Livening up HCS IMAGING

With the aid of recent end-user feedback, this article investigates interest in and use of live cell and kinetic HCS imaging; most wanted application areas: advantages of live cell HCS imaging; live cell HCS assays of greatest interest; what's limiting live cell HCS; what is most needed to grow the live cell and kinetic imaging market; assay requirements; and preferred HCS instrument configuration. These findings provide a setting to review some of the latest vendor product offerings in this area. These include live cell and kinetic HCS imagers; fully automated HCS systems; HCS-related instrumentation; reagents, live cell probes and assay kits; image analysis and data management software. Overall, there is still plenty of scope for further technology enrichment in this area and ample opportunity to shift many of today's fixed cell end-point assays to live cell readouts in the future.

By Dr John Comley

For the most part high content screening (HCS) assays done in drug discovery have involved end-point approaches using fixed cells. This reflects the fact that logistically in screening it has been considered easier to uncouple the cell incubation and assay from the fixation and subsequent microplate imaging if moderate throughput is to be achieved. However, the potential of live cell and kinetic imaging to unlock temporal changes and facilitate the detailed analysis of dynamic cellular processing has featured highly when considering this alternative[1,2]. Recent advances in HCS imaging platforms have provided the environmental and optical stability needed to explore the automation of live cell assays in microplates, which, when coupled with on board liquid handling capabilities, have contributed to much innovative investigation and to raising the profile and expectations for live cell and kinetic HCS. With this in mind, HTStec recently undertook an end user survey to understand current interest and requirements for live cell and kinetic HCS imaging. The survey also attempted to identify emerging application requirements, market opportunities, unmet needs and future demand for live cell imagers. In this article we highlight some of the main findings of HTStec Live Cell & Kinetic HCS Imaging Trends market report[3] and contrast these with some of the latest vendor offerings in this area.

For the purposes of this article and in HTStec's report, live cell HCS imaging was divided into the following three categories: 1) Live Cell End-Point – involving incubation with a single image after a set time; 2) Live Cell Kinetics – involving incubation with multiple sequential images taken within a well; and 3) Live Cell Fast Response Kinetics – involving a dispense shortly before imaging, with exact timing between dispense and subsequent sequential imaging.

Interest and use of live cell HCS imaging

To put live cell imaging into context the survey found that only 27% of all HCS assays were made today using live cells (ie they do not image fixed cells). Of these live cell HCS assays only 8% involve a liquid dispense followed by fast response kinetic imaging. In **Figure 1** we have examined current interest and use of live cell imaging among persons actively involved in HCS assays. For live cell end-point HCS imaging the majority (31%) of respondents were interested in it, and have plans to use in the future, although only 23% of are actually using it today. For live cell kinetic HCS imaging the majority (38%) of respondents are interested in it, and have plans to use in the future, although only 18% are actually using it today. For live cell fast response kinetic HCS imaging the majority (48%) of respondents are interested in it, but have NO plans to use it in the future, and only 1% of respondents are actively using it today.

Application areas

Survey respondents rated live cell analysis on individual cells providing cell-specific results and drug toxicity and mode of action studies, to distinguish between drugs that appear similar based on end-point analysis as the application areas where they most want to use live cell and kinetic imaging (**Figure 2**). This was closely followed by to undertake extended duration longer term studies, including time lapse experiments and automating live cell HCS assays over a long time course, eg in basic/academic research.

Advantages of live cell HCS imaging

The ability to automate live cell incubation and image capture was ranked by survey respondents as the most relevant advantage of live cell and kinetic HCS imaging (**Figure 3**). This was closely followed by ability to make kinetic (sequential) measurements on live cells and then the ability to perform cell tracking with live cells.

Live cell HCS assays

The HCS assays where survey respondents were most interested in applying live cell and kinetic HCS imaging today are given in **Figure 4**. Greatest interest was shown for toxicity – cell health assays, followed equally by toxicity – proliferation assays, and receptor – internalisation and recycling assays. Of the assays selected as of interest to respondents, only 21% were given a 'must have' confocal imaging requirement, and 42% a 'nice to have' confocal imaging requirement.

Figure 1: Current interest and use of live cell imaging

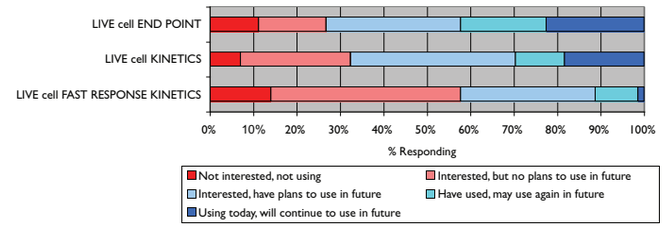


Figure 2: Application areas where respondents most want to use live cell and kinetic imaging

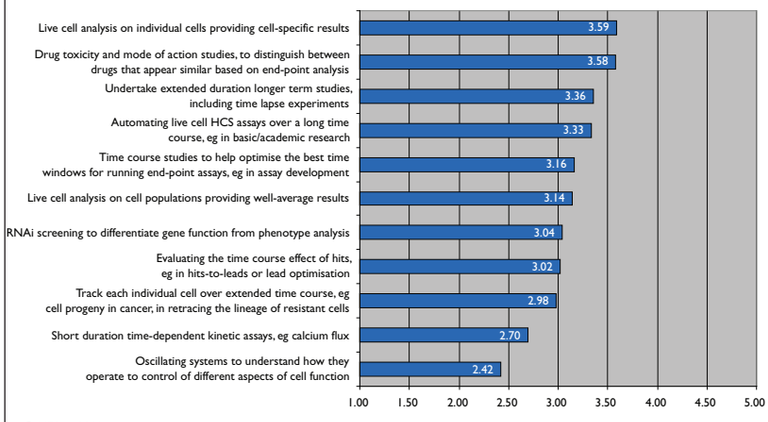


Figure 3: Advantages of live cell and kinetic imaging of greatest relevance to respondent's research

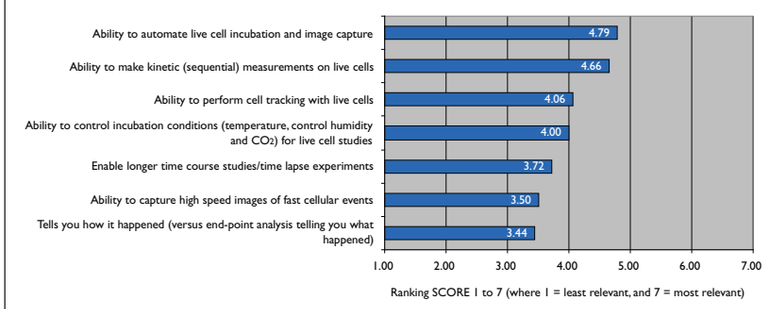


Figure 4: HCS assays where respondents are most interested in applying live cell and kinetic imaging

