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Technology Review

Application of Laser-Scanning Fluorescence Microplate Cytometry in High Content Screening

Wayne P. Bowen and Paul G. Wylie

Abstract: The resolution of cell-based assays down to the cellular level has created new opportunities for the drug discovery process. Aptly named high content analysis, such an approach is enabling new methods of analysis for the broad range of therapeutic targets emerging in the post-genomics era, and offering alternative multiparametric readouts for some traditional analyses. Microplate cytometry is one of the technologies that is being applied to a broad range of assays utilizing fluorescent labeling, at throughputs compatible with primary screening campaigns. Cellular resolution is achieved using scanning laser excitation coupled to photomultiplier detection. This configuration results in area-based scanning across a large field of view, plus simultaneous detection of up to four emission colors for efficient multiplexing. Microplate cytometry is being used most extensively in the field of oncology research because of its usefulness for numerous applications, including protein kinase activity, cell cycle analysis, and cell colony formation. The review focuses on the Acumen Explorer® microplate cytometer (TTP LabTech Ltd., Melbourn, Hertfordshire, UK), detailing the principal components of the instrument and providing an overview of its use in high content screening.

Introduction

HTS OF COMPOUND LIBRARIES remains the fundamental approach used to identify chemical leads against emerging therapeutic targets within the pharmaceutical industry. Most screens employ homogeneous biochemical assays based on cell extracts or purified components such as enzymes and their cognate cofactors and substrate, or receptors and their ligands. While such approaches control the escalating costs of research while maintaining both quality and throughput, pharmaceutical and biotechnology organizations are continually seeking new ways to enhance their HTS initiatives. This is driven by a demand to profile the expanding range of therapeutic targets and a desire to assess the activity of new compounds versus targets in their native environment. High content analysis (HCA) is such an approach, one that is rapidly gaining a foothold in the screening environment as the benefits become apparent.

Arguably, the most established cell-based screening assay platform (for G-protein coupled receptors [GPCRs]) is the FLIPR® system (Molecular Devices, Sunnyvale, CA), which principally monitors release of calcium from intracellular stores using fluorescent reporter dyes.[1] Although FLIPR assays are reliable and applicable to screening compound libraries, analysis of receptors not coupled to calcium requires either construction of chimeric receptors or co-expression of genes encoding promiscuous G-proteins (*e.g.*, Gα15, Gα16). In addition, the readout from each well is a kinetic bulk flo-

TTP LabTech Ltd., Melbourn, Hertfordshire, UK.

ABBREVIATIONS: CCD, charge-coupled device; CHO, Chinese hamster ovary; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; HCA, high content analysis; HCS, high content screening; PMT, photomultiplier tube.

rescence measurement, making it unable to differentiate responses from subpopulations of cells.

HCA is not a new concept. However, recent developments in instrumentation, software, and fluorescent probes have now made the supply of reliable and relevant biochemical assay information possible.[2] HCA is the measurement of complex cellular responses in a population of whole cells in parallel. It provides additional knowledge that can be critical when determining which targets to investigate and which lead compounds to pursue. One approach uses whole-cell-based assays to assess complex, multicomponent targets that cannot be addressed in biochemical assays. This had led to the development of multiplexing capability and high content readouts in cell-based assays (Table 1). These are focused mainly in the area of oncology because of the compatibility between available assays and the therapeutic targets being investigated in the search for new cancer therapies.

The principal advance in cell-based screening has been to generate single-cell data from cultures grown in microplates. Currently, high content instrumentation falls into three categories: flow cytometry, microscope-based charge-coupled device (CCD) imaging, and microplate cytometry. Flow cytometry offers high sensitivity for a number of validated research and clinical protocols. For screening purposes, flow cytometry has several shortcomings: most notably, low throughput, the requirement for large number of cells, and the inability to analyze adherent cell lines *in situ*. Microscope-based CCD imaging offers high optical resolution (including confocal), particularly for morphological readouts, and can provide high information data. The downside is that it can generate large quantities of data (up to terabytes), requiring expensive storage and retrieval solutions, plus sample throughput is generally low compared to established screening platforms. Microscope-based CCD imagers are thus best applied for target identification and validation. In contrast, microplate cytometry enables high throughput, whole-well HCA without generating terabytes of information. These features make such instruments ideal for sustained use of HCA in hit identification and hit-to-lead optimization screens.

Table 1. Examples of High Content Screens

Cell adhesion
Neurite outgrowth
Micronucleus formation
Protein translocation
Cell cycle analysis
Cell colony formation
Adipogenesis
Reporter gene analysis
Cell viability
Cell spreading
Cell proliferation
Apoptosis
Angiogenesis
Receptor trafficking
Protein kinase activation
Mitotic index
Proteasome activity
Oligosaccharide synthesis
Cell migration
Membrane potential
Gap junction formation
P-glycoprotein activity

A prerequisite for HTS using high content assays is their transfer from the development to the screening laboratory. Much progress has been made through the availability of commercial reagent kits and the consolidation of assay protocols among different laboratories and manufacturers, including software algorithms. However, what is often overlooked is the fact that instrumentation used for HCA in assay development does not necessarily meet the different requirements of high content screening (HCS) (Table 2). The emphasis of HCA is providing as much information as possible on each cell analyzed, and most often for target validation, secondary screening, or lead optimization. The *modus operandii* of HCA commonly involves profiling small numbers of well-characterized compounds in assays where the chemistry and biology are well understood. The low sample number allows high-resolution analysis of limited numbers of cells (typically about 1,000) with the associated storage of large data files. For these reasons, HCA is predominantly performed using microscope-based CCD imagers because they meet all the criteria, especially when performing assays based on immunocytochemistry, whose origins lie in fluorescence microscopy.

In contrast, HCS is geared to profiling compound libraries as fast and economically as possible to generate hits against new therapeutic targets. Detailed information is not required—a simple yes/no readout will suffice—since 99.9% of wells in a typical HTS assay do nothing. To meet these demands, high content assays may require significant modification when transferred from development. They need to be robust ($Z' > 0.5$), ideally fixed end point to permit batch processing of large numbers of plates, and preferably offer whole-well analysis to account for intrawell variation in cell distribution and responsiveness. In addition, the number of cells required should be low, and data file sizes should be kept small to cope with the increased throughput and the lack of requirement for re-analysis. Currently, no single instrument meets the requirements of both HCA and HCS for all biological applications. Many laboratories are choosing to integrate the multiple platforms to maximize their individual capabilities. For more efficient cell-based primary

TABLE 2. COMPARISON OF THE KEY FEATURES OF HCA AND HCS

High Content Analysis	*High Content Screening*
• compound capacity is in the 100–1,000s	• compound capacity is in the 10,000–100,000s
• requires multiparametric data from each cell, often from multiple fluorescent colors	• requires yes/no answer—only interested in hits
• readout may be kinetic or single end point assay performed on live or fixed cells	• readout is fixed end point for signal stability and ease of automation
• high-resolution analysis of subpopulation of cells in each well	• ideally whole-well analysis to account for possible variations in well
• large data files produced to store all information for detailed analysis	• small data files needed to cope with increased throughput

screening, microplate cytometers were developed, which, unlike flow cytometers, use a scanning laser for *in situ* analysis of cells located in the bottom of clear-bottomed microplates. Here, we outline the key features of a microplate cytometer, the Acumen Explorer® (TTP LabTech Limited, Melbourn, Hertfordshire, UK),[3] and how it generates HCS data.

