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New directions in kinetic high information content assays

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Kinetic high information content assays can greatly inform our understanding of cell signalling. A new generation of fluorescence and electrical detection instruments facilitates the routine use of kinetic assays within drug discovery. New biosensors enable the detection of a wide variety of signalling molecules in real-time and the potential applications of new assay approaches, such as protein fragment complementation, new biosensors, and imaging techniques, such as fluorescence lifetime imaging, broaden the range of experimental options. Greater use of kinetic, compared with snapshot, cell screening assays will enable subtle, discrete effects of compounds to be detected, aiding the interpretation of compound action and leading to a better understanding of key signalling pathways.

Instruments with the capability of detecting cellular changes with high spatial and temporal resolution have been available for many years to scientists working towards a detailed understanding of cellular behaviour. Optical and electrical detection of rapid, transient changes in cell signalling has facilitated our understanding of transmitter release, long-term potentiation, stages of cell death and many other key physiological and pathological events. An interesting recent development has been the transition of such studies and assays into the industrial screening environment [1,2], whereby subcellular spatial and high temporal resolution are now available on robust high-throughput-compatible readers [3–5]. Situations in which there is a clear need for measuring detailed cell kinetics in drug discovery now present themselves as screening opportunities, rather than requiring the development of a more crude surrogate assay as the primary screening approach [6].

So-called 'black box' screening for desired biological events, such as phenotypic changes, have been a key use of endpoint high content screening (HCS) assays. Endpoint, or snapshot, determinations empowered the first generation of HCS assays such as the identification of compounds that prevented (or caused) cell death, increased neurite outgrowth, and so on. [1,2,7–9]. However, snapshot measurements provide only a limited view of cellular biology; screening approaches that allow an understanding of the kinetics of cell events can drive improved decision making when evaluating compound activities. Temporal resolution is intrinsic to many assay types – for example, only by using kinetic detection can rapid, oscillatory and/or transient events, such as ion channel activation, inactivation, modulation or desensitization, transient changes in transcription factor expression or location, and oscillations and/or waves of release of cell signalling molecules [6], become applicable to the screening environment.

What gets missed in an endpoint screen?

One of the first routinely used HCS assays, pioneered on the Cellomics ArrayScan system, was the translocation of nuclear factor-κB (NF-κB) from cytoplasm to nucleus. NF-κB normally resides in the cytoplasm of cells, sequestered in a complex with NF-κB inhibitor (IκB) and other proteins. Cell stimulation, for example by tumour necrosis factor-α (TNF-α), results in the phosphorylation and degradation of IκB, releasing NF-κB thereby unmasking its nuclear localization sequence. This event results in the translocation of NF-κB to the nuclear compartment. Endpoint assays on early HCS platforms were usually performed to measure NF-κB translocation in response to various pathway stimuli and inhibitors; a small number of time points were typically measured to characterize its translocation into the nucleus [8,10].

This was, and remains, a valuable screening approach. However, by kinetic imaging using fluorescence protein (FP)-linked signalling

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molecules, it is now well established that, in multiple cell types, NF-κB translocation is not an endpoint event, but rather occurs as a dynamic oscillation between cellular compartments [11,12]. HeLa and neuroblastoma cells transiently transfected with plasmids containing p65 coupled to the red fluorescent protein DsRed and NF-κB-inducible IκB coupled to green fluorescent protein (GFP) showed NF-κB oscillations that were controlled by feedback loops. NF-κB increases expression of multiple genes, including that encoding for IκBα; newly synthesized IκBα enters the nucleus and binds NF-κB and the complex then relocates to the cytoplasm by nuclear export. In HeLa cells, TNF-α causes translocation of the p65 form of NF-κB to the nucleus within 1 h, but by 5 h most has returned to the cytoplasm [11]. Oscillations between these cellular compartments (with a period of ~100 min) occur for >20 h in the presence of TNF-α [12]. This pattern of oscillation appears to differ significantly between individual cells, making population studies inadequate. As NF-κB becomes dephosphorylated and inactivated within the nucleus, persistent NF-κB oscillations deliver new active NF-κB to maintain induction of gene expression amplitude [12]. Multiple components of the NF-κB signalling pathway display interrelated oscillatory behaviours [13], such that even subtle modulation of the localization of some key mediators can have marked downstream consequences. The kinetics of transcription factor oscillations are likely to have significant effects on gene transcription events [14,15].

Other transcription factors are also now known to have oscillatory subcellular localizations [16]. To capture the full complexity of the impact of a compound or event on a signalling cascade, dynamic and unsynchronized oscillatory events will best be investigated and implemented as biosensor-based imaging assays using kinetic readers. This is just one example of an assay type in which the conversion of endpoint high-content assays (for cellular localization) into kinetic assays (tracking the behaviour of proteins throughout the course of their cellular response) enables far greater understanding of the regulation of key signalling events. FP-based biosensors, commercialized as Redistribution® (www.bioimage.com), are now available to track many aspects of cycling and signalling behaviour within cell subcompartments [17–20].

Instruments for complex kinetic biological responses

The transition of kinetic assays on single cells into an HTS environment has been facilitated in recent years by the development of novel optical and electrophysiological instrumentation. Voltage-clamp and patch-clamp methods give extremely high resolution measurements of cellular or individual ion channel activity. However, the manual and highly skilled nature of these methods limits the amount of information that can be gathered, and they are very low throughput. Several instruments have been developed to study electrical activity in a systematic, automated fashion. This technology has been based around the innovative breakthrough of being able to use planar array-based systems for carrying out patch-clamp electrophysiology [21]. Higher throughput electrophysiology instruments utilize planar patch-clamping in multiwell formats in combination with multiplexed amplifiers to carry out simultaneous electrophysiological recording from up to 96 cells in parallel. Currently these systems are amenable to studying voltage-gated ion channel activity and drug effects on channel kinetics. The highest throughput is the Molecular Devices Ionworks HT. The latest version of this instrument, the Ionworks Quattro, averages single-cell recordings across each well to give a more accurate measure, in 96- or 384-well formats. Molecular Devices PatchExpress, QPatch from Sophion, as well as instruments from Flyion and Nanion, are similar systems that utilize up to 16-channel chips for simultaneous recording from individual cells.