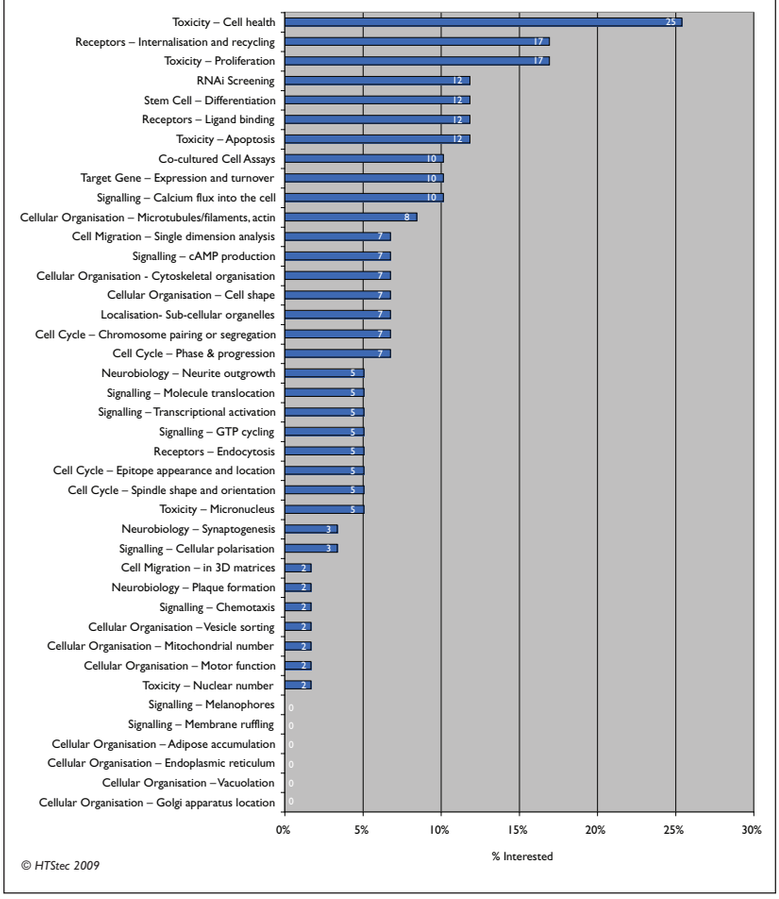
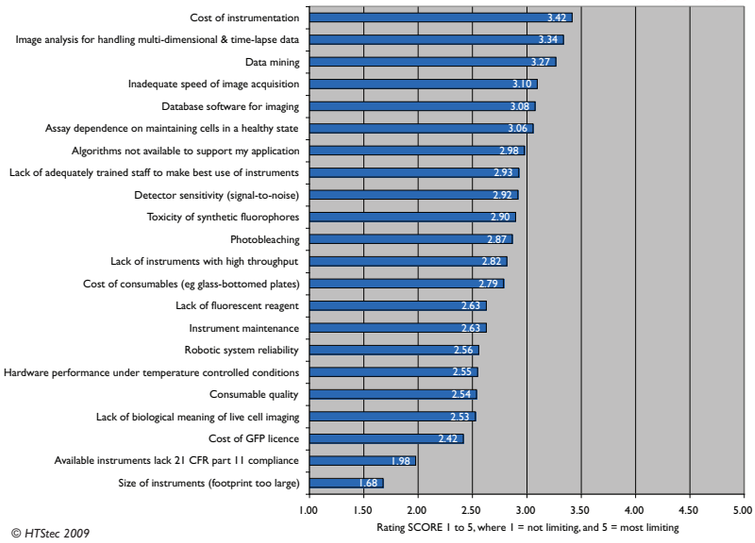


Figure 5: Factors that currently limit ability to make maximum use of live cell and kinetic HCS imaging



What's limiting live cell HCS

The cost of instrumentation was rated as the factor that most limited respondents' ability to make maximum use of live cell and kinetic. This was closely followed by image analysis for handing multi-dimensional and time-lapse data and data mining (**Figure 5**). However, as the difference between the rating values for the various limiting factors considered was not particularly marked multiple factors are at play here.

Growing the market

Improved ways of delivering probes into live cells and targeting specific proteins or organelles was rated in the survey as the factor most needed to grow the market (increase use and adoption) of live cell and kinetic imaging (**Figure 6**). This was closely followed by better reagents, new fluorescent probes better suited to live cell imaging, and then improved image analysis and informatics and lower priced instruments.

Assay requirements

Respondents' throughput and assay expectations for live cell and kinetic imaging today were 96-well plate format; <10 plates/8h day; and 2 different live cell and kinetic HCS assays/year. Maintaining optimum cellular function is critical in all live cell studies and the environmental control requirements wanted by survey respondents for these assays were: ambient temperature to 37°C; 5% CO_2; and >95% humidity. The time course requirements when undertaking kinetic analysis of live cellular events without a liquid dispense was a data read interval of once every one minute, with a total experiment duration of up to 24 hours. When undertaking fast response kinetic analysis of live cellular events after a liquid dispense the time course requirements were a data read interval of once every 1 sec, with a total experiment duration of 10 minutes.

Preferred HCS instrument configuration

Around 40% of survey respondents want to see additional or improved live cell or kinetic HCS imaging instruments, believing the current systems to be inadequate. The HCS instrument configuration of greatest interest to survey respondents is given in **Figure 7**. The preferred main system was for an HCS imager integrated into an automated HCS system, although a third of respondents could see the merits of an HCS imager integrated into a fully automated high speed high throughput system. The preferred capabilities almost equally

split between fully configured for all aspects of HCS, including live cell analysis and add-on modules for live cell and kinetic available. There was limited interest in a system with capabilities specifically optimised for live cell and fast response kinetics only, suggesting respondents want to keep their options open to explore both fixed cell and live cell HCS.

Table 1 summarises the main vendor offerings that currently support live cell and kinetic HCS imaging.

Latest vendor offerings

The following snapshots provide details of some of the latest advances vendors have made in provision of live cell and kinetic HCS imagers; fully automated HCS systems; HCS-related instrumentation; reagents, probes and assay kits; image analysis and data management software.