Principles of Microplate Cytometry

Automated cell analysis, the beginning of cell-based screening, began in the early 1990s with the release of the first flow cytometer with 96-well plate aspiration by Becton-Dickinson (Franklin Lakes, NJ). Flow cytometry had already established itself as the benchmark of high content cell analysis, having been initially developed for clinical analysis of non-adherent human peripheral blood monocytes in the mid-1980s. In flow cytometry, cell analysis is performed by passing a stream of suspended cells through a fixed spot of laser light. However, as the technology moved into the research laboratory where the primary cell models were mainly adherent, flow cytometry was found not to be optimal. Microplate cytometers have enabled *in situ* visualization and analysis of fluorescent cells in microplates by using a scanning laser and highly specialized optics. Consequently, microplate cytometry is sometimes described as flow cytometry without the flow.

Instrument configuration

The Acumen Explorer microplate cytometer is composed of three major components: a laser unit housing the laser and power supply, a scan head unit incorporating laser optics and plate alignment systems, and an external PC for system control and data processing (Fig. 1). The instrument is fitted with either an argon-ion air-cooled laser emitting 488 nm (blue) light or a 405 nm (violet) solid-state laser. Nominal power for each is

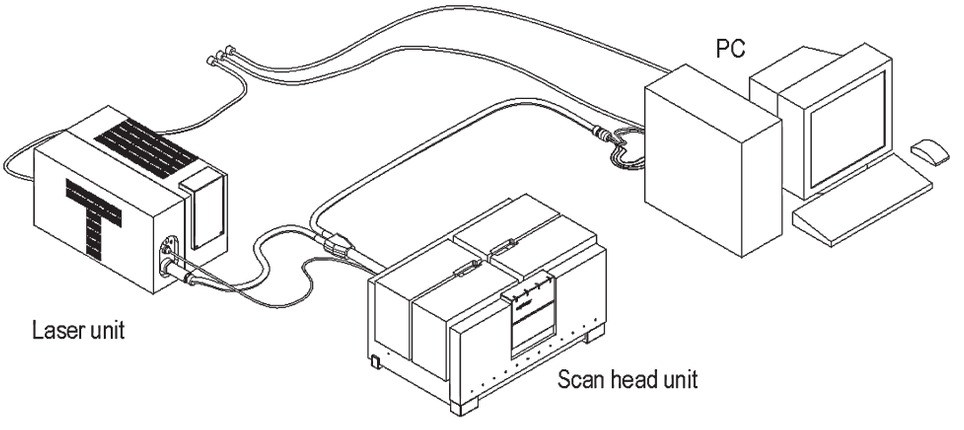

FIG. 1. Major components of an Acumen Explorer microplate cytometer. Microplates are scanned within a scan head unit containing all the precision optics, fluorescence detectors, and plate movement mechanisms. Lasers and the power supply are housed in a separate unit with laser light being delivered to the main optical bench within the scan head unit via a flexible fiber optic cable. The entire system is controlled by an external PC that also processes and reports the fluorescence data.

around 20 mW, but in routine use a power setting of 6 mW is applied when scanning. Laser light is delivered to the main optical bench within the scan head unit via a flexible fiber optic cable. Here, the width of the beam is expanded using a series of lenses before it is reflected off the main dichroic beamsplitter and onto the high-speed galvanometer mirrors, which control the path of the laser as it traverses the scan area at speeds of up to 5 m/s (Fig. 2). A specialized *F*-theta scan lens receives the light and focuses it onto the bottom of the microplate being analyzed. In addition, the scan lens "flat-fields" the arcing laser spot to maintain focus across a large field of view (a central 20- × 20-mm area is used for scanning). The entire surface of the bottom of a Society for Biomolecular Screening standard format microplate is analyzed by moving the plate relative to a stationary scan lens 24 times and scanning the field of view. The *F*-theta scan lens has a large depth of focus (about 25–30 μm depending in the wavelength of light), which compensates for variations in the bottom of the microplate being scanned. This design does not require re-focusing between wells, as is the case for microscope-based CCD imaging. A focus position for each microplate used on the instrument is determined using fluorescent beads, and this is set in the analysis protocol (Fig. 3).

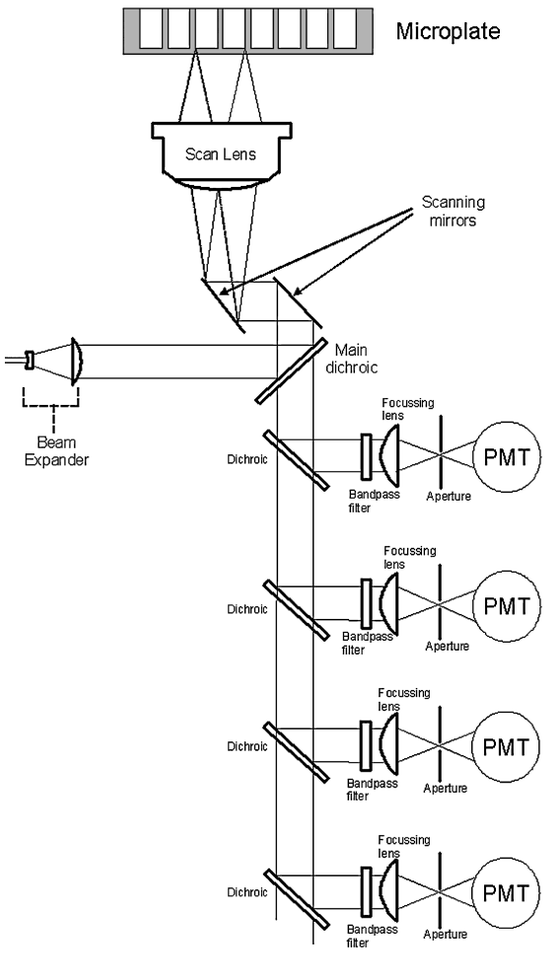

FIG. 2. Optical configuration of an Acumen Explorer microplate cytometer. Scanning of microplates is performed through a specialized *F*-theta scan lens, which focuses incident laser light onto the bottom surface. High-speed galvanometer mirrors control laser movement across a scan area of 20 × 20 mm at speeds of up to 5 m/s. Emitted fluorescent light passes back through the scan lens and a set of filter blocks containing bandpass filters and dichroic beamsplitters. PMT detectors monitor the light passing through each filter set providing simultaneous detection of four colors.

