Chapter 3

Live Cell Imaging: An Industrial Perspective

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Abstract

The analysis of live cells using automated fluorescence microscopy systems on an industrial scale is known as high content screening/analysis (HCS/A). Its development has been driven both by the demands of compound screening in the drug discovery industry and by the promise of whole genome functional analyses using siRNA knockouts. This chapter outlines the primary applications of HCS/A within the drug discovery process and in systems cell biology. It discusses specific issues which must be addressed when undertaking HCS/A, such as choice of cells, probes, labels, and assay type. Drawing from information gathered from surveys of key users of HCS/A in industry and academia, it then provides a detailed description of HCS/A user issues and requirements, before concluding with a summary of the imaging instrumentation currently available for live cell HCS/A.

Key words: Live cell imaging, automated microscopy, high content screening, high content analysis.

1. Introduction – An Industrial Perspective to Live Cell Imaging

The term "high-content screening", or "high-content analysis", (HCS/A) has been used since the mid-1990s to describe the processes and technologies that allow the automated imaging and analysis of many samples of cells at greatly increased throughput than had previously been possible. This automation of the labour-intensive processes of microscopy imaging and image analysis enabled the pharmaceutical industry to utilise HCS/A to accelerate aspects of the drug discovery process.

A key aspect of HCS/A is the labelling of cells with multiple fluorescent probes. The combination of multiple probes with automation renders HCS/A incredibly powerful as a tool because

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it permits the analysis of multiple parameters at the level of individual cells. This, in turn, enables interdependent cellular processes to be investigated simultaneously, with assays being conducted on large populations of cells. With its revolutionising of the way in which cellular behaviour can be investigated, over the last 10 years HCS/A has become an indispensable tool in both academia and the pharmaceutical industry.

This chapter considers the use of HCS/A with an emphasis on live cell applications. The term "industrial" is used here to emphasise the scale of what is possible with HCS/A technologies, rather than the body or organisation undertaking the work. Thus, in this chapter, both academic and commercial labs are regarded as undertaking live cell imaging on an industrial scale. In offering an industrial perspective on live cell imaging, this chapter starts by describing the research needs that have driven the increases in scale for cellular imaging and explains the various applications for HCS/A technology. Following this, some of the requirements and constraints for live cell HCS/A are explored, together with the technological solutions available to meet these requirements. The chapter then highlights three key issues that must be taken into account when using HCS/A for live cell imaging and concludes by summarising the commercial sources of imaging instrumentation.

1.1. Why Increase the Throughput of Live Cell Imaging?

What is the driving force behind the need to increase the throughput of live cell imaging? In short, it is because there is so much to do. Pharmaceutical companies will be spending 10–15 years and in excess of $1 billion to develop a new drug (S Paul, Executive VP, Eli Lilly & Co, 2006). One effective way to accelerate the process and to reduce the cost is to ensure that the drug candidates that enter pre-clinical and clinical development have the best chance of success. Undertaking compound screening and characterisation in cellular systems with high biological relevance, using assays that allow simultaneous elucidation of mechanistic, toxic, and off-target effects, can achieve this. For basic research applications in academia and the pharmaceutical industry, new ways of manipulating the expression of hundreds or thousands of genes simultaneously using RNAi technologies have created the need for instruments that can screen cellular gene expression libraries rapidly.

Two distinct benefits derive from using cell-based assays. First, cells provide a more physiological environment for analyses. Decisions and conclusions based on data from cell-based assays will therefore be more biologically relevant than those based on results from assays that use purified components or cell fragments. Second, and more profoundly, modern techniques of cellular analysis permit the use of multiparametric approaches to assay development. This means that much more complex

questions can be addressed in a single assay and that greater insight into the mechanisms and pathways underlying a response can be acquired, and acquired rapidly. This multiparametric approach – HCS/A – is being increasingly adopted, firstly by the pharmaceutical industry as an improved means for selecting compounds with the highest potential to become successful drugs, and secondly by academic institutes when applying systems biology approaches to the investigation of gene function at the cellular level.

The motivation for using live cells in high-content assays is the same as in "low-content" experiments, and is related to the dynamics of the pathway under investigation: rapidly changing cellular behaviour cannot easily be elucidated using fixed endpoint assays. Furthermore, when alterations to the time course of an event are being observed, a live cell assay greatly reduces the number of cells/experiments required, since each time point does not require a separate set of experiments. These benefits of live cell experiments are especially important in drug discovery assay development in which the parameters of a screening assay are being determined, and in functional genomics studies investigating the sequential and/or causally related induction of cellular phenotypes. Another benefit of live cell high-throughput assays is the simpler protocols, with fewer washing steps, that permit fast and robust screening assays. Use of fluorescent proteins and live cell-compatible fluorescent probes has made such assays feasible. Other chapters in this book will describe in more detail the advantages of using live cells in imaging experiments.

