (PEG) were recently shown to enable reproducible intestinal organoid formation and demonstrate how hydrogel properties could be tuned to control stemness and differentiation in cultured organoids[3]. The viscoelastic profile of hydrogels has also been shown to define the mechanical confinement of growing organoids[66]. In other examples, the activity of adult stem cells can be controlled using PEG hydrogels with photo-degradable moieties[67]; biomimetic polymers can be modified to incorporate

essential ECM signals to generate organoids with tailored features[3]. Tunable PEG hydrogels can promote intestinal crypt budding[68]; while dextran-based GMP-compatible hydrogels support expansion of cells for longer passages[69]. Lastly, microfabricated arrays were recently reported to enable the uniform production of crypt-villi-shaped epithelium[70]. Even the recent advancements using the synthetic matrix to grow organoids, organoid growth by the synthetic matrix is still less efficient than Matrigel-cultured organoids. There is an unmet demand to develop a better matrix.

Organoids can arise either from round colonies generated by single cells[7,35] or, from initial multicellular structures such as intestinal crypts[30,63], cell aggregates[24] or micropatterned cells[26]. The aim of the latter approach is to establish a cellular niche which involves other cells of the same or different type from the beginning. To form cell aggregates, the simplest method is to use an ultra-low attachment dish coated with hydrophilic hydrogel[71] to prevent cell attachment; subsequent centrifugation can promote aggregate formation, enhancing cell-cell contacts[72]. The size and compaction of cell aggregates can be tuned and controlled, such as through the use of microwell arrays[72,73]. Another well-established method to form cell aggregates is the hanging drop method, where aggregates form at the bottom of the drop. In a recent example, droplet microfluidics was used to aggregate murine cholangiocytes to form complex organoids with liver mesenchymal cells[74]. Droplet microfluidics can print one organoid per well and enable the rapid generation of intra-organoid heterogeneity[75]. Another example of microfluidics use in organoid research comes from a recent paper, where authors use droplet-based microfluidics to perform better scRNA-seq analysis of intestinal organoid cell identities during various developmental stages, revealing extensive population heterogeneity[76]. Microwell structures or microfabricated pillar arrays[70] have also been developed to enable enhanced uniformity in cell aggregation[72,73,77]. In one example, microfabricated patterns of Laminin-512 were shown to reproducibly support human pluripotent stem cells to form lumen structures in Matrigel and differentiate into human neural tube-like structures[26]. These examples, amongst others emerging in the field, illustrate how our knowledge of biomaterials and tissue engineering, can be extrapolated to provide precise control over organoid structure and function.

[H2] Soluble factors

Organoid cultures are fundamentally based on our accumulated knowledge of developmental biology[14], where soluble cues are presented to cells in a spatiotemporally controlled manner (Soluble factors used to differentiate TDC and iPSC into various tissue types are listed in **Supplementary Table 1**). Translated to organoid culture, these soluble cues are recapitulated *in vitro* in the form of biologics, mainly as proteins such as growth factors[78], or small-molecule drugs, which can activate or inhibit signaling pathways. While growth factors could be costly and unstable, and many small-molecule drugs

can affect multiple pathways resulting in poor reproducibility[78], some organoid protocols have combined the use of both biologics and small-molecule drugs[79]. The use of conditioned medium from engineered cell lines producing biologically active growth factors, e.g., L Wnt-3A, can replace commonly used growth factors, such as Wnt3a ligands[80]. These conditioned media face a similar issue of batch-to-batch variation and require stringent tests to ensure reproducibility[81]. Thus, novel sorrugate molecules are starting to arise as potential substitutes to condition medium[80,81].

It is critical to consider how and when soluble cues are are added to organoid cultures because soluble cues *in vivo* are typically presented to cells by the ECM or nearby cells, coordinated in time and space. This is the concept of spatiotemporal presentation. For example, it is now known that fibroblast growth factor (FGF) activity and specificity can be regulated by cell surface heparan sulfate proteoglycans, suggesting how the addition of free FGF into cell culture medium may not recapitulate the way FGF is presented *in vivo*[82]. The importance of presenting soluble cues in a spatiotemporally relevant manner is especially important for growing human iPSC into complex structures with multiple cell lineages such as in the case of kidney organoids[83]. Based on previous studies[84], it is known that the ureteric epithelium develops from early migrating presomitic mesoderm cells. To recapitulate this process, the effect of different durations of initial Wnt signaling before the addition of FGF was investigated[83]. The spatiotemporal presentation of soluble factors can be achieved using different tissue engineering approaches. In one strategy, these growth factors can be encapsulated within nanoparticles, and conjugated onto cell surfaces for controlled release[85-88]. To mimic how certain growth factors are bound to the ECM *in vivo*, researchers conjugated polymers with heparin which can bind to growth factors, or conjugated these growth factors to the polymer itself[89]. Surface tethering can also be achieved with nanotechnologies, such as nano-imprint lithography, electron-beam, and electrospinning, can fabricate substrates with nanopillars, nanopits, or nanochannels, mimicking the basement membrane for 3D organoid culture[90]. Lastly, microfluidic systems can be leveraged to create miniaturized niches with precise control over mechano-chemical properties[91]. With directed flow and gradients of gas or small molecules, these systems can finely control environmental parameters within organoids[18]. In one example, a microfluidic neural tube device was developed to present simultaneous opposing gradients of growth factors to direct neural tube patterning, enabling recapitulation of the *in vivo* structure[92].