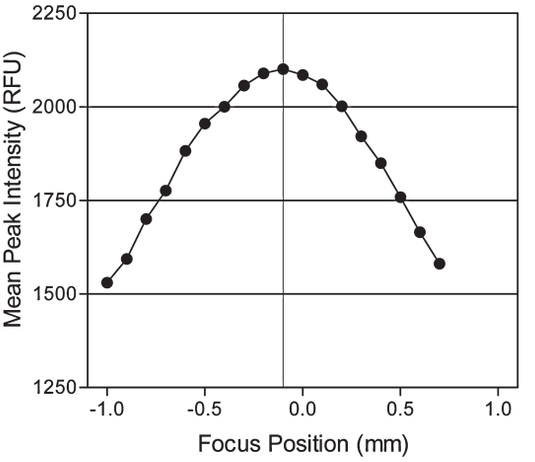

FIG. 3. Effect of focus position on fluorescence intensity. The focus position of a plate is determined using fluorescent beads placed in the number of wells occupying the entire scan area (*i.e.*, four for 96-well and 16 for 384-well microplates). In the example shown, approximately 2,000 green fluorescent beads (5.7 μm in diameter; Bangs Laboratories, Fishers, IN) were pipetted into four wells of a clear-bottomed black-walled 96-well plate (Greiner Bio-One, Stonehouse, Gloucestershire, UK) in 100 μl of phosphate-buffered saline. The mean peak intensity of all beads in the scan area at a range of focus positions was determined using an Acumen Explorer. Optimal focus position is defined as that producing the highest value for mean peak intensity, and was found to be –0.1 mm for this plate type (as indicated by the vertical line).

Fluorescent light emitted from objects in the microplate passes back through the scan lens, mirrors, and main dichroic beamsplitter and on through a set of filter blocks containing bandpass filters and dichroic beamsplitters. The dichroic beamsplitters define the maximum wavelength of light that is reflected onto the bandpass filters, which subsequently narrow the range of light detected and block any reflected laser light. When fitted with standard filter sets, the emissions are divided into the green, yellow, red, and far-red colors of the visible spectrum. Specialized filter sets are available for specific fluorophores such as phycoerythrin and quantum dots and are easily interchanged with the standard sets. Photomultiplier tube (PMT) detectors monitor the light passing through each filter set for true multiplexing across

four colors within assays. Acquisitions cards running at up to 10 MHz provide a maximum sampling resolution of 0.1 μm of laser spot movement.

As a validation tool for assay development, the Acumen Explorer may be integrated with an inverted fluorescence microscope to provide a direct link between the instrument's software algorithms and the cellular staining observed microscopically. This capability assists in the correct classification of cell populations within each well through more effective identification of discriminating morphological and fluorescence object characteristics. Also, the Acumen Explorer is easily integrated with plate-handling robotics to suit all levels of automation from providing a few plates per day up to around 200 $\times$ 1,536-well microplates per day. This scalability allows users to modify their level of automation as their usage changes with minimal disruption and cost. Barcode readers can also be incorporated into the system to ensure strict correlation between plates and the resultant data.

Table 3. Effect of Sampling Resolution on Object Characteristics

	Sampling resolution	
	1 $\mu m \times 1$ μm	*1 $\mu m \times 4$ μm*
Perimeter	167 $\pm$ 43 μm	142 $\pm$ 38 μm
Area	842 $\pm$ 186 μm^2	843 $\pm$ 188 μm^2
Intensity (FLU)		
Peak	5,254 $\pm$ 637	5,036 $\pm$ 650
Mean	1,266 $\pm$ 196	1,267 $\pm$ 201
Total	1,037,455 $\pm$ 115,503	259,763 $\pm$ 29,533

Approximately 2,000 green fluorescent beads (5.7 μm in diameter; Bangs Laboratories, Fishers, IN) were pipetted into a single well of a 96-well plate and allowed to settle for 30 min. A range of object characteristics were determined using an Acumen Explorer at two scan resolutions. Scan times were 13 and 52 s for 1 $\times$ 1 μm and 1 $\times$ 4 μm resolutions, respectively. Data are presented as mean $\pm$ standard deviation. FLU, fluorescence units.

Data acquisition

The scanning resolution is user-defined and sets the spacing between intensity measurements in the *x*-direction as the laser spot traverses the well, and the spacing between successive lines in the *y*-direction. Sampling in the *x*-direction is preset (0.1-, 0.5-, 1-, 2.5-, and 5-μm intervals) since it is configured through combination of laser scanning speed (controlled by the *x* scan mirror) and data sampling. In contrast, *y*-direction sampling is predetermined by the user with no preset values, although it normally lies between 1 and 10 μm, and is controlled by the *y* scan mirror. Sampling resolution has an effect on both the speed of a scan and the amount of data generated. For example, if a whole plate is run at a sampling resolution of $x = 1$ μm and $y = 8$ μm, the file size is about 50 MB, and the scan speed will be approximately 10 min. If the same plate is run at a sampling resolution of $x = 1$ μm and $y = 4$ μm, the file will be around 100 MB in size and will take 20 min to read. Typical assays are run at a sampling resolution of $x = 1$ μm and $y = 8$ μm with no loss of data quality. Often, scans of different sampling resolutions produce equivalent high content information for each cell, and so, with the advantages of speed that lower sampling resolutions give, many users opt to scan using the lower resolutions (Table 3).

One of the key features of the Acumen Explorer is its application of patented thresholding algorithms for object recognition. During scanning, the background level of fluorescence is constantly monitored. Where cell seeding density is low, the space between cells represents a high proportion of the total well area and stabilizes the estimation of background fluorescence. When the laser spot encounters the border of a labeled cell, the fluorescence signal rises above the background fluorescence. When the signal exceeds a predefined value (normally 2 standard deviations of the mean background fluorescence), recording in all enabled data collection channels is triggered and continues until fluorescence returns to background levels on the opposite side of the object. Setting a low theshold value gives a more sensitive scan (good for dim objects), while a higher value requires more light to initiate data collection (ideal for very bright objects to discard dim unwanted data). Where multiple channels of data collection are enabled, the user can set which channels trigger the collection of data. This capability can be used to target objects of a particular fluorescent color, for example, red nuclei within a green cytoplasm for protein translocation studies. A single line of data points collected through an object is termed a feature—collections of these are joined together using objection-recognition software algorithms and displayed as three-dimensional profiles of objects (Fig. 4). These profiles permit the calculation of a range of fluorescence and morphological parameters for each object identified. One disadvantage of using thresholding for cytometric analysis is that it cannot resolve clusters of cells where cytoplasm staining triggers data collection. Clustering can be minimized by keeping the cell seeding density low; however, some immortalized cell lines always grow as small colonies so are not ideal for microplate cytometry. Cell segmentation is more easily achieved when only nuclear staining is used because of the frequent location of the nucleus in the center of the cell.

