Confocal v widefield

high content imaging

A variety of new confocal hardware and software solutions have recently become available to support high content imaging. Most of the solutions have in common the potential of rejecting out-of-focus background fluorescence and of improving the signal to noise of images. Yet awareness and understanding of these alternative approaches and where they can be applied most advantageously is overall deficient. The tendency to rank 'true' confocal imaging solutions in a league superior to widefield optical sectioning alternatives may, in some cases, be unjustified if actual needs are given thorough consideration. In this article we will review the benefits of confocal imaging, the different confocal options, the causes of poor image quality and if confocal imaging is needed for HCS applications. This discussion will provide an introduction against which the reader can appraise the updates of vendors' HCS offerings.

The need to provide confocal alternatives to existing widefield imaging systems has been a cause for concern to some HCS (High Content Screening) instrument vendors lacking such an option. Prompted by a request to contribute to this debate HTStec undertook its 'Confocal HCS Imaging Trends' survey in October 2005. The objective of this survey was to document current understanding of confocal technology; the reasons why confocal optics are chosen over widefield, and the specific imaging assays and requirements where confocal optics are actually needed. In this article we will discuss survey feedback from HCS and HCA (High Content Analysis) users and review the latest developments in, and alternatives to, confocal imaging.

Overall, the level of technical understanding of confocal imaging among survey respondents was moderate. Responses ranged from those individuals who clearly were optical experts, who in one case was insulted by being asked such seemingly trivial questions, to those that suggested a lot lower level of familiarity and some uncertainty regarding the basic concepts. There appears to be an element of 'smoke and mirrors' surrounding confocal instruments and alternative solutions particularly when it comes to the optics (resolution) and the performance (throughput). Many people hear the words 'confocal' and 'laser' and automatically assume that the technology is somehow newer, faster and better!
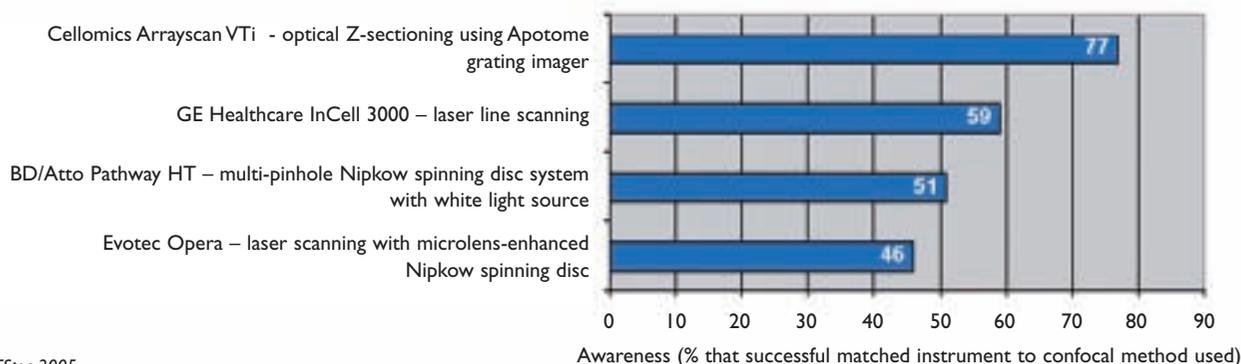
By Dr John Comley

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Figure 1: Perceived HCS benefits of confocal imaging

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Mean rank, where 1 = no importance and 5 = extremely important
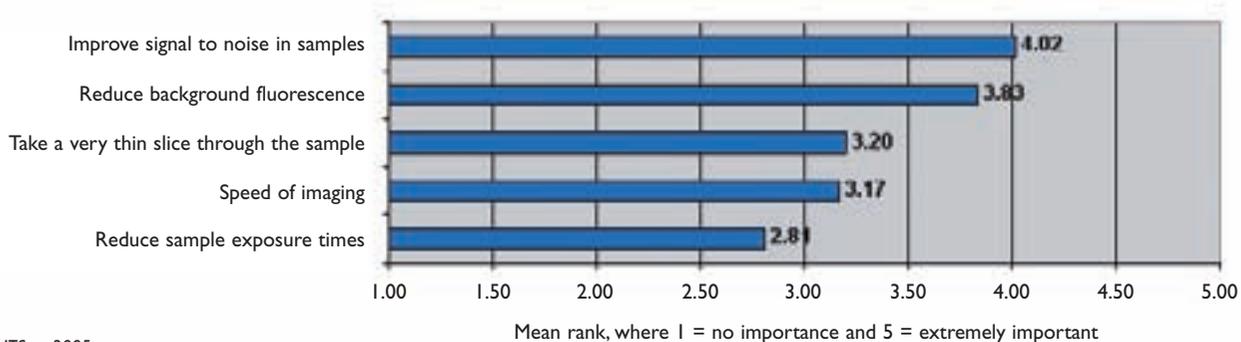
Figure 2: Awareness of confocal imagers and confocal method used

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Awareness (% that successful matched instrument to confocal method used)
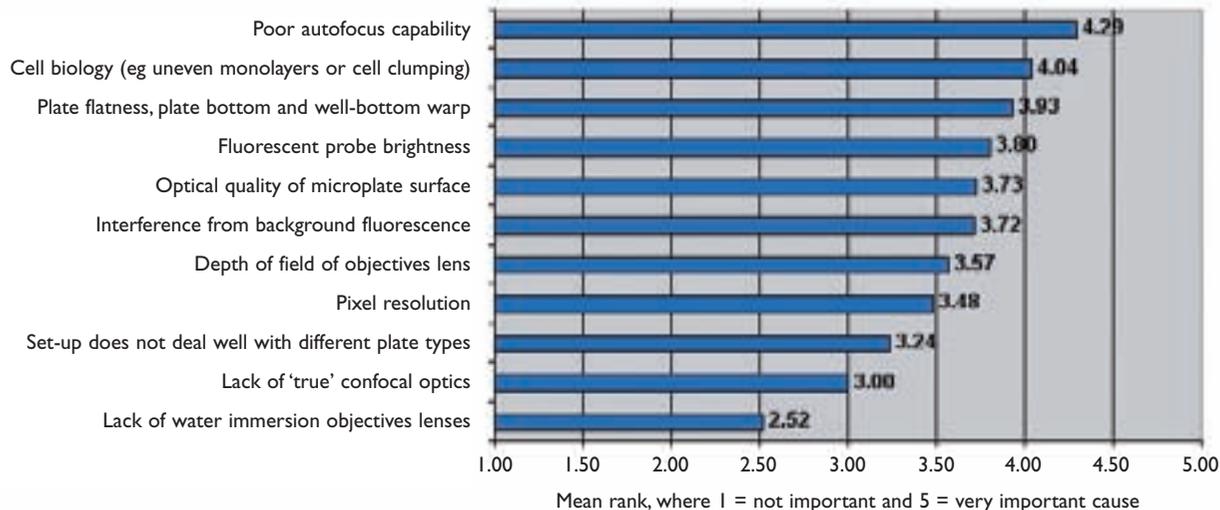
Perceived benefits of confocal imaging

Improved signal to noise in samples and reduced background fluorescence were ranked by survey respondents as the confocal benefits they believed to be most important to HCS experiments (**Figure 1**). The other benefits of confocal imaging, ie taking a very thin slice through the sample; enabling enhanced speed of imaging and reduced sample exposure times (eg resulting in less photobleaching and to prevent phototoxicity in 'live cell' studies) were all ranked of less importance to the HCS users surveyed. In reality it is the ability of confocal imaging to reject out-of-focus background fluorescence which improves the signal to noise.

