Fluorescence readouts in HTS: no gain without pain?

Philip Gribbon and Andreas Sewing

Fluorescence-based detection technologies are frequently applied in biological testing, due to their unique advantages in setting up homogeneous, sensitive assays in miniaturized formats. However, the wide application of these readouts has highlighted challenges in reagent design and problems with interference from biological reagents and compounds. Here, we summarize the current application of fluorescence-based detection methodologies, focusing on the problems faced by assay developers and on solutions to reduce false positive and negative results in fluorescence-based HTS.

Philip Gribbon
Andreas Sewing
Lead Discovery Technologies
Pfizer Global Research
and Development
Ramsgate Road
Sandwich
UK CT13 9NJ
e-mails: philip_gribbon@sandwich.pfizer.com; andreas_sewing@sandwich.pfizer.com

▼ Due to the complexity of biological systems and our subsequently limited ability to make predictions about the biological activity of new chemical entities, HTS is at the centre of today's drug discovery. Within early discovery, the ability to design and synthesize ever more compounds has driven the development of new assay technologies and the use of high-density assay plates. The reduction of assay volumes and the concomitant decrease in the use of biological reagents are perceived as key factors to contain cost at this stage of the drug discovery process; however, assay miniaturization is dependent on multiple factors, including the need for homogeneous assays, the ability to handle ultra-low volumes of liquids and the demand for sensitive readouts. Historically, investigators relied on radiometric and absorbance-based techniques and, although robust assays have been designed, there are clear limitations, with respect to miniaturization. For absorption, Beer-Lambert considerations constrain assay volumes, as the absorption at a given concentration depends on the optical path length. In radiometric assays, reducing radioisotopes almost invariably extends detection times. As the radioactive decay is irreversible and the biological system limits the concentration of radioactive tracer, miniaturization of assays with radiometric detection has proven challenging, even after the introduction of sensitive imaging-based detection methods. By contrast, the inherent efficiency of fluorescence readouts overcomes these boundaries, as a consequence of the multiple (typically many thousands) of excitation (ex.) and emission (em.) cycles that each fluorophore molecule undergoes before photobleaching. Intrinsic scalability enables seamless miniaturization with fluorescence-based methods, right down to the single-molecule level. With the minimum of changes, fluorescence assays can deliver the same results, whether applied in 100 or 5 μl [1,2] because readouts are, theoretically, volume-independent, although, in practice, the geometry of microtitre plates and detection equipment, such as imaging systems, restricts miniaturization for macroscopic measurements. In many cases, however, miniaturization using fluorescence readouts is only restricted by liquid handling, absorption or evaporation effects.

The boundary between definitions of 'fluorescent' and 'non-fluorescent' methods is fluid. In many respects, fluorescence detection resembles 'light'-based technologies, such as scintillation proximity analysis (SPA), where immobilized scintillant transduces the radioactive decays into detectable light. A full appreciation of these similarities is beyond the scope of this review and we will focus on methods involving a fluorescence excitation and emission.

Fundamental principles of fluorescence readouts applied to HTS

For homogenous and cell-based assays, multiple variants of fluorescence-based methods are applicable, including: FLINT (fluorescence intensity or prompt fluorescence), FP (fluorescence polarization/anisotropy), FRET (fluorescence

1359-6446/03/$ – see front matter PII: S1359-6446(03)02895-2

resonance energy transfer), fluorescence lifetime (FL), TRF (time-resolved fluorescence) and single-molecule detection methods, such as FIDA (1D and 2D fluorescence intensity distribution analysis). These methods each exploit different aspects of fluorescence and present distinct advantages and disadvantages, particularly in terms of their sensitivity, robustness and susceptibility to artifacts. The underlying physical principles and applications of fluorescence in bioassays have been expounded in the primary literature and recent reviews [3,4], but there is scope for a brief discussion.

In FLINT, steady state fluorescence intensity is directly related to changes in fluorophore concentrations. Typical applications include direct enzyme assays measuring fluoro-substrate turnover. FLINT can be affected by compound-quenching, auto-fluorescence effects and inner-filter phenomena, particularly at high fluorophore concentrations. By contrast, FP measures the rotation of single biomolecules or their complexes (e.g. receptor–fluoro-ligand mixture) by interrogating the relative polarization state of emitted fluorescence. The sensitivity of rotation rates to molecular hydrodynamic dimensions forms the basis of the signal. Kinetic and equilibrium readouts are accessible but accurate quantification of equilibrium states, such as fluoro-antigen–antibody or fluoro-ligand–receptor systems require a significant mass change upon binding (ideally more than tenfold). Similarly, the reporter fluorophore's fluorescence lifetime must be matched to the mass shift under observation, for example, short lifetime fluorophores (<4 ns) are not suitable for describing rotation rates for macromolecules with a molecular weight (MW) >50 kDa. In HTS applications, characteristic rotation rates are represented by the polarization value, or the mathematically related parameter, anisotropy. FP has become a widely applied method in HTS because the polarization signal is volume-independent and, consequently, several high-density, low-volume (<5 μl) FP assays have been reported [5,6].

The interest in radiation-independent energy transfer techniques has recently been re-ignited, following improvements in dyes and detection technology. In FRET, fluorophore pairs with overlapping emission and excitation spectrum (donor and acceptor) exchange energy via a dipole–dipole vectorial coupling, over a 1–5 nm-length scale. Transfer efficiency falls off at the sixth power of separation distance, making FRET suited for the assay interactions of biomolecules in homogeneous and cell-based formats as the length scales correspond with macromolecule dimensions (antibody–antigen interactions, peptide substrates for proteases etc.).

