Current trends in label-free technologies

Label-free technologies have been used throughout drug discovery for the detection and characterisation of molecular interactions. The implementation of automated systems, increased sensitivity and the development of new technologies have recently broadened the use of label-free methods from primary screening through to mechanism-of-action studies in lead optimisation. The trend is now towards adopting label-free methods earlier in the drug discovery process so that compounds can be tested against native protein or cells resulting in improved biological assessment of compounds more predictive of therapeutic effect.

By Philip Rawlins

Healthcare reform, tougher regulatory hurdles and patent expiry of drugs are putting the pharmaceutical industry under increasing pressure, with R&D costs continuing to increase but numbers of NMEs remaining static. It is clear that for companies to remain competitive, they not only need to reduce project attrition and improve cycle times but also identify technologies that provide more physiological mechanistic data to help identify compounds that translate *in vitro* activity to *in vivo* efficacy.

The majority of drug discovery projects are aimed at protein targets. Over the last decade, the focus has been on developing homogeneous, sensitive, robust and cost-effective assays for screening. However, these assays utilise extrinsic labelling or reporter systems that are prone to non-specific compound interference. Label-free technologies enable the confirmation of direct binding of compounds to a molecular target, thus providing confidence in applying valuable medicinal chemistry resource. Cellular assays are now common for screening and can provide a better functional understanding of the molecular target when expressed in a cellular context. Generic transfection approaches and high receptor expression give acceptable signal-to-noise but can result in artificial systems not representative of receptors in their native environment. Label-free technologies provide a route for measuring endogenous receptor activation from model cell lines, differentiated stem cells or primary cells. The implementation of more relevant assays into the early screening cascade is anticipated to provide a better correlation with human pathology.

Label-free (biophysical) technologies cover a broad spectrum of assays that can interrogate such diverse aspects of molecular and cellular biology as the measurement of binding affinity, rate constants, enthalpy, receptor pharmacology, signalling pathway activation, cell growth/proliferation, cell adhesion, viral replication, GPCR functional selectivity, toxicity screening, ion channel activity, transporter activity and stem cell differentiation. With the exception of Optical Waveguide Grating (OWG) technologies (Corning Epic® and SRU Biosystems BIND®), label-free technologies can be divided into those that measure binding to soluble molecular targets and those that measure cellular function and for this reason these areas are discussed separately.

Label-free technologies for soluble enzymes

The majority of assays for soluble enzyme screening are highly miniaturised, sensitive,

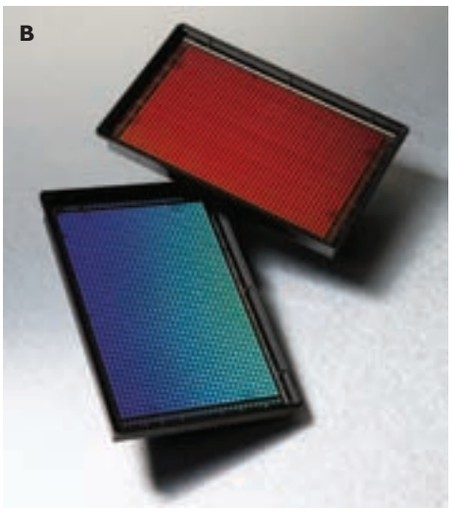

Figure 1
(A) The Corning Epic® OWG high throughput screening instrument capable of both affinity binding assays and real-time cellular functional responses, and (B) examples of 1536 Epic® proprietary microplates containing OWG biosensors within each well

homogeneous formats using fluorescent or luminescent detection amenable to high throughput screening (HTS) and these approaches have been successful in delivering new compound series over the last decade. However, they have a number of disadvantages: tagging or labelling of molecular targets may alter their behaviour, the detection may be prone to interference or quenching, and compound aggregation can cause non-specific inhibition. This can lead to 'real' hits being hidden within the 'noise' of false positives, so alternative methods are required to confirm that compounds bind directly to the enzyme and valuable resources are not wasted. Label-free technologies enable the measurement of binding affinity with the native molecular target without the need for tagging or labelling.

