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journal homepage: [www.elsevier.com/locate/addr](http://www.elsevier.com/locate/addr)Q5 Drug discovery through stem cell-based organoid models<sup>☆</sup>

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## Q7 Q6 1. Introduction

38 The development of new drugs is currently a long and costly process  
 39 in large part due to the failure of promising drug candidates identified in  
 40 initial in vitro screens to perform as intended in vivo. New approaches  
 41 to drug screening are being developed which focus on providing more  
 42 biomimetic platforms. This review surveys this new generation of  
 43 drug screening technologies, and provides an overview of recent

developments in organoid culture systems which could afford previous- 44  
 ly unmatched fidelity for testing bioactivity and toxicity. The challenges 45  
 inherent in such approaches will also be discussed, with a view towards 46  
 bridging the gap between proof-of-concept studies and a wider imple- 47  
 mentation within the drug development community. 48

Drug discovery today is at a crossroads: while increasingly large and 49  
 varied compound libraries are synthesized and tested in primary 50  
 screens, the promise of the identified lead compounds remains largely 51  
 unrealized. Indeed, while tremendous investments in automation 52  
 have enabled the costs and turnaround time for large to medium-scale 53  
 primary screening to fall significantly [1], the gap between lead com- 54  
 pound validation and success in the clinic is still wide, suggesting that 55  
 a process still beset by significant limitations in efficiency. 56

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To a significant degree, this inefficiency in taking lead compounds into the clinic may be due to the discrepancy between the simplified *in vitro* assays currently performed and the complexity of real *in vivo* pathologies. Indeed, while both drug safety and efficacy intrinsically linked to administration into a complex and heterogeneous three-dimensional (3D) physiological system, most primary drug screening campaigns are still carried out with cell lines grown on two-dimensional (2D) plastic, an entirely reductionist approach where important parts of the drug-biology interaction are lost. The outcome of this primary screening process is the identification of “hits”, which satisfy very specific molecular targets or phenotypic requirements. A key problem is that these lead compounds are then validated and optimized in similarly over-simplified culture models. The process of ADMET evaluation (adsorption, distribution, metabolism, excretion, toxicology), while having undergone significant improvements in the last 15 years [2], could still be considered one of the main bottlenecks in the drug development process and could afford the greatest return on technological innovations [3].

As an important additional requirement, regulatory agencies require that identified pro-drugs be tested in two animal models before granting approval to proceed to any human clinical trials. This costly process of validation in animal models often fails due to physiological events linked to fundamental differences between human and animal model physiology. At this increasingly costly step, due to well-known differences in mechanisms of metabolism and toxicology between species, there remains a significant lack of fidelity between current testing procedures and human outcomes, particularly as related to appropriate evaluation of toxicity and drug dose.

These shortcomings have been clearly recognized within the pharmaceutical industry [4], yet few fundamental solutions have currently been implemented. The behavior of cells and their response to drugs continue to be studied *in vitro* mostly in 2D cell cultures that completely fail to mimic the complexity of the microenvironment. Not surprisingly, drug responsiveness in these settings is therefore often not predictive of

the *in vivo* situation, which dramatically increases the costs of drug discovery.

At the same time, a vast amount of research has been carried out in academia to develop more relevant test-beds for screening and validation efforts (Fig. 1). In particular, there has been a push towards the development of multicellular spheroid models [5], notably in cancer modeling [6], as well as a number of miniaturized approaches culminating in organ-on-chip systems [7]. More recently, there has been a tremendous interest in developing increasingly complex multicellular constructs termed “organoids” [8–10] (Fig. 2). These morphogenetic models, often recapitulating developmental programs from embryology or harnessing adult stem cell-based regenerative processes, have allowed molecular and cell biologists to understand key signaling events required for the initiation and maintenance of multicellular organs. By recapitulating not only the form but also the rudiments of function of their *in vivo* counterparts, these constructs have the potential to move from laboratory proof-of-concepts to relevant tools in the drug discovery pipeline. Indeed, such organoids could finally provide a key missing link between compound screening and clinical trials, and could serve as models for testing drug efficacy in target organs, for toxicity in liver models or for bioavailability through intestinal system models. In particular, by using primary human cells, especially patient-derived cells with relevant pathologies in conjunction with cellular reprogramming strategies, these techniques could provide an invaluable link to disease-specific human drug screening models.

Ultimately, the wider implementation of these bio-mimicking approaches within a still-conservative drug development community will require the level of reproducibility and consistency currently achieved with cell lines. Thus, such culture models will require 3D culture conditions which afford the needed flexibility to achieve precise control over the cellular microenvironment as well as a level of scalability. Furthermore, the applicability of such models will be greatly enhanced by adapting to existing infrastructure, notably automatic robotic platforms for experimental setup and assay readouts.

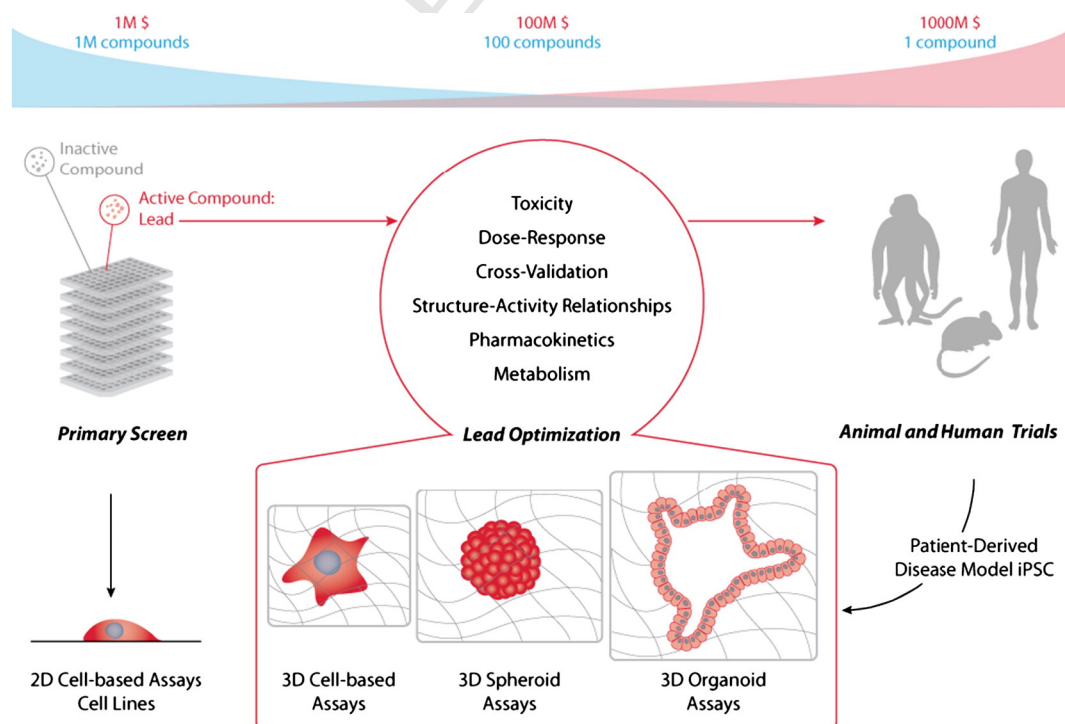
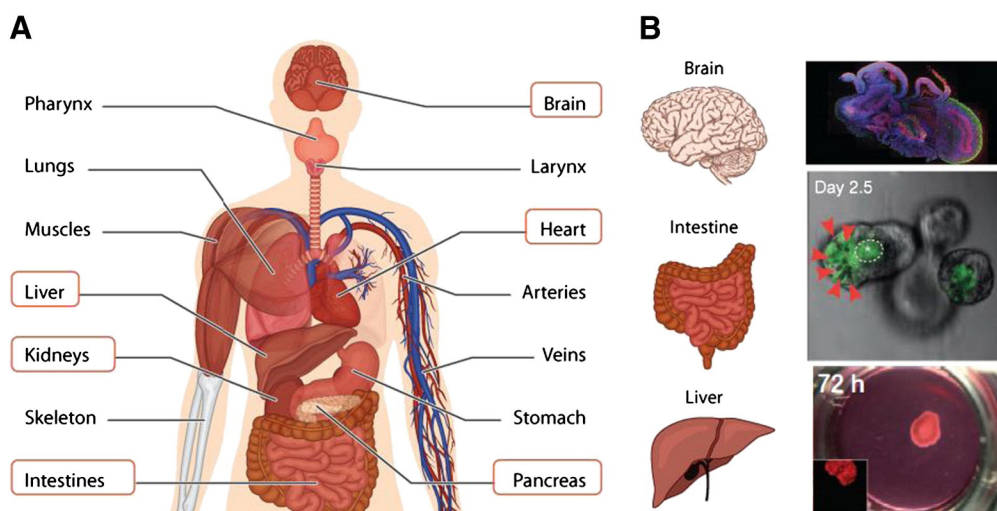