Most applications that have been described so far make use of fluorophores with short lifetimes (10^{-9} s), whereas, time-resolved approaches, such as HTRF (homogeneous time-resolved fluorescence) combine the FRET principle with fluorescence dyes based on the lanthanide chelate, Eu^{3+} cryptate [7]. The long-lifetime lanthanide-based fluorescent probes (>10^{-4} s) enable discrimination between fluorophore and signals from interfering assay components, like biological reagents and compounds. For homogeneous assays, proprietary assay platforms like the Trace™ (CisBio; http://www.cisbiointernational.fr) and LANCE™ (PerkinElmer Lifescience; http://www.perkinelmer.com) have been applied to develop assays for kinases, proteases, cell surface receptors and protein–protein interactions.

A recent addition to HTS formats is fluorescence lifetime-based assays using time-domain measurements. Here, fluorophores are excited by short (picosecond), high-frequency laser pulses and the characteristic lifetime is determined. Whereas methods relying on fluorescence intensity can be influenced by many factors, the lifetime of a fluorophore is an absolute parameter that is determined by the local environment. Although technically challenging, equipment has become available to incorporate lifetime into the HTS arena (Tecan, http://www.tecan.com; Molecular Devices, http://www.moleculardevices.com; Evotec Technologies, http://www.evotec.com). The full benefits of lifetime changes as readouts for biological processes, such as receptor–ligand binding, remain to be established, particularly because the magnitude of changes are difficult to predict and assay development remains an empirical process.

Fluorescence methods that apply single-molecule detection

HTS has recently seen the advent of single-molecule spectroscopy-based readouts as a solution to achieving high sensitivity and information content, along with reduced reaction volumes. The EVOscreen™ platform (Evotec Technologies) exploits confocal optics to give a highly focused sampling region, of the order of size of a bacterium (10^{-15} l). With few molecules instantaneously under observation (typically 1–10), the de-convoluted 1D and 2D photon count distributions give highly informative screening data at a molecular level. Outputs include the absolute concentrations of multiple assay components, 'molecular brightness' (a function of quantum yield) and hydrodynamic parameters. An example of single-molecule multi-parameter approach is shown in Figure 1. In the guise of HTS readout, the method is currently applied in three formats: fluorescence correlation spectroscopy (FCS), where mass changes associated with binding are revealed by changes in translational diffusion times. FCS is a rarely used method in HTS, because tenfold changes in mass are a pre-requisite for manageable data acquisition times. A

