Changing dynamics in dispense and imaging assays

The prospects for a significant shift in dispense and imaging (D&I) assays towards a greater utilisation of calcium-mediated flash luminescence readouts appears to be finally under way. Driving this changing dynamic is the acceptance that assay sensitivity (better quality hits) may be enhanced using flash luminescence and a realisation that the commercial instrument platforms are now sufficiently robust to support high throughput operation. Flash luminescence assays have become even more accessible with the expiration of a key aequorin patent and the availability of a range of aequorin offerings and alternative photoproteins. However, the current use of fluorescent-based D&I assays still predominates and is by no means static from a new development perspective, with increasing diversity in the number of alternative no-wash calcium kits offered and the prospect of more competitive pricing. Adding value to D&I fluorescent readouts looks set to be a theme for the future. Overall, we can expect both flash luminescent and fluorescent-based D&I assays to remain the key tools and principal technologies that screeners will use for GPCR mobilisation and ion channel assays for at least the next five years.

By Dr John Comley

In the Winter 2003/4 Issue of *DDW* we reviewed flash imagers and their prospects for facilitating high throughput functional GPCR assays[1]. At that time we concluded that the emergence of new detection platforms that combine multi-channel liquid handling at the reading position with low light imaging technology were finally providing screeners with the tools to enable high throughput calcium-mediated flash luminescence and the time was right to consider alternatives to the more commonly used fluorescent-based methods for screening functional calcium mobilisation assays. Flash luminescence assays using photoproteins such as aequorin, as indicators of intracellular calcium, are particularly suited to the study of GPCR activation. We previously summarised in detail the many advantages of aequorin flash luminescence and compared the then available detection instruments[1]. Although there have been hardware and software upgrades to these instruments, and even a few subtle name changes since late

Figure 1: Current (2005) use of different probes/technologies in D&I assays

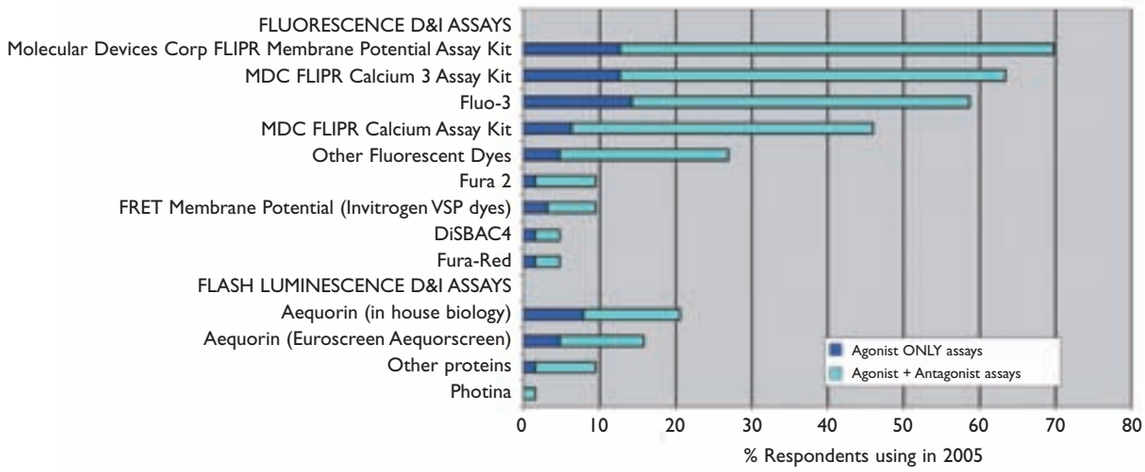


Figure 2: Estimated breakdown of D&I assay type

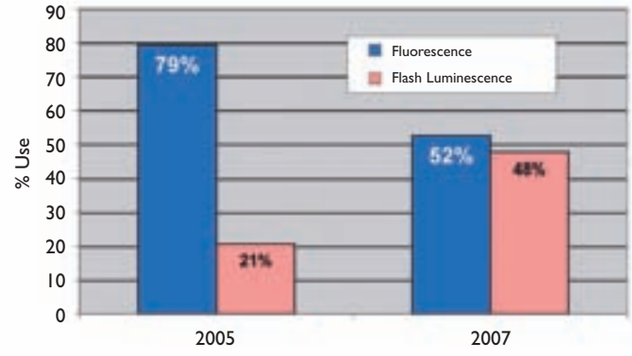
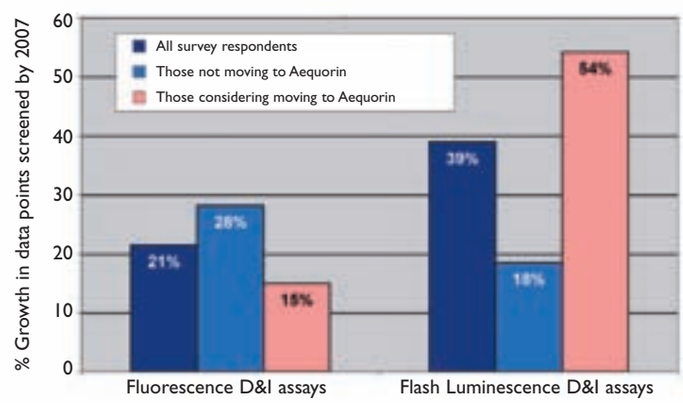


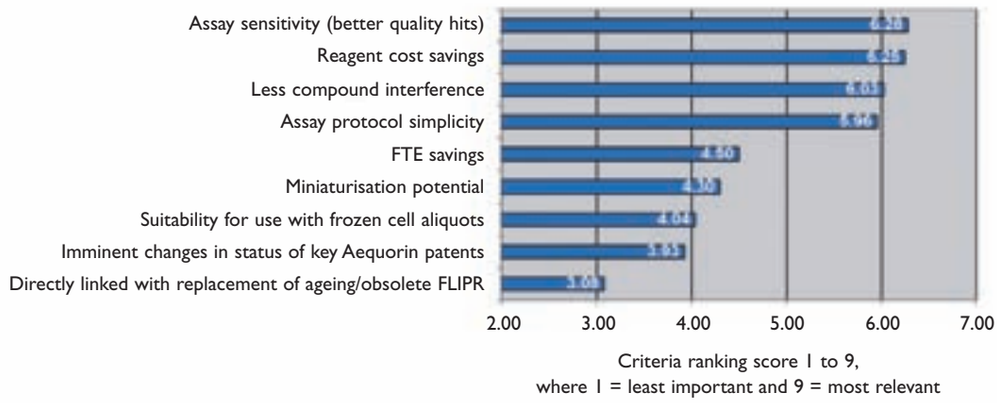
Figure 3: Expected growth in D&I data points screened over the coming years



2003, the main luminescent imaging platforms available today (CyBio CyBi®-Lumax flash HT & HS, PerkinElmer LumiLux and Hamamatsu FDSS6000) have remained essentially unchanged. New instrument innovation has largely been confined to devices that evenly suspend, maintain and dispense ceolentrazine (the substrate for aequorin) loaded cells into microplate wells containing compound. Since the previous article Molecular Devices has introduced the more flexible FliprTETRA. This instrument is planned to replace the ageing and obsolete population of legacy Fliprs worldwide, variously estimated to be around 350 units, some up to 10 years old. The FliprTETRA is however intended mainly to address fluorescent-based dispense and image (D&I) assays and its flash luminescent capability is generally regarded as limiting in some assay protocols.