A good example of the utility of Ionworks HT and the new Quattro system is in the identification of compounds likely to produce QT prolongation. Blockade of the hERG potassium channel is the major contributory factor underlying QT prolongation and cardiac toxicity, so identification of this liability at an early stage of development can be a major benefit [22]. The ability to perform multiple drug applications to a single cell is now being incorporated into systems to study ligand-gated channel activity, for example in the very promising 48-channel QPatch upgrade. This should enhance the capability of these instruments to study the modulation of receptor-ion channel kinetics. The Dynaflow from Cellectricon can apply up to 48 different solutions to a single cell by rapid switching, allowing fast evaluation of drug effects using patch-clamp recording.

Another electrophysiology-based technology allows the evaluation of compound effects on network activity of electrically excitable

BOX 1

Dynamic Ca^{2+} and electrical oscillations

- Regulation of intracellular calcium has spatial and temporal complexity, being locally modified by ion channel permeability, organellar release and uptake, and cytosolic buffering [5,52,53]. The complex structure of primary neurons in culture exacerbates this, as events in synapses, axons, dendrites and cell bodies can have significant temporal and spatial differences.
- Changes in intracellular ion concentration occur in response to exogenous stimuli, or can oscillate intrinsically. The frequency of spontaneous oscillations in intracellular neuronal Ca^{2+} levels is known to depend closely upon synaptic density, buffer composition and culture maturity.
- Initially, spontaneously active cultured neurons fire calcium transients frequently, but out of synchrony with each other. Unsynchronized signals within a population, or signals that occur in only a minor subpopulation of cells, are unsuitable for conventional well-recording HTS readers (Figure 2a); neither snapshot HCS systems, nor population kinetic readers such as FLIPR, usefully capture this kind of signalling. Kinetics-enabled high-content imaging systems that capture behaviour in multiple regions of interest simultaneously enable this kind of complexity to be assessed [34,35].
- Later in culture, cells begin to form synaptic connections with each other, so that Ca^{2+} oscillations increasingly become synchronized with each other and change in frequency and intensity (Figure 2b). Effects of drugs or siRNAs on this synchronization can be studied and quantified.
- Synaptic events involved in neuronal ionic changes are usually characterized by activation of ion channels permeable to cations, so can also be detected using electrophysiology. Multi-electrode arrays (MEAs) enable the detailed characterization of the extracellular field changes simultaneously at many points across a network of neurons [23,27,36,37]. This enables users to assess not only electrical spiking activity at a single electrode, but how this activity is spatially and temporally related across a network of cells (Figure 1). Oscillatory patterns of cell firing can be correlated across a network, and this measure is extremely sensitive to modulation by neuroactive drugs [54].

cells [23,24] (Box 1). Multi-electrode array (MEA) recorders, from Multichannel Systems or Med64, allow simultaneous recording of extracellular activity from up to 60 electrodes, which can be used to record activity in cultured networks of neurons or field activity in a brain slice. An MEA is a glass chip embedded with 64 electrodes forming an 8×8 array (the four corner positions are used for grounding), over which cells can be cultured or thin brain slices can be laid (Figure 1). Synaptic activity can be monitored by measurement of action potentials or field potentials at each electrode and compared to surrounding electrodes. Cultured preparations are viable for many weeks, allowing long-term study of developing connections and cellular activity. As well as the use of MEAs to study drug effects on spontaneous network activity, electrical stimulation of one region can evoke responses in the surrounding area, enabling MEA-based study of drug effects on evoked activity. By using repetitive stimulation, a simple learning paradigm in neuronal cultures has been proposed [25] and mechanisms involved in the development of synaptic plasticity can be followed [23].

Ion transporters are another important drug target, involving measurement of dynamic kinetic responses, and these have also been subject to the development of new potentially high-throughput instrumentation. The Surface Electrogenic Event Reader developed by Iongate offers the potential to measure the electrical currents generated by substrate movement across ion transporters and is currently being developed into an automated multiwell format.

There are some interesting developments in the use of other biophysical readouts as kinetic detection techniques for intact cells. MDS Sciex CellKey measures cellular dielectric spectroscopy in 96-well plates, a readout of transcellular and extracellular impedance across live cell cultures. The kinetics of orphan G-protein-coupled receptor (GPCR) ligand responses in this system can enable the determination of which G protein a receptor couples to, without any prior signalling pathway knowledge. ACEA's RT–CES (www.aceabio.com) is a medium throughput system that also uses impedance as its readout; it records from microelectronic cell sensor arrays integrated into the bottom of wells [26]. The sensors provide a continuous readout reflecting the biological status of cells in each well, so biological changes, such as cellular differentiation, division, or cytotoxicity, can be quantified dynamically. The Corning Epic reader uses microplates containing resonant waveguide grating sensors that reflect a specific wavelength as a function of the combined indices of refraction of all materials within 200 nm of the sensor surface, which can include biomolecules. Addition of a binding ligand results in a change in the refractive index of cells and this is detected as a pM shift in the wavelength of reflection, which can enable users to generate apparent K_d values without labelling of the ligand. These biophysical technologies are currently only at an early stage of implementation within drug discovery, but represent interesting additional tools for studying cell behaviour without manipulation.

