

progress in the implementation of **LABEL-FREE DETECTION**

part 1: cell-based assays

Progress made in the use of impedance and optical grating technologies for label-free detection of cell-based assays were examined in HTStec's recent Cell-Based Label-Free Detection Trends 2008 report. Uniquely all of these label-free offerings are plate-based and are amenable to moderate throughput. From a cost perspective, no price premium has been derived from cellular label-free assays as for the most part the cost of labels has simply been displaced by the biosensor plates. Considerable evidence has now accumulated that label-free has contributed to our understanding in the GPCR area by facilitating endogenous receptor analysis, in some cases using primary or native cells. Like many emerging technologies there has been some over-hyping of applications, which are little more than proof-of-principle studies. An aspect which seems to be emerging is the coupling of label-free with other readouts or technologies. Overall it remains unclear whether label-free will break into main stream cell-based lead discovery or will mostly fill the gaps that other technologies do less well?

Awareness of label-free technologies is currently at an all time high, generating a lot of interest in drug discovery, and was the focus of the recent dedicated SBS Symposium in Dresden, Germany (June 2008). Label-free technologies and knowledge of potential application areas have advanced greatly in recent years, such that it is now realistic to consider grouping them into two distinct categories: 1) cell-based assays, which are almost entirely microplate-based sys-

tems with moderate throughput; and 2) binding analysis assays, which for the most part are still done with single or multiple sensor-based technologies or by measuring the heat capacity of a sample, all at relatively low throughput. These binding assays have proven invaluable in the investigation of biomolecular interactions, and are extensively used for protein applications undertaken during biological characterisation and screening. More recently binding analysis assays have

By Dr John Comley

Lead Discovery

Figure 1: Most significant advantages of label-free cell-based assays

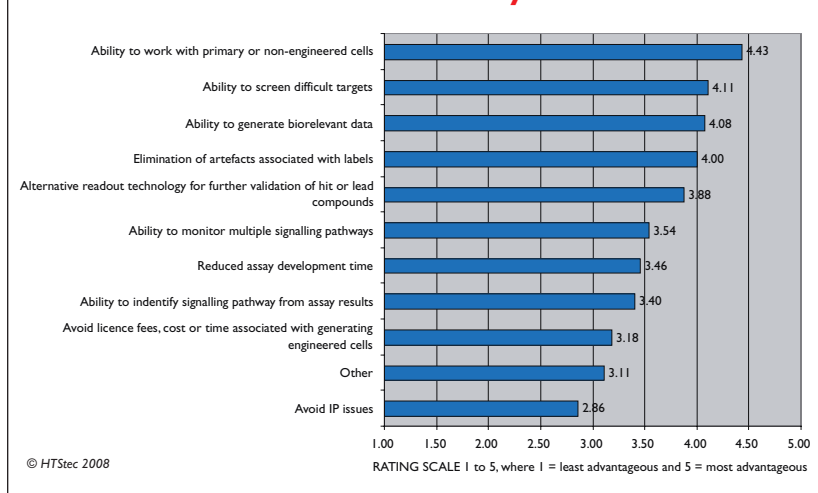


Figure 2: Percentage of all cell-based assays performed using label-free technologies

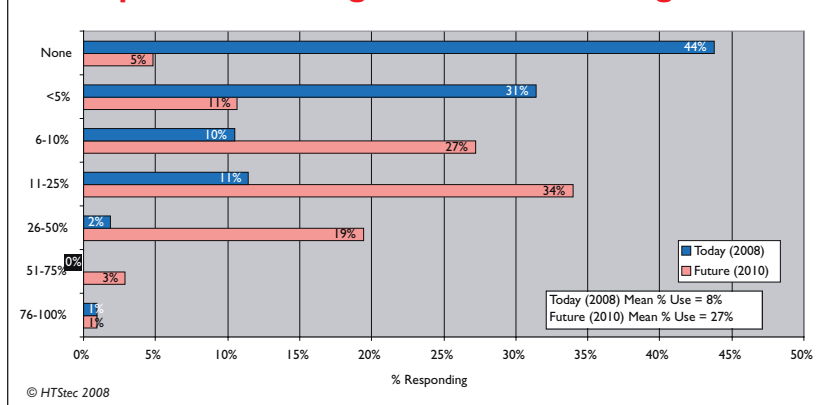
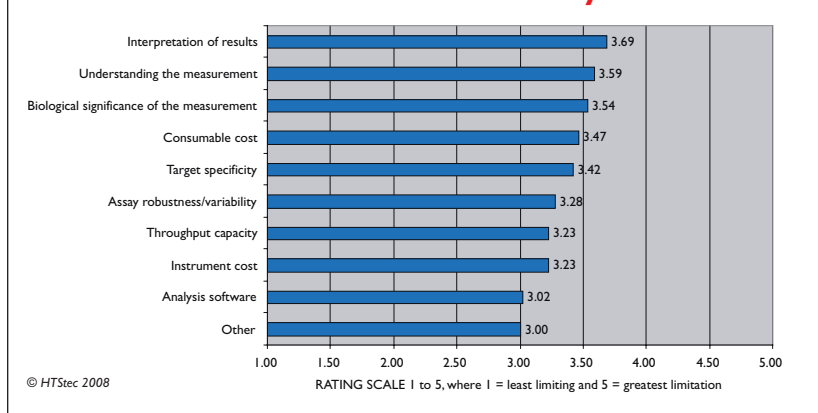


Figure 3: Most significant limitations of current label-free cell-based assays



been enabled using optical sensors at higher throughput and are being evaluated for small molecule and fragment-based drug discovery and development. Both types of label-free assays were the subject of recent HTStec market reports^{1,2}. The label-free cell-based assays are now reviewed in this article and the label-free binding analysis assays will follow in a sister article to be published in the next (Fall) issue of *DDW*.

