High throughput screening, high content screening, primary and stem cells *new techniques now converging*

Over the past decade, the use of cell-based assays has accelerated in modern drug discovery. Indeed, the majority of assays in either target validation or lead identification/optimisation all now employ cell-based technologies. Furthermore, a wide range of target classes are also addressable via cell-based approaches, including G protein coupled receptors (GPCRs), kinases, nuclear hormone receptors and ion channels. Many of these assays routinely use highly miniaturised protocols, accompanied by highly sensitive detection techniques and automated fluid handling instruments. An implicit assumption in all of these approaches is that the functional response of the cell provides a better understanding of both the physiology of the drug target, as well as the pharmacological interaction with novel compounds. However, classical cell-based assays employ cell phenotypes that differ markedly from those found in human pathology, and have historically been chosen based on their ease of use with prevailing screening technologies. This reliance on the use of immortalised clonal cells has raised some concerns in terms of the clinical relevance of either the target validated, or of the identified lead compound selected for subsequent development. Consequently, some drug discovery programmes are employing primary or stem cells in cell-based screening, as well as adopting sophisticated cell-based assays employing high content screening (HCS) techniques.

By Dr Richard M. Eglen

A widely used approach for primary high throughput screening (HTS) employs assays in which immortalised cells are recombinantly engineered to express a discrete molecular target, the functionality of which is assayed using cell responses that are readily detected and quantified using automated fluid dispensing and detection systems. Now broadly used in drug discovery against a range of target classes, cell-based HTS has been the predominant approach for identification of compounds targeting GPCRs, kinases, nuclear hormone receptors and ion channels for many years. In the specific case of GPCRs, most assays measure the receptor-compound interaction using functional responses, ranging from second messenger accumulation to more complex

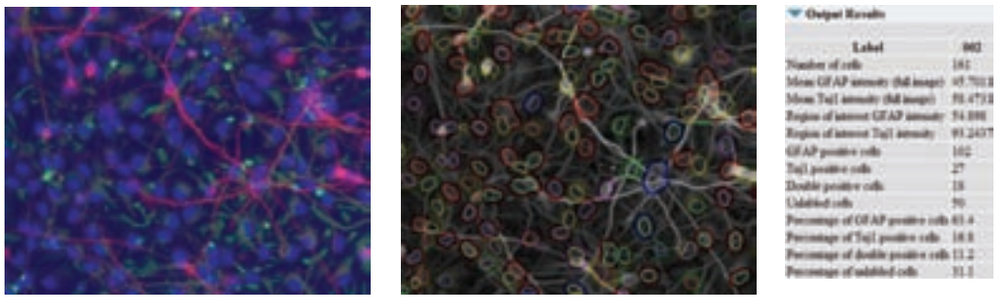

Figure 1
Neuronal and glial cells, differentiated from embryonic stem cells, stained for Tuj1 and glial fibrillary acidic protein (GFAP), imaged on the Opera (PerkinElmer) and analysed using Acapella software (PerkinElmer). The cytoplasmic regions of all cells were identified, allowing quantification of neuronal and glial cell marker. Left: Three color image (Tuj1 = red, GFAP = green, nuclei = blue). Middle: Cell identification and region definition in Acapella. Right: Output parameters, quantifying the numbers and percentages of Tuj1, GFAP and double positive cells

cell responses such as protein translocation (movement of ancillary molecules, including arrestin, or internalisation of the GPCR *per se*), modulation of membrane conductance or activation of kinase signalling pathways[1].

Most often, recombinant immortalised cells are used in the screening of large libraries of small molecules. Putative 'hits' identified are subsequently 'validated' using multiple counter-screens in order to establish compound potency and specificity. Based on sub-structural searching, focused libraries may then be used to optimise lead compounds with respect to several 'druggable' properties including solubility and pharmacokinetic properties, as well as potential cellular toxicities. Usually, this is an iterative process in which one re-screens focused libraries for interactions at the molecular target, eventually leading to the selection of candidates for *in vivo* testing and ultimately clinical study[2].

This standard approach, while rapid, is also expensive due to the extensive chemistries involved in lead optimisation. Additionally, it is inefficient, as little information is generated prior to lead optimisation, in terms of potential human efficacy or toxicity. Despite these shortcomings, these approaches have historically been highly successful in identifying compounds that interact with the validated GPCR target with high affinity and/or selectivity, allowing several compounds to proceed to clinical evaluation. Nonetheless, in some cases, important differences are found between actions of compounds on recombinant molecular targets (often over-expressed in immortalised cells) and their effects on 'physiologically normal' tissues *in vivo*[3].

