High throughput physiological micro-models for in vitro pre-clinical drug testing: a review of engineering systems approaches

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High throughput physiological micro-models for in vitro pre-clinical drug testing: a review of engineering systems approaches

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Abstract
To address the low success rate of new drug discovery, there has been significant growth of in vitro physiological micro-models based on human cells. These may be in the form of cell spheroids, organs-on-a-chip, or multi-cellular tissue cultures, and it is expected that the more biomimetic environment they create will be more accurate than standard cell culture in drug screening prior to clinical testing. However, commercial use of complex co-cultures is still limited. This is due to a lack of validation, low throughput rates, and a lack of compatibility with standard assessment techniques. This review paper focuses specifically on the different engineering approaches used to create, mature and analyse these micro-models, with the aim of exploring which approaches have the potential for high throughput. Active and passive pumping and nozzle based dispensing techniques are considered for fluid handling, with transwells, cell patterning, spheroid cultures and microfluidics considered for establishing and maintaining co-cultures, together with conventional analysis techniques (proteomic and genomic approaches, and immunohistochemistry) and novel sensor systems for downstream analysis are considered. It is concluded that (i) throughput is essential for validation as well as exploitation of the models, and (ii) an integrated approach to model re-design for high throughput is key, with the limitations on throughput at each stage considered in order to develop a system which can deliver and analyse at high throughput rates at all stages of the process.

Keywords:
High throughput; drug discovery; micro-tissue model; organs-on-a-chip; microfluidics; cell culture; co-culture
# Table of Contents

1. Introduction ........................................................................................................................................3  
2. Liquid handling ...................................................................................................................................4  
   2.1 Active pumping systems ................................................................................................................6  
   2.2 Passive pumping system ................................................................................................................6  
   2.3 Nozzle based dispensing ................................................................................................................8  
      2.3.1 Automated pipetting system ...................................................................................................8  
      2.3.2 Inkjet and micro-valve systems ..............................................................................................8  
      2.3.3 Multiple-jet systems ................................................................................................................9  
3. Establishing and Maintaining Co-cultures ............................................................................................9  
   3.1 2D Co-Cultures and Transwell Insert Systems ............................................................................11  
   3.2 Cell Patterning .............................................................................................................................11  
   3.3 Cell Spheroids ..............................................................................................................................12  
   3.4 Microfluidic Platforms ..................................................................................................................14  
      3.4.1 Device material selection & functionalization ......................................................................14  
      3.4.2 Throughput enhancement with well-format-based design ....................................................14  
      3.4.3 Enhanced Organ-on-a-Chip Approaches ..............................................................................17  
4. Analysis .............................................................................................................................................19  
   4.1 Adaption of Conventional Techniques for Micro-Tissues ............................................................20  
      4.1.1 Immunohistochemistry .........................................................................................................20  
      4.1.2 Proteomic assays ...................................................................................................................20  
      4.1.3 Genomic assays .....................................................................................................................23  
   4.2 Novel Sensor systems ....................................................................................................................25  
5. Current Position and Outlook ............................................................................................................27  
Reference ...............................................................................................................................................28
1. Introduction

Less than 1 in 10 drug candidates which enter clinical trials are approved by the Food and Drug Administration (FDA). The high failure rate is attributed to two major causes: (i) nonclinical/clinical safety (accounting for >50% failure) and (ii) efficacy (>10%), which current pre-clinical models do not accurately predict[1,2]. To address this problem, fabricated multi-cellular in vitro tissue models are being explored as a new potential pathway for improved pre-clinical models[3,4]. These models can take a number of forms with common approaches utilising cell spheroids[5], lab-on-a-chip (LoC) or organ-on-a-chip type systems[6-8], and multi-cellular tissue cultures[9,10]. Multi-cellular models are considered to offer environments which are closer in structure and biochemistry to the native environment for cells and tissues, and thereby offer the potential to provide a new set of tools for understanding disease and the effectiveness of specific therapies[5]. Such models can be based on human cells, and so may prove more predictive of response in humans than animal models, reducing or replacing the need for animal testing.

The use of fabricated physiological micro-models in the development of therapies or in any clinical context has been very limited for a number of reasons[11]: (i) many experiments have low numbers of models (or $n = 1$), with no dilution series, replications, or positive and negative controls; and (ii) there is little or no compatibility with standard assessment techniques. As biological processes are to some degree stochastic and vary across populations, there is a clear need for biological and technical replicates. If it is difficult to scale up, with the difficulty exacerbated by a reliance on assessment techniques which are not commonly available, then this make the models slow to develop and validate, and low throughput in terms of their ability to provide information on diseases or therapies. The key to unlocking the widespread use of physiological micro-models is the ability to run more models either quicker or in parallel for higher throughput, as this will facilitate rapid development, validation and use of models.

The aim of this review paper is to give an overview of the different engineering approaches used to develop and fabricate multi-cellular models, and to explore which approaches have the potential to bring higher throughput levels across the process chain.

The main phases of multi-cellular tissue model development are (Figure 1):

(i) establishment of the cultures or co-cultures: positioning of cells and other model constituent materials in order to create starting cultures or co-cultures.

(ii) maturation: ongoing culture of cells to allow the starting cultures to mature and provide the required functionality.
(iii) introduction of compounds: this will commonly involve assessment of a candidate drug, but may also involve the introduction of elements designed to stimulate and simulate disease within a phenotypically healthy tissue.

(iv) analysis: may be ongoing or terminal, single stage or multi-stage, but quantitative or qualitative analysis of the effectiveness of a candidate drug is required.