Advantages of label-free

There are many potential advantages to label-free approaches, most noteworthy is that they can provide direct monitoring of analyte binding to target molecules without modifying the molecules of interest with labels or by using reporter systems (see ³ for a summary of the advantages attributed to label-free). However, from a cell-based assay perspective, HTStec's survey found it was the ability to work with primary or non-engineered cells that was perceived as the most significant advantage of cell-based label-free assays. This was closely followed by the ability to screen difficult targets, then the ability to generate biorelevant data, and only after these factors did elimination of artefacts associated with labels rate. The next factor rated was an alternative readout technology for further validation of hit or lead compounds, which is where label-free assays are generating quite a lot of traction currently (Figure 1). Interestingly 34% of survey respondents stated they have already successfully used label-free detection to perform testing against a difficult cell-based target, the majority of which were GPCRs.

Cell-based label-free assays set to increase, despite concern over limitations

Today (2008) very few cell-based assays are performed using label-free technologies, and the mean proportion done with label-free is estimated to be around 8%. This proportion is however expected to increase considerably (to 27%) over the next few years (Figure 2).

There are still many perceived limitations with cell-based label-free assays that have contributed to the relatively slow introduction of these approaches (Figure 3). Of these limitations the interpretation of the results was rated as the most significant, closely followed by understanding the measurement, the biological significance of the measurement and then the consumable cost.

Survey respondents ranked that they have other technologies which meet their needs as the main reason given for not planning to adopt cell-based

label-free technologies in the next few years. This was followed by unproven technology, other and instrumentation too costly (Figure 4).

Greatest interest in GPCRs

The main key cell-based application area where survey respondents would like to deploy label-free technologies was a universal GPCR screen, closely followed by studies on the mechanism of action of compounds. Other application areas where interest to deploy label-free was intense were high content screening and orthogonal screening (Figure 5)

Survey respondents rated GPCRs as the target class/application area they expect label-free cell-based assays to impact the most. This was closely followed by adhesion molecules (Integrins, cadherins etc), and then by receptor tyrosine kinases and ion channels (Figure 6).

Complementary or displacing role

68% of those surveyed view cell-based label-free technologies to be complementary to existing assay technologies. In addition, 42% of survey respondents do not see label-free displacing any existing cell-based assay technologies. Of those technologies label-free might displace greatest importance was attributed to second messenger assays (eg cAMP, IP1 etc), then radioligand binding, followed by calcium flux assays (eg FLIPR, Aequorin, etc) (Figure 7).

Where label-free will impact the most

The drug discovery areas where label-free approaches have already shown most potential today were assay development, compound profiling and lead identification/secondary screening. The drug discovery areas where label-free approaches are expected to make a significant role in future were hit identification (focused screening), hit identification (primary screening/HTS) and lead optimisation (hits-to-leads). The drug discovery areas showing least potential today or where label-free approaches are expected to show NO significant role were clinical diagnostics and leads-to-candidate (ADME Tox/preclinical research), and to a lesser extent target identification and target validation (Figure 8).

Importance of coated plates

Successful implementation of label-free approaches to cell-based assays is, however, critically dependent on the availability of surface chemistries that promote and support cell adhesion and growth. The majority (41%) of survey respondents believed that the availability of coated plates for label-free

Figure 4: Main reasons given for not planning to adopt label-free technologies in next few years

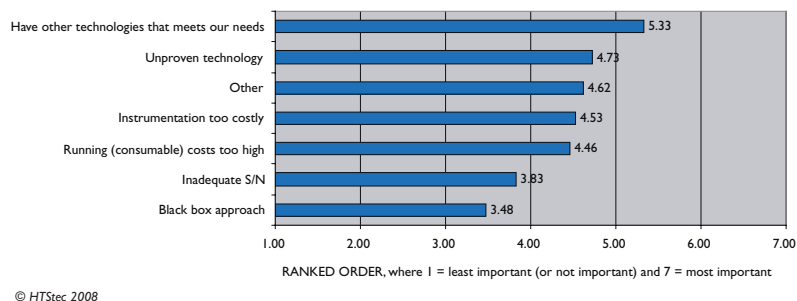


Figure 5: Key cell-based application areas where respondents would like to deploy label-free technologies