Fig. 1. 3D assays could bridge the gap between primary screening and animal and human trials. Drug discovery pipeline typically proceeds from multiple compounds tested at relatively low cost to few compounds in high-cost high-risk trials. The process of lead optimization and validation can benefit from increasingly representative *in vitro* technologies.



**Fig. 2.** Current organoid models. (A) An ever-increasing number of organs have in vitro organoid equivalents. Organoid-based assays present a novel and potentially high-value de-risking strategy, particularly when generated as iPSC cell-derived disease models. (B) Among recently reported self-assembling cellular constructs, cerebral [73], intestinal [43] and hepatic [44] organoids are prominent examples of organoid cultures with potential applications in drug discovery. Reproduced by permission of Nature Publishing Group.

125 Thus the purpose of this review is first to provide a selected survey of  
 126 existing state-of-the-art 3D models of in vitro drug evaluation, then to  
 127 introduce some key recent developments in organoid systems, and fi-  
 128 nally, through a critical evaluation of the limitations of such systems,  
 129 to propose some advances which could lead to the adoption of such  
 130 models by making the case for a real functional value in helping de-  
 131 risk this process.

## 132 2. From phenotype to organotype: high-throughput screening and 133 the 3D paradigm

### 134 2.1. Phenotypic screens: an additional dimension

135 Phenotypic drug discovery has become increasingly popular in early  
 136 stage drug discovery. Unlike target-based screens, in phenotypic screens,  
 137 there is no a priori understanding of the molecular mechanism of action,  
 138 and the effect of compounds on cell phenotype is observed directly. Such  
 139 an approach has emphasized the importance of biologically-focused as-  
 140 says: between 1999 and 2008, in a period where target-based screens  
 141 still predominated the drug discovery process, out of 75 first-in-class  
 142 drugs with new molecular mechanism of action approved by the US  
 143 Food and Drug Administration, the contribution of phenotypic screening  
 144 to the discovery of first-in-class small-molecule drugs exceeded that of  
 145 target-based approaches – with 28 and 17 of these drugs coming from  
 146 the two approaches, respectively [11]. As cell-based assays continue  
 147 to gain prominence and widespread adoption, it is clear that the new  
 148 approaches focused on further enhancing biological relevance are  
 149 necessary.

150 In the last few years, there has been a significant effort to develop 3D  
 151 culture systems which better represent in vivo biology. Within this vast  
 152 field, there have also been numerous approaches focused on high-  
 153 throughput and miniaturized implementations of such technologies.  
 154 Here, we will present such approaches, particularly focusing on most re-  
 155 cent developments pertaining to implementation in high-throughput  
 156 systems. Oncology has been one of the most important targets of drug  
 157 discovery; in this field where the presentation of the pathology is  
 158 often heterogeneous, and drug effectiveness, resistance and toxicity  
 159 manifest itself in many ways. It is therefore in this field that a number  
 160 of advances in the creation of more physiologically relevant approaches  
 161 have been most prominent. Indeed, using a number of established cell

lines, the cancer spheroid model as well as complementary assays for  
 invasion, migration, and angiogenesis have been most readily explored.

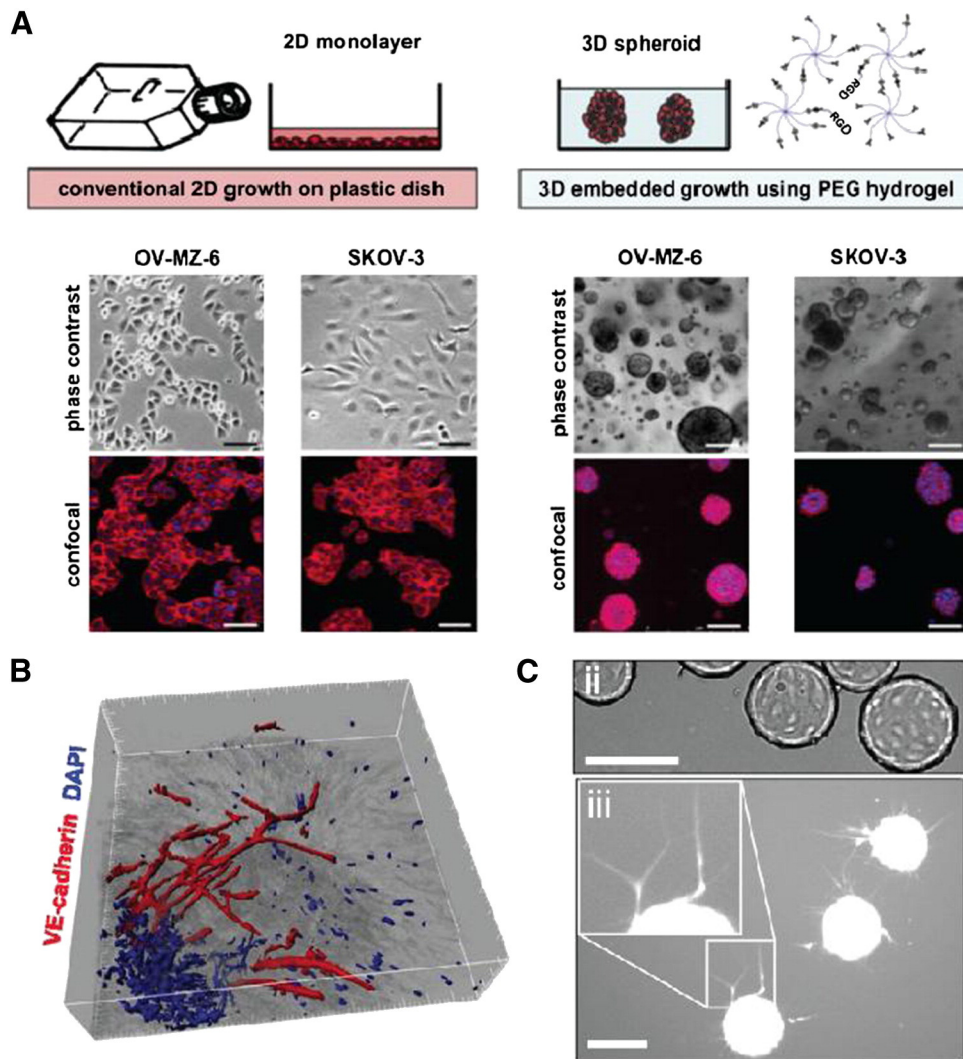
### 2.2. Scaffold-free multicellular cancer tumor spheroids

