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Abstract

There has been a renewed interest in cell-based phenotypic screening in drug discovery with the goal of improving the success and decreasing the clinical failure rate of new therapeutics. This has increasingly led to the development of biomimetic cellular models that more faithfully replicate human disease biology. Human tumour models have advanced to include relevant cell types such as primary patient tumour cells and grown using organotypic and 3D methods. Tissue organoids, which are 3D organ buds displaying realistic microanatomy, are becoming more commonly used in drug discovery to advance in vitro assays which predict drug toxicity and pharmacokinetics. Emerging technologies and cell culture methods are constantly improving the quality of tissue modelling that can be employed during primary phenotypic screening, and this has resulted in the identification of more efficacious and patient-relevant therapeutics.

Keywords: phenotypic, HTS, screening, high-content, high-throughput, three-dimensional, complex, spheroid, drug discovery, ECM, matrix

1. Introduction

This chapter will introduce the concept of complex and advanced high-content phenotypic drug screening. Phenotypic screening is a reductionist approach to modelling a particular aspect of biology and identifying modifiers of that biology. Conventionally, genomics- and chemical-based high-content screening has been performed on single cell types grown on plastic. However, accumulating evidence has shown that those methods are poor surrogates of actual disease biology. Three-dimensional and complex phenotypic screening employs disease-relevant cell types assembled in biomimetic fashion and miniaturized to accommodate a 384- or 1536-well high-content screening plate. Screening platforms for 3D and multi-culture
cell models are typically employed in oncology research to better represent patient tumour biology. Recently, advanced cell culture techniques have made their way into other disease areas such as regenerative medicine and immunology, and the resulting screening platforms have greatly expanded the therapeutic targeting space.

2. What is phenotypic screening?

2.1. Description and historical significance of phenotypic screening

A phenotype is a composite of an organism's observable traits. On a cellular scale, a phenotype refers to a definable characteristic such as morphology, biochemical or physiological properties, motility or cell cycle status. A phenotypic assay is a quantitative measurement of one or more cellular parameters after exposure to a modifying agent or perturbagen such as small molecules, proteins or RNA-interfering reagents. Application of a phenotypic assay to large-scale endeavours where many test reagents are applied to the cellular model is referred to as phenotypic screening. Phenotypic screening is regularly employed in early stage drug discovery by both academic and pharmaceutical institutions where it is referred to as phenotypic drug discovery (PDD). Phenotypic screening is a system-based approach using a target-agnostic assay to monitor phenotypic changes \textit{in vitro} or \textit{in vivo} \cite{1}. PDD is often carried out in a high-content or high-throughput fashion using microtiter plates with 96, 384 or 1536 wells (Figure 1) to enable the analysis of thousands or millions of test compounds.

Phenotypic screening is not a new concept. In fact, before the era of cellular biology phenotypic screening was often carried out in whole organisms. A benchmark example of this process was the large scale systematic screening to find a drug against syphilis. In 1909, the Nobel Prize-winning immunologist Ehrlich et al. synthesized hundreds of organoarsenic derivatives and tested them in syphilis-infected rabbits \cite{2}. The 606th series tested cured the rabbits and was later marketed as Salvarsan, which was one of the most frequently prescribed drugs until its replacement by penicillin in the 1940s \cite{3}. Alexander Fleming, arguably the most well-known microbiologist of recent history and discoverer of penicillin, pioneered the first type of \textit{in vitro} antimicrobial screening technique. He would use small circles of filter paper doused in a test chemical and applied to a lawn of pathogenic bacteria in a Petri dish to look for zones of growth inhibition (Figure 2). This method, eventually optimized in the 1950s as the Kirby-Bauer disk diffusion test, required much less resources than testing in diseased animal models and eventually became an industry standard for the systematic identification of new antimicrobial compounds \cite{3}. This technique is still widely used in academia and industry, though at a much higher throughput.

Modern phenotypic screening in eukaryotic cells arose with the capacity to culture human cells \textit{in vitro}. Although mammalian cells have been propagated \textit{in vitro} since 1907 \cite{4}, cell culture techniques advanced significantly in the 1940s and 1950s to support efforts in virology research. The basis for conventional cancer drug discovery began with the emergence of human cancer cell lines in the 1950s, starting with the well-known HeLa cell line \cite{5}. Since that time, many human tumour cells from all types of solid organs and hematopoietic cancers have
been adapted to *in vitro* cell culture conditions and used to find new drugs that kill cancer cells. Early phenotypic screening from the 1960s through the 1990s relied heavily on cytotoxic assays that identified anticancer drugs in human cell lines that exhibit the phenotype of rapid unrestrained growth [6]. Arguably the most successful examples from those efforts were the discoveries of camptothecin and taxol in the 1960s which are still widely used to treat many types of cancer. However, with the advent of modern genomics and its application to the study of cancer genomes, tumour transcriptional profiles and disease-driving mutations, a revised understanding of the molecular bases of cancer has yielded new classifications of tumour cell phenotypes [7]. This nuanced view of the molecular underpinnings of cancer has facilitated more rapid target-based drug discovery (TDD) but also enabled the definition of more patient-relevant cellular models and phenotypes that can be employed in PDD. Subsequently, modern phenotypic screening initiatives involve somewhat more knowledge of the disease biology and are not entirely target-agnostic compared to earlier “black box” screening efforts.

![Figure 1](http://dx.doi.org/10.5772/65387)

**Figure 1.** Three different assay plate formats used in high-content cell culture. The 96-well plate is rarely used in drug discovery and is only for assays incapable of further miniaturization (screening capacity: 1000s of compounds). The 384-well plate is a standard size for complex phenotypic screening (screening capacity: 100,000s of compounds). The 1536-well plate is mainly used for biochemical and simple cell-based assays (screening capacity: 1,000,000s of compounds).
2.2. Phenotypic screening versus target-based screening

In terms of methods used to discover new drugs, phenotypic drug discovery is in stark contrast to target-based drug discovery (TDD), where the target (phenotype-modifying protein) is already known. Although target-based screening approaches may occur within cells, they often consist of biochemical assays using purified recombinant proteins in artificial environments. TDD is a rational, informed approach to drug discovery that uses molecular tools (compounds or biologics) to modify a particular target's activity or behaviour. Although TDD is the predominant form of drug discovery in big pharma, it relies on the unwavering assumption that the target in question is the elicitor of the relevant disease biology. Opposing this, a principle application of PDD is to identify new, previously unknown targets that may impact a phenotype hypothesized to be linked to disease pathology. Although TDD is a hypothesis-driven approach to identifying new drugs and also may provide criteria for choosing patient populations and setting doses, PDD has likely been more successful at identifying first-in-class medicines through the unbiased identification of novel molecular mechanisms of action (MMoA) [8].