The sections below describe the work and needs of different users of HCS/A, beginning with the pharmaceutical industry; and they highlight the requirements necessary for success. Information has been gathered from several sources, including published articles, and through discussions held with key users in the pharma/biotech industry and within academia as part of a number of user surveys. In these surveys, structured telephone interviews were conducted with leading scientists active in the HCS/A field to determine major requirements and issues in various aspects of HCS/A.

2. The Drug Discovery Process

Pharmaceutical companies develop new drugs from compound libraries consisting of many hundreds of thousands of different potential drugs. Most of the cost of developing new drugs occurs after a compound has been nominated as a "candidate drug" and becomes subject to stringent preclinical and clinical testing.

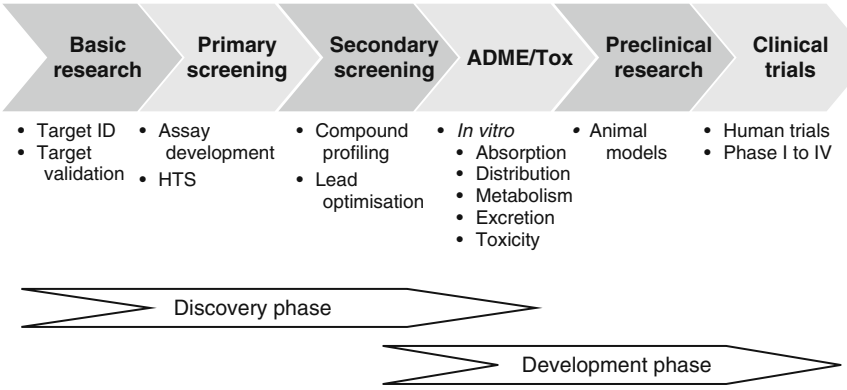

Fig. 3.1. The drug discovery process.

Correctly identifying and rejecting an unsuitable compound before this stage is estimated to save a pharmaceutical company $1–$3 million per compound.

Following selection of a drug target, the process of "compound attrition" begins with the primary screen (*see* **Fig. 3.1**). All compounds from a library are screened in an assay to identify those which might have a beneficial impact on the disease target. It is increasingly common to use a cellular assay at this stage, rather than a biochemical one. Output is from populations of cells in wells, in 384- or 1536-well plates. The positive drugs ("hits") are then further investigated in the secondary screening process. In secondary screening, dose-response relationships are determined, functional mechanisms are examined, and newly synthesised, related, compounds are tested for improved drug properties. Efficacy and safety assessments are then initiated in vitro to determine certain pharmacokinetic and toxicity profiles of the lead compounds prior to pre-clinical development in animal models. The purpose of all of these tests is to identify and discard unsuitable compounds, leaving only the most promising candidates to be nominated as candidate drugs.

2.1. Drug Discovery Phases

2.1.1. Target Identification and Validation

A drug discovery programme begins with the search for potential drug targets (usually proteins) that can be manipulated to modify the expression of a disease phenotype. Potential drug targets must then be validated as truly having an influence on the disease (*see* **Section 3.1** for more discussion of the criteria for validation). Basic research in academia underpins much of the work conducted in pharma/biotech's target identification and validation programmes. The outcome of these projects is a well-characterised drug target and the basis for a high-throughput assay to allow primary screening of compound libraries against this target. Live cell HCS/A tools have become an important component of target identification and validation

because of their capacity to follow changes in cell behaviour over time.

2.1.2. Primary Screening

Researchers involved in primary screening for new drugs test their company's compound library against selected targets, using an assay that will identify those compounds which affect or modulate target function. Compound libraries are commonly full diversity libraries comprising up to one million compounds, although they may be smaller, targeted libraries of a few tens of thousands of compounds. When HCA is used in primary screening, it is commonly against a targeted library. The need to conduct such large numbers of tests creates many of the key drivers that determine the needs of primary screening laboratories.

The purpose of the primary screen is twofold. First, it should identify all those compounds that change the assay signal readout beyond a certain threshold: these are the positive "hits" that will be taken to the next stage of the process. Failure to detect compounds that do actually affect the assay target gives rise to false negatives and reduced sensitivity. Second, the primary screen should reject all compounds that do not alter the assay signal readout. Failure to reject inactive compounds gives rise to false positives, and the assay lacks specificity. The costs of low assay sensitivity and specificity are considerable.

