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Primer

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Organoids

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Abstract

Organoids 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, and can also be used in diagnostics, disease modelling, 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 tumours. To date, numerous organoid engineering strategies that support organoid culture and growth, proliferation, differentiation and maturation have been reported. This Primer highlights the rationale underlying the selection and development of these materials and methods to control the cellular/tissue niche; and therefore, the 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 are also outlined. Lastly, we conclude by elaborating on the limitations of organoids in different applications, and the key priorities in organoid engineering for the coming years.

Sections

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Introduction

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 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 spatio-temporal 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 tumours and used to support in vitro cell culture[5]. Matrigel was later shown to allow breast epithelial cells to grow in three dimensions and form lumens with milk protein secretion[6], and adult intestinal stem cells embedded in Matrigel in the presence of a tissue-specific cocktail of growth factors were also shown to self-organize into 3D crypt–villus structures[7]. Organoid research intertwined with 3D cell culture, stem cell and tissue engineering for over a century, with various debates on the definition, standard and scope.

An organoid is a self-organized 3D 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[8–11]. Cells comprising organoids can be derived from induced pluripotent stem cells (iPSCs) or tissue-derived cells (TDCs), including normal stem/progenitor cells, differentiated cells and cancer cells[12]. Compared with conventional 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[13] than animal models. As such, organoid cultures have been leveraged for a wide variety of applications including drug discovery[14,15], personalized companion diagnostics[15] and cell therapy[13].

Organoid cultures exhibit significant heterogeneity and variable complexity in cellular composition, can undergo poorly controlled morphogenesis in the self-assembly process and often lack stromal, vascular and immunological components[4,12]. 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 microenvironment in the form of 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[16,17]. 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[16]. Beyond biochemical cues, stem cells also experience active and passive forces from their external microenvironment and convert these physical stimuli into biochemical responses[18]. These physical cues arise from the matrix, external forces and/or cell–cell interactions. Rather than relying on a 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[19]. The dynamic biofluidic environment has diverse effects on different cell types depending on the magnitude, direction and frequency[19]. Hence, microfluidic systems and bioreactors can be applied to provide perfusion at both the micro-scale and macro-scale[20–22]. Lastly, it is now known that cells interact with their neighbours and respond to external stimuli in a collective manner[23]; topographical cues, such as the curvature and shape of neighbouring cells, can affect stem cell decisions[24]. 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[25].

It is debatable whether engineered cell-based in vitro models such as organoids need to faithfully recapitulate the structures and functions of the in vivo organ of origin. One trend is to recapitulate as much in vivo tissue architecture and function as possible in vitro in order to demonstrate the physiological relevance of models of increasing complexity. For bioengineers, 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. There is an optimism to creating highly complex models and expecting them to accurately mimic the in vivo organ of origin. For the majority of users, simpler models — such as a model with one or two cells in monolayer or 3D culture — are more robust for mechanistic studies and applications[26–28] than more complex models, such as assembloids, or other multicellular models.

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 experimental considerations for setting up organoid-based cultures, categorized into four major components — 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 are also outlined. Lastly, we conclude by elaborating on the limitations of organoids in different applications, and the key priorities in organoid engineering for the coming years.

Experimentation

Cell source

Under defined physicochemical conditions, tissues such as small intestine[7], colon[29,30], stomach[31,32], oesophagus[29], tongue[33], liver[34–37], lung[14], pancreas[38–40], heart[41], ear[42] and skin[43] have been obtained from iPSCs, adult or fetal cells and either stem/progenitor cells or differentiated cells. The starting cellular population for any given organoid is of prime importance, affecting not only the variability and heterogeneity in the structures obtained but also the function of the tissue they aim to model. To establish tissue-derived organoids or cancer organoids, tissue-resident stem/progenitor/differentiated cells or tumour cells, respectively, are obtained through an optimized tissue dissociation method. For iPSC-derived organoids, iPSC lines are established and fully characterized as the starting cells. Patient/tissue-derived stem cells are obtained through an optimized tissue dissociation method and then embedded into a 3D matrix mimicking stem cell niches. iPSCs can be maintained and expanded as undifferentiated clonal populations on feeder cells. To exemplify the generation of tissue-derived organoids we 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 and then cut into 2–4 mm fragments to increase the surface area for enzymatic digestion or further mechanical dissociation. EDTA treatment is used to chelate calcium, disrupting cell–cell adhesion and tissue integrity[44]. Larger tissue fragments and whole cells are removed 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 are generally obtained from adult or fetal tissue biopsy samples. The most commonly used tissue dissociation method is enzymatic digestion, which dissolves the ECM[45]. The composition of the enzymatic cocktail and the efficacy of the enzymatic dissociation process varies with tissue type[46], and in certain cases DNase can be added to remove excessive DNA released from necrotic cells[47]. 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, the cell yield and viability can be inconsistent[46]. Mechanical and enzymatic dissociation can be combined to generate better cell yield. After tissue dissociation, TDCs for organoid development are identified and collected using known biomarkers or physical characteristics[45]. Tissue-specific stem cell markers are typically used to identify and isolate the desired stem cells to generate organoids[1,2]. Fluorescence-activated cell sorting or magnetic-activated cell sorting isolates cells based on multiple parameters, including size, shape and cell-surface marker expression[45,46]. Other isolation techniques include laser capture microdissection and manual cell picking[45].

iPSCs 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. As single iPSCs do not survive well in vitro, iPSCs 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 the level of cell sensitivity[44] and whether the cultured cells secrete excessive ECM, making it difficult to detach the cells from the cell culture plate (Fig. 2b).

Tumour tissue, derived from either biopsy samples or surgical resections, is also typically processed akin to normal tissue to isolate tumour cells to grow as organoids[15,48–50]. Tumour cells isolated from liquid samples such as peripheral blood[51], ascites[49,52] and pleural effusions[53] can be used as starting material to generate organoids. Patient-derived tumour organoids can be generated from samples obtained from minimally invasive Pap brush material[54,55]. Owing to their low numbers, tumour cells from biopsy samples or liquid samples can be first expanded in animal models as xenografts in order to obtain sufficient cells for organoid generation[56]. In the case of tumour tissue, it is 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. Although for some tumours it may be possible to enrich for tumour-forming cells by sorting a priori[57], for the majority there is currently no robust method to separate normal and tumour cell populations prior to seeding into a matrix for culture. An approach to overcome this issue is to take advantage of culture conditions by using selective media that omits certain factors required for growth of normal organoids, as tumour cells

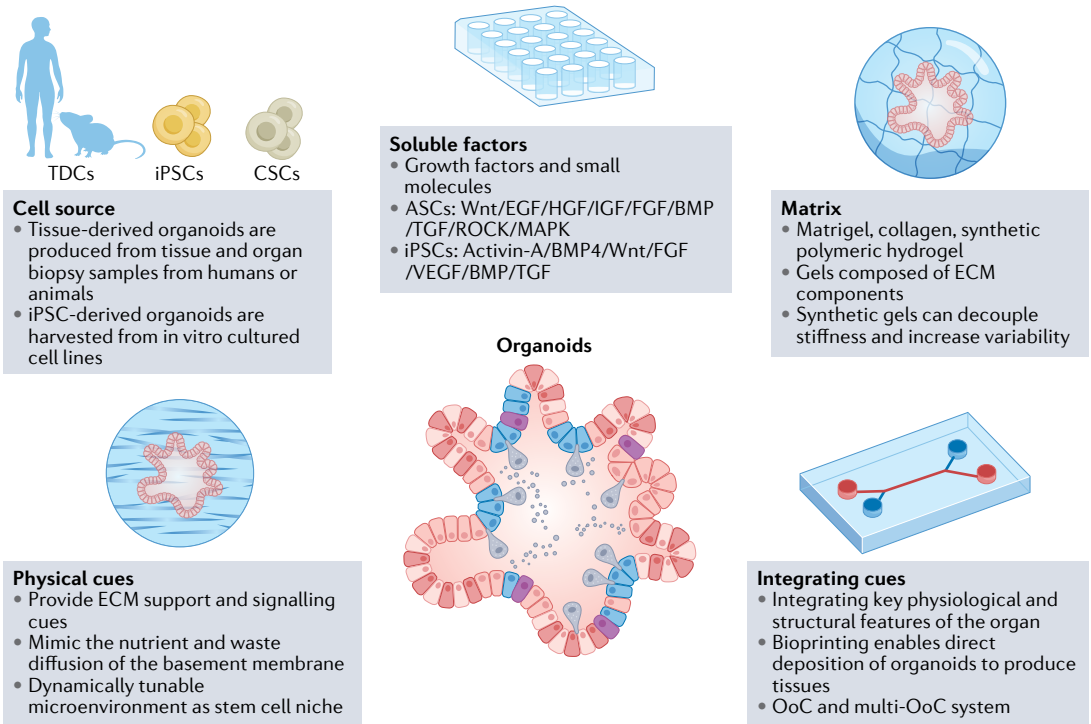

Fig. 1 | Components of organoid engineering. The establishment of organoid-based culture requires considerations about major components that make up organoid cultures — cells, soluble factors and matrix, physical cues — and the successful integration of these components. ASCs, adult stem cells; CSCs, cancer stem cells; ECM, extracellular matrix; FGF, fibroblast growth factor; iPSCs, induced pluripotent stem cells; OoC, organ-on-a-chip; TDCs, tissue-derived cells.