Prompted by support and interest from within the screening and vendor community and a desire to gain a better understanding of the changing dynamics of the market for D&I systems and applications, HTStec undertook its Dispense & Imaging Trends survey in December 2005. The objective was to comprehensively document current trends and experiences of D&I systems (both fluorescent and flash luminescent) and applications, and to establish if the need to replace ageing Fliprs over the coming few years will act as a catalyst in the reappraisal of fluorescent-based approaches for GPCR activation and stimulate interest in switching to aequorin and acquiring flash luminescence detection capability. The study aimed to provide D&I instrument manufacturers and those developing D&I applications (eg fluorescent dye kits,

Figure 4: Criteria motivating respondents to consider Aequorin



aequorin and other photoproteins, etc) with current market information enabling them to make informed decisions as to where the industry will go over the next few years.

Fluorescent-based dispense and imaging assays still predominate

The relative current (2005) use of different probes and technologies in D&I assays is presented in **Figure 1**. The first observation is that the majority of screening includes both agonist and antagonist assays. For the fluorescence-based D&I assays greatest use is made of the MDC (Molecular Devices) Assay Kits, particularly the Membrane Potential, Calcium 3 and Calcium kits. Of the assays run using commercial fluorescent reagents greatest use is made of Fluo-3. Compared to the MDC kits and Fluo-3 other probes (eg Fura 2, FRET Membrane Potential (Invitrogen VSP dyes), $DisBac_4$ and Fura-Red) are used by less than 10% of respondents. For the flash luminescent D&I assays greater use is made of aequorin based on in-house biology versus that derived from Euroscreen (Aequorscreen). Other photoproteins and Photina are rarely used. It is currently (2005) estimated that the majority (79%) of all D&I assays are fluorescence-based, with only 21% using flash luminescence. By 2007 the proportion of fluorescence-based D&I assays is predicted to decline to 52%, while flash-luminescent D&I assays will increase in share to 48% (**Figure 2**). Growth in the number of D&I data points screened by respondents in 2007 (relative to 2005) is also expected to increase, with 21% growth for fluorescent-based assays and 39% growth for flash luminescent assays (**Figure 3**). When survey respondents were split into two groups, based on their interest to move to aequorin from fluorescence-based assays, the growth estimates for numbers of data points screened showed further increases (**Figure 3**).

Increasing interest in moving to aequorin

About 50% of all survey respondents indicated an interest to move to aequorin, ie they either have already switched, were in the process of switching or were considering switching their GPCR calcium activation assays from fluorescence to aequorin. The relative influences of the perceived advantages of aequorin over fluorescence alternatives in this decision are ranked in **Figure 4**. The most important criteria motivating them to consider aequorin were assay sensitivity (better quality hits) and reagent cost savings, closely followed by less compound interference and assay protocol simplicity (**Figure 4**). Contrary to some thoughts the move to aequorin does not appear to be directly linked with replacement of ageing/obsolete Flipr. 50% of survey respondents indicated they have chosen not to consider aequorin. Criteria which may have accounted for why these respondents plan to stick with fluorescent-based alternatives to aequorin are ranked in **Figure 5**. The main reason given for this decision was the need to purchase a flash luminescent imager followed by they prefer tried and tested fluorescent-based methods (**Figure 5**). Interestingly, the lack of suitable commercial flash luminescence imagers is still regarded by some as a significant factor, suggesting some respondents think there is still need for improvement in the commercial instrument offerings.

Figure 5: Criteria influencing respondents not to consider Aequorin

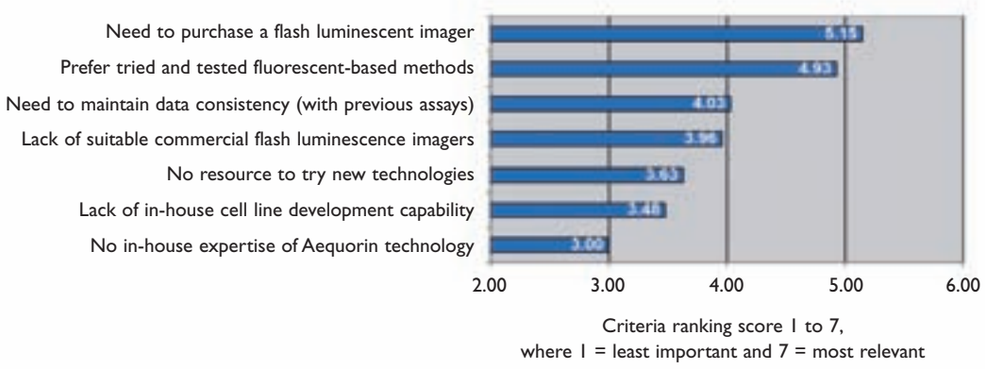
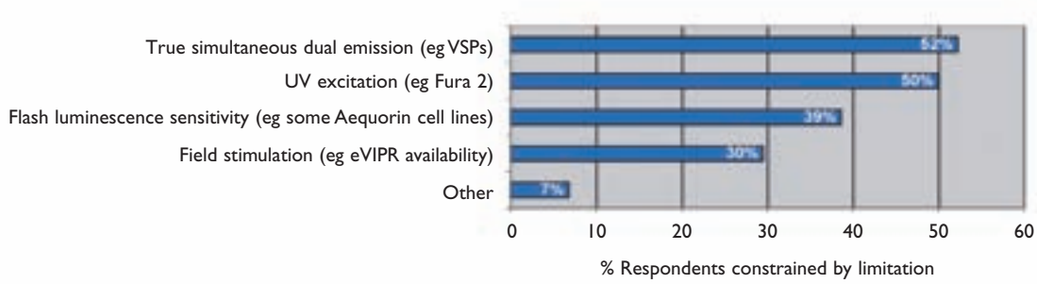


Figure 6: Detection limitations that have constrained the use of particular probes or technologies



New fluorescence-based assay reagents still wanted

When survey respondents were asked if they were satisfied with the reagents offered/available for fluorescence-based D&I assays, 19% said yes, overall they were happy with the current offerings, while a further 54% said yes, but would still like to see a greater diversity of reagents. In terms of the main detection (D&I instrument) limitations that have constrained respondent's use of particular probes or technologies most cited the lack of true simultaneous dual emission (eg for VSP dyes) and UV excitation (eg for Fura 2) as most limiting (**Figure 6**). Flash luminescence sensitivity (eg needed for some aequorin cell lines) was less of a constraint for respondents, although it was the most important limitation for those respondents whose origin was from large Pharma.

Overall the survey provided a wealth of additional information that supports the changing dynamic of D&I instrument and assays market. The global pharma/biotech market for all D&I instrument sales in 2006 is estimated to be around 100 units and worth approximately $40 million. Molecular Devices' market share of new D&I instrument sales (based on the current Flipr TETRA) is expected to decline over the coming years as interest in flash luminescence D&I readouts increases.

Vendor snap-shots

The following are vendor snap-shots that highlight their latest developments that address both fluorescent and flash luminescent D&I instruments and assays.

