

Supplementary Box 1 | A detailed list of SWOT issues

System development**Strengths:**

- **Phenotypic cell differentiation:** 3D multicellular systems are characterized by more accurate phenotypic cell differentiation than 2D cultures^{1,2,3}.
- **Physiological relevance:** It is today well consolidated that 3D model systems are more representative of the architecture and pathophysiology of in vivo tissues including tumours than 2D cell culture assays^{4,5}.
- **Patient-derived models:** 3D models incorporating primary patient tissue biopsies are genetically stable relative to 2D cell line cultures and thus allow personalized treatments and diagnosis^{4,6}.
- **Stem cell models:** 3D cultures of stem cells deliver more differentiated organotypic cultures⁷.
- **Animal model substitute:** 3D model systems can decrease the need for animal experimentation^{8,9}.
- **Biological heterogeneity:** 3D models offer the opportunity of growing several cell types together that will interact in a physiologically relevant manner. For instance, cells of the innate immune system such as macrophages and microglia are known to play crucial roles in several pathologies such as liver toxicity, cancer and neurodegeneration^{10,11,12,13}.
- **Target validation:** Physiologically relevant model systems can be used in low-throughput formats to validate novel therapeutic targets to support drug discovery investments including high-throughput in vitro screening assays.

Weaknesses:

- **Morphological heterogeneity:** The generated 3D model systems are typically shape/size-heterogeneous and this makes the data obtained not perfectly comparable between replicates¹⁴.
- **Size/shape-based viability:** The viability of the cells in the 3D culture is dependent upon the size and the shape of the 3D multicellular aggregates¹⁴.
- **Spatial dependence of physiological state:** The physiological state (“morpho-biological stability”¹⁵) of the generated 3D cell cultures is volume- and shape-dependent¹⁶.
- **Simplified organ models:** Despite resembling characteristics of organs, the 3D model systems available today are generally not fully differentiated and thereby represent just a simplified version of the complexity of an organ^{17,18}.
- **Validation uncertain:** Difficult to validate clinical relevance of the models^{19,20}.
- **No vascularization system:** 3D model systems are typically characterized by the absence of a proper vascularization and relevant immune system resembling in vivo tissue perfusion. This determines a different pattern of viability of the cells composing the 3D model system, based on the distance from the outer surface²¹.
- **Dynamic tuning of tumor ecosystem:** Without a proper fluidics system it is not possible to dynamically change the physical and biochemical properties of the tumour ecosystem²².
- **Long, complicated cell culture protocols:** Most differentiation protocols and 3D cell culture protocols require several weeks of culture and are not fully stable. This requires the establishment of a perpetual production pipeline of the desired cell and reagent components with constant quality control monitoring.

Opportunities:

- **Orthogonal cell type interactions:** Physiological relevance of the 3D model systems increases by mixing distinct cell types to better mimic the cellular heterogeneity of complex in vivo tissues and disease e.g. tumour microenvironments^{23,24}.

- **Ex vivo clinical trials:** Induced pluripotent stem cell and gene-editing technologies enable incorporation of a broader variety of genetically defined disease models which extend in vitro pharmacogenomic studies beyond simple 2D cancer cell line viability assays into more relevant 3D assay formats, across multiple disease areas, to guide personalized medicine strategies²⁵.
- **Fluidic systems:** Micro- and macro-fluidic systems can be used to better approximate in vivo oxygen and nutrients levels. Microfluidics also mimic shear forces, which are important for differentiation^{26,27,28,29,30,31}.
- **Manipulate the extracellular matrix:** With the emergence of hydrogels mimicking the extracellular matrix, it is possible to recapitulate biological processes more faithfully and engineer morphogen sources and sinks³².