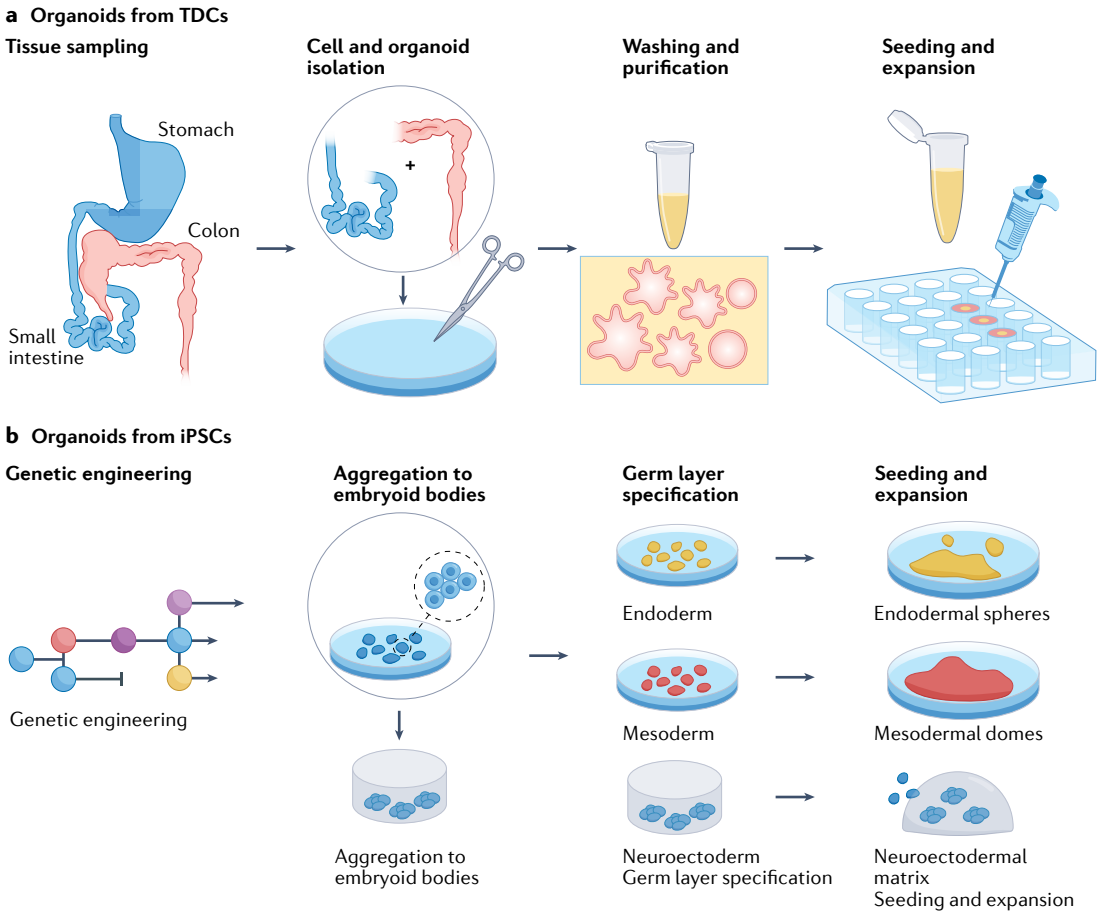

Fig. 2 | Flowchart of the procedures. Organoids can be generated from tissue-derived cells (TDCs) or induced pluripotent stem cells (iPSCs). **a**, To generate organoids from TDCs, tissue samples are obtained from humans or animals, such as the gut and stomach. The intestinal tissue samples are opened, washed and then cut into small fragments (2–4 mm) to increase the surface area for enzymatic digestion or further mechanical dissociation to isolate single intestinal stem cells or crypts. After several rounds of washing and purification, the harvested stem cells or crypts will be used for seeding and generation of organoid cultures for expansion. **b**, To generate organoids from iPSCs using genetic engineering, iPSCs are maintained and expanded as undifferentiated clonal populations on feeder cells or defined extracellular matrix (ECM) substrates to aggregate to embryoid bodies. Typically, iPSCs are harvested as cell aggregates, which preserve cell–cell contact and yield cell populations with higher viability. These aggregates are further induced through germ layer specification to form endodermal spheres, mesodermal domes and neuroectodermal matrix for additional applications.

gradually lose dependence on those factors during malignant transformation[58]. Blood contamination, particularly erythrocytes, can also affect organoid generation and matrix stability, and therefore standard approaches to eliminate these through lysis are typically used[59].

Matrix

Following cell isolation, cells are typically seeded into biologically derived matrices such as Matrigel[7,60] or a natural ECM such as collagen[61], or into synthetic hydrogels[3,62,63]. Matrigel is mainly composed of laminin, collagen IV, entactin, perlecan and growth factors, and is similar in composition to the basement membrane[61]. As a continuation from the above example using intestinal organoids, we now briefly describe how cells are encapsulated into matrices. Isolated intestinal crypts are first re-suspended into cold Matrigel and pipetted into pre-warmed low-attachment well plates for culture, producing a flat semi-sphere gel of cell–matrix construct. The complete composition of intestinal organoid culture medium has been previously reported[29]. Organoids go through passaging, and the proliferation rate is measured by isolating organoids from the Matrigel, removing single cells and counting crypts. The expansion rate is calculated as the number of organoids from each well divided by the number of initial crypts seeded in Matrigel in that well.

Although 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 spatio-temporal cues that are necessary for improving organoid culture. Therefore, other matrices with defined compositions[63] have been explored as alternative matrices to Matrigel, such as recombinant human collagen[61], fibrin[64] or synthetic hydrogels[3,62]. Natural matrices can be recombinantly produced from proteins or polysaccharides to address the batch to batch variability of Matrigel[60]. 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 polyethylene 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[65]. In other examples, the activity of adult stem cells can be controlled using PEG hydrogels with photo-degradable moieties[66] and 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[67], whereas dextran-based GMP-compatible hydrogels support expansion of cells for longer passages[68]. Lastly, microfabricated arrays were recently reported to enable the uniform production of crypt–villi-shaped epithelium[69]. Even with 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[7,34] or from initial multicellular structures such as intestinal crypts[29,62], cell aggregates[23] or micropatterned cells[25]. 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[70] to prevent cell attachment; subsequent centrifugation can promote aggregate formation, enhancing cell–cell contacts[71]. The size and compaction of cell aggregates can be tuned and controlled, such as through the use of microwell arrays[71,72]. 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[73]. Droplet microfluidics can print one organoid per well and enable the rapid generation of intra-organoid heterogeneity[74]. Droplet-based microfluidics has also been used to perform better single-cell RNA sequencing (scRNA-seq) analysis of intestinal organoid cell identities during various developmental stages, revealing extensive population heterogeneity[75]. Microwell structures or microfabricated pillar arrays[69] have also been developed to enable enhanced uniformity in cell aggregation[71,72,76]. 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[25]. 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.

Soluble factors

Organoid cultures are fundamentally based on our accumulated knowledge of developmental biology[13], where soluble cues are presented to cells in a spatio-temporally controlled manner. Soluble factors used to differentiate TDCs and iPSCs into various tissue types are listed in Supplementary Table 1. In organoid culture, these soluble cues are recapitulated in vitro in the form of biologics, mainly as proteins such as growth factors[77], or small-molecule drugs, which can activate or inhibit signalling pathways. Although growth factors could be costly and unstable, and many small-molecule drugs can affect multiple pathways resulting in poor reproducibility[77], some organoid protocols have combined the use of both biologics and small-molecule drugs[78]. The use of conditioned medium from engineered cell lines producing biologically active growth factors, such as L-Wnt-3A, can replace commonly used growth factors, such as WNT3A ligands[79]. These conditioned media face a similar issue of batch to batch variation and require stringent tests to ensure reproducibility[80]. Thus, novel surrogate molecules are starting to arise as potential substitutes for conditioned medium[79,80].

