

## **II**

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### **INSTRUMENTATION, BIOLOGICAL APPLICATION SOFTWARE, AND SAMPLE PREPARATION**

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## 4

### Requirements, Features, and Performance of High Content Screening Platforms

Albert H. Gough, and Paul A. Johnston

#### Summary

High content screening (HCS) platforms integrate fluorescence microscopy with image analysis algorithms and informatics to automate cell analysis. The initial applications of HCS to secondary screening in drug discovery have spread throughout the discovery pipeline, and now into the expanding research field of systems cell biology, in which new manipulation tools enable the use of large scale screens to understand cellular pathways, and cell functions. In this chapter we discuss the requirements for HCS and the systems that have been designed to meet these application needs. The number of HCS systems available in the market place, and the range of features available, has grown considerably in the past 2 yr. Of the two general optical designs, the confocal systems have dominated the high throughput HCS market, whereas the more cost effective wide-field systems have dominated all other market segments, and have a much larger market share. The majority of available systems have been optimized for fixed cell applications; however, there is growing interest in live cell kinetic assays, and four systems have successfully penetrated this application area. The breadth of applications for these systems continues to expand, especially with the integration of new technologies. New applications, improved software, better data visualization tools, and new detection methods such as multispectral imaging and fluorescence lifetime are predicted to drive the development of future HCS platforms.

**Key Words:** Automation; confocal; drug discovery; fluorescence; high content screening; high throughput; imaging; microscopy; systems cell biology.

#### 1. Introduction

High content screening (HCS) is a major new advance in cell analysis technology introduced by Cellomics (Pittsburgh, PA) in 1997. Fluorescence labeling technologies were first combined with electronic imaging technology more than two decades ago and have been used extensively in basic research to study individual cells by light microscopy (1–3). However, the innovation that drove the development of HCS, and what distinguishes HCS systems from the many confocal and wide-field microscopes, is the integration and automation of the entire analytical process. HCS platforms automate the capture and analysis of fluorescence images of millions of individual cells in tens of thousands of samples on a daily basis, and have made fluorescence-based cell analysis compatible with the needs of drug discovery (4,5), and systems biology (6). There are now more than 10 models of HCS imagers established in the market (see Table 1). Used in

**Table 1**  
**Features of HCS Systems**

System vendor	Optics	Autofocus	Optical sectioning	Cost (\$\$)	Other features
<i>Single end point HCS</i> ArrayScan <sup>®</sup> VTI (Cellomics)	WF	Software	Apotome	\$\$	–
Discovery-1 (Molecular Devices)	WF	Software Laser <sup>a</sup>	Software	\$	Brightfield <sup>a</sup>
InCell 1000 (GE Healthcare)	WF	Software laser		\$\$	Dispenser <sup>a</sup> Incubation <sup>a</sup>
Cell Lab IC 100 (Beckman Coulter)	WF	Software video		\$\$	
ImageExpress Micro (Molecular Devices)	WF	Software Laser <sup>a,b</sup>	Software	\$	Brightfield/phase contrast <sup>a</sup>
CellwoRx (Applied Precision)	WF	Software	Software	\$	Uses cellomics bioapplications.
<i>Kinetic HCS</i> KineticScan <sup>®</sup> (Cellomics)	WF	Software laser	–	\$\$	Pipetor incubation
ImageExpress 5000A (Molecular Devices)	WF	Software laser	–	\$\$	Pipetor incubation
Pathway HT (Becton-Dickinson)	WF CF	Software	Nipkow disk	\$\$	Pipetor incubation
<i>HT-HCS</i> InCell 3000 (GE Healthcare)	CF	Laser	Confocal	\$\$\$	Injector incubation
Opera (Evotec)	CF	Laser	Disk scan confocal	\$\$\$	–

The currently available HCS systems can be categorized by target application area, single end point HCS, kinetic HCS, and HT-HCS. Features listed are limited to those features, which distinguish systems. All systems use either wide-field (WF) optics, confocal (CF) optics, or provide both. The approximate system costs are indicated as \$ < \$200K, \$\$ = \$200K–\$500K, or \$\$\$ > \$500K, but will depend on the specific configuration.

<sup>a</sup>Optional features.

combination with appropriate probes, antibodies, fluorescent protein fusion partners, biosensors, environmentally sensitive probes, and stains. HCS systems can be applied to many drug target classes that might be configured for simultaneous multiple target readouts (multiplexing), and can provide information on distributions and cell morphology, in addition to many other fluorescence parameters. Image-based assays therefore provide multiparameter quantitative and qualitative information beyond the single parameter target data typical of most other assay formats, and thus are referred to as high “content” assays. In recent years there has been a growing trend in drug discovery toward the implementation of cell based assays, in which the target is screened in a more physiological context than in biochemical assays of isolated targets (7). Fluorescence microscopy, whether confocal or wide-field, is one of the most powerful tools that cell biologists can use to interrogate biomolecules and investigate the molecular mechanisms of the cell (8). HCS platforms are therefore being deployed throughout the drug discovery process; target identification/target validation, primary screening and lead generation, hit characterization, lead optimization, toxicology, biomarker development, and diagnostic histopathology. Furthermore, these platforms are now spreading into the research markets for applications in high throughput biology (9–11).

The integration of new technologies in drug discovery by the pharmaceutical industry typically occurs in their high throughput screening (HTS) groups. New technology integration is largely driven by a need to increase throughput and capacity, the ability to enable screens for a previously intractable drug target class, or to provide a novel method to rescreen high-priority targets in which the hits from other HTS formats fail to progress to quality leads. Dependent on

the complexity of the assay, automated imaging platforms might only provide relatively modest throughput (10–30 K/d) when compared with other assay formats that are compatible with high (10–100 K/d) or ultrahigh (>100 K/d) throughput (7). However, some high content assays are also high throughput (12). Certainly imaging assays have provided a means to address many intracellular target classes (kinases, phosphatases, proteases, and so on) that were previously challenging in cell-based HTS formats, and have provided novel methods to screen some target classes (e.g., GPCR's) that were well supported by other assay formats (8,12). Owing to the relatively lower throughput and higher complexity of the multiparameter data generated, discovery scientist initially deployed HCS platforms for secondary and tertiary cell-based assays in hit characterization and lead optimization. However, as the HCS platforms and reagents have continued to mature, and as the adopters of the technology have gained more experience and understanding of its capabilities, HCS has been applied throughout the drug discovery pipeline.

The automated, multiplexed cell assays possible on HCS platforms are applicable not only in drug discovery, but also in basic biomedical research, especially in the emerging field of systems cell biology (13–15). The analysis of complex cellular pathways and cell functions requires large scale experimental approaches that take advantage of powerful reagent tools such as siRNA (5) and controllable gene expression systems (16) for manipulating biological systems, in conjunction with HCS systems such as those described here. Although fluorescence imaging microscope systems are nearly ubiquitous in cell biology research labs, the applications have predominantly involved detailed, high resolution analysis of macromolecular structures, localization of cellular components, and measuring the dynamics of cellular functions, in a reductionist approach that has been used to identify and characterize many pathways, and molecular intermediates (17). However, understanding the complex interplay between these molecules and pathways, and the emergent cellular functions, requires running larger numbers of multiplexed assays in a much more automated way than is possible on current research imaging systems. As a result, the application of HCS systems in the academic research market is expected to grow rapidly over the next few years.