The BD Pathway™ systems from **BD Biosciences** (www.bdbiosciences.com/bioimaging/) offers an integrated solution encompassing instrumentation, software and reagents (**Figure** 8). Flexible and powerful, the BD Pathway™ systems enable the researcher to choose between the ease of ready-to-run applications to developing-your-own customised assays. The preset applications take advantage of the live-cell kinetic and confocal capabilities, and can be run in multi-plate high-throughput and/or low-throughput modes. Application wizards help you modify existing applications or develop unique, advanced applications as needed. Additionally equipped with an integrated environmental control module, with active CO_2 concentration and temperature control, the BD Pathway 855 bioimager allows live-cell assays to be performed over extended time periods. In addition, an on-stage, automated liquid dispensing system can carry out scheduled reagent additions – important from an automation perspective, and enabling real-time live cell kinetic assays. The BD Pathway software provides a suite of integrated tools to accomplish the tasks of high-content cellular analysis from image acquisition and analysis to data analysis and visualisation. The image processing, data analysis and visualisation tools included cover a broad range of fluorescence-based kinetic and end-point biological assays. Flexible export of images and data are also supported, allowing you to migrate your data to third party software provider solutions. Of interest for customers accustomed to flow cytometry data, the imaging data can be exported as fcs files (flow data format) for further analysis. Advanced features of the software include: confocal Z stack acquisition;

Figure 6: What's needed to grow the market (increase use and adoption) of live cell & kinetic imaging

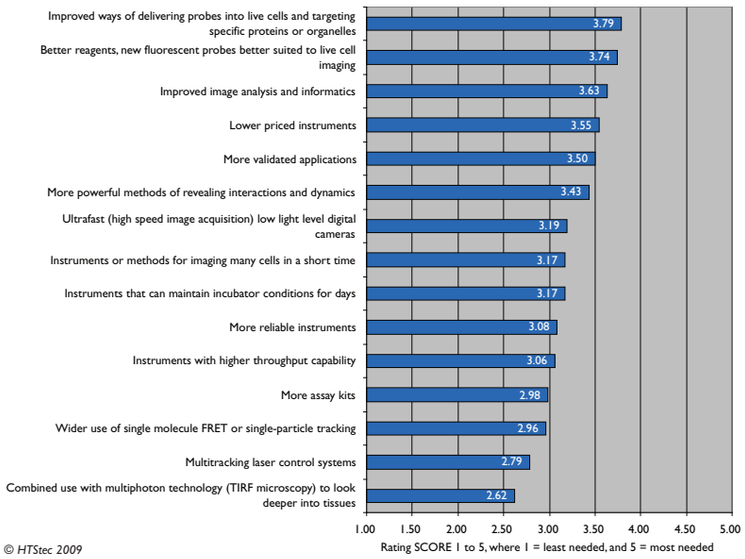


Figure 7: HCS instrument configuration of most interest to respondents

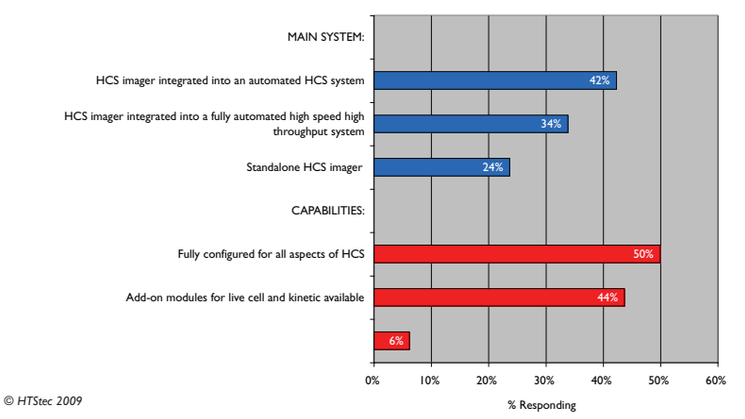


Figure 8
The BD Biosciences Pathway™ 855 high-content Bioimager for analysis of live cell-based assays

3-D visualisation; image montage (tiles); and advanced kinetic control (BD Pathway 855 system), enabling scheduling of compound additions co-ordinated with imaging, allowing live cell dynamics analysis.

The introduction of pharmacologically-validated fluorescent ligands for G protein-coupled receptors (GPCRs) by **CellAura Technologies** (www.cellaura.com) has reopened the debate over the use of ligand-binding versus functional measurements in fundamental drug discovery. In recent years, functional screening has edged ahead because of the growing issues associated with radioactivity, the ease with which functional assays can be undertaken in living cells and the growing realisation that GPCRs can signal via both G protein-dependent as well as G protein-independent

Table 1: Vendors of live cell & kinetic hcs imaging-related technology

VENDOR	LIVE CELL & KINETIC HCS IMAGERS	FULLY AUTOMATED HCS SYSTEMS	HCS-RELATED INSTRUMENT-ATION	REAGENTS, PROBES & ASSAY KITS	IMAGE ANALYSIS SOFTWARE	DATA MANAGEMENT SOFTWARE
BD Biosciences	✓			✓	✓	
CellAura Technologies				✓		
Chipman Technologies			✓			
Definiens					✓	
Essen Instruments			✓			
Fluxion Bioscience			✓			
GE Healthcare	✓			✓	✓	
Invitrogen				✓		
MDS Analytical Technologies	✓			✓	✓	✓
PerkinElmer	✓	✓		✓	✓	✓
Thermo Fisher Scientific	✓	✓		✓	✓	✓
TTP LabTech			✓			
Yokogawa	✓	✓			✓	

(eg beta-arrestin) mechanisms. The implication of this latter point is that the pharmacology at the level of the GPCR is changed by the allosteric actions of the signalling protein (G Protein or beta-arrestin) with which it interacts. However, at the end of the day, functional assays are used to predict the pharmacological properties (ie affinity and efficacy) of drugs acting at the level of the GPCR itself. The use of properly validated fluorescent ligands, now allows ligand binding to be performed in single living cells in real time and provides a direct and unambiguous measurement of binding affinity at the single cell level. This technology also provides the first real potential to make these measurements in primary human cells. However, more significantly, the group at the Institute of Cell Signalling, University of Nottingham and CellAura

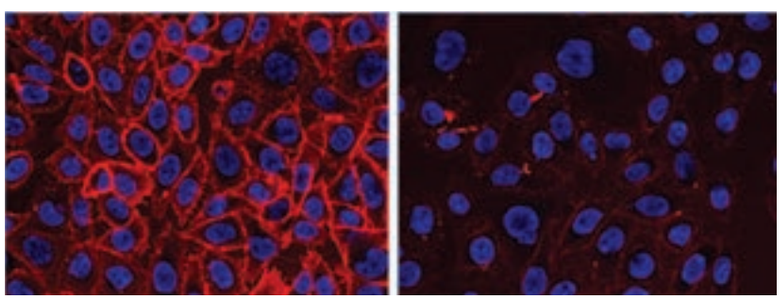


Figure 9: Cell Aura's fluorescent ligand binding in live cells imaged on the PerkinElmer Opera. Left panel: M3-663-AN ligand (30nM) binding to CHO cells expressing muscarinic M3 receptors. Right panel: binding blocked by the unlabelled competitor 4-DAMP (10μM)

