A multi-faceted approach to the advancement of cell-based drug discovery

Cell-based assays, particularly those involving the use of microscopic imaging, are undergoing a renaissance within many pharma and biotech drug development programmes. Much of this has to do with the licensing, partnering and co-development of new ancillary technologies which then provide for a strong underpinning of several lines of novel investigation. The support structure for today's cell biological endeavours includes advancements in DNA manipulation, viral transfection schemes, post-cryopreservation experimentation, automated cell passaging equipment, high-speed HTS/HCS platforms, siRNA and systems biology. Here, the coalescence and integration of several of these advancements is described in the context of a preclinical pharmacological setting, along with details of the enabling features inherent within each measure.

By Dr Ralph J. Garippa

While possibly not the wisest choice for a military campaign, it seems that those persons wishing to integrate cell-based assays into earnest drug discovery strategies have chosen a path which is paying early dividends and promises to yield an even far greater return on investment in years to come. That path is to move the field forward along several different fronts simultaneously. Perhaps it is a decision borne out of necessity rather than choice really. Or perhaps it is the lessons learned from the implementation of high throughput screening (HTS) assays, ie a 'single point of attack' shifting of the bottleneck to a different area of drug screening has yielded only a partial solution at best[1]. What is now needed are ways to dispel a number of ingrained beliefs such as:

- High variability is inherent in cell-based assays.
- Cells are 'temperamental and unpredictable'.
- Cell-based utility is sketchy at best beyond the use of just a handful of 'well-known', 'go-to' cell lines from ATCC.
- The speed and reliability of cell-based HTS cannot compete with biochemical (non-cell-based) HTS.
- Cells are not predictive of *in vivo* outcomes in multi-cellular, multi-tissued organisms.
- Cell passaging and plating will not adapt easily to automation and robotics.
- It is too expensive and time-consuming to retain high content cell-based data for any length of time.
- Cell-based assays are useful only for secondary and tertiary assays but cannot be of use in selecting the most functionally tractable genomic targets.

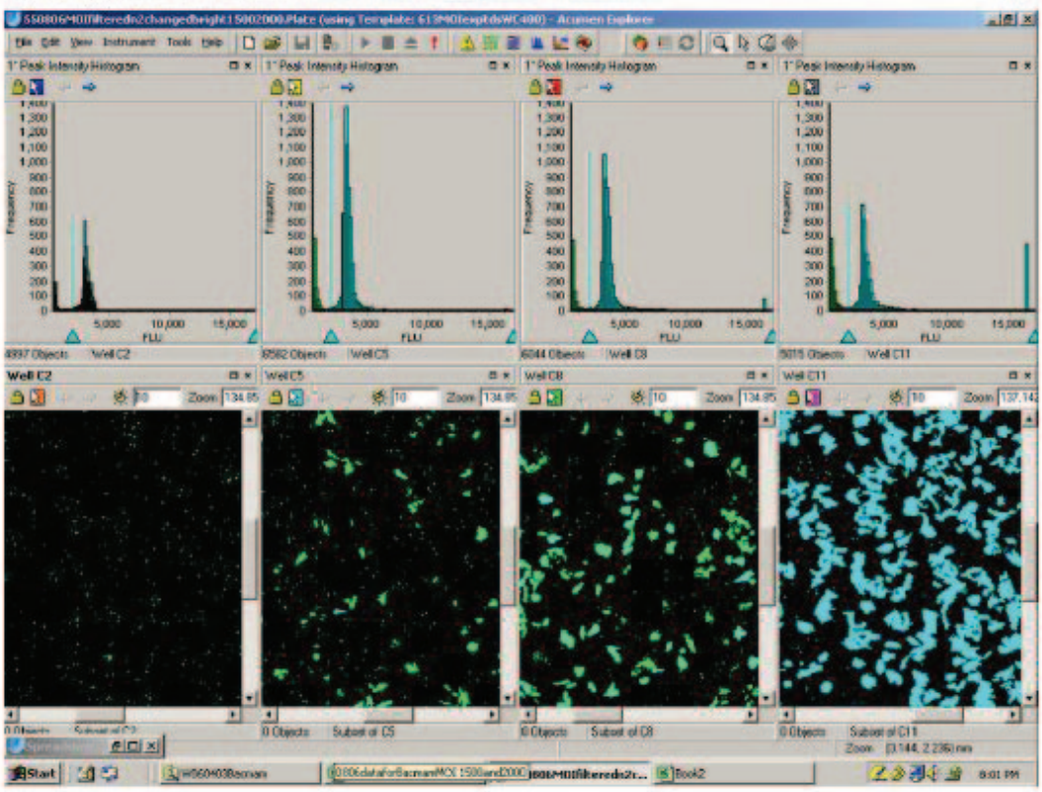

Figure 1
Acumen Explorer software pseudocolor visualisation of U2OS cells transduced with CMV-GFP baculovirus

One by one, each of these so called 'accepted tenants' of Pharma- and Biotech-based cell biology have been quietly dismissed by researchers working hand in hand with vendor companies in the deployment of new technologies to assist in the profiling of nascent drug discovery programmes.

