Phenotypic screening with 3D cell-based assays

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chapter 2

Getting the most out of 3D cell based assays with high content image analysis and phenotypic profiling

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Abstract

Introduction of more relevant cell models in early pre-clinical drug discovery, combined with high-content imaging and automated analysis are expected to increase the quality of compounds progressing to pre-clinical stages in the drug development pipeline. In this review we discuss the current switch to more relevant 3D cell culture models and associated challenges for high-throughput screening and high content analysis. We propose that overcoming these challenges will enable front-loading the drug discovery pipeline with better biology, extracting the most from that biology and in general, improve translation between *in vitro* and *in vivo* models. This is expected to reduce the proportion of compounds that fail *in vivo* testing due to lack of efficacy or due to toxicity.

Introduction

Declining drug success rates and increasing costs suggest that alternative strategies are required in early drug discovery. Traditional drug discovery has favoured a target-based approach where drugs were selected to manipulate a single molecular target. Since good targets are often not identified or inhibition of single targets is often not sufficient for effective therapy, phenotypic screening represents an alternative approach that has proved successful in recent years.[1-3] Phenotypic drug screening techniques typically combine simple single end-point measurements, such as cell viability, with 2D monolayer cultures of cell lines. Such pleiotropic endpoints limit their sensitivity and selectivity for the most promising drugs.[4] Furthermore, cells cultured as a monolayer often respond differently to drugs compared to native tissues.[5]

Reasons underlying the aberrant responses of 2D cultured cell lines compared to tissues include the grossly distorted architecture of cells stretched on rigid plastic, the absence of natural ligands with which to attach to and the lack of multiple different cell types found within a tissue that are typically closely intertwined to regulate cellular behaviour. These cellular interactions can dramatically influence cell differentiation and signalling and thus the ability to accurately recapitulate the situation in the body. For example, cancer cells grown as a monolayer have a deregulated cell cycle, often doubling every 24 hours, while tumours *in vivo* typically show only a few percent of actively cycling cells and only have a marginally higher rate of proliferation compared to healthy tissue. As a result, cancer drugs selected on this basis often show adverse effects in healthy tissues. These problems indicate that different, more biologically-relevant strategies, will be more effective in developing successful medicines.

Three-dimensional cell culture models simulate aberrant tissue organization in pathology

Over the last three decades or so, three-dimensional (3D) cell culture techniques have been developed that have resulted in models that more accurately mimic physiological and diseased states than their 2D counterparts.[6-11] These have the potential to provide a more physiologically relevant context for drug screening, as culturing cells in a 3D environment allows the formation of complex multicellular micro-tissues or organoids that display cell-cell and cell-matrix attachment that drive differentiation and normal tissue function.[12-19]

The resulting biological complexity of these multicellular micro-tissues makes them particularly well suited for phenotypic drug discovery. Traditional end-points, such as proliferation and viability can be combined with 3D assays – either using biochemical assays or specific fluorescent labels.[20] But just as modern histopathology relies on a diverse range of cell and tissue archtectural characteristics of patient material for decision making, maximum leverage of the more complex biology of 3D-cultured tissues can also be gained from analysis of diverse morphological characteristics. This can be of particular value when aberrant tissue organization is directly associated with pathology, for example, with neurodegenerative disorders,[21-22] tissue fibrosis,[23] cancer,[24-27]

and ciliopathies such as polycystic kidney disease (PKD).[28-29] In the context of these diseases, 2D cultured cell lines fail profoundly to capture properties critically associated with the pathophysiology. The modelling of cystopathies is a particularly clear example since cysts, such as those formed in the kidneys of PKD patients, are 3D structures that cannot be recapitulated in 2D cell cultures. Therefore, mechanistic studies and compound efficacy testing can only effectively be studied in a 3D environment or *in vivo*. Similarly, to evaluate tumour dysplasia and invasion, 2D cell cultures lack the required physical environment. These and many other examples underscore the need for the more disease-relevant 3D cell culture models (Figure 1).

Front-loading the early *in vitro* stages of drug discovery with more disease-relevant biological models will inevitably increase the quality of molecules entering the pipeline.[30] A more faithful *in vitro* representation of the pathways and processes in disease *in vivo* will improve drug testing even with simple end-point measurements such as cell viability. However, maximum potential of 3D cultured tissues can be realized by exploiting the phenotypic complexity with high-content endpoints.