Some specific models may have other stages, such as physical damage to simulate an injury, but these four stages are relevant to the majority of models. Liquid handling is a key underpinning technology for many of the operations in these four stages, and for the “tissue maturation” and “introduction of compounds” stages liquid handling is the primary engineering element. In the sections that follow liquid handling technologies are first considered, with an overview of active and passive pumping and nozzle based dispensing approaches. Techniques for the establishment and maintenance of co-cultures are then considered, covering 2D cultures, transwells, cell patterning, spheroid cultures and microfluidics. Then, prior to a review of the current state of the art and future outlook, techniques for downstream analysis are considered, covering proteomic and genomic approaches, immunohistochemistry and also novel sensor systems, often developed specifically for particular system designs.

**Figure 1.** Flow diagram of key stages involved in physiological micro-model development for drug screening, with the main focus areas of this review in blue

2. Liquid handling

The fluid volumes in multi-cellular models are generally small (from nanolitres to millilitres), and so microfluidic systems have been widely developed and used in the design of such models. The progress of microfluidic technologies for biomedical applications has been the subject of a number of review papers in recent years[6-8,12-29]. They include reviews on the general impact of microfluidics on biomedical research[7,25], perspectives on building of microfluidic devices[12,14,30], cell
(co-)cultures[18,24,31], the development of specific biomimetic living models[21,22,26,29], validation of models in preclinical drug discovery[6,16], high throughput screening[17,20], research and commercialization[13], and end-user perspectives[11]. Fluid delivery systems can be seen as being either active (normally with an external pump), passive (acting under gravity or surface tension) or jetted (using droplets or streams of fluid from pressurised nozzles). Table 1 summarises the characteristics of these different modes of fluid delivery, and the sections that follow outline key exemplars of the different approaches.

Table 1 Characteristics of different delivery modes for microfluidic cell culture

<table>
<thead>
<tr>
<th>Mode</th>
<th>Performance</th>
<th>High throughput possibility</th>
<th>Compatibility to existing techniques</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active pumping system</td>
<td>• Needs external pump and tubing system</td>
<td>Normally based on a complex design of multiplexed channels, can be automated</td>
<td>Normally requires expert operator</td>
<td>[32-35]</td>
</tr>
<tr>
<td></td>
<td>• Allows continuous flow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Pneumatic multiplexing requires an elastic construction material (e.g. PDMS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Passive pumping (including gravity, surface tension or osmosis driven)</td>
<td>• Tubeless</td>
<td>Gravity-driven and surface tension-driven pumping has been demonstrated for high throughput study</td>
<td>Compatible with traditional cell culture equipment, automated pipetting tools</td>
<td>[36-40]</td>
</tr>
<tr>
<td></td>
<td>• Needs no electrical power</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• ‘Open’ system to refill liquid for continuous perfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Unsuitable for dynamic flow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Surface-tension driven is limited to low volume flows (nL/s to µL/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nozzle dispensing</td>
<td>• Needs an open accessible microfluidic chip for fluid deposition</td>
<td>Can be automated and suitable for high throughput</td>
<td>Needs integration of perfusion flow for long-term culture</td>
<td>[41-46]</td>
</tr>
<tr>
<td></td>
<td>• Needs external source to generate fluid stream or droplets</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.1 Active pumping systems

Traditional external flow pumping systems are the most common form of actuation used in microfluidic devices to guarantee a perfusion flow mode[32] (Figure 2A). Syringe pumps in particular have been used for non-recirculatory flow and peristaltic roller pumps for recirculatory flow, connected with the microfluidic chip via tubing. To minimise the number of pumps required, arrays are a common architecture[35,47] (Figure 2B and C). A range of approaches to this technique have been described, including new channel architectures to reduce cross-contamination[33,34].
2.2 Passive pumping system

Passive pumping methods typically include gravity, capillary force, osmosis pressure, and surface hydrophilicity[48]. Structurally simple, passively-driven microfluidic systems have proven to be practical and useful for biochemical analyses, cell patterning, and cell sorting systems due to their simple fabrication, straightforward operation, portability, and low cost[40].

Figure 3 Passive pumping systems of: a) gravity-driven flow[40] and b) its demonstrated example in 96-well perfusion microplate for cell culture[36]; c) surface tension-driven flow and d) an array of 192 microfluidic channels for surface tension driven flow, each with two access ports positioned according micro-titer plate standards[38]; and e) osmosis-driven flow[49].
Gravity based passive systems typically use liquid mini-reservoirs setting at different heights to achieve fluid flow from the higher reservoir to the lower one[37,40,50]. One key advantage of this pumping system lies in that the liquid can be re-filled over time to prolong flow and hence it can be considered as an “open” system to enable long-term studies. Arrays of horizontally-oriented reservoirs can be engineered to achieve a constant flow rate (Figure 3A). The system has been demonstrated in providing flow for multiplexed cell culturing and assaying in a standard 96-microwell plate device (Figure 3B)[36].

Surface tension-driven flow can be generated from the difference in pressure inside drops of unequal volume (Figure 3C), and has been demonstrated[38] for a high degree of parallelization (96–192 channels per array) while retaining basic microfluidic operations including routing, compartmentalization and laminar flow (Figure 3D). Such systems are effective and allow refilling of liquid into the inlet for short-term perfusion; however, it is limited to low volume flow rates (in the range of 30 nL/s to 20 μL/s), making it difficult to perform long-term perfusion studies.

Osmosis-driven flow is induced by a difference in solute concentrations across a membrane that is only permeable to the solvent and not the solute (Figure 3e)[39,51]. The flow rate generated is proportional to the difference in the osmotic pressure across the permeable membrane as well as the contact area of the membrane. The most important characteristic of osmotic pumps is that they can provide very slow, near constant flow rates that can last from hours to days, making them suitable for long-term cell cultures. However, it does require a more involved setup than gravity-driven flow or surface tension-driven flow.

2.3 Nozzle based dispensing

Nozzle based dispensing includes traditional pipetting, but the focus here is on automated methods, including automated pipetting and bioprinting techniques. Bioprinting approaches have been extensively reviewed elsewhere[42,52-55], and the focus here is on the utility of these approaches for fluid and cell dispensing.

