High-throughput cell analysis using multiplexed array technologies

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The desire for more biologically relevant data from primary screening has resulted in a dramatic increase of cell-based assays in HTS labs. Consequently, new cell-array technologies are being developed to increase the quality and quantity of data emerging from such screens. These technologies take the form of both positional and non-positional formats, each with their own advantages. Notably, screens using these technologies generate databases of high-quality data that can be analyzed in ways currently not possible. The power of cell-based assays combined with new array and analytical technologies will enable the condensation of serial drug discovery processes, thereby decreasing the time and cost of taking a hit compound into clinical trials. Here, we compare array strategies being developed towards the goal of integrating multiplexed cell-based assays into HTS.

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▼ Historically, biochemical assays have greatly outnumbered cell-based assays in HTS. Although relatively easy to implement in a HTS format, many biochemical assays do not address important biological issues, such as cellular toxicity or the complex cell biology surrounding the target. To bridge this gap, the use of cell-based assays in HTS has increased dramatically in recent years [1]. In the past, cell-based assays were slow, lacked reproducibility and were prohibitively expensive. However, as our understanding of cell biology increases and new technologies emerge, cell-based screens are becoming faster, cheaper, more reproducible and increasingly powerful. This is evident in the vast number of new biosensors, assays and detection systems being developed for cell-based HTS [1–13] (see also http://www.aurorabiosciences.com and http://www.moleculardevices.com]. Subsequently, a desire to increase the amount and quality of information obtained from cell-based assays has emerged (as discussed in an interview with Paul Negulescu *et al.*, available at: http://www.bio.com/newsfeatures/newsfeatures_fochives.jhtml).

Mulitplexed versus multi-parametric assays

One approach to increase data content and quality from HTS, has been to obtain multiple datapoints from each microtiter well. There are two basic ways of obtaining multiple datapoints from a single well: multi-parametric assays and multiplexed assays. Throughout this review, we define multi-parametric assays as those yielding multiple measurements from a single cell type, and multiplexed assays as those producing a single assay measurement for each multiple cell types within a single well.

Performing multi-parametric and multiplexed assays provides significant advantages to drug discovery. In comparison to singlet assays (one datapoint per well), these assay formats provide increased information density, resulting in savings of time and reagents. In addition, as the multiple datapoints come from a single well, they possess inherent consistency and higher quality.

Multi-parametric assays, often referred to as 'high content' assays, have recently captured the interest of many who run cell-based assays. These assays are typically performed using automated, high-resolution microscopy platforms to deconvolve the parameters being measured. An example of a multi-parametric assay has been developed by Cellomics (http://www.cellomics.com) to measure cytotoxicity. In this assay, cells are plated into an optically clear-bottom 96-well microtiter plate and treated with the compounds of interest. Cells are exposed to reagents labeled with spectrally unique fluorophores to assay nuclear and acidic organelle characteristics, as well as plasma membrane permeability. This enables simultaneous quantification of multiple indicators of cytotoxicity: cell density, lysosomal pH and morphology,