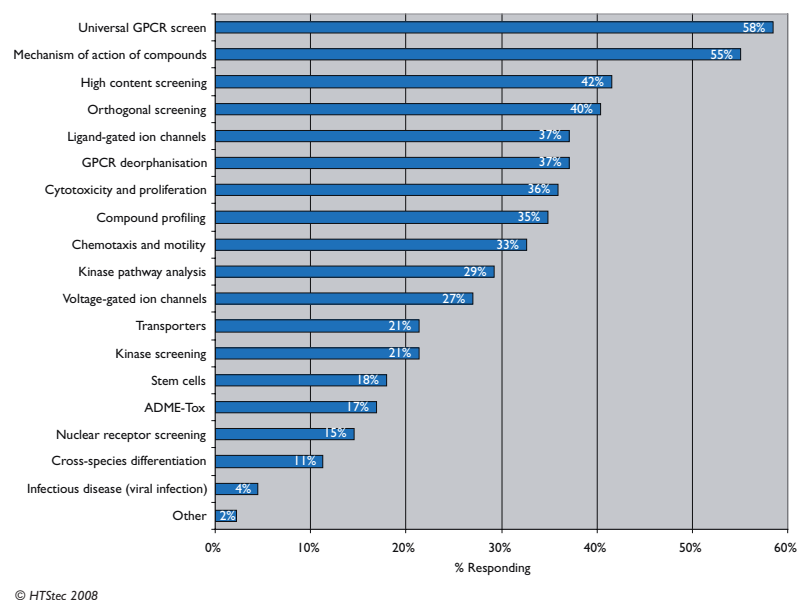
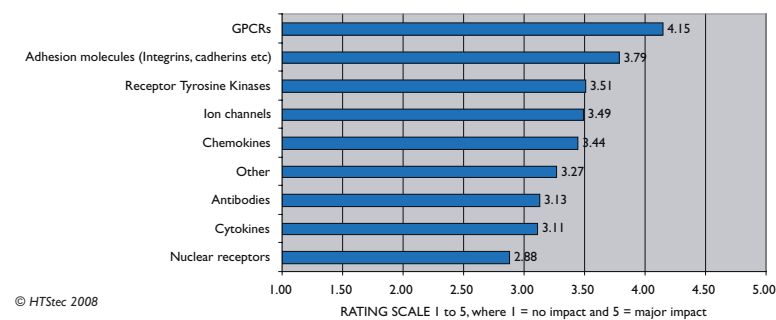
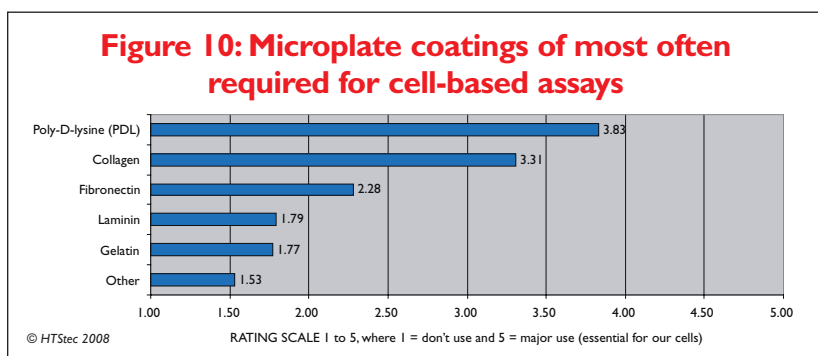
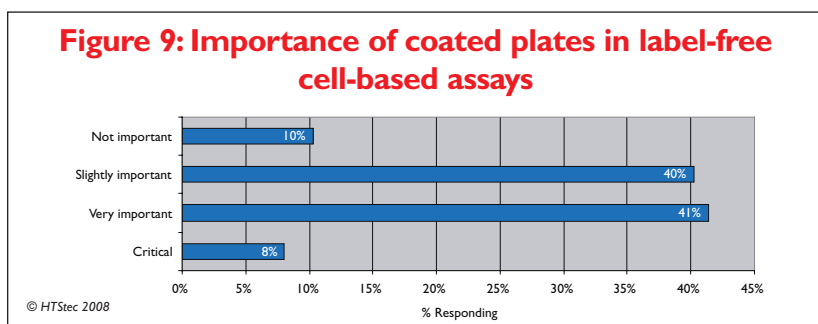
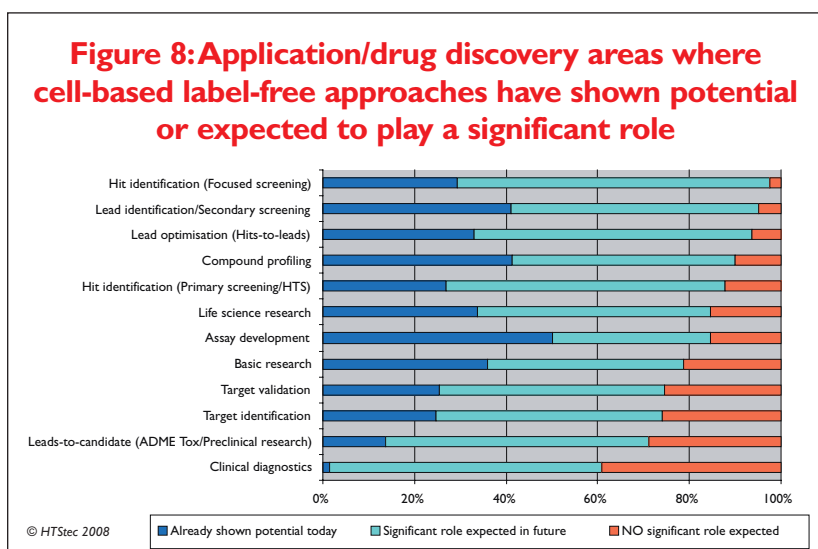
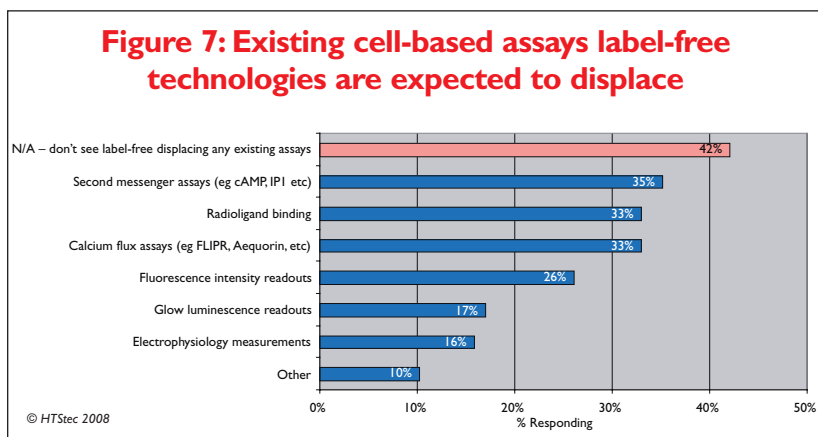


Figure 6: Targets classes/application areas that cell-based label-free assays are expected to impact the most



Lead Discovery



cell-based assays was very important. A further 40% of respondents said it was slightly important (Figure 9).