3D cell culture models for high-throughput screening

Many different options to culture cells in 3D have emerged, each with specific limitations and advantages for evaluation of compound effects.[31-35] 3D culture techniques often make use of immortalized cell lines due to ease of culturing and relative lack of heterogeneity and, while convenient for high-throughput screens, these cells may not accurately represent tissues, since these generally require the interaction of multiple cell types for normal function. This problem may be circumvented by introduction of co-cultures,[36] as has been shown for different co-culture systems.[37-39] However, co-culture systems also introduce an increased level of complexity to the culture system, which can be undesirable for high-throughput screens. For example, cell ratios and cell culture media require optimization to support growth of both co-cultured cell types to obtain functional tissues.[36, 38] It may only be worth considering this approach if the interaction between the co-cultured cell types is of particular significance for the disease, such as the interaction of fibroblasts and epithelial cells in fibrosis.[40-41]

Additional improvement of the relevance of cell models can be gained by the incorporation of primary cells obtained from specific tissues.[12] However, as these can only be passaged a few times before they cease proliferating, their capacity to develop into functional tissues is limited. Furthermore, the cost, logistics and lack of prior characterization of patient tissues limits their suitability for *in vitro* testing.[36]

Induced pluripotent stem cells (iPSCs) are an attractive alternative to the direct use of primary cells in screening, since iPSCs can be generated from virtually any adult cell type reprogrammed with a combination of transcription factors (e.g. Oct4, Sox2, Klf4 and c-Myc[42]). The resulting pluripotent stem cells can be differentiated to generate a desired tissue type. As a result, iPSC-derived tissues have been used to model a variety of different diseases[43] such as cardiovascular, neurological[44] and hepatic[45] disorders.