It is critical to consider how and when soluble cues 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 spatio-temporal 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 that the addition of free FGF into cell culture medium may not recapitulate how FGF is available in tissue in vivo[81]. The importance of presenting soluble cues in a spatio-temporally relevant manner is especially important for growing human iPSCs into complex structures with multiple cell lineages, such as in the case of kidney organoids[82]. Based on previous studies[83], 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 signalling before the addition of FGF was investigated[82]. The spatio-temporal 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[84–87]. 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[88]. Surface tethering can also be achieved with nanotechnologies, such as nano-imprint lithography, electron beam and electrospinning, and substrates with nanopillars, nanopits or nanochannels, mimicking the basement membrane for 3D organoid culture[89]. Lastly, microfluidic systems can be leveraged to create miniaturized niches with precise control over mechanochemical properties[90]. With directed flow and gradients of gas or small molecules, these systems can finely control environmental parameters within organoids[17]. 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[91].

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 re-seeded regularly[7]. Inadequate nutrient and waste diffusion is also problematic in brain organoid culture, where the resulting millimetre-sized constructs often exhibit necrosis within the inner core due to nutrient inaccessibility. This problem can be partially resolved using shaking cultures[22,92], spinning bioreactors or suspension under continuous agitation[22], or in continuously stirred bioreactors[92]. 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[20,21,93]. 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[24]. Topography-directed morphogenesis has been demonstrated using intestinal organoids grown on soft hydrogels[94].

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[95]. Ideally, specific physical or chemical cues should be presented in a spatio-temporally, physiologically relevant manner using simple, reproducible and robust methods (Supplementary Table 2). We 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 exogenously 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 number 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[25] 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 approach for reconstructing tissues is bioprinting[96] This technology uses bio-ink, comprising living organoids encapsulated within a biomaterial, to precisely create 3D biological geometries that mimic those 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, where centimetre-scale intestinal tissues were printed sequentially to mimic boundaries in the gastrointestinal tract[97]. 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, known as the organ-on-a-chip[98] (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[99]. 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)[100] and type 2 diabetes (with human pancreatic islets and liver spheroids)[101]. A two-organoid microfluidic device was recently fabricated with multiple read-outs using cardiac and hepatic organoids[102]. Moreover, OoC devices have improved the characteristics of human iPSC-derived pancreatic islets[103], intestinal[104], stomach[105] and liver[76] organoids by supporting physiological flow rates. Kidney organoids have been cultured in an OoC device that enables fluid flow to simulate shear stress[106] and the generation of a vascular network[107].

Results

It is important to evaluate whether cultured organoids recapitulate key aspects of the representative 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.

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 signalling 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 scRNA-seq profiles all of the cell types in the organoids – undifferentiated and committed – at the whole-genome transcriptome level. These cell types are then compared with 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 or organ[108]. 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[109] are also expected to be observed. For branching epithelial organoids, such as breast organoids[110], distinct branching structures are expected. For intestinal organoids, crypt-like structures[99] should be observed. For more complex organoid culture systems comprising incorporated supportive cells, such as fibroblast[111] and/or endothelial cells (ECs)[106,109], it is expected that the incorporated ECs should form a vascular network[106] that recapitulates the endothelial–epithelial interactions around the organoids.

Organoid functionality

Evaluation of organoid functionality includes, but is not limited to, the generation of mature cells, the formation of the vasculature or neuronal networks[25,43], the accurate response to external stimuli and

the effective secretion of cytokines or hormones, among other factors. Organoids should be compared with freshly isolated tissues as a positive control. Discrete physiological recapitulations are 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.

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 and PP cell) is examined by the expressions of the corresponding hormones — insulin, glucagon, somatostatin and pancreatic polypeptide, respectively — by immunostaining[112,113]. To assess the extent of β-cell maturation, the ultrastructural morphometric analysis of insulin secretory granules is performed by immuno-gold transmission electron microscopy (TEM) imaging[109]. To evaluate the ability of the β-cells in organoid responsiveness to glucose, glucose-stimulated insulin secretion experiments are performed, for example using an insulin or a C-peptide ELISA assay with cyclic high and low glucose incubation[114]. As insulin release is associated with calcium dynamics, calcium signalling traces imaging rapidly increases in response to glucose and returns to baseline[113,115].

Table 1 | Methods used in organoid research to assess/characterize organoid structure and function

Result	Characterization methods	Function	Example applications	Refs.
Organoid structure	Bright-field imaging	Qualitative assessment of morphology and viability; quantitative assessment of organ 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 CC organoids, grape-like structures for hepatocyte and HCC organoids	34–36,41,99, 109,110,124, 125,131,154, 203,205
	Immunofluorescent staining	Quantitation of viable cells; Edu and Ki67 staining to evaluate the survival and proliferation of the cells in the organoids; staining of differentially expressed proteins in a 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 BSEP or MRP2 for bile canaliculi, ALB or HNF4a for hepatocytes), and in some cases bile ducts can also be formed between the cholangiocytes (identified by markers such as KRT19 and SOX9)	35,99,106, 109,110,124,125, 131,154,203,205
	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 organoid glycogen accumulation, Golgi and mitochondria morphology, and bile canaluciuli presence	7,35,40,41, 99,106,108, 116,124
Organoid function	Quantitative PCR, scRNA-seq 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	scRNA-seq can profile all cell types in the organoids; also useful for screening differentially expressed markers under different stimuli	34,36,37, 40,41,99,106, 109,116,124,125, 131,203,205
	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 (for example, for CFTR functionality in gut, lung and pancreas organoids), CMFDA (5-chloromethylfluorescein diacetate) dyes to determine bile transporter functionality or dextran-based fluorescent dyes to investigate permeability of epithelium or endothelium	35–37,40,99, 106,110,113, 124,125,131, 154,203,205
	Alcian blue and periodic acid–Schiff 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	122,123
	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	34,35,37,40, 109,113–115, 124,125,131
	Luciferase assays	Luminescent assays for measuring enzyme activity	Various cytochrome activity for hepatocyte organoids (CYP3A4, CYP1A2, P450)	34,35,124,125
	Calcium signalling	Characterizing the electrophysiology property of the organoid	Used for organs such as the heart, neuron, retina, skeletal muscle and pancreatic islet	26,113,115, 124,125
	Implantation	Testing the in vivo function of organoids for cell therapy	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	34,40,109, 113,115,116, 124,125,131, 154,205

CC, cholangiocarcinoma; HCC, hepatocellular carcinoma; scRNA-seq, single-cell RNA sequencing.