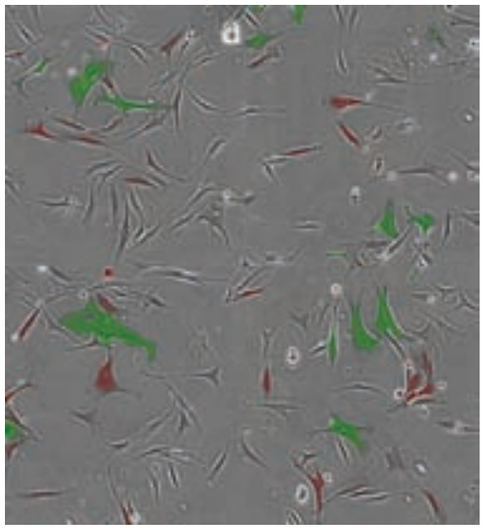


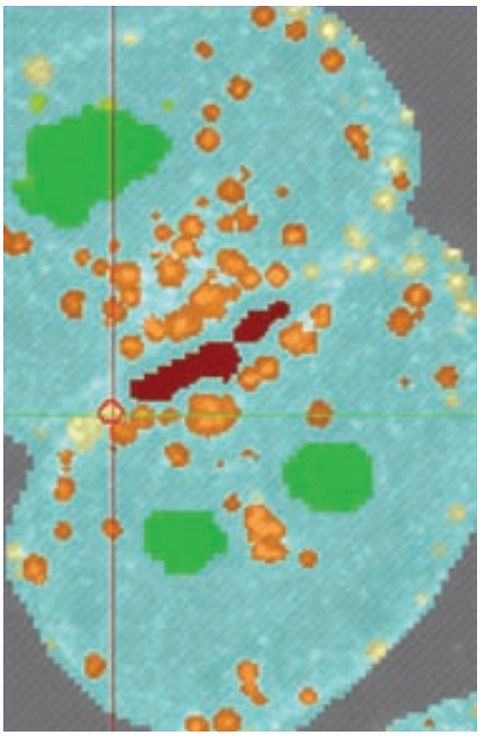
Figure 10: Chipman Technologies Cell-IQ® system with CelLite™ Multi-Label Module (left), Murine dermal fibroblasts transfected with lentivirus for GFP and DSred imaged with Cell-IQ®,(right). Image Courtesy of Dr Tristan McKay, Northeast Embryonic Stem Cell Centre, University of Manchester

has now developed fluorescent agonists that allow both affinity and efficacy to be monitored simultaneously in the same cell[4,5]. The group has also shown that the agonist-bound high affinity active state of a GPCR can be studied using confocal techniques in microdomains of single living cells[5]. The combined use of this fluorescent technology and the availability of high content confocal imaging plate readers (eg PE Opera, MDS Ultra) now opens up the exciting prospect of measuring simultaneously both the affinity and efficacy of ligands acting at GPCRs in single cells and in real time (**Figure 9**).

The need for a system that fulfills the requirements of researchers in the field of HCA combining phase contrast and fluorescence data has been met by a number of suppliers. However as many researchers have found, these systems do not support cell growth for longer than 48 hours, may cause phototoxicity and rely on fluorescence output only for data analysis. Cell-IQ® from **Chipman Technologies** (www.chipmantech.com) has overcome these key issues having been designed specifically to support cell growth under optimum conditions as demonstrated in a number of recent publications where cells can be cultured and imaged for >10 days without problem as seen by researchers at the Weatherall Institute of Molecular Medicine, Oxford. Plates or flasks can be removed to change media but imaging positions are stored so once returned to the system imaging can commence immediately. The dynamic Z-stack allows cells to be tracked in both horizontal and vertical planes making the system suitable for migration studies such as wound healing 'scratch test assay' the movement of cells through membranes or other materials as in invasion or angiogenesis assays, or for studying larger samples such as embryos and stem cell colonies as performed at the Karolinska Institute, Sweden. Analysis can be performed using phase contrast images only due to the proprietary analysis software allowing detailed information to be gleaned and quantified without having to transfect or label the cells. Flexibility in the objective selected enables greater intracellular detail to be derived from images and overlaying of the fluorescence data allows localisation to be determined. The system can image phase contrast and three fluorescence outputs at any one time and filers can be change to increase the range of labels imaged. Cell-IQ can offer not only visualisation of the cells but also provide automatic analysis of that data providing that all-important high content aspect required by today's researchers (**Figure 10**).

Definiens XD (www.definiens.com), released in 2008, is a multidimensional image analysis platform for high content image analysis in 2D and 3D images over time. Definiens XD is built upon Definiens Cognition Network Technology®, which employs domain-knowledge and context information to analyse multidimensional image data automatically. The technology generates semantic networks to describe in detail objects' properties and objects' relationships, providing a greater level of contextual information. However, current image analysis solutions on the market are often narrow in scope, platform-dependent, proprietary and perform well only on simple image analysis tasks. Definiens XD is platform-independent and applicable to high-content screening (HCS). While in HCS workflows the current standard for microscopy involves the production of 2D and 2D elapsed images, the market demand is increasingly moving toward 3D and 3D plus time image acquisition. The Definiens XD image analysis platform enables high content analysis of all multidimensional image data from cell-based assays, including confocal image stacks and confocal image stacks over time. Definiens is a technology partner in the EndoSys/HepatoSys network, a project studying endocytosis and signalling activities in primary hepatocytes. For the project, Definiens Developer XD has been successfully deployed in analysing confocal images from live cell-based assays of hepatocytes. Individual hepatocytes, particles, markers, proteins and organelles were segmented and classified. Distances, distributions, morphological properties and relationships between all these objects were measured and quantified to facilitate the extraction of relevant experimental parameters. Those parameters and relationships are used in experimental design, modelling and simulations concerning endocytosis (**Figure 11**).

Essen Instruments (www.essen-instruments.com) has developed a kinetic imaging product line designed to fit inside a standard cell culture incubator. The motivation for the development of the IncuCyte™ was the need for a non-invasive tool to optimise and standardise cell culture conditions for Essen's internal high-throughput electrophysiology screening assays. The original model, IncuCytePLUS allows for automated acquisition of phase-contrast images in space and time without removing the cells from the incubator environment. The client-server software architecture allows users to log into the system and acquire and analyse cellular images and time-lapse movies from any computer with access to the local network. The integrated image analysis tools provide a convenient method of documenting and quantifying cell growth in a scientific and objective manner.



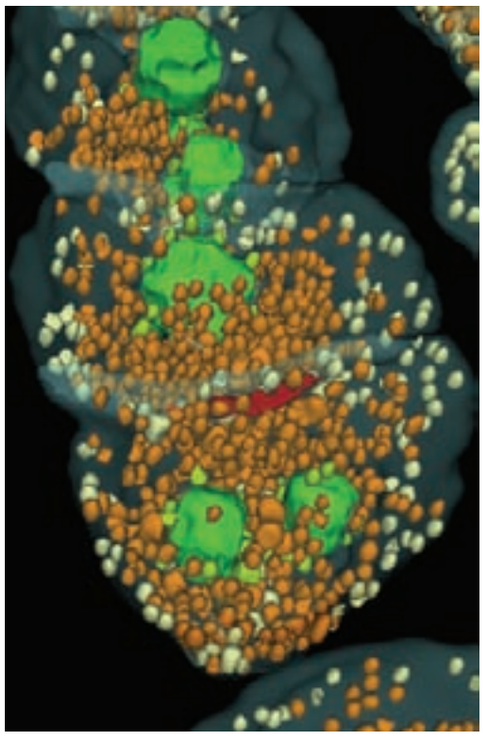


Figure 11
An example of Definiens XD being employed for kinetic analysis of confocal images of hepatocytes, where one confocal image stack represents one point in time. Left image: single slices from the confocal stack are analysed: green: nuclei, red: canaliculi, brown and white: lamp (lysosome-associated membrane protein). Right image: 3D representation of the hepatocytes. Each nucleus is an object. Each endocytotic organelle/particle is an object (single object analysis). Hepatocytes are separated from each other (single object analysis). Each single object carries information about its neighbourhood. For example, clusters of objects are objects themselves and they carry information about their position relative to each other and about the relative positions to other objects and their contents. Distances, distributions, morphological properties of objects and colocalisation measurements are possible and can refer to single objects and interrelations between objects. Images are from the M. Zerial Group MP-CBG, Dresden, Germany, the analysis performed by Definiens