There are different confocal imaging solutions

Based on survey respondents' ability to successfully match the various commercial HCS imaging systems to the method used to produce a confocal image, greatest awareness was of the Cellomics

Arrayscan VTi with ApoTome and least awareness was of the Evotec Opera based on a laser scanning with microlens-enhanced Nipkow spinning disc (**Figure 2**). Understanding of all systems and methods was greatest among those persons that were using or had used a confocal HCS system or who came from large pharma. A confocal solution can be achieved by several methods: 1) a purely hardware solution, such as confocal microscope involving a pin hole or slit or spinning disk or laser line scanning, ie 'true' confocal optics; 2) use a software solution, such as deconvolution of an image stack; or 3) do a combination of hardware and software methods such as used in the Cellomics ApoTome (grating imager). In 'true' confocal imaging the excitation light is focused in a single plane, thus the emission light that is used to generate the resulting image comes from a single optical slice of the sample. There is no emitted fluorescent light from above or below the sample and no bleaching of the sample above or below the

Figure 3: Potential causes of poor image quality

Cause	Mean rank
Poor autofocus capability	4.29
Cell biology (eg uneven monolayers or cell clumping)	4.04
Plate flatness, plate bottom and well-bottom warp	3.93
Fluorescent probe brightness	3.80
Optical quality of microplate surface	3.73
Interference from background fluorescence	3.72
Depth of field of objectives lens	3.57
Pixel resolution	3.48
Set-up does not deal well with different plate types	3.24
Lack of 'true' confocal optics	3.00
Lack of water immersion objectives lenses	2.52

Mean rank, where 1 = not important and 5 = very important cause

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plane of excitation. In contrast, in a widefield imaging systems the excitation light passes through the entire sample and some of the light in the resulting image is from out of focus emission light both above and below the focal plane of the lens. Reconstruction of 3D images can be done with both widefield and 'true' confocal imaging systems. In 'true' confocal instruments the pass through the Z-section is co-ordinated with the depth of field and reconstruction of a 3D image uses all the data collected. Widefield methods acquire a Z-stack of images and typically a decon-volution method is employed that removes or back calculates the position of the 'out of focus' light that came from above and below a focal plane in the stack, repositioning it in the image plane. This method can considerably sharpen an image and reveal signal over noise. Deconvolution relies on careful characterisation of the point spread function of the objective lens used to accurately reposition the out of focus light. The thickness of the image plane and the visual quality of the image differs only slightly with the confocal technology employed. Both methods are quantitative and result in beautiful images. However, deconvolution does require considerable processing time, and the image quality can suffer from bleaching of the probes as the Z-stack is acquired since the entire sample is illuminated in Z-space during the Z-stack acquisition. A widefield solution is, however, cheaper and facilitates a 'use as you need' approach.

Potential causes of poor image quality

Poor autofocus capability was ranked as the most important cause of poor image quality, closely followed by cell biology (eg uneven monolayers or cell clumping) and plate flatness, plate bottom (ie high quality glass bottomed multiwell plates) and well-bottom warp (**Figure 3**). Interestingly, interference from background fluorescence and the lack of 'true' confocal optics were both rated less important, highlighting the fact that the benefits of confocal optics alone will not improve poor image quality unless accompanied by other hardware and biology improvements. To better understand the advantages of 'true' confocal optics it is necessary to have an awareness of several basic concepts:
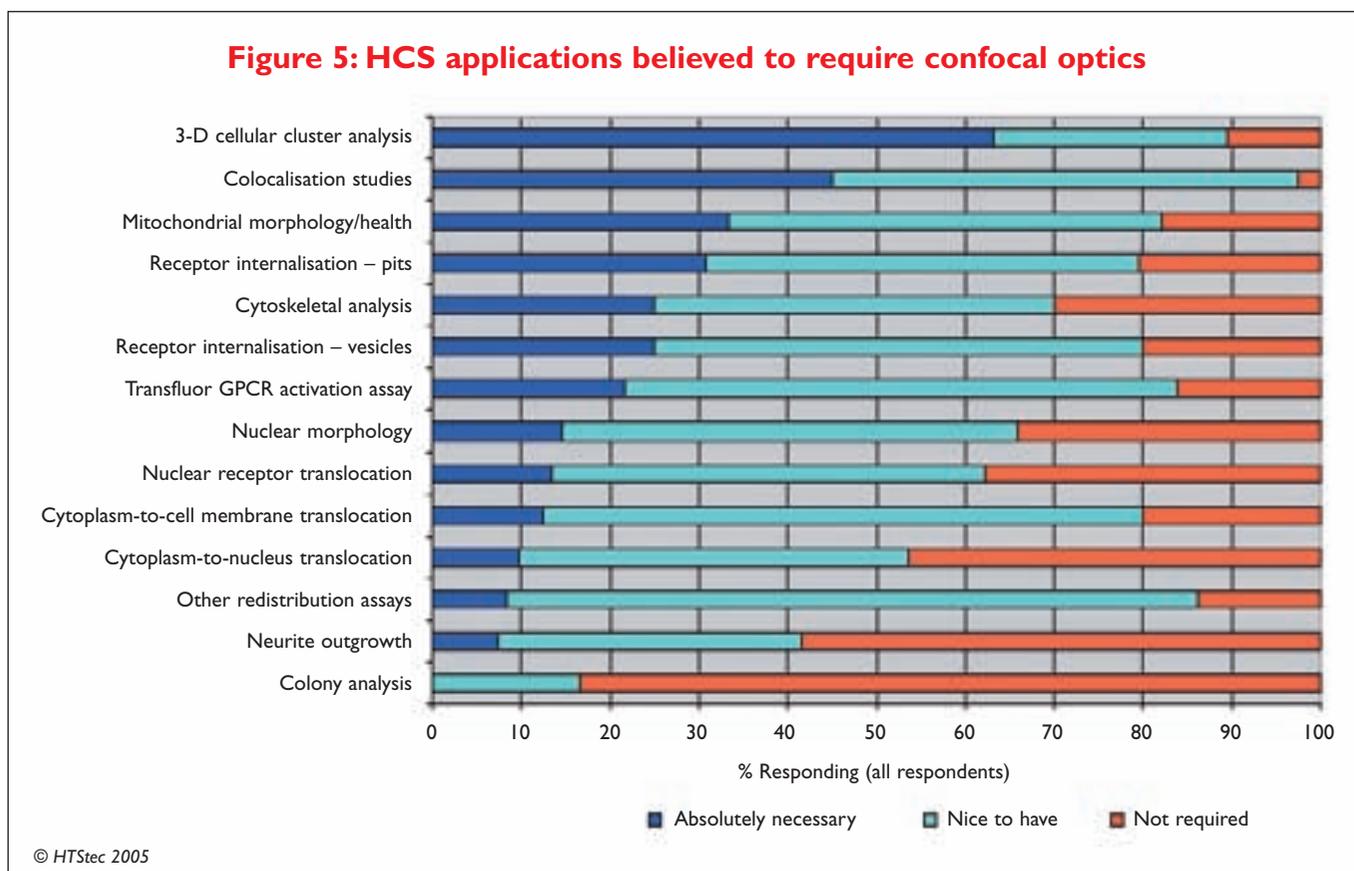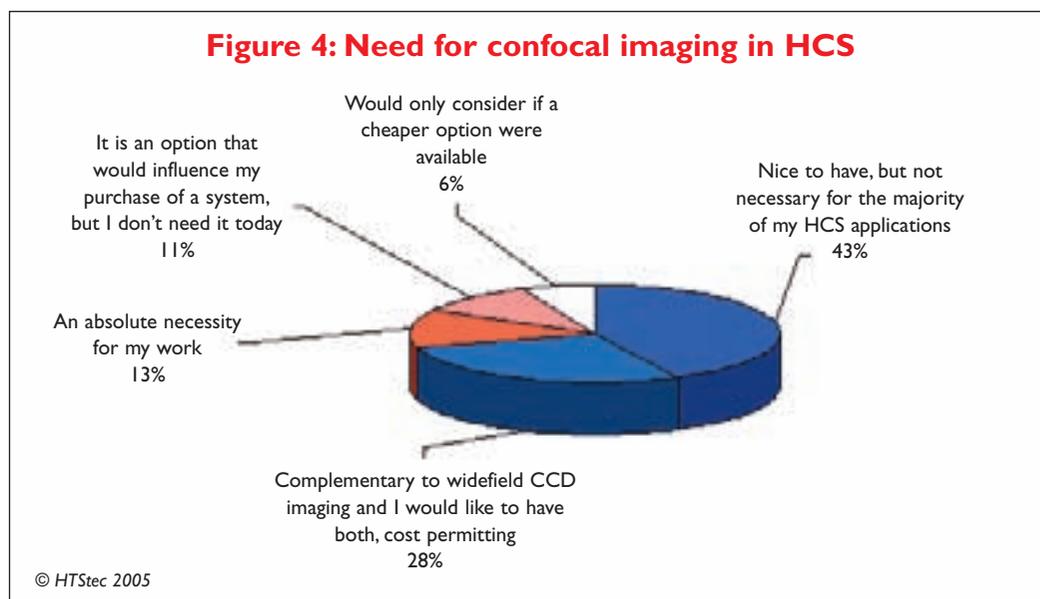
1. Contamination of an image with background out of plane light