The market for HCS technologies has grown to more than \$100M and includes the instrumentation for automated image capture, reagents for sample preparation and staining, image/data analysis software, image/data visualization software, and image/data database applications to manage, and store the very large amount of data that these platforms can generate. In the past 2–3 yr the HCS instrument market has consolidated with many of the large HTS instrument vendors acquiring the smaller independent imaging platform manufacturers to provide their offerings in the HCS field. Amersham Biosciences (Piscataway, NJ) acquired Imaging Research Inc. and Praelux Inc. as the foundation of their In Cell Analyzer (INCA) 1000 and 3000 platforms, respectively, and then was acquired by GE Healthcare (Giles, UK). Molecular Devices (Sunnyvale, CA) acquired Universal Imaging and Axon Instruments as the basis of their Discovery-1 and ImageXpress 5000 platforms, respectively. Beckman-Coulter (Fullerton, CA) acquired Q3DM for their EIDAQ-100 platform, and Becton-Dickenson (Franklin Lakes, NJ) acquired Atto-Biosciences for their Pathway-HT platform. Even Cellomics (Pittsburgh, PA), the last of the independents and the company that created the HCS market, has recently been acquired by Fisher Scientific (Hampton, NH). However, even as this first round of consolidation is coming to a close, a new group of companies like Blueshift Biotechnologies (San Francisco, CA) and Applied Precision (Issaquah, WA) are entering the market with a new crop of novel HCS systems.

Although all of the HCS platforms were designed to meet common functional requirements discussed more in detail later, a diverse array of imaging technologies that have been integrated to meet these requirements. The systems discussed in this chapter will be limited to automated fluorescence imaging microscope systems designed for use in HCS. We will review the functional requirements that drive the development of HCS systems, and discuss the various solutions

that have been used to achieve these requirements. It is important to keep in mind that the HCS imager is only a component (albeit an important component) of a larger assay platform that includes cells, reagents, image analysis applications, informatics, and automation (*see* related Chapters in this volume).

The goals of this chapter are to:

1. Provide an introduction to the fundamental features required of all HCS systems;
2. Identify the key features which distinguish current HCS systems; and
3. Introduce new technologies that are likely to, or should be integrated into HCS systems to provide expanded functionality.

## 2. System Requirements and Components

All HCS platforms require a process for the input and output of multiple microplates, mechanisms to position plates on a stage, the ability to position wells over the optics with precision and reproducibility, a method to capture quality images, image analysis applications (algorithms), data review tools, a mechanism for data management, and further analysis and visualization. The solutions offered by the many vendors of HCS systems vary in the degree to which all these components are integrated and provided as part of a complete screening system. Here we will focus on the HCS imagers and software; discussion of the applications and informatics tools can be found elsewhere in this volume. The basic requirements for an HCS imager are the following:

1. Sufficient resolution and sensitivity to capture and analyze the cellular features of interest;
2. A field of view large enough to image multiple cells;
3. Spectral channels to distinguish multiple fluorescent labels;
4. Adequate speed to meet the needs of the planned screening volume; and
5. Flexibility to address a wide range of assay requirements.

Unfortunately, these requirements cannot be addressed independently, but are interrelated, and therefore, the optimum design and configuration of an HCS system for a particular assay requires an understanding of the relationships between these requirements.

### 2.1. Biological Application Requirements

The basic cellular features of interest for HCS assays (at present) include subcellular localization and distribution; fluorescence intensity, and intensity ratios; texture within regions; cellular and subcellular morphometrics; and the total count of a particular feature, such as nuclei or cells. Numerical combinations of these features are also useful features, as are the correlations between features, for example, colocalization. HCS platforms must be designed to acquire images with sufficient contrast, resolution, and signal-to-noise ratio to allow image algorithms to extract these features of interest. These features span a wide range of sizes, from hundreds of nanometers, to hundreds of microns for colony features. A typical mammalian cell, attached and spread on the bottom of a microplate might be on the order of 20–50  $\mu\text{m}$  across, with a nucleus of about 5–10  $\mu\text{m}$  in diameter. One hundred of these cells in a confluent layer occupy an area of about 400  $\mu\text{m}$  on a side, depending on the cell type, plating density, and amount of spreading. Assays vary widely in the maximum cell density that can be used. A cell-spreading assay naturally requires subconfluent cells, whereas receptor activation assays can often be done using highly confluent cells. The analysis of 100 to as many as 1000 or more cells, occupying an area as large as several square millimeters, might be needed to achieve an adequate level of statistical significance.

The area of a cell 30  $\mu\text{m}$  in diameter is about 700  $\mu\text{m}^2$ , or 700 pixels if sampled with 1  $\mu\text{m}$  resolution. Except for very fine details, 700 pixels per cell provide more information on the spatial distribution of components within the cell. More resolution might make the edges of a feature sharper or measures of the absolute size of an object more accurate, but chances are the

inherent variability in the biological feature or its labeling, will be much greater than the variability resulting from a small degree of under sampling. Optimally then, an HCS system would have a field of view of several square millimeters with resolution around 1 $\mu$ m. Of course, some assays will require more or less resolution than this as discussed later.

## 2.2. Fluorescence Imaging and Multiplexing

Multiwavelength fluorescence imaging, which provides the ability to specifically label and detect multiple cellular components in a single preparation, is a key enabling technology for HCS (15). Although other imaging modes, such as phase contrast or differential interference contrast could be used in the context of an HCS assay, the limited availability of specific molecular markers, and analysis algorithms has provided less incentive for vendors to incorporate these imaging modes into HCS systems, although a few have (*see Table 1*).

All HCS platforms provide the capability of imaging multiwavelength fluorescence, acquiring wavelength channels either sequentially or in parallel. Most systems use sequential acquisition, in which each fluorophore is separately excited and detected on a single monochrome camera. For assays with reasonably bright fluorescent labeling this method can be moderately fast and is the most cost effective. Channel selection is commonly accomplished using a fast excitation filter wheel combined with a multiband emission filter; however, single band emission filters can be used to improve selectivity. The majority of systems come with filters for common fluorescent probes and can distinguish up to four labels in a single preparation with minimal crosstalk between channels. Crosstalk arises from overlap in the spectra of the fluorophores, and the bandwidth and nonideal performance of filters (18). The imaging performance of an HCS system, especially in terms of sensitivity, signal to background and signal-to-noise, will depend strongly on the selection of the fluorophores and the filter performance. The optimum filter configuration would be designed precisely for a specific set of fluorophores, the light source and the detector spectral sensitivity (19). However, systems normally come with filter sets designed for a handful of the most commonly used fluorophores, and thereby meet the needs of most assays.

Two HT-HCS systems, the INCA 3000 (G.E. Healthcare), and the Opera (Evotec Technologies), are available with multiple cameras, which allow simultaneous imaging in three or four emission channels, respectively. The multichannel detection allows these systems to scan multicolor HCS preparations very fast. Taking advantage of this parallel detection capability; however, requires careful choice of fluorescent labels to avoid spectral overlap. This is especially problematic with near UV excited fluorophores like DAPI and Hoechst DNA labels that have broad blue emission bands, which overlap the green fluorescein (or green fluorescein protein [GFP]) emission channel. Of course the blue channel can be acquired independently, but this diminishes the speed, and therefore the value, of the parallel detection capability. Proposed combinations of fluorophores should be carefully evaluated for crosstalk early in the system selection and assay development processes.