Population-based kinetic fluorescence measurements have been available since the mid 1990s on popular instruments such as Molecular Devices FLIPR [5,27], now in its fourth generation as FLIPR Tetra, and on more recent competitors such as the Hamamatsu FDSS6000. These widely implemented instruments are important in front-line drug discovery, although they lack the single-cell resolution necessary for more-detailed investigation of

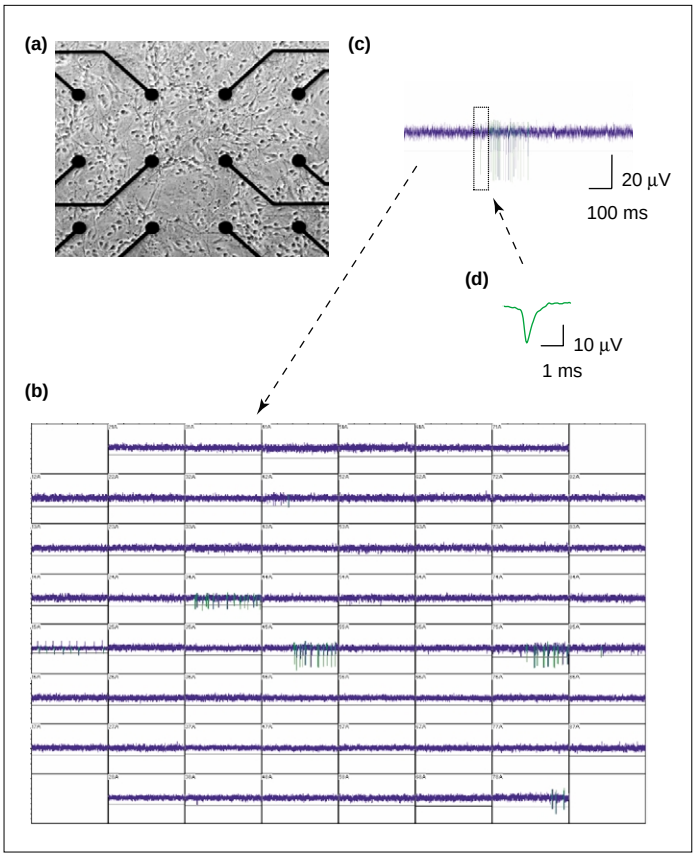

FIGURE 1

Illustration of multi-electrode array (MEA) data. (a) Embryonic neurons are cultured onto glass chips containing an 8x8 array of titanium nitride electrodes. After several days, cells sprout dendrites and axons and form an active network shown as a bright-field image. **(b)** Extracellular activity is simultaneously recorded on 60 electrodes (the four corner positions are used for grounding) and can be analyzed either individually to monitor bursting activity or in relation to neighbouring electrodes as a measure of connectivity. **(c)** A close-up of a single electrode activity with spiking visible as downward deflections from the baseline. **(d)** A single action potential recorded from that electrode, from which amplitude and kinetics can be quantified.

pathway mechanisms. For detailed temporal studies, several fluorescence imaging HCS systems now incorporate kinetic measurements at 1Hz at least, and many of these also integrate on-line liquid handling [3]. These include GE INCell 3000 ([19,28]; www.amershambiosciences.com), BD Pathway HT (www.bdbiosciences.com), Cellomics KineticScan ([29]; www.cellomics.com) and EvoTec Opera ([30]; www.evotec-technologies.com).

High information content assays rely primarily upon fluorescence intensity (FLINT) to determine changes in a detection reagent (Figure 2). New imaging instrumentation [e.g. BlueShift Biotechnologies' IsoCyte (www.blueshiftbiotech.com)] will broaden the range of detection approaches into fluorescence polarization (FPol) and fluorescence lifetime (FLIM) determinations using anisotropy. FPol assesses the rotation of biomolecules by measuring the degree of polarization of emitted light from a molecule after excitation with polarized light. FPol is widely used in well-based high-throughput assays, for example for enzyme inhibition [8], and the IsoCyte cytometer approach, by generating high-resolution two-dimensional fluorescence emission output, should allow its use with single cell or subcellular resolution. Proteins display