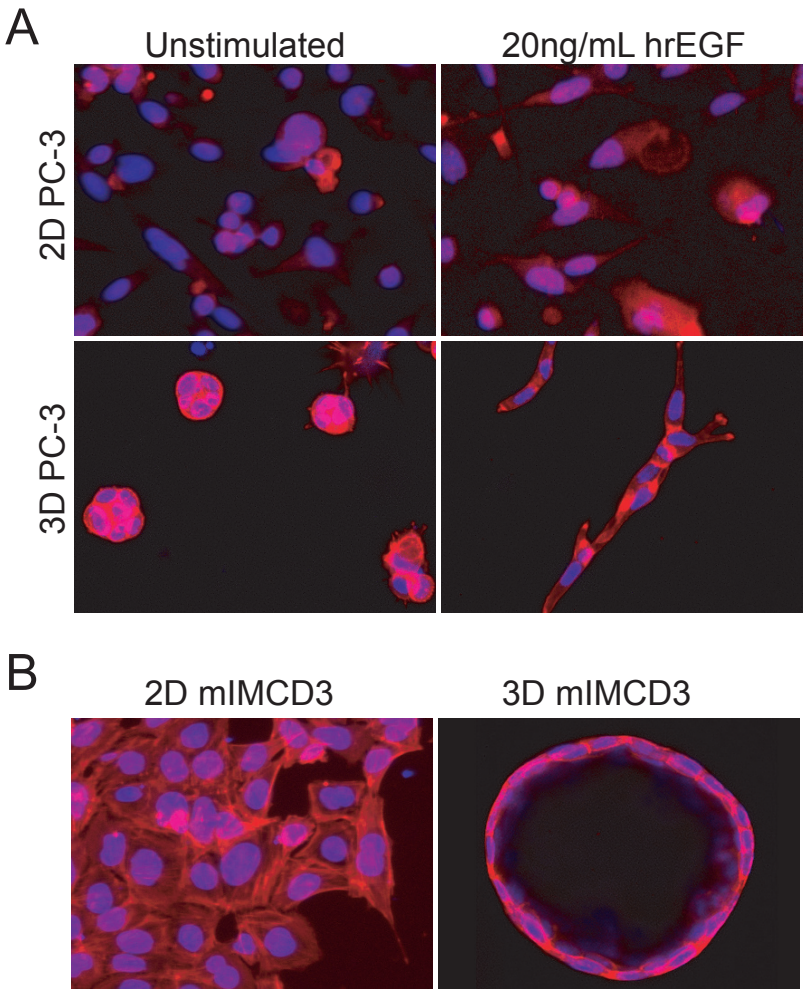


FIGURE 1 3D cell cultures provide a more physiologically relevant context for drug screening. **A)** Prostate Carcinoma (PC-3) cells cultured as 2D monolayer (top) or embedded in 3D hydrogels (bottom) display differential morphology and response to growth factors.[75] Images in top panel obtained using wide-field BD pathway 855 with a 10x objective and images in bottom panel obtained using a Nikon Ti Eclipse confocal microscope with 20x objective. **B)** mIMCD3 cells transduced with a short-hairpin targeting *Pkd1*, deactivation of which is responsible for cyst growth in polycystic kidney disease, cannot form cysts in 2D culture conditions (left panel, BD pathway 855 with 10x objective), whereas they can in 3D hydrogels (right panel, Nikon Ti Eclipse confocal microscope with 20x objective). F-actin (rhodamine-phalloidin), red; Nuclei (Hoechst 33258), blue.

Although the popularity of using iPSC-derived tissues in high-throughput screens is rapidly increasing, significant hurdles for routine use of iPSCs for this purpose are still posed by extensive differentiation procedures that are required and also the possibility of incomplete differentiation.[46] In addition, slow growth[47] and challenging culture conditions can complicate screening procedures.[48] Interestingly, because 3D culturing of iPSC-derived tissues is known to facilitate rapid reprogramming,[49] growing iPSC-derived tissues in 3D assays may overcome some of these hurdles.

In the context of neoplastic disorders, an attractive possibility is the use of patient derived xenograft (PDX) tumour material as a source of cells for 3D culture assays.[50-52] These tumours are typically well characterized genetically with respect to drug sensitivity *in vivo* and the availability is not restricted as with primary patient tumour material. Practically, dissociated tumour cells can be allowed to reform as tumour spheroids in extracellular matrix hydrogels for the screening of small molecules and biologics (figure 2). The use of PDX derived tumour material for *in vitro* tests also offers the possibility to subsequently test compounds in the autologous *in vivo* model. Such approaches are expected to improve the concordance between *in vitro* and *in vivo* data although to what extent remains to be established. Recent advances in tissue culture technology have also enabled the generation of 3D organoid cultures of normal and diseased tissues from stem cells derived from tissue biopsies. Studies on panels of patient derived organoids have shown that these can preserve the histology and genetic profile of the primary tissue and maintain an additional level of physiological relevance by forming more complex structures comprised of cells with different functions.[9-10,53] While expansion of these tissue cultures is demanding compared to standard cell lines, they can still be used for compound screening.[54]

Despite a number of successful studies showing the practical implementation of 3D cultures in routine screening,[55-57] adoption of these model systems in routine drug discovery pipelines has been slow. Generally, high reagent costs and low-throughput experimental procedures have long hampered the development of high-throughput screening platforms, and as a result, 3D cultures have mostly been used for small-scale experimentation and validation with single end-point measurements, rather than for primary screens. Although several technical challenges remain, the appearance of a wide range of new reagents, technologies and published methods have resulted in increasing adoption of 3D cultures for compound screening and testing.

Matrix composition and automation

To provide a physiologically relevant context for 3D-cultured micro-tissues to develop and interrogate the effects of compounds, a micro-environment is required that provides cells with mechanical and physical interactions that normally occur *in vivo*.[58] For this purpose, scaffolds have been used that can mimic the extracellular matrix (ECM).[59-61] The most commonly used scaffolds include hydrogels, which can be natural, synthetic, or a combination.[62] Natural hydrogels are animal-derived basement-membrane (BM) extracts, which have fixed chemical and physical properties, but an un-