The use of thresholding algorithms allows the discarding of any data that are not associated with objects. This results in small file sizes relative to those created by microscope-based CCD imagers, which must retain all data as an image for derivation of high content infor-

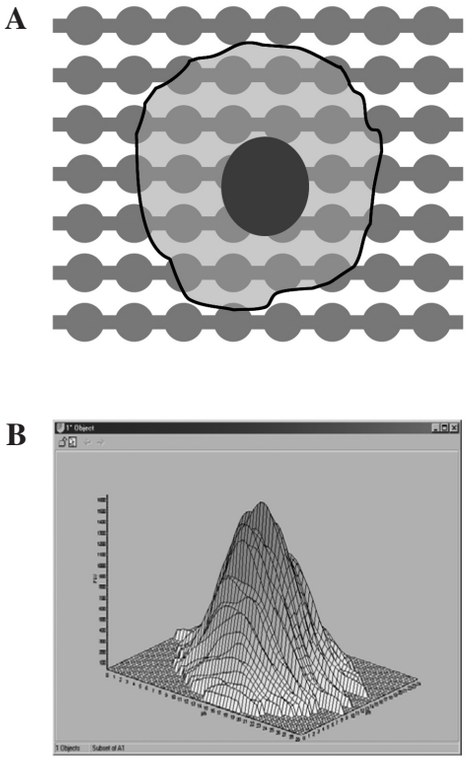

FIG. 4. Laser line scanning of fluorescent objects. As the laser spot moves across the scan field, fluorescence is measured at a distinct location (**A**). Where the amount of fluorescence exceeds a predefined threshold value, data points stored, aggregated using object-recognition software algorithms, and plotted as three-dimensional intensity profiles (**B**). These profiles are used to calculate a range of fluorescence and morphological parameters for each object identified.

mation by software algorithms. The Acumen Explorer software offers three levels of data collection to suit the development, validation, and screening scientist. For example, a typical whole-well scan for a 384-well plate requires the processing of approximately 16 GB of data. This can be reduced in assay development mode to around 250 MB and in assay validation mode to about 80 MB, and in HTS mode, the comma separated values list files generated may be as little as 30 KB. Such dramatic data reduction simplifies transfer of screening results from instrument to offline screening databases, and also alleviates issues of data server implementation and maintenance. Where analysis by third-party flow cytometry software is required (*e.g.*, cell cycle analysis), results can be exported in flow cytometry standard (FCS) version 3.0 file format, in which case all data for every object in each well are made available.

Data analysis

For every object in every well scanned, a range of high content information is calculated for each enabled data collection channel. These so-called object characteristics include both fluorescence and morphological parameters and positional information (x and y coordinates) within the well (Table 4). In addition, any two object characteristics can be divided using a ratio function for increased complexity. This is used extensively to ratio the fluorescence intensity in multiple data collection channels, for example, in cytotoxicity assays where live cells may be stained green (calcein acetoxymethyl ester) and dead cells stained red (propidium iodide). By giving the user access to individual parameters, it is possible to design novel experiments without the requirement to purchase assay-specific algorithms or resort to computer programming.

The object characteristics generated for each object are used to classify populations and subpopulations within a well by introducing a number of population filters to the analysis protocol. Initially, cells are distinguished from fluorescent artifacts (*e.g.*, cell debris, crystals of fluorophore) based on size using width and depth filters. Subpopulations of cells are subsequently defined using additional filters, most commonly a single fluorescence intensity measurement. For example, in cell cycle analysis, cells are classified according to phase (G1, S, and G2/M) based on the total intensity emitted from DNA staining. Optimization of filter values is achieved using a variety of data visualization tools, including histograms, scatter plots, and virtual images of individual wells (Fig. 5). The end result is a very basic but robust protocol using only three object characteristics (width, depth, and total intensity). Thus the predominant readout from microplate cytometry assays is not an intensity measurement that one might associate with microscope-based CCD imaging or bulk fluorescence, but the number of cells in a defined population, for example, live and dead, activated versus non-activated. This transition from fluorescence intensity to object number can permit the setting of large ranges for the filters defining populations where the fluorescence report differs markedly between populations, leading to robust protocols for HTS. Such an approach can also overcome heterogeneity in the reporter gene assays where cells possess different numbers of reporter transcripts (*e.g.*, green fluorescent protein [GFP], β-lactamase).

Area-based scanning

A key design feature of the Acumen Explorer microplate cytometer is the large field of view afforded by the precision optics. At 400 mm^2 (20 $\times$ 20 mm), the field of view is far greater than that offered by the objectives in microscope-based CCD imagers ($\sim$1 mm^2 for a $\times$10 objective), which has important implications for how assays are configured and the data obtained. The small field of view on microscope-based CCD imagers is only suf-

Table 4. Commonly Applied Object Characteristics Derived in the Acumen Explorer Software

Characteristic	*Description*	*Use/applications*
Fluorescence		
Peak intensity	Maximum intensity measurement	Used where punctate staining is present, *e.g.,* Transfluor vesicle assay
Mean intensity	Average intensity measurement	Applied widely where cells either become fluorescent or change the amount of fluorescence upon treatment, *e.g.,* mitotic index
Total intensity	Sum of all intensity measurements	Cell cycle analysis, reporter gene analysis (*e.g.,* GFP)
Color ratio	Ratio of intensity measurements from different color channels	Multicolor assays commonly involving comparison with nuclear staining. Examples include cytotoxicity, apoptosis, and β-lactamase reporter gene analysis
Half-width intensity	Half-width is an object's width at 50% of peak intensity. Half-width intensity is the peak intensity divided by half-width	Single-color translocation assays, *e.g.,* protein kinase activation using anti-kinase antibodies or movement of GFP-tagged proteins
Morphological		
Width and depth	Maximum feature length in the x (width) and y (depth) directions	Generally used together for separating populations of cells from fluorescent debris
Perimeter	Distance around the edge	Reporting of shape change, *e.g.,* cell adhesion
Area	Space within the perimeter	Reporting of shape change, *e.g.,* cell proliferation
Volume	Volume assuming spherical shape	Cell colony formation
Gaussian	Goodness-of-fit of the intensity profile to an idealized Gaussian shape	Reporting of shape change, *e.g.,* cell adhesion
Aspect ratio	Major axis divided by minor axis; rotationally independent	Reporting of shape change, *e.g.,* cell differentiation
x and y coordinates	Absolute location of an object within a well	Selection of a subset of objects within the scan area

ficient to obtain resolved images of around 100 cells at once, which is insufficient to obtain statistically significant results in some assay types. In microplate cytometry, the application of laser scanning over such a large area means that analysis is performed on an area and not a well basis. In practical terms this equates to the simultaneous scanning of four, 16, and 64 wells in 96-, 384-, and 1,536-well format, respectively. Therefore, reconfiguration of assays into higher-density plate formats results in a concomitant increase in throughput up to 300,000 samples per day in 1,536-well microplates. In assays where both the cells and assay response are evenly distributed across the entire well, the scan area can be reduced to decrease the scan time. This facility can also be used to target specific areas within a well such as the center or perimeter.