2.3.1 Automated pipetting system

The development of automation-compatible and reliable high-throughput technologies is a prerequisite to translate micro-tissue development from laboratory assay to industrial applications. Robotic work stations equipped with standard multiple-channel pipette heads are the most automated liquid handling systems currently available, and are routinely exploited for automated cell culture. Numerous commercial robotic platforms are available[56-62], each having advantages and disadvantages, and in addition there are semi-automated pipettors[63]. Systems may be further automated with liquid level detection[64]. For pipetting systems, the sedimentation of cells and associated variations in dispensed cell number is a challenge.

Pipettes also have a limitation in terms of dispensing small volumes. For smaller volume dispensing Zhou et al.[46] developed a PDMS liquid pipette chip, and focused acoustics droplet ejection has been successfully employed to inject droplets of nanoliter and picoliter volumes from a reagent source plate.
to an assay plate without contact, eliminating the wash step[44,65]. More recently, a novel nanoliter centrifugal liquid dispenser has been developed for introducing nanoliter reagents into microwell arrays[45]. Low cost, home-made robotic workstations, have also been developed for automated fluid delivery system for high-throughput experiments, but subject to potential cross-contaminations between sequential samples[66].

2.3.2 Inkjet and micro-valve systems

Inkjet systems enables accurate deposition of single picoliter drop volumes[67] of a large range of materials, materials in solution or cells in media, under digital control at defined spots on the surface of a wide variety of substrates. Inkjet printing is best suited to low viscosity materials[68]. For cell printing, each inkjet drop will typically contain a few cells, with much research effort directed towards a single cell per drop[69], and the major issue which has to be overcome is cell agglomeration leading to nozzle blocking[70]. Single micro-valves can be used to deposit larger droplets (pL to nL sized) than inkjet, with similar restrictions on viscosity[71]. The microvalve system shows advantages in high throughput printing with higher rates of cell density possible[72,73]. A single inkjet or microvalve is essentially a liquid dispensing system, but post-print crosslinking can allow for the creation of more viscous materials, with the crosslinking typically enabled through UV cure[74] or a reactive substrate[75].

2.3.3 Multiple-jet systems

To enable more complex reactions with inkjets or micro-valves some twin jet systems have been developed. On-substrate reactions with two adjacent microvalves has been used[76,77], and multiple cell types can be delivered to surfaces using multiple jets[78]. A twin piezo system can be used to create arrays of gel droplets[79], and impinging droplets from two micro-valves have been shown to offer the ability to deposit gels with very high cell densities[72]. Gels can offer a more physiological environment for cells, as a hydrated environment, but with mechanical properties replicating those of soft tissues[80]. Micro-valve jetting systems[81] can also create encapsulated spheroids through jetting droplets into a stream of crosslinking solution[81].

2.3.4 Extrusion of cell filled gels

The most commonly exploited bioprinting approach is the extrusion of cell filled gels from a syringe-like container[53]. This can be limited in terms of cell density and deposition speed, and ink formulation is key to achieving good quality output[54]. One approach to overcome these limitations is the use of a mixing cartridge which delays mixing the gel components until the point immediately before extrusion[82,83].

3. Establishing and Maintaining Co-cultures

Table 2 summarises the main methods used for establishment of both direct contact co-cultures and physically separated co-cultures, and the sections that follow outline key exemplars of the different methods.
<table>
<thead>
<tr>
<th>Methods</th>
<th>Description</th>
<th>Co-culture</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolayer 2D co-culture</td>
<td>A conventional approach mixing two or more different cell types in a culture</td>
<td>Direct</td>
<td>• Simple and straightforward</td>
<td>• Limited relationship to in vivo environment and response</td>
<td>[84-86]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>contact</td>
<td>• Low-cost and high speed of testing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transwell inserts system/membrane filter system</td>
<td>Polyester or polycarbonate membrane with fixed pore sizes to segregate one culture on the membrane from a second culture below the membrane</td>
<td>Direct or indirect</td>
<td>• Large volume</td>
<td>• Limited to two compartmentalized cultures (except the tri-cultivation case)</td>
<td>[5, 87-95]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Compatible with standard techniques/assays and cell culture robots</td>
<td>• Can have long diffusion times due to the large distance from Transwell membrane to the base of the well</td>
<td></td>
</tr>
<tr>
<td>Cell-patterning</td>
<td>Using a cell-manipulation platform to pattern cultured cells form multiple cell types with desired arrangement according to the cell adhesion to micropatterned surfaces</td>
<td>Direct or indirect</td>
<td>• Can position multiple cell types</td>
<td>• Relies on external conditions (e.g. substrate modification, etc.)</td>
<td>[96-103]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Cell deposition can be non-invasive, contactless</td>
<td>• Potential effect on culture from artificially introduced substrate heterogeneity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Precise control of spatial configuration</td>
<td>• Not always suitable for large-scale process</td>
<td></td>
</tr>
<tr>
<td>Cell Spheroid</td>
<td>Spheroids, spherical aggregate of cells in static or stirred suspension culture, are amenable to the co-culture of different cell types, in particular tumour cells and normal cells.</td>
<td>Direct</td>
<td>• Better mimic the heterologous cellular environment in a solid tumour or at sites of metastasis</td>
<td>• Size and uniformity can be difficult to control</td>
<td>[5, 92-95]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>contact</td>
<td>• Mostly used for co-culture of physical contact cells</td>
<td>• Limited number of human tumour cell lines have capacity to grow in spheroid cultures</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• wide range of preparation strategies (e.g. hang-drop, etc.) available for the co-culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microfluidic platforms</td>
<td>Bespoke design for co-culture, with different compartments separated by either fluid channel or membranes</td>
<td>Direct or indirect</td>
<td>• Precise spatial and temporal control</td>
<td>• Complexity may limit scalability</td>
<td>[8, 13, 15, 16, 26, 29, 104-108]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Design flexibility</td>
<td>• Not always compatible with standard assessment techniques</td>
<td></td>
</tr>
<tr>
<td>3D scaffold</td>
<td>Cells are seeded on or migrating to cell-interactive solid supports as extracellular matrix substitute (e.g. collagen hydrogels)</td>
<td>Direct or indirect</td>
<td>• More effectively replicating cell interactions with extracellular matrix</td>
<td>• Needs pre-mixing of cell types in a suspension before seeding on scaffolds or cross-linking into scaffolds, or sequential seeding</td>
<td>[109-112]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Geometric variations and tolerances in scaffold manufacture a potential additional source of variability</td>
<td></td>
</tr>
</tbody>
</table>
3.1 2D Co-Cultures and Transwell Insert Systems