Baculoviruses to the rescue

A particularly nettlesome problem with being able to consistently integrate cell-based screens into mainline drug discovery schemes has been day-to-day variability of the measured output. For this reason, most researchers would prefer to use a stably transfected cell line which is expressing an appropriate amount of a species-specific protein rather than a transiently expressed protein. Such thinking is being challenged by the successful utilisation of transient viral expression systems. In our hands and in the hands of others, the baculoviral system (BacMam) which integrates a number of mammalian elements into the expression vector has shown much promise[2]. In its essence, BacMam screening begins with the cloning of a gene of interest into a shuttle plasmid and transformation into *E coli*. A round of alkaline lysis then yields recombinant DNA which is ready for transfection into Sf9 insect cells. At this point, viral replication can be quantified (~1 x 10^9 pfu per ml^{-1}) and the material stored as a relatively stable frozen stock which can be used time and time again for cell transduction. **Figure 1** shows the results of using ascending concentrations of CMV-GFP recombinant baculovirus to transduce a GFP signal into U2OS cells, as visually quantified on the Acumen Explorer (The Technology Partnership, Hertfordshire, UK). Without chance of further viral replication in the receiving cells, such BacMam work is often regarded as Biosafety Level I in nature. In practice, this procedure is generally not hampered by issues of cytotoxicity and cell vacuolisation following viral transduction. Furthermore, it is applicable to primary freshly-harvested cells and to difficult-to-transfect cell lines.

Putting the DNA in a safe place

One area on which successful cell-based assays are dependent is the ability to confidently integrate exogenous DNAs into host cell lines. Stable cell integration of the DNA of interest is a vital underpinning of assay development towards reliable cell-based screening. We have found two methodologies to be of particular use. In the first example, we have outsourced the cloning of orphan G protein coupled receptor DNAs (oGPCRs) to Cell and Molecular Technologies

(CMT) of Phillipsburg, NJ (USA). In their paradigm, the gene or interest is cloned into a bicistronic vector in which one consciously makes a choice for either an epitope tag, eg CD8, or a fluorescent protein, eg GFP as a marker to gauge both the positive identification and relative expression level of the translated protein[3]. While not a strict one-to-one correlation of fluorescence intensity and protein expression, fluorescently activated cell sorting (FACS, B-D Biosciences, Billerica, MA) enables one to separate the transfectants into high, medium and low expressing pools of cells. The pools can then go through subsequent rounds of enrichment or be parsed via limiting dilution into stable single clonal populations.

In the second example, we have utilised a Cre/lox system of stable targeted integration of exogenous DNA into a pre-selected host cell line. The LoxP recombinase recognition sequence[4] in the expression plasmid is able to recognise and align with the LoxP sequence, one which is already stably integrated within the host Chinese Hamster ovary (CHO) cell line. Thus, the non-transfected blue fluorescent hygromycin-resistant cells (our arbitrary choice for signal in the non-transfected host cell population) can be easily sorted from the transfected neomycin-resistant cells containing the gene of interest. **Figure 2** demonstrates one example of this powerful technology. In panel A, the stably transfected CHO (dhfr-) cells containing the human melanin concentrating hormone receptor (huMCHR) result in a FLIPR-recorded (Fluorescence Imaging Plate Reader, Molecular Devices, Sunnyvale, CA, USA) calcium flux event of approximately 6,000 relative fluorescent units (RFU) when stimulated with 30 nM MCH agonist peptide whereas in panel B the CHOK1 (Cre/lox) huMCHR1 cell line is able to generate 24,000 RFU in response to 10nM MCH agonist peptide. Overall, the Cre/lox cell line demonstrates less variability, ~4X higher signal amplitude, and ~3X greater agonist sensitivity than the optimised non-Cre/lox CHO cell line.

At this point, the obvious alternative to using transfected cell lines should be mentioned, that is, endogenously expressed protein in clonal cell lines. To aid in finding these proteins, investigators have turned to single target expression profiling (STEP) analysis as a means to identify the cell line(s) of highest interest. A final noteworthy mention goes to immortalised primary human cells (Xenotech, Lenexa, KS, USA) as a way to address issues such as studying the protein of interest in a more 'natural' context[5].