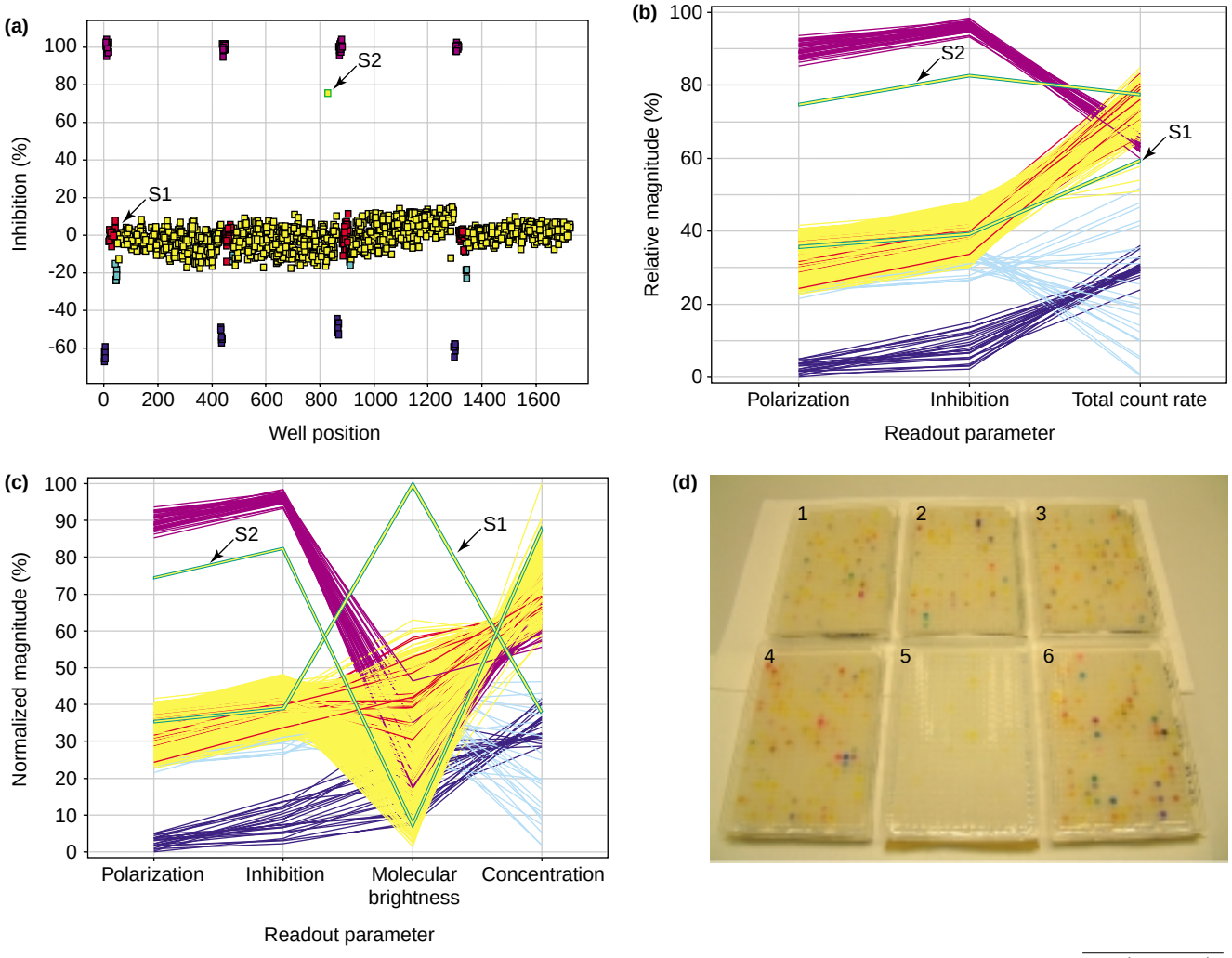

Figure 1. High content screening in homogenous assays. Example of multiparameter analysis applied to a homogenous FP assay with conventional (count rate) and single-molecule (Evotec FCS++) methodologies. Results from one Evotec 2080 well plate (1726 sample wells) for a kinase inhibitor screen. Yellow = compound wells; red = high enzyme activity; light blue = fluoro-ligand only control; dark blue = dye well control; purple = 100% enzyme inhibition. **(a)** Inhibition versus well position across the plate. Two sample wells have been highlighted in grey: S1 – compound well showing no inhibition; S2 - compound well containing a putative active. **(b)** Detecting compound-related artifacts in bulk FP measurements. Shown is a trellis plot of polarization, inhibition and fluorescence count rate. Note that the count rate results for both S1 and S2 fall within the general population distribution, for this property, and would not be detected using standard threshold-based discrimination. **(c)** The same data as in parts (a) and (b), trellis plotted, with the inclusion of the corresponding single-molecule parameters: molecular concentration and molecular brightness. With four parameters now available for comparison, it can be seen that the putative inactive compound, S1, has a high molecular brightness and a low molecular concentration, which suggests a compound-related artifact. Similarly, the putative active compound S2 has a relatively high molecular concentration combined with a low molecular brightness – this signature is indicative of the influence of interference from a weakly auto-fluorescent compound, suggesting S2 is a false positive. **(d)** Images of compound mother plates. Plates 1–5 are the selected putative actives from a primary screen using conventional fluorescence polarization, with no filtering of problematic compounds. It can be seen that there is a high incidence of colored and auto-fluorescent compounds. Plate 6 is a random plate selected from the originally screened file for comparison.

more popular manifestation has been fluorescence intensity distribution analysis (FIDA), which enables a direct measure of ligand-binding state in a homogenous format, and has been applied to receptor–ligand assays [1,8]. 2D FIDA polarization (2D FIDA) is the single-molecule analogue of the standard FP approach and uses the 2D photon count distribution in each polarization plane [9]. It is a particularly powerful methodology in indirect enzyme-based assays for studying kinases (but not restricted to these), where the readout exploits the mass change from antibody recognition of a turned-over substrate [10]. Cross-correlation-based methods, using 2-colour labeling have

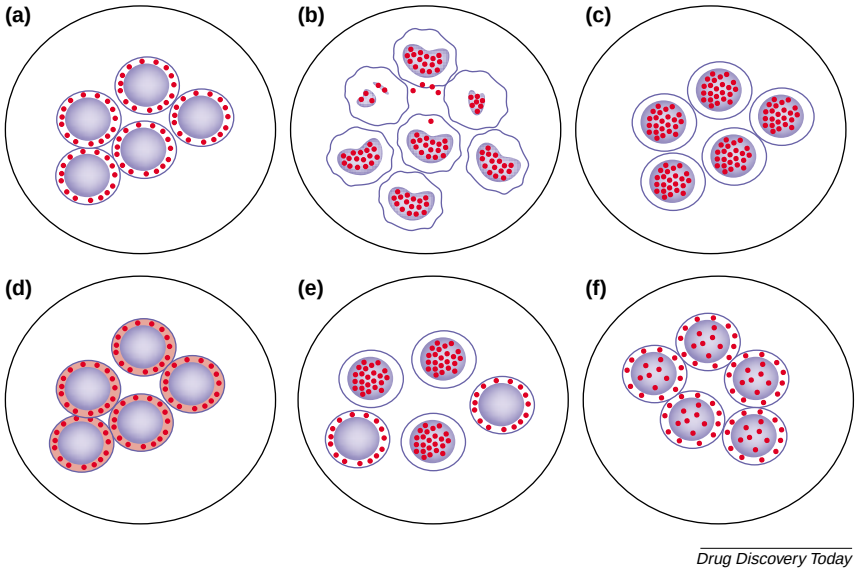

Figure 2. High content screening in cell based assays. The example illustrates possible readouts of a translocation assay measuring the redistribution of a fluorescently labeled molecule from cytoplasm to the nucleus**. (a)** no compound effect; **(b)** translocation but indication of cytotoxicity (i.e. change of morphology, nuclear disintegration); **(c)** complete translocation; **(d)** compound interfering with readout (i.e. auto-fluorescence); **(e)** response in sub-population of cells and; **(f)** partial but homogeneous response.

also been reported [11] but have found limited employment, owing to the significantly lengthy data acquisition times and the linear increase of issues surrounding efficient fluoro-labeling when two probes are required.

Data reduction for single-molecule screening is fundamentally different from the conventional methods. A molecular model of the assay behavior is defined to account for the underlying biological events and, under suitable conditions, the description can be extended to identify, and in some cases correct for, intrinsic compound and reagent effects, such as auto-fluorescence or mis-dispensing by incorporating an additional fluoro-component in the model used to fit the data. However, as with all data-fitting, choices must be appropriate because the modeling of an auto-fluorescent compound where none is present can increase the overall uncertainty in fit results and can subsequently reduce data quality.

