Whole cell hard sell – high content screening in drug discovery

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A previous article ("Space and time in the pharmaceutical universe"), gave an overview of the main technologies associated with the drug discovery process, noting the huge and growing volumes of data being generated by drug discovery activities, and the consequent problems for data collection, storage and analysis. A subsequent article ("Computational challenges for drug discovery – transforming data into knowledge") examined some of the software approaches to the issues raised by the scale and complexity of data analysis. Here we briefly outline another of the trends identified in the first article, namely the increasing use of whole cells at early points in the drug discovery process, and we outline a few of the technological advances supporting this trend.

Cinderella cells

Classically, drug discovery has been perceived as something of a numbers game. There are a certain number of biological targets, and a certain number of accessible chemicals. Applying one to the other may identify chemical compounds that have a therapeutic effect. Therefore (the logic goes) the more of one you apply to the other, the more chance you have of success. Hence there has been a strong emphasis on increasing 'throughput' in drug discovery, ie increasing the number of compounds you can screen against a given target in unit time.

This emphasis on throughput has put constraints on the types of assays that are conventionally employed in the early parts of the drug discovery process. Usually, assays that are suitable for high-throughput screening (HTS) must be robust and reproducible, particularly in small volumes. These constraints have tended to favour relatively simple assays, eg the binding of one molecule to another as measured by an easily accessible indicator (such as colour change in the solution in which the reaction occurs). Whole cells have, until recently, usually been excluded from the HTS ball, as they are complex bundles of constantly reacting and interacting molecules that present corresponding difficulties in assay scale-up and data capture/interpretation.

However, there are distinct disadvantages to the simple assay approach. Reliance on a single parameter, such as binding of two molecules in isolation, may not be a particularly good indicator of what happens in a patient, where there may be issues related to the metabolism of the drug (ie the breakdown of the drug to various products which may themselves have particular effects) drug-associated toxicity, and the small matter of other molecules in the cellular/physiological milieu which might affect the interaction. It would be better to analyse as many relevant parameters as possible in high-throughput, early in the discovery process. Concentrated efforts over the last few years have resulted in a number of technological advances which are allowing 'data-rich' assays earlier in the drug discovery process. Whole cell assays in particular may be coming of age - cells *shall* go to the ball.

The stories cells can tell

There are a number of specific advantages to the use of whole cells in the drug screening process. For example, many drug targets are cell surface molecules, and require anchorage in a

cell membrane, and perhaps interaction with other molecules in the cell, in order to take on their normal (therapeutically relevant) conformation. Thus the binding event between drug and target may require a cellular environment if it is to be reflective of binding events in a patient. Also, investigators usually wish to examine not just the binding of one molecule to another, but to observe the effect that a binding event has on particular cascades of molecular interactions. These cascades may involve *de novo* production of proteins by synthesis from ambient intermediates or by transcription from previously dormant genes, or activation of partner proteins by phosphorylation events, or translocation of proteins from one site in the cell to another. Clearly a functional, whole-cell environment is required for these types of cascades to occur in a normal way. In addition, the whole-cell environment contains some of the critical barriers that drugs will be exposed to in the context of a patient, such as destructive enzymes, lipid-rich membranes obstructing passage into various functional cellular compartments, and so forth. The multi-faceted, three dimensional perspective afforded by the cell gives a valuable insight into the complexities surrounding drug interactions, and may become a prerequisite for effective screening and development of molecular therapeutics.

It is not surprising then that the cell is now becoming a point of focus for drug discovery efforts. As a self-contained, complex biological system, the cell is able to integrate internal and environmental signals into molecular responses that become the recognisable descriptors of a combination of molecular pathways, and which may be surrogate endpoints for a therapeutic effect. Thus, the cell can encompass the optimal biological presentation of a target molecule, enable targets to be seen in context with other potentially competing influences and, perhaps most importantly, present some of the formidable pharmacological hurdles that a successful drug must overcome. Hence, cell-based high content screening (HCS) assays offer advantages over traditional affinity-based screens, particularly as they can be used across nearly all stages of the drug discovery and development process, including target identification and validation, lead selection and optimisation and even preclinical studies.