2D monolayers with mixed cultures of different cells in well plates/flasks are the most simple and straightforward way to create a direct contact co-culture, but they lack interplay with stroma and the 3D architecture of a tissue. Monolayer co-cultures do however introduce the issues related to co-cultures, namely that separating out the cell populations for analysis is difficult, and that most cell types will have optimised media, which may not suit the other cell type in a co-culture. Used in their simplest form, Transwells allow two cell types to be co-cultured without being in direct contact, sometimes in their own media (depending on the transwell design), but whilst allowing exchange of supernatant through a porous membrane[87-89,91] (Figure 4). Complex cultures in either upper or lower chambers may be used to generate more complex transwell based models[89,90,113-115].

![Figure 4](schematic_representation_of_indirect_contact(i),_direct_contact(ii)[88]_and_tri-cultivation_(iii)[89]_co-cultures_using_transwell_system)

3.2 Cell Patterning

Micro-patterned cell co-cultures are generally based on modifications to substrates to produce specific patterns that show different affinities to cells. The modifications may be chemical or structural, in order to change surface chemistry[116] or topology[117,118][119-121], as these allow some control of cell sensing and purpose-specific cell-regulating cues development[122]. Approaches to developing structured patterns include etching based on microfabrication techniques including plasma[123], UV-assisted capillary moulding[120], lithography methods (soft-, photo-, colloidal-, etc.)(121,124), and so on[122,125]. Another important approach is surface coating with cell adhesive biomolecules including, for example, collagen[126], laminin, fibronectin[127], antibodies[128], bovine serum albumin (BSA)[129], gelatin[116], peptide and aptamer ligands[121], and glutaraldehyde[130,131,132]. Surface silanization is often combined with photolithography to provide selective micropatterning for selective attachment of cells to targeted area[133]. In addition the application of both stencils[99,100][101,102] and meshes[97] has been used to give micropatterns, with the former approach having been developed further as “cell sheet technology”[98].

3.3 Cell Spheroids

Multicellular spheroids are more similar in structural and functional terms to tissue than 2D monolayer co-cultures, and have been of particular interest for modelling metastasis and solid tumour growth. A series of techniques have been developed over the years to produce spheroid co-cultures...
(Figure 5), including hanging drop, rotary cultures (e.g. spinner flasks), micropatterned plate (e.g. concave microwell[134,135]), nonadhesive culture wares, and scaffold-based methods, each with particular advantages and disadvantages[93]. Spheroids can be integrated with fluidic networks to establish them within perfusable circuits for nutrient supply, substance dosage and inter-organ metabolic communication between parallel formed models [136]. In particular, cultured in a concave microwell based on standard plasticwares as fabricated through rapid prototyping methods, the spheroids are easy to integrate with a high-throughput workflow for drug screening[134,135].

Figure 5. A) 384 hanging drop array plate and a cartoon of the spheroid formation process. The size of the spheroid is controlled by the number of cells seeded into each hanging drop[137]; B) Schematic showing a two-layer PDMS-based microfluidic device for the generation of uniformly-sized embryoid bodies. The cells are firstly introduced into the upper channel to fully cover the membrane before spontaneously aggregating to form embryoid bodies as the membrane are resistant to cell adhesion.[138] C) scaffold-based method for spheroid formation[93].
Closely placed tissue spheroids undergo tissue fusion — a process that can allow larger organoids to be generated[139]. The limitations of spheroid culture are that not all cell types (including many tumour cell lines) have the capacity to grow in spheroid cultures[95], it can be difficult to form and reliably maintain spheroids of uniform size, or to form spheroids with small numbers of cells, and, as for monolayer co-cultures, analysis of individual cell types can be difficult[140].

3.4 Microfluidic Platforms

3.4.1 Device material selection & functionalization

So far, Polydimethylsiloxane (PDMS) is still the most commonly used material for microfluidic designs, primarily because of the ease of moulding and low cost. However, PDMS is increasingly criticised for leaching of un-crosslinked oligomers that can contaminate culture medium and bind to cell membranes[141], and also the negative effect of PDMS on cell metabolism and proliferation in long-term culture[24]. PDMS structures are also difficult to mass produce. As such, alternative materials ranging from glass, polystyrene (PS), polycarbonate (PC), acrylic, polymethylmethacrylate (PMMA), cyclic olefin polymer, polyaryleterketone (PAEK), polylactic acid (PLA) and other polymers have emerged as chip materials in standard and customised microfluidic cell culture equipment offered by a range of commercial providers[18,142,143]. In particular, PS, being the most common macroscale cell culture material, is popular, readily available in high volumes, and can be processed for cell culture using a range of methods (e.g. micromoulding [144], hot embossing[145] and ‘Shrinky-Dinks’[146]).