In addition to being sensitive and specific, primary screening assays should have high throughput in order to screen a full library as rapidly as possible (hence primary screening is also known as high-throughput screening or HTS). High throughput is usually defined as the ability to test 30,000 or 40,000 compounds per day on a single instrument, although higher throughput of up to 100,000 compounds per day is desirable. To facilitate such high numbers of assays, the pharmaceutical industry uses a standardised microtitre plate format that accommodates 96, 384, 1536 or 3456 wells on a plate. Of these, the low density, 96-well plates do not allow sufficiently high throughput for HTS, while 384-well is the most commonly used format. Live cell HCS/A assays are generally not compatible with primary screening because they do not permit the high throughput necessary for this stage of drug discovery. The highest throughput HCS instruments (e.g. PerkinElmer's OPERA, Molecular Devices' ImageXpress instruments, GE's IN Cell 3000, and TTP Labtech's Acumen ᶜX3 cytometer) have been used for primary screening with end-point assays in fixed cells.

2.1.3. Secondary Screening

Hits identified by the primary screen are subsequently subjected to additional analyses to determine dose-response relationships and to examine functional mechanisms of activity. These hits are also the starting point for the process of designing new, related

compounds with improved drug properties. The number of compounds subjected to secondary screening is much lower than in HTS, with only 0.1–1% of all compounds from a full diversity compound library expected to pass through to secondary screening. Thus the throughput required from secondary screening assays is much lower, but more data points are required from multiple tests on each compound. Assays designed for secondary screening should seek to maximise the biological relevance of the assay, which usually means developing a cell-based assay if the primary screen relies on a biochemical approach, or alternatively transferring to a more relevant cell model, as appropriate. These requirements make HCS/A approaches very suitable for aspects of secondary screening, which is a well-developed application for HCS tools (1, 2).

2.1.4. Safety Screening

The final stage of in vitro drug discovery is known as ADME/Tox (for absorption, distribution, metabolism, excretion and toxicology). These investigations are designed to assess the suitability of a compound for development into a drug and to identify potentially toxic actions in standardised metabolic pathways. In the context of drug safety, HCS/A is becoming an increasingly significant tool, as "off-target" effects (i.e. side-effects) of potential drug candidates can be assessed more effectively in a multiparametric cellular assay.

3. Systems Cell Biology

Systems cell biology is predominantly carried out by universities, governmental facilities, and not-for-profit research institutes in order to increase our fundamental understanding of cellular processes. This work often feeds the drug discovery conveyor belt of the pharmaceutical industry and is, to some extent, continued by the industry in its target identification and validation programmes.

3.1. Applications of HCA in Systems Cell Biology

A major driver for applying HCA technologies to basic research is the desire to build a comprehensive understanding of cellular pathways both in the "normal" and diseased states. It is believed that this understanding of how pathways function will provide insight into how disease pathology is expressed and how a pathway might be manipulated to ameliorate or cure disease. To build an as complete as possible understanding of a system, researchers will integrate data and knowledge from several fields.

In the context of drug discovery and industrial applications of HCS/A, academic research plays a major role in target

identification and validation (3). Target identification is simply the indication that a gene (and its product) is up- or down-regulated in some way in the disease model, compared with in the control. Target validation requires the demonstration of a functional link between the modulated gene and the onset or expression of the disease phenotype. To be able to establish this link in vitro, the assays used must meet several criteria:

- They must use a cellular system that is a faithful model for the relevant aspects of the disease.
- The assay readouts must accurately reflect the activity of the target and must additionally be related to the disease.
- Methods must exist for modulating the activity or expression level of candidate target genes.

HCA can have a significant impact on each of these criteria. First, it uses a cellular format. Increasingly, the cells being used are not highly transformed, modified cells, but are primary cells – from patients – that accurately reflect the cellular pathology of the disease. Stem cell technology is likely to increase the availability of clinically relevant cells for analysis. Second, HCA utilises multiplexed assays that enable analysis of a complex cellular pathway in a single well. One example is a cellular signalling cascade initiated by a receptor-activated kinase. Here, the activity of a kinase (assay parameter 1) causes translocation of a transcription factor or signalling molecule (assay parameter 2) to the nucleus, which leads to the phenotypic outcome such as apoptosis, mitosis, or differentiation (assay parameter 3, e.g. Ref. 4). Third, in concert with RNAi technologies, the microwell plate format of HCA permits the rapid evaluation of gene silencing experiments on a medium- to high-throughput scale (5–8). It is now possible to target druggable gene families, or even whole genomes, with libraries of small RNA molecules that allow highly effective knock-out experiments, using HCS/A assays that have multiple readouts (9).

4. Needs and Constraints for Live Cell HCS/A

Clearly, HCS/A is among the most promising new technologies for cellular research. However, it is also apparent that obtaining useful and meaningful data requires rigorous attention to detail in experimental design, sample preparation, and choice of assay-, imager-, and analysis-strategies. Some of the specific issues surrounding live cell HCS/A will be considered here, beginning with a description of the experimental process. This is then followed by

a more detailed description of user requirements, obtained from recent surveys of users of HCS/A technology.