Figure 12: Essen Instruments' IncuCyte™ Imaging system inside a standard cell culture incubator. The IncuCyte™ system enables around-the-clock automated imaging and analysis without removing cells from the incubator

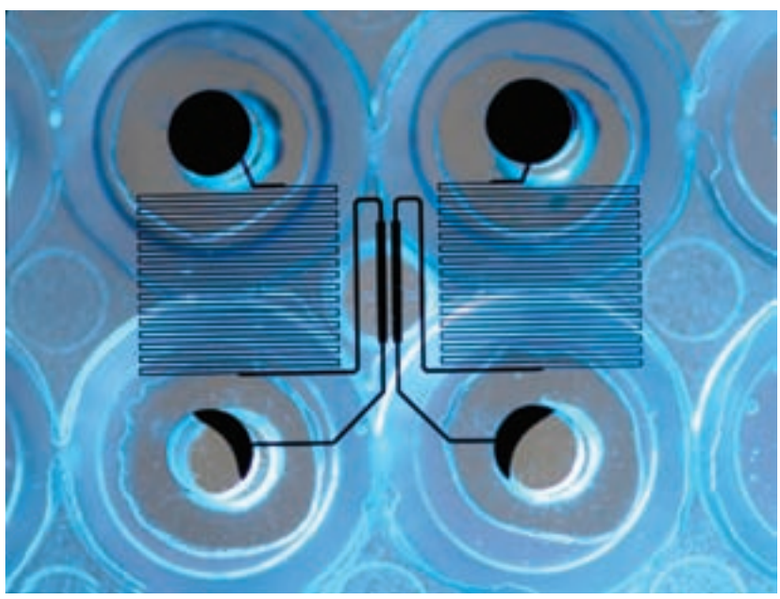


Figure 13: The Fluxion Biosciences BioFlux system utilises a pressure interface to drive flow through microfluidic channels integrated to the bottom of standard well plates. The system includes a fluorescence microscopy workstation adapted for use with BioFlux Plates

IncuCyte™ supports data collection in a variety of cell cultures vessels including slides, dishes, plates and T-flasks. The system also has a multi-vessel tray; allowing users to image multiple vessel types concurrently. Recent innovations include the invention and release of the HD Imaging™ mode. HD Imaging™ is a proprietary imaging technique which allows for high-contrast, non-labelled images even in 96 and 384-well microplates. Traditional phase-contrast image quality is poor in microplates due to fluid-meniscus artefacts. HD Imaging™ is insensitive to these artifacts and can be used to non-invasively assess cell morphology, uniformity and temporal growth characteristics in higher density microplates. Essen has also recently released a fluorescent version, IncuCyteFLR which is designed for long-term imaging of fluorescent reporters. The IncuCyteFLR can be used to continuously monitor a variety of GFP-linked biosensors of cell signalling, growth and differentiation. While the system can be used as an end-point reader, it is most enabling when following reporters over time. Common applications include making GFP cell lines, stem cell differentiation and extended high-content assays such as co-culture angiogenesis (**Figure 12**).

Fluxion Biosciences (www.fluxionbio.com) recently introduced the BioFlux 1000 workstation for high content imaging under biological flow conditions. Like its predecessor, the BioFlux 200, this system offers the ability to run live cell imaging assays under controllable shear flow. It enables the simulation of vascular flow as well as the ability to exchange fluids and compounds across the cell sample in a time-resolved manner. The BioFlux 1000 includes a fully-integrated microscopy workstation which provides added throughput and convenience. Key components of the system include a research-grade inverted microscope, automated stage, cooled CCD camera, automated filter wheel changers, BioFlux pressure controller and a unified software package for hardware control and analysis. The BioFlux system uses BioFlux Plates, which are microfluidic devices integrated in to standard well plate formats. Each device offers the convenience of well plate loading and handling, with the ability to precisely control shear flow across the sample. Up to 96 assays can be run in parallel to accommodate higher throughput applications. The BioFlux 1000 system enables a wide variety of applications in drug discovery and research. Many vascular biology assays will have higher physiological relevance when run under shear flow, as compared to a static well plate assay. These applications include cell

adhesion, transmigration, platelet adhesion/aggregation and high content imaging. Other applications also benefit from flow control and compound exchange, such as anti-microbial screening, stem cell differentiation, cell toxicology and life cycle analysis. BioFlux 1000 software features enhanced image analysis capabilities. In addition to the standard image analysis toolkit, several application-specific modules are available to streamline data processing. These modules include cell scoring, nuclei counting, live/dead analysis, cell tracking, co-localisation and more (**Figure 13**).

GE Healthcare's IN Cell Analyzer (www.biacore.com/high-content-analysis/) continues to improve its live cell imaging capabilities by enhancing its functionality in this area. Live cell imaging is increasingly important in functional studies and the IN Cell Analyzer performs assays using non-fixable probes, capture of rapid events, and environmentally controlled imaging over long periods. The hardware autofocus capability is particularly helpful in cases where there may only be a few stained cells, if any, in the field of view. In environmentally-controlled time course studies, where cell confluence is changing or cells might be expected to die, hardware autofocus is a necessary feature. In addition to the large range of fluorescent live cell markers that can be detected, the platform's transmitted light modalities allow visualisation and analysis of unstained cells. The modalities include standard brightfield, phase contrast and differential interference contrast (DIC) imaging. Customers have the ability to modify the parameters of image acquisition to optimise the degree of contrast and detail required for high content analysis. There are numerous advantages to incorporating transmitted light modalities into high content analysis assays: brightfield analysis, using phase contrast images, can be used for cell counting and morphological characterisation, helpful indicators of cell viability and health. Data obtained using brightfield modalities compares favourably with fluorescent assay results, exemplified in applications such as apoptosis detection and neurite outgrowth. Brightfield imaging can also be readily

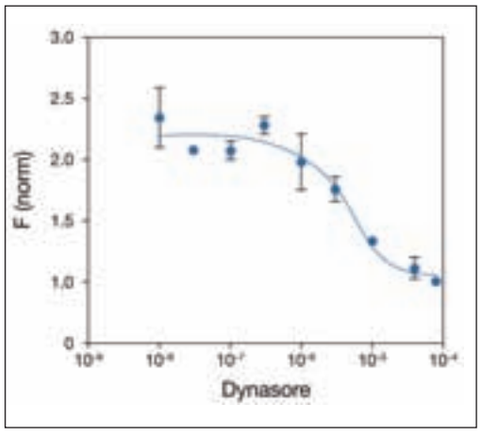
Figure 14: GE Healthcare's IN Cell Analyzer 1000 Autosystem