Cell-based, functional assays using fluorescent probes

Fluorescent methods for cell-based assays have initially been developed using small, highly-fluorescent, organic molecules, monitoring ion concentrations, membrane potential and as intracellular substrates for reporter genes. These assays are now routinely developed and applied across the pharmaceutical industry and have been successfully automated using various platforms [12–15]. For several of these applications, dyes are 'loaded' as membrane-permeable acetoxymethyl esters and are caged within the cell after cleavage by intracellular esterases. Examples of this approach include the calcium-sensitive probes Fluo3 and Fluo4, which have an altered quantum yield upon calcium-binding and are used to measure calcium fluxes after activation of Gq-coupled seven transmembrane receptors [13,14,16,17]. 'Voltage'-sensing dyes [DiBAC series (http://www.dibac.com) and related dyes] can be applied to measure the membrane potential in response to modulation of ion channel function. [18,19]. Scientists at Aurora Biosciences (now part of VERTEX Pharmaceuticals; http://www.vpharm.com) have developed FRET assays to monitor the activity of ion channels and β-lactamase reporter genes. Both applications use fluorophore pairs with overlapping emission/excitation spectra to set up intracellular FRET assays, providing a more robust readout, which is less sensitive to variations in dye loading and cell number [20,21].

In an attempt to enhance the specificity and spatial resolution of cell-based assays, macromolecules like proteins, RNA and DNA have been labeled with fluorophores and introduced into living cells by various techniques, to follow movements within signaling cascades and between organelles. These techniques were not generally applicable to large-scale screening, but the discovery of genetically encoded fluorescent probes, such as green fluorescent protein (GFP), and their optimization through genetic engineering has created a set of tools that has enabled a new type of cell-based assay with high temporal and spatial resolution, based on fluorescence microscopy, which is applicable to a multitude of targets (reviewed in [22,23]). These assays, based on subcellular imaging, provide a multitude of information from single wells, thus, enabling decisions to be made on the nature of the observed effects [i.e. mode of action, specificity and cytotoxicity of compounds (Figure 2)] and are, as a whole, summarized as 'High Content Screening' (see Box 1). To enhance resolution and image quality, new imaging platforms, such as the OPERA™ cell reader (Evotec Technologies) combine features of confocal microscopy with single-molecule detection technologies.

Although functional assays using living cells present several different problems to assay development and

Box 1. High content screening

The term 'high content screening' summarizes cellular assays based on sub-cellular imaging and automated image analysis (Figure 3). Initially, applying immunofluorescence methods on fixed cells, new developments focus on biochemical sensors to follow signaling events in real time (reviewed in [39,40]). Multiple assay parameters can be analyzed in parallel (cell shape, cell viability, translocation events etc.), giving more information than traditional cell-based assay technologies. Biosensors can be derived by labeling macromolecules with small fluorescence dyes, or by applying genetic engineering to construct chimeras of cellular proteins fused to naturally fluorescent proteins, such as GFP, to establish a genetically encoded sensor. One example is the fusion of β-arrestin with GFP, which established a generic procedure to measure activation of a number of different GPCRs through recruitment of GFP-arrestin to the plasma membrane ([41], Transfluor™, Norak Biosciences; http://www.norakbio.com).

To allow sufficient throughput, assays are read on specially adapted imaging systems, loosely based around inverted fluorescence microscopes with signals captured using photomultiplier tubes (PMT) or cooled CCD cameras. Due to the challenges associated with rapid automatic focusing, the quantity of image-derived data and the complexity of algorithms for accurate image analysis, assays were initially applied to second line testing and compound characterization ([42], for example, Cellomics ArrayScan® (http://www.cellomics.com); however, a new generation of imaging systems are specifically targeted to HTS ([43], Acumen Explorer™, http://www.acumenbioscience.com; Amersham InCell, http://www.amersham.co.uk; Evotec Opera™, http://www.evotec.com). Figure I shows the application of this technology to study the binding of endothelin to its cognate receptor and the translocation of NFκB after treatment of cells with IL1α).