Threats:

- **Expensive:** To produce systems at sufficient scale to promote widespread adoption and screening applications is expensive. Cost of complex systems is limiting, especially when incorporating primary cells and organic extracellular matrix substrates.
- **Challenging model validation:** Objective criteria for validation of complex models are lacking. For every application, a clear set of criteria for physiological relevance should be formulated and analysed.
- **Current lack of clinical validation:** There is currently not sufficient evidence of positive predictive value to enable certification by regulatory authorities to support new medicine approvals.
- **Incentives and funding:** Lack of sufficient pressure from translational drug discovery funding schemes, industry and private equity investors to invest in the development and application of new more relevant biological model systems over commercial exploitation of healthcare products/medicines using the well-established traditional preclinical models, which offer short-term speed and cost benefits.

Imaging

Strengths:

- **Current equipment is sufficient:** For small 3D model systems (i.e. multicellular aggregates with diameter < 100 micrometers), the current confocal microscopes yield satisfactory results³³.
- **Clearing protocols exist:** Due to differing refractive indices between medium and tissue, imaging deep into tissue is challenging. SeeDB is an optical clearing protocol compatible with plastic 384 well imaging plates that can easily be adapted to robotic liquid handling stations³⁴.
- **In vivo diagnostic technology analogy:** Similarity between 3D-microscopy solutions and in vivo and/or clinical diagnostic imaging technologies like PET, CT/micro-CT and MRI.

Weaknesses:

- **Limited penetration depth:** 3D multicellular systems are characterized by high density and differences in refractive index that perturb the penetration depth of light and the opportunity to visualize very large spheroids³³.
- **Limited holder solutions:** Today there is a very limited number of solutions available for holding 3D spheroids to be imaged by light sheet microscopy. For instance, there is no “multi-well”-like solution available for HCS.
- **Requirement for object search:** Contrary to 2D cell cultures, which are generally homogeneously distributed, 3D tissues are sparse and rarely in wells of a 384 well plate. There is therefore a requirement to first identify the location of the objects with a low resolution objective

to then carry out high resolution imaging. This requires more expertise and image processing knowledge from the experimentalist.

- **Bleaching of fluorophores:** In conventional widefield or confocal microscopes, the entire volume of tissue is illuminated and the fluorophores are activated leading to bleaching, especially in the last planes of an acquisition stack. Only SPIM imaging activates selectively the fluorophores that are imaged and therefore has the best photon budget and suffers least from bleaching.
- **Multiscale imaging requirement:** When imaging 3D tissues, it is often of interest to both analyse the overall tissue organisation and the subcellular distribution of molecules and organelles. Spatial tissue organisation requires a large field of view with low magnification objectives precluding subcellular resolution, whereas high resolution subcellular imaging typically does not allow global tissue analysis.

Opportunities:

- **Optical physics technology innovations:** Development and investment in academic optical physics to provide broader variety of cost-effective microscopy solutions that promote adoption of quantitative 3D biology for every laboratory.
- **Customized supports:** 3D printing technologies and microfabrication of customized microtiter plate formats and microfluidic devices for image-based sampling of complex 3D tissue culture models.
- **Developing of photostable fluorophores:** New photostable fluorophores such as pDots will be developed in the future³⁵. Also imaging with infrared dyes that penetrate further into tissue will become more common.

Threats:

- **Need for optimized equipment:** For large 3D model systems (i.e. multicellular aggregates with diameter > 500 micrometers), the inner core of the aggregate cannot be visualized using state-of-the-art microscopes^{36,37}.
- **Image quality:** The quality of images of 3D tissues are often poor due to low penetration of dyes and antibodies, due to the optical properties of the tissue and lastly due to the poor resolution in the z-axis of conventional microscopes.
- **Long acquisition times:** 3D stacks take a long time to acquire compared to alternative, faster non-spatial techniques.

Screening

Strengths:

- **High physiological relevance:** Examples: organoid assays that preserve the histological architecture, gene expression and genomic landscape of the original tissue. Spheroid and organotypic assays that recapitulate hypoxic microenvironments and poor drug penetration of in vivo tissue³⁸.
- **Available assays:** Most of the established cell lines and primary cells available for 2D screening assays also work for 3D assay systems and encompass large areas of biological target space^{39,40,41}.
- **Novel 3D tissue models:** New phenotypic screening assays where in vivo models unavailable or deemed unreliable - exemplified by human iPSC neuronal tissue model systems⁴².
- **4D in vitro pharmacology:** New mechanistic insights provided by 4D biology (3D models combined with the dimension of time to enable temporal profiling of dynamic events to advance evaluation of target biology and cell pharmacology).