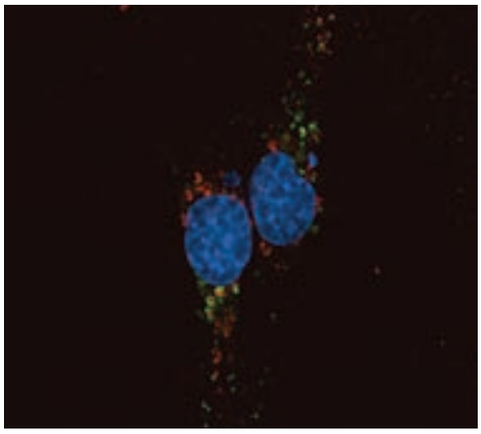
multiplexed with fluorescent images offering advantages for both live cell and fixed cell imaging and analysis. On the horizon for live cell imaging using the IN Cell Analyzer will be the addition of 2- and 3-dimensional image deconvolution. This process produces confocal-like images by removing background fluorescence from sources such as unbound probe, culture medium and plastic plates. The resulting images are of higher resolution and contrast (**Figure 14**).

The powerful combination of **Invitrogen**'s (www.invitrogen.com) fluorescent protein–based Organelle Lights™ reagents and pH-sensitive pHrodo™-labelled proteins, or dextrans, facilitates visualisation of protein internalisation and trafficking in live cells. The pHrodo™ dye is a novel, red-emission pH-sensitive dye that dramatically increases fluorescence as the pH of its surroundings becomes more acidic, making it an ideal tool to study endocytosis and its regulation by drugs and/or environmental factors. Organelle Lights™ reagents are ready-to-use fusion constructs prepackaged in baculovirus particles for highly efficient cellular delivery. Conjugates of the protein of interest (eg, a receptor) are prepared with the amine-reactive pHrodo™ succinimidyl ester. The lysosomes or endosomes are labelled by the addition of the appropriate GFP-based Organelle® Light reagent and overnight incubation to allow expression of the targeted fluorescent protein. GFP is the recommended fluorescent protein, as it can be easily distinguished from the red fluorescence of the pHrodo™ dye. Once the cells are ready, add the pHrodo™ conjugate and incubate while imaging the conjugate as it travels through this dynamic cellular pathway. To study endocytosis using high content imaging pHrodo™, succinimidyl ester was conjugated to an amine-containing 10k dextran and serial dilutions of dynasore were used to create dose response curves for inhibition of endocytosis (**Figure 15**, left panel). More importantly pHrodo can be coupled with other fluorophores to gain a multiparametric analysis of endocytosis. The early endosomes, acidic endocytotic vesicles and internalised transferrin along with the nuclei, were visualised in living HeLa cells using Organelle Lights™ Endosome-GFP, pHrodo™-dextran, Alexa Fluor® 647 transferrin and Hoechst 33342 respectively (**Figure 15**, right panel). In this image it is possible to discriminate acidic and non acidic early endosomes that either contain or lack transferrin molecules.

The ImageXpress^{MICRO} integrated widefield imaging system from **MDS Analytical Technologies** (www.moldev.com) (MDS-AT) includes an optional environmental control (EC) and fluidics module,

Figure 15
Real-time analysis of endocytosis using automated imaging with Invitrogen's pHrodo™ conjugates and Organelle Lights™ reagents





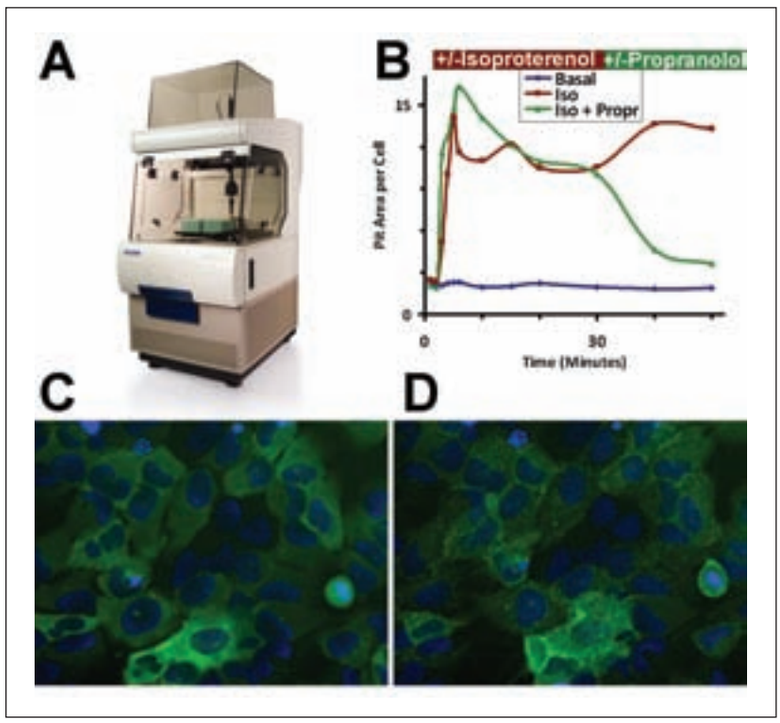


Figure 16: A. The ImageXpress^{MICRO} integrated widefield imaging system from MDS Analytical Technologies including optional environmental control (EC) and fluidics. B: Time course pit formation as U2OS cells are treated +/- isoproterenol (agonist) or +/- propanolol (agntagonist). Pit formation is reversed when live cells are treated with the antagonist. C: Cells at time 0 prior to stimulation show a diffuse staining pattern. D: Cells treated with isoproterenol 30 minutes after treatment