Immortalised cells and HTS – a pragmatic compromise

Obviously, there are advantages to the use of immortalised cell lines in drug discovery. First, cells transfected with the molecular target may be grown in virtually unlimited quantities. Second, they provide a null background for target expression and subsequent measurement of assay responses. Third, such cells provide a consistent, homogenous vehicle for HTS campaigns. Indeed, using standard cloning and expression technologies, not only can the molecular target be stably expressed at physiologically relevant levels, but additional proteins can be engineered into these cells, thereby providing reporter protein readouts of compound/receptor interactions[3].

Nonetheless, emerging data indicate some limitations in screening compounds using these systems, particularly with regard to the applicability of the data generated on the action of the compound in the human physiological setting. This is partly due to the fact that the genetic, and therefore molecular, phenotype of cultured immortalised cells differs considerably from that of native cells *in vivo*. This can be readily highlighted in the case of GPCRs. Thus, clear differences exist concerning the GPCR expression levels in transfected, immortalised cells. The expression levels of these molecular targets in immortalised cell lines employed in HTS are frequently very much higher than occurs with endogenous levels occurring physiologically or even patho-physiologically. Such elevated expression levels significantly change the ratio of GPCR to G protein, and consequently, the inherent efficiency in receptor activation. Therefore, over-expression of GPCRs can amplify the normal physiological activity of these receptors, thereby rendering a partial agonist as a full agonist and a neutral antagonist with agonistic effects.

A second factor with regard to GPCR over-expression in immortalised cells concerns the creation of constitutively active receptors – ie receptors producing a functional response in the absence of activating ligand. Since GPCR constitutive activity is found in several pathologies, compounds that selectively target constitutively active receptors could be useful in treating diseases caused by aberrant activity. Since constitutively active receptors

exhibit an intrinsically high basal activity, antagonists that reduce basal activity are designated as inverse agonists.

A third complexity involved in drug screening on recombinant receptors expressed in tumour cells relates to the multiplicity of signalling pathways coupled to each GPCR. Indeed, most GPCRs couple to multiple G proteins, thereby regulating more than one cellular signalling pathway. Collectively, it appears that the pharmacological properties of ligands acting on the same receptor differ, depending on the response measured, or the cell type used[4]. Potential differences in the pharmacological profile of compounds interacting at receptors raises the question as to which functional response one should measure in a prototypic HTS assay employing engineered tumour cell lines – ie which response is most relevant to identify leads that can generate a desired clinical benefit. Since it is also clear that G proteins affect the pharmacological properties of a given GPCR, and the overall cellular environment profoundly influences differences in G protein coupling properties, the signalling pathways measured in the HTS assays (as well as the cellular background) can profoundly influence the compounds identified in HTS[1].

While most GPCR drug discovery HTS campaigns employ immortalised cells, there are clearly a number of issues with this approach which hinder identification of 'hits' likely to become successful leads candidates for human testing and eventual therapeutic use. One problem arises from the artificial environment of the immortalised cell in comparison to the native cells expressing receptors. This artificiality includes abnormal expression of the receptor, the G proteins which mediate its cellular effects, as well as the cellular signalling systems critical for mediating the physiological actions of the receptor. (Most immortalised cell lines do not express the voltage-dependent ionic conductance channels which normally convey the biological readout of receptor activation.) The artificial environment of the cells can result in drug profiles developed against a GPCR that work in a predictable manner on the cell lines, but whose activity *in vivo* differs considerably[5].

Primary, stem cells and HTS – a step forward?

Several disparities clearly exist between the physiological environments of screening systems using recombinant cell lines when compared to those found in 'natural' tissues. There is, therefore, an emerging interest in the use of primary mammalian cells for HTS. In primary cells the endogenous target is tacitly assumed to be expressed in an environment that more closely resembles that found in the human disease, and at levels that resemble those found endogenously. Consequently, novel drugs characterised using