Hardware / wetware reciprocal evolution

Given the above, one might ask why it has taken so long for cell-based screening to achieve higher prominence in automated drug screening processes. Part of the answer lies in the difficulty in removing the human element from the capture/interpretation of the types of complex data provided by whole cell investigations. For example, one bottleneck has been a dependence on observation by microscopy (that is, by a human microscopist) and the consequent subjectivity of event assessment. Routine application of high-throughput cell screens would require the human element to be replaced at the point of data capture by a high degree of automation. This would include, for example, the development of sophisticated image capture instrumentation and image analysis software which can identify and perhaps interpret changes in morphology, fluorescence distribution within the three dimensional cell, and so on. At the same time, these technologies, in order to realise their full potential, would require the concomitant development of reagents which allow the detection of molecules and molecular events, preferably without at the same time interfering in the events they seek to record. Therefore one would expect a reciprocal evolution of ‘wetware’ (chemical reagents), software and hardware to allow cell-based screening to be carried out at commercially interesting levels of throughput. This indeed appears to be happening.

Although a complete discussion of all aspects of this interdependent advance is beyond the scope of this article, we can point out some of the more obvious developments. One such is the re-evaluation of the flow cytometer in the context of high-content screening for drug discovery. The flow cytometer is essentially a microscopy system configured so that it can rapidly analyse light signals from objects flowing at high speed through a focus of illumination usually supplied by one or more lasers. It has been used as a standard tool in the laboratory for many years. The key advantage of the flow cytometer for drug discovery is the ability to develop high-throughput assays that can deal with multiple cell populations or simultaneous analyses of multiple responses. The importance of this capability lies partly in the fact that development of a drug requires not just optimisation of one parameter (eg affinity of the drug for a target) but

optimisation of many parameters (eg affinity for target, and ability to cross cell membranes, and lack of toxicity to the cell, etc).

The availability of flow cytometer-type hardware and imaging techniques that allow high-speed analysis and sorting, linked with robotics, shifted the emphasis for platform technology evolution onto the development of better (smarter) 'wet' probes. These are chemical reagents which can enter cells and relay information about the presence and location of molecules of interest, as well as identifying fundamental features of the anatomy of the cell. For example, cell-based assays (especially those based on image acquisition) frequently require orientation parameters. There are multiple solutions, but one that also provides an informative parameter in its own right is that of cellular DNA content/nuclear location. This long-established methodology has until recently relied upon the availability of relatively few fluorochrome (fluorescent marker) technologies, which in turn often limited the performance of detection instrumentation.

The situation was even more restricting when the demands of live cell assays were considered. Indeed, up until recently, there have only been two DNA reporters that could effectively report DNA content/nuclear location in live cell analyses – DAPI and Hoechst 33342. Both of these probes are excited by expensive and optically demanding ultra-violet (UV) lasers. The emission range for the UV probe (ie the wavelengths of light it emits during the image capture stage of the assay) reaches across the visible spectrum, resulting in spectral contamination that can limit flexibility and resolution in multi-probe assays. The introduction of the cell-permeant cyanine SYTO nucleic acid stains (e.g. SYTO 17) has provided reagents with convenient red-shifted spectral characteristics but without the required DNA discrimination.

DRAQ5 – accelerating assay development

A solution to this reagent deficit was found through approaching fluorochrome design in an intelligently directed manner, similar to that used for small molecule design in therapeutics, taking into account the demands of reagent delivery to intracellular targets. The aim was to shift the orientation parameter to the far red but to retain DNA discrimination and convenient excitation characteristics. Hence, the novel reagent DRAQ5™ was developed by Biostatus to meet such requirements, with enhanced DNA affinity and intracellular selectivity for nuclear DNA. The dye can be used as a membrane-permeant fluorescent dye for the rapid and convenient staining of the nuclear DNA of organisms, including live and fixed cells, with minimal RNA-associated fluorescence. Unusually, excitation is possible by a wide range of laser light wavelengths (e.g. 488–647nm wavelengths) despite optimal excitation at 647nm, providing flexibility, and applicability to a wide range of diagnostic assays and instrumentation platforms

The dye is also detectable using lamp-based instruments with appropriate infrared (IR)-emission filtration. Emission spectrum beyond 670nm provides minimal overlap with the emissions from visible range dyes including green fluorescent protein (GFP). The novel probe does not photobleach (ie does not become less fluorescent with exposure to light), an important consideration for generating multiple scans of cells – providing flexibility in analysis sequences. Because of the unique co-excitation of green fluorescent protein (GFP - a commonly-used marker) and DRAQ5™ at 488nm, and the clear spectral separation of the emission signals in live cells, DRAQ5™ can be used in live cells or in fix/stain protocols for single-pass laser-scanning. Such single laser excitation also permits higher image resolution than dual scan systems that may require co-registration corrections. This is an important factor in increasing throughput rate (often halving a typical assay from 12 weeks to 6 weeks) and in allowing live cell assays to track agonist/antagonist responses over time frames of minutes.