An example of the importance of label-free technologies is the SIRT1 enzyme, a putative target for type 2 diabetes. Biophysical techniques have been used to demonstrate that a number of compounds, including resveratol, do not activate the native SIRT1 enzyme directly[1]. Instead, they appear to bind the fluorescent TAMRA-p53 peptide substrate used in the screening assay. Although these compounds may yet be found to have *in vivo* efficacy via an alternative mechanism, this clearly demonstrates the dangers of using engineered fluorescent assays alone to identify and characterise compound activity.

The 'toolbox' of label-free affinity screening techniques each has different throughputs, protein requirements and sensitivity so the method employed will depend on the number of compounds to be tested, available protein and the mechanistic questions that need to be addressed.

Orthogonal screening

The objective of orthogonal screening is to ensure that compounds that appear active are binding to the molecular target of interest and not interfering with detection reagents, binding to the substrate or inhibiting non-specifically. One assessment of the output of a β-lactamase HTS put the number of false positives at more than 95%, based on their sensitivity to detergent, unreproducible data and biophysical assessment[2]. An important consideration for orthogonal screening from the output of HTS is the number of compounds that can be tested. OWG technologies use waveguide biosensors in each well of 384 or 1536 proprietary microplates (**Figure 1**). This enables much greater throughput for measuring equilibrium binding and can be used as an intermediary step prior to more detailed characterisation via Surface Plasmon Resonance (SPR), Nuclear Magnetic resonance Spectroscopy (NMR) or Isothermal Titration Calorimetry (ITC). Native mass spectrometry[3], which uses 'soft' ionisation to maintain non-covalent interactions, is also gaining use due to advances in nanoelectrospray microchip technology such as the Advion Triversa NanoMate®.

Fragment-Based Lead Generation (FBLG)

The principle of FBLG is to screen a small but diverse collection of low molecular weight (less than 300Da) highly soluble ligands to identify relatively weak compounds with high ligand efficiency. A fragment-based approach is a much more efficient way of sampling chemistry space than HTS and can identify novel areas of chemistry that might otherwise be missed. NMR is the 'gold standard' for fragment screening due to its

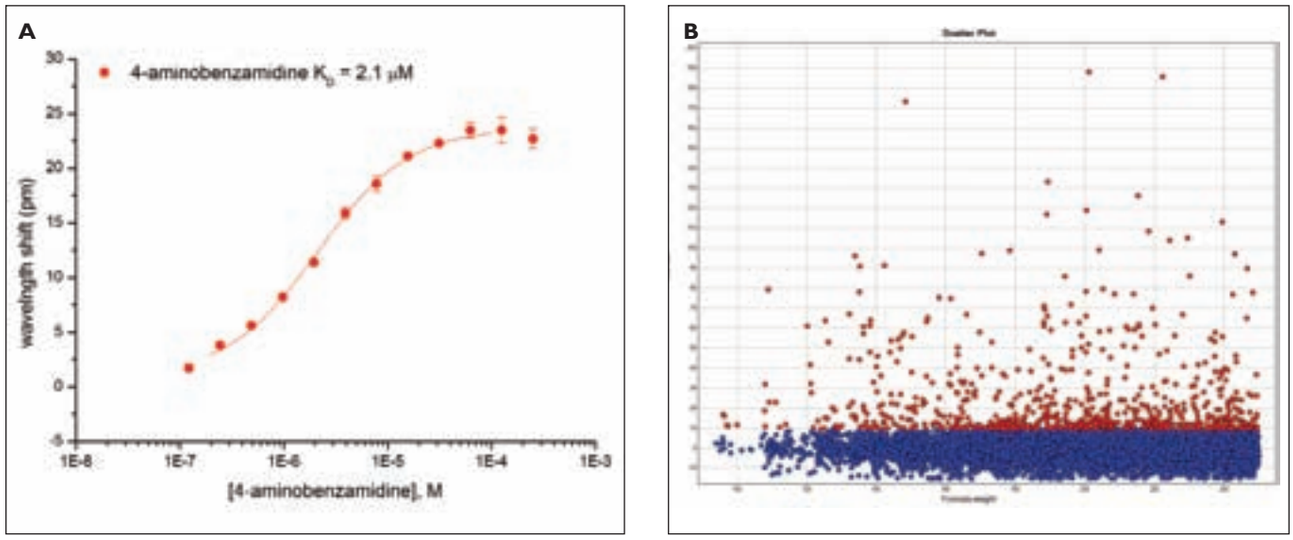

Figure 2: (A) Determination of the binding affinity of a 135Da fragment to human trypsin using the Corning Epic OWG platform, and (B) a complete data set of an 11,000 compound fragment screen for human trypsin tested at 100μM. Data is plotted as a percentage of the signal obtained with amiloride hydrochloride plotted against the molecular weight of the compounds. Red indicates compounds that bind to trypsin, blue indicates non-binding compounds