Light from out of the focal plane contributes to background and therefore decreases signal to background. As a consequence is it harder to make determinations in the lateral (X-Y) resolution because segmentation becomes more difficult. Degradation in the axial (Z) resolution is almost guaranteed since you don't know for sure where in Z space light contributing to an image came from. The edges of the focal plane would add significant background, thus reducing the quantitative value of the Z information in the image.

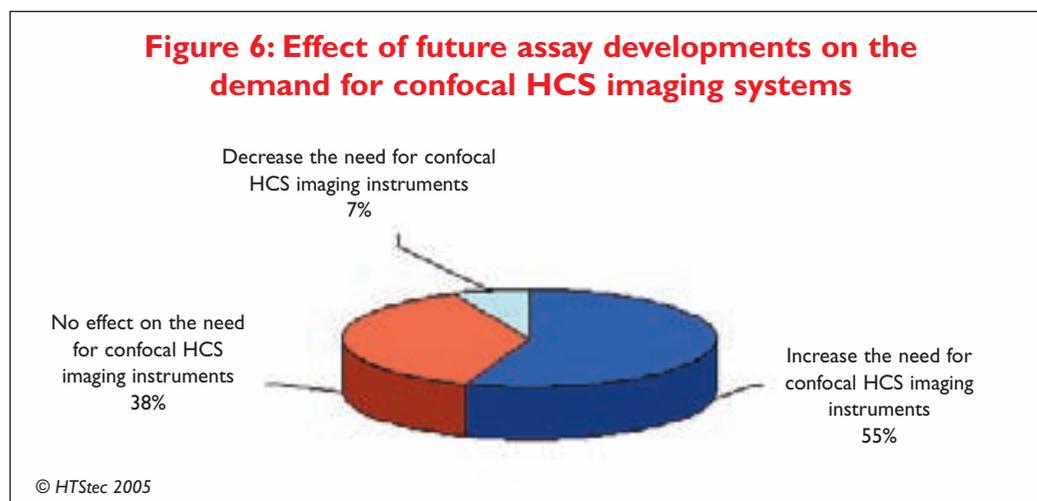**2. Importance placed on the depth of field of the objective lens**

The importance of depth of field is in determining the optimal imaging thickness of the Z-section you

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Figure 4: Need for confocal imaging in HCS

It is an option that would influence my purchase of a system, but I don't need it today
11%

Would only consider if a cheaper option were available
6%

Nice to have, but not necessary for the majority of my HCS applications
43%

An absolute necessity for my work
13%

Complementary to widefield CCD imaging and I would like to have both, cost permitting
28%

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Figure 5: HCS applications believed to require confocal optics

3-D cellular cluster analysis
Colocalisation studies
Mitochondrial morphology/health
Receptor internalisation – pits
Cytoskeletal analysis
Receptor internalisation – vesicles
Transfluor GPCR activation assay
Nuclear morphology
Nuclear receptor translocation
Cytoplasm-to-cell membrane translocation
Cytoplasm-to-nucleus translocation
Other redistribution assays
Neurite outgrowth
Colony analysis

% Responding (all respondents)

■ Absolutely necessary   ■ Nice to have   ■ Not required

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are investigating. Specifically, the depth of field is the distance in Z in your sample that appears in focus in your image. A large depth of field will give a thick slice of the Z-section of the sample in focus, a narrow depth of field gives only a very thin slice in sharp focus. Depending on the sample or desired analysis in Z-space either situation may be beneficial. For example, if one wanted to determine if two cellular organelles were co-localised or situated above or below each other, it would be necessary to

Figure 6: Effect of future assay developments on the demand for confocal HCS imaging systems

Decrease the need for confocal HCS imaging instruments 7%

No effect on the need for confocal HCS imaging instruments 38%

Increase the need for confocal HCS imaging instruments 55%

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have a narrow depth of field and multiple planes of focus to compare their location in Z-space. If one wanted to see as much of a sample as possible in focus in a single image, a large depth of field would be desirable.