## 2.3. Light Sources

To achieve the highest throughput, fluorescence imaging systems require high-intensity illumination sources such as lasers or arc lamps. Confocal scanning systems generally use lasers, which can be provided with as much power as needed to minimize scan times. Because lasers produce sharp emission lines, multiple lasers must be combined to provide the multiple wavelengths needed for multiplexed HCS assays. However, the cost of purchasing and maintaining multiple laser systems can be quite high, limiting the excitation wavelengths provided on laser scanning systems. The majority of HCS systems, the wide-field microscope systems, use one of three common types of broad-spectrum arc lamps, mercury, xenon, or metal halide, all of which provide good intensity, with broad spectral distributions. The broad-spectrum arc lamps, coupled with a wide array of interference filters, provide flexibility to use nearly any fluorescent probe. The high power available from laser illumination allows faster scan times, but limits the choice

of probes to those that fit in with the specific wavelengths available and can cause photobleaching, and induce phototoxicity (see Chapter by Giuliano et al., in this volume).

#### **2.4. Throughput: Balancing Resolution and Field of View**

As HCS systems are designed for the analysis of large numbers of specimens, throughput is always an important consideration. The throughput achieved in a HCS assay will be impacted by many factors; automation and instrument design, the nature and quality of the fluorescent samples to be imaged, and the specific biological requirements for the assay. Although the user has little control over the instrument design, except when selecting a system, there are some important configuration options that can significantly impact the throughput of an assay on an HCS system.

The choice of objective to use for an assay will have the greatest impact on performance and throughput, affecting the resolution, field of view, detection sensitivity, and algorithm performance (20,21). Because resolution and field of view are inversely related, higher resolution results in a smaller field of view with fewer cells per image, and longer scan times. In general, for HCS, the optimum balance between resolution and field of view is usually just enough resolution to reliably measure the feature of interest. This is very different than most basic research applications of cell imaging in which throughput is usually limited by the interactive analysis process, and higher resolution images are needed for visual analysis. Thus, the choice between resolution and throughput is different for HCS and research imaging applications, and therefore the two approaches can be used in concert.

Most vendors supply standard long working distance objectives with magnifications from  $\times 5$  to  $\times 40$ . These objectives are designed for use with thick (0.5–1.1 mm) substrates, like those most often found on clear bottom microplates, and provide sufficient clearance to image all the wells on nearly all plate designs, without mechanical interference. **Table 2** lists the magnifications of some typical objectives used for HCS, and properties related to their performance. The optical resolution of a microscope depends on the numerical aperture (NA) of the objective lens and the wavelength of light, and is commonly defined to be  $0.6 \lambda/\text{NA}$ . For a typical HCS imager with a standard  $\times 10$  or  $\times 20$  long working distance objective, the NA will be 0.3–0.4. When used to image fluorescence emission at 550 nm, the resulting optical resolution will be about 1  $\mu\text{m}$  (see **Table 2**). However, the resolution captured in an image also depends on the sampling resolution of the camera and the magnification of the system. Typically the magnification is simply that of the objective in use, but some HCS systems also include secondary optical magnification elements. Nearly all HCS systems provide a means to vary the sampling resolution of the detector. For the systems in **Table 1**, all of which have a CCD camera as the primary detector, the resolution can be varied by a process of combining the signal from multiple pixels on the chip into a single pixel, a process known as binning. For example, a commonly used CCD camera with 6.45  $\mu\text{m}$  pixels, might be binned  $2 \times 2$  to give an effective pixel size of 12.9  $\mu\text{m}$ . When  $2 \times 2$  binning is combined with a  $\times 10$  objective, the sampling resolution in the specimen plane would be 1.3  $\mu\text{m}$ .

By comparing the optical resolution to the image resolution in **Table 2**, it can be seen that this binning mode results in a small loss of the available optical resolution. Although higher resolution would capture more information, there are several advantages to imaging with the lower resolution. First, binning  $2 \times 2$  effectively increases the signal per pixel fourfold allowing the use of shorter integration times and the detection of lower intensities. Second, imaging with a lower resolution  $\times 10$  objective, instead of a  $\times 20$  objective, gives a fourfold greater field of view, and therefore four times as many cells. Third, the analysis of higher resolution images, especially when using processor intensive functions, can take much longer. However, when the assay simply needs more resolution there are two choices, move to higher magnification or stop binning. The right choice depends on the particular assay constraints. For assays with bright labeling, that

**Table 2**  
**Typical Imaging Performance Parameters of HCS Systems**

Magnification (×)	NA (NA)	Working distance (mm)	Binning (nxn)	Relative sensitivity	Optical resolution (μm)	Image resolution (μm)	Field of view (μm)	Typical cell (#)
Long working distance objectives								
5	0.25	12.5	2	1.9	1.3	2.6	1320	1200
10	0.3	5.6	2	1	1.1	1.3	660	300
20	0.4	7.9	2	0.8	0.8	0.6	330	75
20	0.4	7.9	1	0.2	0.8	0.3	330	75
40	0.6	3	2	1	0.6	0.3	165	20
High NA objectives								
10	0.5	2	2	7.7	0.7	1.3	660	300
20	0.75	0.6	2	9.8	0.4	0.6	330	75
20	0.75	0.6	1	2.4	0.4	0.3	330	75
40	0.75	0.7	2	2.4	0.4	0.3	165	20

The selected optical configuration parameters, magnification (with associated NA and working distance), and camera binning, affect the performance of an HCS system, as indicated by the relative sensitivity, resolution, and field of view. The magnification, NA, and working distance presented here are typical the microscope objectives available for use on HCS systems. The most common camera binning modes,  $2 \times 2$  and  $1 \times 1$  affect the relative intensity and image resolution as follows. The relative intensity is proportional to  $(\text{binning})^2 \times (\text{NA})^4 / (\text{Mag})^2$ , and is calculated relative to the  $10 \times 0.3$  NA objective in Binning mode 2, which is assigned a value of 1. The image resolution is calculated as  $(\text{binning}) \times (\text{pixel size}) / (\text{magnification})$ , in which pixel size is taken as  $6.45 \mu\text{m}$ , a common camera pixel size on many HCS systems. The optical resolution is calculated as  $0.6 \lambda / \text{NA}$ , using a wavelength,  $\lambda = 550 \text{ nm}$ . The Field of View is the width and height of the region imaged in the specimen, assuming a square array of 1024 pixels and is calculated from  $1024 \times (\text{pixel size}) / (\text{magnification})$ . Typical cell number per field is provided for reference and assumes cells are confluent with a  $25 \mu\text{m}$  diameter.

can tolerate the fourfold decrease in sensitivity as a result of not binning, staying at  $\times 10$  and using the full resolution of the camera maintains a larger field of view. But if signal is limiting, it might be necessary to move to  $\times 20$ , to achieve higher resolution whereas maintaining sensitivity, and accept the extra time required to image four times as many fields. Optimization of the HCS system configuration can yield significant gains in overall assay throughput.

The Acumen Explorer (TTP Labtech, UK) is a laser-scanning device with a wide field of view and just enough spatial resolution to distinguish between cytoplasm and nuclear localization, but not much more. Four fluorescent channels are recorded using photomultiplier tubes, which are digitized in sync with the scanning. The Explorer is a good example of the extreme in giving up resolution for field of view and speed. Scan times are very fast, on the order of 10–20 min, to scan the whole area of all the wells of a microplate, regardless of format (96, 384, or 1536). It also uses a unique software approach in collecting a defined set of features from every plate, extracted on the fly, without actually creating and saving images. Certainly, the Explorer is not a multipurpose HCS imager, but it can provide a cost effective solution in which speed is the primary requirement, and only very low subcellular resolution is needed.