Photina™, developed and marketed by **Axxam** (www.axxam.com), is the Ca^{2+} activated photoprotein that represents a new and improved tool for the development of precise Ca^{2+} mobilisation assays for High-Throughput Screening (HTS). The inherent flash luminescence of Photina™ offers several advantages over the use of the classical fluorescent Ca^{2+} dyes: 1) a large dynamic range due

to low background levels; 2) the possibility to measure Ca^{2+} concentrations at specific cellular sites; 3) short incubation time with tested compounds; and 4) the opportunity to follow reaction kinetics. The expression and functionality of Photina™ has been tested in various cell lines most frequently used in HTS processes, such as CHO and HEK293. The results obtained show that the expression of the protein is stable over time, does not affect cell growth and viability and no toxicity is detected – even after long periods of cell culture. While Photina™ is well-suited for measuring GPCR activation, it also is ideal for any cellular targets capable of increasing the intracellular Ca^{2+} concentration. Several different targets, like Ca^{2+} permeable ion channels or Na^{+}/Ca^{2+} exchangers, have been transfected in CHO cells that express either the mitochondrial or the cytoplasmic version of Photina™. The results obtained clearly demonstrate that the functional activation of these targets can be detected easily and reliably by Photina™ (**Figure 7**). The choice between cytoplasmic or mitochondrial localisation of the photoprotein greatly expands the range of targets addressable by the use of flash luminescence. Axxam researchers have run fully-automated HTS for ion channel targets expressed in CHO-Photina™ cell lines on the CyBio Cellight workstation. A 384-MTP format with adherent cells and a library of 200,000 compounds were utilised. The light signal intensity generated by Photina™ was robust and reproducible over the complete screening campaign, resulting in an average Z' factor of 0.7. Axxam has recently expanded its range of assay services to include carrying out full primary screening and compound profiling campaigns for their customers applying Photina™ technology across a range of different target classes.

Chemicon's (www.chemicon.com) Aqualite® is a recombinant aequorin derived from the jellyfish Aequorea victoria that provides an excellent tool

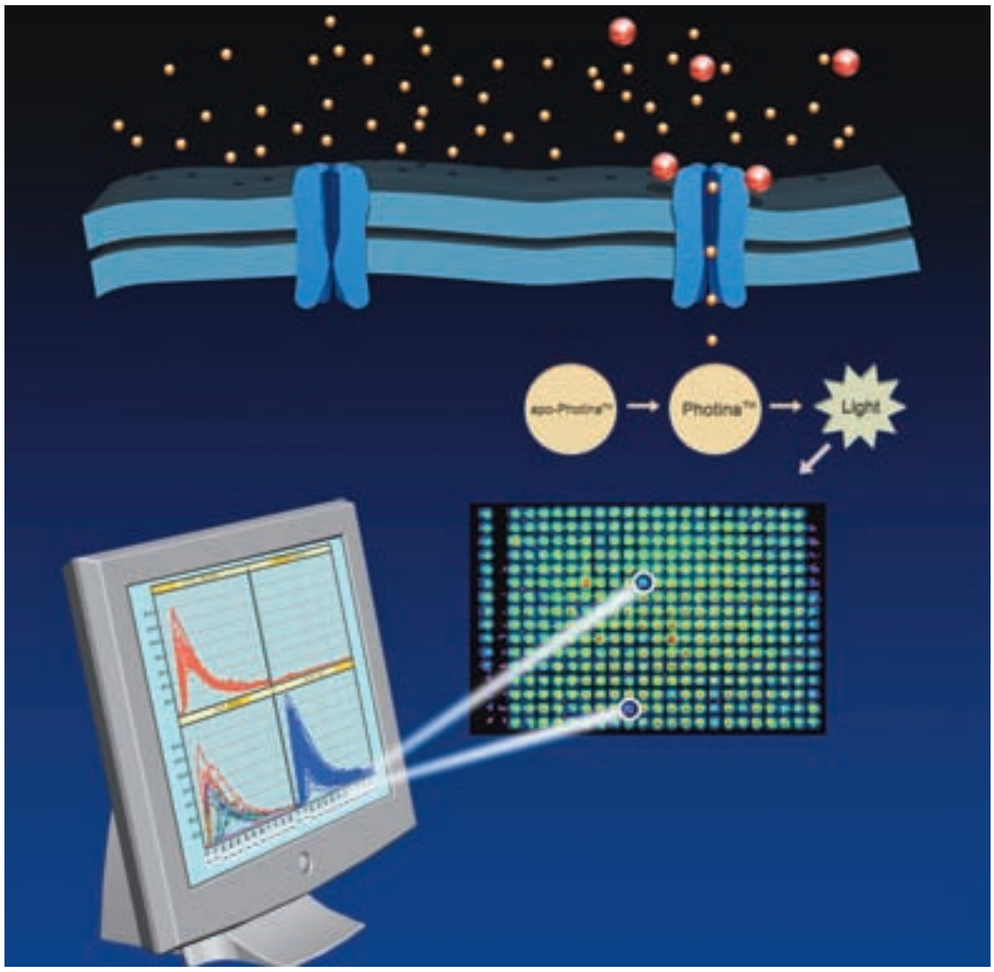


Figure 7: Application of Photina™ for the screening of Ca^{2+} permeable ion channels. Upon opening of the ion channel by either its ligand or by a membrane potential change, Ca^{2+} from the extracellular space enters into the cytoplasm of the cell. There Ca^{2+} binds to Photina™ and within seconds triggers the productions of photons, which are captured and recorded by a CCD based camera system. Therefore ion channel activity can be accurately measured by monitoring the photons produced by Photina™ in HTS even for very fast acting ion channels

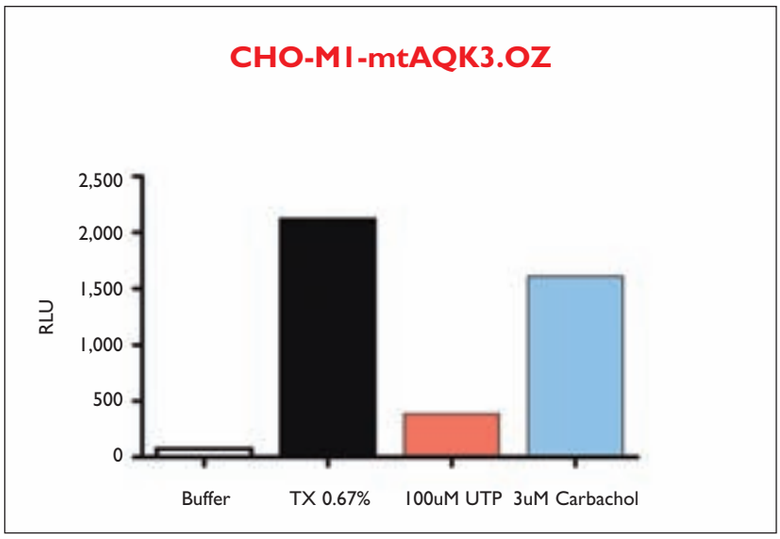


Figure 8: A stable CHO cell line expressing the M1 muscarinic acetylchline receptor and mitochondrially targeted Aqualite® displays high signal-to-background flash luminescence after stimulation with the M1 ligand, carbachol