[H2] Physical cues

Beside biochemical cues, it is also important to consider when and whether it is necessary to provide appropriate physical cues to cultured organoids. Nutrient supply and waste removal, which are diffusion-dependent, become less efficient during organoid growth into larger tissue structures. This is the

reason why intestinal organoids need to be fragmented into smaller cell clusters and reseeded regularly[7]. Inadequate nutrient and waste diffusion is also problematic in brain organoid culture, where the resulting millimeter-sized constructs often exhibit necrosis within the inner core due to nutrient inaccessibility. This problem can be partially resolved using shaking cultures[23 93], spinning bioreactors or suspension under continuous agitation[23], or in continuously stirred bioreactors[93]. These bioreactors can monitor pH, temperature, oxygen, and glucose levels to maximize mass transfer while minimizing shear stress. In this regard, perfusable microfluidic chips have also been developed to promote the long-term culture of organoids[21,22,94]. Lastly, it may be useful to consider providing topographical cues to control organoid culture *in vitro*; the topography of the substrate is known to modulate cell area, shape, and cell-cell interactions, resulting in biochemical signals that can affect stem cell fate[25]. Topography-directed morphogenesis has been demonstrated using intestinal organoids grown on soft hydrogels[95].

[H2] Integrating cues

In contrast to the original self-organizing organoid model, the above described cues can be integrated to confer greater control over organoid morphogenesis. Integration of cues is a commonly employed strategy in the field of tissue engineering to construct tissues *in vitro* and *in vivo*[96]. Ideally, specific physical or chemical cues should be presented in a spatiotemporally, physiologically relevant manner using simple, reproducible and robust methods (**Supplementary Table 2**). We will illustrate this using the example on intestinal organoids[7]. Following the identification and isolation of *LGR+* intestinal stem cells residing in crypts, biologically-derived Matrigel was leveraged to mimic the laminin-enriched crypt environment, supplemented with exogeneously added growth factors in the cell culture medium. Most intestinal stem cells could survive, proliferate, and form organoids[7]. However, given the stochastic nature of organoid development, the resulting organoids vary in size, bud numbers and function. In one example of how environmental cues (mechanical and biochemical) were integrated to exert control over organoid morphogenesis, the organoid maturation process was dissected into different stages, and biomaterials engineering was used to identify the optimal mechano-chemical environment at each stage[3]. It was shown that mechanically dynamic matrices that could switch from high to low stiffness over time, enabled control over the morphogenesis process[3]. In another example, human neural tube morphogenesis[26] was simulated using micropatterning which created mechano-chemical gradients to regulate cell-cell/matrix interactions, and soluble factor presentation to orchestrate morphogenesis.

Besides these examples, other engineering methods have been used in organoid culture to control cell proliferation, differentiation and morphogenesis. A popular bioengineering approaches to reconstruct tissues 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-

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

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

[H2] Validation of organoid functionality

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

[H3] Pancreatic islet organoids

We use the example of mouse pancreatic islet organoids to discuss various characterization and validation methods (**FIG. 3**). The formation of four endocrine differentiated cell types (β-cell, α-cell, δ-cell, PP-cell) is examined by the expressions of the corresponding hormones, Insulin (Ins), Glucagon (Gcg), Somatostatin (Sst), or Pancreatic polypeptide (Ppy), by immunostaining[113,114]. To assess the extent of β-cell maturation, the ultrastructural morphometric analysis of insulin secretory granules is performed by immune-gold transmission electron microscopy (TEM) image[110]. To evaluate the ability of the β-cells in the organoids' responsiveness to glucose, one performs Glucose-stimulated insulin secretion (GSIS) experiments, for example insulin or C-peptide ELISA assay with cyclic high and low glucose incubation[115]. As the insulin release is associated with the Ca^{2+} dynamics, the calcium signaling traces imaging rapidly increased in response to glucose and returned to baseline[114,116]. To test the full potential of the islet organoids, the *in vivo* functional evaluation is to ameliorate the hyperglycemic phenotypes of streptozotocin (STZ, a toxic to the insulin-producing β cells of the pancreas in mammals)-induced type 1 diabetic mouse model upon organoid transplantation[110,114,117]. The characterization parameters include normalized and stable blood glucose level, normal plasma insulin level and maintained body weight.

[H3] Intestinal organoids

Intestinal organoids are among the first organoid types that were successfully established *in vitro*[7,30]. The formation of classical crypt-villus-like architecture is regarded as an important symbol of the successful organoid establishment, exhibiting the proper branching morphology with robust multicellular composition and location reflecting the organoids differentiation and maturation level[118,119]. The maintenance of intestinal stem cells (ISCs) can be analyzed by the expression of Lgr5 maker by real-time qPCR or by imaging using a Lgr5- fluorescent reporter[7,120]. The differentiated cells are assessed by the staining of their markers, including Lysozyme (Paneth cells), Villin (enterocytes), Mucin 2 (goblet cells), and Chromogranin A (enteroendocrine cells)[7,100,121]. These various cell types can also be distinguished by TEM according to their characteristic subcellular structure[7,121]. Furtherly, functional intestinal organoids exhibit a relatively thick mucus layer due to mucus secretion from mature goblet cells[122], which can be detected by Alcian blue (AB) and periodic acid-Schiff (PAS) staining[123,124].

[H3] Liver organoids

In the case of the liver, both cholangiocyte (ductal cell of the bile duct)[35,36] 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

organoids established from microsatellite instable tumors had higher *de novo* mutations after long-term culture than stable ones[59]. Some of the differences observed may be attributable to how different clones and subclones adapt and expand in culture. Using deep targeted sequencing of 500 cancer-associated genes, truncal mutations were shown to be retained while subclonal ones are gained or lost, with mutational changes at each passage[127]. Most mutations and CNVs are are preserved in the late passage of lung cancer organoids, while *de novo* mutations can be attributed to the sub-clonal expansion of small subsets of cells present in the tumor of origin[15]. Overall, these studies showed that genetic drift can occur in organoids cultured for extended time, influencing their reliability as models for functional and drug discovery studies[133]. This reiterates the importance of validation in the context of tumor organoid establishment, characterization and testing. As an example of analysis, crucial steps in characterisation of liver cancer organoids are demonstrated (**FIG. 4**). Lastly, normal tissue contamination is an issue for establishing tumor organoids and should be addressed when validating patient-derived models. Presence of normal cells can be estimated by histological comparison and IHC as well as sequencing[55,134].