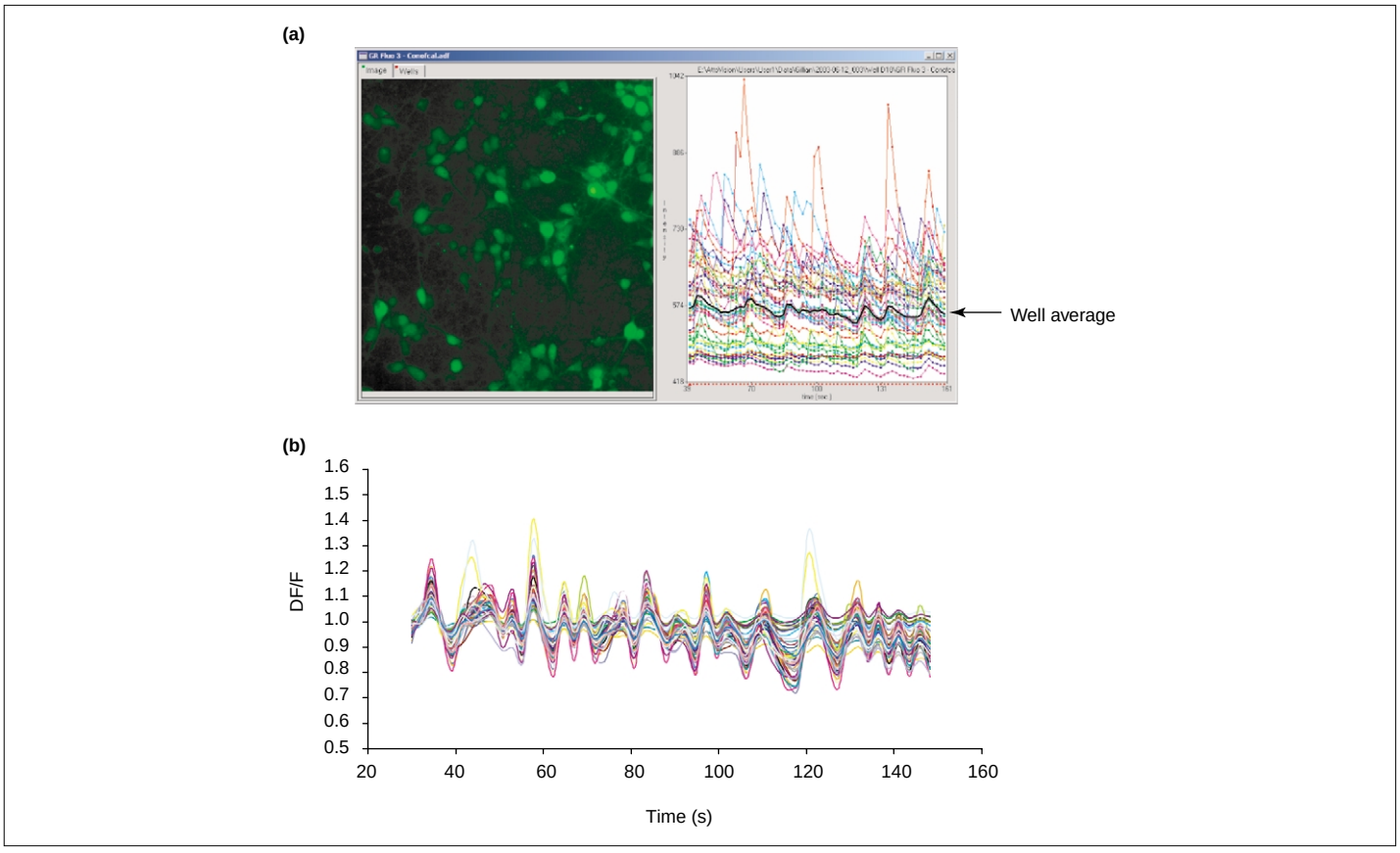

FIGURE 2

Neuronal Ca^{2+} oscillation data acquired using BD Pathway HT. (a) Fluo3-loaded embryonic cortical neurons plated onto 96-well microtitre plates display spontaneous, unsynchronized calcium spiking activity at early stages in culture (<7 days *in vitro*). If the activities of all cells are averaged, there is very little diversion from baseline (black line), as would be detected by a conventional high-throughput Ca^{2+} detection device such as FLIPR. The ability of kinetic HCS detection systems such as BD Pathway HT to identify regions of interest based on their intrinsic fluorescence, and track changes in these regions separately, enables the ready delineation of individual cellular activity and characterization of spontaneous or evoked kinetic responses. **(b)** Later in culture neuronal cells will become networked and display smaller, synchronized oscillations; the transition from unsynchronized to synchronized activity can be characterized and the effects of compounds on this transition studied.

altered FPol as a result of changes in conformation, fluorescence-based energy transfer (FRET), or subcellular location. One advantage of kinetic imaging by FPol, or closely related anisotropy approaches, could be to improve signal to noise ratios – anisotropy is less affected by quenching, auto-fluorescence and other interfering factors than FLINT assays.

Another detection option that is becoming available on HCS imaging systems, such as IsoCyte and EvoTec Opera, is FLIM [30], which is based on the average time a fluorophore remains in the excited state after excitation [31]. This is significantly affected by local environmental factors, including pH and viscosity, but is independent of fluorophore concentration, so it can be used to derive information on the biophysical environment of the labelled protein. For example, this technique has recently been used to evaluate a GFP-tagged protein within natural killer cells, to differentiate the expression of the labelled protein specifically at the immune synapse from expression in the unconjugated cell membrane [32]. This technique is already available on some HTS and HCS readers, and it will be interesting to see whether this technique is sufficiently robust to provide high-quality information within a HCS environment [8,30]. Frequency, rather than time, domain-based FLIM measurements can increase the data-acquisition rate to enable more-rapid kinetic acquisition. Such approaches could be used in cellular assays to deduce information about a molecule's binding status and local environment – small, freely rotating molecules with long FLIM have low anisotropy (i.e. very little perpendicular emission), whereas larger or bound molecules, especially if they have high FLIM, show higher anisotropy. FLIM can also be used for recording rapid changes in levels of a signalling molecule such as Ca^{2+}, with the advantage over FLINT measurements of being insensitive to dye concentration and photobleaching [33].

Why measure complex kinetic biological responses?

In a typical kinetic-based fluorescence screening assay, the scientist adds a stimulator of signalling to a mixed population of cells, and dynamically tracks the changes in each cell simultaneously using a kinetics-enabled HCS reader (Box 1); then they can use either fluorescence proteins or subtype-selective immunocytochemistry to identify the different cell types and assess whether they respond differentially [6,29,34,35]. So, for example, in a culture of cells in which multiple cell types (e.g. progenitors, neurons and glia) co-exist, compounds that have a discriminating effect on a single population can be identified using kinetic imaging, in preference over compounds that lack cell-type selectivity and so are

likely to have off-target liability [6]. The high-resolution data generated requires sophisticated data analysis and storage solutions in order for this approach to be routinely applied within drug discovery. Appropriate data analysis solutions for large kinetic datasets are available from other research fields; currently their application to kinetic cellular screening is largely implemented in-house, and improved commercial solutions are required [34]. However, by automating kinetic high-content readers onto robotic systems [35], data generation is easily sufficient for use in selecting functionally validated hits for further chemistry optimization, as well as in combination with siRNA for novel target identification or validation studies. For example, compounds identified from endpoint HCS library screening for their positive effects on neurite outgrowth or synaptogenesis endpoints, can be confirmed to have the desired functional outcome of increasing neuronal communication by their kinetic evaluation in a network Ca^{2+} oscillation study (Figure 2), as described in Box 1.

Kinetic electrophysiological detection is critical for modern drug discovery for ion channel targets. Higher-throughput systems are moving electrophysiology higher up the drug discovery cascade, and MEA-type assays enable their deployment for understanding how compounds affect interactions between cells rather than just individual cells in isolation. Many kinetic responses, for instance bursting and oscillatory firing in electrically active cells, are dependent on their connectivity within a network. MEAs enable the recording of electrical activity within a network of cultured cells (Box 1). Changes in cellular activity can be measured over the course of minutes to days, as activity can be compared from one array over many days in culture. In addition, drug effects on stimulated responses can be measured by stimulating individual electrodes. Several types of excitable cells can be cultured onto MEAs [36,37]; a 96-well version specifically focused on cardiac myocytes can be used as a high-throughput assay for cardiovascular activity [27].