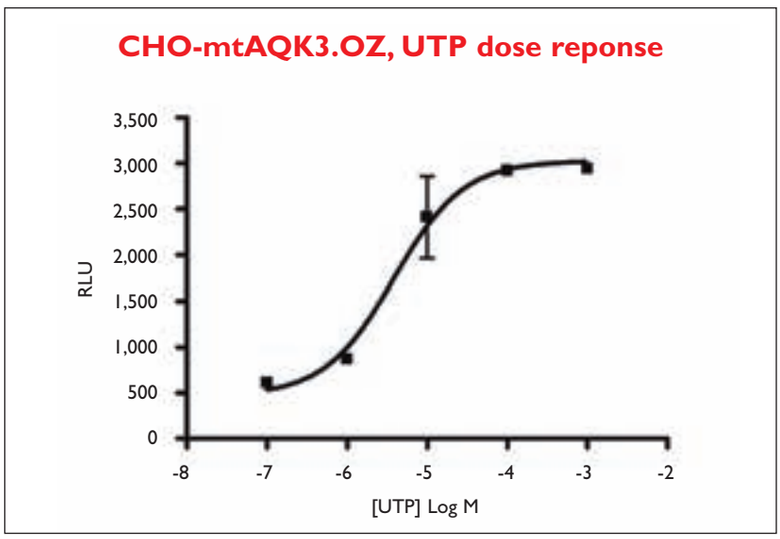


Figure 9: A stable CHO cell line expressing mitochondrially targeted Aqualite® displays the high sensitivity required to detect calcium fluxes induced by endogenous GPCRs, such as the UTP purinergic receptor

for luminescence assays. The active form of Aqualite® consists of a complex of a 22,000-dalton apoaequorin protein, two molecular oxygen and the luminophore coelenterazine. When the active Aqualite® complex encounters calcium at high nanomolar levels or greater, an oxidation reaction occurs that results in a two-second flash of light with a maximum emission at 486nm. Chemicon's Aqualite® is a licensed variant of aequorin that exhibits four-fold greater luminescent intensity than that of Euroscreen. The high quantum yield of Aqualite® makes it a highly sensitive detection system for immunoassays and other binding assays. Chemicon has recently employed Aqualite® for the detection of intracellular calcium fluxes stimulated by activation of GPCRs that couple to Gq or G15/16. Aqualite® modified to contain an N-terminal mitochondrial targeting sequence was stably expressed in CHO cells, and clonal cell lines with high levels of aequorin activity were selected. When the mitochondrial Aqualite® is co-expressed with the M1 muscarinic acetylcholine receptor, addition of a ligand for M1 such as carbachol results in a >20-fold increase in luminescence (**Figure 8**). In addition, the luminescent signal from Aqualite®-expressing CHO cells is sensitive enough to detect lower signals of endogenously expressed GPCRs: addition of UTP to activate endogenous P2Y receptors increases luminescence in a dose-dependent fashion (**Figure 9**). Aequorin has a number of advantages over other Ca^{2+} indicators, such as the wider Ca^{2+} concentration detection range, the lower background signal, and the less phototoxicity. Chemicon's Aqualite®-expressing cell lines therefore provide an excellent alternative to fluorescence assays for monitoring intracellular calcium concentrations for GPCR validation and HTS applications.

CyBio (www.cybio-ag.com) has developed a unique D&I technology, the cell flash technology, that enables optimised non-contact dispensing of cells and simultaneously extremely sensitive imaging of flash luminescence signals. With its capability to dispense cells into microplates containing the compounds to be screened, the CyBi®-Lumax flash provides a number of important advantages. It significantly saves time and costs for cell culture and provides superior consistency of results, because the cells can be grown and loaded with coelenterazine (for aequorin assays) in flasks instead of microplates. The risks of edge effects and contamination, inherent with cell growth in microplates, are eliminated by the use of a cell dispenser. In contrast to the use of pipetting tips, which requires time for tip washing between plates and generates additional costs for consumables, non-contact dispensing of cells increases the throughput and prevents cross-contamination. Furthermore, it streamlines the assay process and enables fast collection of agonist and antagonist data from one compound plate. By use of external reservoirs, which can be spinner flasks of any size as appropriate, a unique recirculation function

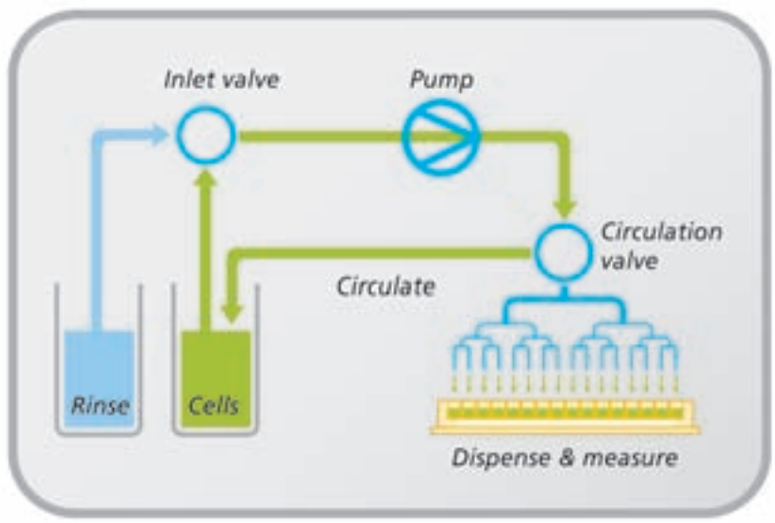


Figure 10: Schematic diagram of the cell circulation function and the patented multichannel droplet generator (EP 1 036 594) of the CyBi®-Lumax flash

and optimised fluid paths (**Figure 10**), the CyBi®-Lumax flash efficiently eliminates cell settling effects without generation of cell stress, and produces levels of uniformity in cell dispensing previously not achievable. CyBio's assay automation solutions for the CyBi®-Lumax flash range from one or several stackers with up to 280 plates capacity, to workstations including a pipettor (**Figure 11**) to screening systems with incubator(s) and scheduling software, and easy integration with third party robotics. CyBio offers two versions of flash luminescence readers: The CyBi®-Lumax flash HT for ultra high throughput, which is achieved with the integration of two 16-channel non-contact dispensers, is capable of generating >200,000 data points within eight hours in 1536-well format. The CyBi®-Lumax flash HS for ultimate sensitivity, which is based on a proprietary lens and high resolution imaging, enables cell-based flash luminescence assays with total assay volumes as low as 2µl and less than 500 cells per well. CyBio is currently working on the next generation of the CyBi®-Lumax flash and is collaborating with Axxam to develop new automated high throughput applications for Axxam's Photina™ Technology.