The majority of microfluidic device materials do not have optimal surface properties for cell adhesion and proliferation, and require surface functionalisation. Both flow rate and shear stress of the laminar flow in microfluidic devices influence the efficiency of cell adhesion and detachment. The application of shear stress can deform cells and enlarge the contact area between cells and microchannel surfaces, hence enhancing the cell adhesion to the surface[130,147-149], while an increased shear stress reduces cell adhesion rates. Surface treatments are also required to inhibit unwanted cellular attachment in the flow channels or the non-specific protein adsorption from the culture medium[18]. PEO (polyethyleneoxide) based coatings[123,150,151] are a common approach. For cell detachment from substrate surfaces, trypsin and dispase are available to remove cells[152], and the process can be influenced by wall shear stresses[130,153].

3.4.2 Throughput enhancement with well-format-based design

Organ-on-a-chip systems are increasingly more application focussed, and conventional well plate formats that are compatible with standard robotic and fluorescent plate readers are increasingly being used as a template for platform design. The microfluidic setup is either integrated to existing industry-standard well format or devised into a multi-well plate analogue, for high throughput assays and for standard lab
equipment compatibility, and a variety of these are summarised in Table 3, with exemplars in Figures 6 and 7.
Table 3 Representative microfluidic platforms interfaced with well plate format

<table>
<thead>
<tr>
<th>Platform</th>
<th>Well Format</th>
<th>Remarks</th>
<th>Organ models</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard-well-format-based</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vulto group: organoplate (MIMETAS)</td>
<td>384-well plate with 96 microchambers</td>
<td>• PDMS-free; • The most compact and throughput organ-on-a-chip system on the market</td>
<td>Neurons, hepatocytes, endothelial cells, kidney proximal tubular cells, cancer cells, etc.</td>
<td>[108, 154, 155]</td>
</tr>
<tr>
<td>Vascularized micro-organ (4Design Biosciences)</td>
<td>96-well plate with 12 tissue units</td>
<td>• PDMS layer is attached to 96-well plate by a chemical gluing method; • Generation of interstitial flow for vascular angiogenesis</td>
<td>Endothelial cells, Microvasculature, tumor cells</td>
<td>[156-158]</td>
</tr>
<tr>
<td>Angiochip2.0: InVADE</td>
<td>96-well plate for multi-organs with up to 20 tissues</td>
<td>• Polystyrene-based multi-well plate; • Allows microvascular perfusion across multi-organ tissues</td>
<td>Supporting various parenchymal tissues such as tumor, liver, cardiac tissues, etc.</td>
<td>[159, 160]</td>
</tr>
<tr>
<td>DAX-1,AIM BIOTECH</td>
<td>Compliance with the SBS ANSI 384-well plate standard with AIM chips fitted</td>
<td>• Non-PDMS plastic with gas-permeability and excellent light transmittance • Compatible with all polymerisable gels, controllable interstitial flow</td>
<td>Direct culture of vasculogenesis and angiogenesis, co-culture with tumor cells</td>
<td>[161, 162]</td>
</tr>
<tr>
<td><strong>Well-format-analogue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µOrgano</td>
<td>Well plate allows more than 100 individual units</td>
<td>• PDMS-based plate and connectors • A platform of multi-organ-chips</td>
<td>Supporting tissues such as heart, liver and fat tissues, etc.</td>
<td>[163, 164]</td>
</tr>
<tr>
<td>PREDICT96(Draper)</td>
<td>96-well</td>
<td>• PDMS-free • A portable and reconfigurable multi-organ • Precise flow control based on electromagnetic actuators • Allows for real-time data collection by integrated microscale sensors</td>
<td>Five organs: ovary, fallopian tube, uterus, cervix and liver with a sustained circulating flow between all tissues</td>
<td>[165, 166]</td>
</tr>
<tr>
<td>LiverChip®: Microfluidic multi-well plate (BioCN)</td>
<td>12-well &amp; 36-well</td>
<td>• Polysulfone-made top plate (contacts cells and media) • Enables extended cell culture ( &gt;1month)</td>
<td>liver</td>
<td>[167, 168]</td>
</tr>
</tbody>
</table>
Figure 6 (A) OrganoPlate® based on a 384-well plate consists of arrays of 96 chip-based microchambers with each microchamber being a three-lane bioreactor glued to the bottom of 4 consecutive wells[108]. The adjacent lanes are separated by a phaseguide technique to build a stratified 3-D cell culture system. Some optical assays are available from an observation window. (B) A vascularized micro-organ (VMO) platform chip based on 96-well plate[157]: 6 tissue units arranged on half of the well plate, with each occupying 6 horizontal wells. One tissue unit consists of 3 tissue chambers (T1-T3) connected to 2 adjacent microfluidic channels, 2 gel loading ports (L1-L2), 2 medium ports (M1 and M2), and 1 pressure regulator unit(PR). (C) Illustration of an integrated vasculature for assessing dynamic events (InVADE) based on a scaffold integrated 96-well plate[159]. Images from left to right and top to down are: Schematic overview of cover, wells and base; SEM (Scale bar 1 mm) of the tissue chamber for the liver model with a scaffold suspended across and of the tissue chamber for the heart or tumor models with a scaffold, attached with four cantilevers, suspended across the tube; magnified SEM (Scale bar 200 μm) showing the main channel and the microholes on the side channel walls of the scaffold; illustration of the scaffold seeded with endothelial cells and parenchymal cells showing the spatial configuration of the co-culture environment.
**Figure 7** (A) Photographs of LiverChip® device based on a perfused multiwell with an array of 12 bioreactors. The left includes inserted photographs of a bioreactor and a scaffold (scale bar on channels with cells image 300 mm). The right shows the built-in connectors and pneumatic lines distributing positive and negative air pressure to individual valves and pump chambers in a partially docked perfused multiwell[167]. (B) Underlying concept of the μOrgano system. Schematics depicting the basic μOrgano components: the master-organ-chip and exemplary plug & play connectors. Conceptual idea of the usage principle of the μOrgano system for the connection of two microphysiological systems (MPSs) in series via a simple linear channel connector with a close-up of the connected system highlighting the resulting media flow[163].