Whole-well analysis allows determination of high content information for every cell in all wells on a microplate. This has many advantages over the restricted reporting on microscope-based CCD imagers, which results from only capturing a few images from a small well area in the interests of sample throughput. First, it allows normalization of biological responses to total cell number, offering a simple toxicity or proliferation readout with every test. Second, it permits the use of low cell numbers (1,000–2,000 per well in 96-well microplates), decreasing the demands on cell culture departments for provision of cells or the number of tests that can be performed on precious samples. Finally, whole-well analysis reports data for every cell within the well, and thus generates statistically robust data from a truly representative cell population, which can overcome problems of variable stimulation and random cell distribution often observed in screening plates prepared using automation. This last point was highlighted in an assay developed to monitor α globin expression.[4] Mouse erythroleukemia stable cell lines, which contain the human α globin promoter driving an enhanced GFP reporter, were used to

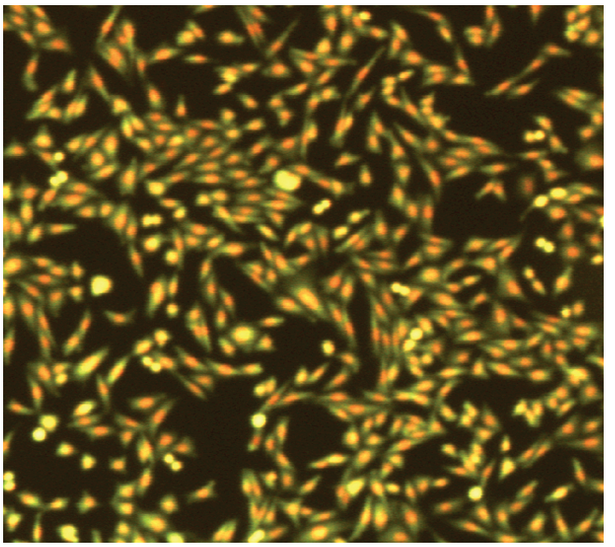

FIG. 5. Virtual image of fluorescent cells. The *x* and *y* coordinates of every fluorescent emission detected during scanning is recorded, which permits the construction of a two-dimensional intensity map. CHO cells were stained with the live cell marker calcein acetoxymethyl ester (0.5 μM; Molecular Probes, Eugene, OR) and counterstained with Syto® 64 (1 μM; Molecular Probes). Cells were scanned using an Acumen Explorer microplate cytometer at a sampling resolution of 0.5 μm × 1 μm.

measure α globin expression after hexamethylene-bisacetamide-induced terminal differentiation. The effect of scan area on Z' was investigated, and as can been seen, a Z' over 0.5 or greater was only achieved with scans of 16 mm^2 or greater (Fig. 6). In this assay, cells were plated out at 50,000 cells per well, so although the sample number was greatly increased with larger scan areas, as the sample size was large even in small areas, the difference is more likely to be attributed to differences in stimulation across the well, which are exaggerated when the user samples a small area of the well. In screening mode, a robust assay with a good Z' is essential, and variations across a well can effect the quality of a screen. Whole-well scanning eliminates this well effect and gives greater confidence in screening data obtained.

HCA—An Enabling Technology

Advantages of analyzing whole cells

The HCA approach uses whole cell-based assays to dissect multiple pathways in a single multiplexed assay that cannot be easily addressed in traditional biochemical assays. This results in a reduction in compound requirement, increases the amount of information gained from the assay, and saves time by identifying better-quality hit compounds. HCA has the ability to measure complex cellular responses, including morphological variations within populations, that are applicable to differentiation and colony assays, plus translocation of proteins in multiple populations of whole cells in parallel. In addition, it can provide supplementary knowledge that can be critical when determining which targets to investigate and which lead compounds to pursue. For example, there are some major key advantages of using HCA as a primary screen over traditional biochemical screens. It is also the only method for determining the activity of proteins that cannot be easily purified. As the assay is carried out in whole cells, HCA can provide secondary information such as compound potency, cell permeability, and cytotoxicity. This information is very valuable, as a great deal of time and money can be lost by validating compound hits from primary screens that are toxic and or impermeable to the cell. All this information can all be obtained simultaneously from a single run of an assay.

Multipopulation analysis: protein kinase

The elucidation of the human genome has thus far identified around 518 kinases and presents a real opportunity for novel kinase pathway identification. In a whole-cell environment, all the components required for protein kinase activation are present in native quantities, thereby eliminating stoichiometric imbalances and physiological locations, giving the potential to find more effective drugs than could have been identified by inhibiting the kinase in biochemical assays. HCA provides an approach to

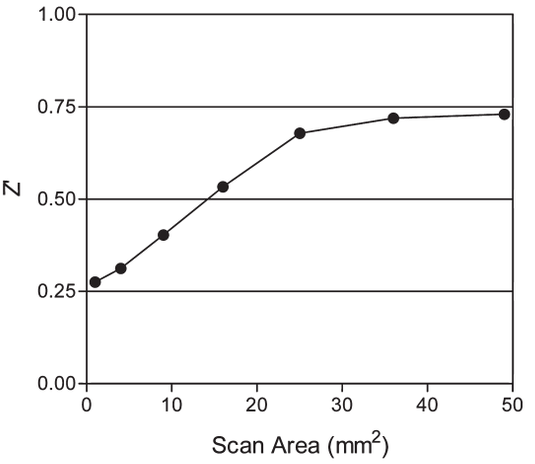

FIG. 6. Effect of scan area on Z' value. Mouse erythroleukemia stable cell lines, which contain the human α globin promoter driving an enhanced GFP reporter, were used to measure α globin expression after hexamethylene-bisacetamide (HMBA)-induced terminal differentiation. Cells were plated into a 96-well plate (50,000 cells per well) and treated with either solvent control or 5 mM HMBA (n = 4) for 48 h. Enhanced GFP expression was quantified using an Acumen Explorer and reported as the total total intensity of green fluorescence. Assay performance (Z') was compared across a range of square scan areas (from 1 × 1 mm to 7 × 7 mm).

identifying kinase modulators using either phosphospecific antibodies that recognize only the active form or anti-protein antibodies to determine kinase translocation from the cytoplasm into the nucleus (indicating activation).[5] Easily applied protocols have already been developed for many proteins, including a large range of phosphoproteins, such as extracellular signal-regulated kinase, to determine the numbers of both active and inactive cells in each well, leading to normalization of active cell number to total cell number in the whole well and greatly enhancing data quality.