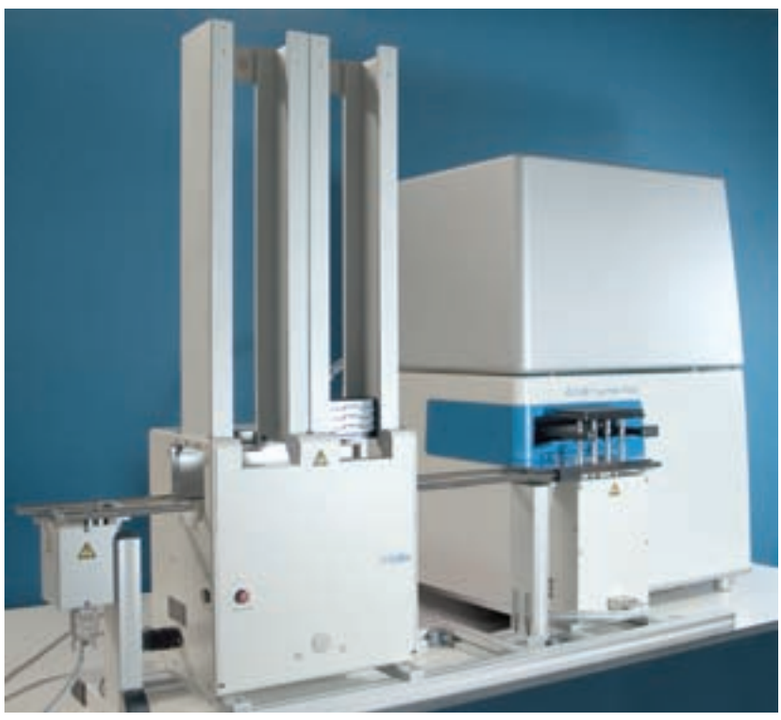


Figure 11: Small and powerful cellular screening workstation from CyBio: CyBi®-Lumax flash with one stacker and a comprehensive range of extension possibilities

DiscoveRx (www.discoverx.com) has also recently developed a rapid and robust method for detection of changes in intracellular calcium occurring in response to GPCR activation. The DiscoveRx Calcium No Wash assay provides a cost-effective method for measuring calcium mediated cell signaling and is entirely compatible with injection capable fluorescent readers and D&I instruments. Cells expressing a GPCR that signals through calcium are preloaded with a calcium sensitive dye and then treated with ligand. Upon stimulation the receptor signals release of intracellular calcium, which then causes the dye to fluoresce. The performance of the assay was examined against several cell types and GPCRs and demonstrated comparable performance and sensitivity to other commercially available assays. Further, the DiscoveRx Ca No Wash assay does not contain a quencher dye, and is therefore not susceptible to quencher interference issues commonly seen with chemokines and other small peptide ligands.

Recent developments at **Euroscreen** (www.euroscreen.com) have made the use of aequorin more accessible to screening laboratories. Many CCD-based flash luminescence readers are now available with cell injections capability and 384 or 1536 well plate capacity. Instruments suppliers such as CyBio, Hamamatsu and Perkin Elmer have optimised their readers to improve the stability of coelenterazine-loaded suspension cells for aequorin testing using different strategies, thus making large HTS campaign with aequorin more practical. Euroscreen extended its range of aequorin cells expressing GPCRs validated for

HTS purposes (AequoScreen™), and introduced a new range of frozen, ready to use division arrested aequorin cells (AequoZEN™). AequoZEN™ cells are available in aliquots ranging from 10 to 100 million cells per tube, and can be used directly after thawing with a much simplified protocol. The irradiation procedure used to prevent cell growing insures that small aliquots of cells can be purchased cost-effectively, and guarantees that cell number is constant after plating thus reducing plate artifacts. Carefully titrated irradiation was proved not to affect aequorin signal and reduces possible risks related to the use of anti-mitotic agents to prevent cell division. AequoZEN™ cells can be stored in -80°C freezers, thawed, washed and loaded with the desired coelenterazine substrate the same day of the test. The selling price of both AequoScreen™ and AequoZEN™ cells includes the licence for the order of addition Euroscreen patent that still applies worldwide (ie the patented addition of aequorin-transfected cell suspensions to compounds during automated assay protocols). Another factor making the use of aequorin more accessible is represented by the fact that the cost of purchasing AequoScreen™ cell lines was recently reduced in Europe and Japan following the expiration of UGARF (University Of Georgia Research Foundation) patents for apoaequorin sequence, exclusively licensed to Euroscreen, that still applies to the United States.

Evotec Technologies (www.evotec-technologies.com) provides a Calcium Flux Kinetics Analyzer tool, which enables using the full information content of kinetic time traces (eg FLIPR data) beyond just using the peak height or area under curve. An application example (**Figure 12**) demonstrates the use of 'kinetic fingerprint' parameters to predict the selectivity of compounds in an agonist screen. The fingerprint is generated by modelling of the curve shape of FLIPR data. The Analyzer application does not need any parameter tuning and has an easy to use graphical user interface. It is based on the Acapella™ Scientific Data Analysis Framework, a platform to flexibly design and run applications for high content data analysis like image and time series data.

Current attempts at multiplexing GPCR calcium mobilisation assays in the same well have been based mainly upon a single fluorescent dye, usually Fluo-3 AM or a derivative thereof. Several strategies have been adopted, these include measuring: 1) the temporal differences in responses of two different cell populations or two receptors

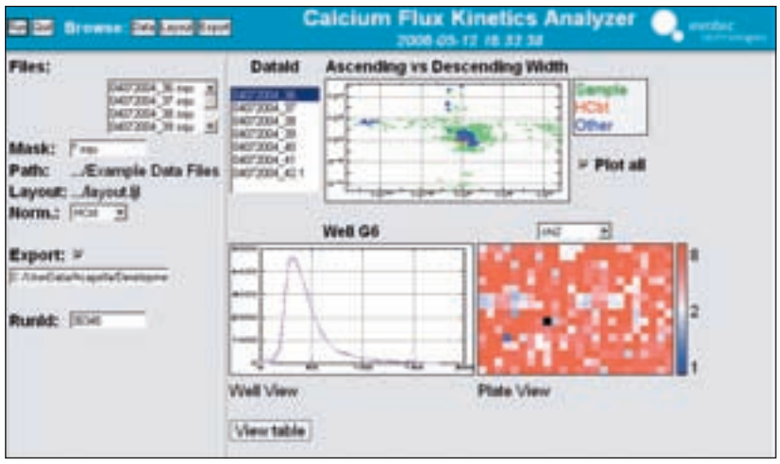


Figure 12: Acapella based Calcium Flux Kinetics Analyzer Application – User interface. On the left frame input and logistic information are specified (input data files, eg FLIPR squ files, plate layout information and output path to pass results to follow-up data analysis and management software, eg idbs ActivityBase, Genedata Screener, etc). The right frame provides an interface for browsing the analysis results for visual control: The bottom left panel shows the kinetic time series and the corresponding fitted curve. Bottom right: Plate map of goodness of fit – indicates wells with measurement artifacts (curve does not match the expected shape). Top panel: Kinetic fingerprint – predictor of specificity and alternative artifact indicator

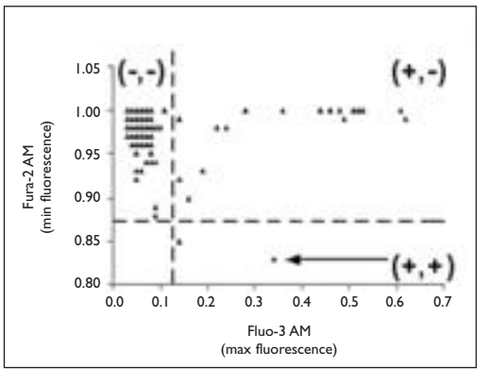


Figure 13
Minimal fluorescence for Fura-2 AM (ex380 em540) plotted against maximal fluorescence for Fluo-3 AM (ex480 em540). Results can be divided into three groups: (-,-), double negative; (+,-), positive in Fluo-3 AM loaded cells, negative in Fura-2 AM loaded cells; and (+,+), positive in both dye loaded cells