high sensitivity, low rate of false positives and ability to provide structural information[4]. However, high quantities of protein are required, throughput is low, users need to be highly trained, equipment is expensive and isotopically labelled protein is necessary for protein observed NMR. Other techniques have been used successfully for FBLG including SPR, ITC, Mass Spectrometry and high concentration screening in standard HTS assays[5].

At AstraZeneca, we have used OWG technologies for fragment screening using both conventional direct binding assays (**Figure 2**) and a competition assay where a target-specific compound is immobilised to establish a label-free competition assay in solution. The direct binding assay will detect all binding sites on a target protein and an immobilised tool compound is not required, whereas the competition assay has the advantages of a larger assay window, greater stability during the assay and a lower hit rate since only a single binding site is interrogated.

Binding kinetics

There is increasing evidence in the literature that measuring drug residence time (ie dissociation rate) may be important for predicting clinical outcome. A long residence time, for example, may give greater efficacy and a better safety profile for off-target effects; short residence times may help minimise mechanism-based toxicity[6]. Therefore, the measurement of binding kinetics, in addition to providing affinity data, can provide information to differentiate compound series.

Since the launch of Biacore instruments (now part of GE Healthcare) in 1990, SPR has become the most common method for measuring binding kinetics. Since then, SPR has progressed in terms of both sensitivity and throughput, and there is now a broad range of validated applications and many instruments available including the Biacore 4000 (GE Healthcare; **Figure 3**), Proteon XPR36 (Bio-Rad), AP-3000 (Fujifilm), SR7000DC (Reichert), SensIQ Pioneer (ICx Technologies) and SPR-2 (Sierra Sensors).

Figure 3: The latest Biacore SPR instrument, the Biacore 4000, features four independent flow cells and five measurement spots enabling the analysis of up to 16 targets in parallel. This instrument also has lower background noise and increased resolution for faster off-rate determination than previous instruments

Figure 4
The fully integrated Cellkey™384 impedance instrument with temperature control up to 37°C, on-board pipetting and tip wash capability

Other optical approaches include Biolayer Interferometry (BLI; ForteBio) with the Octet range of instruments for measuring binding kinetics. The Octet Red384 is an integrated system using 16-tips to measure samples in 384 microplates. This technology has the advantage that it is compatible with crude samples and has low protein requirements. Also, Farfield has developed the Analight 4D instrument, which can measure changes in protein density and thickness at the surface and thus detect conformational changes via Dual Polarisation Interferometry (DPI). It is also very sensitive; molecules below 50Da can be observed binding to immobilised macromolecules of up to 100kDa with a mass resolution of <5Da.

Binding thermodynamics

ITC can determine the change in Gibbs free energy of binding and measure the change in enthalpy. Enthalpic binding represents favourable specific hydrogen bonding interactions whereas entropic binding is produced from mostly non-specific hydrophobic interactions. There is evidence from analysis of HIV protease and HMG-coA reductase drugs that favourable binding enthalpies differentiate best in class from first in class[7]. Choosing compound series with favourable enthalpies or optimising chemistry based on enthalpy may contribute towards reducing attrition at later stages of drug discovery.

The use of ITC is often restricted to small numbers of compounds due to throughput limitations and protein consumption. The automated ITC instrument from GE healthcare, Auto-ITC200, reduces the amount of protein required approximately five-fold and automates the time-consuming steps of sample loading, thus increasing throughput. Palo Alto Research Centre (PARC) is developing a nanocalorimetry technology (Enthalpy Arrays) which has the potential to dramatically increase throughput of thermodynamic measurements using significantly less protein.