In many assays, not all the cells in a well respond in a homogeneous manner, because of effects such as poor mixing of compound or fluorescent dye within the well. These effects can be more common and pronounced in primary screening because of the heavy reliance on automation. By analyzing the whole well in HCS, these effects can be easily identified. It also enables researchers to add different cell lines to a single well and determine cell line-specific effects to treatment. Cell-specific proliferation in a single well has been demonstrated on the Acumen Explorer using Chinese hamster ovary (CHO) cells and SH-SY5Y cells labeled with different Qtracker labels.[6,7] These types of assays can be readily adapted to other kinases or transcription factors such as p70 ribosomal S6 kinase and AKT (protein kinase B).

Tracking protein movement: translocation assays

Another of the key advantages of HCA is the ability to monitor the movement of specific proteins in living cells upon treatment with compound. Many cell signaling proteins move to different cell compartments in response to specific stimuli. One such application of translocation assays is to determine protein kinase activation, where the movement of the protein or the downstream transcription factor from the cytoplasm into the nucleus is identified. Using extracellular signal-regulated kinase as an example, in the inactive form it is located in the cytoplasm; however, upon phosphorylation and activation, the protein translocates into the nucleus. Other examples of these types of assays are SMAD and nuclear factor κB translocation from the cytoplasm to the nucleus. The other major form of translocation is to identify movement of the target protein between the cytoplasm and the membrane, for example, protein kinase Cα. Depending on the type of fluorescent dyes used, these types of analysis can be carried out in live cells or fixed cells. For example, using a reporter gene construct such as GFP, the analysis can be carried out in live cells, and translocation of the protein can be recorded over time, although it is more typical to use fixed cells and antibodies to the target proteins in a screening environment. As with many types of HCA assays, multiple protein targets or responses can be simultaneously detected. These translocation assays are reserved for microscope-based CCD imaging and microplate cytometry instrumentation, as flow cytometry systems are unable to determine translocation of proteins within a cell.

Morphology: cell colony formation

The use of tumor cells in colony formation assays for research and clinical applications to assess the functional integrity of cells in oncology research is extensive, including modeling for pharmacological and chemotherapy research programs. Colony formation and cell cluster assays are ideally run in a HCA format as they require the cells and colonies to be analyzed in their "native form." That is, they maintain comparable biochemical and morphological characteristics that are observed in cancerous tissues, which results in more biologically relevant information for parameters such as proliferation and differentiation. Subsequently, this type of assay cannot be analyzed on flow cytometry instrumentation since they require dispersed suspended populations of cells. Traditionally, enumeration of colonies has involved laborious and subjective counting by hand using a microscope. Subsequently, this has been automated using microscope-based CCD imagers looking at the area of all the colonies or estimating the number of cells within a colony. However, even these types of HCA systems have their limitations. For very large colonies ($>$1 mm), microscope objectives are not easily able to fully visualize single colonies, thus making analysis with microscope-based CCD imagers slow and impractical for screening purposes. Microplate cytometry provides an automated high content readout of the effects of cytostatic agents on either the formation of colonies, or their destruction. Colonies containing the designated number of cells are discriminated and identified from small clumps of cells using a fluorescent microscope integrated with the microplate cytometer.[8] Because of the large field of view, analysis is rapid and easily identifies large colonies. The assay provides a simple, high content, high throughput method for predicting the response of tumors to chemotherapeutic agents.

Integration of HCA Technologies

As the high content research area continues to mature, there is a growing consensus that there is not a single instrumentation platform that can perform all high content assays and at the range of throughputs being applied. Therefore, researchers are being forced to integrate the three main technologies of flow cytometry, microscope-based CCD imaging, and microplate cytometry, as either single or multiple units to match the therapeutic targets under study and the laboratory's role, for instance, target identification and validation, hit-to-lead optimization, or HTS.

Integration of flow and microplate cytometry

Flow cytometry represents the most sensitive and multicolor high content platform. Such attributes result from analysis of a well-characterized stream of suspended cells using multiple laser lines and PMT detectors, a configuration that is capable of capturing a high percentage of emitted fluorescence. It also offers cell sorting where required. A key use of flow cytometry is in oncology research, where cell cycle analysis is a common application. The cell cycle is a target for many anti-cancer drugs, making the ability to monitor the effects of such agents on the cell cycle an important part of the drug development process. The most standard method of determining the cell cycle phase of individual cells is by quantifying their total DNA content using flow cytometry following staining with a fluorescent dye such as propidium iodide.[9,10] The measurement of DNA content was one of the first major applications of flow cytometry and is still one of the most common uses today. This is unlikely to change because of the inherent high sensitivity of flow cytometers and the number of validated protocols for research and clinical use. However, the inability of flow cytometry to analyze cell lines *in situ* can limit its use in the research laboratory where the primary cell models are mainly adherent.

Microplate cytometers have proved to be extremely effective for enumerating DNA content in cells and offer new possibilities for cell cycle analysis. Since propidium iodide staining provides a strong emission profile when excited with a 488 nm laser line there are no sensitivity issues. Of major significance is the ability to analyze adherent cells *in situ*, unlike flow cytometry, which requires their suspension for processing. *In situ* reading preserves morphological changes that may have occurred during drug treatment, thus giving valuable secondary information, especially when analyzing highly differentiated cells such as neurones. Finally, in multiplex assays where cell cycle analysis is being combined with other readouts such as receptor or pathway signaling, analysis within microplates eliminates the secondary effects associated with the harsh process of suspending the cells for flow cytometry investigation. One disadvantage of analysis using microplate cytometers is that the number of cells and thus events observed is limited by the size of the well being used. This can result in insufficient data being produced for the application of curve-fitting algorithms for estimation of cell cycle distributions (*e.g.*, ModFit, Verity House Software, Topsham, ME).