New biosensors

Whereas calcium, membrane potential and transcription factors are among the most-studied kinetic signals within cells, many other intracellular signalling molecules can also now be studied dynamically using fluorescent biosensors (Figure 3; Table 1). There are several reported biosensor approaches for cAMP, one being a PKA-based FRET assay in which the α and β subunits of protein kinase A (PKA) are fused to yellow and cyan fluorescent proteins, respectively [38]. In another recent development, commercialized as ACT:One (www.bdbiosciences.com), a rat olfactory cyclic nucleotide gated (CNG) channel has been mutated to enhance its cAMP-binding affinity and reduce the cGMP-binding affinity, to build a biosensor. Cell lines expressing this mutated channel display selective responses for cAMP that can be detected kinetically through Ca^{2+}- or membrane potential-responsive dyes on a plate reader or imaging system. A positional biosensor approach for cAMP has been reported by Cellomics, now licensed to Cellumen (www.cellumen.com). A GFP-labelled binding domain transfected into cells contains a dominant nuclear localization element along with a cytoplasmic targeting signal. Activation of PKA within cells causes the catalytic PKA subunit to interact with the GFP-labelled binding domain, which masks the nuclear localization element, thereby resulting in redistribution of GFP fluorescence with a cytoplasmic bias.

Evaluation of signalling pathways upstream of calcium release has become much more sophisticated, with several biosensors of the phosphoinositide pathway available [39,40] (Figure 3). In particular, real-time measurements of inositol trisphosphate ($InsP_3$) using a pleckstrin homology (PH) domain of PLCδ1 has empowered our understanding of the control of intracellular calcium release over time [41]. GFP-tagged PH-PLCδ1 (www.amershambiosciences.com) binds with high affinity to phosphatidylinositol 4,5-bisphosphate. Increases in the level of $InsP_3$ compete for pleckstrin domain binding, causing the construct to translocate from the plasma membrane to the cytosol. The translocation of the GFP fluorescence thereby corresponds to a dynamic kinetic measurement of the level of the intracellular signalling molecule $InsP_3$ [39,41], which has revealed oscillations usually closely synchronized with cytosolic changes in Ca^{2+}. A combination of Ca^{2+}- and $InsP_3$- signalling biosensors is now being used to better understand microdomain signalling within synapses and other subcellular structures [39]. Upstream of these signalling molecules, changes in receptor expression patterns can also be tracked. For example, GPCRs that internalize upon sustained stimulation can now be tagged for quantitative imaging by a variety of HCS tools, such as CypHer5A (www.amershambiosciences.com), Transfluor (www.moleculardevices.com), or direct receptor-FP labelling [42–44].

Fluorescent proteins such as GFP have broad emission bands, and are very large molecular tags, raising the concern that they could alter endogenous protein folding or interactions [44]. This has led to the development of a second generation of cell protein biosensor tags that could serve as convenient imaging tools for subcellular detection without disruption of cellular events. Narrower, and flexible, emission bandwidth, although not reduced tag size, is available using a 33 kDa mutant haloalkane dehalogenase, which can be used to generate N- or C-terminal fusions that cells express efficiently. The HaloTag (www.promega.com) forms a rapid and stable covalent bond to cell-permeant ligands, which have been developed containing one of several fluorophores, thus providing a stable means of detecting a protein of interest using a user-selected emission bandwidth. This allows cells to be imaged at multiple wavelengths without requiring changes to the underlying construct, or the fluorophore to be switched to allow, for example, FRET assays or temporal analysis of protein fate.

In another approach, with a smaller molecular tag, a six-amino acid tetracysteine motif sequence can be engineered into a protein of interest and an exogenous ligand added, which becomes fluorescent upon complexation with this motif [8,44–46]. The tetracysteine motif occurs rarely in natural proteins, enabling the specific fluorescent labelling of a protein inside living cells with only moderate background signal [47]. These tetracysteine biarsenical affinity tags (marketed as Lumio; www.invitrogen.com) have been successfully incorporated at either the N- or C-termini of proteins, as well as at exposed surfaces. Advantages of biarsenical fluorophores as molecular tags include: their small size, which makes them less likely to interfere substantially with the function of the protein [44]; their nanomolar dissociation constant for the tetracysteine motif; rapid conversion of the reagent to a fluorescent state upon binding; and rapid binding, which is reversible by addition of ethane dithiol. In one study, fast incorporation of the Lumio tag enabled monitoring of the time-course of protein misfolding [46].