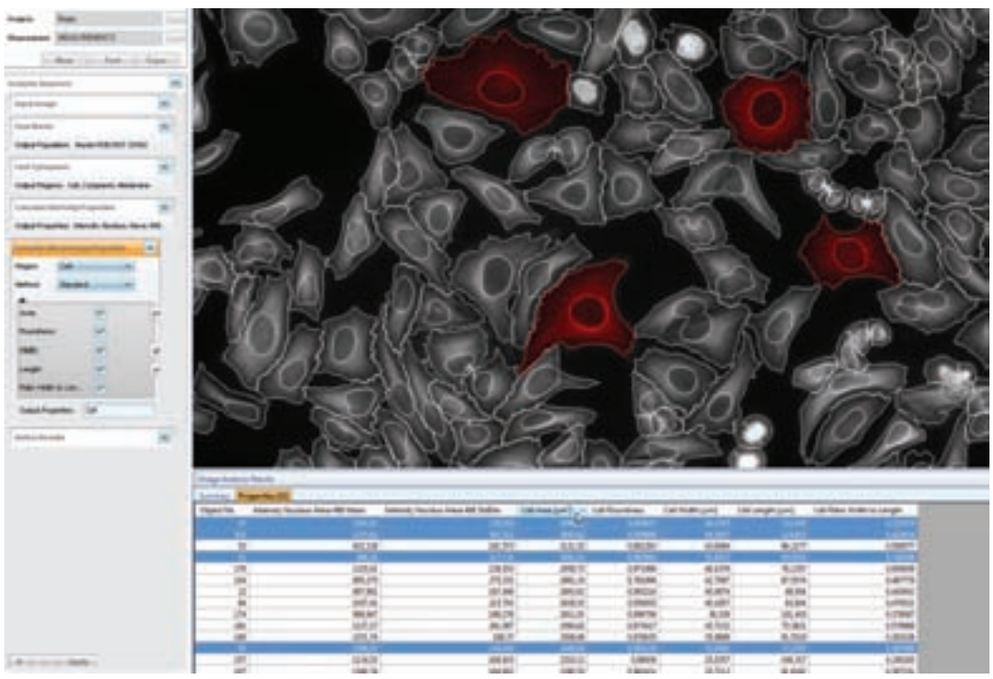

Figure 2
High content analysis of a cell sample using the Harmony software (PerkinElmer), highlighting specific single cells for further investigation (marked red). The cells can be manually selected and analysed, or the results can be sorted according to a specific morphology or intensity parameter and a defined cell population then highlighted

Figure 3
The Opera high content screening instrument dedicated to high throughput image based screening using live cells in kinetic mode. The Opera may include an environmental control sample chamber with full control over temperature, CO_2, and humidity, as well as a dispensing unit plus software for parallel kinetic measurements of several wells. The screening can be conducted in 96, 384 and 1536 well plates. Water immersion objectives with high numerical aperture ensure short exposure time and high image quality

these primary cell systems are presumed to act in a more predictable fashion, as one might find in the disease, than those characterised in immortalised cells[5].

Primary cells often comprise cells derived from embryonic tissues including neuronal cultures, as well as those from adult tissues such as pituitary cells or hepatocytes. Primary cells are used to evaluate endogenous targets for drug discovery, or be modified to express recombinant targets using viral vector systems as well as employing tissues from transgenic animals. As with recombinant cells, assays using primary cells may employ functional responses including transient changes in intracellular calcium, second messenger accumulation or other movement of proteins reflective of cell signalling. These include reporter enzymes via response elements recombinantly engineered into cells derived from transgenic animals, as well as electrophysiological approaches that measure membrane potential changes[6].

A major limitation on the use of standard primary mammalian cells in HTS is their relative scarcity. To an extent this is compensated for by the combined use of highly sensitive assay techniques and miniaturised detection systems, collectively allowing the use of very few cells per assay data point. In addition, the wider availability of embryonic stem cells (ESC) may provide for cells that are grown in relatively high abundance (in a similar manner to immortalised cells), yet which may retain several phenotypic characteristics of the natural cells. Furthermore, ESCs can be induced to differentiate into distinct cell types, each reflective of specific organs and tissues, such as hepatocytes, cardiomyocytes, kidney cells and neurons. In some cases, these differentiated cells develop the characteristics of the mature cell, such as cell-cell networks[7].

Currently, most primary cells and ESCs are derived from rodent tissues, and up to the present, relatively few phenotypes have been used in HTS, being restricted to secondary screening campaigns and lead optimisation studies. To date, primary cells are rarely used in initial HTS programmes for a variety of reasons. Firstly, primary cells are generally grown only in limited quantities, in amounts insufficient for routine HTS techniques. Moreover, they cannot be readily frozen and subsequently thawed for later use in assay screening (in marked contrast to immortalised cells such as CHO or HEK293 cells) since they require great care in handling so as not to disrupt their physiology. Furthermore, while some primary cells are cultured from adult animals (including hepatocytes and pituitary cells) most primary cells need to be cultured from embryonic tissues, particularly neuronal cultures[6,7].