### 3.4.3 Enhanced Organ-on-a-Chip Approaches

The microvascular system uses microvessels and capillaries to transport oxygen, blood and nutrients throughout the entire body. This system enables tissue functionality, supports diverse biological phenomena and contributes to the close interactions among the organs[29]. Lee et al.[170] have
reviewed the latest advances achieved with regard to the microfluidic-based vascularized microphysiological systems (MPS), which can address lumen structure formation\cite{171}, the role of interstitial flow in regulating the angiogenic response\cite{172}, blood-brain barrier models\cite{173}, and tumour spheroid development\cite{174,175}.

Depending on the application, physical cues such as mechanical stimulation, electrical stimulation and biochemical stimulation can be useful in improving maturation of the in-vitro micro-tissue\cite{176}. One example is a lung-on-a-chip model\cite{177}, where cyclic stretch was introduced to mimic the effects of breathing on the alveolar epithelium and endothelium, and there are also examples applied to electrical\cite{178-180} and mechanical\cite{181-183} stimulation of in-vitro cardiac tissue. External stimulations have also been investigated for the regulation of nerve\cite{184}, skeletal muscle\cite{185} and liver\cite{186} tissues.

Body-on-a-chip or human-on-a-chip models, integrating multiple organs may be useful in the modelling of systematic interactions between various tissues and organs\cite{187-191}. Organoids for liver, cardiac and endothelial modules have been integrated in microfluidic devices under common media, showing sufficient viability\cite{187,189,190} (Figure 8A). Lee et al.\cite{192} have recently combined a pumpless multi-organ-on-a-chip (operated with gravity-induced flow) to evaluate the metabolism-dependent anticancer activity of a flavonoid, luteolin. As previously noted in Table 3, multi-organ models based on a standard 96-well plate have been developed\cite{159}. In this work, a built-in microfabricated vascular bioscaffold was developed to define vascular space and support self-assembly of various parenchymal mini-tissues including a metabolically active liver, a free-contracting cardiac muscle, and a metastatic solid tumour. Based on the platform, the complete cancer invasion-metastasis cascade has been demonstrated across multiple organs through the common vasculature (Figure 8B). In general, scaling of multiple organ models is considered a complex task\cite{193-195}.

4. Analysis

Analysis of tissue models can be undertaken in a number of ways, with the key logistical consideration being that the rate at which models can be assessed needs to match the rate at which they are produced. Conventional proteomic and genomic bioassays may be used for samples of culture media or cultured tissue, and have their advantages (see Table 4) but for the purposes of this paper we will predominantly focus on reviewing engineering approaches which aim to allow for in-situ assessment of tissue model behaviour. These include adaptations of conventional techniques for in-situ analysis, and the development of new sensor systems. These are considered in turn below.
Figure 8. Multi-organs on a chip. (A) (i) A depiction of a liver, cardiac, and vascular organoid-containing body-on-a-chip platform. Individual organ chips are connected through a central breadboard, with integrated flow control and imaging, and (ii) photograph of a three organoid system[187]. (B) A schematic diagram of InVADE platform. Multiple organ models utilise a common geometry and 96 well platform. Interconnected wells allow for the organ models to be arranged in a linear sequence[159].

Table 4 Comparisons between Conventional and In-situ Bioassays

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Available bioassays</th>
</tr>
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</table>
| Conventional | Readily available and standardized;             | Cell culture volumes are typically quite small (nL to mL scale), rendering the signal-to-noise ratio low in comparison to classical cell culture techniques | • Immunohistochemistry  
• Enzyme-linked immunoabsorbent assays (ELISA)  
• Luminescence liquid/gas chromatography-mass spectrometry (LC/GC-MS)  
• RNA expression and colorimetric assays |
| In-situ | High signal-to-noise ratio as small volume is required | Assays need to be tailored to the microfluidic environment | • Immunohistochemistry  
• Permeability  
• Trans epithelial electric resistance (TEER)  
• Migration assays  
• Angiogenesis and other optical readouts (e.g. calcium imaging, colorimetric and luminescence) |
4.1 Adaption of Conventional Techniques for Micro-Tissues

4.1.1 Immunohistochemistry and proteomic assays

Immunohistochemical staining is the dominant in-situ analysis technique[11]. This is due to its relative simplicity, involving the sequential introduction and removal of liquid reagents, and the fact that it provides information on both the presence and location of target molecules. Once stained and imaged, quantification is also possible based on image analysis software[196].

The principle proteomic approaches of relevance are gel electrophoresis, ELISA and preparation for mass spectroscopy. Further depth on some aspects of proteomics on-a-chip can be found in previous review papers [197,198].

Gel electrophoresis on-a-chip systems have been developed based on having a moving blotting membrane interfaced to a microchip[199], or arranging different antibodies in a series of parallel micro-fluidic channels[200], or through a microchip with electronic control and a novel gel formulation[201] (Figure 9A).

ELISA-on-a-chip systems must address the immune complex reaction and signal readout. The immune complex reaction antibodies can be present in solution[202,203], attached to the surfaces of the microfluidic channels[204], or linked to beads for increased surface area (magnetic[205-207] or otherwise[208-211]; Figure 9B). Magnetic beads can also be used as both the functionalized surface and as a method of mixing fluids within channels[205,206]. For signal readout, optical/fluorescent[212,213], electrochemical (EC)[214,215] and mechanical[216] methods have been integrated into microfluidic platforms. EC immunosensors are usually based on immobilization of antibodies on the surface of EC electrode for antigen detection, which gives a system that is essentially single use[217,218], but disposable microbeads have been used to immobilize antigen-recognition molecules[215], which allows for a continual quantification of biomarkers. Recent work has demonstrated that multiplexed ELISA immunoassays can be carried out on-chip[107,208].