Robots as a second set of hands

With the increase in the number of cell-based assays being employed for primary (HTS) and secondary (subtype selectivity, species specificity, or functional HCS readout) assays, investigators have turned to robotics/ automation on the one hand or cryopreservation methodologies on the other as a means to facilitate cell handling. **Table 1** shows a summary metric sheet for one cell-based handling technician at our Nutley NJ site. As can be seen, there has been double-digit growth in the number of 384-well microtiter plates generated for cell-based HTS. This increase is currently being managed by the use of

Figure 2
FLIPR kinetic tracing of agonist-induced calcium signalling via MCH agonist stimulation in two different clonal cell lines

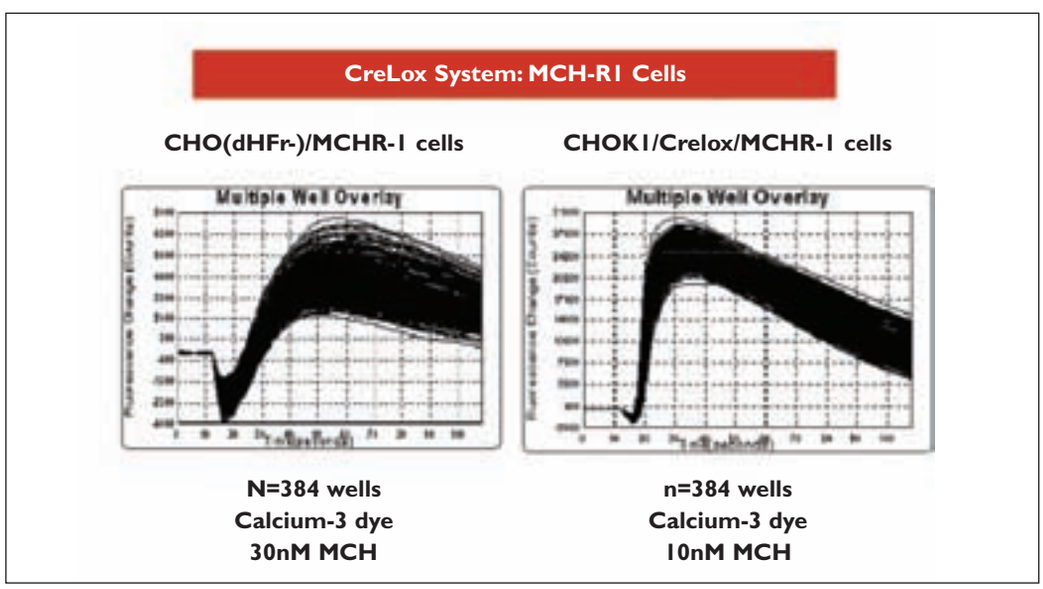

Table 1: Increase in the number of cell-seeded 384 well plates requested for screening

YEAR	# OF CELL LINES HANDLED	# OF CELL-BASED ASSAYS SUPPORTED	CELL-BASED ASSAY OUTPUT:	OUTPUT:
			# OF 384-WELL PLATES DELIVERED	% CHANGE FROM LAST YEAR
2000	3	3	4,024	n/a
2001	13	7	3,982	-1%
2002	6	5	5,430	36%
2003	12	7	7,939	46%
2004	6	6	12,129	52%

multilevel stacking 'cell factories' (Nunc, Rochester, NY, USA) and the assignment of additional FTEs. However, at our Roche Basel facility we have rolled-out the SelecT from The Automation Partnership (TAP, Cambridge, UK). With this automation-assisted cell culture, one technician can schedule plating (96/384/1536) and passaging of dozens of cell lines simultaneously. Although the cell lines, disposables, media and dispersal agents (trypsin or versene) have to be manually loaded, the scheduling feature allows for several hours of unassisted operation. TAP is not alone in this area of endeavour, as the AcCellerator (RTS Thurnal, Manchester, UK), Cellerity™ and Lancellot™ (Tecan, Mannendorf, Switzerland) are now marketing robotic-based cell handling products.

From the freezer to the lab bench

Another myth currently being dispelled is that cells do not function well in screening assays until they have been allowed to 'equilibrate' for several passages following thaw from cryopreservation. Ironically, many who have held to this tenant were perfectly willing to screen adherent cells 16-24 hours post-trypsinisation, a procedure that is