Future developments

Issues remain for many of the label-free affinity technologies described. Techniques such as ITC and NMR require high quantities of protein and optical technologies require the target protein to be immobilised on the detection surface, and sensitivity is reduced for large proteins. In addition, solubilised membrane receptor proteins are particularly challenging despite recent successes with SPR[8], frontal affinity chromatography-mass spectrometry[9] and differential static light scattering[10].

An affinity screening technology that reduces protein consumption and measures interactions in solution would be a major step forward. Back Scattering Interferometry (Molecular Sensing Inc) has some

major advantages over other affinity-based methods[11]. For instance, binding affinity can be measured in solution (immobilisation is still required to measure binding kinetics), much smaller concentrations of protein are required than for most other affinity methods, and targets of any size can be studied. Although at an early stage in development, this technology has some promise in addressing many of the key issues in biophysical characterisation.

Label-free technologies for cellular applications

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 established screening technologies. Artificial reporters are commonly employed and receptors are often expressed at very high levels to ensure a good signal to noise ratio. This provides a robust assay for screening and Design-Make-Test cycles but may lead to pharmacology that is not predictive of *in vivo* activity. A

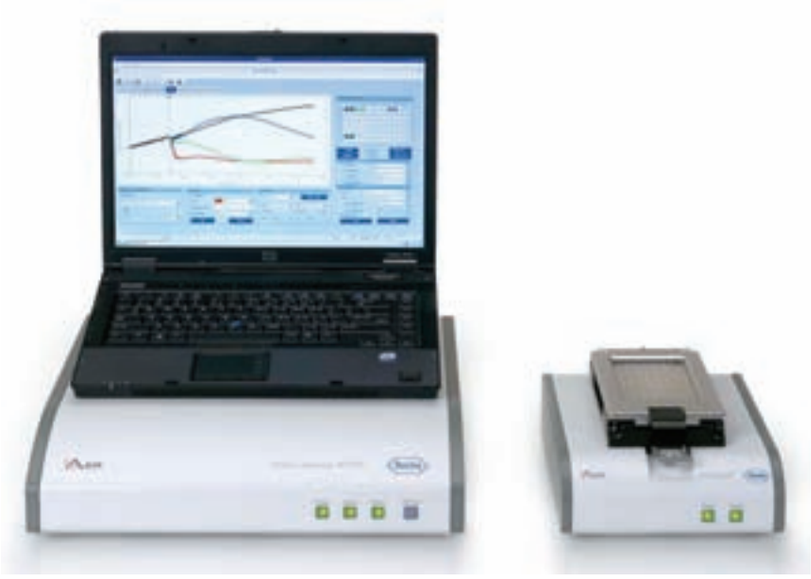

Figure 5: The RTCA SP xCELLigence instrument from Roche Applied Science. The compact instrument design enables continuous measurement of cellular responses within a standard incubator over minutes or hours