4.1. HCS/A Workflow

4.1.1. Cells, Probes and Labels

The first requirement for HCS/A is cells. The cell type chosen must clearly be suitable both for the purposes of the assay and for the screening programme. Cell lines are dynamic entities, susceptible to variations that can affect the conclusions drawn from experiments using them. Provision of well-characterised, stable, phenotypically relevant cells for HCS/A requires significant effort and is costly. The pharmaceutical industry has recognised cellular variability as an issue, and has sought to minimise it in several ways: for example, assay-ready cells can be purchased in bulk, saving significant time and resources on maintaining cell line stocks and the associated requirement for quality control. These well-characterised, division-arrested, frozen cells need only to be dispensed into plates prior to assay. However, many projects will require cells with specific characteristics that are not available "off-the-shelf". In this case, automation may be the answer to increasing the quantity of material available. Essentially all cell culture steps, from simple maintenance and passage of cell lines, through plating into 1536-well plates, to clonal expansion, can now be fully automated, bringing greater consistency.

Currently, almost all HCS/A is conducted using fluorescence-emitting probes. These are either chemical-based small molecules or genetically-encoded fluorescent proteins (FPs). The chemical probes are used either covalently linked to a targeting vector, such as an antibody or a peptide ligand or substrate (e.g. Alexa dyes), or as a diffusible chemical that accumulates in certain regions within cells.

The requirement to add a label of some description creates problems for assay design and for data interpretation. The first problem is getting the probe into the cell. For FPs, the cell line must firstly be transfected with a vector expressing the necessary construct(s), and then secondly it must be shown to be stable and to continue to display the required phenotype. Chemical probes soluble in aqueous solution may require special carriers or procedures to enable them to be taken up by cells. Lipid-soluble probes may diffuse to membrane compartments beyond those targeted. In all cases, a second problem associated with fluorescent probes must be considered, namely the potential impact of the probe on both the parameter that it is being used to measure and on cellular function and behaviour in general. These problems may be more acute and pronounced in live cell assays, but the problem is not confined to this group. Issues with probes are dealt with elsewhere in this book.

4.1.2. Fixed Cell or Live Cell Assays

HCS/A users must choose the most appropriate type of assay for their needs. The choice between using an end-point assay, in

which the cells are fixed at a particular time point after treatment, or using a live cell assay, in which biological processes can be monitored as they occur over suitable time periods, depends on several factors. Fixed cell assays may be easier to perform, and the data are available more rapidly: these form the majority of HCS/A assays. Specifically, where throughput is an important issue in a compound screening or gene library silencing scenario, end-point assays are often more suitable.

Live cell HCS/A assays are more commonly used in lead optimisation and assay development, and in functional genomics studies. Here, it is more important to follow the biology of the system, in order to determine drug action on multiple pathways, and to ensure that an assay truly reflects the requirements of the study. As well as taking a longer time (and therefore having lower throughput), live cell assays are much more demanding technically. Cells are relatively delicate things; they do not like temperature variations (to within 0.5°C); they do not like being shaken around (so robotic plate-moving systems must be slow and gentle); and they need to be both provided with the right nutrients and also to be maintained at the correct pH. All of these considerations have to be built into the design of the imaging system used for live cell assays. One additional complication associated with live cell assays is the need to manipulate or activate cells whilst they are being measured. Some imagers are able to add reagents to cells whilst they are being imaged: *see* **Table 3.1** for examples.

4.1.3. Imaging Instrumentation

Imagers provide the data acquisition core of HCS/A experiments. The first systems were developed by Cellomics in the mid-1990s, and Cellomics were largely responsible for the creation of the field.

Imagers must be able to acquire images, at 4× to >40× magnification, of cells in SBS-standard microwell plates (most commonly 96-well or 384-well plates) and on standard (i.e. 25 × 75 mm) microscope slides or coverslips. It must be possible to acquire images at several (at least three) wavelengths compatible with commonly used probes and to overlay these images to produce a composite image of the field of view. Users sometimes need to be able to acquire several images from one well and to combine them together to create a composite image of the well. A system should provide for the automated movement of the sample carrier so that images can be taken from each well (or cell array spot) in turn, and the plate- and well-identification data can be stored with the images. In addition, there should be a means to integrate the imager with an external robot that can supply and retrieve microwell plates to and from the imager, before and after image acquisition. For imagers used in live cell assays, the environmental needs of cells must also be accommodated, as described above. HCS/A imagers create very large volumes of data and