[H2] Data analysis

[H3] Image processing and analyses

ImageJ / Fiji[135], CellProfiler[136] and other software are used for image processing. The number of organoids formed in a certain period, organoid size (area or volume) and cell number (nuclei number) could measure the cell proliferation rate. Cell proliferation could be quantified by the ratio of EdU or Ki67 positive nuclei using the plugins in the software. Morphological evaluation, for example, the bud formation ratio in the intestine organoid system, is also necessary when quantifying the success rate considering functional maturation. Mean/normalized fluorescence intensity of maturation markers (either by live imaging with knock-in reporters or immunofluorescence staining at the endpoint of the experiment) for different tissue types could be recorded to measure their maturation level. As for lineage tracing purposes, long-term live imaging could be analyzed using software to track the cell behavior. Web resources could be used for pathway analysis and visualization of multi-omics data to generate heatmaps for understanding the differentiation stage of organoid[137,138]. Other software, like e.g.: Profiler, GSEA, Cytoscape and EnrichmentMap, have been used for biomolecular interaction networks, pathway enrichment analysis and visualization[139-142].

[H3] Machine learning and Artificial Intelligence data analytics classification of phenotypes

Miniaturization and automation could make it more consistent for high throughput and multidimensional phenotyping of organoids; machine learning and artificial Intelligence-based data analytics showed their

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

[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

breast cancer patient. Progression-free survival was 3.5 times longer on the eribulin regimen than the previous therapy the patient received[171]. This case study is indicative of the potential of organoids to impact clinical care.

[H2] Source material to establish xenografts

An application of organoids that is becoming more popular involves using them as starting seeding material to generate xenografts with high take rates. These involve using intact organoids[15,59], or individual cells after digestion[55,172]. Matano and colleagues seeded human intestinal organoids transformed by CRISPR-Cas9 to carry mutations in APC, SMAD4, TP53, KRAS, and/or PIK3CA in subcapsular kidney space of immunocompromised mice. They found that benign organoids carrying mutations in 1-2 genes could not form tumors while more malignant ones with mutations in 4-5 genes were tumorigenic[173].

Patient-derived organoids can also generate xenografts, confirm their tumorigenic capacity and recapitulate the histology of parental tumors[15,55]. In a study of colorectal cancer, benign tumor organoids showed no or minimal engraftment in mice. In contrast, colorectal cancer organoids derived from metastases were more invasive than those from primary tumors[59]. Hubert and colleagues directly compared the morphology of xenografts formed by glioblastoma multiforme cells cultured *in vitro* either as tumor spheres or organoids. They showed that while cells from tumorspheres displayed a solid growth pattern, organoids were more diffusive, similar to the tumor of origin[172]. Lee and colleagues showed how organoid lines derived from bladder tumors of lumenal origin transplanted in mice gave rise to tumors with the same lumenal phenotype[127].

[H2] Infectious diseases

Organoid models are now widely used in research on host-pathogen interactions. For instance, organoids derived from tissues and organs such as intestine[174,175], livers[176], lungs[175,177], oral mucosa[178], stomach[33,179], have been cocultured with pathogens such as bacteria[33,179,180], viruses[174,176-178,181] and parasites[175]. In human intestinal organoids, differentiated intestinal cells are more susceptible to human rotavirus (HRV) infection than undifferentiated ones. Organoids infected with HRV or rotavirus enterotoxin showed lumenal swelling, a characteristic of rotavirus-induced diarrhea[181]. Human liver organoid was used as a drug screening platform to test anti-HRV drugs and antibodies, identifying mycophenolic acid, IFN-γ and anti-rotavirus-VP7 antibodies capable of blocking rotavirus replication *ex vivo*[176]. Organoids can also be used in studies of oncogenic pathogens. Infection of murine gastric organoids by H. pylori showed increased cytosolic β-catenin levels induced by bacterially originated CagA, which led to increased proliferation in infected organoids[179].

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

complete organoid structures containing different cellular populations (reviewed in[209,210]), such as the recently published endothelial and healthy human colon and human colorectal cancer organoids[211], murine cholangiocyte and mesenchymal organoids[74], and murine pancreatic islet and endothelial organoids[110], will require controlling sources of variation derived from incorporating additional cells to an already heterogeneous system. Exerting control over the variation while generating more functionally relevant structures represents a significant challenge for the field.

Finally, significant heterogeneity is also observed following organoid treatments. While this represents a drawback when trying to use organoids as a tool for investigating molecular mechanism, it is beneficial when aiming to model patient responses. In particular, for cancer treatment, several different patient-derived organoid models tested, including but not limited to: pancreas, colorectal and liver cancer all exhibit heterogeneous responses to known chemotherapeutic and anticancer agents[49,132,212]. This parallels the variability in patient outcomes observed in the clinic and have positioned cancer organoids as potential predictive tool for drug testing (Extensively reviewed in[213]). This heterogeneity in treatment responses is now being exploited for the treatment of cystic fibrosis patients[191], with a large ongoing European multicentre clinical trial study, led by the HIT-Cystic Fibrosis consortium, to identify potential responders[214]. Similar initiatives are lagging behind for cancer patients, though mostly because of disparities in establishment rates and lengthy timelines (extensively reviewed in[215]). In that regard, while this manuscript was under revision, the group of Markus Heim reported the use of organoids to inform the treatment of a patient suffering from a rare form of liver cancer. Unfortunately, the treatment was discontinued due to deterioration of the patient’s general condition, preventing the authors to draw conclusions regarding the predictive value of the organoid system for the drug treatment of that patient[216].