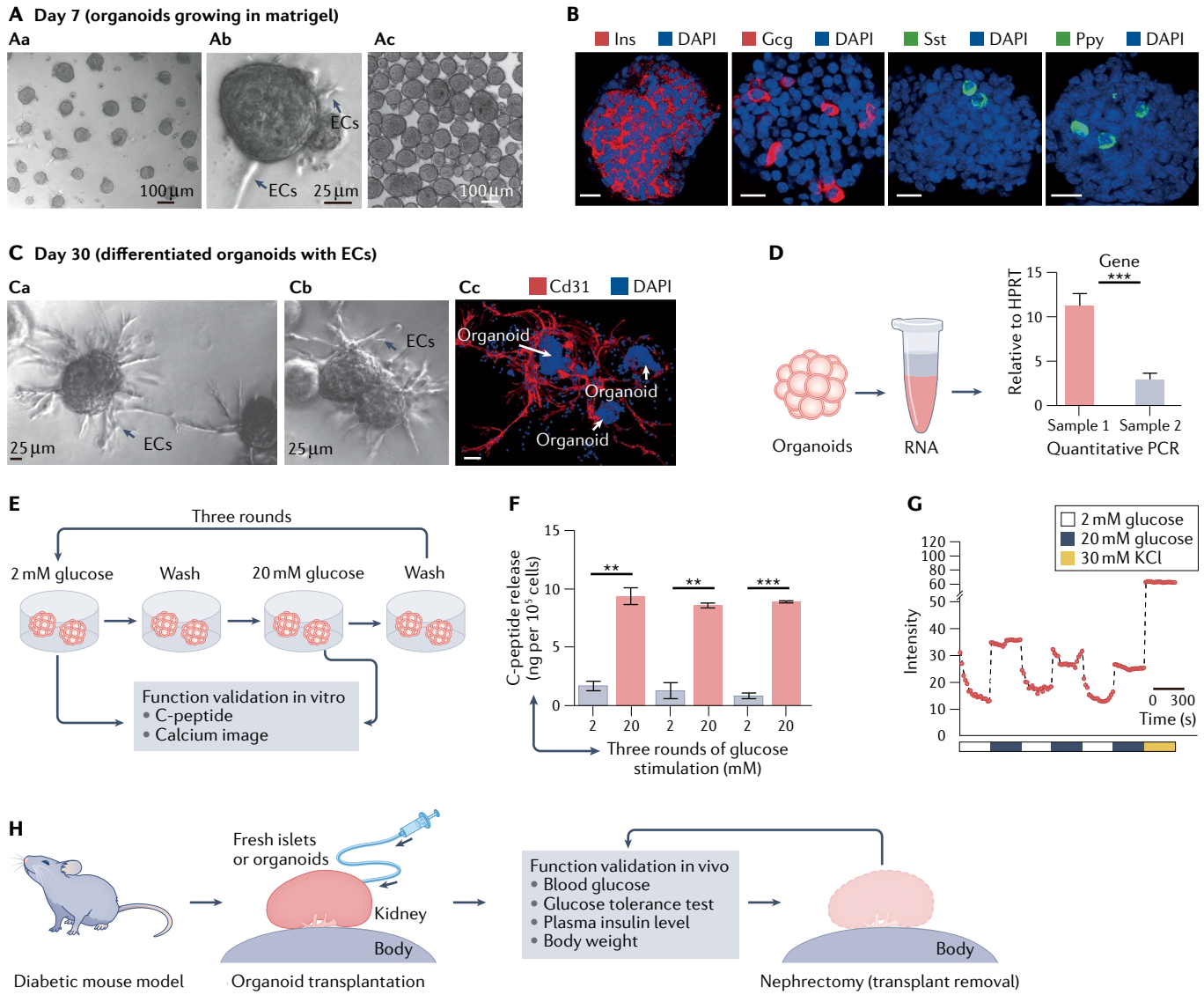

Fig. 3 | Representative results of pancreatic islet organoid validation analysis. **A**, Representative view of pancreatic islet organoids at culture day 7. **Aa**, Organoids growing in Matrigel exhibit spherical shape. **Ab**, Viewed at high magnification, these spherical organoids are surrounded by endothelial cells (ECs) (arrows). **Ac**, A view of the organoids after they are released from Matrigel by dispase. **B**, Cell types and hormone secretion level validation by immunofluorescence or immunohistochemical staining. The expression of insulin (Ins; β-cells), glucagon (Gcg; α-cells), somatostatin (Sst; δ-cells) and pancreatic polypeptide (Ppy; PP cells) can be detected in mature organoids (shown by DAPI; blue). Scale bars, 20 µm. **C**, The morphology of mature organoids induced through prolonged culture for a total of 30 days: the organoids are surrounded by elaborate EC networks (parts **Ca** and **Cb**), shown by Cd31 (endothelium marker; red) and DAPI (organoid nuclei; blue) staining (part **Cc**). Scale bar, 50 µm. **D**, Real-time quantitative PCR analysis for some key transcription factors and differentiation markers. **E**, Pancreatic islet organoid function validation in vitro. Organoids are sequentially incubated with low (2 mM) and high (20 mM) concentration glucose for three rounds, followed by C-peptide ELISA and calcium imaging. **F**, Representative ELISA result of organoid secreting C-peptide measurement. The rise of C-peptide concentration in the media is sharply responsive to high glucose stimulation. $^{**}P < 0.01$; $^{***}P < 0.001$. **G**, Representative curve of intensity of calcium signalling trace imaging, indicating the organoid's capability of responding acutely to glucose, with high/low calcium activity consistent to high/low glucose stimulation. Potassium chloride incubation is used as a positive control. **H**, Islet organoid function validation in vivo. Organoids (or fresh islets as positive control) are transplanted into the kidney capsule of streptozotocin-induced diabetic mouse, followed by monitoring of certain physiological indexes, indicating the rescue of the diabetic phenotypes. Finally, the transplants are removed and the surging blood glucose levels in the mice are expected, further demonstrating that the transplants were responsible for the lowering glucose level effect. Parts **A**–**C**, **F**, **G** and **H** adapted from ref.[113], Springer Nature Limited.

To test the full potential of the islet organoids upon transplantation, in vivo functional evaluation is needed to ameliorate the hyperglycaemic phenotypes of streptozotocin-induced type 1 diabetic mouse models[109,113,116]. The characterization parameters include normalized and stable blood glucose level, normal plasma insulin level and maintained body weight.

Intestinal organoids. Intestinal organoids are among the first organoid types successfully established in vitro[7,29]. Classical crypt–villus-like architecture is regarded as an important feature of successful organoid establishment, exhibiting proper branching morphology with robust multicellular composition and location reflecting the organoids' differentiation and maturation level[117,118]. The expression of Lgr5 marker can analyse the maintenance of intestinal stem cells by real-time quantitative PCR or by imaging using a Lgr5-fluorescent reporter[7,119]. The differentiated cells are assessed by the staining of their markers, including lysozyme (for Paneth cells), villin (for enterocytes), Mucin 2 (for goblet cells) and chromogranin A (for enteroendocrine cells)[7,99,120]. These various cell types can also be distinguished by TEM according to their characteristic subcellular structure[7,120]. Functional intestinal organoids exhibit a relatively thick mucus layer due to mucus secretion from mature goblet cells[121], which can be detected by Alcian blue and periodic acid–Schiff staining[122,123].

Liver organoids. In the case of the liver, both cholangiocyte[34,35] and hepatocyte organoids[124,125] have been established, both from mouse and human tissue samples. Measuring the passaging time and proliferation of organoid cells is used to assess liver organoids. Regarding marker expression, specific cholangiocyte (for example *KRT19*, *KRT7*, *SOX9*) or hepatocyte (such as *ALB*, *HNF4A*, *MRP4*) markers are assessed both on the RNA level (real-time quantitative PCR, scRNA-seq) and on the protein level (immunofluorescence staining). Often, also markers of liver progenitors (such as *LGR5*) are used to assess the differentiation state of the cultured cells. For the assessment of organoid functionality, further markers such as *CYP3A4* and *CYP3A11* for hepatocytes are analysed on the RNA level. Furthermore, albumin secretion, low-density lipoprotein uptake, the presence of glycogen accumulation assessed with periodic acid–Schiff staining, bile acid production and activities of liver enzymes such as CYP3A4 are assessed using chemical assays to confirm hepatocyte function. The ultrastructure of the organoids is also often assessed for typical hepatocyte or cholangiocyte morphology by 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[34,124,125].

Validation of tumour organoids. Patient-derived tumour organoids must maintain the genomic, transcriptomic, morphologic and functional profiles of the tissue of origin. As such, it is important to validate them by comparison with the tissue they are derived from in terms of histology and immunohistochemistry profiles[15], transcriptomics as well as genomics[14,126]. Cellular organization, tissue structure and protein expression patterns of tumour organoids can be easily compared with the cancer of origin[15]. Similarly, organoids are expected to have gene expression profiles similar to the tumour they are established from. This has been shown by RNA-seq on many tumour types including bladder cancer[127] and oesophageal cancer[128] amongst others. Lastly, it is important to confirm whether tumour organoids have diverged from the patient tumour at the genomic level. Several studies have shown concordance of organoids and parent tissue in terms of mutations and copy number variations[14,126]. A greater degree of divergence may be attributed to intra-tumour spatial heterogeneity and sampling bias: tumours can be spatially diverse, and organoids generated from a specific portion may lack the representativeness of distant regions[129,130]. In addition to this topographical issue, culture conditions can contribute to selecting specific clones, possibly altering or reducing clonality over time[128].

Validation of tumour organoids is even more critical when culturing over a long period of time. Whereas some studies have reported that molecular characteristics can be maintained over long-term culture, others have observed tumour evolution after serial passaging[128,131]. For instance, exome sequencing of liver cancer organoids showed that mutation concordance decreased from 92% for organoids cultured for less than 2 months to 80% for those grown for more than 4 months[131]. Colorectal tumour organoids established from microsatellite instable tumours had higher de novo mutations after long-term culture than stable ones[58]. 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 whereas subclonal ones are gained or lost, with mutational changes at each passage[126]. Most mutations and copy number variations are preserved in the late passage of lung cancer organoids, whereas de novo mutations can be attributed to the subclonal expansion of small subsets of cells present in the tumour of origin[14]. 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[132]. This reiterates the importance of validation in the context of tumour organoid establishment, characterization and testing. As an example of analysis, crucial steps in characterization of liver cancer organoids are demonstrated (Fig. 4). Lastly, normal tissue contamination is an issue for establishing tumour organoids and should be addressed when validating patient-derived models. The presence of normal cells can be estimated by histological comparison and immunohistochemistry, as well as sequencing[54,133].

Data analysis

Image processing and analyses. ImageJ/Fiji[134], CellProfiler[135] and other software are used for image processing. The number of organoids formed in a certain period, organoid size (measured as area or volume) and cell number can measure the cell proliferation rate. Cell proliferation can be quantified by the ratio of EdU-positive or Ki67-positive nuclei using available software plug-ins. Morphological evaluation — such as the bud formation ratio in the intestine organoid system — is also necessary when quantifying the success rate considering functional maturation. Mean and/or normalized fluorescence intensity of maturation markers for different tissue types can be recorded to measure the organoid maturation level. Long-term live imaging can be analysed using software to track the cell behaviour for lineage tracing purposes. Web resources are used for pathway analysis and visualization of multi-omics data to generate heat maps for understanding the differentiation stage of the organoid[136,137]. Other software, such as Profiler, GSEA, Cytoscape and EnrichmentMap, have been used for biomolecular interaction networks, pathway enrichment analysis and visualization[138–141].