Table 3.1.
Summary of HCS imaging instruments

Manufacturer	Instrument	Confocal?	Detector	Light source (s)	# λ_{ex}	# λ_{em}	Environmental control	Liquid-handling	Optimised for live cell analysis	Transmitted light	Robotics integration compatible	Auto-focus method	Objectives	Plates and slides
BD Biosciences	Pathway-855	Yes – true confocal	1× CCD	White light	16	8	Yes	Yes	Yes	Yes	Yes	Laser	Olympus – 4× to 60× air	Both
	Pathway-435	Yes – true confocal	1× CCD	White light	8	8	No	No	No	Yes	Yes	Laser	Olympus – 4× to 60× air	Both
Compucyte	iCyte – laser-scanning-cytometer	No	4× PMT	4× laser	3	6	No	No	No	Yes (scatter)	Yes	NA	10×, 20×, 40× air	Both
GE Healthcare	IN Cell 1000	Optional – structured light	1× CCD	White light	6	6	Optional upgrade	Optional upgrade	With upgrades	Optional	Yes	Laser	Nikon – 4× to 40× air	Plates. Upgrade for slides
	IN Cell 3000	Yes – true confocal	3× CCD	2× lasers	3	3	Yes	Yes	Yes	Optional	Yes	"Dynamic"	?	Plates
Molecular Devices	ImageXpress Ultra	Yes – true confocal	PMTs	2 to 4× laser	4	?	No	No	No	No	Yes	Laser	Nikon – 4× to 100× oil	Both

	ImageXpress Micro	No	1× CCD	White light	5	5	Optional upgrade	Optional upgrade	With upgrades	Optional	Yes	Image, laser option	Up to 4 Nikon – 4× to 100× oil	Both
Olympus Europa	Scan^{R}	No	1× CCD	White light	8	6	Optional upgrade	No	With upgrades	Yes	Yes	Image, laser option	Olympus – 2× to 100× air	Both
PerkinElmer	Opera	Yes – true confocal	3× CCD	4× laser	4	4	Optional upgrade	Optional upgrade	With upgrades	No	Yes	Laser	10× to 60× water	Plates
	Opera LX	Yes – true confocal	1× CCD	3× lasers	3	3	Optional upgrade	Optional upgrade	With upgrades	No	Yes	Laser	10× to 60× water	Plates
Thermo-Fisher Cellomics	ArrayScan VTI	Optional – structured light	1× CCD	White light	10	5	Optional upgrade	Optional upgrade	With upgrades	Optional	Yes	?	Zeiss – 2.5× to 40× air	Both
With Applied Precision	cellWoRx	Digital deconvo-lution	1× CCD	White light	4	4	No	No	No	No	Yes	Image	10× or 20×	Both
TTP Labtech	Acumen ^{e}X3 – laser scanning cytome-ter	No	4× PMT	3× lasers	3	4	No	No	No	Yes	Yes	NA	Equivalent to 20× maximum	Both

All data from manufacturers' websites, January 2009. NA: not applicable; ?: no data available.

many images: 1 terabyte per month is an acquisition rate commonly cited by users. The data must be stored on non-volatile media, be readily accessible from the analysis programme user interface, and be easily and securely identified with the experiment and with the plate and the well from which they were acquired.

Three basic types of instrument are in common use for HCS/A assays. Two of these are based on the imaging capabilities of fluorescence microscopy, and they generally rely on CCD cameras for image acquisition. One type uses standard wide-field optics, and another uses high resolution confocal optics. A third type of instrument creates images using a scanning laser beam, in a manner analogous with laser scanning confocal microscopes, but does not use confocal optics. This technology is not commonly used for live cell imaging and will not be described further here. Other approaches to instrument design are discussed below and instrument features are summarised in **Table 3.1**.

The most common type of instrumentation in use is the wide-field CCD imager: this might be described as the default system. The wide-field CCD imager provides sufficient resolution for the majority of HCS/A assays but, where precise colocalisation of probes is required, the image depth of field is too great to provide sufficient accuracy. The relatively thick depth of field does mean that, except for very large cells or 3D aggregates, all of the cellular fluorescence is collected and contributes to the recorded signal. However, there is also the possibility to collect fluorescence signals from the medium bathing the cells, especially if the compounds being screened for activity in the assay are, themselves, fluorescent. This phenomenon of drug-compound fluorescence can significantly both decrease the assay signal-to-noise ratio and increase the between-well variability. Wide-field systems are less expensive than confocal systems, and some manufacturers have designed optional "confocal-like" upgrade components: for example, Cellomics offers the Zeiss Apotome structured light image enhancement system as an upgrade. Such "confocal" upgrades generally both significantly increase image acquisition time and decrease overall system throughput.