### 2.5. High NA Objectives: For Improved Optical Performance

Some vendors offer “high NA” objectives for use on their systems. Often these are high quality, highly corrected objectives. These objectives usually have a very flat field, are typically corrected for focus at three wavelengths, and the high NA provides improved sensitivity. **Table 2** shows a comparison between these “high NA” objectives and the standard objectives. The most striking difference is the 2.4–10  $\times$  increase in Relative Sensitivity. The Relative Sensitivity, which is proportional to the fourth power of the NA and inversely proportional to the square of the magnification (*see Table 2*), is the main reason research microscopists nearly always go for

the highest NA objective available. However, for HCS applications, high NA objectives are not always the best choice. First of all, the high NA microscope objectives provided for use on HCS systems are designed for a very specific optical setup, imaging specimens on a 170  $\mu\text{m}$  thick cover slip glass with a refractive index of 1.515 and air between the objective and cover slip. Unless these conditions are precisely met, the imaging quality will be degraded. Only some glass bottom microplates currently meet these requirements, and they are still relatively expensive (\$15–20/plate). Some 96- and 384-well plates with thin plastic bottoms (0.1 mm) can be used with high NA objectives, although a reduction in imaging resolution will result from the deviation of the thickness and refractive index from the optical design conditions. Second, the short working distance of these objectives (*see Table 2*) limits the ability to access the outer wells of many microplates. Third, the high NA also results in a smaller depth of field (higher axial resolution). On systems that rely on image-based focusing, this shallow depth of focus requires smaller focus step sizes and more frequent focusing when moving from field-to-field within a well, which can add to scan times. In situations in which the constraints on using high NA objectives are acceptable, the significantly higher sensitivity might be useful for assays with low signal intensity.

## 2.6. Autofocus Systems and Performance

Reliable autofocus is essential for all HCS systems and is arguably one of the most difficult challenges. All HCS systems focus well most of the time, but even slightly soft focus 1% of the time can be a problem when screening. There are several challenges to providing reliable autofocus.

1. The autofocus must be fast, because even one second per well adds more than 6 min to the overall scan time on a 384-well plate.
2. The best focus must be precise within a few microns, depending on the objective in use.
3. The bottoms of microplates are universally bowed, typically by more than a hundred microns corner to center. Variability in the z-position of the well, from one well to the next on a 96-well plate, can easily be more than 50  $\mu\text{m}$ .
4. The thickness and surface quality of plastic bottom plates varies. Glass bottom plates provide a much smoother and uniformly thick substrate, but the bottoms are still bowed, and the plates are generally considered too expensive to be used routinely in most labs.
5. Plates are often contaminated with dust or fine fibers that are highly fluorescent when illuminated by UV light, and therefore interfere with focusing. And finally, the z-position of the cellular feature of interest relative to the substrate can vary significantly, especially in a thick confluent layer of cells.

Three different autofocus solutions are in use today on HCS systems, all of which are based on sound principles, but there are certain to be some differences in the performance that depend on the implementation and the specimen preparation. The three autofocus solutions for HCS are based on image analysis, specular reflectance, or video signal processing. In all cases, autofocus involves feedback between the focus drive and a focus parameter, which is to be maximized by changing the focus position. The three solutions differ principally in the focus target and the method used to determine of the focus parameter. Nearly all image analysis based autofocus systems image a target fluorescent label in cells, commonly nuclei but any feature of interest could be targeted, and use an algorithm to measure relative sharpness in the image. Specular reflectance systems project a small spot of illumination onto the substrate and maximize the intensity of the reflected light. This microscope focus method was originally developed for, and is widely used in semiconductor wafer inspection systems. In contrast to the image-based systems, the specular reflectance autofocus systems focus on the substrate, optimally the interface between the water (or media) in the well and the well bottom, the surface to which the cells are attached. The last method, video signal analysis, is normally used in a brightfield-imaging mode such as phase contrast to achieve a good signal-to-noise ratio in a single video frame.

Sophisticated software focus methods have the advantage of focusing on a feature of interest, ensuring sharp focus for that feature. The most sophisticated are designed to ignore dust and other contaminants when finding focus. Laser focus is very fast but the focus quality relies on a smooth substrate and a consistent position of the feature of interest relative to the substrate. Focus based on video signal processing is also very fast and focuses directly on the cells, although not necessarily the cellular feature of interest. All the focus methods should work well on cells, which are well spread on the substrate and therefore very thin, especially with magnification of  $\times 10$  or less, because of the large depth of field. Although laser and video-based focusing are certainly faster than software focus methods, the impact on speed is only significant for HT-HCS, in which upwards of 50,000 assays a day must be run.

### 2.7. Detectors

The ideal detector for quantitative fluorescence imaging has high sensitivity, broad spectral range from the near infrared to near ultraviolet wavelengths, high dynamic range, and a linear response. Scientific CCD cameras have all these features, and all of the systems listed in **Table 1** use a scientific grade CCD camera. Furthermore, many of the systems use the same camera or at least the same CCD detector in the camera. Of the systems, only the camera on the INCA 3000 is of a significantly different design, and that is principally because of the design of the system. Because the INCA 3000 is a line scan confocal system, it uses three line scan CCDs, one for each emission channel, rather than the two-dimensional CCD cameras used on the other systems. Some vendors offer alternative cameras with improved sensitivity and performance. For example, the new ImageExpress Micro (Molecular Devices, Sunnyvale, CA) (**Table 1**) is available with either the CoolSnap ES (Photometrics, Tucson, AZ) or the higher sensitivity, lower noise, lower dark current CoolSnap HQ. The added cost of a higher performance camera will, in most cases, be offset by improved performance, and therefore is usually money well spent.

### 2.8. Summary

In summary, HCS systems provide flexibility to allow the optical configuration to be customized to address a wide range of specific assay requirements. The performance of an HCS system depends not only on the design, but on choosing the optimum configuration to meet the assay requirements. To take advantage of the flexibility in resolution, field of view, speed, and multiwavelength imaging, assay developers need to understand the relationships between configuration options, and determine the critical elements of the imaging requirements for each assay. For example, when running a Transfluo<sup>TM</sup> GPCR assay on the INCA 3000 platform, using Draq 5 to stain the nuclei of  $\beta$ -arrestin-GFP expressing cells avoids crosstalk and allows imaging both fluorophores with a single pass, collecting the emissions on separate red and green CCD cameras. By selecting glass bottom plates with the appropriate seeding density, only a single field of view will be required, and the width and length of the scan can be reduced to speed up image capture further. Binning  $2 \times 2$  will also reduce scan times. Under these conditions, scan times of 8–10 min/384-well plate are attainable. However, scan times will increase significantly for other assays with different combinations of fluorophores in which more than one pass is required for excitation, or multiple channels of fluorescence and fields of view might need to be acquired. When comparing throughputs on different imaging platforms, it is good practice to run as many distinct biology's and combinations of fluorophores as possible, collecting data on instrument performance under a wide range of conditions, not just optimal conditions.

## 3. Imaging Software Solutions

A critical component of any automated imaging platform is the software, it serves to control and set up the instrument for image capture, but perhaps more importantly provides tools for image/data analysis, image/data visualization, and must integrate into a database application to manage and store the large volume of data generated (22).