Data analytics classification of phenotypes. Miniaturization and automation using machine learning and artificial intelligence-based data analytics make high-throughput and multidimensional phenotyping of organoids more consistent. Various software for these purposes have been developed in recent years, including ilastik[142], OrganoidTracer[143] and Phindr3D (ref.[144]). Most of these have Fiji or CellProfiler plug-ins, which make handling large data sets in shorter time frames possible for users without substantial computational expertise. Those software usually contain functions for image segmentation, object classification, morphological characterization,

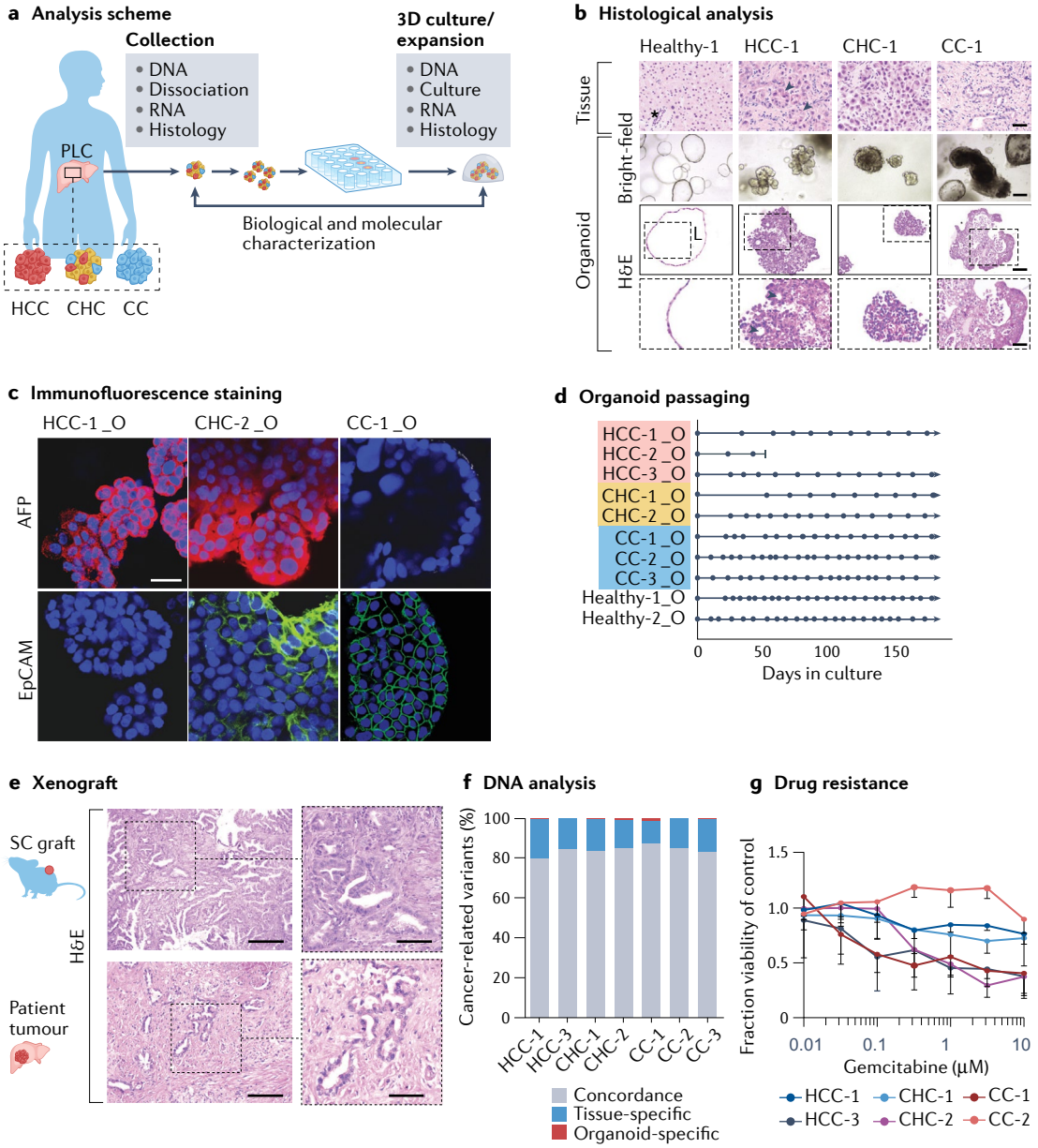

Fig. 4 | Typical characterization of cancer organoids: liver cancer subtype[131]. **a**, Isolation of cells from patient samples and organoid culture, with schematic of tissue isolation and processing. **b**, Histological analysis of liver cancer samples. Top row shows tissue (scale bar: 50 μm), middle row shows organoid bright-field images (scale bar: 100 μm) and bottom two rows show histological haematoxylin and eosin (H&E) staining of organoids (scale bar: 50 μm). **c**, Analysis of specific marker gene expression, including immunofluorescence staining for AFP (hepatocyte/hepatocellular carcinoma (HCC) marker; red) and EpCAM (ductal/cholangiocarcinoma (CC) marker; green). DAPI staining shown in blue. Scale bar: 30 μm. **d**, Organoid passaging shown as growth and splitting curves, where dots represent a splitting time point and arrows represent continuous expansion. **e**, Transplantation of xenografts into immunodeficient mice. Xenograft and histopathology analysis, matching the patient's original tissue sample. Scale bars: 125 μm, inset: 62.5 μm. **f**, Analysis of genetic changes in the cancer organoids and their concordance to the mutations in the original tumour sample. **g**, Half-maximal inhibitory concentration (IC_{50}) curves for gemcitabine treatment showing organoid sensitivity to drugs. CHC, combined HCC/CC tumours; PLC, primary liver cancer; SC, subcutaneous. Adapted from ref.[131], Springer Nature Limited.

fluorescence intensity quantification, cell counting and tracking for hundreds of images. Good image quality is a prerequisite for a robust result, so optimizing the image acquisition process is crucial. At different stages of the data processing, validation of the accuracy of the trained algorithm is recommended.

Applications

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. Here, we summarize a few applications.

Tissue regeneration

Tissue-derived organoids can be a potential source of transplantable material for regenerative medicine. Organoids of murine intestines[30], livers[34,124,125] and pancreas[68,145] have been successfully transplanted into mice with restoration of organ function. For example, liver organoids have been generated from hepatocytes isolated from murine livers[125]. 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[34]. Organoid engraftment rates were as high as 80%, and the engrafted mice survived longer than 100 days after the interruption of nitisinone therapy[125]. 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 hyperglycaemia[146]. Beyond murine organoids, human liver organoids derived from $EpCAM^{+}$ ductal cells from liver samples have been engrafted into immunodeficient mice with acute liver damage induced[34]. Human pancreas organoids can be generated from $ALDH^{high}$ stem cells and have the potential to produce insulin[145] without in vivo evidence yet to confirm engraftment. Intestinal organoids have been shown to fuse into tubular structures resembling in vivo intestines[147,148]. A more complicated intestinal organoid can also be generated in an artificial ECM with modulable biodegradability[3]. Although there is a long way to go for organoids to be used in regenerative medicine, improved protocols to generate normal tissue organoids, good manufacturing practices ensuring high reproducibility, grafting and function can already be established[149].

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[15,150,151]. A screening of 240 tyrosine kinase inhibitors on organoids derived from patients with ovarian or peritoneal tumours provided individualized drug sensitivity profiles within a week from surgery. The study included a carcinosarcoma of the ovary, a sporadic ovarian cancer for which no line therapy was established[150]. Tumour organoids can be screened to select effective drugs from structurally similar candidates[152] and investigate the synergic effects of different drugs for combination therapy[153]. Non-tumour cells from adjacent normal tissue in biopsy samples or surgically resected samples can be used to develop control healthy organoids to confirm the tumour-specific efficacy of the tested drugs[154]. Organoids can be screened with libraries of immune cells engineered to express chimeric antigen receptors against different neo-antigens[155]. Checkpoint inhibitors and other immuno-oncology drugs can also be screened in tumour organoid models that include immune cells[156].

Biomarker research

As organoids can maintain the genomic profile of the parent tissue[157], drug screening can be used to provide connections between genetic mutations and drug responses[36,153,158]. By screening gastric cancer organoids from 7 patients against 37 drugs, tumours with *ARID1A* mutations were shown to be sensitive to ATR inhibitors[158], supporting a previous study[159] and functionally identifying an intervention for a class of mutations that were considered undruggable.