True confocal HCS/A imagers provide the highest resolution available for cellular analysis and are able to take "optical slices" from the sample. Some of these systems are compatible with water-immersion objectives (to 60×, PerkinElmer Opera) or oil-immersion objectives (to 100×, Molecular Devices ImageXpress^{ULTRA}). As suggested above, there are two primary advantages to the confocal format. First, precise colocalisation (or separation) of probes is possible, and this can enhance the performance of certain types of assay. Second, almost all of the fluorescence collected comprises signal emitted from the probes because the optical sectioning rejects any background fluorescence emitted by screening compounds. This means that the signal-to-noise

ratio is enhanced, even though the signal itself is somewhat attenuated. Of the confocal imagers currently available, two use multiple cameras to capture images at different wavelengths simultaneously (PerkinElmer's OPERA and GE's IN Cell 3000). This feature means that these systems have among the fastest imaging speeds and highest throughput of all HCS/A imagers. The OPERA uses Yokogawa's Nipkow disc-based multibeam scanner to enable high-speed imaging, whilst the IN Cell 3000 uses a slit-scanner system. The other CCD-based confocal imager (BD's Pathway855) also uses a Nipkow disc, but uses a white light source and is without microlenses. The Pathway855 has only one CCD and does not match the acquisition speed of the OPERA and IN Cell 3000. Molecular Devices'' ImageXpress^{ULTRA} confocal HCS/A imager uses a conventional point-scanning confocal beam and is also marketed as a high-throughput system for primary screening.

4.1.4. Image Analysis and Data Management

Critical to effective utilisation of HCS/A are both the software tools for extracting information from images and the informatics and data storage solutions used to process and store the images. Usually, several images are collected from each well of a 96- or 384-well plate, and each is processed with software routines that recognise features and extract data regarding those features. These objective, numerical data are then used to quantify and compare the effects of compounds on the biological assay output. Such detailed measurements of cellular behaviour define the phenotype expressed by the cell, and describe the changes elicited by compound addition or by RNAi-mediated gene silencing.

The complex process of describing quantitatively the phenotype of a cell requires image analysis algorithms optimised for the biology under study (cell type, assay format, etc.). Also necessary are sufficient computing power to ensure fast processing of many images; fast and efficient data storage and retrieval systems; and an intuitive, easy-to-use interface that allows biologists to interact with the system to extract useful and meaningful information from it. HCS/A users require several key elements to implement an effective image analysis and data management programme. These include the following: fully validated algorithms that accurately describe the biology; access to new algorithms; and the ability to develop new (or adapt existing) ones themselves. Data storage solutions must be scalable to meet future needs (current data accumulation rates can be 1 terabyte per month) and must allow for image storage for 5 years or more. A commonly cited (but seemingly unmet) need is for effective image databasing tools that allow full integration between the images, their associated metadata, and complementary data such as genomic or proteomic data sets.

5. Users' Requirements and Issues

Information on users' requirements was obtained from discussions with experts active in the field of HCS/A, from both academia and the drug discovery industry. **Table 3.2** summarises the key attributes for HCS/A and the most commonly expressed requirements associated with each attribute.

There is a clear consensus from discussions with HCS/A users that the areas of image analysis, data mining, and data management provide the greatest frustration for users and that vendors have so far failed to meet users' needs in these areas.

5.1. Users' Issues

The technical issues raised in discussions with users cover many aspects of HCS/A workflow. These discussions suggest that successful implementation of HCS/A requires developments in several areas, including improvements to instruments, to increase productivity. These technical issues are summarised in **Table 3.3**, and selected points are discussed in more detail below.

5.1.1. Maintaining Cell Viability

For some kinetic applications, cells need to be maintained in a healthy and functioning state whilst being illuminated in the presence of organic fluorophores and/or fluorescent proteins.

Table 3.2
Key user requirements for HCS/A

Attribute	Requirement
Sample volume/throughput	At least 5000 data points/day and, for many users, >10,000 data points/day is preferable. No difference in needs between basic research and drug discovery.
Resolution	All users want to be able to resolve subcellular structures at high resolution. Most also want the flexibility to use lower resolution if assay permits it.
Number of wavelengths/ probes	At least four detection channels are strongly desired, with significant emphasis on flexibility of wavelength selection.
Image quality	Absolutely critical to have excellent image quality, as all analysis depends upon it.
Live cell imaging	Currently around 25% of HCS/A experiments use live cells.
Kinetic analysis	Essential element of live cell analysis, mostly in the "minutes" and "hours" time frames.
Image analysis algorithms	Image analysis algorithms should be both simple to use and customisable.
Data management	A source of great frustration for users is inadequate data management systems. Currently there is great scope for improvement on existing systems.