165 Multicellular cancer tumor spheroids (MCTS) represent a well-  
 166 established in vitro model for avascular tumor growth and this model  
 167 has become a classic reference for 3D studies [5,12]. By forming an aggre-  
 168 gate of cells which form cell–cell interactions, striking changes in mor-  
 169 phology and gene expression are evidenced [13] (Fig. 3A) as a number  
 170 of pathophysiological characteristics of an in vivo tumor are recreated,  
 171 notably, oxygen gradients, glucose distribution, lactate accumulation,  
 172 DNA strand breaks, ATP distribution and histomorphology/proliferation  
 173 characteristics [14]. It is also particularly appropriate as numerous cancer  
 174 cell lines, particularly those from the NCI-60 DTP human tumor cell line  
 175 screen established by the NIH, have been shown to form spheroids with-  
 176 out the addition of exogenous matrix materials [14].

177 For screening purposes, spheroid-forming assays have been devel-  
 178 oped in a most rigorously standardized manner within liquid media  
 179 cultures. A key consideration in such studies has been the control over  
 180 spheroid size, with the intention to establish a spheroid-based screen  
 181 with clear pathophysiological gradients but without central necroses  
 182 at the onset of treatment [15]. Indeed, a spheroid size of 400  $\mu\text{m}$  was  
 183 found to be ideal to recreate hypoxic conditions at the core, as well as  
 184 proliferative gradients, which have significant impact on radio and  
 185 drug-resistance as well as indirect effects of hypoxia-driven gene ex-  
 186 pression. Monitoring of spheroid growth kinetics to determine growth  
 187 delay and regrowth upon drug administration consists of the primary  
 188 analytical endpoint, and is performed by standard phase-contrast imag-  
 189 ing. The acid phosphatase assay (APH) has also been established to  
 190 monitor cell integrity and viability, with IC50 values estimated from  
 191 dose–response curves determined through such an APH cell viability  
 192 assessment.

193 Such a basic approach has been enhanced with the use of specifically  
 194 designed high-density plates which have been engineered to allow for  
 195 high-throughput hanging drop culture systems. Indeed such systems  
 196 have been optimized for droplet stability [16], have been shown to be  
 197 robust in fluorescence- and colorimetric-based assays through Z-factor  
 198 calculations [17], and have allowed for the determination of differential  
 199 effects on growth arrest with drugs. For example, fluoracil (5-FU) was  
 200 determined to be more effective as an anti-proliferative agent in 2D,





**Fig. 3.** State-of-the-art 3D assays allow for evaluation of multicellular tumor spheroid proliferation and quantification of angiogenesis and lymphangiogenesis sprouting. (A) Cancer cell line morphology and proliferation are clearly different between conventional 2D plastic culture and within synthetic PEG hydrogel (actin filaments stained with rhodamine phalloidin, nuclei with DAPI) [13]. Reproduced by permission of Elsevier. (B) Isosurface rendering of characteristic VE-cadherin immunofluorescence staining for VE-cadherin from ex vivo 3D (3D) assay of sprouting angiogenesis with arterial explants from human umbilical cords in Matrigel™ [74]. Reproduced by permission of American Society of Hematology. (C) Lymphatic endothelial cell-coated beads showed cellular protrusions that sprouted into the collagen gel [30]. Effect of lymphangiogenesis inhibitors was quantified using identification of sprout number per bead by in-house developed software. Reproduced by permission of National Academy of Sciences of the United States of America.

whereas hypoxia activated drugs such as tirapazamine (TPZ) were seen to be more effective in 3D hanging drop cultures [18]. Hanging drop culture systems have also been recently used in an elegant high-throughput manner to show how co-culture models of cancer and stromal cells could unveil novel regulatory pathways [19].

### 2.3. Scaffold-based multicellular cancer tumor spheroids

The generation of large-scale liquid media spheroids has been the focus of technological advances in up-scaling; by creating “droplet microarrays” with the possibility of generating thousands of droplets with defined geometry and volume using superhydrophilic–superhydrophobic patterned surfaces [20], or enhancements in hanging drop spheroid manipulation through incorporation of magnetic-based technologies [21].

While these techniques utilizing hanging drop or liquid suspension cultures provide an undeniable ease of use, such a free-growing construct certainly does not recapitulate the physical constraints experienced by in vitro tumors and may miss important mechanisms of extracellular interaction-mediated drug resistance. In order to address

this issue, multiple platforms have been developed to allow cancer spheroids to be grown within simplified extracellular matrices (ECM). To cite only a few recent examples, methylcellulose has been used as a simple 3D culture system for pancreatic ductal carcinoma cells and compared to standard 2D culture conditions; in the 3D case cell metabolism was seen to shift towards glycolysis, and, notably, while most drugs tested were shown to be less effective in 3D, two were identified as having particularly significant effects in this model [22]. In another study a 3D soft agar matrix was adapted to high-throughput screening, and 1528 natural product compounds were screened against colorectal carcinoma colonies [23]. Notably, this study showed how by comparing tumor-only cells with a co-culture model incorporating colon epithelial cells, it was possible to distinguish tumor-specific agents from general cytotoxic ones. A number of these technologies are now commercially available and have been shown to be compatible with a number of downstream assays beyond imaging. For example, in alginate-based scaffolds while cytotoxicity was measured by AlamarBlue® assay and drug effectiveness was measured by imaging, additional readouts such as apoptosis were evaluated by immunohistochemistry and RT-PCR, where cellular uptake of drugs and nanoparticles could also be

239 evaluated [24]. Again, it was shown that IC50 values for a number of  
240 cancer drugs were significantly higher in a spheroid model as compared  
241 to 2D. Thus, as demonstrated in these selected examples, the multi-  
242 cellular tumor spheroid model, despite its simplicity as a first approxi-  
243 mation of a 3D tumor microenvironment, has already shown value in  
244 a high-throughput drug discovery pipeline.