Of those surface coatings available for other microplate applications, Poly-D-lysine (PDL) was rated by survey respondents as the coating most often required for their cell-based assays. This was followed by collagen and then fibronectin. It remains to be seen if these coatings will be offered on microplates suited for label-free applications (Figure 10).

Table 1 summarises the main vendor offerings that currently support label-free cell-based assays.

Vendor snapshots

The following snapshots provide details of the progress vendors have made in implementing their plate-based label-free technologies, and the cellular applications where they have achieved greatest success.

The founders of Applied BioPhysics (www.biophysics.com) pioneered label-free monitoring of cells in 1984 using weak AC current to measure complex impedance of small cell-covered gold electrodes. ECIS (Electric Cell-substrate Impedance Sensing) has been sold to several hundred laboratories worldwide, and data appears in more than 275 peer-reviewed, scientific papers. Applied BioPhysics continues to innovate, and its newest ECIS system measuring complex impedance using a 96 well format will be released shortly. By interpreting the complex impedance data from cell-covered electrodes, the barrier function, the cell membrane capacitance and the spacing between the cells and the electrodes can be determined. Future use of ECIS will capitalise on this modelling capability, particularly in label-free studies of endothelial cell monolayer permeability and its response to various drugs and other experimental conditions. In recent years, non-invasive ECIS monitoring has been coupled with higher electric fields to produce a fully automated wound-healing assay to measure rates of cell migration. This assay can follow changes due to the presence of drugs and different medium composition, and recent research demonstrates the effects of different adsorbed extracellular matrix proteins upon cell migration. These higher fields can also be applied for a fraction of a second to allow the entry of non-membrane-permeable molecules via electroporation. By returning the instrumentation to its monitoring mode, the effect of these compounds upon cell behaviour can be discerned. We believe this assay will increasingly find

Table 1: Some of the plate-based label-free systems being used for cell-based assays

VENDOR	SYSTEM NAME	BIOSENSOR	WELL-FORMATS	CELL-BASED APPLICATION FOCUS
Applied Biophysics	ECIS	Impedance-based	16- & 96-well and arrays	Exploration of cell migration, invasion, permeability and signal transduction
Corning	Epic®	Optical Grating	384-well	Endogenous receptor analysis, signaling pathway analysis, mode of action studies
IonGate	SURFE ² R	Electrogenic	96-well	Transporters, ion pumps using plasma membrane fragments
MDS Analytical Technologies	CellKey™	Impedance-based	96-, LV 96- & 384-well	Biorelevant screening and analysis of transfected and endogenous cellular receptors
Roche Applied Science	xCELLigence	Impedance-based	16- & 96-well	Cell quality, proliferation, viability, cytotoxicity, adherence and spreading, functional monitoring receptor signalling
SRU Biosystems	BIND®	Optical Grating	8-, 16-, 96-, 384-, & 1536-well	GPCR assays, endogenous receptor analysis, adherent and suspension cells, primary cells, ion channels

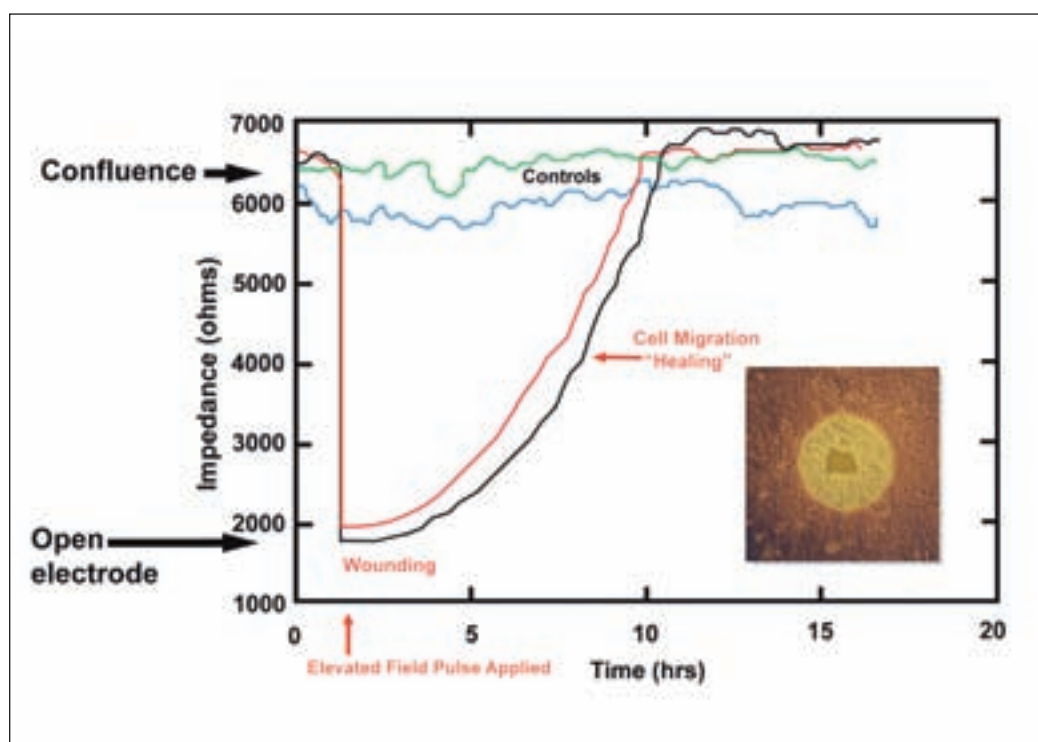


Figure 11
 Typical ECIS wound healing data from two experimental wells with controls. The inset shows a small electrode during cell migration. At time zero the impedance of the small (250 micrometer diameter) ECIS electrode covered with cells at the base of four different wells. At the red arrow, a high field pulse is applied to two of the wells to wound (kill) the cells upon the electrode resulting in a rapid decline in the impedance to that of cell-free (open) electrodes. Over the next few hours, as the healthy cells on the periphery of the wound migrate inwards, the impedance of these electrodes slowly rises until it is back at the level of the non-wounded controls. By noting the time of this recovery (~9hrs) and knowing the size of the wound (125 micrometer radius) one can calculate the migration rate – in this case ~16 micrometers/hr