### 3.1. Acquisition and Control Software

During the development and optimization of image-based assays, a number of experimental parameters will be established that impact how the instrument must be set-up to capture the images. The number and types of fluorescent probes to be imaged will determine how many separate channels or passes will need to be collected, and which excitation and emission filters are required. The quality, reproducibility, and intensity of the fluorescent signal can affect whether auto-exposure or fixed exposure will be utilized, and the optical resolution required will determine, which objective will be employed. The cell seeding density and/or the relative frequency of the response being measured in the total population of cells will impact the number and/or the size of the image fields that need to be captured. For instruments with confocal capability, the appropriate focal offset must be selected based on a previous Z-stack analysis. Many of these instrument settings are either controlled by the software or have to be selected in the software, along with other experimental details such as the plate density, and number of wells to be imaged. It is important that the instrument setup procedure and software should be both intuitive and straightforward.

### 3.2. Image Analysis Software

There are multiple levels of image analysis/processing; at the pixel level, the object level, the semantic concept level, and the pattern and knowledge level (22). Raw and filtered digital images are made up of pixels, or gray values captured by a CCD camera. The pixels are assigned to objects established through segmentation. Segmentation methods include marker-based, object-based, or contour-based segmentation. Information about the objects in an image is condensed into features such as intensity, color, shape, and texture. Objects and regions can be classified into user-defined categories based on these features or properties. A variety of object classification approaches are available; decision rules defined by the user as a set of boundary conditions, fuzzy decision rules that permit a gradual transition between classes, clustering methods that distinguish groups of similar objects based on a similarity measure defined on a set of features, and supervised learning methods such as neural networks or support vector machines (22). Image analysis algorithms derive quantitative and qualitative measures of features such as object count, width, length, spatial distribution, motion, and behavior over time, and feature ratios, which are calculated on a per cell basis and/or as a well (population) average (*see* Chapter 6).

Research fluorescence microscope imaging platforms typically utilize software packages such as Image-Pro (MediaCybernetics, Silver Spring, MD) or MetaMorph (Molecular Devices, Sunnyvale, CA), for the acquisition, analysis/processing, archiving, and retrieval of raw and enhanced digital images. These software packages are designed for interactive image analysis and provide the high levels of flexibility and complexity that are required in a research environment. Most HCS platforms are operated by scientists that are not image processing experts, and therefore require more user friendly, or turnkey solutions to image analysis. More importantly, HCS is a production operation requiring methods that perform consistently and efficiently, and therefore requires applications that are designed, and validated to be robust and efficient. Once again, research fluorescence microscopes and HCS systems can be used very effectively in combination to address a wide range of biological applications (for more information, *see* Chapter 1).

HCS platforms typically provide a number of canned algorithms to address specific biological applications (*see* Table 3). These automated image analysis algorithms produce a set of relevant features that might be tailored to specific biological applications (22). The user identifies the objects and features to be extracted automatically from every image by adjusting algorithm parameters before running the analysis procedure. Although these canned algorithms limit the power of the image analysis application, too many options might be bewildering to the inexperienced user, or might significantly impede the assay development process. It is important therefore, that the

**Table 3**  
**Software Applications Available From HCS System Vendors**

Category	Cellomics	GE-Healthcare	Molecular devices	Becton Dickinson	Beckman-Coulter
Translocation	Cytoplasm to nucleus translocation	Nuclear trafficking analysis module	Nuclear translocation	Nuclear translocation	Nuclear-cytoplasmic translocation
	Cytoplasm to cell membrane translocation	Plasma membrane trafficking analysis module	Translocation		Invasion and motility
Cell cycle	Molecular translocation				
	Kinetic molecular translocation				
	Cell motility				
Cell cycle	Mitotic index	Cell cycle analysis module	Mitotic index		Cell cycle
	Cell cycle analysis		Cell cycle		Regulation of protein expression in the cell cycle
GPCR	Receptor internalization	Granularity analysis module	Transfluor®		GPCR validation report
	GPCR signaling				GPCR activation
Neurite outgrowth	Spot detector				
	Neurite outgrowth I	Neurite outgrowth	Neurite outgrowth		
Morphology	Extended neurite outgrowth				
	Cell spreading		Angiogenesis tube formation		
Cell health	Morphology explorer		Monopole detection		
	Live/dead		Live/dead	Live/dead	Proliferation and apoptosis
General applications	Multiparameter necrosis		Cell health	Apoptosis	
	Multiparameter apoptosis		Cell proliferation	Mitochondrial dysfunction	
	Cell health profiling			Steatosis	
	Micronucleus detection			Ion channels and ratio imaging	Aggregate formation
	Compartmental analysis	Object intensity analysis module	Granularity	cAMP	Transiently transfected cell populations
	Kinetic compartmental analysis	Bead analysis module	Count nuclei		
			Cell scoring		

automated image analysis software is intuitive and easy to navigate so that setting and validating the parameters for image segmentation, object identification/classification, and feature calculation are user friendly. The selection and optimization of the final image analysis parameters typically involves the use of a training set of images, most commonly the assay controls for the top and bottom of the signal window. Assay development might be done independently of the instrument, if the fully functional software is provided for offline use, such as the Cellomics vs HCS toolbox. At the time of writing, the number of image analysis algorithms and the variety of biological applications addressed varied significantly between the HCS platforms, with the Cellomics ArrayScan™ having the most extensive portfolio (**Table 3**). However, as users become more experienced or require methods for biological applications not adequately addressed by the available image analysis algorithms, it will become a requirement that image files can be exported to more powerful image analysis software to develop customized algorithms for analysis offline. Equally important, platforms will need to provide a process to import customized algorithms, and other software tools into their software. Another level of complexity is associated with the image analysis of multiplexed target readouts. Most of the basic canned image analysis algorithms require that separate target channels be analyzed independently, whereas many of the more advanced algorithms, such as Cellomics Compartmental Analysis application, have been designed to handle multiplexed target readouts (*see* Chapter 5).

### 3.3. Data Mining

In addition to the image analysis algorithms, the automated imaging platform software must provide an integrated environment that supports visual data mining (22). There are several levels that the software must achieve. First, when the instrument is acquiring images from a set of screening plates the software must be able to monitor and display the progress of the run in real time. This should include views to assess how the screening run is progressing at the plate level, plate views that illustrate which well is being imaged together with some representation, for example, a heat map, of the results for wells already imaged, and image views of the fields being captured for that well. These views serve to provide the operator a degree of confidence that the robotic plate loader is functioning, the instrument has been set up correctly and is acquiring quality images, the image analysis algorithm has been appropriately optimized, and that the data for the plate controls are consistent with expectations. Second, the software must provide a means to inspect and interpret the multiparameter analysis results in the context of the images, the raw data, the experimental conditions and procedures utilized. The user needs interactive software that allows them to assess the quality of the experiment and programmed analysis (22). In addition to the specific target readout that the algorithm provides, the software should allow the user to mine the data to extract additional parameters such as morphology features, apparent cytotoxicity, or potential artifacts such as fluorescent compounds. The ability to toggle between the images (both fields and individual cells) and the data views is critical, and some software packages like that provided with the Cell Lab IC 100 platform (Beckman-Coulter), and others, allow the user to pick points or groups in a data viewer and pull back the individual cell images in a gallery. These tools enable the user to view the images from obvious outliers in the data set and can often provide a means to identify a plausible underlying cause, for example, cells that are in mitosis. Third, the software must provide tools to visualize, manipulate, and compare the multiparameter data and images, to help recognize high-level patterns and relationships that might assist interpretation of the data (8,15,22). If the data visualization tools are limited, they should provide user friendly methods to extract and export multiparameter data, at both the cell and well levels, to more powerful external data visualization and analysis packages such as Spotfire® (Spotfire, Somerville, MA) or S-plus® (Insightful, Seattle, WA). If images are to be analyzed postacquisition, rather than in real time, additional fully functional software seats beyond that loaded on the instrument will be required. Additional copies of software might also be required

to facilitate data sharing both on site and in a global organization. Although this might require more copies of the fully functional software, for some purposes simpler data viewer functionality might be adequate. The number of copies and/or cost of software seats that the vendor provides beyond that loaded on the instrument should be considered when selecting a platform to purchase (*see also* Chapter 23).