The advantages of the characteristics of DRAQ5 summarised above become obvious when it is considered that changes in the specific location of a molecule within a cell may betray significant shifts in response and behaviour without changes in absolute amount. Here time is critical and assays demand high-performance dyes to increase assay resolution. Hence, DRAQ5™ nuclear recognition technology is becoming incorporated into a series of new 'reporter translocation'

assays in which the movement of a response molecule can be tracked in relation to the cell's nucleus. Assay systems which have exploited DRAQ5™ nuclear recognition for translocation event monitoring in drug discovery include the Amersham Biosciences cyclin B1 reporter system and the Redistribution® technology of BioImage® A/S (Søborg, Denmark).

In addition, cell and nuclear orientation using DRAQ5™ has been used in the Transfluor® assay, on the Amersham IN Cell analyser system. DRAQ5™ nuclear stain with Transfluor® has allowed the simultaneous red/green image capture rate of 14 minutes per plate with a throughput of 90 x 384 well plates per day providing 35,000 wells per day with a two to three hours of downtime for data transfer. A 950K compound screen can be run on a 24-hour cycle, seven days a week for a six-week campaign for primary HTS (communicated by Dr Emma-Louise Cooke, Senior Research Scientist, AstraZeneca R&D Charnwood, UK).

Probing the future

The process of reciprocal evolution noted above suggests that, along with the development of sophisticated new probes, the power of image cytometry platforms also is set to increase, closing in on the goal of high throughput image-based assays on non-adherent cell types for drug discovery. For example, the laser scanning cytometer developed by CompuCyte Corporation has found wide research applications, particularly in the field on monitoring cell death (apoptosis) in heterogeneous populations, in addition to enabling automated laser-based image cytometry for preclinical safety and toxicology testing.

In general, however, high capacity laser scanning technologies are limited by the acquisition time for fields that contain sufficient numbers of informative events especially in plate-formatted assays. This is a particularly critical problem when live cell scanning is attempting to capture, for example, agonist/antagonist effects that occur over a matter of minutes. Here, the rapid DRAQ5™ nuclear staining technology requires only a single laser pass to capture both nuclear location and GFP expression in live cells and avoids the loss of data due to UV-excitability of a candidate molecule. Amnis Corporation's ImageStream™ technology for the multispectral imaging of cells in flow is a step towards the linking of flow capacity with a platform that could address reporter translocation-based assays. ImageStream™ employs a detection technology called time-delay-integration (TDI). TDI operates electronically to track the motion of the objects being imaged permitting realtime image processing and therefore the morphological analysis of cells in flow as well as the localisation, identification and quantification of reporter molecules such as DRAQ5™.

Cell and tissue-based microarray technologies and optical biochips incorporating live cells will continue to develop and offer new opportunities for high throughput validation of drug targets. 'Seeing is not necessarily believing' and biochip and MEMS approaches that 'sense' rather than image could involve the phenotypic analyses of cells growing on top of a surface of arrayed molecules including interfering RNAs, as well as candidate agents generated by combinatorial chemistry.

With all imaging technologies there is the problem of converting the 'picture' into a 'thousand words'. Image recognition routines are increasing in complexity and performance but so is the throughput capacity of new high content screening platforms. Rapid analysis with no retention of raw data is one solution but may result in the loss of information not analysed by first intent. Perhaps the most daunting prospect is the integration of data into predictive modelling where the quality and pertinence of the data are paramount. Hence, an important issue is the detection of 'informative cells' for a reporter-based assays on such non-imaged chips Such informative cells may represent only a fraction of a population at any one time but be capable of providing the critical test of drug bioaction.

A constant and significant challenge for drug screening is to seamlessly link the early and later steps in the discovery route. This challenge will require modular tools, exemplified by the live cell

probes discussed briefly here, to report on the dynamics of drug-target interactions and associated cell responses often shifted in time and space. Meeting this challenge will provide an important feed-back loop for drug design and a knowledge base for future *in silico* drug discovery efforts. Importantly, whole cell studies can also provide a rich source of design concepts for diagnostic assays – witness the impact of cytometry in immunophenotyping. Development of such assays will provide indicators for individualised medicines and advance pharmacogenomics at the level of clinical decision making, and help to maximise their wider clinical and commercial impact. Thus, combining therapeutic targeting and diagnostic strategies in new drug discovery and development programmes will continue to gain ground. New probe technologies, dictating detection-platform design, are being actively developed to meet these multiple needs.

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