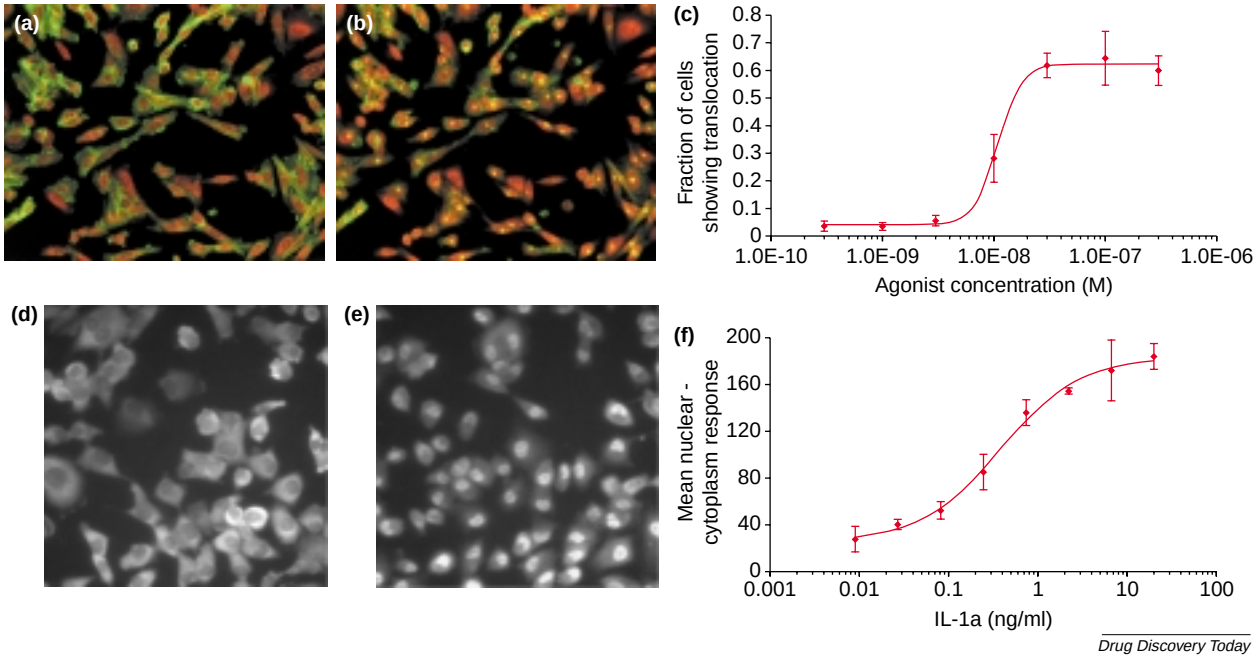

Figure I. Application of sub-cellular imaging. **(a–c)** Receptor internalization assay. The internalization of G-Protein coupled receptors after agonist binding can be quantified to set up functional, cell-based assays for HTS and secondary profiling. The example illustrates the internalization of endothelin receptor (labeled with an auto-fluorescent homologue of GFP) after binding of endothelin in live cells. Image **(a)** is taken prior to the addition of endothelin; image **(b)** shows the internalization of the receptor ca. 180 min after ligand addition. **(c)** Image analysis can be applied to quantify internalization and dose response curves can be derived. Images were analyzed using the Evotec OPERA™ cell reader (unpublished images courtesy of Evotec OAI). **(d–f)** Nuclear translocation assay. The figure shows the activation and subsequent nuclear translocation of NFκB, in response to IL-1α in fixed A549 cells (Cellomics NFκB HitKit); **(d)** untreated cells; **(e)** 30 min treatment with 20 ng x ml^{-1} IL1α; **(f)** dose-response curve for Il-1α-mediated translocation. Images were taken on a Cellomics Arrayscan and analyzed applying the Cellomics nuclear translocation algorithm. The response on the Y-axis is derived by substracting the mean fluorescence intensity in the cytoplasm from the mean fluorescence intensity in the nucleus (unpublished images courtesy of Paul Hayter, Pfizer).

screening in comparison with biochemical assays using isolated targets, the fluorescence readout itself shares the same challenges with its biochemical counterpart, namely the accurate prediction of the molecular properties of probes and the interference of compounds and biological reagents, due to auto-fluorescence and light-scattering. As

a result, the same strategy to reduce these readout artifacts can be applied to both assay types.

Problems in the practical application of fluorescent methods

The widespread application of fluorescence-based readouts has highlighted several problems. The most frequently observed effects are interference with the emission signal from the reporter fluorophore, which can lead to false negatives and positives. Interference can be additive, such as auto-fluorescence and light-scattering, or subtractive, such as quenching or absorption. The degree of interference is related to the concentration of reporter fluorophore and the excitation and emission wavelengths, with lower wavelength dyes (e.g. fluorescein and coumarin derivatives) being particularly susceptible [24,25]. The situation is not improved by the fact that the fluorophores in many assays are used in the nanomolar range, whereas screened compounds are typically tested in the micromolar range. Recent studies have suggested that a significant proportion of compound collections show interference in fluorescent HTS assays that is unrelated to any intrinsic biological activity [26].

Strategies for dealing with compound artifacts in HTS follow two general approaches: (i) engineering the assay so that interference is minimized, via the choices of dye wavelengths, concentrations and so on, or (ii) identification and, if appropriate, correction of the artifact's contribution to the signal. In the first approach, longer wavelength (ex. >600 nm), 'red-shifted' dyes are applied to reduce auto-fluorescence from compounds [24,27] and are a first-line defence against a false hit 'overload'. Examples of these dyes include MR121 (ex. 640 nm, em. 670 nm), as well as the family of Cy dyes (Cy3 ex. 550 nm, em. 570 nm; Cy5 ex. 649 nm, em. 670 nm). The application of time-resolved methods can further reduce interference. Long-lifetime lanthanide fluorophores filter the contribution from interfering compounds and assay components, simply by delaying the measurement until the interfering short-lifetime signal has decayed [7]. Another approach uses FLARE to reduce compound interference and is based on the observation that most fluorescence events caused by compounds have a very short lifetime, whereas the lifetime of the fluorophores used for assay development is much longer. First comparative studies have shown FLARE technologies to decrease the relative proportion of false positive compounds by comparison to FP, but the full benefits of this approach have yet to be demonstrated in large-scale HTS, especially because the instrumentation is currently less sensitive in FLARE mode [26].

Bulk fluorescence polarization is a ratiometric technique that appears to be less sensitive to artifacts in general; however, this method has pitfalls, aside from the obvious viscosity and temperature effects. Shifts in fluorophore quantum yield can occur upon binding, which, if uncorrected, will underestimate the apparent concentration of a bound fraction. Although auto-fluorescent compounds in HTS screens should mimic the low polarization of the free fluoro-ligand, insoluble scattering aggregates have large polarizations and exaggerate an apparent bound fraction. Gross changes in polarization as a result of compound effects can normally be discriminated by defining appropriate cut-off criteria and employing multi-parameter analyses; the identification of subtle effects, where significant fractions of scattering aggregate and solution forms of an auto-fluorescent species co-exist, is more challenging (Figure 1).

By exploiting modern fluorescence readers, the measurement and analysis of multiple parameters from assay wells can be used to construct profiles of 'real actives' and provide an information technology solution to compound interference. By comparing the profile of test compounds, the number of 'actives' can be dramatically reduced (Figure 1). These methods are most effectively applied when using single-molecule detection methods, but more basic variants can be applied with bulk measurements. It should be noted, that although problems with compounds can be reduced and monitored, no method is able to distinguish real activity from fluorescence properties of a molecule. If a molecule is both highly fluorescent and active, only a secondary assay, using an alternative readout, can provide a definitive answer.