The target-based approach (TDD) can be thought of as molecularly driven and involves the identification of chemical or biological reagents that modify the activity of one specific protein. Target-based drug discovery begins with a validated target protein that has been shown to convey an important aspect of the relevant disease biology. Therefore, in essence, the target-based approach is only as strong as the evidence produced for the characterization of that
target. Perhaps, the most classic and well-defined example of target-based drug discovery is the discovery of Gleevec (imatinib) for the treatment of chronic myelogenous leukaemia (CML). In 1960, a chromosomal abnormality was discovered in the white blood cells of CML patients and dubbed the Philadelphia chromosome by the two researchers in Philadelphia who made the discovery [9]. However, it was not until 1973 that the Philadelphia (Ph) chromosome was characterized as a translocation between chromosomes 9 and 22 [10]. A further twelve years later, in 1985, the Ph chromosomal rearrangement was shown to yield the BCR-ABL fusion protein which was identified as the genetic driver of malignant neoplasia [11]. Finally, in 1993, a clinician in haematology/oncology named Brian Drucker teamed up with the Ciba-Geigy pharmaceutical company (now Novartis) to find a low molecular weight compound that could inhibit the BCR-ABL fusion protein and kill CML cells. The product of those efforts, a compound called STI571 and eventually known as imatinib, would go on to save thousands of lives and effectively cure Ph+ CML [12]. The Gleevec story is a textbook example of how target-based drug discovery is carried out in well-defined sequential steps: (1) a genetic abnormality in a diseased population is identified, (2) that genetic abnormality is shown to produce a mutant protein that drives the disease, and (3) a screening campaign identifies a chemical modulator of the mutant protein. Although the current field of molecular genomics now enables a faster turnaround time between the identification of a mutant protein and the chemical screen for a therapeutic, there still must exist a substantial body of work around the protein of interest to launch a full-blown drug discovery campaign.

Phenotypic drug discovery (PDD), on the other hand, is a discovery process that begins with an observable and quantifiable change in biology (phenotype) without prior knowledge of a causal target or mechanism of action. Due to the fact that modern phenotypic screening in drug discovery was only recently industrialized and the length of time needed to progress a drug from the bench to the bedside (10–15 years by most estimates), there are few examples of drugs currently being used in the clinic that were discovered from purely phenotypic-based approaches. Although taxol and camptothecin were discovered using cancer cell viability assays, a particularly inspiring example of PDD in recent history is the identification of vorinostat (Zolinza) for use in haematological malignancies. In 1971, an academic investigator at the Sloan-Kettering Institute for Cancer Research in New York made the observation that dimethyl sulfoxide (DMSO) had the properties of being able to induce erythroid differentiation in erythroleukaemia cells [13]. As leukaemia cells are often characterized by their lack of differentiated state, a compound capable of restoring differentiation in these cells is highly desirable. DMSO is an organosulfur fluid that is frequently used to dissolve both polar and non-polar compounds and is one of the most widely used reagents in chemistry and pharmaceutical discovery. The initial phenotypic observation led to the assembling and screening of DMSO-related and –derived compounds that had similar chemical structures. Although the set of compounds synthesized and screened in this effort would be considered small by today’s comparison, the investigators were able to find one molecule, suberoylanilide hydroxamic acid (SAHA) that was able to induce cytodifferentiation and growth arrest of erythroleukaemia cells much more potently than DMSO. After many years of trial and error, SAHA was eventually moved to preclinical development after the discovery that its target was histone deacetylase (HDAC) [14]. RNA transcription and subsequent protein expression is regulated
by acetylation of histone proteins, and HDACs have been shown to contribute to the development and progression of cancer through their silencing of tumour suppressor genes and/or activation of oncogenes. HDAC inhibitors and other epigenetic modifiers are now widely used in the clinic to treat a variety of hematopoietic malignancies and solid organ tumours. However, at the time of vorinostat preclinical development, HDAC inhibition was viewed as a completely novel approach to treating cancer. Several successful clinical trials showed that vorinostat was efficacious in treating patients with cutaneous T-cell lymphoma (CTCL), and the drug was approved by the FDA in 2006 [15]. Ensuing clinical trials showed that vorinostat is successful in treating other types of lymphoma, glioblastoma and non-small cell lung cancer and this has paved the way for other HDAC inhibitor development programs. Therefore, this drug, vorinostat, was derived from a common reagent present on the laboratory bench of nearly every pharmaceutical researcher and was shown by PDD to elicit a therapeutic mechanism completely novel to medicine.

Figure 3. Discovery of first-in-class drugs approved by the US FDA from 1999 to 2013. Most drugs were discovered through target-based approaches (TDD) with more small molecule drugs (compounds) than biological ones (proteins). Most system-based approaches (e.g. PDD) originated from a known compound class (chemocentric) and relatively few were discovered by pure black box PDD. Adapted with permission from [1].

The true measure of which drug discovery approach is more successful, target-based or phenotypic-based, is how many drugs currently used in the clinic originated from each approach. Since the late 1990s, most pharmaceutical discovery has focused on target-based approaches, so there has been a heavy bias towards TDD compared to PDD. However, in terms of first-in-class drugs that target “new molecular entities” (NMEs), phenotypic approaches have been shown to be more successful than the target-based approaches that typically involve follower drugs or “me too drugs” [8]. Me too drugs are structurally similar to existing drugs and share the same target class, though they are distinct enough to escape patent infringement. Although these types of drugs may create competition between pharma companies and may drive drug prices down, within the research and development space, they may hamper creativity, innovation and ultimately, productivity. Conversely, a more recent review of the
origins of 113 first-in-class drugs approved by the FDA from 1999 to 2013 revealed the majority (71%) of first-in-class drugs were discovered through target-based approaches (Figure 3) [1]. Regarding the systems-based approaches (e.g. PDD) for NMEs during this time frame, most drugs originated from a known compound or compound class (chemocentric approach) and only a few were discovered through purely target-agnostic phenotypic screening-based efforts (Figure 3) [1].

In the practical sense and from a pharma perspective, most drug discovery falls somewhere between TDD and PDD. Although a large portion of exploratory screening is performed in phenotypic models, the reagents that are screened are mechanistically informed. This has led to a newly defined approach that still falls under the category of phenotypic screening but is not entirely target-agnostic. Mechanism-informed phenotypic drug discovery (MIPDD) is screening against targets that are known or reported to be involved in the relevant disease pathology [6]. For example, screening ion channel inhibitors in cardiac assays or modifiers of extracellular matrix (ECM) remodelling for cartilage regeneration assays would be MIPDD. In essence, this approach restricts the scale of reagents tested but subsequently allows for easier data deconvolution due to the limited range of MoAs. This concept of MIPDD becomes especially important when designing and screening complex and 3D phenotypic cellular models, as will be discussed later.

2.3. Benefits and liabilities of high-content phenotypic screening

One problem in particular that plagues PDD but not TDD in high-content compound screening is target deconvolution. PDD is accompanied by the challenge of identifying what molecular entities are engaged by the hit compounds and what the phenotype-modifying molecular mechanism of action might be. Deconvoluting a compound’s MMoA may not prove to be difficult assuming there are biomarkers or pharmacodynamic (PD) markers of compound action. For example, receptor internalization, reduced kinase phosphorylation or downregulated oncogene expression may explain a general MMoA, but it does not reveal the actual target of the compound. There are a variety of technologies available to identify the target(s) of a compound; for example, affinity chromatography, protein microarrays or chemical proteomics, though they each have their respective benefits and liabilities [16]. There are two main approaches to target deconvolution following phenotypic screening; the direct approach where the target is identified physically bound to the compound and the indirect approach that relies on cellular profiling. The direct approach method that provides the most confident data is chemical proteomics. Chemical proteomics involves the modification of one part of the compound so that it can be immobilized onto a purification bead (Figure 4A and B). The compound is then mixed with cellular extract and a pull-down assay followed by mass spectrometry reveals the most likely proteins that are bound to the modified compound (Figure 4C). Although this approach is the most straightforward, it is strictly dependent on knowing the active site(s) of the compound. The compound must be tethered to the bead in a manner that maintains its target recognition properties or the mass spec results may be misleading [16] (Figure 4).
Figure 4. Chemical proteomics method of target identification. (A) Lead compound with unknown target must be tethered to purification bead by one of its side groups (boxed in red). (B) Compound-bead conjugates are synthesized to maintain target binding during lysate exposure and purification (not to scale). (C) Mass spectrometry of proteins attached to modified compound-bead conjugates may reveal target protein of interest.