Table 3.3
Technical issues identified in user discussions

Issue	Description	Potential Solution(s)
Speed: image acquisition	Kinetic analysis requires the acquisition of several images per second to gain an accurate view of cellular responses. It should be possible to add drugs while images are being taken.	Acquisition of multiwavelength images requires multiple detectors *and* on-board dispensing technology to add compounds whilst imaging.
Speed: throughput	The throughput demands of both drug discovery and basic research continue to rise. Throughput is particularly an issue for kinetic experiments, where time courses must be acquired from each well.	Consider parallelisation of imaging – but this would add significant cost.
Image analysis	Many issues to address: image formats; throughput; availability of new algorithms; ease of development of new algorithms; lack of effective data-mining tools; availability and analysis of metadata.	Requires consensus on image formats – OME appears to be only contender. Multiprocessor computers are needed. Several vendors are addressing these issues.
Image quality	Poor image quality seriously affects image analysis capabilities. The main cause of low image quality is variable, imprecise autofocus. Other causes cited were as follows: cell biology (uneven monolayers and cell clumping); plate flatness; and poor discrimination of fluorescence channels.	Laser-based autofocus is the most effective: it is increasingly available on instrumentation. Stringent quality control is required on plates and cells prior to assay. Laser-scanning systems could implement spectral separation such as that seen on point-scanning confocals.

These applications enable detailed analysis of cell proliferation, differentiation, and cytotoxicity. However, these applications also demand mechanisms for assaying cell plates over long time periods (hours and days), with full environmental control. In addition to the environmental conditions that must be maintained to keep cells alive, the cells must also be protected from excessive illumination by the fluorescence excitation source. There is considerable stress imposed on live cells by the light used for illumination, since fluorophores are prone to photodegradation and, once destroyed, are lost for imaging within a cellular sample. Furthermore, the breakdown products of photodegradation can seriously damage cellular processes. These "photobleaching" and "phototoxicity" effects can affect reproducible imaging for screening quite critically, and must be minimised.

Two aspects of imaging must be considered in minimising the unwanted effects of illumination. First, determining the

correct focus position in the *z*-axis can increase phototoxic and photobleaching effects. There are two methods for automated focussing. Image-based focussing uses a z-stack of images taken through the focal plane to determine the z-position of maximum intensity or contrast. This approach requires many subsequent exposures in every position to be imaged, and it is not well suited for live cell imaging because it takes considerable time and bleaches the sample. The preferred option for live cell screening is to employ an infrared laser as a light source, and a detector to observe reflections from the interfaces between sample carrier and medium. The second consideration is the mode of excitation during image acquisition: the more the sample is illuminated, the greater the extent of phototoxic effects and loss of signal. These effects are cumulative and can be a significant problem in long-term live cell assays. The optimum mode of illumination for maintaining cell viability is to use high-frequency pulses (~300 Hz to ~1 kHz) of relatively low intensity illumination, rather than continuous illumination (wide-field microscopy) or low frequency (1–10 Hz) illumination of high intensity (point scanning confocal, *see* Ref. 10). In practical terms, the optimum illumination mode for long-term imaging is a multi-beam confocal configuration, such as provided by Nipkow disc systems including the PerkinElmer OPERA and the BD Biosciences Pathway. Whichever mode is selected, imaging systems for live cell HCS/A should utilise high NA optics and sensitive detectors, to minimise the illumination required. Intelligent scheduling of imaging steps is also important, to ensure that cells are exposed to the illumination beam for the shortest possible time.

5.1.2. Improving Speed

There are three main technical requirements for high-speed imaging in HCS/A. First, it is important that data from different emission wavelengths can be collected simultaneously. Second, the detectors should have high sensitivity to allow them to detect signal in the very short time available for image acquisition. Finally, the signal should be well discriminated from the background noise. It follows that a high-speed HCS/A imager should have multiple detectors and that these should be of sufficient sensitivity for simultaneous acquisition of images at several wavelengths. Confocal optics will improve the signal-to-noise ratio by rejecting out-of-focus light and have been shown to improve the performance of high-speed imagers, such as GE Lifesciences' IN Cell 3000 and PerkinElmer's Opera.

Further increase of the throughput of HCS/A, especially of live cell HCS/A, can probably only come through the parallelisation of detection channels, i.e. using more objectives. However, this is likely to be a very expensive solution, and it is not clear whether there is sufficient demand for it at present.

5.1.3. Image Quality

HCS/A users who participated in the surveys discussed here identified several factors contributing to poor image quality. Some of these factors were quality control issues relating to cell biology and consumables, such as the clumping of cells and the design of microplates with insufficiently flat well bottoms. Two issues relevant to instrument design were problems with autofocus and the poor discrimination of fluorescence emission. Inconsistent performance of autofocus image-based systems was the most frequently cited cause of poor image quality. Manufacturers appear to be responding to this complaint, with more recently introduced systems benefiting from higher performance laser-based autofocus, or offering this as an optional upgrade. A second problem identified by the user surveys is poor discrimination of wavelengths. This is usually a problem for fluorescence emission but, where white light is used as an excitation source, proper discrimination must also be implemented in the excitation path. Emission wavelength selection in HCS/A instrumentation is universally made with high-quality band-pass filters. In principle, laser-scanning instruments have the option of adopting spectral separation of emitted light, as seen in many point-scanning research confocal microscopes. Using spectral separation should provide better discrimination of emitted fluorescence signals than is currently possible. For camera-based systems the options are more limited, and selecting alternative probes with larger Stokes' shifts may be the best route to improving wavelength discrimination.