3. Relationship between objective magnification and depth of field

While both numerical aperture and magnification affect the depth of field, the situation is actually more complicated and for most high-end confocal imaging systems (eg Evotec Opera and GE IN Cell Analyzer 3000) the dominant factor in defining the depth of field is the numerical aperture of the objective lens employed. Furthermore, users of the Evotec Opera can use high NA immersion objectives, so here the depth of field is really dependent on the numerical aperture. Most HCS practitioners therefore concern themselves primarily with the numerical aperture in determining the depth of field, since the magnification can be changed by other components of the optical path that manipulate the final image magnification, eg coupler lenses, or shrinking the scan area. In a situation where instead of imaging a thin discrete object, one is imaging continuous fluorescence (such as a whole cell stain, or very large structure), then magnification would play a bigger role. However, most HCS images are of more discrete objects, eg cytoskeleton, plasma membrane, small organelles, etc.

Is confocal imaging needed in HCS applications?

When questioned on the need for confocal imaging in HCS the greatest proportion (43%) of survey respondents thought that confocal imaging was nice to have but not necessary for the major-

ity of their HCS applications (**Figure 4**). A further 28% of respondents thought that confocal imaging was complementary to widefield CCD imaging and they would like to have both cost permitting. Only a small minority (13%) thought that confocal imaging was an absolute necessity for their work. The remainder either do not need it today, although it is an option that would influence their purchase of a system, or would only consider it if a cheaper option were available. Most (62%) respondents felt that some of their assays would benefit from a confocal imager and 48% had generated data that validated a confocal optics requirement for selected HCS assays. The HCS applications which respondents believed require confocal optics most are presented in **Figure 5**. 3-D cellular cluster analysis and colocalisation studies were ranked most absolutely needing confocal optics by the largest number of respondents. For all of the other HCS applications listed in **Figure 5** most respondents thought confocal optics were either nice to have or not required. The confocal approach seems to work best when there are limitations of signal strength in the sample, as a result of target abundance or the performance of the reagents used. It was also suggested that confocal imaging may open up the way for higher throughput FRET-FLIM type analysis, but the time course of cellular responses when trying to capture data from 96-384 well plates may be an issue. Other respondents were more uncertain about the benefits of confocal solutions, concerned about the artifacts deconvolution algorithms may introduce; the lack of robust image analysis software for rapid object measurement of assays that do not use cells, eg zebrafish embryos and larvae; the need for better

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focusing algorithms, better primary optics and not forgetting improved plate optics.

As HCS moves forward and new assays are developed, more than half (55%) of survey respondents predict there will be an increased need for confocal HCS imaging instruments (**Figure 6**). Only a very small number (<7%) predict a decreased need for confocal HCS imaging instruments. So we can expect reagent improvements will drive the need for high speed confocal instruments in order to enable researchers to query their specific biological application.

Vendor updates

BD Biosciences (www.bdbiosciences.com/bioimaging) has extended its product line through the introduction of two new endpoint imaging platforms and by upgrading the existing BD Pathway™ platform. The new entry-level BD Pathway™ 415 is equipped with high-speed eight-position excitation and emission filter wheels combined with an independent high-speed five-position dichroic wheel. Illumination is provided by a long-life, alignment-free metal halide lamp source. The system is provided with a newly designed high-per-

formance laser autofocus system that can be used with a variety of plate types, and can be turned off or combined with software-based focusing technology. Brightfield imaging is also possible using a unique LED-based light canopy housed above the plate tray. The new BD Pathway 435 shares the same optical bench as the Pathway 415 but includes BD's proprietary spinning disk confocal technology. The confocal technology allows real-time resolution enhancement as well as fully functional 3D modelling capabilities including automated collapsed Z-stack image capture. The upgraded BD Pathway™ 855 now includes a high performance laser autofocus system. The imager includes a custom designed confocal optical bench coupled to an environmentally controlled imaging chamber and on-stage liquid handling for imaging living cells. The design permits imaging while reagents or compounds are being added to the cells, and the system's ability to capture several images per second assures important early kinetic events are not missed. Two independent mercury arc lamps, 16 excitation and eight emission filter positions are brought together by two independent five-position dichroic filter changers, enabling
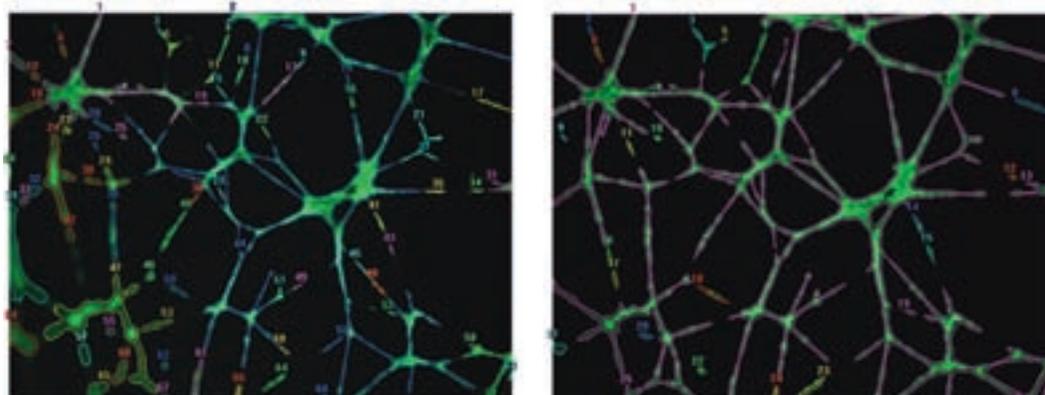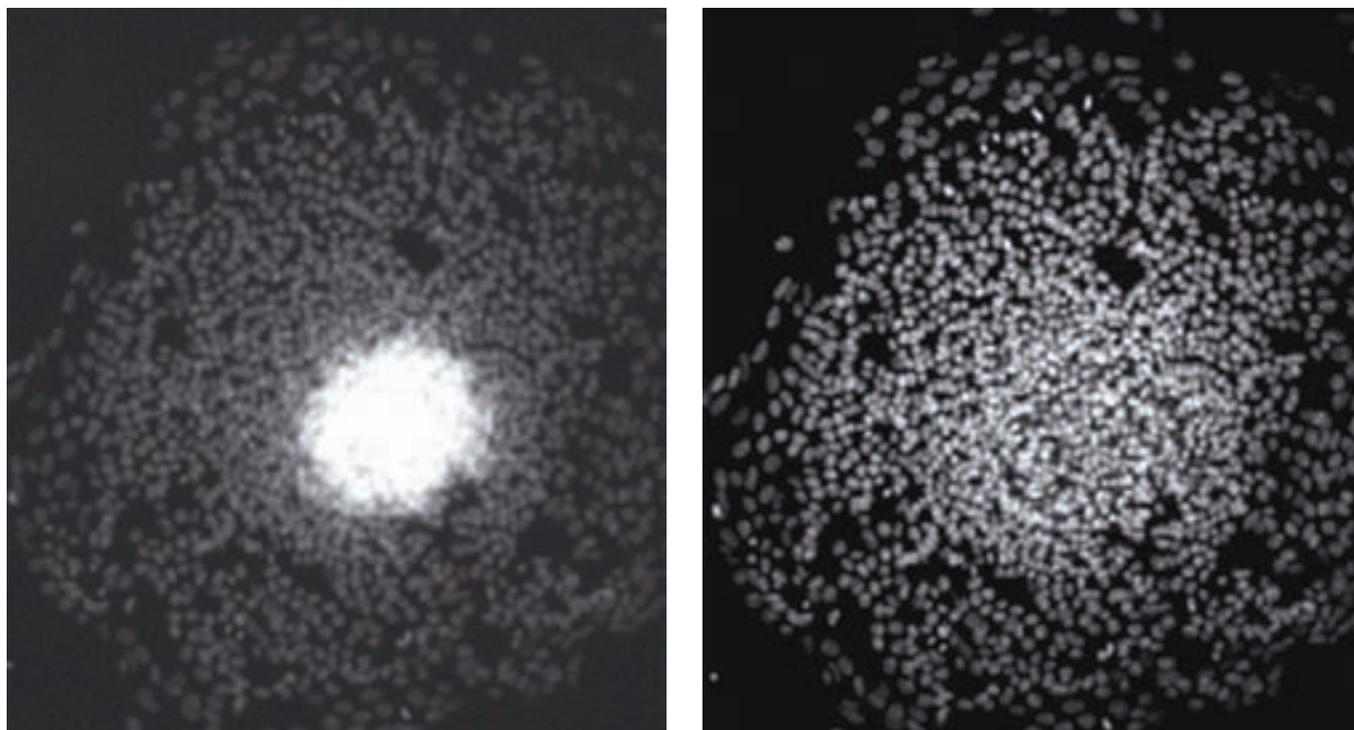
High Content Screening