Direct methods of target identification such as chemical proteomics tend to be resource-intensive and time-consuming [17]. Therefore, pharma investigators have made a concerted effort to develop new technologies that are able to reduce the time and increase the success rate of small molecule MoA determination. Classified as “systems biology” methodologies, these indirect approaches to target deconvolution rely in comparing “signatures” or “finger-
prints” of compounds to other chemical entities with known or predicted activities/targets. These compound signatures, which may be in the form of gene expression, protein or metabolite profiles, can be compared to the signature of the unknown test compound and similar patterns may reveal a potential mechanism or, in the best case scenario, the actual target. Gene expression profiles are the most common signatures for these types of profiling approaches and proteomics profiles can be employed when the action of a drug has no impact on RNA levels [16]. However, these methods are both costly and low throughput so pharma investigators created new profiling platforms that maintain their MoA predictive powers but can be run in high-throughput for negligible cost. A benchmark example of these technologies is pathway reporter screening that is widely used by industrial drug discovery investigators. This screening platform consists of an extensive series of reporter gene assays (RGAs), where a luciferase is under transcriptional control of promoters responsive to different transcription factors involved in various aspects of cellular biology [18]. For example, promoters driving the luciferase reporters might be derived from metabolic genes, inflammatory genes, extracellular receptor genes, hypoxia responsive genes, etc. The activity profiles of an unknown compound are then compared to other compounds with known targets/MoAs for similar signatures (Figure 5A). Compound activity is then tested orthogonally in an enzymatic assay alongside the compound with known activity to confirm the prediction (Figure 5B). The goal of the RGA platform is to cover all focal aspects of cellular signalling that might be affected by compound treatment. The RGA technology has proven to be very informative at predicting cellular targets of unknown compounds in an automated, inexpensive and time-efficient manner [18].

Figure 5. Pathway reporter screening. (A) Terprenin is a compound with unknown target/MoA so it is profiled for activity on pathway reporters. The activity profiles are hierarchically clustered against compounds with known targets/MoAs revealing brequinar, a known dihydroorotate dehydrogenase (DHODH) inhibitor, as a very close match. (B) An enzymatic assay confirms terprenin as a DHODH inhibitor. Reprinted with permission from [18].
From an early stage drug discovery perspective, it is more desirable to “fail early”, than to progress a drug to later evaluation stages which are more time and resource consuming [18]. What this translates to is better selection of drug candidates early in the discovery process, possibly at the primary screening stage. One important reason invoked to play a role in the benefits of PDD over TDD is that PDD enables the testing of drug candidates in the context of the cell, and not in a biochemical assay using purified recombinant proteins as is typical with TDD. Since cell models are used in the prioritization of drug candidates based on potency and toxicity, it is only rational to bring these models forward to primary screening efforts to minimize late-stage expensive failures [19]. Therefore, it is critical that cell models of human disease used for primary high-content screens are as predictive of in vivo cellular biology as possible.

3. Paradigm shift in cell culture: 2D–3D

3.1. Recognizing the shortcomings of 2D cell models

If the purpose of drug discovery is the identification of novel chemical entities that alleviate a burden of infection or disease, then the diseased tissue in question should be accurately represented during the discovery process. What this translates to on the benchtop is a cellular model that is intended to faithfully replicate important aspects of disease as seen in a patient. Cells in the human body grow in 3D and are surrounded by other cells that continuously communicate to maintain organ function and homeostasis. Further, a variety of different extracellular matrices are found throughout the body that support cellular structure and organ integrity. The stimuli and responses experienced by cells in vivo is lost when those cells are purified and cultured in 2D on plastic or glass surfaces. Although 2D cell culture is relatively easy, robust and inexpensive, it may often misrepresent the biology of a phenotype. The key difference between 2D and 3D cell culture is cell-to-substrate interactions versus cell-to-cell interactions. Cells cultured on plastic assume a more flat and geometrically-constrained structure due to the interactions with the rigid substrate. This cell flattening can affect the spatial distribution of cell surface receptors and prevent the polarized morphology as seen in vivo [19]. Many important cell signalling pathways are downstream of cell surface receptors, and their misalignment can have serious and discrepant consequences. Integrins, for example, are cell surface receptors that communicate cell-to-cell and cell-to-ECM interactions and regulate the cytoskeleton. Integrins sense the extracellular microenvironment and activate protein signalling pathways inside the cell which results in proliferation, shape or motility changes and enables a rapid and flexible response to events occurring on the cell surface [20]. When cells are cultured on plastic, integrin expression and, subsequently, cellular behaviour can be drastically changed [20, 21]. These discrepancies can be further highlighted by comparing gene expression between the same cells grown as a 2D monolayer, 3D spheroid or subcutaneous tumour implant in a mouse (Figure 6). Comparative RNAseq studies highlight the massive batteries of genes that are turned off during 2D monolayer cell culturing and, thus, differentiate 2D samples from 3D and in vivo samples (Figure 6, red areas). Moreover, during the drug discovery process of treating cells with a test compound, in 2D, all of the cells are
equally and highly exposed to the reagent and constituents of the media. In 3D cell culture, compounds and nutrients are subjected to diffusion gradients such as those seen in human tissues. Intuitively, there have been numerous studies that have shown differential compound efficacy when comparing the same cells grown in a 2D or 3D environment [22–24].

Figure 6. Comparison of melanoma cell gene expression in 2D, 3D and in vivo. RNAseq heat map of SK-MEL-30 human melanoma cells grown in 2D monolayer, 3D spheroids (day 7 and day 11 time points) and subcutaneous xenograft murine tumour implants. Hierarchical clustering of gene expression reveals similarities between 3D spheroid growth and in vivo growth. Two-dimensional monolayer growth results in massive downregulation of many genes (red areas).

As mentioned previously, phenotypic screening has been thoroughly integrated into modern drug discovery since its inception in the late 1990s. However, these screening efforts have mainly occurred in cells grown on plastic using a single parameter readout. Compounds and targets identified through 2D screens often do not translate their efficacies to in vivo animal models. Consequently, the use of 2D cell models puts into question the physiological relevance and translational applications of simple phenotypic screening models used in high-content drug discovery. Of course, nothing short of an animal model is expected to fully replicate in vivo biology, but more complex cell models growing in 3D may be able to address some of the shortcomings of conventional 2D cell models and may be more predictive of in vivo behaviour [25].
3.2. 3D cell models: development in academia and implementation in industry

The development of 3D and organotypic cell models has been rapidly expanding since the late 1990s. In particular, investigations at the Lawrence Berkeley National Laboratory by Mina Bissell and colleagues on breast cancer modelling revealed that 3D tumour cultures are more predictive of in vivo cellular behaviour than conventional 2D models, and these predictive powers typically extend to mouse xenograft tumour studies [26]. A steady but exponential increase in 3D cell model research has led to an abundance of literature on the subject over the last 10 years. In 2005, there were an estimated 135 papers reported in PubMed on “3D cell culture” and in 2015 that number jumped to 781 papers. Accordingly, there are currently several competing marketplaces for 3D cell culture reagents and in vitro tissue modelling services that did not exist 10 years ago. Consequently, there has been an overall recognition by academia and industry alike that modelling cells in 3D more closely mirrors organism biology and this has resulted in a revised understanding of the methods with which we practice drug discovery [25].