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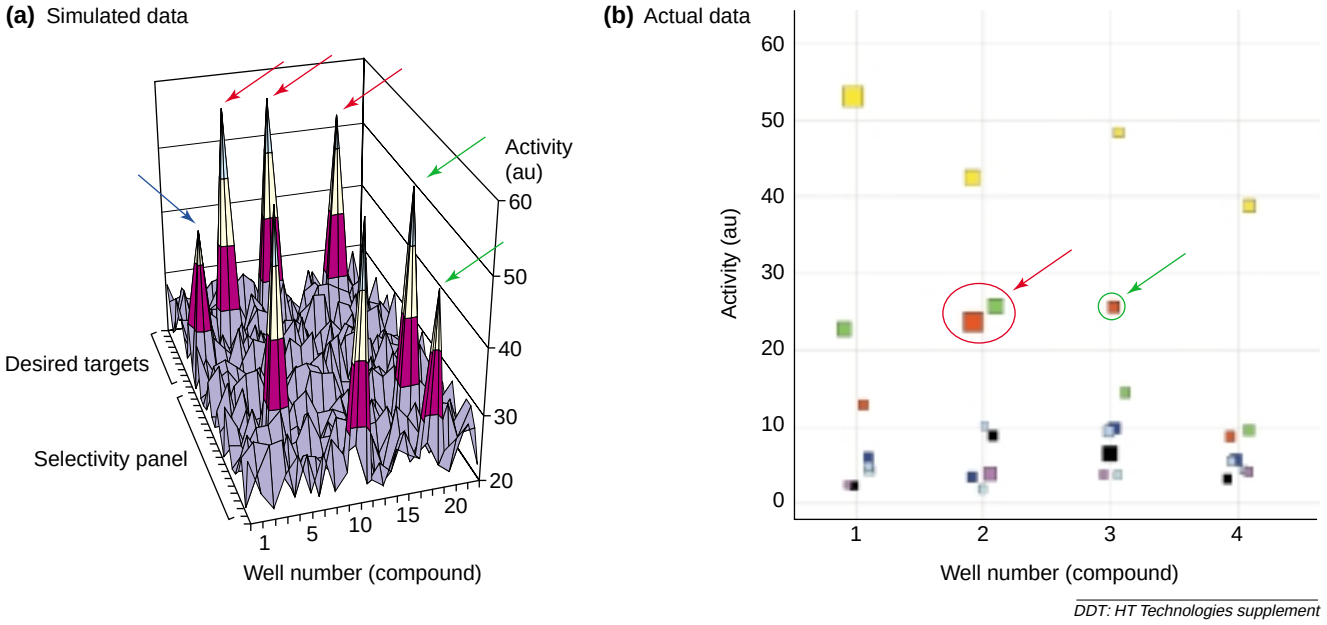

Figure 1. High-throughput selectivity screening (HTSS™). **(a)** Simulated data generated from a screen run on a platform that enables the multiplexed analysis of multiple cell lines. Well numbers 5, 11, and 17 show compounds that have very potent effects (red arrows) on the desired targets with significant cross reactivity to the selectivity panel (green arrow). However, well number 3 displays a less potent hit that is very selective (blue arrow), which might have been missed using traditional HTS screening techniques. This condensation of primary and selectivity screening would provide a powerful tool to aid the decision-making process as to which hits should be followed up as leads. **(b)** Actual data generated from a multiplexed G-protein-coupled receptor (GPCR) assay run on a Virtual Arrays platform (http://www.virtualarrays.com) and visualized using SpotFire software (http://www.spotfire.com). The cells were exposed to various compounds and agonist activity was measured. Each cell line is designated by a different colored square. Well number 2 shows non-selective activation (red arrow) of two cell types while well number 3 shows selective activation of a single cell type (green arrow). The yellow and pink squares represent positive and negative control cell lines, respectively.

nuclear morphology, and membrane permeability. By generating multiple datapoints per well with this multi-parametric analysis, it is possible to compare the effects of a compound on these separate indicia to better understand and predict the toxic effects of compounds.

As mentioned previously, multiple datapoints can also be generated from a single well by multiplexing a single assay measurement across different cell lines. How data from this type of multiplexed cell-based assay can be generated and deconvolved from positional and non-positional arrays is the primary focus of this review.

Advantages of multiplexing: high-throughput specificity screening

Multiplexing cell-based assays is providing new potential for cell-based assays. Multiplexing cell lines facilitates the interrogation of multiple targets and enables the ability to include assay controls within each well [14]. This not only removes the uncertainty regarding pipetting errors and reagent mixing, but also provides increased statistical robustness. By including positive and negative controls in each well, the datapoints within the well can be normalized, not only to control wells but also to controls within the well. As a result, some of the well-to-well variability caused by inconsistencies in pipetting or evaporation should decrease. Although this remains to be rigorously proven, the concept of including controls within each well to generate inherent assay consistency provides a unique advantage to multiplexed assays.