Figure 9: The nuclei of stem cells in a large spherical colony were labelled with Hoechst 33342. Images were collected without (left) and with (right) the ApoTome on the ArrayScan® VTI HCS Reader using a 10X/0.3NA objective. ApoTome sectioned images display reduced background and enable resolution and identification of the individual cells in the centre of the colony
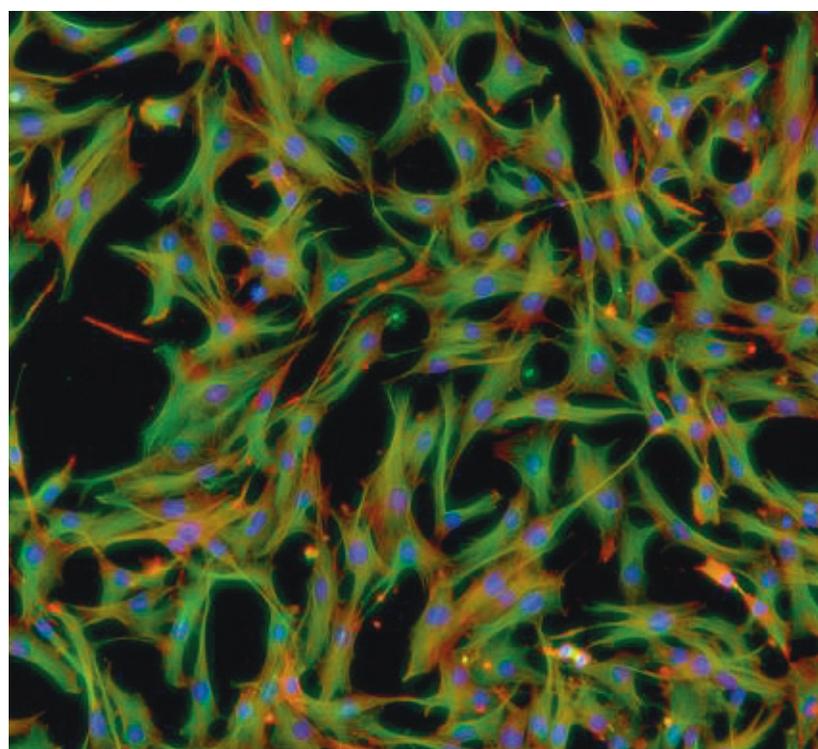

Figure 10: A pseudocoloured composite image of cells labelled with fluorescent markers for nuclei (blue), f-actin (red), and tubulin (green) and imaged on a cellWoRx™ High Content Cell Analysis System. Whereas the optical resolution is 0.74 micron, the deconvolution algorithm applied to the images enables the elucidation of the fibres

imaging from 340nm up to near-IR. All of the Pathway bioimagers are built around a high precision (under 100nm) x, y, z objective positioning system that keeps the plate motionless during imaging. The high precision allows multiple sequential images to be taken throughout a well to virtually extend the field of view, and the motionless plate provides suspension cell imaging capabilities. (**Figures 7** and **8**).

Cellomics (www.cellomics.com) and **Applied Precision** (www.api.com). Most biologies do not require the additional efforts and expense required from confocal approaches; however, there are three main reasons that this approach is selected by researchers. The first is speed, which is achieved primarily in point-scanning laser confocal systems by increasing laser power to reduce exposure times. This time saving is generally only realised when switching through multiple lasers is not required. The second is higher contrast than can generally be achieved with traditional wide-field, epi-fluorescence microscopy. The third is the requirement for optical sectioning in thick and densely labelled specimens. The Cellomics answer to the question, widefield or confocal, is to offer the researcher optimised instruments that are tailored to their

biologies. This best-in-class total solution approach includes two options.

● Option 1: The ApoTome system, engineered by Carl Zeiss and integrated into the ArrayScan® VTI HCS Reader, is a hardware/software module for optical sectioning. The ApoTome utilises a grating imager to acquire a number of images, which are then combined and filtered to provide an optically sectioned image. The depth of field of such images is in the order of, and varies with, the depth of field of the objective used, allowing you to capture a significant portion of the cells' depth in a single section. The resulting images have better signal-to-background due to the removal of out-of-focus light. The ability to resolve objects such as cytoskeletal fibres, small punctuate objects, cytoplasm of round cells and identification of the individual cells in the centre of a colony (**Figure 9**) is also improved. Thus, the ApoTome Module is a very affordable option for those seeking optical sectioning for the purposes of background reduction or fine structure elucidation.