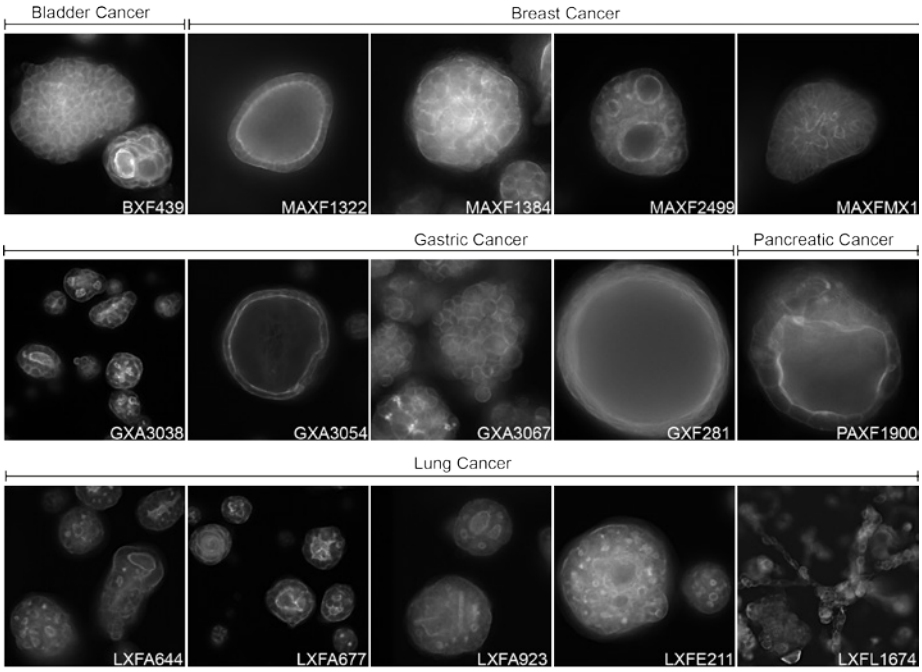


FIGURE 2 3D cultures of patient-derived xenograft (PDX) material. PDX material from different tumours can be cultured in 3D hydrogels to form complex micro-tissues that can be used for compound screening in a physiologically- and patient-relevant context. Actin cytoskeleton visualised with rhodamine-phalloidin. Image materials provided by OcellO B.V. (Leiden, the Netherlands).

defined composition that varies between batches with unforeseen consequences. Examples of such natural hydrogels are collagen or the laminin-rich extracts produced from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (Matrigel). These gels contain many endogenous factors that can support viability of cell cultures.[63] Synthetic hydrogels, in contrast, are well-defined and can be readily modified and manufactured, thereby overcoming many problems associated with natural hydrogels. However, synthetic hydrogels often do not support cell growth since these lack endogenous factors and first require remodelling to support cell adhesion and other cellular behaviour.[64] In order to adequately recapitulate the natural microenvironment, these gels need to present various different ligand types at different densities.[65] Probably most critical is the absence of functional laminin mimetics which are required to engage at multiple sites on the laminin binding integrins[66] and subsequently drive differentiation.[67] Cells that grow under conditions where integrin-mediated interactions with the extracellular environment are compromised, such as in synthetic hydrogels, but also in hanging-drop, suspension media, or ultra-low adhesion systems, fail to differentiate adequately or require extended culture periods to enable secretion of endogenous ECM proteins to support differentiation.

Automation of liquid handling for 3D culturing techniques is a more technical challenge that can hamper the adoption of 3D micro-tissues in primary high-throughput screens. While liquid handling for suspension media and ultra-low attachment microplates can be conveniently automated, this can be challenging for more viscous liquids such as collagen- and Matrigel-containing hydrogels.[68] The polymerization of these gels is typically temperature sensitive, requiring extensive environment control to avoid premature polymerization and rapid liquid handling to avoid blocked pipette tips. While automation of 3D culturing techniques can often be achieved for 96- or 384 well plates, further miniaturization may be problematic due to pipetting of smaller volumes.[69]

Sample preparation

Additional challenges arise due to the environment in which cells are cultured. For example, for the detection of fluorescent signals, or for absorption measurements, the culture matrix often interferes with measurement, and this can be especially important for colorimetric measurements of cell viability or proliferation. Also, protein- or RNA/DNA sample preparation techniques are often not compatible with the use of natural hydrogels that contain many endogenous factors, as the presence of matrix proteins can interfere with antibody labelling of protein or purification and detection of RNA and DNA.

Alternatively, the specific composition of the matrix can interfere with free diffusion of certain compounds, especially large molecules, such as antibodies, or molecules that bind to ECM-proteins. For sample preparation, this means that standard procedures for immunofluorescent labelling have to be modified to allow sufficient time for diffusion of antibodies through the gel. Similarly, washing steps need to be prolonged to allow excess antibody removal. Importantly, these properties of gels can also mimic specific physiological processes, such as poorly perfused tissues.

Developments in 3D culture reagents and liquid handling technology will help to overcome these challenges and the adoption of 3D cell cultures in high-throughput screening, will inevitably continue to grow.