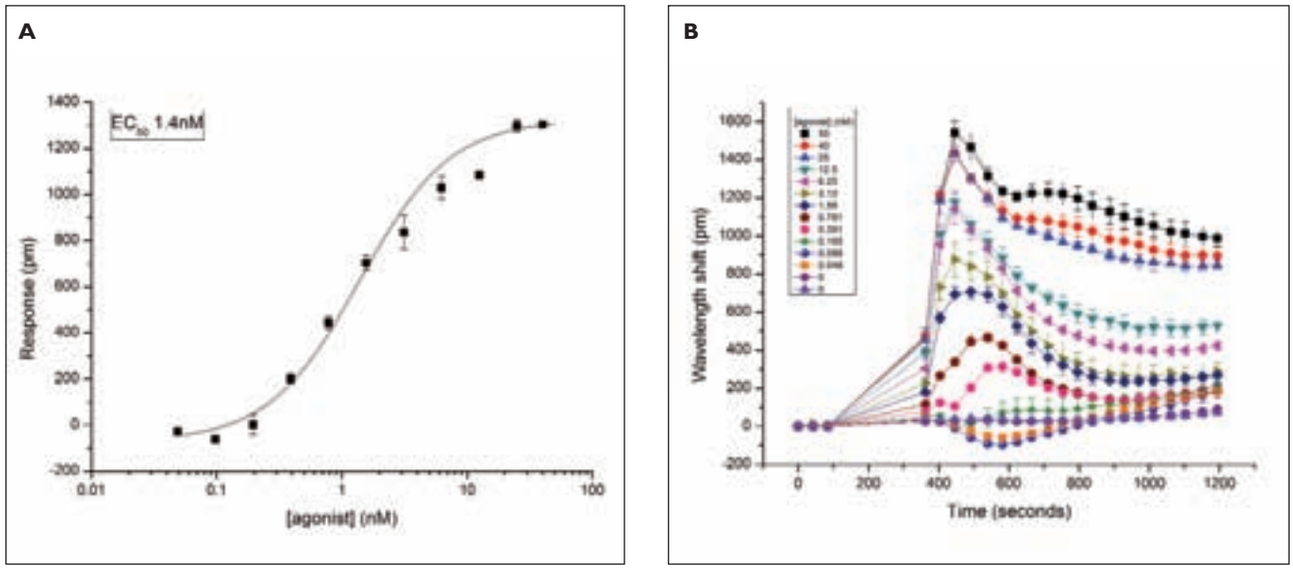

Figure 6: (A) Kinetic traces of an agonist concentration response from freshly isolated human neutrophils measured on the Corning Epic over approximately 20 minutes, and (B) an EC_{50} determination for this agonist giving a comparable potency for this agonist compared to other functional responses in neutrophils

simple example is high expression of GPCRs that can make partial agonists appear as full agonists or neutral antagonists appear as partial agonists. Receptors are also commonly transfected into immortalised CHO or HEK cells that are easy to transfect and grow but may not contain the relevant signalling machinery for the target of interest, potentially leading to spurious results. The use of native cellular screening systems would provide a better physiological model and therefore be more predictive of *in vivo* effects.

Figure 7: The SRU Biosystems BIND® Scanner instrument. The development of a label-free imaging instrument will enable significant reduction in cell numbers and measure individual cell responses from a heterogeneous population. These features combine to increase the accessibility of label-free primary cell applications

OWG technologies measure subtle changes in cell adherence or morphology in close proximity to the biosensor surface[12]. Impedance-based technologies,such as Cellkey™ (Molecular Devices; **Figure 4**) and xCELLigence (Roche Applied Science; **Figure 5**) measure electrical impedance across a cell monolayer, which is highly sensitive to changes in morphology, adherence, volume or cell-cell contacts[13]. GPCR activation is a good example of how OWG and impedance technologies can be used. There are a number of advantages of these technologies over more conventional assay formats that measure specific signalling pathway events such as calcium mobilisation or cyclic-AMP stimulation. These label-free technologies are non-invasive so cells can be used for other assays or treated with sequential ligand additions. They are also 'universal' for GPCRs, so selectivity across GPCR classes can be screened in the same assay format and the signal profile can give an indication of receptor coupling.

GPCR functional selectivity

It is becoming clear that GPCR signalling is extremely complex since GPCRs can interact with

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more than one G-protein and some may signal via G-protein independent pathways such as activation of β-arrestin recruitment. In addition, ligand efficacy may be dependent on cell background and the signalling pathway being measured. For example, propranolol has been found to be an inverse agonist for cyclic-AMP stimulation but an agonist for ERK1/2 activation[14]. This phenomenon has been termed 'ligand-bias', 'agonist trafficking' 'GPCR functional selectivity' and 'ligand directed signalling'. If the signalling pathway important for the required downstream physiological effect is unknown, it would require screening compounds through four or five different assays. In contrast, all ligands could be simultaneously identified from one label-free assay. The concept of GPCR functional selectivity suggests that if a label-free technology is used as the primary screen in HTS it would potentially identify novel compounds that may be missed in a single pathway screen. This principle has been demonstrated at AstraZeneca using the Corning Epic® system. A high throughput screen has been completed for a muscarinic antagonist using a recombinant cell line[15] and compared to data for the same compounds in a fluorescent calcium mobilisation assay. Approximately 70 compounds identified from the label-free assay were not active in the calcium assay, around 50 of these inhibited binding in a radioligand assay. The mechanism of these compounds is yet to be determined but this demonstrates that label-free assays open up the possibility of identifying novel compound series that may be missed with other assay formats.