Lead Discovery

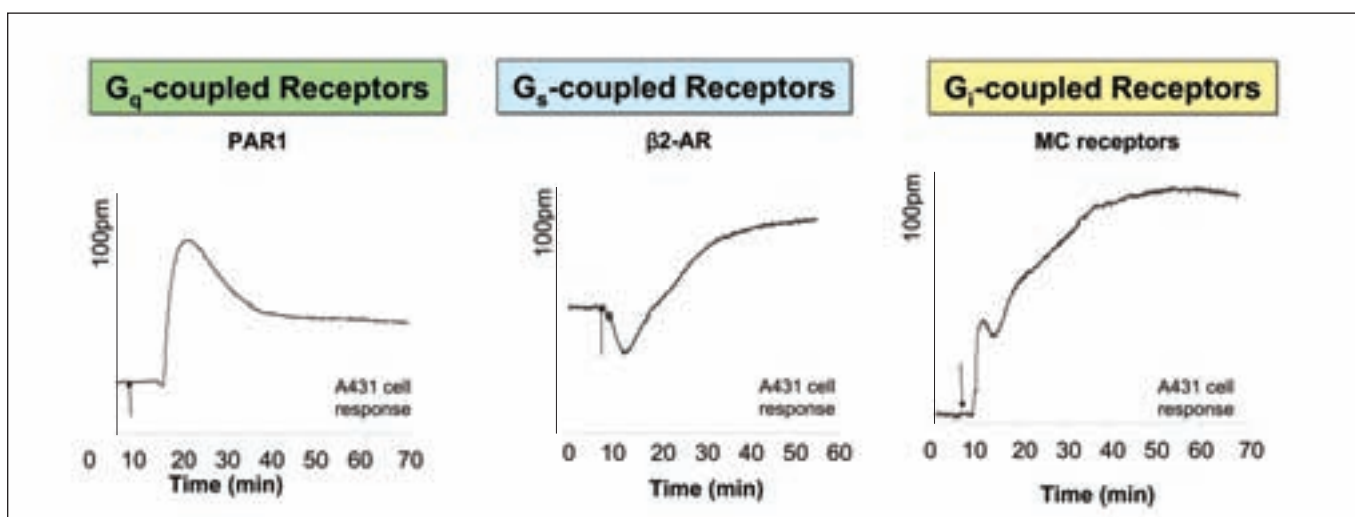
use in studies of cellular response to normally membrane-impermeable compounds. As the ECIS product line evolves, different configurations of electrodes are being developed to tailor the measurement for specific applications. ECIS accessories are now available permitting impedance measurements to be coupled with microscopic observation of the cells, and new instrumentation has been developed to allow monitoring of cells under various flow conditions and also under hyper and hypoxia conditions (Figure 11).

Since the launch of the Corning® Epic® System (www.corning.com/epic) in 2006 major pharmaceutical and academic institutions have repeatedly demonstrated the value of the world's first high throughput label-free technology to create detailed, quality information from more biologically relevant samples. Whether performing a small molecule biochemical assay or studying the complex functional characteristics of endogenously expressed G-protein coupled receptors, the label-free Epic system is promising to improve the quality and speed of the drug discovery pipeline. Pharmaceutical and academic organisations such as AstraZeneca, University of Bonn, Amgen and Roche have each publicly presented the key benefits of label-free assays at major conferences around the world – most recently in Dresden, Germany in June 2008 at the first ever Label-free Symposium hosted by the Society for Biomolecular Screening. Most notably, Paul Lee et al⁴ of Amgen recently demonstrated the ability of optical biosensors to study a ligand's mechanism of action, specifically distinguishing inverse agonists from neutral antagonists. The results of this double-blinded study profiling 12 compounds against two

recombinant cell lines suggest that Epic's dynamic mass redistribution (DMR) shift is capturing significant details about how ligands down-regulate receptor signalling – a detail that conventional label-based assays may miss. Other researchers⁵ have demonstrated Epic's ability to study ligand-directed functional selectivity in endogenous GPCRs. The ability of the Epic system to characterise functional receptor biology in endogenously-expressing cell lines allows researchers to study complex biology which may not be evident with conventional methods. Epic's ability to detect the tightly regulated and highly specific redistribution of intracellular proteins is effective across all major classes of GPCRs (G_s , G_i , G_q) (Figure 12). Overall, the drug discovery community is very excited about the ability of label-free optical biosensors to acquire detailed, quality information from more biologically relevant samples.

IonGate's (www.iongate.de) SURFE²R technology allows the label-free investigation of electrical activity of membrane proteins bound to gold electrodes. Proteins are activated by means of a substrate concentration jump applied via rapid solution exchange. A wide variety of membrane preparations containing functional proteins is suitable for SURFE²R measurements, eg liposomes, cell membrane fragments or tissue membrane preparations. A new development by IonGate now allows the use of cultured cells for direct preparation of sensors. Cells are grown under controlled conditions, harvested and centrifuged on to the gold sensor. These sensors are then mounted to the SURFE²R device. By applying a strong fluidic stress, the cells are broken. Plasma membrane fragments, however, stay attached to the sensor surface