● Option 2: The cellWoRx™ High Content Cell Analysis System is a wide-field imaging system from Applied Precision and Cellomics. The cellWoRx System provides high contrast images of samples commonly used in HCS. Since it is a wide-field system, the depth-of-field is not restricted as it is in confocal systems. In addition, the cellWoRx™ system provides a unique optical axis integration methodology (Z-Scan) which, when combined with the provided on-the-fly deconvolution, permits users to collect an extended depth-of-field without the concomitant loss of lateral resolution. This can be of particular use when the application calls for measuring the entire mass of a biomolecule throughout the volume of the cells. The contrast (signal-to-background) in images for the cellWoRx System is increased relative to conventional epi-fluorescence HCS systems by using a proprietary illumination system that separates the illumination light from the detection pathway. In repeated comparisons with competitor systems, the cellWoRx system produces 3-5 times more contrast than traditional epi-fluorescence systems

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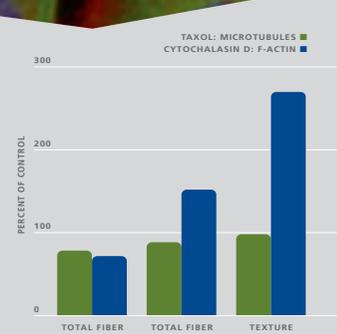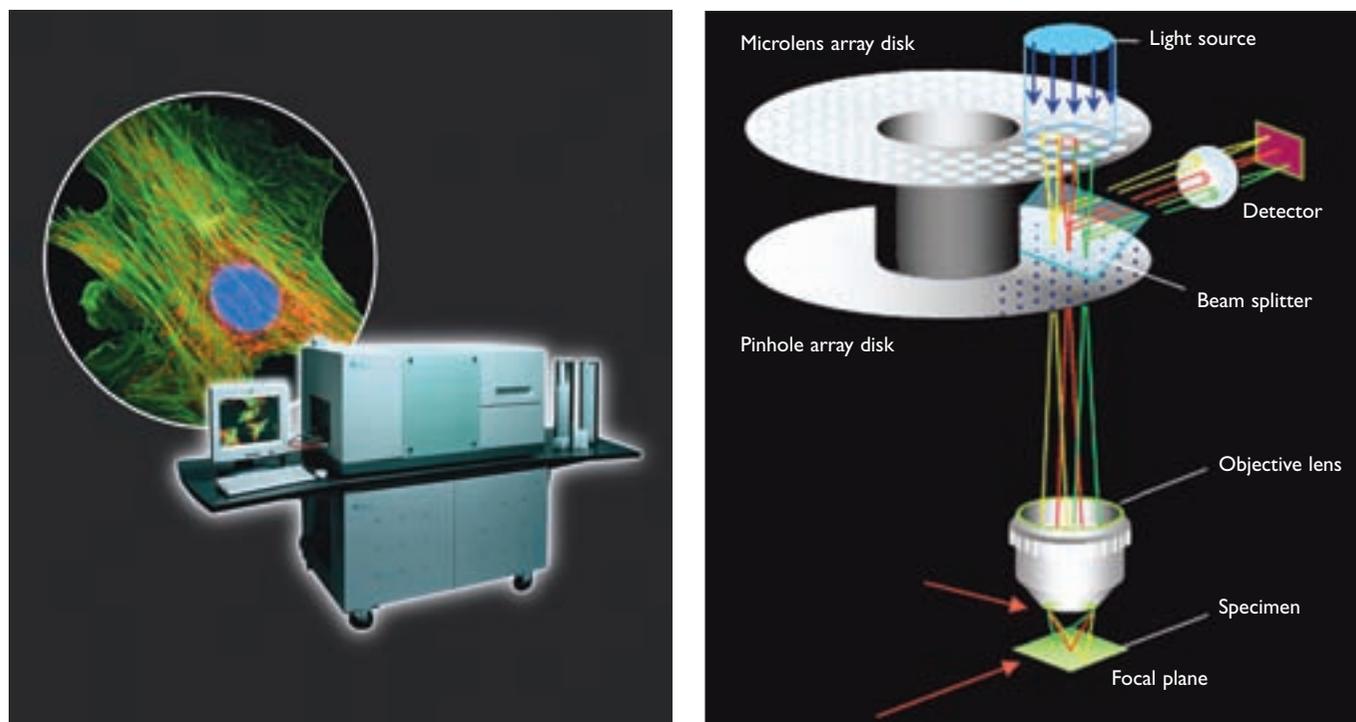

Figure 11: Evotec Opera (left). A schematic (right) of the microlens-enhanced Nipkow disk scanning technology principle (developed by Yokogawa Electric Corporation) used in Evotec's Opera confocal scanning unit. By using a disk containing a microlens arrays in combination with the Nipkow disk, it has succeeded in dramatically improving the light efficiency and thus successfully made high throughput real-time confocal imaging of live cells possible. The method scans the field of view with approximately 1,000 laser beams simultaneously and is capable of very high-speed scanning relative to conventional confocal scanners

and comparable contrast to even the best confocal systems that cost several times more. This approach has been validated and used in a variety of research areas, including studies of G-protein signalling in neurons, mitosis in tumour cells and cytoskeletal structure (**Figure 10**). Both of Cellomics' approaches have been proven to give equivalent data as compared to confocal systems and in some cases the data was obtained in similar timeframes. As previously stated, one of the perceived advantages of confocal systems is speed, but this is also a limitation as to the types of labels that can be selected as well as the types of biologies. So is confocal better? Cellomics believes the answer lies in providing the market with the best possible hardware and software. Using the ArrayScan HCS Reader with the ApoTome Module it will address certain biologies such as quantifying cytoskeletal rearrangement, identification of individual cells in a colony of stem cells and translocation of PKCa from the cytoplasm to the membrane in round Jurkat cells. Using the embedded software algorithms, proprietary optical system, and Z-Scan capabilities found in the cellWoRx system will allow for high quality data imaging with quantitative data analysis.

Whereas for some cellular assay applications wide field imaging may be sufficient, adequate identification of cellular compartments such as plasma membrane areas, lipid rafts, clathrin coated pits, vesicles and intracellular organelles, endosomes, mitochondria, actin filaments etc requires increased spatial resolution in the imaging technique as can be found in confocal imaging. **Evotec Technologies**' (www.evotec-technologies.com) Opera™ is a high throughput plate reader using a laser scanning confocal principle (microlens-enhanced Nipkow spinning disc) (**Figure 11**). This method combined with the use of automated water immersion lenses which are unique for the Opera enables high speed confocal imaging of cell samples. Last year the Opera received one additional confocal detection channel making it possible to acquire three colours simultaneously in confocal mode. In particular, the correct localisation of events in the plasma membrane which is essential for a variety of signal transduction and receptor activation phenomena can only be done in confocal mode. Evotec has recently released extensive detection libraries for such events, eg the translocation from cytosol to plasma membrane, in its

Acapella software providing a set of algorithms from which one can choose the one best suited for the particular application. In the future, Evotec believes that monitoring interactions at the molecular level will increase in importance in drug discovery, eg for compound mode of action studies. High resolution techniques will be required, but most likely it will not be sufficient to detect co-localisation with widefield or confocal imaging. The tasks will have to be addressed by methods which can overcome the limitation of optical resolution of light microscopy-based HCS. The use of fluorescence energy transfer (FRET) probe pairs will help to overcome this limitation, especially when used in combination with the fluorescence lifetime information of the dyes. Opera versions employing methods for FRET and fluorescence lifetime imaging (FLIM) will be available in the near future.