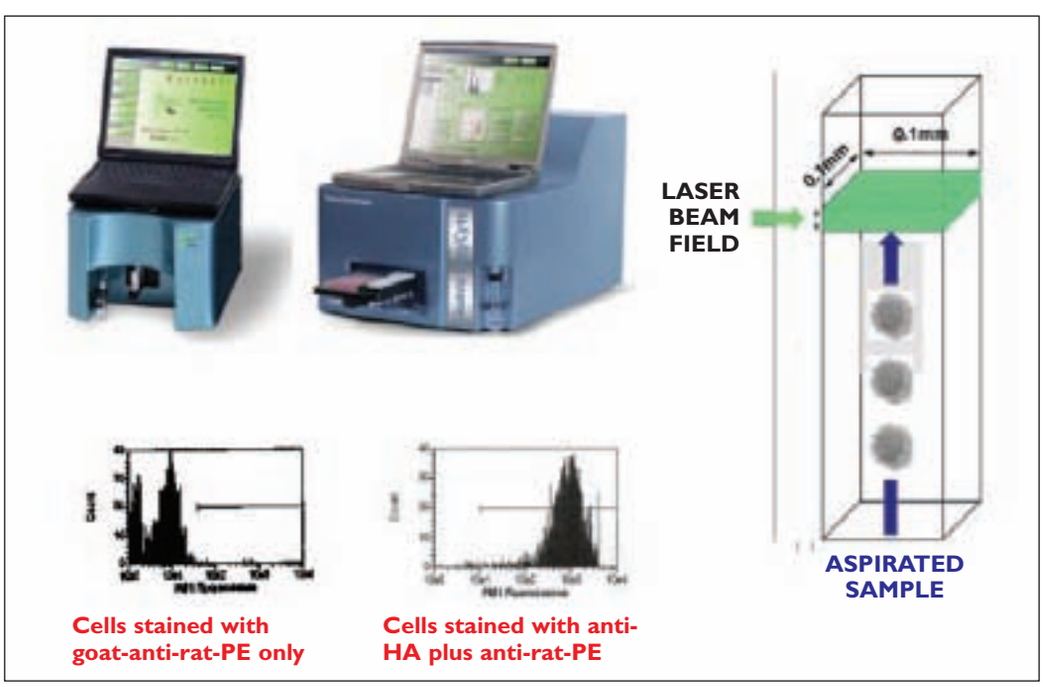

Figure 3
The Guava Personal Cell Analyzer, shown with optional 96 well plate feeder, schematic representation of the sampling principle, and an example flow gram used for quality control

arguably equally stressful to the cells. Recent data[6] have shown that U2OS cells grown in roller bottles to near confluency, trypsinised, pelleted and resuspended in standard freezing media (10% FBS, 10% DMSO) and frozen at -170˚C can be thawed and plated to yield agonist dose response curves that are superimpossible with non-frozen, traditionally passaged U2OS cells. This result has great significance for laboratories wishing to conduct cell-based screens with consistently high Z' values and signal-to-noise ratios. Another benefit is the ability to thaw cells upon demand, like other accepted temperature-protected reagents such as enzymes and antibodies, for use intermittent assays (selectivity screens) or in bona fide HTS. Thus, for the first time, we see the possibility to uncouple the actual cell production steps from their absolute synchronisation with the cell-based screening event.

In as much as it is sometimes desired that the cells being assayed stay at a specified cell density or confluence, CMT has now devised a way to utilise division-arrested cells in a manner similar to the one described above with the additional feature that the frozen cells will not 'overgrow' for several days following thaw and plating[7]. Thus, the overall picture for drug screeners and cell passaging technicians alike is much brighter by virtue of the flexibility afforded by either robotically-assisted or cryopreservation techniques, and one can envision even these two separate approaches being united at one point in the future.

Quality control for cell behaviour

The ability to quickly and easily monitor stably integrated protein expression at the benchtop on the day of a given experiment was dealt a significant boost by the launch of the Personal Cell Analyzer™ (PCA, Guava Technologies, Hayward, CA). The PCA is a microcapillary flow cytometry system that requires a very small amount of fluid volume (~15ul per sample) and cell numbers (~1,000) while fitting into a footprint no bigger than a conventional laptop computer. It requires no sheath fluid and minimal