Endogenous receptor detection

A key advantage of these label-free technologies compared to traditional screening formats is that they are sufficiently sensitive for measuring endogenous receptor activation. We have demonstrated that the Corning Epic® can be used to measure functional responses from freshly isolated human neutrophils (**Figure 6**). The implementation of higher density format microplates for the Epic® and BIND® not only make HTS more efficient but will also help make precious primary cells go further. Although it may be possible to use neutrophils for primary screening, the use of other primary cells may be limiting, since certain human cell types are in very short supply and a suitable surrogate cell line may not exist. The availability of rodent stem cells or adult pluripotent stem cells (iPS cells) that can be reprogrammed to specific cell lineages opens the door for broader native cell screening. Examples of this have been demonstrated with GPCR functional responses from murine neuronal

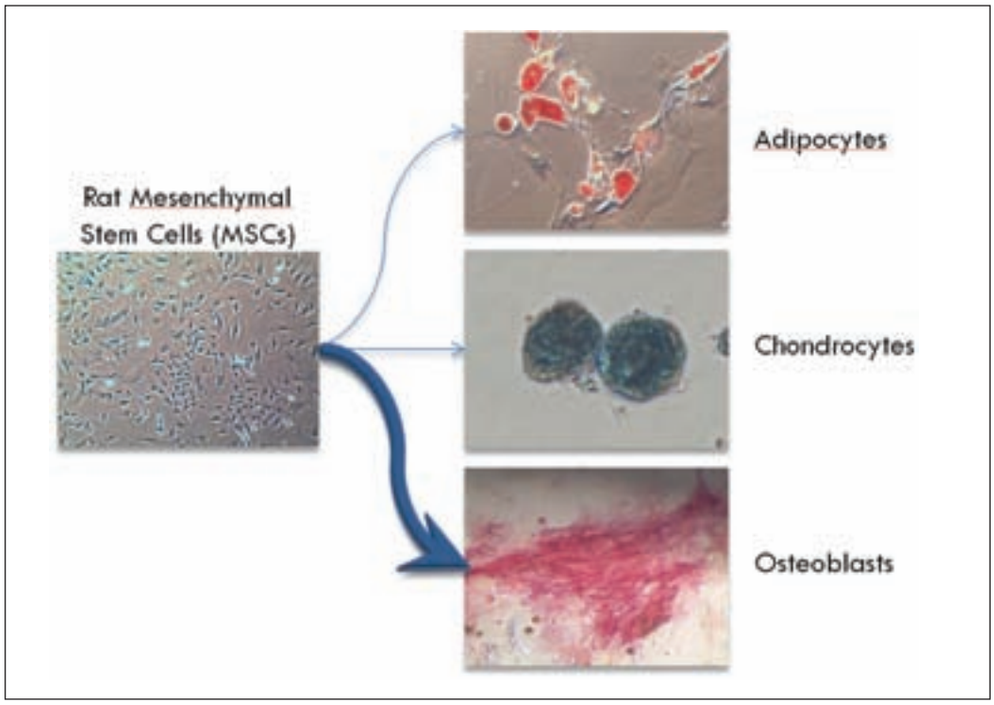

Figure 8: Diagram demonstrating rat mesenchymal stem cells (MSCs) differentiation into adipocytes, chondrocytes or osteoblasts

cells (Corning) and cardiotoxicity screening using iPS cardiomyocytes (Roche Applied Science).

Label-free imaging

Even with high density assay formats, the use of primary cells or stem cells will be limited unless new methods can be developed to further reduce cell numbers. SRU Biosystems has now launched an imager reader small enough to fit into an incubator (**Figure 7**). It has differentiated human mesenchymal stems cells into osteoblasts (**Figure 8**) and demonstrated that differentiation can be expedited in the presence of a GSK3 inhibitor (**Figure 9**). Development of software algorithms enables single cell analysis of functional responses in heterogeneous cell populations. The combination of miniaturisation and new technologies may make it possible in the future to use iPS cells or primary cell types for HTS.