For screening purposes, the throughput of a microplate cytometer for cell cycle analysis is unparalleled, being able to analyze an entire 384-well plate in about 10 min—approximately sixfold faster than miniaturized screening protocols available on specialized flow cytometers. Since all the cell processing is performed with microplates, it is also more amenable to automation. The utility of microplate cytometry for compound profiling has been demonstrated using a multiplex protein reporting cell cycle and mitotic index data using standard agents, such as vinblastine[11] (Fig. 7). In another study, an Acumen Explorer microplate cytometer has been used to simultaneously correlate the effects of a protein kinase interfering RNA library on cell proliferation and cell cycle analysis in a multiplexed assay protocol.[12] Such studies are not easily

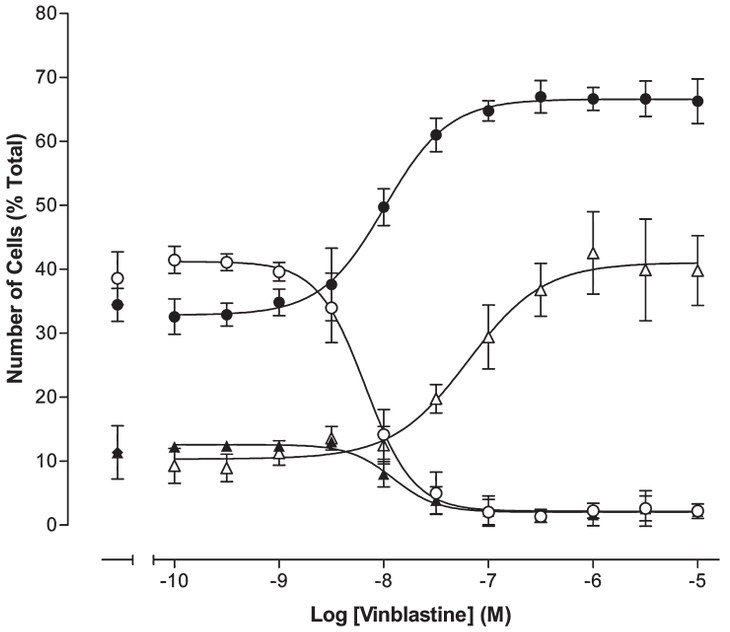

FIG. 7. Multiplex measurement of cell cycle and mitotic index analysis. CHO cells were plated in a 96-well plate (2,000 cells per well) overnight and treated with solvent control or vinblastine. After incubation for 22 h cells were fixed and stained with anti-phosphorylated histone H3 (fluorescein isothiocyanate secondary conjugate) to determine mitoitc index and with propidium iodide for cell cycle analysis. Two-color analysis was performed on an Acumen Explorer at a scan resolution of 1×2 μm using the following object characteristics: mitotic index, green fluorescence peak intensity; cell cycle analysis, red fluorescence total intensity for G1 phase (○), S phase (▲), G2/M phase (●), and mitotic (△).

performed on flow cytometers because of difficulties in estimating total cell numbers, especially where marked reductions in cell numbers are present.

Integration of microscope-based CCD imaging and microplate cytometry

As discussed previously, microscope-based CCD imaging uses microscopic objectives to obtain cellular and sub-subcellular high content information at high optical resolution. The level of detail observed depends on the magnification factor of the objectives applied, with ×10 and ×20 magnification being commonly used to maximize the field of view, and thus number of cells recorded by the CCD camera. Some assays require very high magnification (×40–×100) where the cellular structures being analyzed are small (<1 μm) or high spatial resolution is an assay prerequisite—such assays are not ideal for microplate cytometry. Examples include micronuclei formation, Transfluor® (Xsira Pharmaceuticals, Research Triangle Park, NC) GPCR redistribution, and neuritogenesis. For these assays, sample throughput can be very low (several hours per 96-well microplate), making the technology unsuitable for sustained primary screening of compound libraries. The application of both microscope-based CCD imaging and microplate cytometry can be very powerful for some of these applications, especially where the high sample throughputs of microplate cytometers can be used for lead generation in primary screens and the high optical resolution of microscope-based CCD imagers reserved for high-information secondary profiling studies.

This is exemplified by the analysis of neuronal cell differentiation where the production of neurite outgrowth is observed. Microplate cytometers can detect the differentiation of neuronal cell lines (*e.g.*, SHSY-5Y) through a marked decrease in the peak intensity value of membrane staining upon differentiation. Such an assay can provide a throughput of a single 384-well plate in 8 min, sufficient for lead generation given the complex biological process being evaluated. However, what is lacking is detailed information on the neurite outgrowth itself because of an inability of microplate cytometers to resolve such structures optically. Integration with a microscope-based CCD imager thus permits far higher interrogation of screening hits because of the higher optical resolution they enable. Thus the initial screening result can be supplemented with data for the number of neurites per cell, neurite length, and the number of cross and branch points.

HCA versus bulk fluorescence measurement

Homogeneous fluorescence-based biochemical assays are the mainstay of HTS, offering high sample throughput at low cost. In spite of this, cell-based assays are being increasingly adopted to provide functional readouts from live cells. Examples include measurement of calcium flux (FLIPR) and reporter gene analysis. Some microscope-based CCD imagers can measure calcium flux and thus report data on a cell and population basis. The responses are too rapid, however, for quantification using microplate cytometry because of the use of a scanning laser.

In contrast, many reporter gene assays can be quantified by microplate cytometry, including those based on fluorescent proteins and substrates. The high content readout provided has many advantages. First, the ability to define subpopulations within each well can increase the robustness of assays. This can be important for methods in which only a defined population of cells responds to the stimulant or possesses the required amount of reporter gene activity, for example, in assays using transiently transfected cell cultures. Second, use of the number of cells (including subpopulations) as a primary

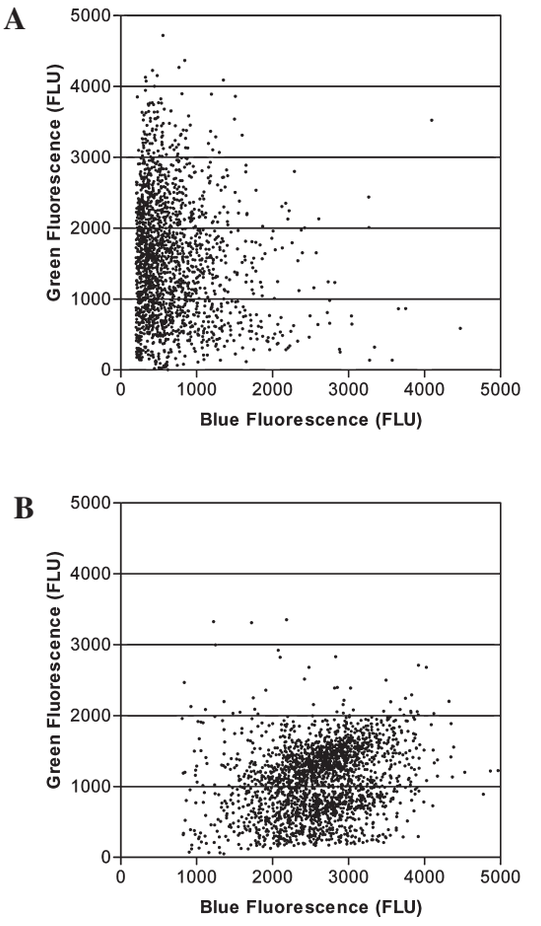

FIG. 8. HCA of β-lactamase reporter gene expression. Scatter plots show blue versus green fluorescence (mean intensity) for inactive (**A**) and active (**B**) D1 CRE-bla CHO-K1 cells expressing β-lactamase reporter gene. Note the heterogeneity in green and blue fluorescence for each population. FLU, fluorescence units.