[H2] Minimum reporting standards

To improve the reproducibility and reliability of the results, it is essential to account for the variability between different cell isolations and intra-organoid heterogeneity. While one could consider that at least 3 independent experiments with at least 3 different biological replicates are a minimum requirement, they might not always be sufficient. In fact, the number of organoids to study per biological replicate as well as the number of biological replicates will very much depend on the question asked, the specific experiment and the variability of the phenotype observed. Whenever possible, to calculate the number of independent organoids per biological replicate, sample size calculations similar to the ones used for mouse experiments, that take into account statistical power and variance of the experiment, would be highly recommended. The data should be robust, meaning repeated in at least three independent biological replicates, which

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

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

[H2] Data Deposition

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

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

A consensus in organoid nomenclature is also missing. Aside from a couple of notable examples, where leaders in the intestine, liver and pancreas addressed the nomenclature of gut, hepatic, pancreatic and biliary organoids[229,230], the field is waiting for a more clearly-defined nomenclature system. Similarly, there is no repository where to submit organoid data. For transcriptomics-related data, such as gene expression, non-coding RNA, ChIP, genome methylation, high-throughput RT-PCR, SNP arrays, SAGE, protein arrays, Gene Expression Omnibus (GEO) is generally recommended[231,232]. For code used to analyze organoid datasets, GitHub is recommended (https://github.com). We envision that data and organoid depositories will arise shortly to fill in the presently missing gap in data repositories for organoid work.

[H1] Limitations and optimisations (814/1,000 words)

The reproducibility, both morphological and functional, of the obtained 3D organoid systems, remains a major bottleneck. In this section, we will elaborate on the limitations and the recent developments in organoid research, providing a path towards a more optimal pipeline for developing, characterizing, and benchmarking organoid systems.

[H2] Limited level of maturity and function

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

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

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

[H2] Limited control of organoid heterogeneity

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

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

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

[H2] Optimizing ECM composition

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

control over composition and stiffness, and the other is to take decellularized tissue and create tissue-specific matrices[205,243]. There are significant efforts to identify chemically-defined, GMP-compatible ECMs that enable the growth and the long-term expansion of human organoids. In that regard, some advances were shown with human pancreas organoids, intestinal organoids and colorectal cancer organoids, which could grow in a dextran-based fully defined ECM; however, they would not expand long term[3,69].

[H2] Organoids meet organs-on-a-chip

It has been shown that by maximizing the mass transfer and minimizing the shear stress in the perfusive soluble microenvironment, cells grown in organ-on-a-chip set-ups upregulate their functions, getting a step closer to a native tissue[94,244,245]. A more recent example shows how the presence of fluid flow enhances kidney organoids' maturation and favors their vascularization *in vitro*[107]. Nikolaev et al. engineered physical constraints into the organoid environment and intestinal cells when provided with boundaries through engineered scaffolds self-organized in crypts of the same size. At the same time, they overcame the inaccessibility of cystic organoids and clearance of cell debris by creating a perfusable culture of mini-intestines where cells are arranged to form tube-shaped epithelia and similar spatial arrangement as the *in vivo* tissue[100].

[H1] Outlook (989/800 words)

Moving forward, the trend is to develop more complex models that recapitulate *in vivo* structure and function as faithfully as possible, in terms of the recapitulating cell types over time, tissue architecture, measurable molecular events and phenotypic functions. Rather than focusing exclusively on the most prominent markers or functional assays, architectural benchmarking of native tissue should also be performed. In the example of hepatocyte organoids[125], hepatocyte functions are preserved, yet the liver tissue architecture does not match the native tissue where hepatocytes are arranged in cords. Similarly, organoids like pancreatic or colon cancer organoids grow isotropically, forming a cyst instead of the tubular structure they would form in their native tissue. To derive more complex functions, organoids with multi-cellular and multi-tissue structures will be important, especially in the context of studying cell-cell interactions[246]. Along this vein, assembloids and organs-on-chips are also becoming increasingly complex and more broadly adopted.

On the other hand, the engineer’s (cell as a machine) approach[28,247] is to pursue simpler reductionist models defined by the minimal functional modules (MFMs) that drive a complex cellular or tissue FOI, to study the mechanobiological causation in development or repair, or to develop a robust system for high throughput screening. The basic premise is that a complex biological function is executed by coordinated operation of a limited number of functional modules, each described by a small set of molecules,

and chemical reactions driving physical attribute changes of mesoscale (sub-cellular or inter-cellular tissue/multi-cellular) structures associated with FOI in the specific spatiotemporal stage/phase/step. For example bile canaliculi in liver exhibit hourly cycles of expansion and contraction. To study the causative contraction events with high resolution, only regions of adjacent hepatocytes forming the bile canaliculi are directly studied in the context of the entire regulatory machinery of adjacent hepatocytes[27,248]. One can choose to create a much larger structure involving cholangiocytes than the one operated by the MFMs but the model will be noisy and costly. Each functional module is coupled to another and can be modeled together or independently at different length scale. Simple reductionist models have been useful for high resolution mechanistic understanding of tissue morphogenetic events such as defects[29,240,249].