The next step may be to combine label-free imaging with high content imaging. Phase contrast imaging instruments such as the Incucyte™ (Essen Bioscience; **Figure 10**) or Cell-IQ® (Chipman Technologies) can be used to monitor cell growth in a non-invasive manner in a variety of formats such as microplates and flasks. These technologies have been used to develop novel applications such as the CellPlayer™ kinetic assays from Essen Bioscience. They have developed an automated, 96-well scratch wound assay for measuring cell migration which is a fully integrated, kinetic, label-free assay contained within an incubator providing significant benefits over conventional assays. The Incucyte and Cell-IQ instruments can be combined with fluorescence imaging via additional modules to enable monitoring of labelled cell populations over time. These label-free technologies have been used to develop novel, specific applications of therapeutic relevance. The further development of label-free imaging technology combined with high content imaging would enable multiplexing of functional responses, tagging of different cell populations and increased understanding of the specific signalling events activated from a given receptor.

Summary and conclusions

The use of assays amenable to miniaturisation using sensitive fluorescent detection technologies have been implemented over the last decade to rapidly prosecute large compound files against many molecular targets. This approach has been successful in generating many new leads but the identification of false positives is a recurring issue. Label-free methods are complementary to these approaches by enabling the characterisation of compounds against native protein or cells. The current trend is towards adopting label-free technologies earlier in the drug discovery process with the aim of increasing efficiency by focusing effort on fewer, higher quality compounds series. In addition, the routine incorporation of binding kinetics and enthalpy measurements may aid differentiation and selection of compound series by scientific rationale or allow compound series with different mechanisms to be taken forward in parallel. While label-free assays are currently employed in certain niche areas, they will increasingly be used as novel hit finding approaches with broader applications.

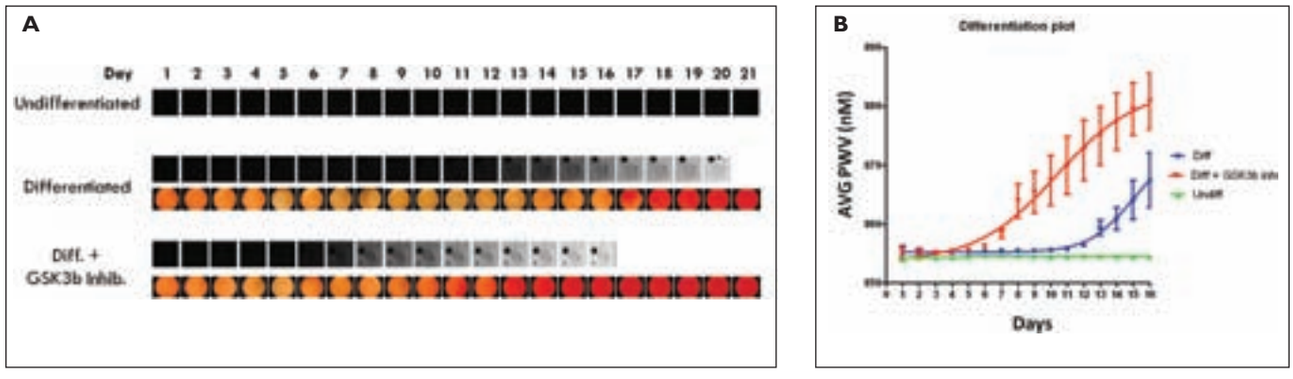

Figure 9: Rat MSCs were seeded in 384-well biosensors at 100 cells/well and treated with osteoblast differentiation media in the absence or presence of an inhibitor of glycogen synthase kinase 3 (GSK3b). (A). Daily images were acquired on the BIND SCANNER and baselined to the Day 0 cell attachment signal. One well per day was fixed and stained with Alizarin Red. (B). Average Peak Wavelength Value (PWV) plotted over 16 day period. A large PWV shift is gradually detected as bone-like minerals are deposited on the sensor surface, as indicated by alizarin red staining of parallel wells. Consistent with previous reports, an inhibitor of GSK3b expedites MSC-osteoblast differentiation. Data demonstrates increased sensitivity of the label-free assay compared to the standard alizarin red assay

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Figure 10: The Incucyte phase contrast live cell imaging system (bottom shelf) fitted within a standard tissue culture incubator. The Incucyte is compatible with both microplates and flasks for cell culture QC, cell-based assay optimisation and long-term, non-invasive, kinetic assay readouts

Further development could one day result in functional cell-based HTS using iPS cells or primary cells to bridge the gap between *in vitro* and *in vivo* pharmacology. Overall, the evolution of label-free technologies will result in a more innovative and efficient process enabling improved biological assessment of lead series. DDW

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