#### 245 2.4. Migration, invasion and angiogenesis in 3D

246 While aberrant cellular proliferation may be the primary manifesta-  
247 tion of solid tumor cancers, it is via remodeling and migration through  
248 the tissue microenvironment that malignant cells metastasize to spread  
249 to adjacent tissues or distant sites via lymphatic or angiogenic means. A  
250 better understanding of the role of the microenvironment in initiating  
251 and promoting these processes is critical if the prevention of metastasis  
252 is to be used as a target for oncologic therapeutics. With appropriate 3D  
253 culture models, it is possible to monitor not only bulk volume increases  
254 of spheroids (*i.e.* tumor proliferation), but also, given appropriate matrix  
255 conditions and chemotactic cues, the outgrowth of individual cells from  
256 such spheroids (*i.e.* cell migration). A number of approaches to assay  
257 migration and invasion exist, with varying degrees of physiological rele-  
258 vance and ease of implementation [25]. A prominent example includes  
259 filter-based transwell assays, which can be additionally coated with a  
260 layer of ECM components such as collagen or Matrigel™. More complex  
261 models incorporate a level of dimensionality, such as “sandwich assays”  
262 where a monolayer of cells is entrapped between two layers of ECM.  
263 Most relevantly, cellular spheroids such as the ones described above  
264 can be seeded onto a relevant matrix, or completely embedded within  
265 a 3D matrix [25]. Angiogenesis is one of the central hallmarks of cancer  
266 progression, and significant efforts have been carried out to determine  
267 ways to understand and inhibit this process in increasingly biorelevant  
268 model systems [26–28] (Fig. 3B). For example, a 3D vascular network  
269 assay showed considerable sensitivity to several angiogenic inhibitors,  
270 including kinase inhibitors and monoclonal antibodies and led the  
271 development of a 3D model of tumor-driven angiogenesis, in which an-  
272 giogenic outgrowth was sustained by spheroids of prostate cancer cells  
273 in the absence of exogenous growth factors [29]. Another notable use of  
274 3D-based spheroid assays was developed to screen for inhibitors of  
275 lymphangiogenesis [30], a process akin to angiogenesis and highly im-  
276 plicated in tumor progression (Fig. 3C). In this study, spheroids were  
277 formed by coating lymphatic endothelial cells around cytodextran  
278 microcarrier beads, and were then tested in a 3D high-throughput  
279 sprouting assay over the LOPAC collection of pharmacological com-  
280 pounds. By enhancing the power of such a screen by deploying auto-  
281 mated microscopy in conjunction with custom-developed advanced  
282 image processing software, novel regulators of lymphangiogenesis  
283 could be detected. In particular, a previously unknown link was  
284 established between statins and the inhibition of lymphangiogenesis,  
285 which has potential implications not only directly in the treatment of  
286 cancer but also for interactions with and management of cardiovascular  
287 disease.

#### 288 2.5. 3D assays for toxicity evaluation

289 While cancer is a primary target for the deployment of advanced  
290 high-throughput screening strategies, and has been seen in examples  
291 above to benefit from a 3D approach, it is by no means the only area  
292 where spheroid-based assays have been used. Indeed the spheroid  
293 assay has found significant use in organ- and cell-type-specific toxicol-  
294 ogy studies, constituting another important step in the drug validation  
295 process. While dose response and toxicity must clearly be evaluated in  
296 the cell type of interest, systemic toxicity, and particularly liver toxicity,  
297 is also of primary concern. As such, toxicity studies are routinely  
298 performed in HepG2 cells, a hepatocarcinoma cell line which has been  
299 frequently used as a model system to study liver metabolism and cyto-  
300 toxicity. 3D spheroids of HepG2 and closely related HepaRG™ cells have

been cultured in hanging drop suspension cultures and compared to 2D  
301 cultures. In one study, it has been shown that activity of CYP4A4, a  
302 member of the P-450 monooxygenases involved in the metabolism of  
303 a broad range of compounds from steroids to drugs and toxins, was  
304 higher in the 3D cultures compared to 2D [31]. Moreover, 3D cell  
305 cultures were more sensitive to a drug that is only toxic upon metabolic  
306 activation in the liver (aflatoxin B), suggesting that such an organotypic  
307 system better represents *in vivo* liver metabolism. Notably, within such  
308 an organotypic culture, the EC50 of acetaminophen was similar to  
309 *in vivo* toxicity, a phenomenon which could not be reproduced in 2D,  
310 demonstrating once again the importance of a 3D model for capturing  
311 *in vivo* response [32]. In a further advance, liver microtissues have  
312 also recently been constructed from primary human hepatocytes and  
313 liver-derived non-parenchymal cells [33]. Interestingly, significant  
314 species-specific differences in drug hepatotoxic response were found  
315 between rat and human microtissues [34], highlighting the importance  
316 of developing human cell-based 3D culture systems.