Phenotypic profiling of 3D-cultured micro-tissues

High throughput screens typically use single-endpoint measurements for hit selection, such as cell viability or a single fluorescent reporter. This can compromise the quality of the selected hits, since only a narrow view of the cellular response to a treatment is reported. Automated microscopy and image analysis enables multiple features to be measured and allows a better differentiation of biological responses. The greater morphological complexity of tissues cultured in 3D make this type of high content analysis particularly valuable, retrieving rich information that would be overlooked by single end-point assays. The fluorescent reporters in 2D assays can also typically be incorporated in the 3D assays. Recent years have witnessed the development of (ultra-)high content phenotypic screening and multiparametric analysis techniques that can fully exploit the complex cellular response patterns to classify compound effects.[70-74] While currently used extensively for 2D cultured cells, high-content screening of 3D cell-based

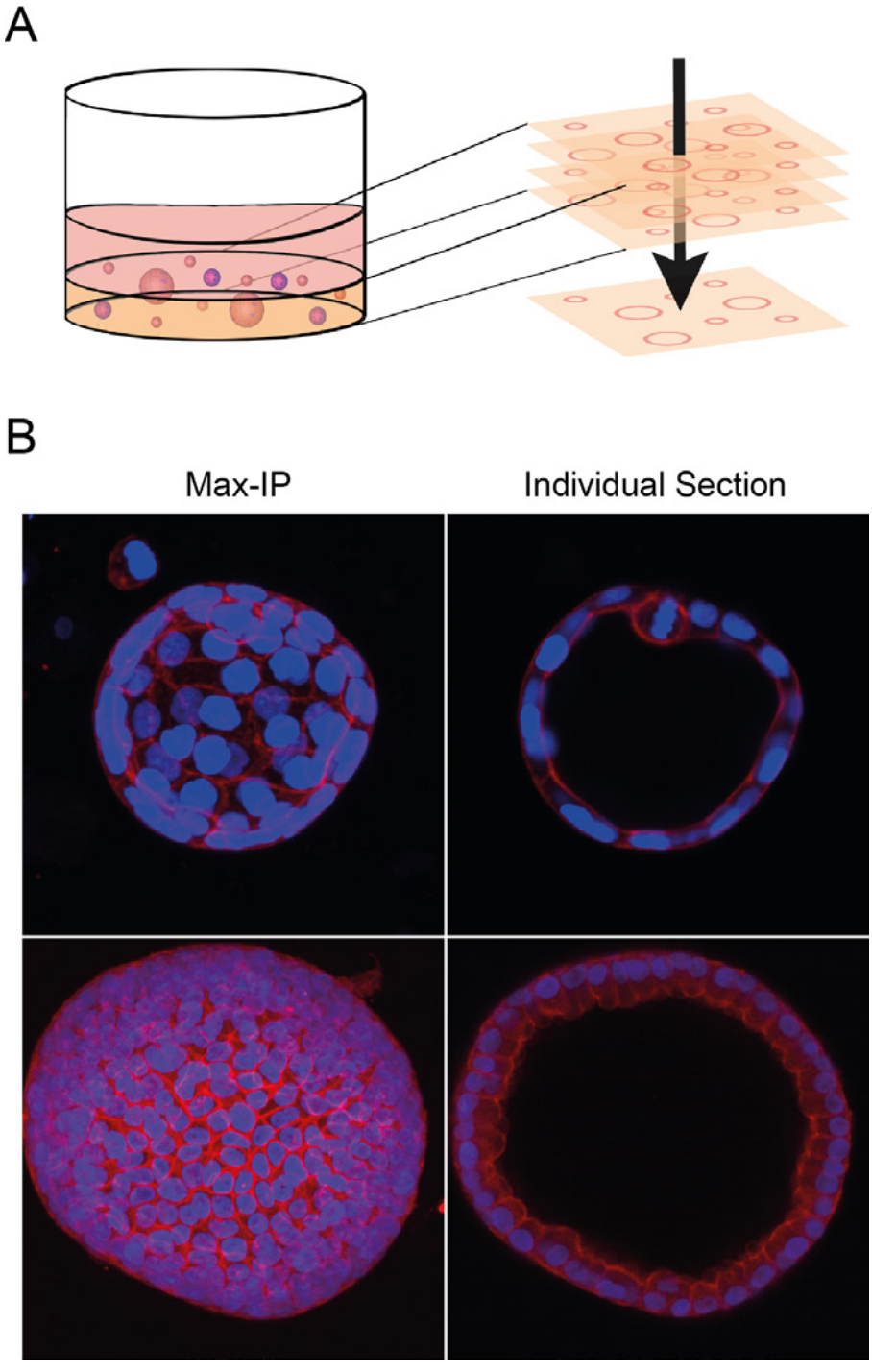
assays presents challenges for imaging, image analysis, computation and data storage and also data visualisation.