Mass spectroscopy is a key proteomic technique, and microfluidic reactors functionalized with pepsin-agarose have been designed to enable rapid digestion of proteins prior to on-line analysis by electrospray ionization mass spectrometry (ESI-MS)[219]. Microfluidic proteomic reactors have also been designed to allow for parallel analysis of multiple protein samples with capture, reduction, alkylation and digestion simultaneously completed on the same device[220], and enzymatic reactors with trypsin and pepsin immobilized inside a microchip have been recently used to accelerate protein digestion and proteolysis[221-223].
Figure 9. (A) Comparative schematics between conventional Western blotting (i) and µWestern blotting (ii) assays, and a scalable electrode array accommodating 48 blots per chip to interface with a standard microscope slide-sized chips (iii)[201]; (B) Schematic diagram and pictures of a point-of-care ELISA-like assay[224]. (i) A preloaded sequence of multiple reagents passively delivered over a series of four detection zones, each characterized by dense meanders coated with capture proteins, before exiting the chip to a disposable syringe used to generate a vacuum for fluid actuation. (ii) Illustration of biochemical reactions in detection zones at different immunoassay steps. The reduction of silver ions on gold nanoparticle–conjugated antibodies yields signals that can be read with low-cost optics (for quantification) or examined by eye. (iii) Picture of cassette with a tube filled with sequence of reagent plugs (here, colored dye) and syringe for generating vacuum. No other peripherals are needed to run the mChip. Silver signals can be read by eye (similar to rapid tests), or with the use of a sensitive absorbance reader, which can aid objective determination of positive and negative results based on optical density.
4.1.2 Genomic assays

The two main techniques of interest for genetic analysis are polymerase chain reaction (PCR) for DNA amplification (which has been recently reviewed[225]) and fluorescent in-situ hybridization (FISH).

To take a fluid through the PCR thermal cycles, serpentine channel designs with three distinct built-in temperature zones are a common design, with the fluid repeatedly passing through the temperature zones[226], although radial designs are also possible[227,228]. Figure 10A-i shows a recent microfluidic PCR device made of polyimide with three resistive copper heaters integrated beneath the microchannel[229]. Stationary systems rely on temperature profile design to facilitate fast heating/cooling or accurate temperature control. Various active heating approaches have been employed for PCR thermal cycling, including light (tungsten lamp[230], LED[231] or modulated laser[232]), acoustic waves[233], Peltier elements[234], micro machined Joule heat-based systems such as thin film heaters[235] and heat exchangers[236]. For active cooling fans[237], flowing media (e.g. water or propylene glycol/water)[238] and heat exchangers are popular. Figure 10A-ii shows a recent example of a stationary µPCR system using an ultrafast photothermal light-to-heat conversion for PCR thermal cycling[231]. It is worth noting that the electrophoresis on-a-chip techniques described for proteomics can potentially also be integrated with DNA amplification[239].

Cao et al[240] review advances in digital PCR (dPCR) and note that this technique is well suited to microfluidic approaches, and in addition, loop-mediated isothermal amplification (LAMP) PCR[241] has been shown to be an approach which lends itself to miniaturization[242,243].

Microfluidic approaches to FISH are emerging, with the need for flow and temperature control over extended time periods making these quite complicated devices[244], but FISH platforms have been demonstrated for various sample types, including cells[245-248] and tissue sections[249-251]. Flow is of particular value in increasing the hybridization efficiency[251] (Figure 10B).
Figure 10. (A) Example figures of microfluidic PCR (i-a) Photograph of a fabricated PCR device. The three temperature zones defined by the three copper (Cu) microheaters beneath the meandering microchannel, are shown. In the inset, a part of the device is shown in magnification, where details of the microchannel and the Cu microheaters are visible at the top and bottom side, respectively. (i-b) Experimental setup for testing the PCR chip for DNA amplification.[229] (ii) Ultrafast photonic PCR. (ii-a) Schematic of the plasmonic photothermal light-to-heat conversion and subsequent heating of the surrounding solution (here, the PCR mixture) through ultrafast photon–electron–phonon couplings. When light is turned off, fast cooling of the heated solution can be achieved by the heat dissipation through the thin Au film. (ii-b) Schematics of the ultrafast photonic PCR using a thin gold (Au) film as a light-to-heat converter and excitation light from the LEDs. Thermal cycling, consisting of two or three discrete temperatures for denaturation, annealing and extension, is required for nucleic acid amplification through the PCR. For multiple PCR reactions, each LED could be modulated separately so that there are unique annealing temperatures for each primer design.[231] (B) (i) Principle of FISH assay.[252] (ii-iii) FISH microfluidic platform for detection of HER2 amplification in cancer cells. (ii-a) Schematic diagram of the FISH chip for use with clinical tissue samples. (ii-b) A photograph of the microfluidic chip (2.2 cm x 5.7 cm). The blue colour indicated the liquid layer, and the red colour indicated the air layer. Ø: diameter. (iii-a) Results of fluorescence pictures compared between positive and negative cases of HER2 over-expression[244].