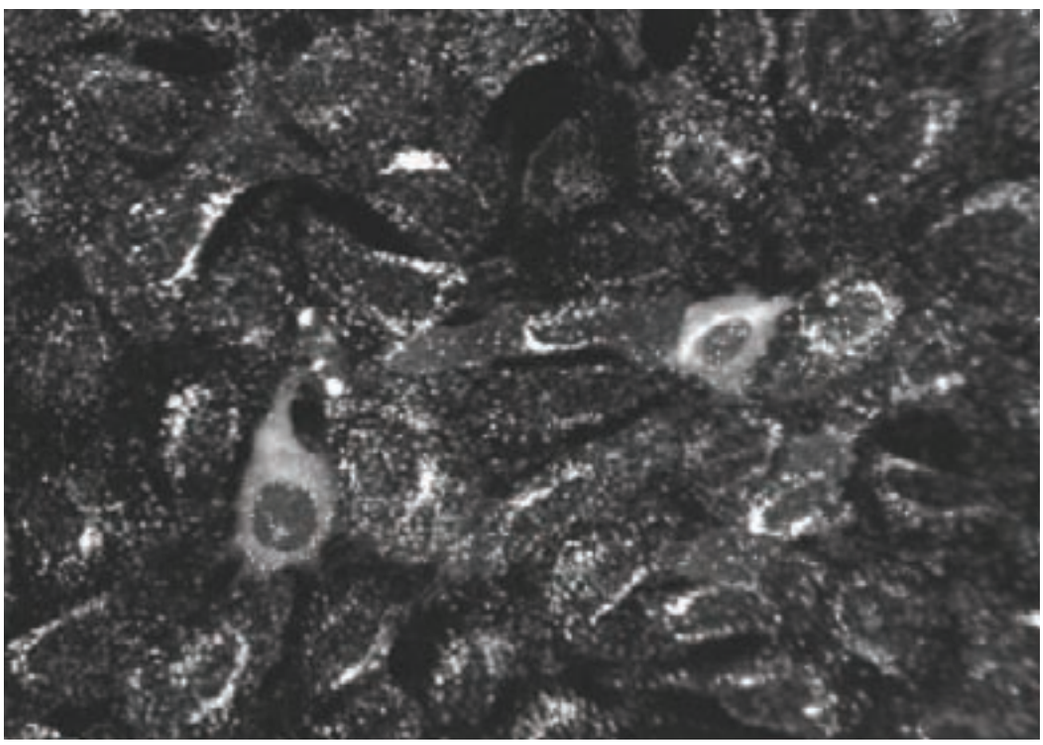

Figure 4
An Evotec Technologies Opera TM -generated confocal image of an orphan GPCR agonist hit identified during a Transfluor™ high throughput screen

maintenance of the flow cell. The instrument is particularly adept to providing a platform for a fluorescently linked antibody-based detection system (primary or secondary to an epitope-tagged HA or FLAG protein or directly to a defined antigenic sequence in that protein) that can be used as a daily quality control (QC) measure in a screening laboratory (**Figure 3**). Consistency in cell-based screening can be affected by cell density, media compositional changes, intra-lot variability of serum sources, temperature and pH changes and a host of other discrete physical stimuli which, of themselves, do not produce any overt morphological warning signs of cellular variability that can be assessed microscopically. But by monitoring protein expression the morning of a given screen, we have been able to de-risk cellular assays for GPCRs and ion channel transporters[8]. Following this QC protocol, compound plates are not thawed and diluted on days in which the cells have not met a pre-established Guava PCA metric, a practice which has saved us thousands of pre-measured compounds as well as pieces of disposable labware which otherwise would have been wasted during a 'predestined to fail' cell-based screening day.

High throughput cell-based assays

While cell-based assays have long been a staple of primary screening in drug discovery, recent years have seen an increase in both the percentage of cell-based HTS as well as the rollout of new screening technologies. Platforms such as scintillation proximity assays (SPA, GE-Amersham, Piscataway, NJ) or FLIPR and VIPR™ (MDC, Sunnyvale, CA USA) have made it possible to test 30,000-50,000 data points per day. Even lofty uHTS metrics have been approached on integrated robotics platforms (Carl Zeiss, Jena, Germany) for receptor signalling assays measuring the generation of cAMP in cells (DiscoveRx, Fremont, CA). Perhaps one of the most promising methodologies to come forth in recent memory is the use of the Norak Transfluor™ assay (Norak Bioscience/ Xsira, Morrisville, NC, USA) to query compound libraries for the presence of GPCR modulatory compounds. In its essence, Transfluor™ utilises a stably transfected U2OS cell line bearing a GFP-labelled beta arrestin[9]. By taking advantage of the universal desensitisation process employed by non-olfactory GPCRs, the investigator is able to overexpress a GPCR of interest in the stable background. GFP-B Arrestin U2OS cells can then

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detect agonist or antagonist compounds by virtue of their ability to elicit coated pit/internalised vesicle morphology (**Figure 4**) or dispersed cytoplasmic localisation in the presence of a known agonist, respectively.

In a further extension of this technology, oGPCRs have been expressed and demonstrated to be functional by use of the Lite™ system, where a constitutively-active G protein-related kinase (GRK) causes non-compound-stimulated movement of 'activated' receptor into pits and vesicles. Since there is little change in the overall well fluorescence in the stimulated versus unstimulated states, investigators have turned to high throughput confocal imaging systems (**Figure 5**) such as the Opera (Evotec Technologies, Hamburg, Germany), the InCell 3000 Analyzer (GE-Amersham, UK) or non-confocal laser line scanning instrumentation such as the Acumen Explorer to speedily and objectively quantify these types of translocation events. Given the early success of the Transfluor™ approach, one may wonder as to the future possibilities for tracking experiments using other stably expressed, fluorescently-tagged transport/signalling molecules.