The adaptation of 3D cell models into high-content drug discovery has been relatively slow compared to their development and usage by academic investigators. There are several obvious reasons for the restricted employment of 3D models in pharmaceutical drug discovery, the most obvious being cost and labour. Three-dimensional screening platforms are relatively expensive compared to 2D platforms. The sources of the extra cost are often specialized 3D plates, ECM components and reagents required for multi-parametric phenotypic readouts; for example, antibodies or cell tracking dyes. Primarily, the main hurdle in adapting a 3D cell model to high-content PDD is technical logistics. Building a 3D screening platform is considerably more labour-intensive than a 2D cellular model. For large-scale screening efforts, the costs and labour associated with screening in 3D may be inhibitory. An important consideration when developing screening platforms for big pharma PDD is the workflow involved in the screen and its adaptability with automation. Something as straightforward and inconsequential as plating cells, for example, becomes considerably more complicated when transitioning from 2D to 3D. Traditional liquid handlers and cell dispensers that are used to create 2D cell models may not be compatible with the intended 3D model. For example, mixing and plating a cell/ECM suspension often requires precise temperature control that may not be possible using standard cell dispensers. Matrigel, a commonly used ECM in 3D tissue modelling, is viscous at cold temperatures but becomes rigid and fixed at 37°C. This means that the Matrigel/cell mixture must be kept cold during plating to ensure the matrix does not polymerize prematurely. Similarly, soft agar is another 3D matrix often used in tissue and tumour modelling and is viscous at warm temperatures but forms a rigid matrix when cooled to room temperature. Consequently, a soft agar/cell mixture must be kept warm during plating to prevent premature matrix formation. Translating these temperature-controlled logistical challenges to automation is not trivial. Although it is relatively straightforward to keep matrix/cell suspensions temperature-controlled in flasks or vessels, the temperature must be maintained during the movement of the mixture through the lines (tubing) of the instrument. Therefore, the lines must be jacketed in some fashion to maintain either a cold or warm temperature, depending on the matrix used, to prevent the mixture...
from polymerizing and clogging before reaching the dispenser. In a practical sense, this can be difficult to achieve, which is why many synthetic ECM reagents are currently being developed that do not require precise temperature control (discussed later). In a similar respect, cell dispensing instruments often use peristaltic pump devices to dispense cells. Peristaltic pumps can be abrupt in their action and may not be amenable to the careful dispensing required for a 3D cell model [25]. Consequently, a different type of cell-dispensing device, for example, a syringe-based system, may need to be engineered into the instrument to achieve the level of precision needed to create automated 3D cell models. In addition to modifications in automation, complex cell models that require feeder cells, gel matrices or scaffolding also provide for logistical challenges and complicated workflows [25]. Finally, complex 3D cell models often require long incubation times to manifest a particular phenotype or may require media changes or other manipulations needed to coax the model into the desired geometry. This type of precision may prove technically taxing compared with simpler 2D approaches, particularly in an automated format and at large scale [25].

An important aspect of complex and 3D cell models that is often overlooked in publications reporting their beneficial characteristics is that of variability. Two-dimensional monoculture screening platforms require few reagents for use and, subsequently, demonstrate minimal variance when assayed in PDD. Upon increasing the reagents involved for a complex assay (ECM, multiple different media or cell types), the variability is equally increased. The increasing number of variables that often accompany complex 3D cell models lead to an accumulation of potential variance. Further, 3D structures themselves, by virtue of their higher dimensional nature, are characterized by an increased level of heterogeneity than 2D systems [25]. With respect to assay readout, data acquisition of 3D structures is tremendously more challenging than for cells grown on plastic. This results in larger standard deviations for 3D cell models compared to 2D (personal observation). In order to overcome this inherent heterogeneity and account for the observed deviation, it becomes necessary to include more replicates within an assay. Including replicates within a primary screening assay results in a three to fourfold increase in cost or, alternatively, restricting the size of the library to be screened (discussed later).

In conclusion, 3D cell models are more expensive, technically challenging and labour-intensive to integrate into automated drug discovery at large scale compared to 2D models. However, in the discovery of novel targets and MoA that authentically represent patient disease biology, 3D models would seem to be superior to 2D models. Importantly, 3D models are often used to triage hit compounds from a 2D assay to an in vivo animal study. If that is the case, then those same gating 3D assays should be moved to the primary screening effort in order to reduce the quantity of false positive hits that investigators spend countless hours hunting down (Figure 7A). Another importance of primary screening in 3D is a reduction in time spent between the primary screen and the in vivo validation study. In this complicated climate of lengthy drug discovery programs, any shortening of timelines is extremely desirable and cost-effective. Most importantly, it has been observed that although many compounds may demonstrate comparable activities on cells grown in 2D or 3D, a large percentage of screened compounds (possibly 25%) may only demonstrate 3D-specific
activity ([24] and reviewed in Ref. [27]). A benchmark example of this type of behaviour is Zalutumumab, an epidermal growth factor receptor (EGFR)-binding monoclonal antibody that only demonstrates efficacy in 3D in vitro and in vivo tumour models. These types of compounds would be missed under 2D screening conditions and may be important in addressing relevant disease biology (Figure 7B). Therefore, lately, it has become prudent for industrial drug discovery scientists to adapt into their high-content screening workflows, the 3D cell models created by academic researchers. The most apparent hurdle in this process is the miniaturization of complex and 3D cell models to accommodate a well of 384-well high-throughput screens (HTS) assay plate which is the size of two uncooked grains of rice. Ultimately, the goal is to progress from good models of tissue complexity and function into models that can be standardized and incorporated into high-content drug discovery [28].

Figure 7. Three-dimensional screening may reduce drug development time and false positive rate. (A) Time between primary screening and in vivo modelling may be reduced using 3D primary HTS. (B) Primary 3D HTS may significantly reduce rate of false positives by as much as 25%. 
4. Oncology research pioneers 3D cell modelling

4.1. An abbreviated history of 3D cell models in oncology research

It is estimated that within their lifetime, a person runs a 39.6% risk of being diagnosed with some form of cancer (National Cancer Institute, 2010–2012). This incredibly large patient population has driven the research and development functions in oncology faster and more furiously than any other disease field. In fact, 3D tumour modelling has been in constant use since the early 1970s. The multi-culture tumour spheroid (MCTS) model, which are tiny microtumours of self-assembled cancer cells, was developed in 1970 by Sutherland [29] and is still considered a crowning achievement in 3D tumour modelling. Similarly, the soft agar colony formation assay, which quantitates a cancer cell's anchorage-independent growth and self-renewal, was developed in 1976 by Courtenay [30] and continues to be widely employed as a gating assay for new experimental chemotherapeutics. Since that time 3D culture models developed for oncology research can be loosely grouped into three categories: (1) cells cultured as multicellular aggregates, (2) cells embedded within an extracellular matrix support (which might be natural or synthetic) and (3) cells cultured on inserts [28].