Figure 17: An example set-up of PerkinElmer's High Throughput/High Content platform cell::explorer™. The set-up is modular and scalable, including dispensers, pipettors, washers, centrifuges, incubators and cold plate storage, in addition to a variety of plate readers. A wide variety of plates can be used, from 96 to 1536 well

enabling single-channel pipetting of compound addition and media exchange for rapid kinetics and extended time-lapse experiments. The fluidics option allows precise control of each pipetting event. The system uses 96-well and 384-well format pipette tips to accurately dispensing volumes between 3 and 200µL. The system maintains humidity, temperature and CO_2. The Transfluor® technology, exclusively available from MDS-AT, is an HCS assay that tracks G-protein coupled receptor activation by quantifying GPCR desensitisation and recycling. The Transfluor Assay is an accepted mode of GPCR screening which has been validated on more than 90 different GPCRs and is used in several pharmaceutical drug discovery programmes. The desensitisation and recycling of GPCRs is a 'universal' pathway for all GPCRs, independent of downstream G-protein signaling. The Transfluor assay utilises image analysis to quantify the internalisation of GFP-tagged β-arrestin associated with a GPCR following its activation: unstimulated cells display diffuse cytoplasmic β-arrestin-GFP fluorescence. Upon activation of the GPCR, β-arrestin targets the receptor for internalisation, resulting in the appearance of small fluorescent clathrin-coated pits and subsequently fluorescent endocytic vesicles (if the receptor has a high affinity for β-arrestin). Traditionally Transfluor assays are run as end-point assays. With the addition of the fluidics module to the ImageXpress^{MICRO} system, kinetic experiments can be used to study the formation and regression of pits and vesicles in the Transfluor Assay. The MetaXpress imaging software fully automates both the image acquisition and the image analysis of the resultant image data (**Figure 16**). U2OS cells expressing β-arrestin-GFP and wild-type β2AR (pit-formers) were treated with assay media only or isoproterenol (agonist) plus or minus propranolol (antagonist). The time-dependent responses of the cells were measured. Pit area per cell dramatically increased within one minute of ligand addition and had saturated by five minutes. Pit formation could be reversed by addition of the antagonist propanolol. These results demonstrate that the ImageXpress^{MICRO} equipped with the EC and fluidics module in conjunction with the Transfluor assay is a robust tool in conducting kinetics experiments in screening GPCR modulators.

The Opera™ platform from **PerkinElmer** (www.perkinelmer.com) provides two essential technologies for live cell imaging-based screening: A Nipkow spinning disc, including a microlens enhanced pinhole array for confocal imaging and

Figure 18: Thermo Scientific ArrayScan VTI with Live Cell Chamber and Liquid Handling Module and CataLyst Express Plate robot for live cell high content imaging

high NA water immersion objectives. Both result in very low exposure times, meaning less exposure of the cell sample to light, thus minimising phototoxicity and photobleaching. As an additional benefit, the confocal images deliver unrivalled resolution to high content screening. All versions of the Opera are available with environmental control (control of temperature, CO_2 and humidity), on-board dispenser and software for high throughput kinetic imaging. For repeated measurements of plates over hours or days, PerkinElmer offers a complete Live Cell Workstation including an external incubator and plate handling capabilities. High throughput live cell screening can be achieved using its scalable cell::explorer™ screening platform with various options for plate reading, plate storage, plate incubation, compound and reagent liquid handling, plate washing and centrifugation (**Figure 17**). Live cell high content screening experiments pose specific challenges for image and metadata management. For kinetic measurements using live cells, a series of images need to be taken within a given period of time, which can mean that measurement in subsequent wells are started before the previous well is completed (as with the Opera Kinetics Extension). The demands of acquiring images simultaneously from different wells and of repeating plate measurements for long term kinetics, means that data points and images must be assigned to the specific well, location and time point. The Columbus™ data management system from PerkinElmer, as a true database, deals with these challenges and offers intuitive visualisation tools for time series.

Just like the nature photographer in the wild, capturing cellular responses at specific, physiologically relevant states is a major goal of high content imaging. As the creator and leader of the High Content revolution in cell assays, **Thermo Fisher Scientific** (www.cellomics.com) understands the importance of cell physiology on assay design, implementation and data generation. Although fixed endpoint assays (post biology imaging) have been the workhorse in high content for the last decade, the requirement to image live cells is on the increase in the industry. We categorise these needs by functional requirements in order to more effectively communicate internally and externally. 'Live Endpoint' (single timepoint), 'Kinetic Endpoint' (multiple timepoint), 'Serial Endpoint' (multiple assays in time), 'Combinatorial Endpoint' (multiple methods) represent assay classes where specific hardware, software, and sometimes wetware developments are needed. Thermo's Cellomics High Content Platform, based on the ArrayScan VTI, includes modules to address all these assay classes. Of course, keeping cells alive and happy inside the imager for extended periods is a prerequisite and its Live Cell Chamber Module with full temperature, humidity, and dynamic gas mixing has been in use for several years. A recent advance for the latter three classes is the Liquid Handling Module (LHM) providing functionality to add and remove liquids from wells being imaged using disposable tips. Compound and dye additions, media exchanges, and harvesting can now be automated simultaneously with imaging. Applications from single cell calcium flux assays, to harvesting media for a cytokine ELISA are possible. All modules are cross compatible so adding a CataLyst Express Plate robot and an automated Cytomat 2C incubator creates a complete 'pharmacology tool' for multiple plates (**Figure 18**). Future developments for live cell experiments will focus on optimising the hardware, software and wetware to further minimise phototoxicity while increasing the long term stability of the imaging environment. Beyond the imaging platform itself, Thermo Fisher Scientific uses its core expertise in cell biology to design novel tools that can be applied to the design of live cell assays. Recent advances include Accell, a new way to use siRNA in primary cells over extended timeframes, Upcell, a new growth surface with 'trypsin free' cell release, and a growing portfolio of Redistribution biosensor cell lines to track signalling pathways in live cells.

Microscope-based, high-content instruments offer high optical resolution and are the instrument of choice for single cell kinetic imaging. Live cell analysis, however, is not restricted to single cells

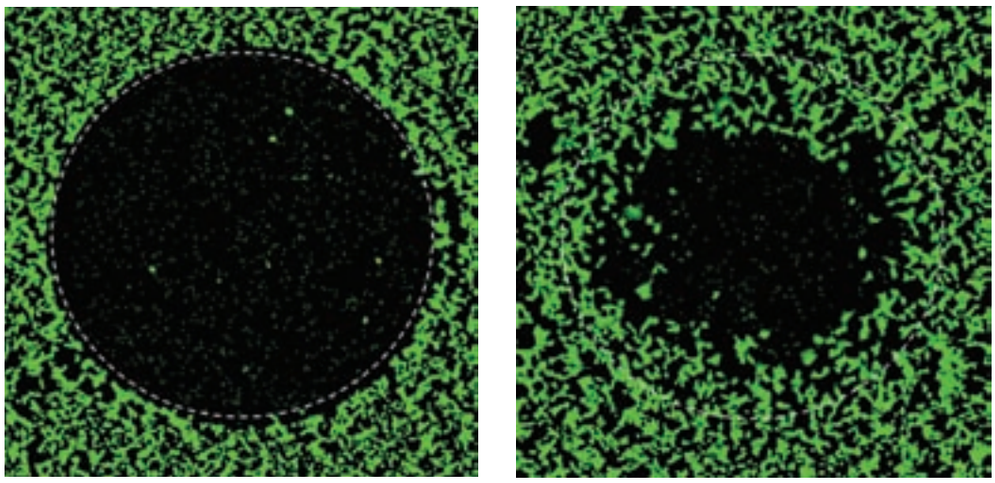


Figure 19
Migration of live HT-1080 cells stained with calcein AM imaged with TTP Labtech's Acumen® eX3 microplate cytometer1. A migration zone was created using a stopper and migration quantified by microplate cytometry. A: 0 hours; B: 16 hours