Imaging of 3D-cultured micro-tissues

To analyse cellular phenotypes, fixed and stained cultures are typically imaged using conventional wide-field or confocal fluorescence microscopy. Because single *xy*-images taken from gel-embedded micro-tissues capture only a fraction of the objects in a well (with the majority captured in a sub-optimum plane), to retrieve sufficient information from 3D cultures it is necessary to capture a series of *xy*-images at fixed steps in the vertical direction using automated microscopes,[75] to obtain a *z*-stack from each well (Figure 3A). Although the entire well of a 384-well plate is typically captured with a 4x objective, stepping up to a 10x lens to capture more (sub)cellular detail multiplies the number of *xy*-fields and *z*-planes required to capture the same number of objects – increasing the image capture time perhaps 10-fold. Using multiple fluorescent channels similarly multiplies image capture time. Wide-field fluorescence imaging can speed up image capture time compared to confocal imaging, but requires post-imaging deconvolution to reduce out-of-focus signal.

Since 3D cultures in multi-well plates require the capture of multiple *xy*-images, often with multiple image channels, data volumes can rise considerably. For example, a 384 well plate of 3D cultures imaged with a 4x lens can typically yield 50GB-100GB of image data. Maximum focus or –intensity projection algorithms are available in several software packages such as ImageJ[76] and CellProfiler[77] and convert 3D image stacks to 2D images, dramatically reducing data volume and the complexity of analysis (Figure 3A). However, collapsing a 3D image stack to a single *xy*-image results in a significant corruption of architecture, mis-measurement of objects blended from different *z*-planes and loss of the spatial association of objects between fluorescence channels, compromising co-localisation measurements; analysis of intact 3D image stacks is necessary to retain this phenotypic information (Figure 3B).[78-79] 2D cell cultures typically provide thousands of cells for phenotypic analysis. 3D cultures, however, often only provide one object (in the case of spheroids generated using the hanging-drop technology or ultra-low attachment plates[20]) or perhaps a hundred objects per well (cells embedded in gel) for analysis. Low object numbers, coupled with heterogeneity of cell seeding and growth, can be potentially problematic when measuring single-endpoints such as cell viability. Multiparametric high-content analysis can overcome these problems by allowing for normalization to object (spheroid) number and can additionally exploit heterogeneity to study the effect of treatments on specific cellular subpopulations.[78-79]

While it is clear that adding a third dimension increases the image capture and computational demands, including live-cell 3D imaging in a multi-well screening format pushes the demands beyond the capacity of the available technology. However, such techniques could provide valuable information on tissue dynamics over time in more relevant biological systems.[80] With advances in image analysis software and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‹ FIGURE 3 Maximum-intensity projections can cause loss of phenotypic information in 3D cultures. A) Schematic representation of 2D maximum-intensity projections modified from Booij et al, 2016.[75] Structures embedded in hydrogels are captured in xy and z directions using automated microscopy and in-focus regions from all sections are projected into a 2D reconstruction. **B)** 2D projections from 3D structures can cause loss of important phenotypic characteristics. These images display human kidney cyst-derived organoids, cultured in Matrigel and stained for F-actin (rhodamine-phalloidin, red) and nuclei (Hoechst 33258, blue) and imaged on a Nikon Ti Eclipse confocal microscope. Maximum-intensity projection performed with ImageJ software prevents lumen and cell shape detection.

automated microscopy systems and the increases in computational power, live 3D image capture is also expected to become accessible.

Image analysis and multiparametric end-points

Despite the availability of advanced image analysis tools through software such as ImageJ[76] and CellProfiler,[77] the true phenotypic complexity of 3D-cultured micro-tissues is often not exploited to its full extent.[4] Although often requiring the use of high magnification lenses and multiple *z*-planes when imaging, it is relatively straightforward to capture single-cell-resolution images from cells cultured in a monolayer and apply this in an automated high throughput format. However it is not yet feasible to achieve this with 3D cultures – largely due to the inability of imager software to detect objects 'on-the-fly' and home in for high magnification image capture. However, this may be compensated by the additional features that can be measured from multicellular organotypic structures using lower magnification lenses in a high-throughput format.