4.2. Screening the tumour microenvironment

Tumours (neoplasia) are complex tissue structures that harbour myriad cellular components similar to an organ. Neoplasia begins with transformed cancer cells that are often epithelial in origin. After the initial transformation and unrestricted cellular growth, cancer cells recruit neighbouring cells to feed tumour development and maintenance. These mesenchyme-derived cells, referred to as fibroblasts, then assume an activated state through the stimulation by cancer cells of fibroblast growth and secretory pathways. These activated “cancer-associated fibroblasts” (CAFs) further feed tumour development and actively participate in the recruitment of other types of cells to contribute to tumorigenesis. For example, CAFs are able to prevent immune recognition and T-cell-mediated tumour killing by secreting immunosuppressive cytokines. These cytokines further blunt the innate immune response and stimulate the invasion of protumourigenic regulatory T-cells and M2 macrophages [31, 32]. Once tumours enlarge beyond 1–2 mm in diameter, they require oxygen to sustain viability because this is the maximal distance that oxygen and nutrients can diffuse without a blood supply [28]. CAFs stimulate neoangiogenesis by recruiting vascular endothelial cells and pericytes that form immature blood vessels, which then sustain tumour enlargement [33]. At this point, the tumour microenvironment (TME) has matured and contains many types of stromal cells (mesenchyme and hematopoietic) that all contribute to tumour survival and immune evasion (reviewed in Ref. [34]).

As one can imagine, accurately replicating the \textit{in vivo} tumour microenvironment and all of its constituents in a Petri dish is not currently possible. That being said, there are elements of the tumour milieu that can be faithfully mimicked \textit{in vitro} for the dissection of important cellular biology. The two most important features of \textit{in vitro} tumour modelling are 3D growth and cell-to-cell communication. As mentioned earlier, there are currently three methods that dominate 3D tumour modelling: multicellular aggregates (spheroids), cancer cells embedded within an
ECM (colonies) and cancer cells cultured on inserts or transwells (skin, lung models and migration assays). Many of these complex models are at various stages of integration into high-content drug discovery.

4.3. The microtumour spheroid model

The spheroid model has gained a lot of popularity of late due to its tumour-like characteristics and ease of use in high-content screening. Spheroids can be thought of as tiny microtumours, in that they are self-assembled cancer structures organized into a hierarchical arrangement where cell-to-cell contacts create a 3D spherical structure. Spheroids often display properties and characteristics found in human tumours. For example, due to their complex structure, spheroids display gradients of oxygen and nutrients such that the outer shell of the spheroid contains rapidly proliferating cells (Figure 8). The proliferating shell surrounds a zone of quiescent cells, which further mantels a hypoxic area (Figure 8). The hypoxic zone is relatively large and results from a lack of oxygen penetration, mirroring in vivo avascular tumour physiology. In the centre of the spheroid is a necrotic core that contains dead or dying cells resulting from a large accumulation of metabolic waste products (e.g. lactate) and characterized as having low pH (Figure 8) [25]. The microtumour spheroid model has been widely adopted in high-content oncology drug discovery due to its emulating several important features of patient tumour biology that are not observed in cancer cells grown in 2D; namely, drug penetrance and hypoxia. Assaying for compound efficacy on cancer cells is equally as important as assaying for drug penetration; as one begets the other. Similarly, hypoxia-inducible factor (HIF) genes that respond to low oxygen concentration have been found to drive cancer stem cell behaviour in a variety of solid organ tumours [35]. There has been a barrage of literature around spheroids as models used in anticancer drug screening [36, 37], and further studies have shown how these models can be adapted to high-content drug discovery [38–40].

Figure 8. The 3D microtumour spheroid cell model. Composite of immunohistochemistry images showing the necrotic core characterized by large lacunae of necrosis (haematoxylin and eosin stained), hypoxic area (pimonidazole duct staining) and zones of low proliferation (Ki67 low) and high proliferation (Ki67 high).
From a first-hand practical perspective, the spheroid platform represents an elegant biomimetic model for the identification of new molecular entities (NMEs) and MoAs. This is particularly true for finding targets and pathways that are only activated in a 3D context. There are several different methods that can be used to generate spheroids in 384-well high density format and they each have their respective benefits and liabilities. Numerous competing technologies exist for creating 384-well hanging drop spheroids (e.g. Perfecta 3D Hanging Drop Plates from 3D Biomatrix or the GravityPLUS System from InSphero), and these plates are easily adapted to high-content drug discovery [40, 41]. Similarly, low-attachment or round-bottom plates can be used to generate spheroids that are less technically cumbersome than the hanging drop system (e.g. 384-well Spheroid Plates from Corning or Ultra Low Attachment (ULA) Plates from SCIVAX). If cost is prohibitory, then ULA spheroid plates can be made in-house using standard U-bottom plates coated with 2-hydroxyethyl-methacrylate, which acts as a hydrogel in water and can induce 3D cellular aggregation [42]. Although spheroids are relatively easy to generate as far as 3D cellular structures go, they are more difficult to analyse effectively. The standard approach to quantifying changes in spheroid phenotype involves high-content confocal-based imaging. This can be achieved using antibodies that recognize a particular protein of interest or, more commonly, a chemical sensor that reports a biological phenotype (e.g. cell viability, cell death, caspase cleavage). The benefit of utilizing an imaging-based approach for the endpoint assay is that spheroid size and structure measurements can also be incorporated into the metrics to provide comprehensive and multi-parametric data [43]. However, as spheroids are several hundred micrometres thick and are rarely found on the exact same focal plane between wells, an autofocus feature on the imaging instrument is highly desirable. The other option is to assemble a Z-stack of images to address the focus problem, though that adds significant time and data storage issues to the readout process, particularly for large screens. Similarly, cell tracking using chemical sensors can be difficult to achieve over a week-long period of time as these dyes often degrade or become diluted with cell proliferation. What is increasingly being used for spheroid-based screens is a simple and straightforward CellTiter-Glo assay. CellTiter-Glo (Promega) is a luminescent viability assay that quantitates a cell’s ATP and, thus, the amount of cells that are metabolically active. Recently, Promega developed a 3D-specific CellTiter-Glo 3D assay specifically designed for measuring spheroid viability, and this assay has been shown to be robust, sensitive and scalable to high-throughput screens [44]. Further, bioluminescent ATP detection assays offer relatively simple workflow and data analysis [44]. This may seem a low-tech readout for a high-tech cell model, but there are significantly fewer problems to overcome working with HTS bioluminescent viability assays compared to HTS imaging assays. Although you lose the benefit of a multi-parametric readout with CellTiter-Glo assays, the data are more robust and demonstrate less variance (personal observation).