High content cell-based imaging assays

The ability to quantify subcellular or whole cell-based events on a cell-by-cell basis rather than a well-by-well basis has opened avenues of functional data gathering that were poorly accessible in previous years. As seen in **Figure 6**, we have experienced significant growth in the number of HCS cell-based assays performed in Roche Nutley in recent years, as tracked by the resulting data files. While the definition of 'HCS' has been used by some to describe several non-cell-based technologies that can be performed in multiplexed or higher throughput mode, the comments here will be limited to cell-based assays that have been read on automated imaging equipment, with or without preset, pre-optimised algorithms. Two of the first companies to offer these 'turn-key' bioapplications were Cellomics (Pittsburgh, PA USA) and Universal Imaging (now a part of MDC, Sunnyvale, CA USA). These systems take advantage of the fact that 'resting' cells are in a state of dynamic equilibrium, open to responses to physical and chemical stimuli alike.

Perturbations direct the cell into states of activation, inactivation, or modulation of

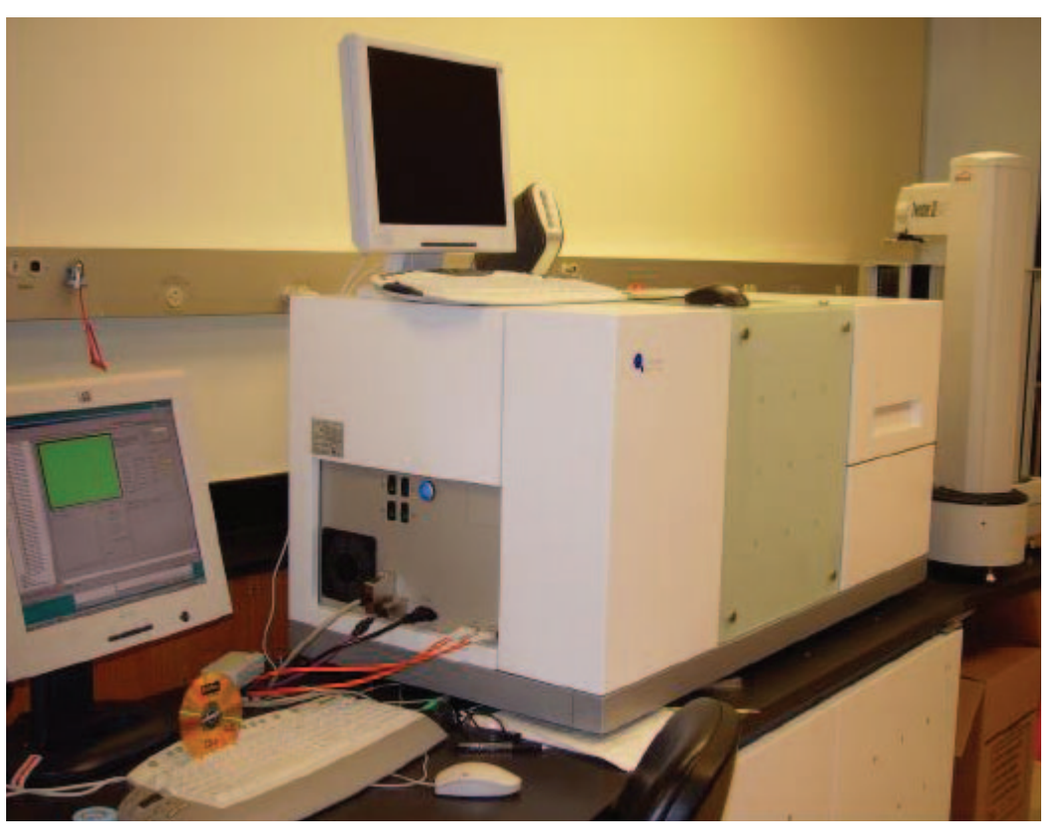
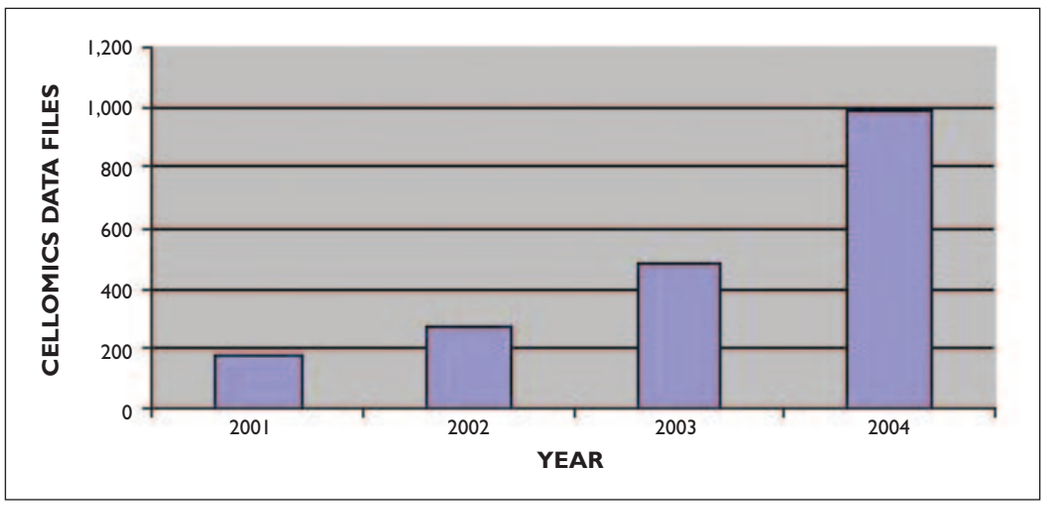