For many research questions, a simple parameter, such as spheroid size, may be adequate to discriminate a treatment response. As an advantage of using only a few parameters, readouts are generally simpler to interpret. But the use of a limited number of parameters ignores an abundance of the information that can be extracted from the 3D image stacks. We showed previously that the integration of multiple phenotypic descriptors can improve classification of compounds according to phenotypic response.[79] The analysis of high-dimensional data (often containing over 500 different phenotypic measurements) requires the use of more advanced data processing and visualisation software, such as KNIME, R and Spotfire. As a result of using hundreds of phenotype-derived parameters, it can be difficult to extrapolate individual parameters to biological observations.[81] To integrate high-dimensional data and generate meaningful visualisations, dimensionality-reduction methods such as principal components analysis (PCA) can be useful. PCA linearly transforms high-dimensional data to a space of fewer dimensions, while retaining most of the variance of the data. We have previously applied this dimensionality reduction technique to differentiate between c-Met and epidermal growth factor receptor (EGFR)-specific inhibitors in a cancer cell invasion assay[75] and also, more recently, to identify new potentially drugable targets for PKD.[82]

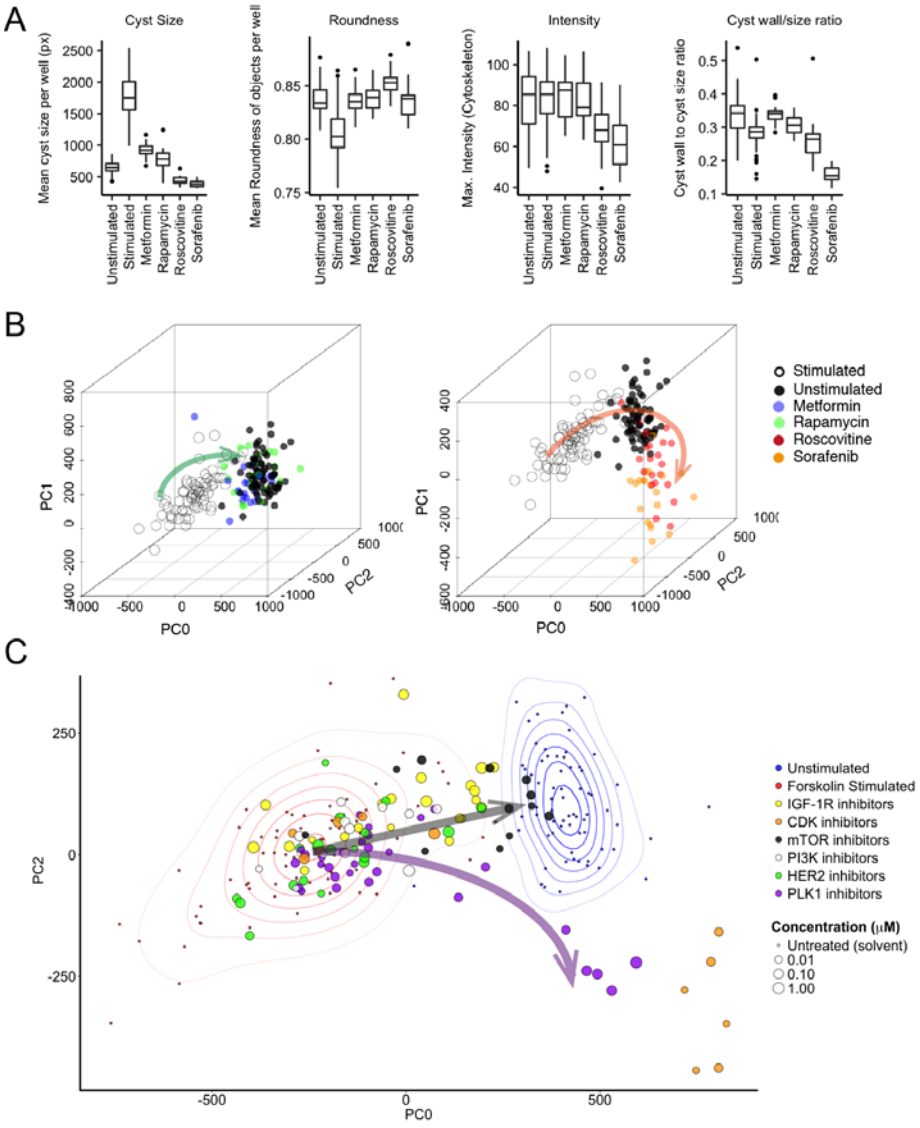

FIGURE 4 Extracting the value of 3D-image stacks with multiparametric end-points. **A)** Metformin, rapamycin, roscovitine and sorafenib inhibit forskolin-induced ('stimulated') cyst growth and roscovitine and sorafenib would be classified as the most potent inhibitors on the basis of single parameters (left to right: cyst size, roundness, perimeter and distance of cyst wall to cyst centre). **B)** Three principal components summarizing 84% of variance in the data show a desirable phenotypic change (left panel, green arrow) from stimulated (empty circles [2.5μM forskolin-exposed large cysts]) to unstimulated (black circles [solvent-exposed small cysts]) for exposure to 5mM metformin + 2.5μM forskolin (purple) or 10nM rapamycin (green) + (legend continues on next page)

2.5μM forskolin. The right panel shows that despite efficacy of roscovitine and sorafenib on the basis of single parameters as shown in A, multiparametric analysis reveals additional phenotypic alterations (orange arrow).[82] **C)** Two principal components from figure B showing inhibitors for cyclin-dependent kinases (CDK), mammalian target of rapamycin (mTOR), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), human epidermal growth factor receptor 2 (HER2) and polo-like kinase (PLK1). Density estimations in red and blue shown to emphasize the locations of 2.5μM forskolin-stimulated and unstimulated controls, respectively). Grey arrow represents desirable compound efficacy (from forskolin-stimulated control to unstimulated) and purple arrow represents novel phenotypes that are observed after treatment with PLK1 inhibitors or high dose CDK inhibitors.