4.4. HTS ECM assays

Another prevalent 3D screening platform used in oncology drug discovery is the colony formation assay that employs cells grown within an ECM. ECM strongly affects cellular organization and function and 3D cell models that incorporate ECM arguably help to better mimic in vivo biology, as they allow for cell-to-ECM interactions [45]. Though there are a variety
of ECM-based assays employed in cancer cell modeling, the colony formation assay indisputably gets the most use. The assay enables the quantitation of a cell’s anchorage-independent growth, through its ability to proliferate in 3D space. Additionally, as colonies are clonal resulting from the growth and proliferation of one particular cell, the colony formation assay also quantitates a sample’s cancer stem cell population; as only cancer stem cells possess the property of self-renewal and clonal growth [46]. Historically, the 3D colony formation assay has been relegated to a secondary or tertiary screening platform by academics and industry scientists alike, where it has served as a gating step for moving forward new experimental therapeutics. Recently, investigators at Abbott Laboratories developed a soft agar colony formation assay that can be adapted to high-content screening, thereby bringing an important secondary assay to the forefront of primary phenotypic screening [47]. This 384-well 3D assay then opened the door for other big pharma screening projects such as the one by Sanofi-Aventis which involved screening 300,000 compounds on five different Kirsten RAAt Sarcoma viral oncogene homologue (the most highly mutated and undruggable oncogene in human cancers) (KRAS)-dependent cancer cell lines grown in 3D ECM to identify pathways, targets or chemical matter with selective KRAS antitumor activity [48]. Researchers at Novartis have taken the 384-well colony formation assay even further, mixing normal colon fibroblasts together with colorectal carcinoma cells to achieve therapeutic indices of experimental test compounds [24]. The therapeutic index is a powerful metric for the simultaneous identification of a compound’s therapeutic efficacy and potential toxicity.

There are a variety of matrix options commercially available for the design and implementation of HTS 3D ECM tumour assays. Soft agar is arguably the most common and least expensive ECM available and can be tittered to achieve the appropriate tensional force; an important characteristic when custom tailoring an ECM assay to different tissue and organ types. Matrigel (BD Biosciences), derived from the basement membranes of mouse sarcoma cells, is widely employed in HTS formats due to its easy-to-use thermal labile properties. However, Matrigel frequently contains cytokines and growth factors that demonstrate batch to batch inconsistencies which may result in unwanted or unpredictable variability [49]. In the past several years, there have been other ECM products developed for 3D assays that are similar to Matrigel such as ECL Cell Attachment Matrix (Millipore) and Geltrex (Life Sciences), which may offer advantages over conventional reagents. There is also a human placenta-derived ECM for 3D assays, HuBiogel (Vivo Biosciences), which has been shown to be a superior product for modelling human tissues due to its composition of collagens and laminins in biologically relevant proportions. Further, HuBiogel ECM is completely devoid of extraneous growth factors and cytokines, leading to more robust and reproducible 3D structure formation [50].

As opposed to naturally derived ECM substrates, there are also synthetic hydrogels that may be specifically engineered with chemical handles or attachment proteins to enable custom matrix conditions while still accounting for the heterogeneities present within the in vivo microenvironment. Chemically defined bioinert hydrogels can be customized with biomimetic and tissue-specific peptides to promote cell attachment and degradation in a robust format that may mitigate the need for naturally derived but ill-defined ECM [51]. These types of synthetic hydrogels are often not temperature labile and can be manipulated at
room temperature, making them suitable reagents for use with automation in that there are no line- or tip-clogging problems to address. Some of these hydrogels (e.g. hyaluronic based hydrogels) can be systematically manipulated with distinct wavelengths of light (e.g. UV) to create custom ECM stiffness and density [52]. From a practical perspective, the synthetic ECM option may be more convenient for high-content approaches in that the cell/matrix mixture can be easily dispensed without the need for strict temperature control in the dispensing instrument. After plating, the ECM is cured through a quick exposure to UV light which solidifies the matrix and locks the cells in place. Synthetic ECM reagents are still currently in development by academic laboratories and have not yet been thoroughly integrated into high-content 3D drug discovery platforms.

4.5. Co-culture assays

Three-dimensional growth that addresses cell-to-ECM interactions is a key parameter for creating biomimetic tumour models. However, a parameter that is equally important to model in oncology drug discovery is cell-to-cell communication and this can only be accomplished through the use of co-cultures. Co-cultures are mixtures of two or more cell types within one assay with the goal of dissecting cellular crosstalk that may be important for modulating a particular phenotype. Co-culture assays are extremely relevant in oncology investigations in order to deconvolute biological signalling that occurs between different cell types within the TME. Cellular communication within the TME remains poorly understood and involves complex networks of secreted factors as well as direct ligand-to-receptor cellular interactions [53]. The most common co-culture assays used in oncology studies incorporate transformed cancer cells with cancer-associated fibroblasts (CAFs). The co-culturing of these two cell types often reveals important mechanisms by which fibroblasts can affect tumour cell behaviour and morphology. As CAFs often constitute the bulk of a tumour mass, it has become increasingly important to better understand their role in promoting and sustaining tumorigenesis, catalysing epithelial-to-mesenchymal transition (EMT; metastasis), suppressing the anti-tumorigenic immune response, and supporting drug resistance. In contrast to cancer cells, de novo acquisition of genetic mutations is less common in stromal cells than in malignant cells, so CAFs may be less prone to escape or resistance to a new therapy via genomic instability or epigenetic modifications. In the parlance of drug discovery, this means that a therapy targeting stromal components of the tumour may be more efficacious than targeting the transformed cells themselves and, moreover, may be more ubiquitously applied to many different types of neoplasia. Indeed a plethora of research investigating stromal targets for use in chemotherapy has surfaced during the last few years, supporting a more nuanced view of the contribution of stromal components to neoplastic transformation (reviewed in Ref. [54]). Prominently, CAFs have been shown to actively participate in tumour immunomodulation; CAF-secreted factors have been shown to suppress inflammation, antagonize T-cell invasion and recruit protumourigenic myeloid-derived cells to the tumour [32, 55].

There are a variety of methods to employ CAFs and tumour cells within the same 3D culture, and the approach is often dictated by the biological mechanism under investigation. For example, CAFs and tumour cells may be incorporated into a co-culture tumour spheroid to
scrutinize direct cell-to-cell (ligand-receptor) communication between these two cell types. Alternatively, CAFs may be plated in 2D with cancer cells grown in 3D atop an ECM overlay, which may faithfully replicate secreted protein-based signalling between the different cell types. Using a genomics-based approach to study CAF-tumour cell interactions, genes may be knocked down using genomics reagents (siRNAs, virally-encoded shRNAs) within the CAFs and then assayed for a resulting phenotype within the cancer cells. This type of investigation may reveal CAF-specific genes or proteins involved in paracrine signalling that are crucial for tumour maintenance.

Continuing with the theme of tumour immunology, primary immune cells are also being used in co-culture with tumour cells to ascertain new mechanisms of immune-based targeted killing. Many of these elegant models employ 3D tumour spheroids and primary T-cells or natural killer (NK) cells in a high-content format to identify chemical reagents that can facilitate immune cell tumour recognition and killing [56]. These types of complex phenotypic screens may greatly expand the targeting space of conventional chemotherapeutics to include members of the tumour stroma such as CAFs and immune cells.

As with any complex phenotypic screening assay, co-culture systems present their own unique set of technical complications that must be overcome and optimized. Important considerations when developing a co-culture assay include the source of each cell type used (primary versus immortalized), ratios of cell types (biologically relevant proportions), length of culture time and data deconvolution. In terms of assay development, the cell culture media used may represent the most challenging hurdle. As different cell types require different types of media; glucose, amino acids, insulin, vitamins, serum, etc., all titrated to specific proportions, determining which media to use for a co-culture may require extensive empirical testing [53].