Factors influencing assay development with fluorescence probes

Appropriate design and production of reagents to match the capabilities of each fluorescent readout is fundamental in achieving a tractable HTS assay. For example, many FP, 2D FIDA and FCS enzyme-inhibition assays involve quantifying mass changes when antibodies recognize enzyme products. The requirement for significant mass changes necessitates the use of small peptidic substrates, rather than holo-proteins; therefore, comparative kinetic parameters (e.g. the Km's of the native and analogue substrates) must be determined. For fluorescence polarization assays, the appropriate design of 'stiff' linkages between the reporter fluorophores and biomolecules is important to reduce possible 'propeller' effects. Labeling DNA with DTAF (diaminotriazinylamino-fluorescein) introduces a stiff six-carbon-ring structure linker between the DNA strand and fluorescein, which prevents spinning of the fluorophore and improves the polarization shift that is observed in hybridization assays [28]. The length of peptidic

substrates and ligands, and the position and identity of a fluorolabel can have a profound effect on enzyme kinetics or ligand–receptor affinities, although the design and labeling of peptidic assay reagents are, themselves, well-described processes. Site-specific labeling reagents, such as biarsenic fluorescein derivatives, are becoming more widely available (F1AsH, PanVera LLC). These reagents bind selectively at Cys-Cys-Xaa-Xaa-Cys-Cys motifs. This sequence can be engineered into proteins of interest and is, itself, intrinsically stiff, thereby enhancing shifts in FP signals. Labeling-related problems are multiplied when applied to smaller biomolecules, such as aminergic neurotransmitters, where the fluorescent dye can match the size of the ligand itself, causing dye steric effects to dominate the underlying biology. All of these factors converge in the design of efficient FRET pairs, which are required to avoid the precipitous drop in energy transfer efficiency with distance, but maintain biological function. Similarly, success in engineering GFP chimeras for 'live' cell-based assays, such as protein translocation, where the bulkiness of GFP can be an important factor, remains difficult to predict. Overall, forecasting the ultimate biological performance of a fluoro-labeled biomolecule often remains a 'hit and miss' process and multiple approaches are best tested in parallel.

Assays are frequently developed in 'indirect formats' (i.e. the product of an enzymatic reaction is measured by competing off a fluoro-labeled product from an antibody), either because of the demands of the readout (e.g. single-molecule applications restrict the fluorophore concentration) or to make use of generic reagents in a 'molecular tool box approach' to enhance assay development. The antibody–antigen recognition systems that are frequently applied in indirect assays can suffer from batch-to-batch variability and, thus, need to be carefully characterized, and this can prolong assay development. In general, indirect assays introduce greater complexity into assay development and potentially, also introduce another target for 'active' compounds.

Correlating results from classical and fluorescence methods

Considering the increasing number of assay formats and technologies that are being applied to HTS, and the profound consequences of the choice of assay format, it is, perhaps, surprising that there have been only a few attempts to systematically compare the readout of the different assay formats on a larger scale. Most comparisons are restricted to pharmacological characterization within assay development, as high throughput screens are rarely run in duplicate using different assay formats. For kinase assays, it has been demonstrated in several systems that Km's for ATP and substrate are in agreement, whether radiometric or fluorescence methods are applied and IC_{50} values for known kinase inhibitors are within the expected range when statistical variation is taken into account [26,28–32]. For receptors, there is the added complication that the properties of small ligands can be altered by the size, or even the position, of the fluorescence label. Where the labeling of receptor ligands has been successful without a larger change in the D_d of the ligand, it has been shown that K_I values or rank order of potencies generated with fluorescence techniques are compatible with results from classic radioligand binding assays and do not differ as a consequence of the fluorescent readout itself [25,27,33]. As with every method, these assay properties must be established at the onset of a screening campaign. One pitfall when using FP in receptor–ligand displacement assays is the high concentration of receptor–ligand that some commercial assays apply to maximise the assay window (ligand depletion up to 70–80%). When applying high concentrations of receptor and/or ligand (>D_d), K_i values should not be derived by simply applying the Cheng-Prusoff calculation [29], but should result from a modified calculation derived by Kenakin (discussed in detail in [28]). It should be noted that these conditions also have a significant impact on assay sensitivity.

Limited pharmacological studies for assay characterization do not necessarily predict performance in a screening set-up where hundreds of thousands of compounds are tested. Sills *et al.* [34] describe the screening results for 30 000 compounds in three different assay formats (SPA, HTRF and FP) in an attempt to answer the question of which methodology was most suitable for the kinase targets under investigation. In these experiments, only a limited set of compounds could be identified in all three methods, indicating that screening results were dependent on the method applied, with a general trend suggesting that fluorescence methods identify more compounds. Although assay sensitivity might have a role, a more likely explanation for this observation is that a high number of compounds detected can be attributed to fluorescence artifacts. The compound interferes directly with the steps for signal generation via a non-biologically relevant mechanism, for example, by binding directly to the reporter antibody in an FP-based kinase assay. This is in agreement with our own experience, which shows that, without correction, up to 80% of 'hits' identified in assays that apply fluorescent probes are active due to their physical, rather than biological properties (Figure 1).

Conclusions and outlook

Fluorescence-based methods are available for all target classes and for both biochemical and cell-based assays.