### 318 3. Organoids as *in vitro* organ models: promise and challenges

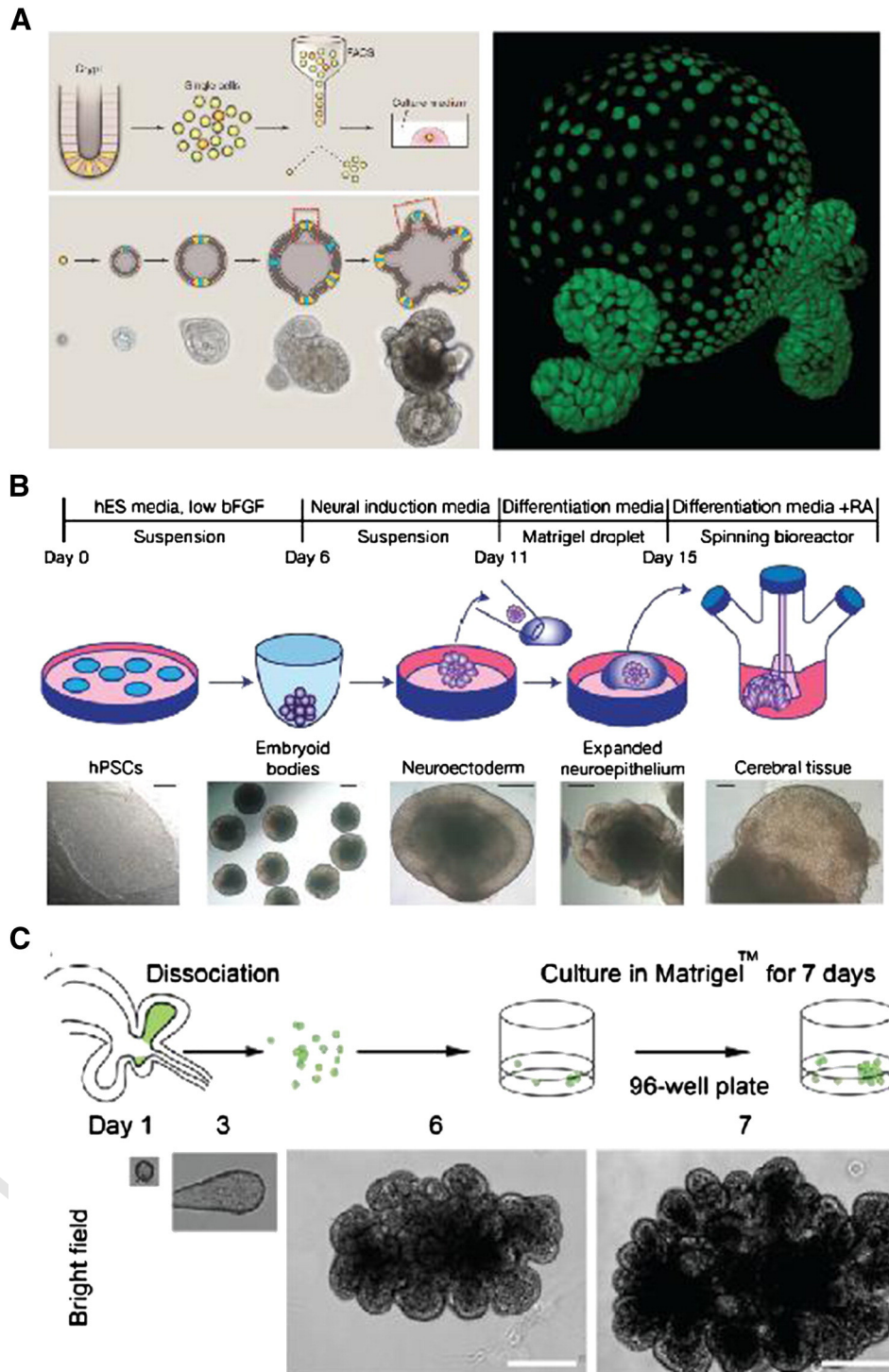
319 Despite the increasingly acknowledged value of *in vitro* 3D culture,  
320 of which some examples were reviewed in the previous section, animal  
321 models have remained as the immediate next test bed for promising  
322 new compounds after an initial primary 2D screen. However there is  
323 a significant gap between the still highly simplified models of spher-  
324 oids and the systemic effects seen in an animal, with all the possible  
325 confounding effects which cannot be clearly deconvolved. Moreover,  
326 there have also been some significant data over the years indicating  
327 substantial differences between animal and human modes of drug  
328 response. For example, in a survey of a dozen pharmaceutical com-  
329 panies, with data from 150 compounds with 221 human toxicity  
330 events, data from rodents failed to predict 57% of incidents of  
331 human toxicity [35]. Furthermore, a recent study showed a complete  
332 lack of correlation in the genomic response to acute inflammatory  
333 stress between human subjects and murine models, suggesting that  
334 the use of such unrepresentative models may have accounted, thus  
335 far, for the failure of all compounds which have been put through  
336 clinical trials intended to block the inflammatory response in criti-  
337 cally ill patients [36].

338 An ideal *in vitro* analysis system would therefore comprise of human  
339 cells, in a construct complex enough to demonstrate physiologic-like  
340 composition, morphology and heterogeneity and, ideally, the rudiments  
341 of functionality, yet simple enough that it could still be readily assayed  
342 *in vitro*. Such a construct, capturing some of the complexity of a  
343 human organ in a dish, has been termed “organoid” [37] (Fig. 2). The  
344 idea of creating *in vitro* organoids is not a new phenomenon: leveraging  
345 cells' intrinsic ability to self-assemble into organized structures has been  
346 envisaged at least since the early generation of teratocarcinomas in  
347 1954 [38]. Indeed, when embryonal carcinoma cells were transplanted  
348 into a host mouse, malignant tumors were found to develop and could  
349 form tissues of all germ layers, and surprisingly, could even in rare  
350 cases develop into complete organs. This became even more relevant  
351 with the advent of embryonic stem cells (ESC), which, under the same  
352 condition, *i.e.* reimplantation into a host animal, could generate equally  
353 histologically and morphologically complex structures. These early  
354 studies clearly demonstrated the potential of stem cells grown *in vitro*  
355 to recreate complex and organized structures, albeit when placed in  
356 the context of a complex host microenvironment. As well, these studies  
357 early on demonstrated the need for extrinsic microenvironmental regu-  
358 lation for growth and development. In the context of more relevant  
359 physiological studies *in vitro*, clearly there was an interest and a need  
360 to understand how such processes could be regulated, in order to then  
361 attempt to recreate these processes *in vitro*.

362 While the field of tissue engineering has made significant attempts to  
363 recreate *in vitro* organs in the last 30 years, these approaches have gen-  
364 erally focused on scaffold-based cell seeding techniques, and arguably

365 have found limited success in recreating the complex and heterogeneous  
 366 cellular organization found in vivo. More recently, new approaches  
 367 based on developmental biology have focused on recreating morpho-  
 368 genesis underpinned by a more sophisticated molecular understanding,  
 369 with the intent of harnessing the differentiation potential intrinsic in  
 370 stem cells to allow for self-organization. Given the right cues, a number

of increasingly complex structures have been recreated in vitro 371  
 (Fig. 2A), which for the first time may allow for “function-in-a-dish”. 372  
 While their potential uses as replacement organs in regenerative medi- 373  
 cine is the clearest and ultimate objective, a more likely and tractable 374  
 shorter-term goal is to make use of such constructs in the context of 375  
 drug discovery. 376



**Fig. 4.** Organoid culture systems require Matrigel as a 3D matrix for structural support and instructive signaling. (A) Development of an intestinal organoid from a single sorted Lgr5-GFP cell and architecture of a mini-gut organoid expressing histone 2B-GFP, with five crypts budding from a large central body containing the main lumen [8,75]. Reproduced by permission of The American Association for the Advancement of Science and Elsevier. (B) Schematic of cerebral organoid multi-step culture system including aggregation of ESCs in embryoid bodies, suspension in neural induction medium to form symmetric neuroectoderm, followed by expansion of neuroepithelium in Matrigel™ and final tissue maturation in spinning bioreactor [73]. Reproduced by permission of Nature Publishing Group. (C) Morphogenesis of pancreatic organoids from mouse pancreatic buds composed mainly of bipotent pancreas progenitors by seeding in growth factor-depleted Matrigel™ [53]. Branched organoid after 7 days contain ~10,000 cells. Reproduced by permission of The Company of Biologists Limited.