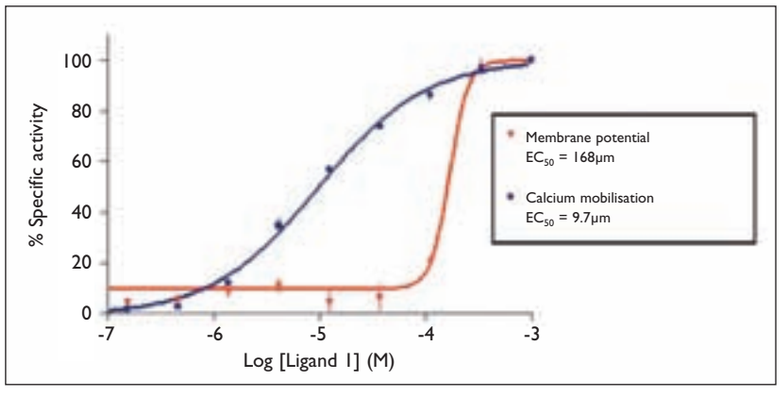


Figure 14: Dose response curves comparing the potency of the same agonist (Ligand1) using either a calcium mobilisation or membrane potential (hyperpolarisation) readout in a multiplexed double dye assay of the same cells loaded with Fura-2 AM and a no wash membrane potential dye. Note Ligand 1's potency depends on the readout

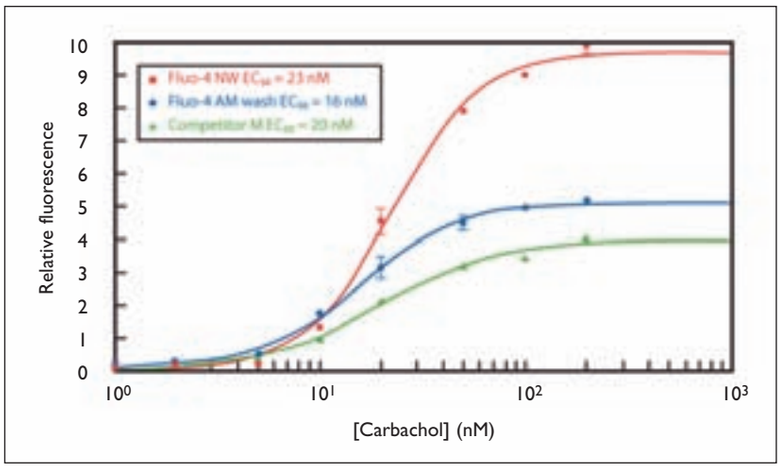


Figure 15: CHO M1 dose response curves. Cells were stimulated with carbachol agonist over the indicated concentration range. Cells were then analysed using the assays indicated. The Invitrogen Fluo-4 NW Calcium Assay Kit shows a greater magnitude of response even without a quencher dye than other commercially available calcium assays, while all assays displayed similar EC50 values

expressed in the same cell, ie cells are sequentially exposed to compounds with time intervals sufficient for cell recovery; 2) the total emission signal and deconvoluting it into two signals, ie the summation of two independent responses recorded at a single wavelength; or 3) cells that have been double dye loaded to concomitantly measure calcium and membrane potential changes, reported dye combinations include Fura-2 AM and $DisBac_2$, Indo-1 AM and $DisBac_2$, and Fura-2 AM and $DiBac_4$. To date most of these assays have been read using plate readers, equipped with injectors and single channel optics. **Hamamatsu**'s (www.fdssdrug.com) FDSS 6000 D&I workstation configured with FRET optics has been used to demonstrate high throughput whole plate multiplexing of GPCR calcium mobilisation assays. The FDSS's xenon flash lamp has a broad excitation capability (300-650nm) that enables UV excitation of Fura-2. Protocols evaluated include mixing Fura-2 AM loaded cells with Fluo-3 AM loaded cells (same target receptor, same cell line) to concomitantly measure calcium mobilisation. Calcium activation of Fura-2 AM (ex380 em540) is associated with a signal decrease while activation of Fluo-3 AM (ex480 em540) results in a fluorescent signal increase. Multiplexed assays that identify compounds giving a positive response for one dye and a negative response for the other are suggestive of compound auto-fluorescence at the positive dye's excitation wavelength, ie the result is an artefact (see **Figure 13**, (+,-) quadrant). In another multiplexing permutation, native cells loaded with Fluo-3 AM and recombinant cells expressing the receptor of interest dye loaded with Fura-2 AM are mixed and sequentially assayed (multiplexed) for agonist activity. A positive result in both native and recombinant cells would then be evidence of a non-specific effect, ie the agonist is affecting the cells in a manner not related to the recombinant receptor. In an additional multiplexing protocol double dye loading of the same cells with Fura-2 AM and no wash membrane potential dye can be used to differentiate potency of an agonist on cells responding to both calcium mobilisation and changes in membrane potential (**Figure 14**). Further multiplexing studies are ongoing with the FDSS6000 to investigate additional dyes including Indo-1 AM and Rhod-2 AM (calcium), JC-1 (mitochondrial membrane potential), and Voltage Sensitive Probes (membrane potential).

Ion channel and GPCR screening is now more tractable due to the availability of three high throughput instrument platforms that can make use of **Invitrogen**'s (www.invitrogen.com) Voltage-Sensor Probes (VSPs), ie Hamamatsu's FDSS6000, Molecular Device's FliprTETRA, and PerkinElmer's CellLux. Because each can utilise Invitrogen's gold standard intracellular calcium indicators, such as Fluo-3, Fluo-4, and Fluo-4 NW Calcium Assays, ion channel screeners now have the ability to reliably identify tractable hits for targets that flux calcium (eg, VGCCs, AMPA, NMDA and KA receptors and TRP channels); those that do not lead to measurable calcium flux can be assayed with the VSPs. Reports of VSP-based assays for NaV channels[2] and two GABA receptors[3] illustrate the performance of VSPs in HTS campaigns for voltage- and ligand-gated ion channels with excellent correlation of potency values from VSP and patch-clamp assays. The recent description of a VSP-based assay for the pain target NaV1.8 on the Flipr TETRA[4] revealed that for eight compounds assayed, the potency of block of the high-affinity inactivated state of the channel in patch-clamp assays and the VSP assay was within two-fold for seven of the eight; the remaining compound had a four-fold difference. Calcium indicators such as Fluo-3 and Fluo-4 have been used extensively to study Gq coupled receptors, and with the use of promiscuous or chimeric G proteins this has been extended to all categories of GPCRs. Developed specifically to meet the requirements of automated screening applications, Invitrogen's newest addition to its calcium indicator portfolio, the Fluo-4 NW Calcium Assay, provides superior performance without a quencher dye along with