Precision medicine applications

There is growing interest in tailoring cancer therapy to each patient. Precision medicine is often synonymous with genomics[160]. However, as shown in several clinical trials[160–164], only a small proportion of patients have an actionable alteration that can be coupled to a specific intervention[160]. In the NCI-MATCH trial that evaluates broad-scale implementation of sequencing-driven therapy, only approximately 37% of patients were found to have an actionable mutation[165]. This and other precision medicine trials have shown limited clinical benefits and modest response rates for many of the targeted interventions, uncovering a large degree of complexity[165–167]. For instance, the SHIVA trial that compared the outcome of genomic-guided interventions with conventional therapy selected by the oncologist for heavily pretreated patients with cancer showed no difference in progression-free survival[160,161]. Although the clinical efficacy of genomics-based precision medicine interventions can be debated, it is clear that most patients with cancer do not have a targetable genetic to guide therapy. Functional precision medicine – based on testing drug responses on patient tumours to identify treatment regimens – has been proposed as a more direct alternative, as it does not require any a priori knowledge of the tumour molecular profile or other characteristics[160,168]. Organoids are ideally suited for functional precision medicine because they are straightforward to establish, have high similarity with the tissue of origin, are tractable[157,169] and have been deployed to identify therapeutic leads in numerous tumours[170], including rare cancers that tend to be understudied and poorly characterized at the molecular level[15,150,171,172]. Recently, organoids established from patient-derived xenografts were used to identify eribulin as therapy for a patient with recurrent triple-negative breast cancer[173]. The patient's progression-free survival was 3.5× longer on an eribulin regimen than on the previous therapy received[173], indicating the potential of organoids in clinical care.

Source material for xenografts

Organoids are increasingly being used as seeding material to generate xenografts with engraftment rates. This process involves using intact organoids[14,58] or individual cells after digestion[54,174]. Human intestinal organoids transformed by CRISPR–Cas9 to carry mutations in *APC*, *SMAD4*, *TP53*, *KRAS* and/or *PIK3CA* were seeded into the subcapsular kidney space of immunocompromised mice. Benign organoids carrying mutations in one or two genes were shown as unable to form tumours, whereas organoids with mutations in four or five genes were tumorigenic[175].

Patient-derived organoids can also generate xenografts, confirm their tumorigenic capacity and recapitulate the histology of parental tumours[14,54]. In a study of colorectal cancer, benign tumour organoids showed no or minimal engraftment in mice. By contrast, colorectal cancer organoids derived from metastases were more invasive than those from primary tumours[58]. Comparison of the morphology of xenografts formed by glioblastoma multiforme cells cultured in vitro either as tumour spheres or organoids showed that whereas cells from tumorspheres displayed a solid growth pattern, organoids were more diffusive, similar to the tumour of origin[174]. Organoid lines derived from bladder tumours of lumenal origin transplanted in mice were shown to give rise to tumours with the same lumenal phenotype[126].

Infectious diseases

Organoid models are now widely used in research on host–pathogen interactions. For instance, organoids derived from tissues and organs such as the intestine[176,177], liver[178], lung[177,179], oral mucosa[180] and stomach[32,181] have been co-cultured with pathogens such as bacteria[32,181,182], viruses[176,178–180,183] and parasites[177]. 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 displayed lumenal swelling, a characteristic of rotavirus-induced diarrhoea[183]. Human liver organoid was used as a drug screening platform to test anti-HRV drugs and antibodies, identifying mycophenolic acid, IFNγ and anti-rotavirus VP7 antibodies capable of blocking rotavirus replication ex vivo[178]. Organoids can also be used in studies of oncogenic pathogens. Infection of murine gastric organoids by *Helicobacter pylori* showed increased cytosolic β-catenin levels induced by bacterially originated CagA, which led to increased proliferation in infected organoids[181].

Since the start of the COVID-19 pandemic, several laboratories have investigated the effects of the SARS-CoV-2 virus on organoids. SARS-CoV-2-infectable distal lung organoids were generated with *ACE2*-expressing lumenal cells[184]. SARS-CoV-2 was found to target goblet cells rather than ciliate ones, in contrast to a previous study in a 2D air–liquid interface model[185]. Studies in intestinal organoids show that SARS-CoV-2 can also infect enterocytes[185,186], and CRISPR–Cas9 and drug screenings have shown that knock-outs of either *TMPRSS2* or *TMPRSS4*, or treatment with Camostat, can reduce viral infection[186].

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 is a genetic disorder caused by mutations in the gene *CFTR*, which expresses a transmembrane chloride and bicarbonate transporter. More than 2,000 mutations in *CFTR* associated with cystic fibrosis have been reported, yet small-molecule therapeutics deployed clinically only target a subset of these[187,188]. Mutations in *CFTR* cause multi-organ dysfunctions affecting the lungs, pancreas, liver and intestine[189]. Cystic fibrosis organoid models of the colon[190], liver and bile ducts[191], rectum[192,193], the respiratory system[179,194] and the small intestine[190,195] have been derived from adult stem cells of relevant organs of either mouse models or patients, with applications ranging from investigating the biology of disease to mirroring patients' drug responses[192]. Forskolin, a *CFTR* activator, has been used to induce organoid swelling due to chloride and fluid flux into the lumen[193], with reduced swelling of patient-derived rectal organoids correlated with clinical response parameters of patients, which could mimic individualized clinical responses to specific therapeutic regimens[193]. Cystic fibrosis organoids have also been used to investigate gene therapy interventions and are able to rescue the effect of *CFTR* mutations in intestinal organoids using CRISPR–Cas9 (ref.[195]).

Inflammatory bowel diseases such as ulcerative colitis and Crohn's disease have also been studied with organoid models[196]. Organoids derived from intestinal epithelial cells of paediatric patients with inflammatory bowel diseases had inflammatory bowel disease-specific DNA methylation signatures despite retaining similar morphologies[197]. Liver organoids were generated to model liver diseases such as α1-antitrypsin deficiency[35], Alagille syndrome[35] and primary sclerosing cholangitis[198]. Lung organoids have been established to investigate diseases such as goblet cell metaplasia[199].

Reproducibility and data deposition

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). This heterogeneity is valuable to recapitulate the individual to individual differences, especially in the context of human diseases, and is valuable in cancer research to mimic patient-specific cancer development for personalized medicine[126–128,131,154,158,169,200–202]. Similarly, the intra-organoid variation reflects the complexity of cellular composition of the tissues. This is advantageous for modelling tissue development or regeneration, with cells forming an organoid encompassing different cellular states, from stem/progenitor cells to more differentiated cells[35,124,203]. Although these levels of variation and heterogeneity reflect the complexity of biological systems, they can jeopardize the reproducibility and robustness of the system when left uncontrolled.

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[119,204–206]. Regarding the cells of origin, the batch and quality of the iPSCs/pluripotent stem cells 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[12]. The media are usually optimized for cell proliferation whereas differentiation towards certain cell fates can be largely influenced by the culture conditions, thus increasing variability and heterogeneity. Refining of culture conditions has been demonstrated to increase the diversity of differentiated cell fates in human small intestinal organoids[119]. Undefined ECMs (such as Matrigel) have unknown composition and batch to batch variability, making reproducibility a significant concern[12,207]. Another important factor becomes apparent when looking into transcriptomic changes during organoid development[208] or studying gene expression changes during organoid passages[34,204]. When organoids mimic development, these changes can lead to confounding effects, for example, 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 scRNA-seq, to help delineate the different populations of organoid-forming cells. Many single cell-resolved data sets are emerging, with several notable examples in more homogeneous organoid production[3,69,99,209] and resolving organoid heterogeneity during growth progression[206,210]. Pooling together different cells and/or organoids for bulk analysis can lead to inaccurate results[3,69,99,206,209,210]. Therefore, good record-keeping about what was pooled in the 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. Efforts to generate more complete organoid structures containing different cellular populations (reviewed elsewhere[211,212]) – such as the recently published endothelial and healthy human colon and human colorectal cancer organoids[213], murine cholangiocyte and mesenchymal organoids[73] and murine pancreatic islet and endothelial organoids[109] – 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. Although 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) exhibit heterogeneous responses to known chemotherapeutic

and anticancer agents[48,131,214]. This parallels the variability in patient outcomes observed in the clinic and has positioned cancer organoids as a potential predictive tool for drug testing (reviewed elsewhere[215]). This heterogeneity in treatment responses is now being exploited for the treatment of patients with cystic fibrosis[193], with a large ongoing European multicentre clinical trial study, led by the HIT-Cystic Fibrosis consortium, to identify potential responders[216]. Similar initiatives are lagging behind for patients with cancer, although mostly because of disparities in establishment rates and lengthy timelines (reviewed elsewhere[217]). Organoids were recently used to inform the treatment of a patient suffering from a rare form of liver cancer, although unfortunately the treatment was discontinued due to deterioration of the patient's general condition, preventing the authors from drawing conclusions regarding the predictive value of the organoid system for drug treatment[218].