Table 1. Commercially available platform technologies using fluorescence-based detection

Technology	Target class	Assay principle	Readout	Supplier
Lance™	Various	Multiple	TRF	PerkinElmer Lifescience
Trace™	Various	Multiple	TRF	CisBio
IMAP™	Kinases, phosphatases	Specific binding of phosphorylated peptides to beads	FP	Molecular Devices
IQ™	Kinases, phosphatases	Iron quench	FRET	Pierce
Z'-Lyte™	Kinases, phosphatases	Protease cleavage of phosphorylated peptides	FRET	Panvera
β-lactamase	Multiple signaling pathways	Reporter gene with membrane-permeable substrate	FRET	Panvera
FLIPR	GPCRs, ion channels	Kinetic analysis of calcium fluxes Membrane potential	FLINT	Molecular Devices
VIPR	Ion channels	Measurement of membrane potential	FRET	Panvera
β-arrestin	GPCRs	GFP-arrestin recruitment	SCI	Norak
CypHer™	GPCRs	Receptor internalisation pH sensitive fluorophore	SCI	Amersham

Abbreviations: FLINT, fluorescence intensity; FP, fluorescence polarization; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; HTRF, Homogeneous time-resolved fluorescence; SCI, subcellular imaging.

They have enabled scientists to construct highly sensitive assays in miniaturized formats. In themselves, they have not delivered more or better leads than conventional detection technologies, because these outcomes are, primarily, a function of the compound file and assay principle, but not the readout *per se*. At the same time, it has become apparent that, without strategies to counteract interference of compounds and biological reagents, fluorescence-based detection methods can suffer from artificial results. Various approaches are being employed to minimize, detect and correct these problems; however, there is no solution that provides a complete safeguard. As for assay development, designing fluorescence probes is not always predictable and most companies rely on specialized providers or 'off the shelf' products (Table 1).

Nearly all of the fundamental principles of fluorescence that are exploited today were described as early as 1900. Today's technology enables us to insert these principles into real applications and efforts are continuing to further improve these methods. There is a current trend to avoid 'population averaging' or 'bulk measurements', to exploit information about single molecules or single cells. By measuring multiple parameters, these methods can simultaneously deliver information about the activity of a compound, as well as its mode of action and, therefore, potentially reduce the downstream workload. New developments are focused on the provision of more interference-resistant assays, generating simplified toolboxes for assay development, as well as new fluorescent probes to enable multiplexing of assays.

As described previously, fluorescence lifetime is one such technique that is actively pursued by several commercial organizations producing HTS assay kits. It can be applied to the measuring of biochemical interactions and also, in a modified form, to the imaging of cells and tissues [Fluorescence lifetime imaging (FLIM)] [4,35,36]. Another area of intense discussion is the application of new fluorescent probes – the so-called 'Quantum Dots'. Quantum dots are based on semiconductor nanoparticles (reviewed in [37]). Two characteristics make them potentially useful in biological applications: first, their size is in the range of biomolecules and the emission wavelength changes simply with particle size, and second, they can all be simul taneously excited with white light. The first examples of the application of Quantum dots to biological systems, such as the study of hybridization of nucleic acids in living cells [38], have been described but, despite the clear potential, mainstream application is still distant.

As we come to expect, all developments will come at a high cost and increased complexity, at least in the short term. It is not only the cost of equipment but, more importantly, the expertise and skills needed in assay development and screening to fully exploit these new technologies. Past examples highlight the observation that the real benefit of these advances will not necessarily be seen by companies at the cutting edge of technology development

but by those organizations that apply the matured technology swiftly to their target portfolios.

Acknowledgements

We thank Chris Williams and Frank Stuhmeier for helpful discussions; Kurt Herrenknecht, Achim Kirsch and Adrian Siemers from Evotec OAI for providing data for the Endothelin receptor-internalization assay and; our colleagues Paul Hayter, Sasha Sreckovicz and Sandra Marcos from the Pfizer Hit Discovery Group for the NFκB translocation dataset.