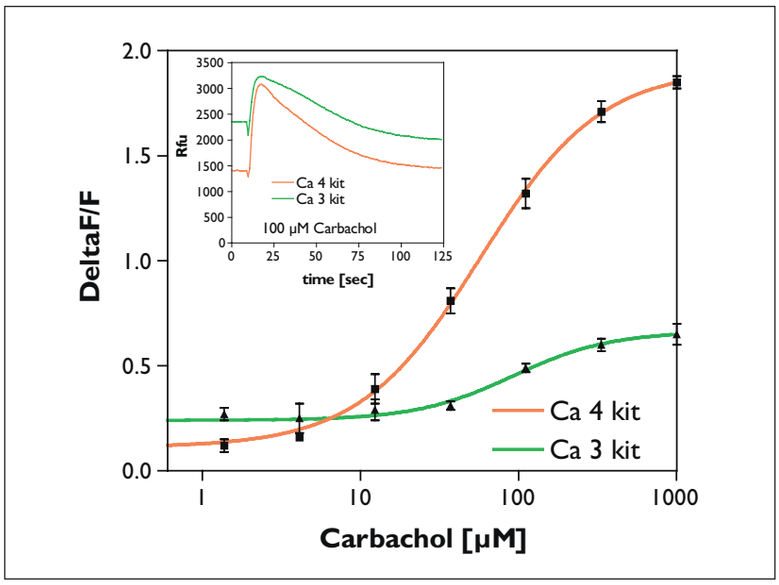


Figure 16: Background reduction in Calcium 4 kit (Ca 4) leads to improved DeltaF/F values when compared to Calcium 3 kit (Ca 3). Assay: endogenous response to Carbachol in parental HEK cells using Calcium 3 and Calcium 4 kits from MDC 384 well plate, 10,000 cells/well, media removal before assay on FLIPR Tetra

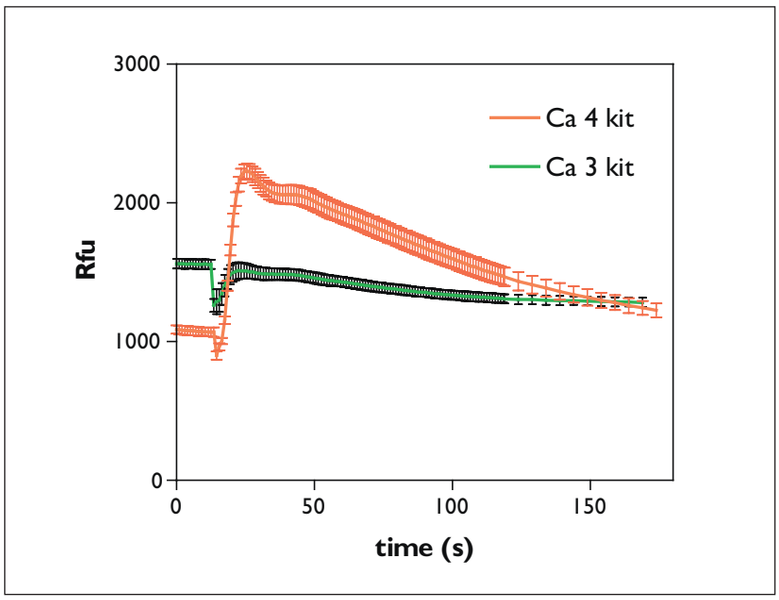


Figure 17: MDC Calcium 4 kit reduces the addition artifact caused by intrinsic media fluorescence in parental HEK without media (DMEM) removal. Assay: endogenous response to 333 M ATP in parental HEK cells using Calcium 3 and Calcium 4 kits, 384 well plate, 10,000 cells/well, no media removal before assay on FLIPR Tetra

the convenience of a no-wash format. Pharmacological validation of the new Fluo-4 NW calcium indicator has routinely shown consistent results with the widely-used Fluo-4 and Fluo-3 products (**Figure 15**).

Molecular Devices' (www.moldev.com) cell-based no-wash calcium-flux assay kits, first introduced in 1999 and designed for the FLIPR® fluorometric imaging detection systems, have become a standard for GPCR screening within the pharmaceutical industry. These kits, based on the proprietary masking technology exclusively licensed from BAYER AG (patent nos US 6,420,183, EP 0906572), continue to demonstrate their capabilities as the platform for many successful GPCR screens. In sustaining the quest for on-going innovation with this technology Molecular Devices has introduced the next generation of these kits, FLIPR Calcium 4 kit. The FLIPR Calcium 4 kit contains a novel masking dye, which combines higher absorptivity with an even lower tendency of interaction compared to FLIPR Calcium 3 kit. This feature contributes to an overall lower background and an increased DeltaF/F value that benefits assays with especially small Calcium responses (**Figure 16**). With this increased absorptivity of the masking dye, the addition artifact (also called 'the dip') is virtually eliminated as shown in **Figure 17** for HEK 293 cells when used without media removal, the litmus test for this artifact. Furthermore, the inert nature of this novel masking dye results in reduction of possible interference between ligands or targets and the masking dye. The FLIPR Calcium 4 kit, along with FLIPR Calcium and Calcium 3 kits, offers the most comprehensive line of homogenous Calcium assay kits. Addressing the researcher's need to identify the ideal Calcium kit for each specific target, an evaluation kit containing the FLIPR Calcium, Calcium 3 and Calcium 4 products is available. An example for such an evaluation done by a customer on a non-adherent cell line is shown in **Figure 18**.

PerkinElmer's (www.perkinelmer.com) LumiLux™ luminescence cellular screening platform based on patented fibre-optic CCD imaging technology is now being used for assay development and SAR profiling, as well as high-throughput screening in 384 and 1536 well formats for flash luminescence assays (**Figure 19**). The LumiLux workstation approach, housing all of the necessary components to run flash luminescence Aequoscreen™ and Photina™ assays (compound

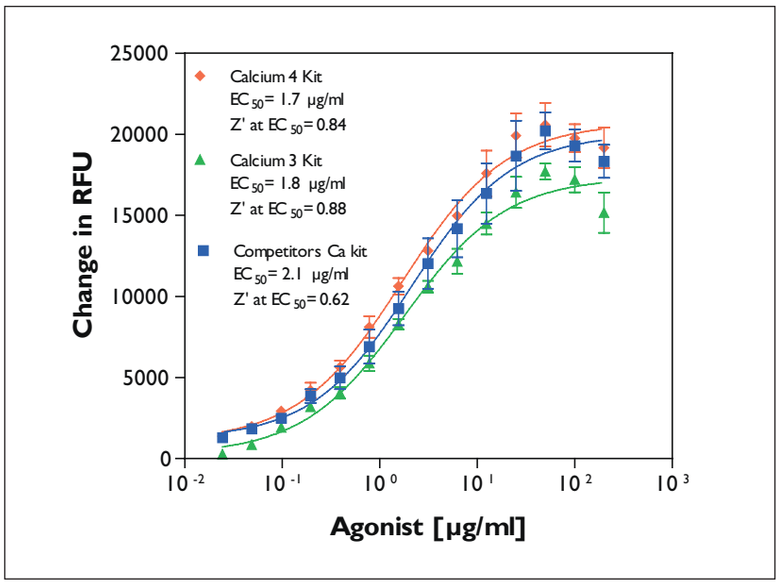


Figure 18: Calcium 4 and Calcium 3 kits from MDC perform in many cases equally well while at the same time outperforming competitive kits as shown by an MDC customer. Assay: undisclosed non-adherent cells stimulated with a protein activator, no media removal, assay was done on a FLIPR instrument