Figure 5
The Evotec Technologies Opera integrated with a Zymark Twister2 plate feeder

Figure 6
Year to year increase in the number of HCS assays run on the Cellomics ArrayScan™ at Roche's Nutley NJ facility



pre-existing processes, each of which can be maintained at a steady-state level. Such processes include cytoplasm to nuclear translocation of nuclear transcription factors, cytoplasm to plasma membrane transport of surface molecules, endocytic and exocytic processing of resident proteins or cellular cargo, morphological events involving actin and tubulin, and discrete phases of the cell cycle. A second set of cellular processes revolve around the cell's ability to contract or expand its plasma membrane, or cell extensions, to spread flat or to ball-up, to anchor itself to a solid substrate (or other cells) versus motility towards or away from a stimulus. A third set of processes may be grouped as cytotoxicites or (the beginnings of) terminal events such as apoptosis, micronucleus formation, necrosis, membrane permeability and mitochondrial integrity.

In each of these cases, there are now established protocols for the objective quantification of such events via automated imaging systems which track molecules via fluorescent microscopy (**Figure 7**) or in brightfield mode (Maia Scientific, Geel, Belgium). Thus, the use of cellular microscopy in drug discovery has advanced beyond the point of being simply a low throughput 'cross-check' of the functional effect of a few compounds to being a proven screening strategy in assembling structure activity relationships for hundreds of compounds. With the development of new dyes and non-cytotoxic (silent) fluorescent proteins linked to proteins of interest at particular junctions in signalling pathways, ie so called 'positional biosensors', we clearly are at the beginning point of full utilisation of cell-based imaging assays[10-12].

Interpreting and revisiting the images

Beyond the development of new algorithms to query cell function, the obvious involvement of informatics in the storage/retrieval of cell-based imaging data as well as subsequent rounds of data mining are areas that have generated much interest. There exists a fairly uniform number of rather familiar algorithms that can be licensed with the purchase of most any HCS instrument. However, researchers are now looking beyond these common 'pre-canned' algorithms for programmes which will help define more intricate biology. Even with the existing algorithms for common applications, eg cytoplasm to nucleus translocation, it is difficult to make a fair across-the-board comparison of one company's algorithm performance with another's. Ilya Ravkin, Founder and CTO of Vitra Bioscience (Mountain View, CA, USA) has stated the problem quite eloquently: "Ideally there should be an open library of images representative of different assays and a mechanism of submitting and publishing image analysis results from different developers. To make algorithm comparison practically useful and not just an academic exercise we need to open (document) file formats for images, metadata and results of processing used in the imaging systems. The effect of it would be far more than comparison of existing algorithms. It would open up the software market for small developers, which could address some less-common assays that do not represent a justifiable opportunity for large vendors[13]." While the image storage issue has been addressed by a number of companies such as Cellomics and Scimagix, the full exploitation of HCS data mining is still in its nascent stages[14].