In addition, multiplexing cell lines for the purpose of screening enables the simultaneous interrogation of multiple targets. Theoretically, this will enable researchers to perform concurrent primary screens as well as selectivity screens on multiple targets. The result is a high-throughput selectivity screen (HTSS™; Fig. 1a). To demonstrate the feasibility of generating these type of data, a Virtual Arrays (http://www.virtualarrays.com) platform was used to multiplex eight cell-types, each representing a different receptor (Fig. 1b). Indeed, as cell-based multiplexing platforms are developed, the ability to generate robust selectivity and potency data within the same well will become a reality.

Furthermore, HTSS™ has the potential to fill a large hole in the structure–activity relationship (SAR) database. Selectivity screening traditionally encompasses only those hits chosen from the primary screen for further characterization. Therefore, the

Box 1. Considerations for multiplexed cell-based assays

Culture conditions
Culture conditions must be compatible with all the cell lines to be multiplexed.

Potential cross-talk
The presence of one cell line might have an influence on the response of another cell line.

Assay development
The assay conditions need to be the same for all of the cell lines.

selectivity information only exists on this subset of compounds, which represents only a small percentage of the entire library. However, in the case of HTSS™, the primary screen is directed towards both the primary targets and the selectivity panel generating selectivity information on the entire library. This would result in a large database containing both selectivity and potency information for each compound in the library. This database would be a very powerful tool that can be used to generate accurate SAR data to guide the chemist towards lead optimization.

As one might expect, there are unique considerations that one must take into account when deciding if a multiplexed cell based assay is appropriate (Box 1). Obviously, for this type of experiment to work, the different cell lines must be able to grow under the same culture conditions. Similarly, as the different cell lines share the same extracellular environment there is also the possibility of cellular cross-talk. That is, there could be secondary effects on a cell line because of the physiology (e.g. cytokine secretion) of another cell line in the same well. Finally, the assay development required to multiplex a cell-based assay is unique. The signal being generated from each individual cell line must be optimized to the same conditions because all the cell lines are analyzed simultaneously. The relative weight of these concerns will vary with assay type and the cell lines to be multiplexed. For example, if the assay involves multiple stable cell lines all generated from chinese hamster ovary cells, the concern about culture conditions and cross-talk will be minimal, but the assay development might be more significant. There could also be other concerns and considerations that are assay specific.

Positional arrays

Array technologies are commonly associated with nucleic acids and microarrays. Within these types of array, the identity of each element of hybridization information is determined by its position within the array. The hybridization data are first gathered on a scanner and then deconvolved via the X,Y coordinates of each datapoint. These positional arrays are widely used in pharma as well as basic research and have revolutionized the field of gene expression analysis.

Recently, two interesting approaches have been developed that can generate positional arrays of different cell types. The first is the CellChip™ technology being developed by Cellomics [15]. This technology is based on the specific capture of different cell types via specific affinity reagents. In this technique, a positional array of cell-specific affinity reagents is generated (Fig. 2a). Next, the different cell classes are passed over the array with each cell type specifically attaching to the position at which the cognate affinity reagent was deposited, thereby generating a positional array of different cell types. In theory, these arrays could be generated in a microtiter plate and would therefore be amenable to high-throughput analysis. However, multiplexing more than a small number of cell lines would involve identifying specific affinity reagents with little or no cross reactivity for each cell line. Indeed, this could be a daunting and expensive task.

Ziauddin and Sabatini [16] have taken another approach (used by Akceli; http://www.akceli.com) to generate positional cellular arrays. In this approach, expression vectors are arrayed onto microscope slides by standard spotting methods such as those used to make nucleic acid arrays (Fig. 2a). The slide is then treated with a lipid transfection reagent (or alternatively, the spotted cDNAs could be premixed with the transfection reagent [7]) and cells are plated on top of these arrays. The cells take up the DNA and become 'reverse' transfected after 24–48 h of culture, thus resulting in a positional array of cells expressing the recombinant genes of interest (additional protocol information available at http://staffa.wi.mit.edu/sabatini_public/reverse_transfection/frame.htm; see also the review by Bailey and Sabatini in this supplement). In theory, this approach is not limited to expression vectors because any transfectable entity could be used to generate these arrays. Assuming that this new technique is sufficiently robust, it has the potential of being very useful for target validation as well as for functional genomic experiments. However, because this technology only generates transient transfections, its use in HTS remains to be proven.