### 3.4. Data Storage and Management

Automated imaging platforms that are operated for HCS purposes generate large amounts of data and it is critical that these data be securely stored and effectively managed in a database (22). In addition to the large raw image files, the data model also needs to capture and integrate the associated metadata together with the data generated by the image analysis/processing algorithm and information from corporate databases to provide an effective data-mining environment (22). The metadata includes the nature of the samples, the experimental conditions, and the procedures used to acquire and analyze the images. The image-derived information includes the objects, features, classifications, and calculated data. In addition to the sheer amount of image data that needs to be managed and stored, the integration of these different data sources presents a significant challenge (22). As the raw and derived data might be utilized to draw important conclusions about the actives, hits, and leads from drug discovery programs, these data must be archived, and stored in an unmodified form for scientific and regulatory reasons. The database should therefore provide user friendly and efficient methods to query and retrieve the images and data for review, and potentially for reanalysis. At the time of writing, only Cellomics and Molecular Devices were offering complete database solutions with their HCS platforms (*see also* Chapter 20).

## 4. Comparison of HCS Platforms

All of the HCS imagers available today are optical microscope systems principally designed for fluorescence imaging, but in some cases providing additional imaging modes. For the lab looking to add capability in HCS, as well as for the HCS veteran looking to add capacity, the array of choices for HCS systems today can be daunting. **Table 1**, lists the established HCS readers at the time of writing. Although an extensive comparison of all the features and options for every system might help drive the choice, interpreting the matrix of features would be complicated. Instead the systems will be compared based on design features and target application areas.

### 4.1. Optical Designs: Confocal Vs Wide-field

The HCS readers can be broadly divided into two optical design types, confocal scanning or wide-field imaging. All confocal scanning systems work by illuminating the specimen in one or more small regions (spots or lines) and building up an image by scanning the illumination through the specimen while measuring the emission in synchrony with the scanning. Confocal HCS systems can be further divided based on illumination scan design, with systems available which use point scanning, line scanning, and multipoint scanning (e.g., spinning disk). In contrast, wide-field imaging systems illuminate a “large” area of the specimen, and directly image that area all at once. A direct comparison of the performance of wide-field and confocal microscope systems concluded that wide-field microscope systems perform better (have a higher signal-to-noise ratio) on thin specimens such as monolayers of cells, whereas confocal systems perform better on thick specimens such as tissue sections and multilayer cell preparations (23). When imaging the microtubule cytoskeletal organization in *Toxoplasma gondii*, the same authors reported that the submicron, weakly fluorescent structures could not be reliably captured by point scanning laser confocal imaging, but were successfully imaged by wide-field microscopy (24). It is difficult to draw a definitive conclusion from these limited comparison studies, especially given the number of different confocal systems and confocal designs available in the market place, but

some useful observations can be made. Confocal systems have a definite advantage in rejecting background fluorescence from material outside the plane of focus, either because of the specimen being significantly thicker than the depth of field, or because of some fluorescent component in the surrounding media, such as excess label. However, on dim specimens, wide-field systems have an advantage in much longer integration times per pixel. For example, the integration time per pixel for a megapixel image acquired on a wide-field microscope will be a million times longer than the dwell time per pixel on a point scanned confocal image with comparable total acquisition time. Line scanning confocal systems and multipoint disk scanning systems are somewhat better in this regard, but still the dwell time per pixel is relatively short. Increasing the illumination intensity compensates for the short dwell time, but is ultimately limited by saturation of the fluorophore, and also results in undesirable photobleaching and phototoxicity (25). Longer integration times can be used to improve the signal-to-noise of weaker fluorescence, but this compromises the throughput of the system.

Of the 11 HCS systems in **Table 1**, only three have confocal capability; the Pathway HT, the INCA 3000 and the Opera. The ArrayScan VTI offers a unique option, the Apotome (Carl Zeiss, Jena, Germany), which uses a grating illumination device to generate optical sections (26). This device can easily be inserted when optical sectioning is needed and the cost is nominal compared with true confocal imaging systems. Although it does not reject background fluorescence like confocal systems, it does subtract it, and for occasional assays requiring measurement of a single cell layer in a clump or colony, isolating cells grown on feeder layers, or moderate solution background, the Apotome is certainly a cost effective solution.

Confocal HCS imagers are more complex, require more expensive light sources, and therefore are typically more expensive than wide-field HCS systems. Even though the majority of imaging applications perform well on wide-field HCS imagers, there is a perception that confocal capability is desirable, perhaps because many assays have their genesis on stand alone confocal microscope platforms. The decision on whether the HCS platform should be a wide-field or confocal imager should largely be driven by the scope and nature of the biology's that will need to be addressed. For assays on single layers of cells, confocal detection might not be necessary, and results in some loss in signal. For live cell imaging, the lower illumination intensities of wide-field imaging will cause less perturbation of the cells. For assays with a high solution background, confocal detection will be an advantage, but in most cases, solution background can easily be washed away. For thick, multilayer cell preparations, confocal imaging will certainly be an advantage. It is fair to say that both types of systems have a wide range of applicability in cell and tissue analysis.

#### **4.2. HCS Systems Optimized for End Point Assays**

The HCS readers designed for fixed cell assays are generally the simplest in design, from both the hardware and software perspective. These readers can be easily integrated with a wide range of automated plate loaders to create a screening platform with a capacity to analyze on the order of 10–25,000 wells per day, depending on the assay complexity. All are of the wide-field optical design, owing to its well-deserved reputation for flexibility and high performance to cost ratio. These include the least expensive HCS systems, listed in **Table 1** for <\$200K (CellwoRx, Discovery-1, and ImageExpress Micro) and three somewhat higher cost systems for >\$200K but <\$500K (**Table 1**). However, it should be noted that in addition to the listed instrument costs, the actual cost of any HCS system will depend on the options selected, the additional image analysis algorithms purchased, the number of software seats provided, and the purchase of hardware and software for a database solution, all of which contribute significantly to the bottom line.

The fixed end point cell reader is the largest category of HCS systems and by far the largest installed base. The available systems (*see* **Table 1**) consist of five relatively compact bench-top units, and one floor standing unit. The fifth generation ArrayScan VTI platform, the Discovery 1

and the INCA 1000 might be considered standards against which all other systems will be compared. The CellwoRx has been in production for less than a year, and the ImageExpress Micro is a recent launch.

The optical designs of five of these six imagers are essentially the same, including the illumination systems, lenses, and cameras, suggesting there will be very little difference in the imaging performance. The CellwoRx system from Applied Precision offers an innovative new optical design. The illumination system uses light guides to deliver the fluorescence excitation directly to the specimen in a “dark-field”-like arrangement. The principle advantage of this design is that there is very little backscatter, and it eliminates the need for a dichroic mirror. The result is fluorescence imaging with very low background. Although this technology is new to HCS, Applied Precision pioneered this illumination system for use in their ArraywoRx chip readers, which have outstanding performance.