GE Healthcare's (www.gehealthcare.com/life-sciences) updated IN Cell Analyzer 1000 is an automated cellular and subcellular imaging system for fast, automated multi-wavelength imaging and analysis in fixed and live cells (**Figure 12**). The basic end-point system can be upgraded to enable kinetic assays, transmitted light imaging, microscope slide handling, optical Z-sectioning and environmental control. The Optical Z-sectioning Module is based upon advances in the technique of structured light imaging coupled to proprietary image reconstruction algorithms. The module is a combination of hardware and proprietary software that removes out-of-focus light above and below the focal plane, allowing the generation of high-quality 2D and 3D images of fluorescently labelled cellular and tissue samples, for analysis where high background is problematic. This optional module adds an optical-Z-sectioning dimension to the standard widefield capabilities of the IN Cell Analyzer 1000. GE Healthcare's IN Cell Analyzer 3000 is a purpose-built high-throughput, line-scanning confocal subcellular imaging system. Combining sophisticated scanning and optical technologies with high-throughput screening capacity, the system provides advanced multiplexing capabilities and real time, cell-by-cell image analysis routines for live- and fixed-cell assays. The combination of continuous tracking-laser autofocus, line scanning technology and a large field of view, enables rapid acquisition of data for high-speed imaging. Automated cell imaging devices running complex subcellular assays generate high-volume data. Effective analysis and data interpretation tools are required to address the breadth of analysis activities necessary for cellular research and screening. The IN Cell Investigator Software suite provides a comprehensive solution for automated high-content image and data analysis, by combining the latest versions of Developer Toolbox (enables construction of user specific image analysis protocols for specialised applications) and IN Cell Analysis Modules (provide a comprehensive range of preconfigured and fully
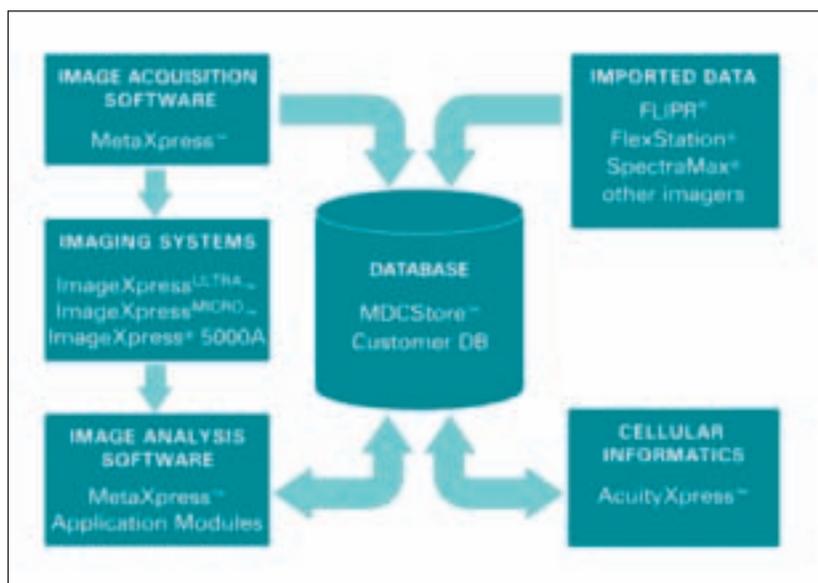
High Content Screening

Figure 13: Molecular Devices' complete imaging solutions include ImageXpress^{MICRO}™(left), ImageXpress^{ULTRA}™ (right), and MetaXpress software

Figure 14: Molecular Devices' integrated HCS platform provides researchers with a complete solution for image acquisition, analysis, database management and informatics


validated image analysis routines) with Spotfire™ DecisionSite™ visualisation software (interactive visualisation of results from Developer Toolbox or image analysis modules). In addition, the IN Cell Analyzer platform can be integrated with laboratory information and data integration tools and leading commercially available data management, analysis and visualisation tools.

Molecular Devices (MDC) (www.moldev.com) has a complete toolset for HCS, consisting of two fully-integrated high throughput imaging systems and a suite of imaging software for image acquisition, automated analysis and cellular informatics. At the centre of MDC's HCS solution is a new generation of automated microscopes: the wide-field ImageXpress^{MICRO}™ and the confocal ImageXpress^{ULTRA}™. These instruments, developed to address the limitations of older generation systems, are cost-effective bench-top systems combining state-of-the-art engineering with industry-leading imaging software. Both systems share high-speed laser auto-focus for increased throughput and better than 100nm resolution for sample positioning and focus. ImageXpress^{MICRO} is a high-resolution, modular, CCD-based imager. Modules include objectives, filters, laser auto-focus, environmental control, phase contrast and brightfield illumination and robotic microplate handling. The ImageXpress^{MICRO} is also a research-level imager with compatibility with microscope slides and oil-immersion objectives. ImageXpress^{ULTRA} is a modular, true point-scanning confocal imager utilising solid-state lasers, photomultiplier tubes (PMTs), and self-aligning optics, providing simultaneous multi-channel acquisition for up to four different wavelengths. In common with other laser scanning cytometers (LSC), the ImageXpress^{ULTRA} constructs a two-dimensional image by taking a series of point samples using a PMT (not a CCD), the difference being it is confocal with axial and lateral resolutions that are comparable to those of research-grade confocal microscopes. The ImageXpress^{ULTRA} has a fully configurable pinhole and adjustable scan length. Modules include choice of objectives, emission filters and robotic microplate handling. Both systems include MetaXpress image analysis and acquisition software (**Figure 13**). Built on MetaMorph®, MetaXpress is optimised for high-content screening. MetaXpress optionally includes more than 15 validated application modules, enabling turnkey image analysis of common assays, with custom tools available for novel assays. MetaXpress is
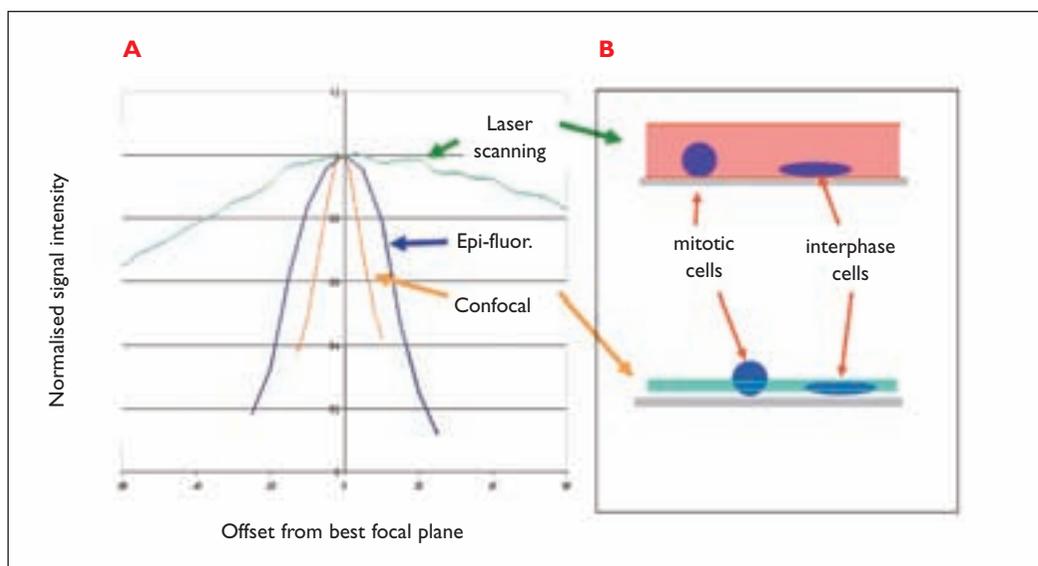
seamlessly integrated with MDCStore™, an enterprise-level database for data management and image storage. The AcuityXpress cellular informatics platform provides novel tools in data mining and visualisation. AcuityXpress includes curve fitting, quality control and clustering tools, as well as the ability to drill down from analysis to original image. AcuityXpress is seamlessly integrated with MetaXpress and MDCStore (**Figure 14**). Finally, the Transfluor® assay for GPCR activation is exclusively available from MDC. This technology, validated on more than 90 different GPCRs, works across all classes of GPCRs, orphan receptors and requires no receptor modification. MDC's Transfluor reagents include cell