Figure 12
Corning® Epic® System has the ability to detect the tightly regulated and highly specific redistribution of intracellular proteins across all major classes of GPCRs (G_s , G_i , G_q)



and can be investigated in the inside-out configuration. The time from cell culture dish to electrical measurements is below 15 minutes, thereby allowing the study of cellular regulation of transport activity. In a number of validation experiments the new SURFE²R application was used to investigate induction of transporter expression in different inducible cell lines. Cells were characterised with regard to background expression level, maximum expression level and time course of induction of functional transporter expression. A second series of experiments was performed to select cell clones from a large number of potentially stable cell lines. To achieve this, approximately 100 different cell clones were grown in 6-well dishes; cells were harvested and investigated as described above. Using the SURFE²R Workstation 5000, (Figure 13) a system operating with 96 well sensor plates and 8-channel activation, a throughput of up to 100 clones per hour was achieved. Comparison of functional expression measured by peak current amplitude, with Western blot analysis showed excellent correlation. Thus, application of cells to prepare SURFE²R Sensors enables the fast analysis of regulation and expression with transporter mediated currents. The method including all necessary components can be purchased from IonGate, which also offers it on a fee-for-service basis.

In April 2008, MDS Analytical Technologies (www.moleculardevices.com) announced the first high throughput cellular impedance assay instrument, the CellKey™384 System (Figure 14). The CellKey384 system brings the simplified workflow of a label-free assay, the biorelevance of endogenous receptor analysis and the rich information of integrated cellular responses to high throughput screening. This new instrument is based upon proven CellKey™ System technology, and expands the deep experience gathered on the original 96-well system. Impedance assays are highly informative, robust and easy to perform, and the CellKey384 system extends the same benefits to primary screening applications. Like its 96-well counterpart, the CellKey384 system is sensitive to endogenous levels of cellular receptors and requires no labels or artificial pathway mediators. The flexibility and universal nature of the technology allows measurement of multiple targets classes, and the ability to screen adherent and non-adherent cell lines and primary cells with simplified assay development and streamlined workflow. The system is comprised of the physical instrument, a custom 384-well microplate and a comprehensive software package capable of



Figure 13: IonGate's SURFE²R Workstation 5000 allows the label-free investigation of electrical activity of plasma membrane fragments from cells harvested on to the gold sensor



Figure 14: The MDS Analytical Technologies CellKey™ 384 System for high throughput cellular impedance assays

Lead Discovery

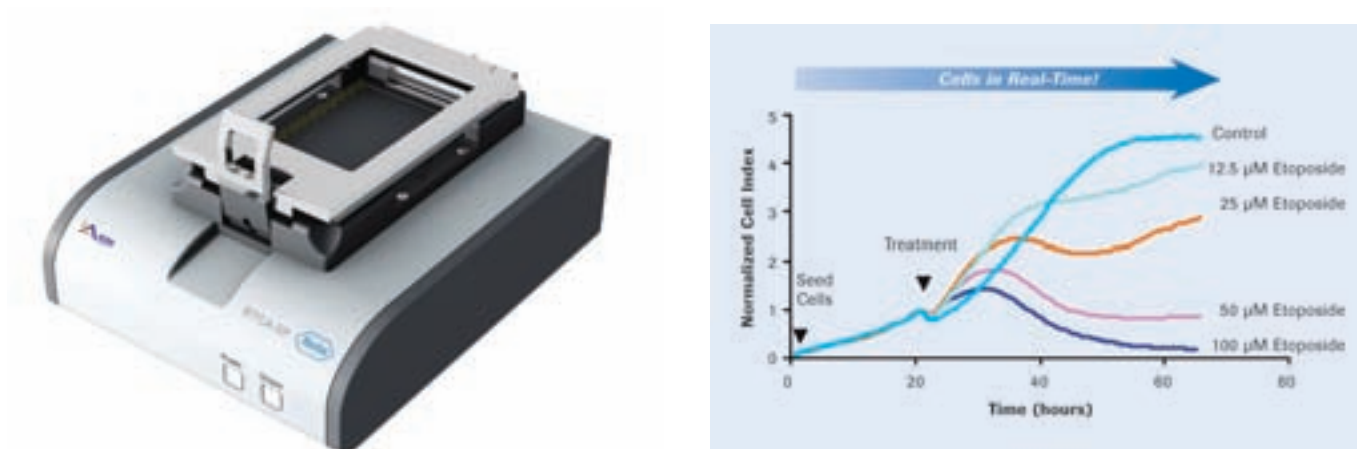


Figure 15: The xCELLigence System RTCA SP instrument (left panel) from Roche Applied Science utilises impedance to monitor cells in the E-plate 96 throughout the entire time course of the experiment, as the plate station sits inside an existing tissue culture incubator for environmental control. The real-time data (right panel) obtained by the xCELLigence System can be used to optimise time of treatment, calculate parameters such as rate of cytotoxicity, and obtain real-time IC₅₀ values

controlling all aspects of assay design, set up, execution and analysis. To monitor fast cellular responses in a high throughput environment, the system delivers compounds to all 384 wells of the cell plate while simultaneously capturing the kinetic impedance data, thus providing insight to cellular responses from the moment of receptor activation. Thermal control and an integrated tip washer further enhance the new system's utility to high throughput analyses. Finally, the CellKey384 system can be easily integrated with third party automation to exchange plates and tips, thereby extending the unattended throughput of the system. The CellKey384 system now generates rich impedance assay information in a simplified manner at the earliest steps of the screening process, resulting in the greater promise of identifying quality lead compounds.