References

1 Ruediger, M. *et al.* (2001) Single molecule detection technologies in miniaturized High Throughput Screening: Binding assays for G Protein-Coupled Receptors using Fluorescence Intensity Distribution Analysis and luorescence Anisotropy. *J. Biomol. Screen.* 6, 29–37
2 Kowski, T.J. and Wu, J.J. (2000) Fluorescence polarisation is a useful technology for reagent reduction in assay Miniaturization. *Comb. Chem. High Throughput Screen.* 3, 437–444
3 Pope, A.J. *et al.* (1999) Homogeneous fluorescence readouts for miniaturized high-throughput screening: theory and practice. *Drug Discov. Today* 4, 350–362
4 Emptage, N.J. (2001) Fluorescent imaging in living systems. *Curr. Opin. Pharmacol.* 1, 521–525
5 Turconi, S. *et al.* (2001) Real experiences of uHTS: A prototypic 1536-well fluorescence anisotropy-based uHTS screen and application of well-level quality control procedures. *J. Biomol. Screen.* 6, 275–290
6 Huang, W. *et al.* (2002) A fluorescence polarisation assay for cyclic nucleotide phosphodiesterases. *J. Biomol. Screen.* 7, 215–222
7 Bazin, H. *et al.* (2002) Time resolved amplification of cryptate emission: a versatile technology to trace biomolecular interactions. *J. Biotechnol.* 82, 233–250
8 Scheel, A.A. *et al.* (2001) Receptor-ligand interactions studied with homogeneous fluorescence-based assays suitable for miniaturized screening. *J. Biomol. Screen.* 6, 11–18
9 Kask, P. *et al.* (2000) Two-dimensional fluorescence intensity distribution analysis: Theory and allocations. *Biophys. J.* 78, 1703–1713
10 Wright, P.A. *et al.* (2002) Development of a 1 µl scale assay for mitogen-activated kinase kinase 7 using 2-D fluorescence intensity distribution analysis anisotropy. *J. Biomol. Screen.* 7, 419–428
11 Kohl, T. *et al.* (2002) A protease assay for two-photon crosscorrelation and FRET analysis based solely on fluorescent proteins. *Proc. Natl. Acad. Sci. U. S. A.* 99, 12161–12166
12 Kunapoli, P. *et al.* (2003) Development of an intact cell reporter gene beta-lactamase assay for G protein-coupled receptors for high throughput screening. *Anal. Biochem.* 314, 16–29
13 Johnston, P.A. and Johnston, P.A. (2002) Cellular platforms for HTS: three case studies. *Drug Discov. Today* 7, 353–363
14 Chambers, C. *et al.* (2003) Measuring intracellular calcium fluxes in high throughput mode. *Comb. Chem. High Throughput Screen.* 6, 355–362
15 Falconer, M. *et al.* (2002) High-throughput screening for ion channel modulators. *J. Biomol. Screen.* 7, 460–465
16 Kuntzweiler, T.A. *et al.* (1998) Rapid assessment of ligand actions with nicotinic acetylcholine receptors using calcium dynamics and FLIPR. *Drug Dev. Res.* 44, 14–20
17 Wood, M. *et al.* (2000) Antagonist activity of meta-chlorophenylpiperazine and partial agonist activity of 8-OH-DPAT at the 5-HT7 receptor. *Eur. J. Pharmacol.* 396, 1–8
18 Baxter, D.F. *et al.* (2002) A novel membrane potential-sensitive fluorescent dye improves cell-based assays for ion channels. *J. Biomol. Screen.* 7, 79–85
19 Farinas, J. *et al.* (2001) A microfluidic device for measuring cellular membrane potential. *Anal. Biochem.* 295, 138–142
20 Gonzalez, J.E. and Maher, M.P. (2002) Cellular fluorescent indicators and voltage/ion probe reader(VIPR (TM)): Tools for ion channel and receptor drug discovery. *Receptors Channels* 8, 283–295
21 Zlokarnik, G. *et al.* (1998) Quantitation of transcriptional and clonal selection of single living cells with b-lactamase as reporter. *Science* 279, 84–88
22 Chiesa, A. *et al.* (2001) Recombinant aequorin and green fluorescent protein as valuable tools in the study of cell signalling. *Biochem. J.* 355, 1–12
23 Lippincott-Schwartz, J. and Patterson, G.H. (2003) Development and use of fluorescent protein markers in living cells. *Science* 300, 87–90
24 Turek-Etienne, T.C. *et al.* (2003) Evaluation of fluorescent compound Interference in 4 fluorescence polarization assays: 2 kinases, 1 Protease, and 1 phosphatase. *J. Biomol. Screen.* 8, 176–184
25 Allen, M. *et al.* (2000) High throughput fluorescence polarization: A homogeneous alternative to radioligand binding for cell surface receptors. *J. Biomol. Screen.* 5, 63–69
26 Fowler, A. *et al.* (2002) An evaluation of fluorescence polarization and lifetime discriminated polarization for high throughput screening of serine/threonine kinases. *Anal. Biochem.* 308, 223–231
27 Banks, P. *et al.* (2000) Impact on a red-shifted dye label for high throughput fluorescence polarisation assays of g protein-coupled receptors. *J. Biomol. Screen.* 5, 329–334
28 Burke, T.J. (2003) Development and Application of fluorescence polarisation assays in drug discovery. *Comb. Chem. High Throughput Screen.* 6, 183–194
29 Cheng, Y. and Prusoff, W.H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 22, 3099–3108
30 Seethala, R. and Menzel, R. (1998) A fluorescence polarization competition immunoassay for tyrosine kinases. *Anal. Biochem.* 255, 257–262
31 Park, Y.W. *et al.* (1999) Homogeneous proximity tyrosine kinase assays: Scintillation proximity assay versus homogeneous time-resolved fluorescence. *Anal. Biochem.* 269, 94–104
32 Ohmi, N. *et al.* (2000) Development of a homogeneous time-resolved fluorescence assay for high throughput screening to identify Lck inhibitors: Comparison with scintillation proximity assay and streptavidin-coated plate assay. *J. Biomol. Screen.* 5, 463–470
33 Gagne, A. *et al.* (2002) Use of fluorescence polarization detection for the measurement of fluopeptide™ binding to G protein-coupled receptors. *J. Recept. Signal Transduct. Res.* 22, 333–343
34 Sills, M.A. *et al.* (2002) Comparison of assay technologies for a tyrosine kinase assay generates different results in high throughput screening. *J. Biomol. Screen.* 7, 191–199
35 Calleja, V. *et al.* (2003) Monitoring conformational changes of proteins in cells by fluorescence lifetime imaging microscopy. *Biochem. J.* 372, 33–40
36 Suhling, K. *et al.* (2002) Imaging the environment of green fluorescent protein. *Biophys. J.* 83, 3589–3595
37 Chan, W.C.W. (2002) Luminescent quantum dots for multiplexed biological detection and imaging. *Curr. Opin. Biotechnol.* 13, 40–46
38 Sokol, D.L. (1998) Real time detection of DNA-RNA hybridisation in living cells. *Proc. Natl. Acad. Sci. U. S. A.* 95, 11538–11543
39 Trask, O.J. and Large, T.H. (2001) Automated imaging: applications to drug discovery. *Curr. Drug Discov.* 1, 25–29
40 Milligan, G. (2003) High-content assays for ligand regulation of G-protein coupled receptors. *Drug Discov. Today* 8, 579–585
41 Barak, L.S. *et al.* (1997) A beta-arrestin green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J. Biol. Chem.* 272, 27497–27500
42 Giuliano, K.A. (2003) High-Content profiling of drug-drug interactions: Cellular targets involved in modulkation of microtubule drug action by the antifungal ketoconazole. *J. Biomol. Screen.* 8, 125–135
43 Grepin, C. *et al.* (2003) Improving quality: High content screening with Acumen Explorer. *Curr Drug Discov.* 3, 37–42