The other differences between these systems are principally in a few specific features and the available options. The Discovery 1 and INCA 1000 both offer laser autofocus as a standard feature, whereas on the new ImageExpress Micro it is an option. The Cell Lab IC 100 has a unique and fast video autofocus system. Although fast hardware focusing is an essential feature on HT-HCS systems, for low to medium throughput applications, a well-implemented image autofocus algorithm might be more reliable, if not as fast. The two systems from Molecular Devices also offer a transmitted light option, although at present there are few validated algorithms available for this imaging mode. A few of the systems also offer an environmental control option, allowing these systems to be used for simple kinetic studies with manual or off system pipeting, as well as live cell end point assays. Overall, the fixed cell HCS systems offer excellent performance and are the lowest cost systems.

### 4.3. Live Cell Kinetic Systems

The live cell kinetic HCS systems are significantly more complex. In addition to simply scanning a plate, the live cell systems require on board liquid handling for stimulus–response assays and an environmental control system. They also require sophisticated acquisition software with provision for scheduling various sequences of reads and liquid additions, scan sequences that accommodate a wide range of biological timing, and sophisticated software applications for analyzing the time-course of the response. The assay developer must be concerned with the status of the cells during the assay, whether the reagents impact the biological processes being assayed, and whether the system itself, through optical, or even mechanical stimulation, might affect the biological response. However, with careful system and assay design, these difficulties can be addressed and valuable information collected. Access to live cell kinetic capability can significantly enhance the assay development capability for fixed end point HCS assays. Although, live-cell systems could double as single time-point fixed cell imagers, the additional features, and cost might not be warranted unless a significant proportion of live cell assays will be run.

Of the 11 HCS systems in **Table 1**, four have been designed for live cell capability; the KineticScan<sup>®</sup>, the Pathway HT, the ImageXpress 5000A, and the INCA 3000. The KineticScan pioneered the integration of a general purpose pipeting device with an HCS imager in order to provide flexibility for single cell kinetic analysis of many cells in many wells. The ImageExpress 5000A provides a similar configuration of pipetor and reader. The Pathway HT presents a variation on this concept, with a single channel pipet and a unique optical design in which the plates remains stationary, whereas the optical system moves to scan the plate. The goal of this design concept is to avoid movements, which could cause mechanical stimulation or motion of unattached cells. The Pathway HT also provides the ability to switch to a Nipkow disk confocal acquisition mode for assays that require sectioning, background rejection, or for three dimensional analysis during assay development. The INCA 3000 utilizes two peristaltic pumps for

making additions to wells, but with a 7 mL dead volume and a limited flexibility in pipeting, the live well imaging capability is severely restricted.

Because live cell kinetic assays are more challenging to implement, requiring interaction between liquid handling and imaging, and more complex algorithms, the selection of a system is better made on the basis of software, and supported applications rather than specific hardware features, the importance of which might be difficult to assess. Demonstration of a validated assay is always the best evidence that a system will provide all the required features, and that the vendor is ready and able to support customers' assay development needs.

#### **4.4. HT-HCS**

Achieving high throughput, which routinely scans more than 50,000 wells/d, on an HCS system, is by no means an easy assignment. In fact, stopping to take images is one of the main impediments to fast scanning. Taking multiple images per well, if they are of different wavelengths or fields, further slows the scanning process. Finally, taking time to focus, and mechanically switch components takes even more time. The INCA 3000 and Opera systems have been designed to minimize these time wasting operations and thereby quickly and efficiently scan a plate. However, there is a significant premium to be paid for this technology, and the solutions are not without their tradeoffs, as discussed later. These are the two most expensive HCS systems costing between \$800K and \$1M.

The INCA 3000 is a confocal line scanning reader, which projects a line of illumination into the specimen, and images the fluorescence emission simultaneously on three CCD line cameras. This system is capable of high resolution in X, Y, and Z. The multiwavelength line imaging allows for continuous scanning of the specimen from one end of the plate to the other; however, most often the system is used to image one field at a time. The speed of the INCA 3000 depends on the use of fluorophores with little or no crosstalk between channels, binning the camera, and an appropriate cell seeding density that allows imaging only a single field of view, and a reduction of scan length and width. If the use of a particular fluorophore requires taking a second image to avoid crosstalk, the scan time will essentially double, and the speed advantage will be lost. The INCA 3000 acquires and saves images, which can be analyzed on the fly, or postacquisition, using standard image processing routines to produce feature sets appropriate to the assay being run. The Raven operating software was not designed to function as a slave to a robot, which makes the integration of automated plate handlers challenging. The INCA 3000 has an environmental chamber, two peristaltic pumps for pipeting, uses three independent lasers, and three CCD cameras, all of which contribute to largest footprint for all of the HCS platforms.

The Opera is also a confocal imaging system, that uses a high performance spinning disk with integral lenses to provide significantly higher sensitivity than that achieved by standard Nipkow disk confocal designs, like that used on the Pathway HT. Coupled with three (optionally four) area CCD cameras, the Opera provides very rapid confocal imaging. On the positive side, the Opera images a whole field at a time, so if a second image is required to avoid crosstalk, the extra acquisition time will increase the overall scan time by only a fraction. A choice of laser and arc lamp illumination provides more flexibility for compatibility with a wide range of fluorophores.

With careful selection of fluorescent labels, proper configuration of the scan parameters, and the right microplates, either of these systems can provide the throughput needed to scan more than a hundred 384-well plates in a day.

## **5. Installation and Operation Considerations**

The integration of an HCS platform into the drug discovery process is a complicated and time-consuming process that will involve a significant investment by multiple components of an organization; facilities engineering, automation, research scientists, and IT. The process will involve considerable educational, technical, and customer service support from the vendor, all of

which are critical components of the decision process, when considering which platform to purchase.

The vendor should provide specific facilities requirements for the instrumentation in advance, factory QC testing, and acceptance criteria, perform the installation, and conduct postinstallation performance testing to meet acceptance criteria. The vendor should also provide training on routine diagnostic procedures with criteria that ensure the instrument is calibrated and operational. It will be critical that the vendor support the integration of the HCS platform with the user's automation, network and database systems. A dedicated telephone hot line and E-mail address should be provided with clearly stated technical support response times to reports of hardware and/or software problems, and requests for trouble-shooting assistance.

As part of the installation of the HCS platform specific training should be provided for instrument setup, image acquisition, image analysis, data visualization, and data management through the database. The training should optimally be structured in two phases; the first phase to cover training users to the point in which, they can perform all of the functions required to implement the technology, and the second phase to follow some 6 mo later, to address the more detailed aspects of the platform, the image analysis tools, and/or to implement specific requirements or applications that the users have requested. The educational process and training should be a continuing collaboration between the user and vendor, with support provided whenever new applications become available, or if new hardware and software upgrades are installed. The level of educational, training, and postinstallation support, together with policies for hardware and software upgrades, should all be critical components of the platform selection process.

### **5.1. HCS Reference Standards**

HCS systems are designed for production cell imaging and analysis, and therefore must perform in an accurate, consistent, and robust fashion. However, despite careful component selection on the part of system developers, there are a number of sources of variability between systems, and in a system over time. System components like arc lamps and interference filters exhibit variability in output and throughput, respectively, between components, across the spectrum and over time. System design and construction factors contribute variability in features such as illumination nonuniformity, scan mechanics, and stray light. In addition, system setup and operational factors such as optical alignment, confocal slit width or pinhole size further affect the reproducibility from system to system. Users working in a single instrument environment will be primarily concerned with variability over time, for example, plate-to-plate and day-to-day. Users operating in a multiple instrument, multiple platform or multiple site environment, will also be concerned with system-to-system variability. Presently, the tools and procedures available for characterizing or calibrating microscope system performance (27–29) are not designed for automation and are not designed to address the wide variety of algorithms used in HCS. HCS standards and standardization methods would be useful for:

1. Routine quality control of a production HCS system;
2. Troubleshooting assay performance issues;
3. Comparison of the performance of two or more HCS systems; and
4. Calibrating the outputs of a HCS imagers.