As an example of this approach, in figure 4A we show the efficacy of four control molecules at inhibiting forskolin-induced cystogenesis.[82] On the basis of single parameters such as cyst size and perimeter, all inhibitors show inhibition of cyst growth, with roscovitine and sorafenib being most potent. However, if a PCA-based visualization is used, such as shown in figure 4B,[82] the inhibitory effects of metformin and rapamycin can be discriminated from those of roscovitine and sorafenib, which induce a novel phenotype indicative of toxicity (figure 4B). This type of approach can also be useful in the classification of previously untested compounds (figure 4C).[73, 75, 82]

The use of multiparametric end-points to profile compounds therefore represents an opportunity to extract more information from primary 3D screens and exploit this phenotypic information to better discriminate promising compounds at the earliest stage of the discovery process.

Conclusion and perspectives

We propose that inclusion of biologically relevant *in vitro* model systems early in pre-clinical development will aid in selection of the best drugs, especially when these model systems are coupled to multiparametric phenotypic analysis strategies. Based on the progress in the development of tissue culture matrices, improvement of cell culture techniques and the incorporation of laboratory automation equipment over the past decade, we anticipate a steep rise in the popularity of 3D cell culture techniques in primary high-throughput screens, and also expect a move away from immortalized cell lines in favour of more physiologically relevant iPSC-, PDX-, co-culture- and organoid models, or even *in vitro* -generated and -cultured organs. It is likely that the current switch from single cell lines to more challenging iPSC-, PDX-, co-culture- and organoid models, or even *in vitro* -generated and -cultured organs, will also increase the demand for high-content analysis methods due to increased tissue complexity that cannot be exploited when using well-based measurements.

With the increases in computational power and improvements in data storage, but also now that better image analysis tools are available, we anticipate that morphological analysis of 3D cell cultures will become more accessible and will eventually allow object-based phenotypic analysis and classification, perhaps eventually also allowing 3D-volume analysis of multi-well plates, rather than analysis of image stacks.

However, there currently is a high need to validate these technologies and to demonstrate that using biologically relevant *in vitro* systems actually improves the efficiency of early drug discovery. Rather than waiting to see sufficient evidence, a comparison of the predictive value of 2D and 3D models for *in vivo* efficacy is required. Ideally such an effort should include collections of molecules that have previously passed and failed in pre-clinical and clinical studies to determine the phenotypic footprint of successful medicines, and apply this knowledge in our search for more effective medicines.

Acknowledgments and conflicts of interest

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