377 While spheroid-based approaches may be sufficient for testing drug  
378 efficacy in ablating tumor growth, mitigating migration and modeling to  
379 some extent angiogenesis, such approaches do not allow the equally im-  
380 portant assessment of cytotoxic effects of drugs on a multitude of or-  
381 gans, nor the investigation of issues such as bioavailability when  
382 crossing the intestinal lining, liver metabolism, or blood–brain barrier  
383 effects. As key examples, we will focus here on recent developments  
384 in creating brain, liver and intestinal organoids (Fig. 2B), which could  
385 help in assessing these whole-organ effects.

#### 386 4. State-of-the-art organoid culture systems: Matrigel™ as 387 critical component

##### 388 4.1. Intestinal organoids

389 In recent years, the organoid system that has gained the most atten-  
390 tion is the mini-gut construct, or intestinal organoid (Fig. 4A). In land-  
391 mark studies by the Clevers group [39–41], it was first shown that the  
392 transmembrane protein Lgr5 marks stem cells in the intestinal crypt,  
393 and that such cells exclusively contribute to the rapid self-renewal of  
394 the intestinal epithelium. Based on this *in vivo* knowledge, experiments  
395 were then carried out to demonstrate how an epithelial cell fragment  
396 isolated from the mouse intestinal crypt, when placed in an appropriate  
397 *ex-vivo* 3D culture system, could generate a 3D construct with some of  
398 the key characteristics of the intestine, including the establishment of a  
399 crypt–villi architecture as well as a lumenized interior [42]. Clearly, the  
400 stem cell niche concept plays a significant role in this system: factors  
401 such as R-Spondin, EGF, and Noggin are essential for the maintenance  
402 of the organoids in culture, and Matrigel™, the matrix used as 3D sup-  
403 port, provides a set of structural and biochemical cues. Notably, it has  
404 been found that even a single Lgr5 cell could be sufficient to regrow  
405 an entire organoid, but this process occurred at very low efficiency  
406 (circa 5%) [42]. Significantly, it has been shown that organoid-forming  
407 efficiency was greatly enhanced when Paneth cell–Lgr5 cell doublets  
408 were used, instead of single Lgr5 cells, suggesting that factors secreted  
409 by the Paneth support cell are crucial for regulating the intestinal  
410 niche [43]. Such intestinal organoids have also been derived from  
411 human ES cells [44], thereby greatly enhancing the applicability of  
412 such a system. In turn, it is now possible to envisage that such intestinal  
413 organoids could begin to be used to detect drug–intestine interactions,  
414 and, more specifically, to investigate bioavailability and aspects of  
415 drug pharmacokinetics. Indeed, intestinal organoids could certainly be  
416 imagined as complement or alternative to the commonly used Caco-2  
417 monolayer transwell assay, which is now the norm as an *in vitro*  
418 model of human small intestinal mucosa's ability to absorb orally  
419 administered drugs [45].

##### 420 4.2. Cerebral organoids

421 The developing human brain acquires its complexity through a myr-  
422 iad of developmental steps, with various cell types and regions acquir-  
423 ing their fate in a tightly regulated and sequential manner. *In vitro*, it  
424 has been possible to establish 2D cell culture protocols to generate  
425 bulk populations of neuronal subtypes from ESCs for screening purposes  
426 in procedures which have become fairly standardized and even in some  
427 cases deployed in primary drug screening assays [46]. With the adop-  
428 tion of 3D culture techniques, it has been possible in recent years to gen-  
429 erate increasingly complex neural subsystems which, to some extent,  
430 preserve their highly restricted *in vivo* spatial arrangement. Indeed,  
431 the formation of an optic cup from ESCs, including a multilayered neural  
432 retina containing rods and cones, was shown to occur using a multi-step  
433 protocol involving the creation of aggregates in a floating culture in  
434 serum-free and growth-factor-reduced medium, named SFEBq culture,  
435 or serum-free culture of embryoid body-like aggregates with quick ag-  
436 gregation [47]. The extraordinary self-organization seen in the optic  
437 cup construct involves nonetheless, both in mouse and human systems

[48], the embedding of aggregates in Matrigel™. Indeed, while it may be  
438 claimed that the process is entirely driven by spontaneous self-  
439 organization orchestrated by local cellular interactions, it is quite possi-  
440 ble that there are instructive matrix-derived cues at critical points in the  
441 process, which may perhaps even be the initiating impetus for key  
442 symmetry-breaking events within the homogeneous aggregates. More  
443 recently, a similar SFEBq-based protocol has been utilized to develop a  
444 human ESC-derived 3D organoid, termed cerebral organoids, where ex-  
445 tensive patterning of brain regions can be seen, including characteristic  
446 cerebral cortex zones with mature cortical neuron subtypes [49]  
447 (Fig. 4B). In an elegant demonstration of how such an approach could  
448 be used for disease modeling, such a cerebral organoid was modeled  
449 from induced pluripotent stem (iPS) cells derived from a patient pre-  
450 senting with microcephaly, a disorder which has yet to be suitably  
451 reproduced in a mouse model. Such organoids were less developed  
452 than their normal counterparts, with an analysis of the constructs re-  
453 vealing a potential mechanism for disease progression rooted in defec-  
454 tive, premature neuronal differentiation. Proof-of-principle studies  
455 such as this one confirm the promise that patient-derived iPS cells can  
456 serve to better understand disease and to identify potential molecular  
457 targets from a function perspective. In particular, cerebral organoids  
458 could also be further developed to study the blood–brain barrier and  
459 help overcome the difficulties in delivering pharmacological agents  
460 into specific areas of the brain. Such an approach, which would require  
461 at least some measure of vascularization, could potentially be achieved  
462 within a co-culture system, and could potentially identify novel molec-  
463 ular paths to entry or specific regions of the brain more sensitive to drug  
464 delivery. 465

##### 466 4.3. Liver organoids

467 Indeed, such a co-culture system has been employed in an approach  
468 focused on generating a liver organoid. A liver bud exhibiting similar  
469 markers to its *in vivo* counterpart was generated after aggregation of  
470 three cell types at very high cell densities (human umbilical vein endo-  
471 thelial cells (HUVEC), human mesenchymal stem cells (MSC) and iPS  
472 cell-derived hepatic cells) and embedding in Matrigel™ [50]. As in the  
473 neural and intestinal systems, self-organization occurred within the  
474 organoids; here a notable advance involved the additional development  
475 of nascent endothelial networks, which, helped by MSCs thought to  
476 function as a source of pericytes promoting vessel stability [51], allowed  
477 the limb bud to integrate into the host vasculature when implanted into  
478 ectopic extrahepatic sites in a mouse. Within two months *in vivo*  
479 these organoids matured and resembled adult liver histologically, and  
480 had developed bile canaliculi (though not bile ducts). Importantly,  
481 multiple transplanted liver organoids were able to rescue mice  
482 from subacute gangyclovir-induced liver failure. As with other  
483 newly developed *in vitro* organoid systems, a fully mature miniatur-  
484 ized organ with a complete set of functional features has not yet been  
485 achieved, but it can be speculated that a better understanding of  
486 complex signaling pathways involved in establishing morphogene-  
487 sis, co-culture approaches and longer maturation times could lead  
488 to the types of function seen here in this case after *in vivo* implanta-  
489 tion. Still, even immature human-cell based organoids such as the  
490 one depicted here, may be more responsive and predictive of acute  
491 liver injury which is difficult to detect in the course of the current  
492 drug discovery pipeline. 492