Another application of a co-culture assay is to identify potential and unwanted toxicity of a compound. In this example, fibroblasts and tumour cells may be mixed and assayed to obtain a therapeutic index of a test compound. Incorporating one target cell type with one normal cell type within the same screening well condenses the experimental workflow so that differential toxicity can be quantitated and used to triage hit compounds for further study [24]. This approach may complicate the primary assay but may also yield more therapeutically important data. In terms of assay readout, it may be crucial to distinguish between two different cell types. From an imaging- or flow cytometry-based perspective, this can be accomplished through the use of cell-specific reporter vectors; i.e. an enhanced green fluorescent protein (EGFP) reporter in tumour cells and a DsRed reporter in CAFs. A potentially easier approach is to label the different cell types with cell tracking dyes, though these types of chemical sensors tend to degrade and dilute over long time courses or may be swapped between different cells in close proximity (personal observation). Alternatively, if a luminescent screening platform is used, the different cell types may be engineered to express different forms of the luciferase enzyme. For example, tumour cells might express firefly luciferase and fibroblasts might express Renilla luciferase and a Dual-Glo Luciferase assay (Promega) can reveal differential luciferase signals and thus efficacy on tumour cells versus toxicity on fibroblasts. This approach requires a lengthy time of cell engineering but may produce robust and straightforward data for an HTS drug discovery effort. In short, the development of physiologically relevant co-
culture assay systems for industrial drug discovery is challenging, but there are many technological innovations available to provide a scientific/technical tool box for the advancement of multi-culture primary phenotypic screening assays and improvement of early stage drug discovery [53].

4.6. Transwell models

The transwell plate system (also referred to as modified Boyden chambers) consists of a series of permeable supports inserted into wells of a cell culture plate. Cells can be plated in the lower chamber and/or the upper chamber, and the porous membrane can be exploited to study chemotaxis or cell migration, making it a versatile assay platform applicable to a variety of phenotypic screening experiments. In oncology research, the transwell system has been used to model the air-liquid interface for several different indications. Skin studies in particular are well modelled using the transwell system, where collagen and dermal fibroblasts are plated upon the insert and melanoma cells are layered on top. Melanoma cell invasion into the dermis is then quantitated by low-throughput methods such as histology of membrane cross sections. Transwell plates are also used for invasion assays of cancer cells. In this type of assay, cancer cells are plated in the top chamber over a layer of Matrigel (or another type of ECM) and a chemoattractant is added to the lower chamber to induce trans-membrane migration of cancer cells. Cancer cells that migrate through the ECM and invade the lower chamber can be quantitated by simple staining and counting [57]. Transwell plates are also used for immune cell migration assays. For example, in place of ECM, transwell inserts can be coated with vascular endothelial cells and immune cells (leukocytes) are plated on top. A chemoattractant is added to the lower chamber and transendothelial migration of immune cells is quantitated by cell staining and counting or, alternatively, antibody labelling for specific cell surface markers that distinguish the migrated population (e.g. T-cells or neutrophils). These methods can be modified to include tumour cells in the lower chamber and monocytes in the upper chamber. As monocytes migrate through the endothelial layer and invade the tumour cells they may differentiate into macrophages and become adherent, an enabling characteristic for quantitating invasion into tumour cell monolayers.

Transwell plates are typically used in low-throughput formats such as 12- or 24-well inserts. Currently, the most high density transwell plate is the 96-well plate made by Corning. The lack of high-content screenable formats has restricted the employment of transwell assays in phenotypic drug discovery. At present, the most common application of the 96-well transwell plate is for cell-based drug absorption assays [58]. In these approaches, CaCo-2 gut epithelial cells are plated as monolayers on the permeable inserts. Experimental test compounds are then added to this mock intestinal lining and permeability and transport characteristics of the compound are calculated. This component of Absorption, Distribution, Metabolism and Excretion (ADME)/tox (absorption, distribution, metabolism, and excretion) testing often determines whether a compound continues in the drug discovery process [59]. Development and implementation of a high density 384-well transwell plate may significantly expand phenotypic drug discovery for a range of different disease indications.
5. Complex phenotypic screening in other disease areas

5.1. Cellular differentiation and flow cytometry in PDD

Complex phenotypic screening assays do not necessarily require 3D cellular growth. Assuming the assay replicates an important and patient-relevant aspect of disease pathology, any multi-parametric phenotypic screen may be considered complex by comparative standards. For example, flow cytometry represents an unparalleled advance in the quantification of multi-parametric measurements on single cells. As mentioned earlier, one of the hallmarks of leukaemia is a block in differentiation. Rapid proliferation of immature, undifferentiated hematopoietic progenitor cells (blasts) leads to blast crisis which often takes the lives of acute myeloid leukaemia (AML) patients. Restoring the natural process of hematopoietic differentiation in these immature myeloid precursor cells usually results in a concomitant decrease in their proliferation. Flow cytometry is a technique well-suited to leukaemia research as identification of cell surface markers is the most common method used to characterize differentiated hematopoietic subtypes. Two leading researchers of leukaemia therapeutics, David Sykes and David Scadden at Massachusetts General Hospital, recently used a flow cytometry-based phenotypic screening approach to identify ML390, a compound identified from a collection of 330,000 compounds, which was able to restore differentiation of several human myeloid leukaemia cell models [43]. From a different assay perspective, flow cytometry can be used to multiplex viability readouts. Different cell types may be screened and then subjected to a fluorescent barcoding scheme where each cell type is given a unique tracker dye barcode, then pooled together and run through one flow cytometry readout (e.g. viability or apoptosis). This cellular barcoding method enables assay multiplexing and condensing multiple assays into one readout, which may often be the bottle neck of the screening experiment [44]. New technologies such as the high throughput sampler system (Becton Dickinson) and the HyperCyt platform (IntelliCyt) have recently enabled the application of 384- or 1536-well flow cytometry to large scale drug discovery phenotypic screening efforts [60]. Although flow cytometry is employed for single cell resolution, large particle flow cytometers (e.g. COPAS from Union Biometrica) are now capable of analysis and sorting of large macrocellular structures several hundred microns thick, such as spheroids [45]. Large particle flow cytometry may be ideally suited to the rapid analysis of spheroids or microtissues in suspension, a cumbersome task for high-content imagers [24].

The cellular differentiation phenotype may also be quantitated using an imaging-based approach, which is equally suited to high-content drug discovery. In a manner similar to flow cytometry, assayed cells are stained with antibodies that recognize cell surface markers, fixed and subjected to HTS imaging analysis. Imaging data are then analysed for the intensity and frequency of antibody staining and/or the co-localization of stem/differentiation markers. This method has been employed extensively by researchers in the field of regenerative medicine and has been shown to yield high quality robust data in primary HTS for compounds that expand hematopoietic stem cells (HSCs) for use in transplant therapy [61] or induces the selective differentiation of multipotent mesenchymal stem cells for cartilage repair [62].
5.2. Cell migration and wound repair

Cell migration and motility are important biological features common to different diseases. Cell migration assays are routinely used in the study of wound healing to identify therapeutics that can increase fibroblast, endothelial cell or epithelial cell migration. The so-called scratch assay is a convenient and inexpensive method that has been routinely employed for decades to study cell migration in vitro [63]. This method is based on the principle that, upon creation of an artificial gap (scratch) on a confluent cell monolayer, the cells on the edge of the scratch will move toward the opening, to close the scratch until new cell-to-cell contacts are established [64]. The scratch assay is overly simple and can be accomplished using common and inexpensive supplies routinely found in most cell culture laboratories. Employing an imaging-based readout to the assay, the width of the scratch is measured at the beginning of the assay and then at subsequent intervals throughout the assay until the scratch is closed. The scratch assay can be integrated into high-content wound healing screens for the discovery and validation of small molecule leads and other perturbagens that affect cell migration [65]. However, at smaller screenable formats such as 384- or 1536-well, making scratches in wells becomes much more difficult to achieve with consistency and reproducibility (Figure 9A) which is why other migration-assaying technologies have been developed.