The xCELLigence System from **Roche Applied Science** (www.xcelligence.roche.com) is an impedance-based, dynamic real-time, label-free cellular analysis platform. The xCELLigence system (Figure 15) is the result of the co-development agreement between Roche and ACEA Biosciences Inc. The interdigitated microelectrodes on the bottom of the E-Plate® cover 80% of each well-bottom's surface area and provide broad dynamic range and applicability in diverse applications. The plate-stations are designed with a compact footprint so that they can be placed directly into existing incubators for control of temperature, humidity and CO₂. These attributes combine to allow for real-time kinetic measurement over the time-course

of the experiment that is determined by the researcher – not the system. The impedance measurement is reported in real-time as the Cell Index, a dimensional parameter which is indicative of cell number, cellular adherence and morphology. Kinetic profiles generated throughout the experiment can be used to monitor proliferation, viability, adherence and spreading, functional monitoring of cell surface receptors, barrier function and viral cytopathic effects. Compound-mediated cytotoxicity and functional monitoring of cell surface receptors will be key applications for compound and target discovery in therapeutic development. Real-time kinetic profiles can be used to infer mode of action for a compound within a given cell line. This has significant potential in secondary screening and pre-clinical toxicology for grouping like compounds and identifying potential off-target effects. Functional monitoring of cell surface receptors such as GPCR, tyrosine kinase and nuclear hormone receptors provides information regarding activation pathways and compares well to traditional assays such as calcium and cAMP measurements. Multiple throughput options will be available in 2008. The RTCA SP system will have a 1 x 96 well plate capacity, the RTCA MP will have a 6 x 96 well plate capacity, and the RTCA DP will have a 3 x 16 well plate capacity. The RTCA DP instrument will be capable of monitoring both the standard E-plate® and novel IM-Plate, which can be used for migration or invasion assays. The IM-Plate is based on a Boyden chamber, in which electrodes are incorporated on the bottom surface of an 8μm membrane between two chambers.

SRU Biosystems (www.srubiosystems.com) has introduced two new instruments in its BIND® platform and demonstrated significant advances in label-free cell-based and biochemical assays. The new BIND® Reader Turbo offers 1536-well capability, a significant increase in scan speed, and new software for integration with popular laboratory automation equipment. In kinetic mode, the Reader can monitor cellular responses across an entire 1536-well plate every 45 seconds, or in simple end point mode, can sample nearly a million wells in an eight-hour day. These enhancements enable monitoring of cell responses on a short time scale using very low numbers of endogenous or primary cells. Data has been presented by BIND users reporting comparable data obtained for BIND® to that obtained on the FLIPR for GPCR assays. Additionally, users in drug discovery have demonstrated ligand-dependent activation on primary rat astrocytes. In a cell-based assay, reproducible IC_{50} values have been achieved for an endogenous receptor agonist with as few as 600 THP-1 monocytic suspension cells per well (Figure 16). In addition, chemokine receptor responses were obtained using the THP-1 cells on BIND. This provides validation of the BIND technology for screening and compound profiling using small numbers of cells per assay. Of particular note, SRU Biosystems has described one of the first reports of detection of ion channel activation, and inhibition, using a label-free system with a Millipore cell line that over-expressed the sodium channel, Nav1.3. The performance of the cell-based applications has been enhanced by the introduction of CA-1 BIND Biosensor plates, which feature a proprietary extracellular matrix coating for cell attachment. In order to address the needs of label-free users requiring lower throughput and smaller budgets, SRU Biosystems has introduced a low cost Cartridge Reader based on the same biosensor technology aimed at assay development and compound profiling.

Summary

The potential for label-free technologies to impact on cell-based assays is high, as uniquely almost all the label-free offerings directed towards cells are plate-based. As such they are amenable to medium throughput, and in some cases the throughput is comparable to that achieved using alternative labelled technologies. Compatibility with the microplate world is a big plus for adoption, particularly the 384-well systems, as it allows for a simpler interface with existing infrastructure, current processes, sample preparation and liquid handling

instruments and liquid compound libraries, the majority of which are in 384 today.

From a purely cost perspective, currently there is no or little price advantage associated with label-free as for the most part the cost of the cell labels has simply been displaced by an expensive consumable, the biosensor plate. However, if greater volumes are achieved, the potential for cheaper plates