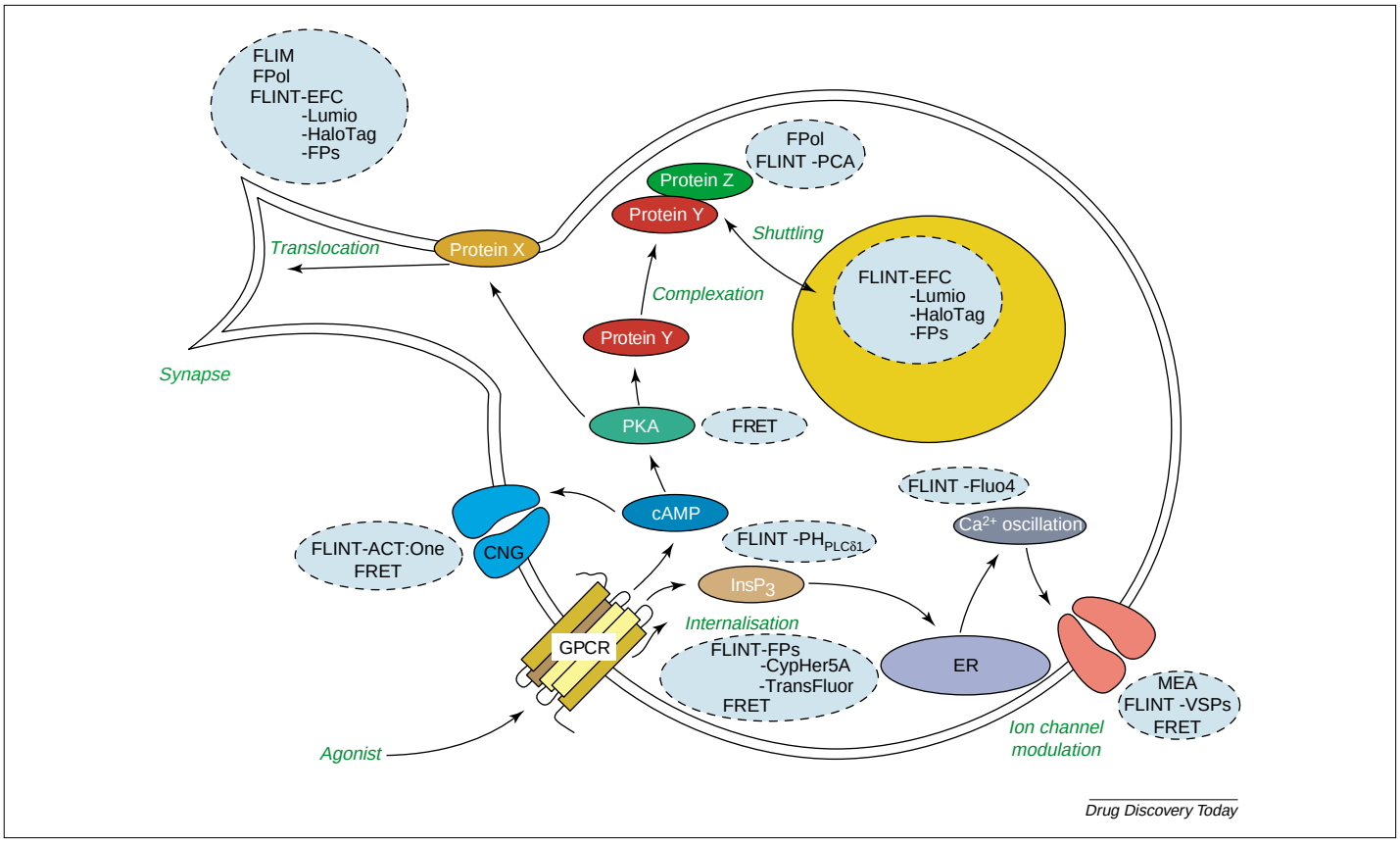

FIGURE 3

Kinetic detection techniques to track events within a hypothetical cell screening cascade. A GPCR activated by its agonist changes configuration, which can be detected by FRET, and could internalize, detectable using the pH-sensitive dye CypHer5A, which will fluoresce more intensely within endosomes. Tagging of the GPCR, or imaging of β-arrestin using the TransFluor technology, can also facilitate subcellular quantification. $G_{q/11}$-coupled GPCRs generate inositol 1,4,5-trisphosphate ($InsP_3$), which can be detected using the pleckstrin homology domain of PLCδ1 ($PH_{PLC\delta1}$). $InsP_3$ generates Ca^{2+} release from the endoplasmic reticulum (ER), often setting up a dynamic Ca^{2+} oscillation, which can be detected using dyes such as fluo4. Change in intracellular cation levels is one way a signal cascade might influence ion channel activity; consequent changes in membrane potential can be detected by electrophysiology techniques, or using fluorescent voltage sensor probes (VSPs). Other GPCRs change the level of cAMP, which can be detected by an ion channel, mutated to be preferentially sensitive to cAMP (ACT:One), using VSPs. cAMP activation of protein kinase A (PKA) can be detected by FRET between tags on different subunits of PKA. If a protein in the signalling cascade (Protein X) translocates to a distinct membrane compartment, this change in its local environment can be detected using FLIM or FPol. Movement within cells can also be detected using enzyme fragment complementation (EFC) when one part of the enzyme is tagged to the cellular compartment of interest, and the other part tagged to Protein X, or by direct tagging of the protein and applying appropriate analytical algorithms. Other proteins might form complexes when activated (Proteins Y and Z); these interactions between proteins can be detected using FPol or protein-fragment complementation assays (PCA). Proteins that translocate into the nucleus or other organelles can be detected using a variety of biosensor tools and analysis algorithms.

Enzyme fragment complementation (EFC) has been used for detecting a variety of cell signalling pathway components [48] and is now being applied and commercialized as PathHunter (www.discoverx.com), as a higher-throughput means of detecting subcellular changes. A 55-amino-acid N-terminal fragment of β-galactosidase (ProLabel) is recombinantly fused to the signalling molecule of interest. ProLabel can complement to another enzyme fragment, the enzyme acceptor, to form active β-galactosidase enzyme. So, in the presence of enzyme acceptor and substrate, the level of the ProLabel-tagged molecule of interest can be determined. It is possible to tag one of the enzyme components to a subcellular region using, for example, a nuclear localization sequence, so that a signal is only generated when the molecule of interest translocates into that compartment. There is no functionally reconstituted enzyme before relocation of the second enzyme component into the nucleus, so the readout for protein relocation is a simple measure of total enzyme activity by measuring conversion of a fluorogenic (or luminescent) enzyme product. 1536-format rapid, automation-friendly readers can therefore be used for subcellular relocation screening. This innovative approach might, therefore, provide an avenue for converting kinetic HCS assays into easier, genuinely high-throughput screens.

EFC requires cell permeabilization for the substrate to enter cells, whereas a related technique involving protein-fragment complementation assays (PCA; www.odysseythera.com) enables truly kinetic imaging of protein interactions. With PCA, two proteins are expressed, linked in-frame to complementary fragments of FPs. Physical association of the two proteins enables refolding of these fragments into an active biosensor, which then generates a quantifiable fluorescence signal. Each of these complementation approaches has been used in an endpoint manner to assay the NF-κB pathway, by monitoring cellular levels of IκB, or interaction of p65 with IκB [49,50]. The flexible nature of PCA technology means that it should prove suitable for live cell-based determination of location

TABLE 1

Some commercially available biosensor tools

Biosensor	Readout	Suitable readers	Additional information
Translocation tools			
Transfluor	FLINT	2–4	www.moleculardevices.com
CypHer5A	FLINT	2–4	www.amershambiosciences.com
PathHunter (EFC)	FLINT or Luminescence	5	www.discoverx.com
PCA	FLINT	2–4	www.odysseythera.com
Ion detection			
Ca^{2+} dyes e.g. Fluo-4, Fluo-LoJo	FLINT or FLIM	1,3,4	www.invitrogen.com www.teflabs.com
VSPs	FLINT, FRET or FPol	1,3,4	www.invitrogen.com www.moleculardevices.com
Multiple use tools			
FPs	FLINT, FPol, FLIM or FRET	2–5	www.invitrogen.com www.bioimage.com www.bd.com
HaloTag	FLINT or FRET	2–4	www.promega.com
Lumio	FLINT	2–4	www.invitrogen.com
Positional biosensors	FLINT	2–4	www.cellumen.com