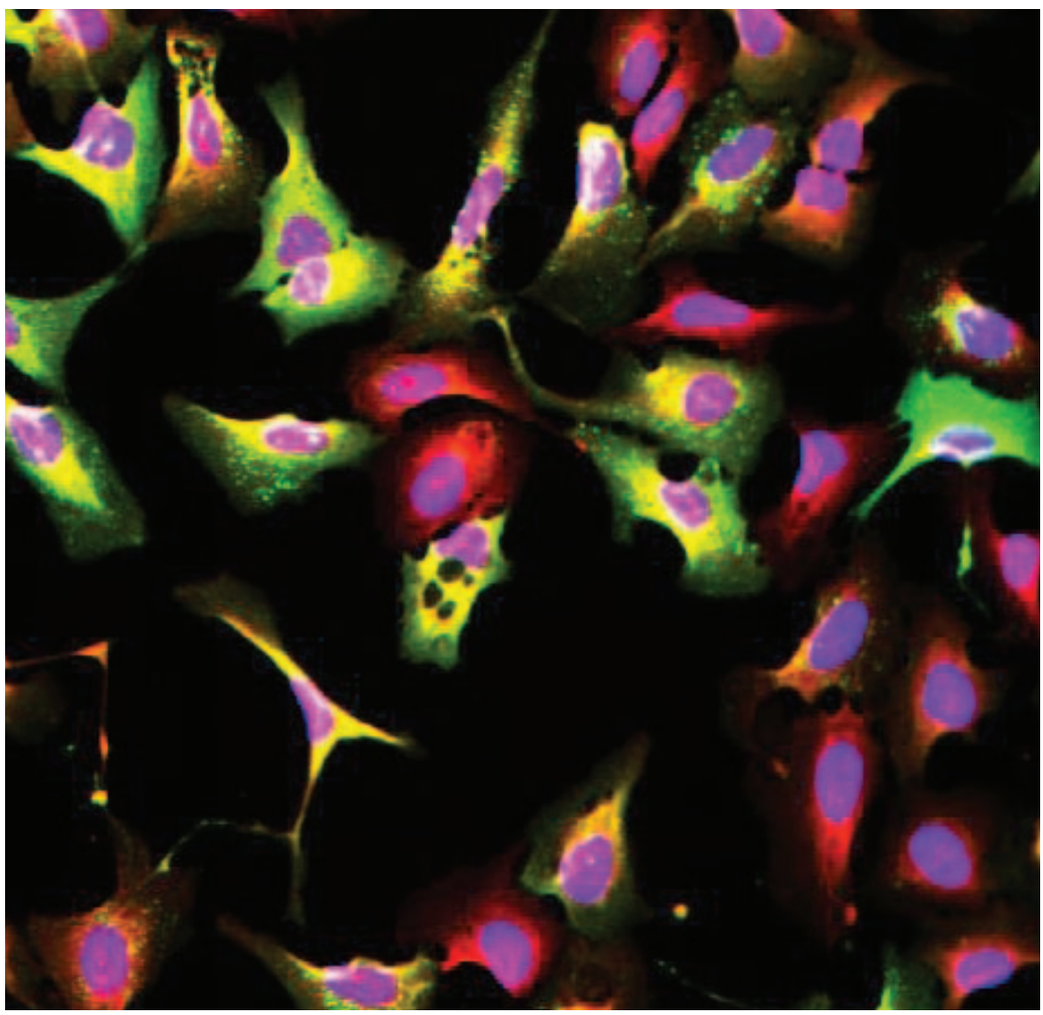


Figure 7
GE Biosciences InCell 3000™ digital image of U2OS cells stained with Hoechst nuclear dye and Mitotracker red stain

Picking the right drug targets

Proponents of siRNA technology and cell-based assays have begun to find a tailor-made partnership in recent years[15]. At the basis of this mutual admiration and synergy has been the discovery that cell-based readouts provide a useful functional context for the gene product knockdown experiments. For example, the metric of assessing the mitotic index (the ratio of cells held in mitotic arrest following a timed siRNA or compound exposure versus non-arrested cells) provides a tool with which investigators can assess the importance of a particular target or family of targets in the cell cycle. A second approach in combining siRNA knockdown with automated visual assessment would be to specifically inhibit specific gene (or gene family) expression and subsequently evaluate the anchorage-dependent three-dimensional growth of cancer cells in soft agar. The automated dispensing of agar and cells is made possible by the C-2 dispensing unit (Titertek, Huntsville, AL, USA) and the automated visual quantification is possible in any number of imaging platforms, making precise measurement of colony diameter possible just a few days following cell seeding. A third example, non-oncology in nature, would be to screen for molecules involved in the translocation of the insulin-responsive GLUT4 transporter. Such proteins would hold significance in the regulation of glucose transport in the diabetic and non-diabetic states. As a recent poll of pharmaceutical executives would suggest[16], there is a need to sort through the many genomic targets becoming available and to then initiate projects involving candidate molecules that have been 'de-risked' as much as possible. The parallel deployment of siRNA and HCS technologies provides one such option to determine disease relevance, particularly for 'one-off' or novel targets.

Conclusion

In a recent year-end communication to me from good friend John Williams of Pfizer[17], he advised the following: "Let us welcome the frantic rush to material celebration as a worthy contrast to highlight the quieter, deeper serenity we gain as we evaluate both our achievements and failures during the past year." I was struck by the profound nature of this statement, of the possibility of being able to harness the undesirable chaos of the holiday season and to channel it constructively into the more desirable pursuits of quiet introspection and intellectual renewal. In assessing the scorecard of cell-based screening in drug discovery, one similarly is challenged to make a concerted, integrated effort to go beyond the obvious shortcomings of the past and to foster the enablement of diverse technologies which will serve to harness the data from these vitally important 'living tools' of drug discovery. DDW

Dr Ralph Garippa is a Research Leader in the Department of Discovery Technologies at Hoffmann-La Roche's Nutley, New Jersey (USA) facility. His cell-based group's mandate within drug discovery is to conduct assay development, run HTS/HCS screens, and provide automation/robotics expertise. He holds a PhD degree from Columbia University in New York City.