Non-positional arrays

As an alternative to positional arrays, new technologies are beginning to emerge that generate non-positional arrays of cells (Fig. 2b). As the name implies, non-positional arrays do not rely on X,Y coordinates to deconvolve the data. By contrast, each datapoint is associated with a different 'code'. The exact natures of these codes are diverse, ranging from colors to radio frequencies (Table 1) [17–23]. In theory, each of the technologies outlined in Table 1 is able to create an unlimited number

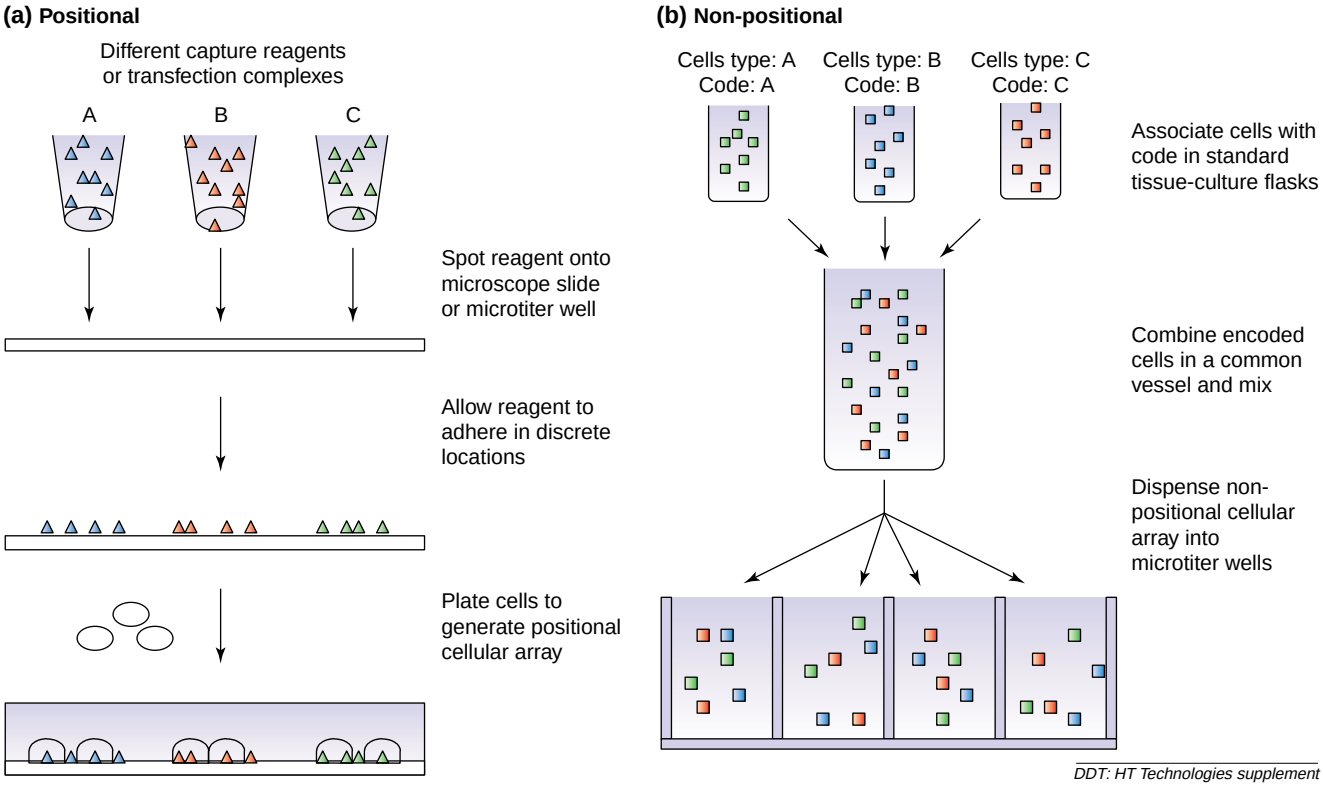

Figure 2. The generation of cellular arrays. (a) Positional cellular arrays are generated by localizing unique cell types to specific locations. For the Cellomics CellChip™ technology (http://www.cellomics.com), capturing agents are first spotted onto a substrate and a population of cells is plated onto them. For reverse transfected cellular arrays, transfection reagents are first spotted and the cells plated onto them. (b) When generating non-positional cellular arrays, the different cell types are first associated with a unique code. These encoded cells are then mixed and aliquoted into a microtiter well for subsequent analysis.