4.2 Novel Sensor systems

The most common in-situ sensor systems are optical sensors, coupled with fluorescence- and absorbance-based measurement systems[253], which have been used to obtain structural and functional information with regard to various cellular activities such as cell viability, cytotoxicity and
cell apoptosis[254-256]. There is also growing interest in a label-free sensing of bio-molecular interactions based on surface plasmon resonance (SPR) as a result of collective charge density oscillation on a metallic surface[257,258]. This allows monitoring of morphological changes in cultured cells, detection of the distance between cells and metallic substrates in cell culture chambers, and quantitative analysis of mass/area cell changes using phase contrast and fluorescence images[259]. The sensitivity can be enhanced with localized SPR (LSPR), which extends the optical measurement to spatiotemporal, quantitative and real-time mapping of proteins secreted from cells and the cellular function immunoanalysis[260,261]. (Figure 11A)

![Figure 11. (A) Principle of nanoplasmonic sensing platform-integrated microfluidic cell culture devices[260]. (B) Scheme of a microfluidics-based setup for oxygen and glucose measurements. Tissue-embedded oxygen sensors in a bioreactor mounted on a microscope are excited via OPAL unit controlled LED signal modulation, and the signal are analysed through a photomultiplier readout. Bioreactor outflow was connected to a microfluidic switchboard containing a series of pressure-controlled micromechanical valves that introduced samples into a unit (controlled by a potentiostat, PSTAT) containing electrochemical sensors for glucose and lactate. Optical, pressure, and electronic sensors were connected to a single microprocessor that synchronized the signal. Right top: Jablonski diagram describing the generation of phosphorescence with Ru-CPOx beads under the influence of oxygen. The quenching of the phosphorescence by triplet oxygen leads to a decrease in signal intensity and phosphorescence decay time (T1). Right bottom: H$_2$O$_2$ is created in equivalent amounts of the analyte as an intermediate product by the activity of glucose oxidase (GOx) or lactate oxidase (LOx)[105]. (C) Multisensor-integrated organs-on-chips platform for automated and continual in situ monitoring of organoid behaviour[11]. (D) Cardiac-on-a-chip model with functional readout of cardiac contractility[262].]
Electrochemical approaches can provide information on glucose, lactate, oxygen and pH, and temperature[105]. Typically, an amperometric sensor is designed in a two/three electrode configuration to measure the current generated in the system that can be proportionally related to the concentration of the analyte (e.g. glucose or lactate). Such amperometric sensors have been widely integrated into microfluidic platforms, sometimes functionalized with oxidase enzymes for sensitivity enhancement, for glucose and lactate monitoring and quantification[105,263] (Figure 11B). They can be made as a separate sensor plate to be plugged in microfluidic chips[263]. Voltage and conductivity measurements have also been utilized for sensor designs[264], and systems have been developed to sense cell secreted biomarkers[215,265]. In contrast to optical measurement, the electrochemical sensors require frequent recalibration and demonstrate significant decay over time, which can complicate the microfluidic design with an additional setup to wash and recalibrate the sensors[105].

A further approach for oxygen sensing is based on a reversible quenching of luminescence or phosphorescence of ruthenium-based dye in the presence of oxygen[266]. In particular, luminophore Ru \((\text{Ph}_3\text{phen})\text{Cl}_2\) (Ruth) dye has been used for fluorescent excitation[267,268] and ruthenium–phenanthroline-based dye (CPOx-50-RuP) has been used for phosphorescence[105]. To be integrated into a cell culture system for real-time monitoring, the dyes are often coated on a bead or micro-particle. The use of particles can yield a higher signal-to-noise ratio for a better readout as a higher dye concentration can be accommodated without inducing self-quenching effects[269], and micro-size oxygen sensing particles have recently become commercially available[270,271]. Opto-chemical process have been utilized to design pH sensors for on-line pH monitoring, mostly based on the optical adsorption of dyes such as phenol red with an optical fibre light source[272].

Multi-sensor systems to be integrated into organs-on-chip platforms for automated and continual in-situ monitoring of biophysical and biochemical parameters[1,273], with a fluidics-routing breadboard/switchboard often introduced to connect the sensors and the organs in a reasonable sequence to realize the operation in a continual, dynamic and automated manner (Figure 11C)[1]. In addition, biosensors can be assembled with cultured micro-tissues to provide online monitoring of the biomechanics and maturation status of the tissue[262] (Figure 11D). These devices require a complex 3D architecture, with 3D printing technologies commonly used to create these[274-276].

5. Current Position and Outlook

A wide range of technologies have been explored to create, maintain and analyse physiological micro-models, driven by the lack of overall functional prediction obtained from existing in vitro approaches. However, there is still little clinical uptake and a lack of clinical validation for models. It is perhaps worth re-iterating that “all models are wrong, but some are useful”[277], and that validation is key to
understanding the usefulness of any model. High throughput and validation are to some extent coupled problems in this context: without high throughput approaches the multiple models required to give the replicates, controls and serial dilutions required for validation cannot be created, and so throughput remains important to unlocking the wide-scale use of physiological micro-models.

The primary fluid handling challenges relate to (i) repeatably dispensing low volumes of cells in media, and (ii) dispensing high viscosity materials with high cell densities. Both of these challenges can be complicated by the need to deposit different materials in a spatially gradient manner. Meeting the different dispensing requirements of the different constituents of models will likely require a range of dispensing techniques to be deployed in parallel.

In moving towards high throughput models the adoption of standard well-plate formats, compatible with existing automated and semi-automated cell culture and biological assessment techniques, can be considered the “second wave” of tissue-on-a-chip techniques, in contrast to the “first wave” of more complex experimental set-ups with low numbers of replicates.

The need for high throughput analysis and characterisation has led to a number of innovative on-chip characterisation techniques. Most of the approaches reported in the literature rely on a single analytical technique for technological validation, whereas in practice the use of a cascade of techniques, for example to filter down a large number of replicates to give a sub-set for further investigation may be a more useful approach. Recent work embedding multi-sensor systems on-chip offer an alternative approach to scaling assessment, allowing read-outs to be combined to more effectively filter down results to those of interest.

In conclusion we can say that the drivers for more predictive in vitro modelling remain clear, that models based on complex co-cultures are continuing to emerge with an increasing focus on usability and integration with commonly available equipment. Systematic approaches to translating models from low to high throughput are required, together with the specification and validation of models being better understood, and with further development, integration and alignment of model creation, maturation and analysis.

Acknowledgements

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32


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