The important outputs from an HCS system are the cellular features derived from the images through the application of image processing algorithms. Optimally then, system performance and reproducibility would be evaluated based on measurement of these outputs. However, the many different algorithms used, and features measured, complicates the development of standards and methods to evaluate all the possible measurements. Presently, HCS practitioners commonly use control wells on each plate to monitor system performance. In addition, some systems offer an autoexpose method that can be used to adjust integration times to produce target image intensities in each fluorescent channel. The adjustment of integration time based on control wells

provides a basic level of system calibration that compensates for some system and sample variables, and improves the reproducibility of measurements. However, using this simple approach to calibration can mask significant sample preparation issues, which would be also a source of variation in assay performance, even with system calibration. Clearly, stable, reliable standards would be a better choice for measuring and compensating for system variation, independent of sample variation. In all cases the optimum standard design will be consistent with the geometry of the specimen and feature to be measured, incorporate stable fluorescent materials, and be constructed in a format compatible with the microplate scanning capabilities of HCS systems.

It is useful to consider three levels of standardization and three types of standards and procedures for HCS system monitoring and calibration. First, are system correction and calibration, such as flat field correction and intensity calibration. For example, a uniform fluorescent specimen is required to correct the nonuniform illumination and response inherent in microscopes, a process known as flat field correction. For thin cell layers, the ideal flat field correction standard would be one or more thin fluorescent layers (30) that cover all the fluorescence channels. Presently, all HCS systems provide a procedure for calibrating and applying a flat field correction to the images, typically using a fluorescent solution. More precise calibration of the measured intensities in terms of fluorophore concentrations could be accomplished using standards calibrated in terms of molecules of equivalent fluorescence, as has been done in flow cytometry (31,32). A second type of standard would model the geometry and intensity patterns in cells that are to be measured, using fluorescent objects of consistent size and uniform or calibrated fluorescence intensities. For example, in flow cytometry, fluorescent calibration beads are commonly used (33) because they mimic the shape of a suspended cell and can be prepared with consistent brightness. Fluorescent beads are also useful as HCS standards, as models of nuclei and other compartments, as well as for their availability in many sizes, colors, and labeling levels (29,34). Additional standards are needed, which simulate the features that are measured in the assays, but are manufactured to precise specifications and incorporate stable fluorescent materials. Traditional microscope resolution slides, such as the Richardson test slide (35), provide a wide range of feature shapes for evaluation of microscope performance. However, there are no automated analysis routines to support the routine use of the slides for HCS system performance evaluation. Furthermore, the slides are designed for transmitted light imaging, and would need to incorporate a stable fluorescent material for use as an HCS standard.

A third type of standard for HCS are labeled cells. These can be on-plate control wells, or plates prepared along with the screening plates, and run before and after, or intermixed with the screening plates for monitoring assay performance throughout a screening campaign. Currently, standard plates such as these are not available commercially, and therefore must be produced and validated in house.

Control wells provide a means to monitor system, sample preparation, and biological repeatability. However, precise standards and methods that could be used to independently compare, monitor, and calibrate the performance of HCS systems, including the algorithms, would provide a more reliable and convenient approach to ensuring optimum performance and accurate measurements.

## 6. New HCS System Technologies on the Horizon

The HCS systems available today and presented here, are powerful tools for high-throughput cell biology in research and drug discovery. The systems, control software, and analysis software have matured to a point in which they can be used for production applications, although they still require a high level of knowledge and experience in order to develop or optimize assay performance. HCS systems will continue to evolve, driven in part by the implementation of more sophisticated assay formats, and in part by the development of new technologies. The timing of integrating support for new HCS capabilities will, to large extent, be driven by the demands of end users.

### **6.1. Improved Software for Automated Assay Development**

The application software available on the HCS systems discussed here covers a wide range of biological applications, but certainly there are more to come, and users will want to combine features from several applications for multiparameter cell analysis. To address these needs, the platforms should provide an easy mechanism to export image files to more powerful image analysis software to develop customized algorithms for analysis offline. Equally important is that platforms provide an easy process to import customized algorithms into their software. Alternatives to custom algorithm development are needed, like supervised learning methods such as neural networks or support vector machines to automated image analysis (*see* Chapter 6). One of the biggest challenges to the effective implementation of HCS technology is making effective use of the multiparameter capability of these systems. It is anticipated that HCS platforms will evolve to provide an integrated environment for visual data mining tools to manipulate and compare the multiparameter data and images, and to identify high-level patterns and relationships in the data.

### **6.2. Multispectral Imaging**

The present generation of HCS systems all depend on sophisticated interference filters to separate the emission from each fluorophore into a distinct detector channel, whether they are acquired simultaneously or sequentially. As mentioned, crosstalk between fluorophore emissions limits the ability to use some combinations of fluorophores. One solution to this limitation is the use of multispectral detectors and linear deconvolution. Briefly, multispectral detectors collect a spectrum of 16 or more wavelength bands for each pixel in an image. Knowledge of the emission spectra of the fluorophores in use allows separation of the overlapping emissions to produce images for each of the fluorophores (**18**). This method has been successfully used to separate emissions from GFP and fluorescein, as well as separating EGFP and YFP (**36**). Spectral deconvolution is certainly a powerful tool for multiparameter analysis, but is not without the limitations. Sensitivity will be somewhat lower as the fluorescence signal is divided into multiple channels, and file sizes will be much larger, at least four times the size of a typical four-channel data set. Multispectral imaging is expected to be a significant new capability in HCS and will almost surely be offered as an optional imaging mode on future HCS readers.

### **6.3. Fluorescence Lifetime Imaging Technologies**

The fluorescence lifetime is a measure of the average time delay between the absorption of a photon and the emission of a photon by a molecule. This delay is typically on the order of 0.5–20 ns, although there are fluorophores with lifetimes outside this range. The fluorescence lifetime of a fluorophore is characteristic of the molecule and its chemical environment, and in contrast to fluorescence intensity, is independent of the number of molecules in the measurement volume. These properties provide additional information on the local environment, and together with other detection methods such as fluorescence anisotropy (**37**) and fluorescence resonance energy transfer, provide opportunities for additional HCS assay formats (**38,39**). Fluorescence lifetime imaging systems have been available for quite some time, but the added cost, and complexity has limited their use to very specialized research applications. However, technology developments are bringing down the cost of adding this capability to imaging systems (**40**). Evotec currently offers a fluorescence lifetime option for the Opera, and HCS imaging vendors are expected to include this mode on future systems, although the general availability is still likely to be several years off.

## **7. Conclusion**

The HCS systems available today provide a broad range of choices, allowing the customer to select a system most appropriate to their needs. However, this selection and the optimal use of the system requires a significant understanding of the features, functions, and performance of the

many system options. As platforms mature, it is expected that the systems will be provided with more sophisticated control software that will simplify the operational decisions to be made, and more sophisticated application software that will simplify assay development, and improve the quality of the analysis. Presently although, the platform development focus has been on adding new features and functions, and this will likely continue as new technologies are applied to HCS and integrated into existing or entirely new platforms.

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