493 The strikingly complex organoid model systems described here rep-  
494 resent only a selection from the rapidly expanding organoid literature.  
495 Indeed, there have also been in the last year reports of such diverse  
496 organoids as the pituitary gland [52], inner ear [49], pancreas [53,54]  
497 (Fig. 4C) and hair follicle [55]. For all these systems, whether derived  
498 from single cells or from pre-aggregated pluripotent stem cells, the  
499 overarching feature has been a significant level of self-organization  
500 over time mediated by a 3D matrix.

501 **5. ECMs and 3D screening: towards synthetics and scalable approaches**502 **5.1. Engineering better hydrogel systems**

503 A number of challenges present themselves in the pursuit of an  
 504 effective translation of these organoid culture systems from an academ-  
 505 ic laboratory proof-of-concept to the kind of robust and reliable assay  
 506 required for a drug discovery program. The first of these challenges is  
 507 the need for a reproducible, well-defined and scalable 3D gel system. In-  
 508 deed, all the organoid cultures presented in the previous section made  
 509 use of the commercially available Matrigel™ system, which is an extract  
 510 from Engelbreth–Holm–Swarm (EHS) mouse sarcoma, a tumor rich in  
 511 ECM proteins. Its main component is Laminin-1, an abundant extracel-  
 512 lular component found in basement membrane, while other compo-  
 513 nents of Matrigel™ include a mixture of collagen IV (30%) and  
 514 entactin (6%) as well as heparin sulfate proteoglycans and a variety of  
 515 growth factors in varying proportions, including transforming growth  
 516 factor beta (TGFβ), epidermal growth factor (EGF), fibroblast growth  
 517 factor (FGF), tissue plasminogen activator, as well as residual matrix me-  
 518 talloproteinases (MMPs) and growth factors occurring naturally from in  
 519 the tumor [56]. It appears clear that the organogenic bioactivity of this  
 520 matrix therefore derives both not only from its three-dimensionality  
 521 but also from the complex mixture of signaling cues. Yet, Matrigel™ re-  
 522 mains a natural extract with relatively imprecise composition and un-  
 523 quantifiable batch-to-batch variability, rendering it unsuitable for  
 524 reproducible and large-scale assays. Furthermore, Matrigel™ presents  
 525 a number of practical limitations in handling and processing: it re-  
 526 quires careful manipulation and must be maintained at constant  
 527 cold temperature throughout cell-encapsulation processes, which  
 528 is incompatible with current implementations of large-scale  
 529 robotics, which have generally been adapted to handle cell culture  
 530 reagents within a different temperature range. Matrigel™ also  
 531 tends to have a widely variable degradation profile, which, depend-  
 532 ing on handling procedures as well as cell-mediated responses, can  
 533 partially degrade in an uncontrolled manner within the time span  
 534 of the assay.

535 Thus, to begin to consider the implementation of organoids as suit-  
 536 able in vitro drug discovery tools, a better-defined matrix which  
 537 would minimize or preferably completely eliminate animal-derived  
 538 components as well as be easy to handle and reproducibly degraded is  
 539 necessary. While other purified natural materials such as alginate and  
 540 collagen I have also been shown to support cell encapsulation, it is  
 541 doubtful whether such materials could allow for the complex morpho-  
 542 genesis seen in Matrigel™, due to their relatively poor abilities to be  
 543 functionalized with additional required signaling cues and lack of inde-  
 544 pendent modulation of structural and chemical properties.

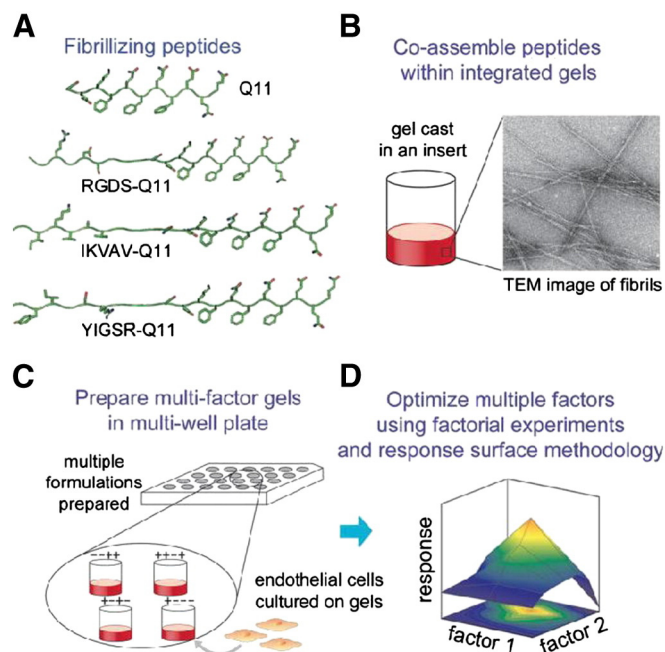
545 Currently, synthetic and highly tuneable approaches to materials en-  
 546 gineering can provide hydrogels with the versatility and consistency  
 547 which would be required for large-scale compound screening  
 548 (reviewed in [12,57]). Such artificial ECMs can allow the experimenter  
 549 to determine in an independent and highly reproducible manner both  
 550 the physical and biochemical properties of such matrices. For example,  
 551 poly(ethylene glycol) (PEG)-based hydrogel approaches allow us to  
 552 create biophysically active materials. These materials allow for mod-  
 553 ulation of mechanical properties as well as cell-mediated degrada-  
 554 tion in response to matrix metalloproteinase (MMP) secretion [13,  
 555 58,59]. For example, in a model of epithelial ovarian cancer, it was  
 556 shown by exploiting the design flexibility of the hydrogel character-  
 557 istics, that proliferation in 3D was dependent on cell-integrin en-  
 558 gagement and on the ability of cells to proteolytically remodel their  
 559 immediate extracellular microenvironment while maintain hydrogel  
 560 stability in long-term culture [13]. Additionally, it is possible to  
 561 chemically tether instructive ECM-derived signals directly onto the  
 562 PEG backbone, thereby modulating the biochemical microenviron-  
 563 ment. Indeed, by engineering protein or peptide constructs compat-  
 564 ible with the chosen cross-linking system [60–62], it is possible to

build a material toolbox whose elements can potentially be used in 565  
 a set of combinatorial rearrangements [63]. 566