and for some assays the limited field-of-view afforded by their objectives can significantly affect analysis times and throughput for screening. For example, the detection of rare events ideally requires the rapid acquisition of a whole well of cellular data. In such circumstances, limited field-of-view coupled to slow image capture can be prohibitive without environmental control. In these cases a laser-scanning fluorescence microplate cytometer offers an alternative approach. Instruments such as **TTP LabTech**'s (www.ttplabtech.com) Acumen® eX3 microplate cytometer[6] perform cytometric analysis across entire wells at throughputs of about 10 minutes per microplate. Although sometimes regarded as low resolution devices, TIFF images (8 or 16-bit) generated from unprocessed fluorescence readings correlate well with those captured using a 20x microscope objective. The whole well scanning capability and rapid read times of microplate cytometers are ideal for many live cell imaging applications since they remove the need for environmental control. During primary screening, cell culture plates may be transferred from an automated plate incubator and robotically-loaded into the microplate cytometer. Up to three excitation wavelengths are available in microplate cytometers making them compatible with the growing number of live cell stains and fluorescent proteins that enable the monitoring of cell health and cell signalling events. Increasingly, high content assays are being used to report phenotypic changes associated with disease rather than target-specific end-points. Examples of live cell activity include cell aggregation, differentiation and chemotaxis. A recent innovation is the Oris™ Cell Migration Assay (Platypus Technologies) enabling higher throughput determination of cell migration in microplates (**Figure 19**). Finally, microplate cytometry permits imaging of live multicellular organisms such as zebrafish and *C.elegans* often after sedation to reduce movement during data acquisition.

Yokogawa Electric Corporation (www.yokogawa.com) a world leader in live cell imaging with its real-time confocal microscopy systems, has developed a high-throughput cytological discovery system, named CellVoyager™, which is capable of high-speed and high-resolution imaging and analysis of biological reactions in live cells (**Figure 20**). Yokogawa is a leading company in the process automation and semiconductor tester industry, and has acquired cutting edge technologies for advanced control, mechatronics and reliable system design. CellVoyager™ utilises Yokagawa's core technologies to create a fully integrated system consisting of imaging system, incubator, plate handler, dispenser and analysis unit. This integrated system enhances the efficiency of screening without difficulties in connecting various instruments. The key imaging technology is based on Yokogawa's CSU (confocal scanner unit), a microlens enhanced dual Nipkow disk confocal unit, which allows high-speed, high-resolution imaging with minimal photo bleaching and laser damages, and is highly suited to the analyse cytological changes in live cells in real-time. Equipped with an incubator, and precision plate handler, it is easy to repeatedly monitor various changes in live cells over an extended time period. For example, effects of chemical compounds over live cells could be traced for an extended incubation period which is useful for drug toxicity tests. A single-channel dispenser with disposable tips is useful to monitor

fast biological reactions right after dispensing compounds, such as calcium flux. Measurement of all 96 wells within one minute becomes possible by the integration of high-speed precision positioning and auto-focusing technologies, and simultaneous imaging with three cameras. In addition, quick plate exchange with plate carriers enables high throughput measurement for large scale screening. Since it is easy to switch between confocal, wide-field and phase contrast imaging, users can get appropriate images and results depending on the application. Two types of software are provided; a simple ready-to-use software solution without the necessity of deciding various parameters upfront, and an advanced analysis software package with easy-to-use navigation to set each parameter for complex analysis. CellVoyager™ was released in Japan in the early part of 2009 and will be offered worldwide in the near future.

Summary

There are clearly many obstacles associated with acquiring live cell and kinetic HCS images. Not least among these are: dye toxicity; light-induced effects (phototoxicity, photobleaching, UV excitation); inadequate cell-culture conditions; modification of cell physiology by addition of either dyes or expression of fluorescently tagged proteins (over-expressing phenotypes); slow image acquisition parameters; the sheer volume of data (images) generated; and considerable computer processing requirements[7]. This article has shown that progress is being made in the provision of tools to overcome many of these obstacles and to support live cell and kinetic HCS imaging studies. These include a range of available HCS imagers all equipped with environmental control chambers and on board dispenser options, plus the ability to derive confocal images, features designed to maintain cell health and maximise incubation while minimising light-induced effects (BD Biosciences, MDS AT, GE Healthcare, PerkinElmer and Thermo Scientific). Nearly all these HCS imagers are offered by vendors as part of a wider integrated solution covering imaging instrumentation, software, assays and reagents. Some also offer simple automated workstations linking imagers with an external incubator and/or plate handler (GE Healthcare, Thermo Scientific, PerkinElmer). A few vendors (PerkinElmer, Thermo Scientific) have developed fully automated live cell screening platforms, some specifically developed to enable higher throughput, by upscaling imaging acquisition and the analysis of live cell assays (Yokogawa). There are also several live cell systems (Essen Instrument and Chipman Technologies) which aim to support cell growth under optimum conditions enabling prolonged incubations, but also have the ability to non-invasively acquire images of the growing cells *in situ* in several types of labware, using both brightfield (phase-contrast) and fluorescence imaging. Other systems now offer the ability to run live cell imaging assays under controllable shear flow (Fluxion), which may be particularly relevant to vascular biology assays. Even microplate cytometers, usually associated with lower resolution rapid whole well scanning, have recently been shown to have a role in live cell migration assays (TTP Labtech). The latest instrument software include modules to address the setup and image acquisition from all classes of live cell experiments and image analysis capabilities that increasingly supports 2D and 3D elapsed images, including deconvolution of confocal image stacks over time (Definiens and HCS imager vendors). Live cell HCS experiments and time series

Figure 20
Yokogawa Electric Corporation's CellVoyager™, a high-throughput cytological discovery system capable of high-speed and high-resolution imaging and analysis of biological reactions in live cells

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also pose specific challenges for image and metadata management, these are specifically addressed by several vendors' databases (MDS Analytical Technologies, GE Healthcare, PerkinElmer and Thermo Scientific). Finally, the importance of novel tools and probes that can be applied to the design of live cell assays and delivered into living cells should not be overlooked. For example Invitrogen's fluorescent protein-based Organelle Lights™ reagents and pH-sensitive pHrodo™-labelled proteins, or dextrans, facilitate visualisation of protein internalisation and trafficking in live cells and CellAura Technologies' fluorescent ligands have potential in measuring affinity and efficacy of ligands acting at GPCRs in single cells and in real time. In addition, the investigation of traditionally applied end-point approaches in live cell experiments has proved enabling in conducting kinetics experiments in screening GPCR modulators (eg Transfluor from MDS AT) and tracking signalling pathways in live cells (eg Redistribution biosensor cell lines from Thermo Scientific). In conclusion, if you are determined live cell and kinetic HCS imaging can be done today. However, as was highlighted above, growing this market would seem to depend on many additional technology enhancements and cheaper instruments and if you are to attempt screening mode it will typically require adjustment of many of the parameters pertaining to the image acquisition[7]. Overall, there remains ample opportunity to shift many of today's fixed cell end-point assays to live cell readouts in the future. DDW

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