readout rather than a fluorescent measurement can overcome the significant heterogeneity observed in the fluorescent intensity of active and inactive cells in some assays. For example, β-lactamase reporter gene activity has conventionally been analyzed by either bulk fluorescence readers or flow cytometers, both of which require very high numbers of cells ($>10^4$). Microplate cytometers such as an Acumen Explorer equipped with the 405 nm laser line can simultaneously detect both the blue (β-lactamase-expressing) and green (β-lactamase-negative) regions of the spectrum to discriminate active from inactive cells. The subsequent reporting of the number of active and inactive cells thus overcomes the scatter in the fluorescent intensity seen in the blue and green emissions, routinely resulting in increased fold activation and assay robustness (Z') (Fig. 8). β-Lactamase reporter gene assays using bulk readers have difficulty differentiating between cytotoxicity and antagonism since both can result in the same reduction in fluorescence signal—this increases the number of false-positives returned by the assay. Moving to a high content output reports the total number of cells in each well without the introduction of addition fluorescent markers, which enables cell loss associated with toxic compounds to be positively identified and differentiated from antagonism where the cell number remains constant (Fig. 9).

Conclusions

The adoption of HCS is not yet complete within the pharmaceutical industry, with only an estimated 450 instruments placed worldwide. The slow speed of many HCA instruments (relative to traditional HTS methods) has pushed companies towards using smaller targeted libraries. The high level of information generated by some systems and subsequent data storage and evaluation are a concern. In addition, instrument cost remains a barrier to entry for many organizations, particularly academic institutions. Nevertheless, some of the disadvantages of HCA are outweighed by the advantages from such an approach.

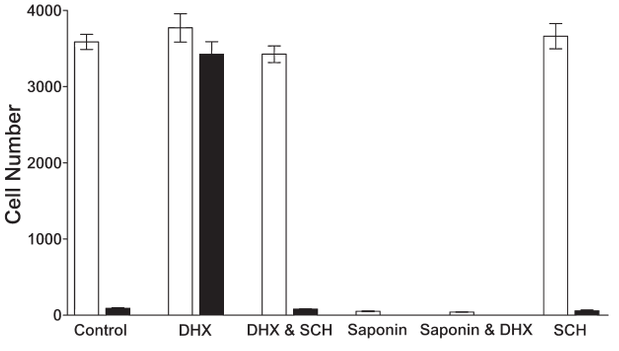

FIG. 9. Use of cell number as a toxicity indicator in β-lactamase reporter gene expression assays. D1 CRE-bla CHO-K1 cells expressing β-lactamase reporter gene were stimulated with the dopamine agonist dihydrexidine (DHX; 1 μM) in the presence or absence of SCH-23390 (SCH; 100 nM) antagonist or saponin (0.01%) detergent. Both the total number of viable cells (open bars) and the subpopulation that was active (filled bars) in each well were determined using an Acumen Explorer microplate cytometer. Note that wells treated with SCH contained control levels of viable cells, thus demonstrating that antagonism can be distinguished from cytotoxicity (*cf.* saponin).

Microplate cytometers, such as the Acumen Explorer, were developed to meet the demand for higher throughput screening at the cellular level. Commonly, they form part of a multilevel screening workflow containing other distinct solutions. Where speed is paramount, they provide rapid primary screening capability for lead identification, with "hit" compounds being validated using microscope-based CCD imagers or flow cytometry. Alternatively, they can be placed after homogeneous HTS screens where they allow follow-up investigation of lead compound activity in a whole-cell environment plus an early indication of cytotoxicity liability. The most widely used high content instruments are those based on automated fluorescent microscopy, namely, the microscope-based CCD imagers. These offer high-resolution imaging of cells, including confocal analysis, making them ideal for morphological readouts. The downside is that they can generate terabytes of information (predominantly CCD images) requiring expensive data storage and retrieval solutions, and sample throughput is generally relatively low. These shortcomings make them unsuitable for sustained use in primary screening unless high optical resolution is required to perform the assay (*e.g.*, neurite outgrowth, micronuclei detection). Evidence suggests that the scanning resolution offered by microplate cytometers is comparable to at least the optical resolution provided by a $\times 10$ microscope objective (possibly even that of a $\times 20$ objective in some applications). Such resolution is perfectly applicable to whole-cell fluorescence assays performed by flow cytometry where sensitivity is not an issue, and subcellular analyses such as protein translocation run on microscope-based CCD imagers.

The Acumen Explorer is most commonly used for protein kinase activity measurements in whole cells using either anti-phosphokinase or anti-protein kinase antibodies.[13] For single color assays, the readouts used include number of fluorescent cells detected (anti-phosphokinase) or fluorescent translocation (anti-protein kinase). When combined with nuclear staining and whole-well scanning, both methods report the total number of cells in each well for data normalization. This can overcome the problems of variable stimulation and random cell distribution thatare often observed in screening plates. Recently, the application of microplate cytometry to cell cycle analysis has become more prevalent. Normally regarded as a flow cytometry method, the DNA staining

used to differentiate cells in each of the main divisions of the cell cycle (G1, S, and G2/M phases) is easily quantified using the PMT detectors in microplate cytometers. Such assays can be formatted in 384-well microplates using adherent or non-adherent cells to enable throughputs of 1,000 samples in under 25 min. When coupled to targeted identification and validation technologies such as interfering RNA, this throughput permits genome-wide profiling or parallel assessment of multiple cell lines.

The number of fluorescent reagents (*i.e.*, dyes, probes or proteins) used in an assay does not necessarily limit the level of multiplexing that can be achieved. Microplate cytometers can contain up to four PMT detectors for parallel multicolor assessment, but their use is not mandatory. For instance, staining the DNA of fixed cells with propidium iodide or Hoechst enables at least four readouts: cell proliferation from cell number; cell cycle analysis using the total intensity of staining; cytotoxicity based on the total cell number; and nuclear morphology for adverse effects such as micronuclei formation.

HCA has shown it can improve the quality of hits identified from primary screens, by the ability to evaluate more than one readout.[14,15] It is possible to evaluate a compound targeted against a specific kinase and simultaneously determine the compound's cytotoxicity. Effects that are undesirable enable compounds to be discounted at this early stage. The potential for reducing false-positives makes secondary screening and "hit to lead" more efficient and cheaper. In an industry preoccupied with the quest for the next blockbuster drug, the advantages and benefits of HCS-enabled lead discovery cannot be ignored. Microplate cytometry is a practical solution for the implementation of high content cell-based assays in HTS, and is especially powerful when integrated with persisting instrumentation such as flow cytometers and microscope-based CCD imagers.

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Address reprint requests to:
Wayne P. Bowen, Ph.D.
TTP LabTech Ltd.
Melbourn Science Park
Melbourn, Hertfordshire SG8 6EE, UK

E-mail: wayne.bowen@ttplabtech.com