Cell migration and motility also play vital roles in the process of tumour metastasis. However, in this case, the goal is the identification of anti-migratory agents that might be used in the clinic to restrict metastasis. Due to the inherent heterogeneity of the scratches made during an HTS scratch assay, a technology slightly more sophisticated was developed that is more robust during high-content imaging. The Oris™ Pro assay system (Platypus Technologies) is a novel, multi-parametric cell migration assay that is available in 384-well format and is fully compatible with automated microscopy and high-content screening [66]. This technology employs a circular plug in the centre of the well around which cells adhere and grow. The plug then dissolves revealing a perfectly centred circular zone of no cell growth. High-content imaging measures the diameter of the circle at day 1 and all subsequent time points until cells grow over the zone. The benefit of this assay over the scratch assay in studying metastasis is that cells are not physically disrupted and sheared as they would be in the scratch assay which is a more appropriate model of wound generation/repair. The Oris™ platform was recently used to identify compounds that inhibit cell motility in human breast cancer cells in an HTS format [66]. A similar type of assay platform was developed by collaborating biologists and engineers and consists of a 384-well silicon plug system that fits into the assay plate [67]. As opposed to the Oris™ system, the ZonEx system demonstrates robust consistency and reproducibility between wells (Figure 9B). Further, it is a reusable technology in contrast to the Oris™ system that is a one-use product [67].

Most currently available migration and motility assays exist only for 2D cell culturing, which may not necessarily mimic the complex mechanical and biochemical interplay between various cells and the ECM microenvironment that occurs in human patients. To address invasion dynamics in 3D culture, a multi-parametric 3D HTS platform for cell motility and invasion was recently developed [68]. In this vertical gel invasion assay, cells are seeded on top of a collagen matrix and their migration/invasion into the gel is quantitated from a Z-stack taken
with a laser-scanning confocal microscope. This approach is more biomimetic than traditional 2D scratch and zone exclusion assays and may reveal important perturbagens of 3D cell migration, for example, integrin-modulating agents. Unfortunately, a drawback to this approach is the requirement of Z-stack image assembly at multiple time points which can result in terabytes or even petabytes of data storage for a large scale compound screen.

Figure 9. Comparison of two HTS cell migration assays. (A) The standard scratch assay in 384-well format demonstrates wide variability of the position and width of the scratch, resulting in poor reproducibility. (B) The ZonEx assay makes perfectly circular zones of the same dimensions and at the same place in every well of a 384-well plate, yielding robust data. Images courtesy of Nicholas Ng and Orzala Sharif.

Overall, there are a wide variety of different assays available to screen for modulators of cell migration and motility either in 2D or 3D (reviewed in Ref. [69]). The different assay platforms range from simple and inexpensive to technically demanding and costly and the suitability of
a particular method may be limited when considering a specific research question [69]. However, the continued integration of these sophisticated and complex phenotypic screening platforms into industrial drug discovery may significantly advance the quality of pro- and anti-migratory therapeutics progressed to the clinic.

6. Future outlook of complex phenotypic screening

The goal of phenotypic screening is the identification of new molecular entities, targets and mechanisms that can be exploited to create better disease-specific therapeutics. As opposed to target-based screening that occurs in an artificial biochemical environment, phenotypic screening takes advantage of the native cellular environment, a necessary quality when assaying for novel disease biology. Improving upon this, complex phenotypic screening expands the cellular environment further to include the extracellular environment, which actively participates in cellular disease pathology. Three-dimensional and complex HTS is relatively new to industrial drug discovery and has yet to prove its impact in big pharma. Although a wide range of products, technologies and services are currently available to facilitate 3D/complex HTS drug discovery, there are three key components that must be incorporated and addressed to ensure maximum success for future screening endeavours: (1) screening disease-relevant cells, (2) incorporating microfluidics and (3) decreasing assay capacity.

First, induced pluripotent stem cells (iPS) or patient-derived primary cells should be used for screening. Advancements in iPS technology, where adult somatic cells are reprogrammed into a pluripotent state similar to an embryonic stem cell, have provided a renewable source for relevant cell types for a wide variety of diseases [25]. Similarly, patient-derived iPS cells are able to recapitulate the characteristics of the disease phenotype from a patient and may open the door for personalized disease modelling. This, in turn, should improve the predictive value of complex in vitro cell models used for drug discovery [25].

Second, converting static cultures to perfused cultures using microfluidics devices will be crucial for optimizing organotypic cell models. Microfluidics represents a potentially revolutionary cell culturing approach using laminar fluid movement that better mimics the physiology of living tissues and organs. Further, microfluidic devices can support 3D cell culture making them excellent surrogates for the in vivo extracellular microenvironment (reviewed in Ref. [70]). However, microfluidics-based cell models require more miniaturization and engineering to create HTS-compatible assay platforms that incorporate active perfusion of media, growth supplements and test reagents. Microfluidics devices are not currently adapted into drug discovery screening and this may be largely due to the need for peristaltic pumps and other valves and mixers that accompany the plates. The ONIX system developed by CellASIC® incorporates a clever workaround for pumps, instead using gravity and surface tension to facilitate fluid flow through the plate. Continuous perfusion of the wells is maintained by refilling the inlet and emptying the outlet [71]. Although these plates can currently only accommodate 32 wells, the concept of fluid flow without the need for active pumping is
a great technological advancement for the field. Another microfluidics plate which does not require use of a pump is the Iuvo Microchannel 5250 system (BellBrook Labs) that uses a passive pumping technology to move fluid between two inlets connected by a channel. Iuvo plates come in 192-channel formats making them potentially useful for high-content phenotypic screening. Early stage drug discovery could greatly benefit from the integration of microfluidic tools into primary platforms which, in most cases, represents an improvement upon existing screening technologies [25].

Third, shifting the current screening paradigm from assay capacity to assay relevance may improve the quality of new therapeutics. Technological advancements that facilitate screening of complex cell models will undoubtedly be associated with a lower throughput than current simple 2D cell models. This translates to fewer 1536-well formatted cell models and, thus, smaller compound and reagent libraries that can be screened. Smaller focused screens sample chemical space instead of blanket coverage, but provide more insightful information when combined with multi-parametric, multi-time point assays [25]. By employing the concept of mechanistically informed drug discovery, smaller, more focused screens that allow multiplexed dynamic readouts may produce data of much higher quality with respect to predicted patient response [25], and this should ultimately result in the discovery of more successful therapeutics.

7. Conclusion

During the process of industrial drug discovery where new therapeutics are being tested in cell-based phenotypic screening assays, the culture methods used should mimic the most natural in vivo representative form possible [45]. In order to maximize success in the current drug development space, new technologies and methods must continue to evolve. Emerging phenotypic assay platforms must be critically compared and evaluated and, most importantly, must share extensive likeness with real tissue or tumour architecture. Incorporating 3D and complex phenotypic cellular assays into high-content drug discovery screening may effectively reduce the false positive hit rate, accelerate preclinical in vivo animal disease model studies and ultimately yield more efficacious and less toxic treatments for disease.

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