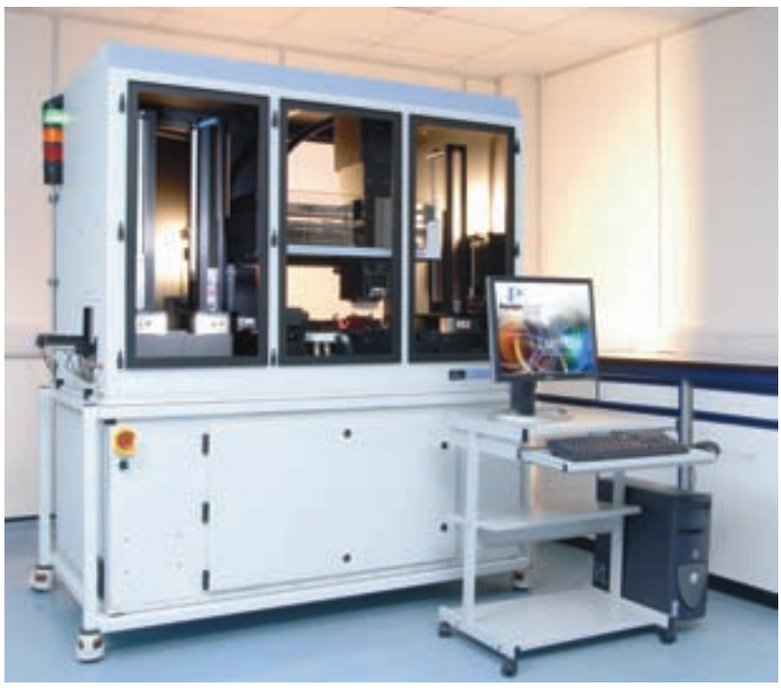


Figure 19: The LumiLux is based on patented fibre-optic 'contact imaging' technology, proven liquid handing and proprietary AssayPro™ data analysis software

plates, novel cell stirrer, control agonists, tip washing and tip loading capabilities) has now been used to screen agonist, combined agonist and antagonist, as well as allosteric modulator GPCR screens. The proven flexibility of the LumiLux platform also enables flash luminescence assays to be run using suspension cells (injecting cells into compound plates), semi-suspension cells (injecting cells into an empty plate followed by the compounds) or as an adherent assay (injecting compounds on to cells seeded into microplates 24 hours prior to the assay). New to the LumiLux workstation are smaller cell stirrer vessels which enable users to validate several cell lines per day for SAR profiling, with reduced dead volumes (**Figure 20**). The option to automate changing tips or washing tips in between plates can be included in the assay protocol, enabling assays to be run without any manual intervention. In addition, the liquid handling functionality on the LumiLux platform is being used to dilute test compounds immediately prior to use and can be included in the flash luminescence protocols. Improvements in plate throughputs have also been demonstrated through utilisation of the multi-tasking capability on the LumiLux instrument. Validation of FlashBlue, SPA and Imaging beads has recently been demonstrated on the LumiLux using 125I, 3H, 33P and 35S to improve the throughputs of radiometric assays compared to conventional PMT detector instruments*. It is also PerkinElmer's intention to evaluate the cell stirrer suspension assay approach for fluorescence calcium assays on the CellLux™ cellular fluorescence workstation, as a precursor to running multiplexed assays.

Summary

The prospects for a significant shift in D&I assays towards a greater utilisation of calcium-mediated flash luminescence readouts appears to be finally under way. Of importance in this changing dynamic is the acceptance that assay sensitivity (better quality hits) may be enhanced using flash luminescence and a realisation that the commercial instrument platforms (eg CyBio CyBi®-Lumax flash; Hamamatsu FDSS 6000 and PerkinElmer LumiLux) and their enhanced cell dispensing peripherals are now sufficiently robust to support high throughput operation. Furthermore, the

* Practice of assays may require a licence from General Electric (GE) Healthcare and/or others. Please consult your legal counsel for advice.

number of instrument options available will increase, with new systems planned for launch over the coming year. Flash luminescence assays have become even more accessible with expiration of a key aequorin patent; with Euroscreen's new range of frozen ready to use division arrested aequorin cells (AequoZEN™); the availability from Chemicon of a licensed variant of aequorin (Aqualite®); and the optimisation of Axxam's alternative photoprotein (Photina™) for flash luminescent monitoring of some ion channels targets. However, the current use of fluorescent-based D&I assays is still predominant and by no means static from a new development perspective. Not only are fluorescence-based D&I assays now well supported by a range of robust instruments (eg Molecular Devices FliprTETRA, Hamamatsu FDSS 6000 and PerkinElmer CellLux) but their attractiveness is further facilitated by the number of alternative no-wash calcium kits (from Molecular Devices, Invitrogen and DiscoverX) customers now have to choose between. This increase in diversity of reagents should address one of the key constraints identified among respondents to HTStec's survey and open the door to more competitive reagent pricing. Adding value to D&I fluorescent readouts looks set to be a theme for the future as Hamamastu FDSS takes the lead in investigating multiplexed readouts; Invitrogen's VSP dyes enable the assay of targets that do not lead to measurable calcium flux; and Evotec Technologies Calcium Flux Kinetics Analyzer tool facilitates extracting the full information content from FLIPR-like kinetic time traces. In conclusion, we can expect D&I assays to remain the key tool and principal technology that screeners will use for GPCR mobilisation and ion channel assays for at least the next five years. DDW

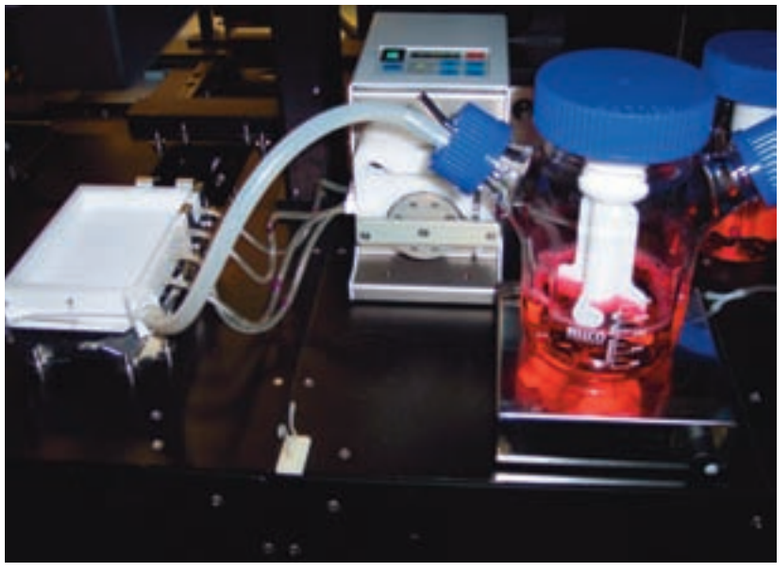


Figure 20: The new LumiLux cell stirrer mounted on a magnetic stirrer maintains cells in homogeneous suspension until ready for use. Cells sufficient for one microplate are pumped into the dispense tray and aspirated using the 384 tip dispense head. The remaining cells are returned to the cell stirrer flask or waste flask when the dispense tray tilts. 1000mL, 500mL, 250mL and 100mL cell stirrer flasks are now available for use in both assay development and screening

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