Weaknesses:

- **Quantification:** Integration of robust quantification of 3D model phenotypes with chemical structure to effectively guide structure–activity relationships and lead identification/optimization programs.
- **Storage capacity:** One or more *z*-stacks of images are typically acquired to analyse a 3D model and thus data storage capacity becomes a bottleneck.
- **Computing power:** Analysing large amount of *z*-stacks is computationally expensive and requires massive parallel computing.
- **Limited access of assay reagents:** For large 3D models, reagents such as antibodies do not penetrate well into the core to support robust assay development.

Opportunities:

- **Intelligent screening:** Given the amount of data to be acquired, intelligent systems that could determine conditions of interest early on and avoid acquired *z*-stacks of objects of little interest would be advantageous. This is a call to develop more sophisticated microscopes and intelligent image-acquisition solutions which operate across *x*, *y* and *z* dimensions.
- **Innovations in biological target space:** Identification and validation of novel target mechanisms under appropriate multicellular 3D biological contexts.
- **Innovations in pharmacological target space:** Discovery of more novel first-in-class hits and novel drug combinations.
- **Clinical-like conditions:** Development of new microfluidic 3D tissue screening platforms incorporating perfusion, gas exchange and physical shearing forces⁴³.

Threats:

- **Speed limit:** Relative to 2D image-based screening assays, speeds for 3D cellular models are significantly lower.
- **Expensive:** Costs per well and costs per compound are often too high for conventional HTS campaigns.
- **Low adoption:** Often poor compatibility with standard screening platforms and lack of standardized assay validation criteria and increased complexity coupled with reduced robustness.
- **In vitro to in vivo translation:** In vivo validation of hits may not be possible were animal models are not available, resulting in lack of confidence and investment to progress into clinical development.
- **Data reproducibility:** Need for a preselection of homogeneous 3D cell cultures and comparable 3D models to obtain reproducible data¹⁴.

Data analysisStrengths:

- **New information-rich data sets:** Multidimensional datasets which link multicellular tissue phenotype, with genotype, and chemical structure to better inform drug discovery investments^{44,45}.
- **Spatial organisation analysis:** Imaging is particularly important as an analytical method for 3D objects as the spatial distribution of cell types, self-organisation of tissue and the location of physiological events is most informative^{46,47}.

Weaknesses:

- **3D parameter understanding:** Difficulty to understand the meaning of parameters in 3D and the biological relevance.
- **Challenging volumetric analysis:** Due to heterogeneous image quality through a large 3D object, the quality of the analysis also varies throughout the object^{33,48}.
- **Ease of use:** In spite of powerful algorithms, there is a lack of user-friendly, intuitive software tools.

Opportunities:

- **Exploit computational biology methods:** Development of highly quantitative 3D phenotypic data using new software tools, including emerging deep learning approaches⁴⁹.
- **Accessible low-cost technology innovations:** i.e. open source image analysis, machine learning, visualization and data downstream analysis pipelines that support distributed parallel processing across clusters/clouds without licence restrictions.

Threats:

- **Limited know-how:** Due to the difficulty of 3D computational methods, many laboratories will not be able to readily access the skills and technology to implement customized 3D data analysis pipelines.
- **Computationally demanding:** Computing resources, including costs, time and hardware capacity required for analysis, are increased due to the size of the dataset.

COMPETING FINANCIAL INTERESTS

P.H. is the founder and shareholder of Single-Cell Technologies Ltd., J.T. is employed by PerkinElmer Ltd. Single-Cell Technologies Ltd. and PerkinElmer Ltd. were not directly involved in the writing of the manuscript. This does not alter the author's adherence to all the Nature policies on sharing data and materials.

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