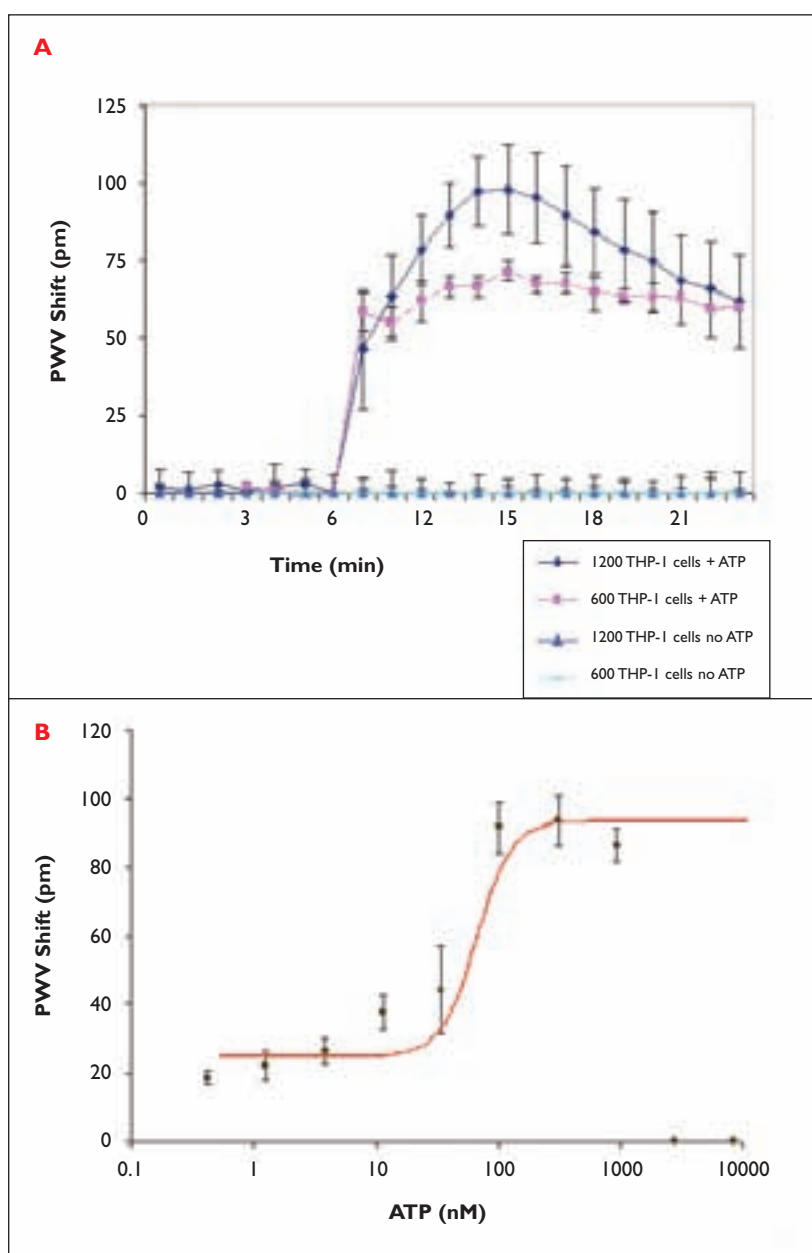


Figure 16: Cell monitoring with SRU Biosystems BIND biosensors. The suspension cells, THP-1, were dispensed at 600 and 1200 cells per well on a fibronectin coated LV 384-well BIND® biosensor surface. After four hours of attachment, the cells were stimulated with ATP.

A A robust signal at 25 μ M ATP is obtained for 600 and 1200 cells/well.

B The EC_{50} value (82 nM) obtained for ATP on 600 THP-1 cells correlated well with values obtained at higher cell numbers (4K cells EC_{50} = 104 nM)

Lead Discovery

References

- 1 Cell-Based Label-Free Detection Trends 2008 Report, published by HTStec Limited, Cambridge, UK, January 2008.
- 2 Label-Free Binding Analysis Trends 2008 Report, published by HTStec Limited, Cambridge, UK, March 2008.
- 3 Comley, J. Label Free Detection – new biosensors facilitate broader range of drug discovery applications. *Drug Discovery World* 2006; 6: 63-74.
- 4 Lee, PH, Gao, A, Staden, CV, Ly, J, Salon, J, Xu, A, Fang, Y and Verkleeren, R. Evaluation of Dynamic Mass Redistribution Technology for Pharmacological Studies of Recombinant and Endogenously Expressed G Protein-Coupled Receptors. *Assay and Drug Development Technologies* 2008; 6(1):83-94.
- 5 Fang, Y, Ferrie, A. Label-free optical biosensor for ligand-directed functional selectivity acting on β_2 adrenoceptor in living cells. *FEBS Letters* 2008; 582: 558-564.

exist, eg SRUs BIND plates are based on a low-cost plastic substrate which is manufactured in rolls, this process should lend itself to lower cost production.

There is now a considerable body of evidence that label-free can usefully contribute to endogenous receptor analysis, as a universal receptor deorphanisation platform and in mechanism of action studies of receptor ligands. This receptor niche has driven much of the recent label-free efforts by vendors and their first customers and it is not surprising that most of the difficult cell-based targets that have been enabled by label-free were GPCRs. Label-free technologies have also proved advantageous in the study of primary cells, where quantities available are often limiting. The main attraction is the ability to use a relatively small number of native non-engineered cells and then to be able to use the same technology to directly compare with an engineered cell line. This should enable analysis of the functional response of native cells to test compounds with native levels of receptors (ie cells expressing more physiologically relevant surface levels of receptor and the associated receptor reserve) with no perturbation by label and no IP issues. It may also facilitate the dissection of the signalling pathway of the receptor and permit the identification of pathway specific antagonists in a single assay. However, the interpretation and robustness of the data still leaves many a little skeptical.

Some attempts have been made to promote label-free as a universal assay platform or panacea for all and every cell-based assay. Evidence that the label-free technologies could measure ion channels without patch clamping or fluorescent dyes, transporters without membrane isolation or other more challenging responses, eg chemotaxis, would be highly welcomed. However, universal claims may prove counter-productive in the short term if they are premature and lead to a lack of focus. Like many emerging technologies there has been a tendency for applications to be over-hyped, when they are little more than proof-of-principle studies, where it is not completely understood what the label-free readout actually represents.

Another aspect which seems to be emerging is the coupling (or multiplexing) of label-free with other readouts (eg microscopic imaging) or technologies (eg electroporation) or systems (eg invasion through membranes/migration chambers) or configurations (eg flow cells). The possibilities seem endless, for example if electrical contacts were added to optical grating biosensors it might facilitate fluorescent dye-free monitoring of field-stimulated ion channels.

In conclusion, there are undoubtedly grounds for optimism with respect to label-free and cell-based assays and much progress has been made in their implementation, particularly in the GPCR niche over the past few years. The majority of these studies have been in assay development, compound profiling and secondary screening. However, the question still remains – can label-free achieve more than just fill in the gaps that other technologies do less well and break into main stream lead discovery? **DDW**

*Dr John Comley is Managing Director of HTStec Limited, an independent market research consultancy whose focus is on assisting clients delivering novel enabling platform technologies (liquid handling, laboratory automation, detection instrumentation and assay reagent technologies) to drug discovery. Since its formation in 2003, HTStec has published 35 market reports on drug discovery technologies and Dr Comley has authored 24 review articles in **Drug Discovery World**. Further information on accessing the market report 'Cell-Based Label Free Detection Trends 2008' can be obtained by visiting www.htstec.com or by emailing john.comley@htstec.com to receive a free copy of the Report's Executive Summary and Table of Contents.*