of codes. However, there are many practical constraints that limit each of the approaches, including (but not limited to) manufacturing and decoding ability. Of these approaches, only that of Virtual Arrays and Quantum Dots (http://www.qdots.com) has demonstrated the feasibility of multiplexing whole-cell assays. Regardless of the encoding technique, these technologies result in an array that is considerably more flexible and potentially more amenable to high-throughput analysis than the positional technologies mentioned previously. Of these technologies, only two have demonstrated feasibility in applying their strategy to cellular analysis (described in the following section).

The strategy used by Quantum Dots is simple; different cell types are labeled with spectrally unique nanocrystal-based fluorophores called Qdots™ (see image at http://www.qdots.com) [20–22]). The unique nanocrystals are associated with the different cell types (e.g. by internalization), which are then mixed, pipetted into a microtiter well, and subjected to the standard assay protocol. Using either flow cytometry or microscopy, the assay results are gathered and the code (fluorophore) analyzed. The spatial correlation of the data with the code results in the deconvolution of the multiplexed data to determine the response of each individual cell type. Unfortunately, increasing the number of fluorophores used to multiplex means there is less room in the fluorescent spectrum to gather data. Although the Quantum Dots particles can be manufactured to have distinct emission peaks, the practical multiplex capacity of this QDot™ approach is limited by the overlaps in other aspects of the emission spectrum of each particle. Therefore, if the QDots™ remain cell autonomous throughout the experiment, and are non-toxic, this technology would be applicable for those interested in multiplexing a relatively small number of cell lines.

The second approach that has demonstrated feasibility in generating non-positional cellular arrays has been developed by Virtual Arrays. This technology uses encoded particles on which cells are grown. These particles can be manufactured using different proprietary materials and contain an expandable barcode. The encoding strategy uses colored bars, which do not interfere with data collection, located at various positions. Therefore, increasing the number of codes can be achieved by increasing either the number of colors or the number of coding bars. Importantly, the design of the particles is such that the barcode does not interfere with the cellular analysis. Each unique

Table 1. Comparison of encoding strategies for the generation of non-positional arrays

Company	Encoding strategy	Theroretical multiplex factor	Practical multiplex factor	Applicable to whole-cell analysis?[a]
Virtual Arrays (http://www.virtualarrays.com)	Color and/or positional barcode	Unlimited	100s	Yes
Quantum Dot (http://www.qdots.com)	Fluorescent nanocrystals	Unlimited	~5	Yes
Aclara (http://www.aclara.com)	Electrophoretic mobility	Unlimited	100s	n.d.
Luminex (http://www.luminexcorp.com)	Dye ratios	Unlimited	100s	n.d.
Surromed (http://www.surromed.com)	Reflective barcode	Unlimited	100s	n.d.
Pharmaseq (http://www.pharmaseq.com)	Radio frequencies	Unlimited	100s	n.d.

[a]Abbreviation: n.d., not determined.

cell line is grown on a differentially encoded particle and is therefore physically associated with the code. These encoded particles plus attached cells are then mixed and subsequently dispensed into a microtiter plate where the assay is performed. This non-positional array is then decoded and the cells associated with each particle analyzed using a charged-coupled-device-based imaging system. By virtue of the imaging system used, this platform is applicable to many assay formats. Assuming adequate cell adhesion to the particles, the development of this technology will result in a platform capable of multiplexing a diverse range of cell-based assays.

Concluding remarks

The increasing desire to obtain more biologically relevant data has drawn drug discovery towards cell-based assays. In an attempt to increase data density and quality, multiple new technologies designed to multiplex these cell-based assays are beginning to emerge. The integration of these multiplexing technologies into HTS will make HTSS™ a reality and significantly condense the time required to take a compound from primary screening into the clinic.

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