While not intended to be a comprehensive list, this illustrates some of the available biosensors, with some of the detection formats in which they have published to work (it is likely that many of the biosensors will also work in alternative readouts, such as FLIM and FPol, in which they have not yet been rigorously evaluated), and some suitable readers. 1 = kinetic population readers (e.g. Molecular Devices FLIPR, Hamamatsu HDSS 6000); 2 = endpoint HCS readers (e.g. Cellomics ArrayScan VTi, Molecular Devices Discovery-1, GE IN Cell 1000); 3 = conventional fluorescence microscopes; 4 = kinetic HCS readers (e.g. EvoTec Opera, GE IN Cell 3000, BD Pathway HT); 5 = non-kinetic fluorescence plate readers (e.g. GE LeadSeeker, PerkinElmer ViewLux, Tecan Ultra).

and interactions of many proteins to better delineate signalling cascades.

The high sensitivity of neuronal cultures on MEAs to modification by pharmacological agents has led to the proposal that these systems can be used as biosensor readouts [27]. By measuring and categorizing the effects of several known neuroactive compounds, the activity pattern produced by novel compounds can be compared and classified as similar or dissimilar to previous classes of compound. Taking this one step further, researchers are now developing methods to culture neurons onto silicon-based arrays, controlling how and where cells form projections and connect to each other [51]. These techniques should allow the development of higher information content assays for drug effects on synapse formation, assembly of networks and neuronal growth.

The state-of-the-art

Advances in tools, assay design and instrumentation now enable more-widespread implementation of temporally-based, single cell-level assays within a drug discovery environment. Electrophysiological and FLINT assays still predominate, however a range of other fluorescence techniques are beginning to diversify the HCS approaches available to the drug discovery scientist. Most aspects of a cell signalling screening cascade can now be assayed in real-time using commercially available tools (Figure 3). These new assays and technologies should empower a re-evaluation of the relative use of kinetic, compared with snapshot, cell screening assays, so that subtle or discrete effects of compounds are detected. Kinetic data can now be used up-front within drug discovery projects for interpreting and prioritizing compounds, and for better understanding the signalling pathways that underlie key cellular responses.

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References

1 Taylor, D.L. *et al.* (2001) Real-time molecular and cellular analysis: the new frontier of drug discovery. *Curr. Opin. Biotechnol.* 12, 75–81
2 Ramm, P. (2005) Image-based screening: a technology in transition. *Curr. Opin. Biotechnol.* 16, 41–48
3 Zemanová, L. *et al.* (2003) Confocal optics microscopy for biochemical and cellular high-throughput screening. *Drug Discov. Today* 8, 1085–1093
4 Allison, K. (2003) The first automated high content screening system. *J. Assoc. Lab. Automation* 8, 27–29
5 Monteith, G.R. *et al.* (2005) Techniques: high-throughput measurement of intracellular Ca^{2+} - back to basics. *Trends Pharmacol. Sci.* 26, 218–223
6 Simpson, P.B. (2005) Getting a handle on neuronal behaviour in culture. *Eur. Pharm. Rev.* 10, 56–63
7 Simpson, P.B. *et al.* (2001) Retinoic acid-evoked differentiation of neuroblastoma cells predominates over growth factor stimulation: an automated image capture and quantitation approach to neuritogenesis. *Anal. Biochem.* 298, 163–169
8 Gribbon, P. and Sewing, A. (2003) Fluorescence readouts in HTS: no gain without pain? *Drug Discov. Today* 8, 1035–1043
9 Richards, G.R. *et al.* (2004) Quantitative assays of chemotaxis and chemokinesis for human neural cells. *Assay Drug Dev. Technol.* 2, 465–472
10 Ding, G.J.F. *et al.* (1998) Characterization and quantitation of NF-κB nuclear translocation induced by Interleukin-1 and Tumor Necrosis Factor-α. Development and use of a high capacity fluorescence cytometric system. *J. Biol. Chem.* 273, 28897–28905
11 Nelson, G. *et al.* (2002) Multi-parameter analysis of the kinetics of NF-κB signalling and transcription in single living cells. *J. Cell Sci.* 115, 1137–1148
12 Nelson, D.E. *et al.* (2004) Oscillations in NF-κB signaling control the dynamics of gene expression. *Science* 306, 704–708
13 Ihekwaba, A.E.C. *et al.* (2004) Sensitivity analysis of parameters controlling oscillatory signalling in the NF-κB pathway: the roles of IKK and IKBα. *Syst. Biol.* 1, 93–103