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. Although one could consider that at least three independent experiments with at least three different biological replicates are a minimum requirement, this may not always be sufficient. In fact, the number of organoids to study per biological replicate as well as the number of biological replicates depends on the research question, the specific experiment and the variability of the phenotype observed. Whenever possible, calculating the number of independent organoids per biological replicate, sample size calculations similar to those used for mouse experiments, taking 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 research question for each experiment should be determined in advance to avoid hypothesis fishing and increasing replicates to get a significance between conditions[219,220]. Data should be reported correctly as a mean of each replicate, with each replicate 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[221–223]. SuperPlots is recommended for data visualization, as it shows both the mean of each independent biological replicate and the spread of the individual data points[224,225]. This type of plot is ideal for organoid reporting, as it allows immediate visualization of any potential artefact concerning batch variation. Journal collections that deepen all aspects of statistics used in biological sciences are valuable resources to facilitate accurate reporting[226].

Organoids should be characterized in full, not only checked for the expression of markers but also benchmarked to the tissue of origin. It is crucial to not only determine whether cells have similar morphology and expression patterns as the tissue of origin and that they have a similar function (using, for example, ELISA for albumin, bile acid production by liver/hepatocyte organoids). Whenever possible, results between compared conditions should be normalized against DNA content (for genomic analysis) or structural proteins (such as actin, for western blotting), whereas functional data such as from ELISA should be normalized to the total number of cells or area of organoid structures in each well being compared. Improving reporting standards is the first step towards increasing reproducibility, thus enabling the field to move forward.

Data deposition

Standardized organoid data repositories are currently missing. A notable exception is a patient-derived repository for cancer organoids 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' OCG) (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, indicating 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[227], is the first step in that direction. For transcriptomics-related data, such as gene expression, non-coding RNA, ChIP, genome methylation, high-throughput PCR with reverse transcription, SNP arrays, SAGE and protein arrays, the Gene Expression Omnibus (GEO) is generally recommended[228,229]. For code used to analyse organoid data sets, GitHub is recommended. We envision that data and organoid depositories will arise shortly to fill in the presently missing gap in data repositories for organoid work. When depositing data from human organoids, it is important to consider all ethical regulations and the necessity of obtaining patient consent and keeping patient anonymity. Critical points have been extensively reviewed elsewhere[230,231].

A consensus in organoid nomenclature is also missing. Aside from a few notable examples, where leaders in the intestine, liver and pancreas addressed the nomenclature of gut, hepatic, pancreatic and biliary organoids[232,233], the field is waiting for a more clearly defined nomenclature system.

Limitations and optimizations

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

Limited level of maturity and function

None of the present organoid model systems reproduces the entire physiological repertoire of cell types, maturation level and/or function 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[73]. 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[234]. However, this is at the expense of giving up control over the formed tissue constructs. Meanwhile, optimizations on differentiation protocols are 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.

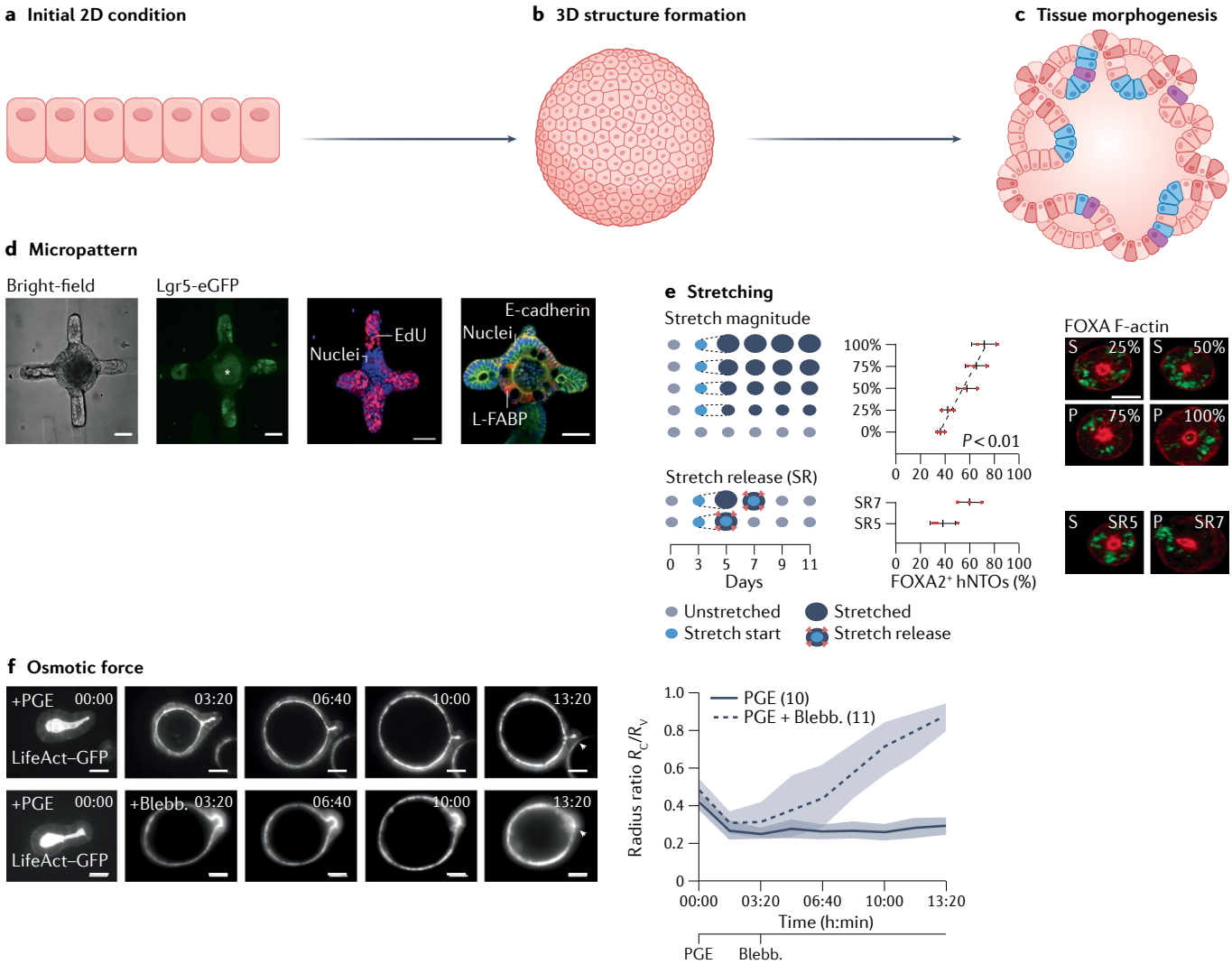

Fig. 5 | Reducing the heterogeneity with complexity reduction. a–c, The organoid development process includes the formation of an initial 2D condition (part **a**), growth into a 3D structure (part **b**) and tissue morphogenesis (part **c**). **d,e**, Simpler models of reduced dimensions to recapitulate the essential tissue structures and functions of interest are gaining momentum. Micropatterned 2D mono-culture or co-culture allows the formation of a reproducible initial 2D condition which can further form the initial 3D structure. For example, crypt formation can be controlled by localized matrix softening and geometry can induce patterned differentiation with enterocytes (L-FABP stain) in the centre region and proliferating LGR5 cells in the buds[69] (part **d**). Subsequently, a high degree of spatio-temporal control can be applied to direct certain tissue morphogenesis. For example, neural cell differentiation pattern can be modulated by stretch magnitude and timing[242]. Graph represents the stretch magnitude form 0 to 100% and scattered (S) and patterned (P) are the two configurations of differentiation patterning. **f**, Osmotic forces can control the crypt morphology in intestinal organoids[243]. Scale bars: 30 µm (part **d**), 50 µm (parts **e** and **f**). Blebb, blebbistatin; hNTO, human neural tube organoid; PGE, prostaglandin E2. Part **d** adapted with permission from ref.[69], AAAS. Part **e** adapted from ref.[242], CC BY 4.0. Part **f** adapted from ref.[243], Springer Nature Limited.

As organoids grow in size, the nutrient supply to cells localized in the centre of the organoid gets restricted, resulting in cellular death. This 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, pluripotent stem cell-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[235].

Limited control of heterogeneity

Once cells form an organoid, we have minimal input in cellular behaviour 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[236].