Nonetheless, cultured primary cells provide a more physiologically relevant environment for the molecular target under examination than that same target recombinantly expressed in an 'artificial' immortalised, cell environment. This is notably the case with primary neuronal cells, for which the complex interplay of endogenously expressed ion channels, second messengers and other cell signalling proteins, can be better recapitulated in a primary cell than in transformed immortalised cell lines. Therefore, HTS campaigns using primary neurons, notably neuronal networks, may be an essential step in the identification of novel compounds to treat neurological disorders that could not be as easily identified using the more standard screening approaches of the past[6].

ESCs in HTS – really?

As discussed above, limitations of cell abundance restrict the use of primary cells in HTS. This may be overcome with the use of ESCs. Pluripotent ESCs are grown in almost unlimited supplies – even frozen in stock solutions, much like immortalised cell lines. Several studies have now shown that these cells can be induced to differentiate into selective cell types such as neurons, hepatocytes or myocytes. By recombinantly engineering into ESCs, protein reporters can be used to isolate and purify homogenous cultures of specific cell types. Insertion into undifferentiated ESCs of antibiotic resistance genes under cell type specific promoters also allows selection of lineages. These transfected ESCs can be selected, expanded, and then, using specific growth factors, induced to differentiate into populations enriched for a selective lineage such as neurons, myocytes and hepatocytes. Human ESCs provide obvious advantages in drug screening, but they have some disadvantages – including the fact that they do not grow as well and are difficult to maintain and expand[8]. One alternative is to use human adult pluripotent stem cells, which can be obtained from cord blood, bone marrow and other tissues.

The use of stem cells in HTS is now only emerging in drug discovery. Stem cells feature several advantages, including the use of previously unavailable cell types, and the ability to study cellular regeneration and differentiation. Small molecules have historically been recognised to reproducibly impact the differentiation of stem or progenitor cells. The increased expertise in human stem cell cultures to facilitate HTS has allowed the identification of many new chemical series that serve to direct cellular renewal, regeneration, expansion, and differentiation, particularly when used with adult pluripotent stem cells (iPS cells). Unsurprisingly, the mechanism of action of these molecules may be as agonists or antagonists of developmental biology pathways – like Hedgehog, Wnt and Notch – all of which regulate stem cell differentiation and signalling networks[9].

Primary cells, stem cells and high throughput imaging – sharpening the focus?

Primary human cells are used in several therapeutic areas, and such biologically relevant cell assays are becoming increasingly recognised as robust and amenable screening tools for HTS. Cellular imaging is emerging as a crucial tool that

integrates biological complexity into drug discovery. Current imaging systems allow high-resolution analysis of single cells, high throughput and kinetic studies on live cells, and are linked to efficient data storage systems via user-friendly image analysis programmes. The key feature of modern cellular imaging systems in drug discovery is to provide a multidimensional aspect for each experiment performed, allowing measurement of multiple parameters, which in turn enables analysis of cellular responses against different stimuli[10,11]. Embryonic stem cells permit the development of predictive screening assays that can deliver higher-quality leads. The emergence of high content imaging instruments coupled to plate handling equipment also allows high throughput assays to be carried out successfully by imaging target cells within heterogeneous cultures. One area that is benefitting from screening via automated confocal imaging systems is the ability to undertake a cellular phenotypic approach to drug discovery. The use of HCS techniques to permit phenotypic profiling of compounds based on changes in cellular activity will undoubtedly grow in drug discovery. However, this will require the development of techniques to analyse large image datasets, as well as precise correlations of phenotypic changes and compound mechanism of action[11].

Summary and conclusions

The use of cell-based assays in all phases of drug discovery, and notably in HTS, has accelerated in the past five years. Frequently, in many screening campaigns, the cell phenotype has been subservient to the assay technology, instrument, or liquid handling systems in the laboratory. Consequently, heterologous expression in immortalised cell lines of GPCRs, ion channels or kinase targets, as well as their ancillary signalling partners, provides the mainstay of most cell-based screening assays. Clearly, such cells are poor substitutes for cells reflecting human diseases.

Furthermore, current cell-based ADME/Tox assays, now moving into HTS, are relatively poor predictors of the human response due to the nature of the cells being used in the studies. Because of these limitations in standard HTS programmes, the growing interest in the use of primary cells – and ultimately stem cells – in drug discovery is increasingly justified. The use of primary cells in secondary screening assays also increases apace, particularly in studies where confocal imaging is used, as well as in target validation studies in conjunction with gene silencing techniques. The convergence of high throughput confocal imaging, classical automated, primary and stem cells will collectively allow better and earlier identification of novel clinical candidates. DDW

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