6. Instrumentation

Since the inception of HCS/A, the predominant imaging format for instrumentation has been based on the wide-field fluorescence microscope light path, using a CCD camera detector, and filters and dichroic mirrors to select and discriminate wavelengths. When coupled with automated microplate-handling hardware and suitable acquisition and analysis software, this arrangement meets effectively the basic requirements for automated high-content image acquisition and analysis.

Recent product launches have seen the wider availability of confocal (or confocal-like) technology, either as upgrades to existing systems or as newly developed instruments. Cellomics has incorporated Carl Zeiss's Apotome structured light image enhancement system into its ArrayScan system and has also partnered with Applied Precision to develop a high-resolution cell analyser using deconvolution. GE has introduced an optical sectioning module as an accessory for the IN Cell 1000. This mod-

ule also uses a structured light system to increase resolution. BD Biosciences has launched lower cost instruments, which have spinning disk confocal capability. Arguably the most innovative development in confocal HCS/A imaging is Molecular Devices' ImageXpress^{ULTRA} which, paradoxically, is based on standard, single beam, point-scanning confocal technology that was first commercialised in the 1980s. This instrument uses a galvanometer for scanning in the *x* co-ordinate and the movement of the stage to scan in the *y*. Like standard research beam-scanning confocals, it uses multiple PMT detectors to perform simultaneous detection of light from several different wavelengths: this provides the potential for high-speed imaging.

There is a range of suppliers and instruments available for HCS. They are summarised in **Table 3.1**, and a brief description of those suitable for live cell imaging follows.

6.1. BD Biosciences

BD markets two instruments for high-content imaging, both of which utilise single CCD camera detection and a Nipkow disc-based confocal system. The Pathway 855 system is designed for all types of analysis, including live cell kinetic experiments. Features include the ability to make compound additions to the plate as it is being imaged, along with full environmental control. The Pathway 435 is a bench-top instrument designed primarily for endpoint assays with fixed samples.

6.2. GE Healthcare

GE's IN Cell 1000 and 3000 instruments incorporate options to support live cell imaging, including environmental control and integrated liquid-handling. The IN Cell 1000 may also have a structured light image enhancement module added on, to provide confocal-like images. GE's other HCS/A system is the IN Cell 3000: this uses multiple CCD camera detectors and a confocal slit scanner to provide high-throughput automated imaging.

6.3. Molecular Devices

The ImageXpress product range comprises a CCD camera/white light illumination system (ImageXpress^{MICRO}) and a point scanning confocal system with up to four laser lines (ImageXpress^{ULTRA}). The ImageXpress^{MICRO} has several upgrade options for live cell imaging, including a more sensitive camera, full environmental control, and single-channel pipetting for kinetic experiments. These options are not yet available for the ImageXpress^{ULTRA}. Both instruments are compatible with high-NA oil-immersion objectives up to 100× magnification, providing high resolution.

6.4. Olympus

Olympus' Scan^{R} system was developed by EMBL in Heidelberg and is based on Olympus microscopy components (automated microscope and white light source) and Hamamatsu CCD cam-

eras. It has environmental control options for live cell imaging, but it is available only in certain countries.

6.5. PerkinElmer

The Opera system is a multi-beam confocal system with four CCD detectors and laser illumination. Multiple detectors confer high acquisition speeds for multiparametric assays; optional environmental control and dispensing accessories provide facilities for automated live cell imaging applications. Fluorescence lifetime imaging and FRET analyses are also a possibility with this system. The Opera LX system is very similar but has only one detector, so multiple wavelength images are acquired sequentially. The LX has the same live cell imaging capabilities as the Opera. The systems are compatible with high-NA water-immersion objectives to 60× magnification.

6.6. ThermoFisher Cellomics

The ArrayScan V^{TI} HCS reader is the fifth generation of Cellomics' instrumentation. It is a white-light, single-CCD detector system compatible with objectives up to 40× magnification. For live cell applications, upgrade modules are available for environmental control and liquid handling.

7. Summary

Technologies are commercially available that enable live cell imaging on an unprecedented scale, permitting the imaging and analysis of thousands of samples in a single day. Such HCS/A instruments have been in use in the pharmaceutical industry in one form or another for a decade; they are now extremely well-proven. HCS/A has additionally found applications in academic institutions, often used in concert with RNAi gene-silencing libraries, for functional genomics analyses.

Several manufacturers produce instruments, with associated accessories and consumables, mostly designed around a standard epifluorescence light-path with a CCD detector. Some systems include laser scanning confocal optics and multiple CCDs or PMTs. Further increases in the scale and consistency of live cell imaging are expected to come from the automation of both cell line maintenance and the preparation of cells for HCS/A assays.

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