Geometrically constraining the size of the initial 2D seeding pattern and 3D formation by micropatterning and supporting 3D cell growth using Matrigel, Karzburn et al., induced tissue-like neural tube morphogenesis and produced highly reproducible neural tubes. They identified the mechanisms of neural tube folding and modeled neural tube defects[26]. They focused on neural tube formation and characterizing a selected subset of relevant molecular events. In another example, symmetry breaking in a uniform sphere of cells and the emergence of a Paneth cell is a critical event in the early stage of intestinal organoid formation. The mechanism was unknown until Serra et al. showed that it is caused by transient activation of mechanotransducer YAP1, which induces NOTCH-DLL1 lateral inhibition events[204]. Gjorevski et al. translated this knowledge to control YAP1 activation by applying geometrical constraints in hydrogel scaffolds and producing a uniform and reproducible intestinal microtissues[3].

Organoids can be constrained by reducing the 3rd dimension in a 2.5D culture. 2.5D is 3D culture with restricted 3rd dimension. Typical examples are culturing cells on curved or patterned surfaces, flattened or constrained cellular construct[250], and overlaying ECM on a flat cell monolayer at high confluency which would pull the cells upward, forcing more cell-cell interactions to adopt 3D cell morphology. For example, hepatocytes in a collagen sandwich have sufficient contact area to attain polarity and form a bile canalicular lumen that contracts in the same periodic cycles as *in vivo*, although missing the 3D network, and lunes are wider and cholestatic compared to the native tissue. This cell-based model enables high-resolution dissection of the mechanism of bile canaliculi contraction into steps and an understanding of the molecular machinery regulating the phase transitions[27,251]. 2.5D culture reduces the depth-driven variabilities of a typical organoid: diffusional constraints in the hypoxic core, limited accessibility for drugs/transfection agents, and impeded imaging 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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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

2124

	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
	Immuno fluorescent 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
	Immuno fluorescent 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

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

Figure legends:

Fig. 1 Components to engineer organoids

The set up of organoid-based culture requires considerations about four major components that make up organoid cultures – cells, soluble factors, matrix, and physical cues and how to integrate these components

Fig. 2 Flowchart of the procedures

Organoids can be generated from TDC or iPSC.

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

control, such as stretching[241] and osmotic forces[242] can be applied to direct certain tissue morphogenesis.

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

Top panel: Accummulation of dead cells and cell debris inside of cystic organoid lumina (left) has been overcome by (right) designing a perfusable open-end structures that use inducible flow to wash out cell debris, which are compatible with long-term experiments.

Middle panel: Organoids grown in Matrigel domes display high-variability of cell heterogeneity and morphology (left) which can be overcome by utilising (right) grids with patterned synthetic ECM which provide cues for cell differentiation. These platforms are additionally compatible with high-throughput screenings.

Bottom panel: Single-cell type derived organoids do not recapitulate the cellular and physiological complexity of native tissue (left), but (right) combining organoids with organ-on-chip (OoC) as novel technology would enable creating controlled micro-envirorment, suitable for multiple cell types

Figure legends:

Fig. 1 Components to engineer organoids

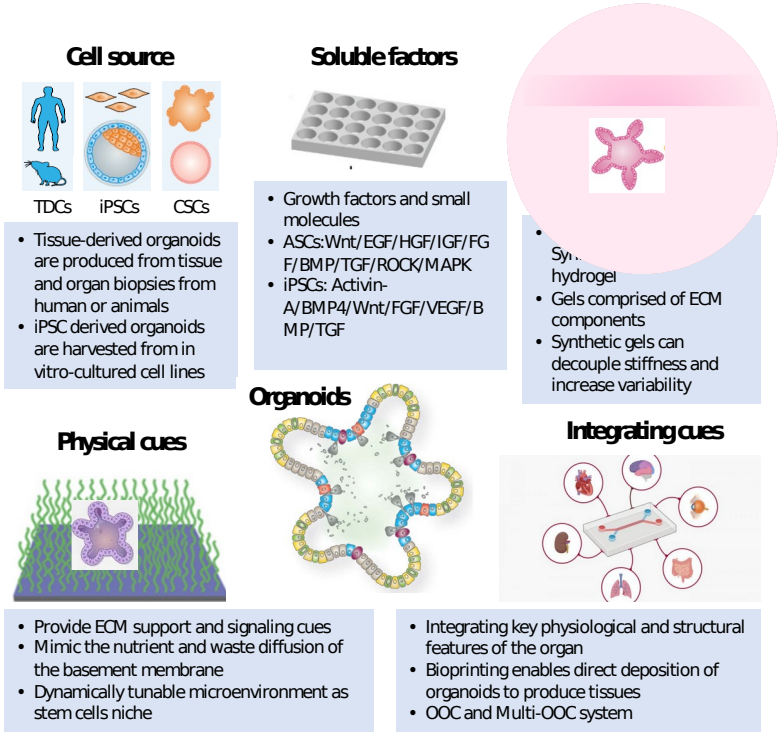
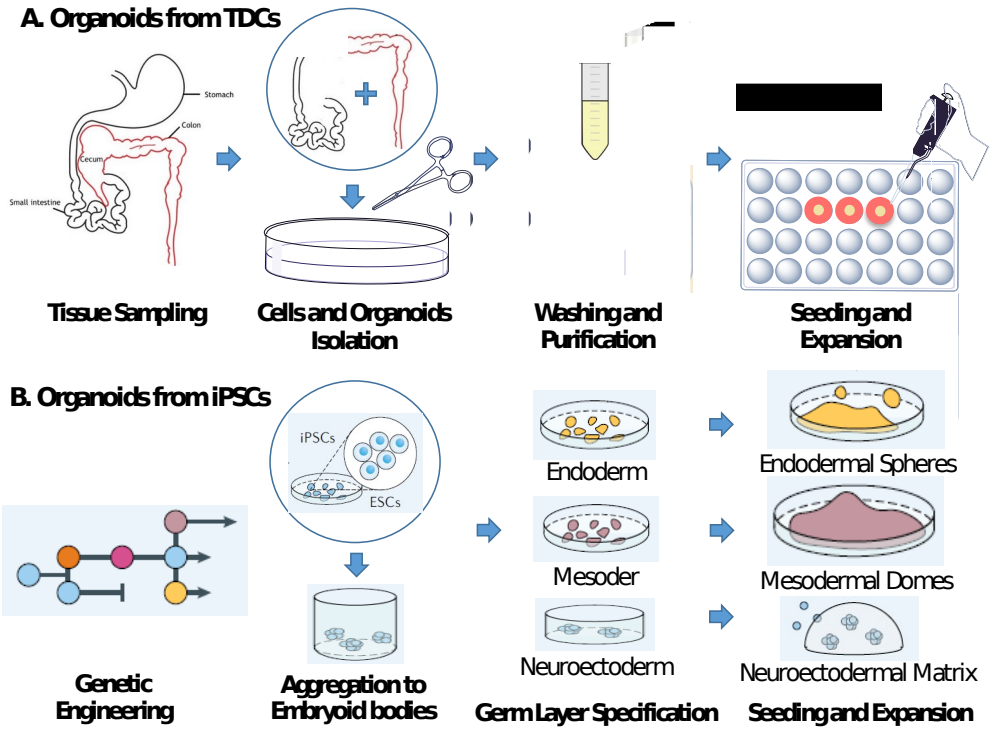