14 Dolmetsch, R.E. *et al.* (1997) Differential activation of transcription factors induced by calcium response amplitude and duration. *Nature* 386, 855–858
15 Nelson, D.E. *et al.* (2005) Response to Comment on "Oscillations in NF-κB Signaling Control the Dynamics of Gene Expression". *Science* 308, 52
16 Lahav, G. *et al.* (2004) Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nat. Genet.* 36, 147–150
17 Almholt, D.L.C. *et al.* (2004) Nuclear export inhibitors and kinase inhibitors identify using a MAPK-activated protein kinase 2 Redistribution® screen. *Assay Drug Dev. Technol.* 2, 7–20
18 Knauer, S.K. *et al.* (2005) Translocation biosensors to study signal-specific nucleo-cytoplasmic transport, protease activity and protein-protein interactions. *Traffic* 6, 594–606
19 Lundholt, B.K. *et al.* (2005) Identification of Akt pathway inhibitors using redistribution screening on the FLIPR and the IN Cell 3000 Analyzer. *J. Biomol. Screen.* 10, 20–29
20 Watson, P. *et al.* (2005) Intracellular trafficking pathways and drug delivery: fluorescence imaging of living and fixed cells. *Adv. Drug Deliv. Rev.* 57, 43–61
21 Schroeder, K. *et al.* (2003) Ionworks HT: a new high-throughput electrophysiology measurement platform. *J. Biomol. Screen.* 8, 50–64
22 Kiss, L. *et al.* (2003) High-throughput ion-channel pharmacology: planar-array-based voltage clamp. *Assay Drug Dev. Technol.* 1, 127–135
23 Arnold, F.J. *et al.* (2005) Microelectrode array recordings of cultured hippocampal networks reveal a simple model for transcription and protein synthesis-dependent plasticity. *J. Physiol.* 564, 3–19
24 Gramowski, A. *et al.* (2004) Substance identification by quantitative characterization of oscillatory activity in murine spinal cord networks on microelectrode arrays. *Eur. J. Neurosci.* 19, 2815–2825
25 Shahaf, G. and Marom, S. (2001) Learning in networks of cortical neurons. *J. Neurosci.* 21, 8782–8788
26 Solly, K. *et al.* (2004) Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay Drug Dev. Technol.* 2, 363–372
27 Hodder, P. *et al.* (2004) Miniaturization of intracellular calcium functional assays to 1536-well plate format using a fluorometric imaging plate reader. *J. Biomol. Screen.* 9, 417–426
28 Maiolo, J.R. *et al.* (2005) Effects of cargo molecules on the cellular uptake of arginine-rich cell-penetrating peptides. *Biochim. Biophys. Acta* 1712, 161–172
29 Abraham, V.C. *et al.* (2003) High content screening applied to large-scale cell biology. *Trends Biotechnol.* 22, 15–22
30 Eggeling, C. *et al.* (2003) Highly sensitive fluorescence detection technology currently available for HTS. *Drug Discov. Today* 8, 632–641
31 Oida, T. *et al.* (1993) Fluorescence lifetime imaging microscopy (flimscopy). Methodology development and application to studies of end of cell fusion in the single cells. *Biophys. J.* 64, 676–685
32 Treanor, B. *et al.* (2005) Imaging fluorescence lifetime heterogeneity applied to GFP-tagged MHC protein at an immunological synapse. *J. Microsc.* 217, 36–43
33 Agronskaia, A.V. *et al.* (2004) Fast fluorescence lifetime imaging of calcium in living cells. *J. Biomed. Opt.* 9, 1230–1237
34 Richards, G.R. *et al.* Measurement and analysis of calcium signalling in heterogeneous cell cultures. *Methods Enzymol. -. Automated Microscopy Screening* (in press)
35 Chan, G.K.Y. *et al.* (2005) High content kinetic assays of neuronal signalling implemented on BD Pathway HT. *Assay Drug Dev. Technol.* 3, 623–636
36 Hescheler, J. *et al.* (2004) Determination of electrical properties of ES cell-derived cardiomyocytes using MEAs. *J. Electrocardiol.* 37(*Suppl.*), 110–116
37 Meyer, T. *et al.* (2004) QT-screen: high-throughput cardiac safety pharmacology by extracellular electrophysiology on primary cardiac myocytes. *Assay Drug Dev. Technol.* 2, 507–514
38 Evellin, S. *et al.* (2004) Measuring dynamic changes in cAMP using fluorescence resonance energy transfer. *Methods Mol. Biol.* 284, 259–270
39 Nahorski, S.R. *et al.* (2003) Visualising phosphoinositide signalling in single neurons gets a green light. *Trends Neurosci.* 26, 444–452
40 Bartlett, P.J. *et al.* (2005) Single cell analysis and temporal profiling of agonist-mediated inositol 1,4,5-trisphosphate, Ca^{2+}, diacylglycerol, and protein kinase C signalling using fluorescent biosensors. *J. Biol. Chem.* 280, 21837–21846
41 Nash, M.S. *et al.* (2001) Receptor-specific messenger oscillations. *Nature* 413, 381–382
42 Conway, B.R. and Demarest, K.T. (2002) Biosensors to study GPCR function: applications for high-content screening. *Receptors Channels* 8, 331–341
43 Milligan, G. (2003) High-content assays for ligand regulation of G-protein-coupled receptors. *Drug Discov. Today* 8, 579–585
44 Hoffmann, C. *et al.* (2005) A FlAsH-based FRET approach to determine G protein–coupled receptor activation in living cells. *Nat Methods* 2, 171–176
45 Griffin, B.A. *et al.* (1998) Specific covalent labeling of recombinant protein molecules inside live cells. *Science* 281, 269–272
46 Ignatova, Z. and Gierasch, L.M. (2004) Monitoring protein stability and aggregation *in vivo* by real-time fluorescent labeling. *Proc. Natl. Acad. Sci. U. S. A.* 101, 523–528
47 Cavagnero, S. and Jungbauer, L.M. (2005) Painting protein misfolding in the cell in real time with an atomic-scale brush. *Trends Biotechnol.* 23, 157–162
48 Eglen, R.M. (2002) Enzyme Fragment Complementation: a flexible high-throughput screening assay technology. *Assay Drug Dev. Technol.* 1, 97–104
49 Zhao, X. *et al.* (2003) Homogeneous assays for cellular protein degradation using β-Galactosidase complementation: NF-κB /IκB pathway signaling. *Assay Drug Dev. Technol.* 1, 823–833
50 Yu, H. *et al.* (2003) Measuring drug action in the cellular context using protein-fragment complementation assays. *Assay Drug Dev. Technol.* 1, 811–822
51 James, C.D. *et al.* (2004) Extracellular recordings from patterned neuronal networks using planar microelectrode arrays. *IEEE Trans. Biomed. Eng.* 51, 1640–1648
52 Simpson, P.B. *et al.* (1995) Neuronal calcium stores: activation and function. *Trends Neurosci.* 18, 299–306
53 Simpson, P.B. (1998) Ca^{2+} waves as a form of glial excitability. In *Integrative aspects of calcium signalling* (Verkratsky, A. and Toescu, E.C eds), pp. 359–379, Plenum Press
54 Keefer, E.W. *et al.* (2001) NMDA receptor-dependent periodic oscillations in cultured spinal cord networks. *J. Neurophysiol.* 86, 3030–3042