502 **5.2. High-throughput approaches for microenvironment optimization** 567

While Matrigel™ presents multiple and potentially interacting cues, 568  
 defined synthetic matrices would need to be precisely tuned to achieve 569  
 the required optimal properties. In such a reductionist and defined ap- 570  
 proach, the faithful recreation of organoid microenvironments would 571  
 have to go beyond the “one-size-fits-all” approach. A Matrigel™-like 572  
 organ-specific synthetic analog would certainly require an as yet un- 573  
 known combination of biophysical properties and biochemical signaling 574  
 cues, and it would be necessary to establish a screening paradigm to sys- 575  
 tematically identify unique microenvironments which would optimally 576  
 support robust and reproducible organoid development. 577

A number of approaches have been proposed to begin to assess the 578  
 effect of large-scale combinatorial biomaterial libraries on cell behavior. 579  
 The repurposing of DNA microarray printers to produce what have been 580  
 termed “cellular microarrays” has been a particularly popular techni- 581  
 que. In one implementation, a combination of synthetic polymers 582  
 with different material properties including wettability, surface topog- 583  
 raphy, surface chemistry and elastic modulus were arrayed onto glass 584  
 slides and were assessed for their ability to maintain human ESC self- 585  
 renewal [64]. In another example, ECM proteins and soluble factors 586  
 were assessed in combination to determine optimal conditions for pri- 587  
 mary hepatocyte maintenance and early hepatic differentiation of 588  
 ESCs [65]. These platforms focused on directly functionalizing micro- 589  
 scope slides have relied on cell adhesion to provide a cellular readout. 590  
 Other approaches have focused on the creation of structured microwell 591  
 arrays which served to entrap cells and could for example track individ- 592  
 ual cell fate in a more precise manner via time-lapse microscopy, and 593  
 platforms where the simultaneous physical and biochemical properties 594  
 of the matrix (*i.e.* hydrogel substrate stiffness and surface protein 595



**Fig. 5.** Modular 3D scaffolds enable multifactorial experimentation [68]. Different ligand-bearing peptides were synthesized (A) and co-assembled into fibrillar hydrogels (B). HUVECs were cultured in combination of gels with different combinations of peptides (C). Full factorial experiments and response surface methodology were used to explore the direct and interactive effects of various immobilized ligands on cell growth, and optimum formulations that maximized proliferation were identified (D). Reproduced by permission of RSC Publishing.



functionalization) that could be modulated have enabled truly multifactorial explorations of extrinsic microenvironmental control [66].

### 5.3. Challenges in implementing engineered organoids

While much can be learned from the combinatorial technologies seen above, a major limitation has remained that only adherent cells or cellular aggregates such as neurospheres [67] in liquid media could be assayed in such systems. Despite technical difficulties which have hampered the use of high-throughput combinatorial studies in 3D, the deployment of rational approaches based on design of experiment (DOE) methodologies has proved to be instructive in ways to assess a combinatorial space for 3D cellular response. In one notable example, multiple peptide ligands were incorporated into engineered self-assembling peptide hydrogels. An iterative process consisting of single-factor experiments for setting initial bounds followed by factorial experiments for identifying main effects and interactions between ligands served to identify previously unknown antagonistic interaction between the laminin-derived peptide mediating HUVEC cell attachment and growth. In a final step, response surface methodology experiments were carried out to identify optimal formulations of these ligands, which led to endothelial cell growth equivalent to that on native full-length fibronectin [68] (Fig. 5).

Ultimately, once such synthetic matrices would be defined for an organoid of interest, it is possible that such artificial extracellular microenvironments could be widely deployed, either in standardized formats using liquid handling robots, or via miniaturized arrays such as the ones described for toxicity testing [69]. With organoid morphology and function being significantly more complex than spheroids or single cells, one important challenge will be in the systematic assessment of function. More advanced readouts would have to be developed to address these issues; while confocal microscopy currently provides the standard imaging tool for assessing cellular function within such constructs, it is certainly limited in throughput. As such, newer techniques currently being developed for whole animal imaging allowing for rapid and highly accurate scanning of large areas, such as light sheet fluorescence microscopy or high-resolution optical coherence tomography could be deployed in this context. Light-sheet microscopy, where only the fluorophores in the light sheet's plane contribute to the image, is particularly useful in reducing out-of-focus blur from three-dimensional samples [70]. Additionally, a technique known as biodynamic imaging, which uses short-coherence dynamic light scattering to evaluate intracellular motions [71], has recently been specifically applied to study the multicellular cancer spheroid model. However, the key for such promising new imaging technologies to gain wider application will be to modify them in appropriate ways in order to conform to the standardized formats and throughput required in the drug discovery process. Furthermore, image analysis tools focused on simple cellular morphologies would have to be adapted and customized to yield relevant and quantitative data. Beyond imaging, a number of multiplexed techniques could also be used to interrogate for functional outputs and to maximize sample value. For example, Luminex/XMAP bead-based assays can be used to detect hundreds of proteins or genes of interest within a single sample, while gene expression can be assessed by such technologies as the Fluidigm qPCR gene expression profiling system [72] as well with a battery of increasingly inexpensive sequencing technologies. Today, some pharmaceutical companies are actively.

## 6. Conclusions and outlook

Numerous studies have now shown the advantages of 3D cell culture, in particular in the context of the multi-cellular tumor spheroid model, with notably different drug responses compared to 2D contexts which in some cases compare favorably to the *in vivo* observations. Such 3D cultures have also been used to demonstrate more physiological responses in other contexts such as migration, invasion, angiogenesis and

lymphangiogenesis, as well as in toxicology. We have proposed in this review that complex self-organized organoids, which have recently come to the fore as striking proof-of-concept examples of *in vitro* developmental biology, could be appropriate test platforms for future drug discovery efforts. Indeed, such miniaturized proto-organs could be used as a significant validation bridge between primary high-throughput screening and costly animal and human trials. Safety and efficacy of lead compounds could be tested directly on *in vitro* organs for the target pathology. It could also be imagined that in the context of oncology, cells and tumor response could be assessed not only in isolation but in a more realistic co-culture system within an organ of interest. Ultimately, such organoid systems could be systemically linked in order to begin to build a "human in a dish" as a technology with unprecedented fidelity to human disease and drug response. Today, the promise of such transformative advances is largely limited by issues of historical importance in the drug discovery process: reproducibility, standardization, validation and quality control. For these far-reaching objectives to be accomplished and for such technologies to move from the university laboratory to a broader use in the commercial drug discovery process, we have emphasized the need to focus on implementations based on synthetic and tailored 3D matrices amenable to medium to large-scale automation, as well as meaningful multiplexed readouts.

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