Fig. 2 Flowchart of the procedures



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2206 **Fig. 3 Representative results of pancreatic islet organoids validation**
2207 **analysis**

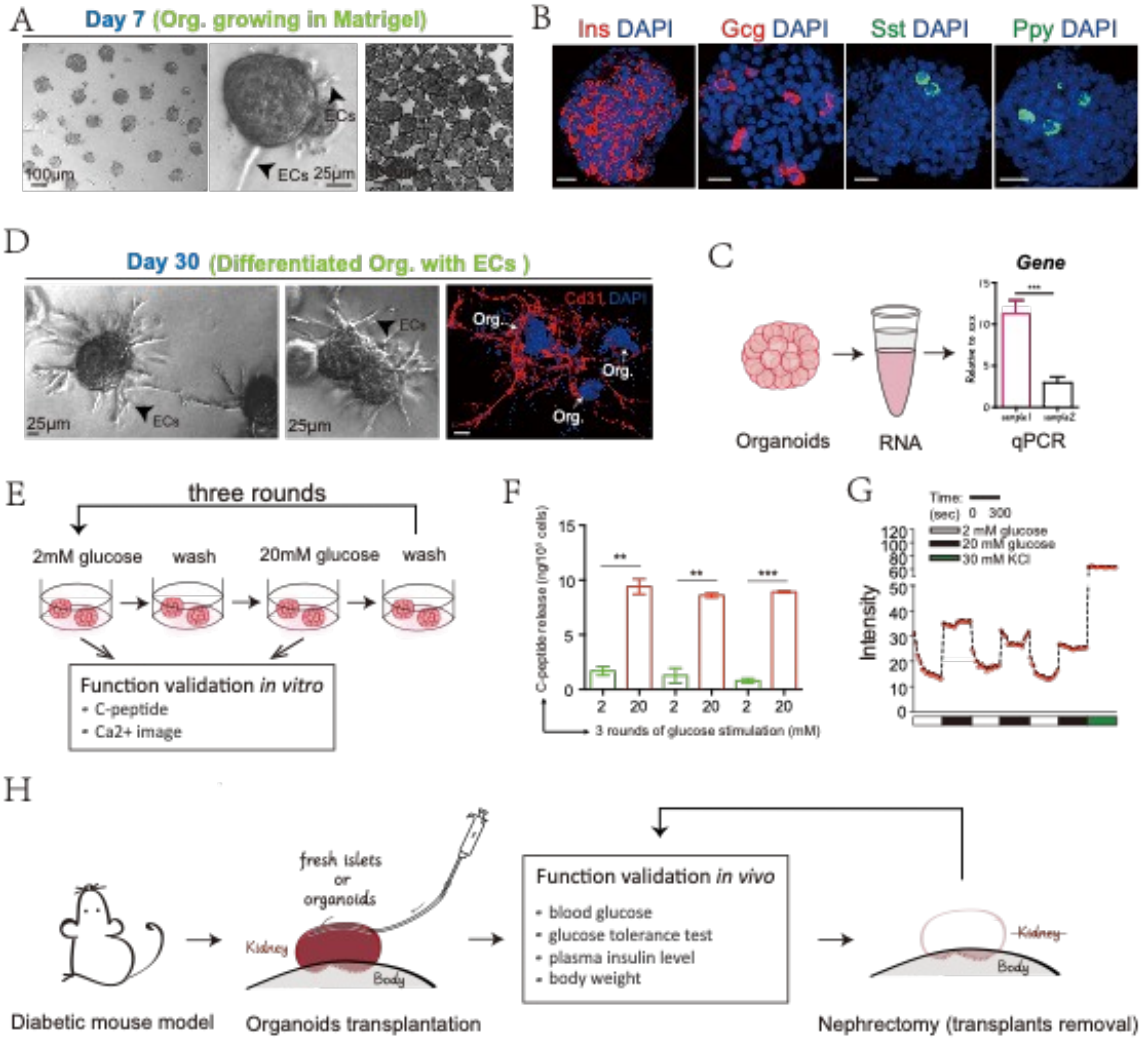

Figure 1. Representative results of pancreatic islet organoids validation analysis

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]
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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.
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G) Organoid sensitivity to drugs: IC50 curves for gemcitabine treatment.