To generate more complex multicellular mature and functional structures, the organoid field has started to create assembloids as demonstrated for human cortico-motor assembloids[237]. Such effort allows the creation of more complex structures, connecting multiple types of tissues with a defined interface such as connecting cerebral cortex, 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 organoids[233], reproducibility in multicellular 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 spatio-temporal 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[238,239], cell sheets[240], stacked 3D structures[25] and micro-positioned ECM substrates[69,241] allow the formation of reproducible tissue structures and functions with a high degree of spatio-temporal control such as stretching[242] and osmotic forces[243] (Fig. 5).

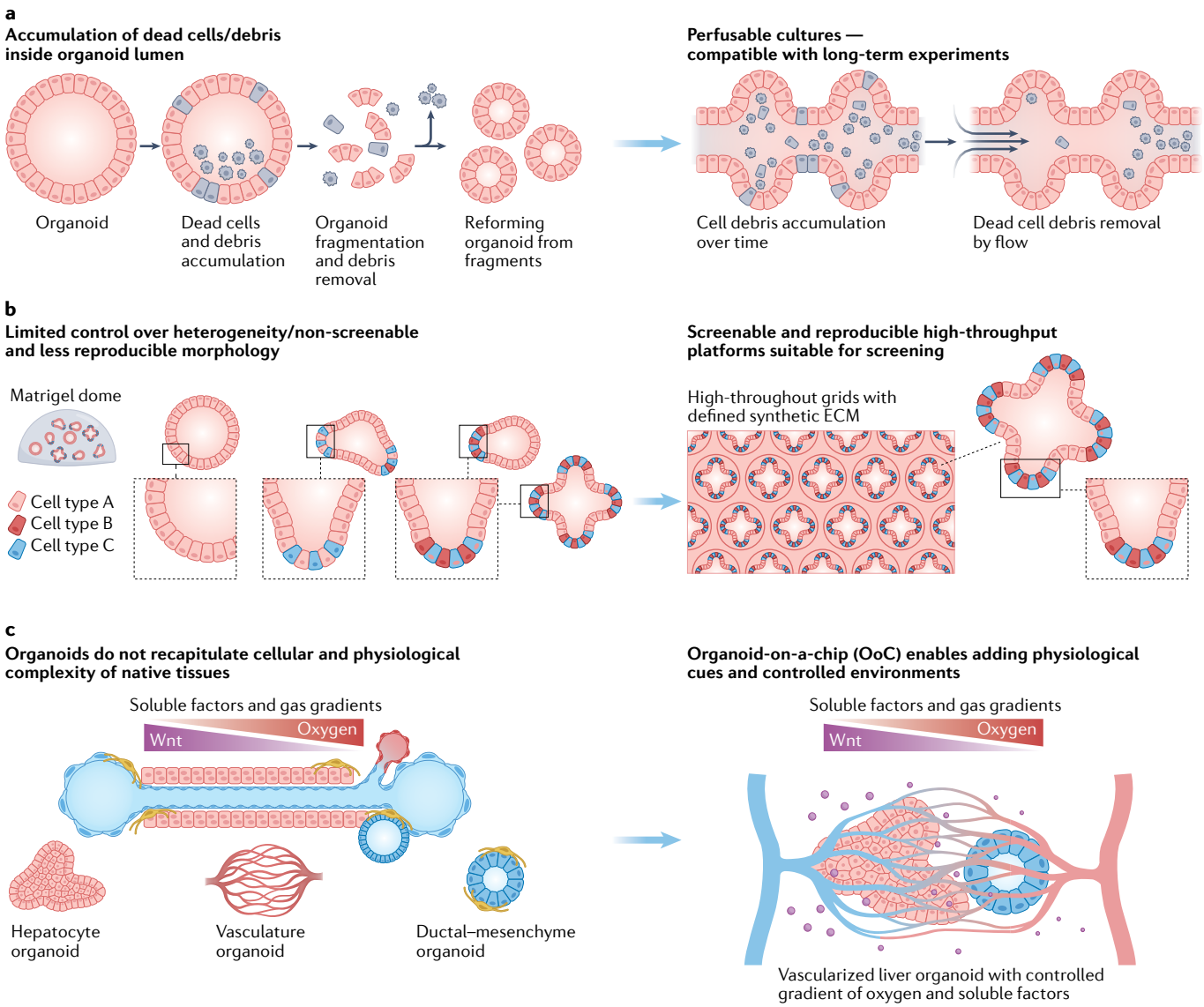

Fig. 6 | Side by side comparison of the current limitations for organoid culture and approaches to overcome them. a, Accumulation of dead cells and cell debris inside cystic organoid lumina (left) has been overcome by designing perfusable open-end structures (right) that use inducible flow to wash out cell debris, which are more compatible with long-term experiments. **b**, Organoids grown in Matrigel domes display high variability of cell heterogeneity and morphology (left), which can be overcome by using grids with patterned synthetic extracellular matrix (ECM) (right) that provide cues for cell differentiation. These platforms are additionally compatible with high-throughput screenings. **c**, Single cell type-derived organoids do not recapitulate the cellular and physiological complexity of native tissue (left), but combining organoids with organ-on-a-chip (OoC) (right) as a novel technology would enable creating a controlled microenvironment, suitable for multiple cell types.

Glossary

2.5D culture
3D culture with a restricted third dimension.

Assembloids
Complex 3D structures combining several separately pre-generated cellular compartments and/or entities.

Bioprinting
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.

Engraftment rates
Degrees of organoid retention in a host tissue after transplantation.

Organogenesis
The series of organized integrated processes that transform an amorphous mass of cells into a complete organ in the developing embryo.

Passaging
The subculturing of organoids by creating either smaller organoid fragments or single cells using mechanical dissociation or enzymatic digestion.

Stem cell niche
A specific tissue microenvironment where stem cells both reside and receive stimuli that regulate cell fate.

Streptozotocin
An agent toxic to the insulin-producing β-cells of the pancreas in mammals.

Temporal heterogeneity
Here, differences in the composition and changes during the time of passaging the organoids.

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[207,244]. 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[3,68].

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 OoC set-ups upregulate their functions, getting a step closer to a native tissue[93,245,246]. A more recent example shows how the presence of fluid flow enhances kidney organoids' maturation and favours their vascularization in vitro[106]. Physical constraints were engineered 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 to the in vivo tissue[99].

Outlook

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[124], hepatocyte functions are preserved, yet the liver tissue architecture does not match the native tissue where hepatocytes are arranged in cords. Similarly, organoids such as 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 multicellular and multi-tissue structures will be important, especially in the context of studying cell–cell interactions[247]. Along this vein, assembloids and organs-on-chips are also becoming increasingly complex and more broadly adopted.

On the other hand, the engineer's approach[27,248] is to pursue simpler reductionist models defined by the minimal functional modules that drive a complex cellular or tissue function of interest, to study the mechano-biological 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 (subcellular or intercellular tissue/multicellular) structures associated with functions of interest in the specific spatio-temporal stage/phase/step. For example, bile canaliculi in the 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[26,249]. One can choose to create a much larger structure involving cholangiocytes than the one operated by the minimal functional modules but the model will be noisy and costly. Each functional module is coupled to another and can be modelled 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[28,241,250].

Geometrically constraining the size of the initial 2D seeding pattern and 3D formation by micropatterning and supporting 3D cell growth using Matrigel enabled the induction of tissue-like neural tube morphogenesis and production of highly reproducible neural tubes. This also allowed identification of the mechanisms of neural tube folding and modelled neural tube defects[25]. 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 recently elucidated: symmetry breaking is caused by transient activation of the mechanotransducer *YAP1*, which induces *NOTCH-DLL1* lateral inhibition events[206]. *YAP1* activation has since been controlled by applying geometrical constraints in hydrogel scaffolds and producing a uniform and reproducible intestinal microtissue[3].

Organoids can be constrained by reducing the third dimension in a 2.5D culture. The 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[251]. Typical examples of restrictions in the third dimension include culturing cells on curved or patterned surfaces, a flattened or constrained cellular construct[251] and overlaying the 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. 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 without the 3D network, wider and cholestatic

compared with 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[26,252]. We would also envision more engineered organoid models based on CRISPR-edited cells for disease modelling, although these cells and models are synthetic.

Besides technological advances in creating more physiologically relevant, robust and easier to use organoid models, we envision seeing greater impact in applications. In the past two decades, although there have been discussions to replace animal testing, these efforts have not yet led to concrete actions. However, this is rapidly changing, with regulatory hard stops now established on multiple fronts[253]. Organoids that can recapitulate the complex physiological functions in vivo have 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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Competing interests
M.H. is inventor on several patents on organoid technology. A.So. and L.L. are inventors on a patent on organoid technology. A.So. is founder and owner of Icona BioDx. H.Y. is inventor on several patents on cell-based models and founder of Ants Innovate and Invitrocue. The remaining authors declare no competing interests.

Additional information
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