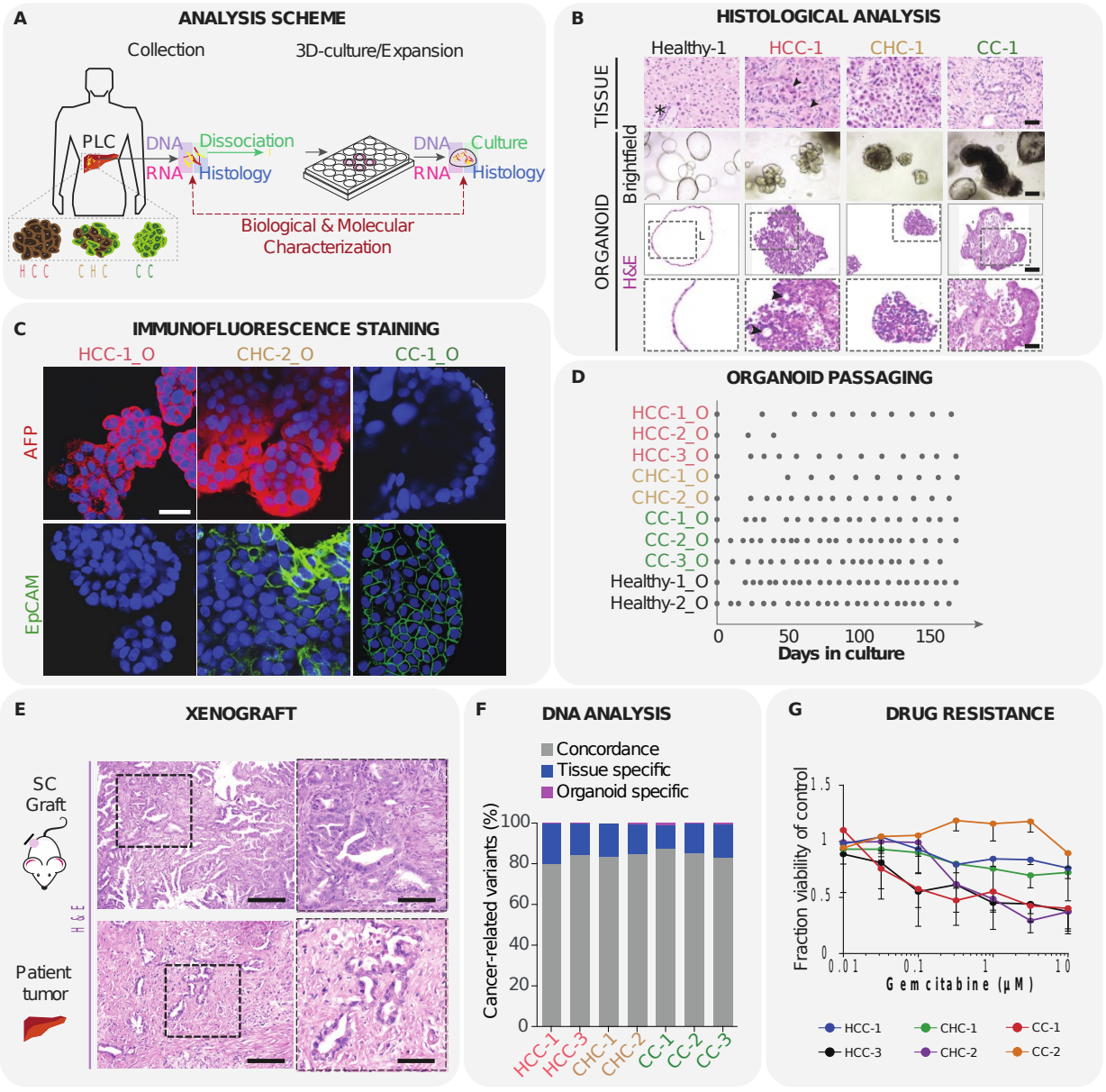


Fig. 5 Reducing the heterogeneity with complexity reduction

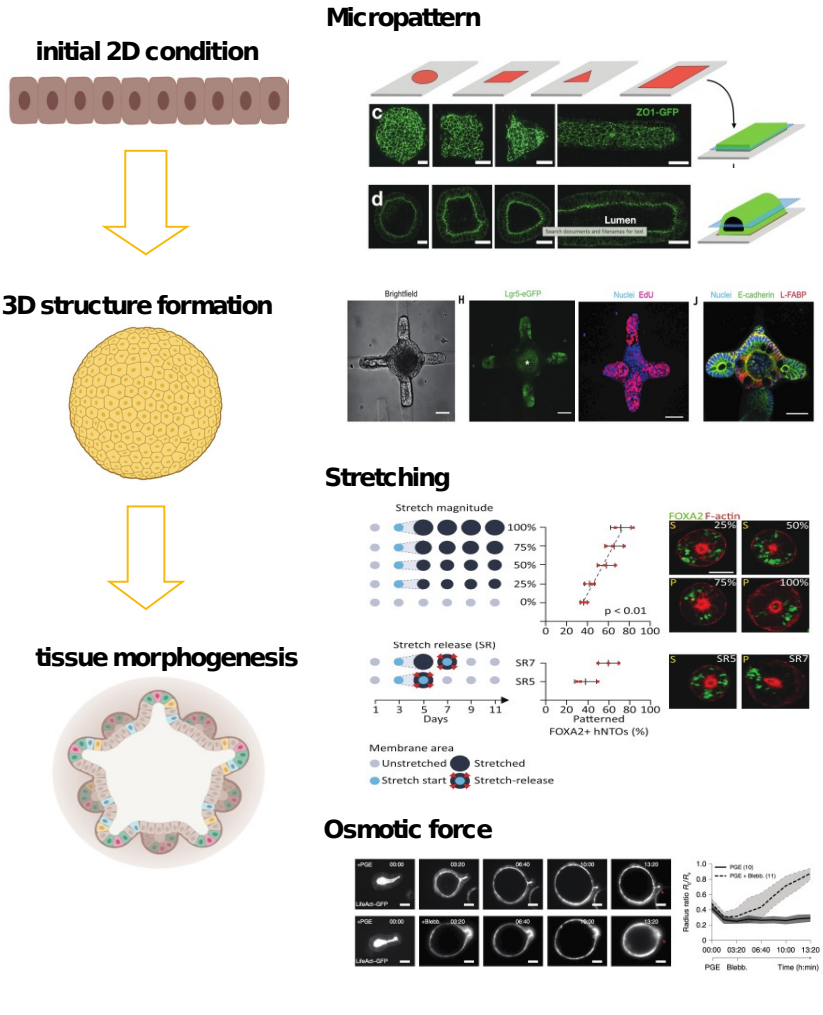
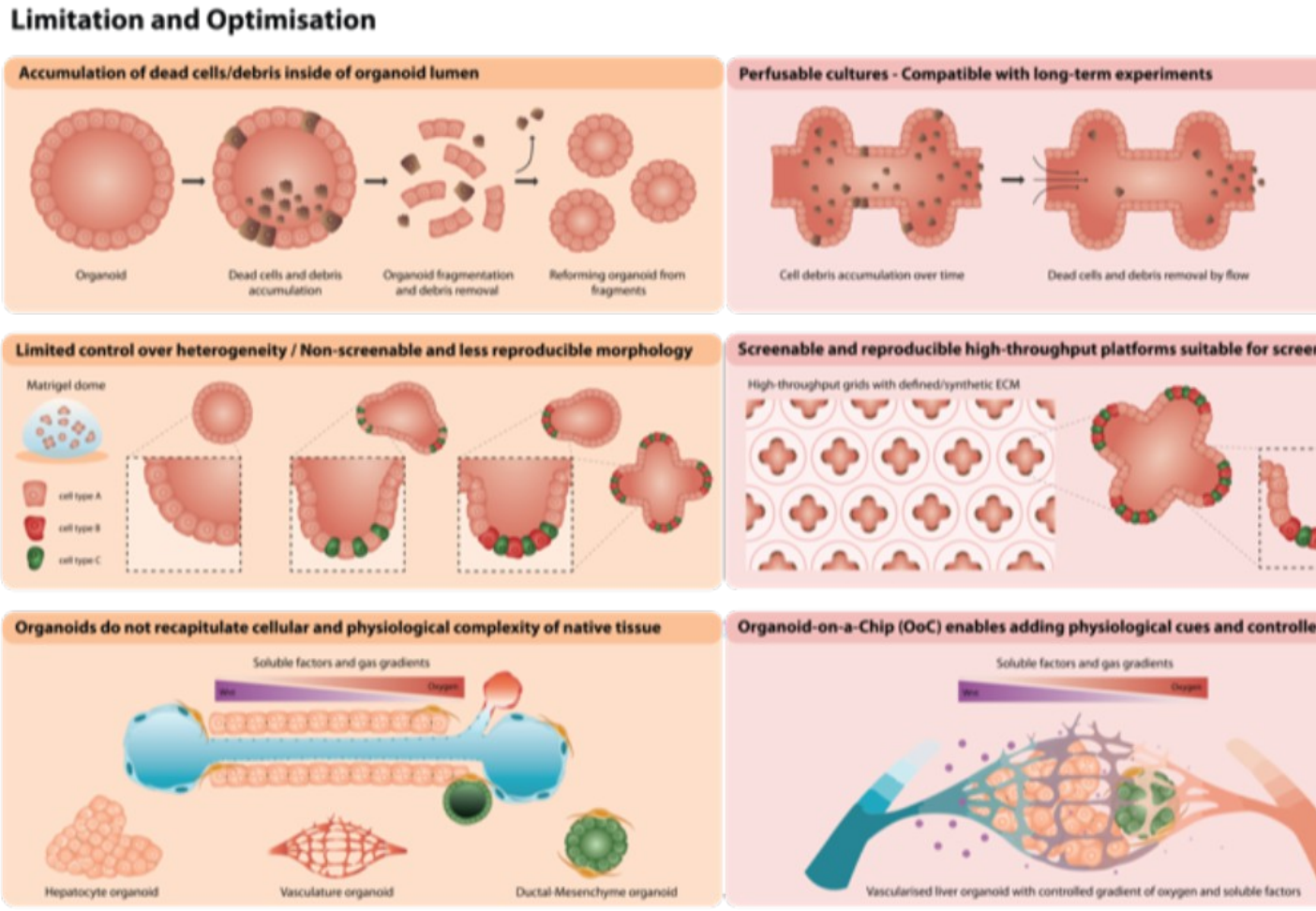

initial 2D condition
3D structure formation
tissue morphogenesis
Micropattern
ZO1-GFP
Lumen
Brightfield
Lgr5-eGFP
Nuclei EdU
Nuclei E-cadherin L-FABP
Stretching
Stretch magnitude
100%
75%
50%
25%
0%
p < 0.01
FOXA2 F-actin
Stretch release (SR)
SR7
SR5
Days
Patterned
FOXA2+ hNTOs (%)
Membrane area
Unstretched
Stretched
Stretch start
Stretch-release
Osmotic force
PGE
Bleb.
Time (h:min)

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



Glossary terms:

1. Stem cell niche: A specific tissue microenvironment where stem cells both reside and receive stimuli that regulate cell fate
2. Extracellular matrix (ECM): A large network composed of an array of (glycol)proteins and other macromolecules that provides structural and 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