lines expressing β-arrestin-GFP and plasmids for custom cell lines.

CompuCyte's (www.compucyte.com) laser scanning cytometers (LSC) are non-confocal by design, enabling quantification on a par with flow cytometry, the current gold standard in cytometric measurement. Robust analytical performance is made possible by a deep focal range which allows signal acquisition through the entire depth of the sample. Confocal imaging limits signal to a narrow plane (**Figure 15**), providing very high spatial resolution. However, confocal quantification comparable to LSC requires multiple passes at different focal planes and subsequent assembly
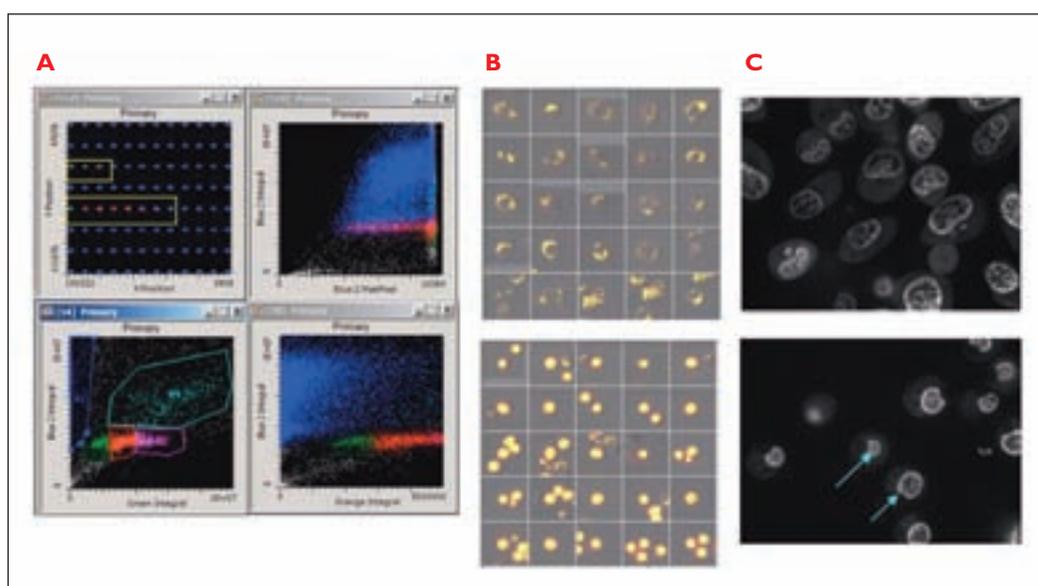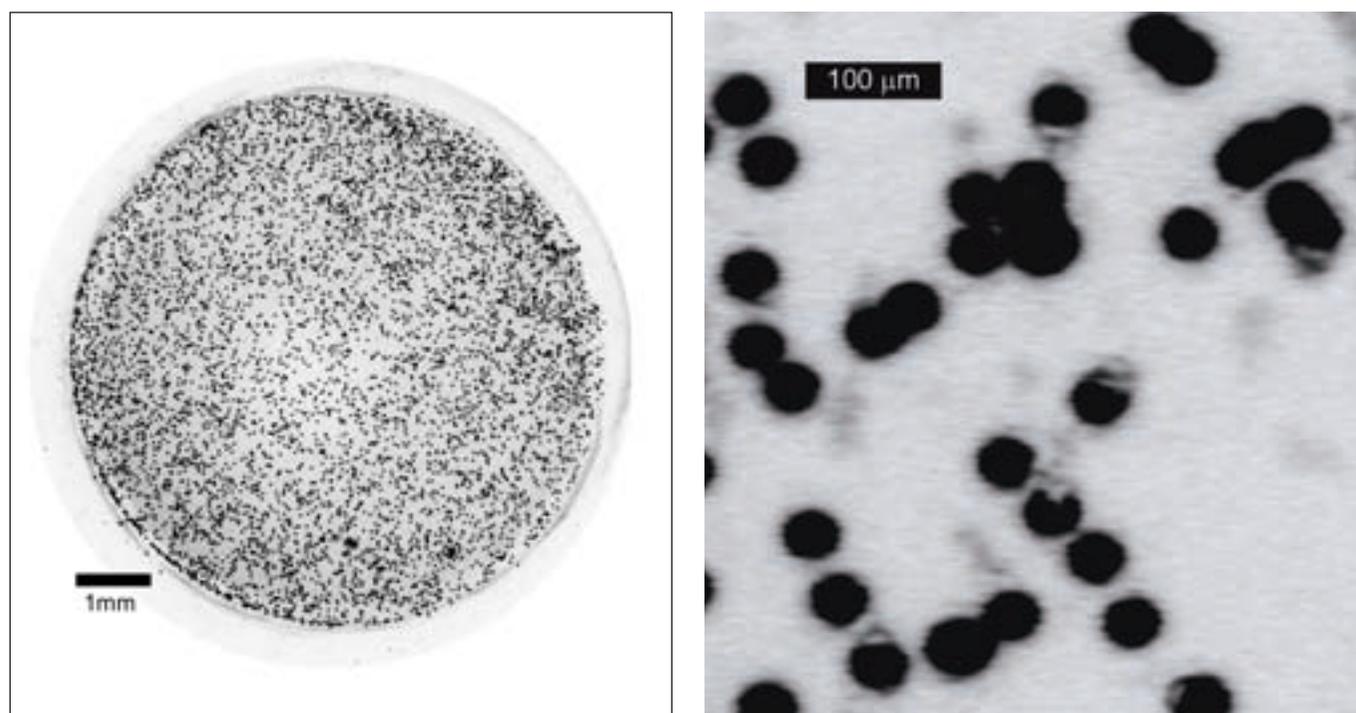
High Content Screening

of the images obtained – a cumbersome, time-consuming process. This is rarely done in routine practice. Pseudo-imaging with LSC, although sufficient for many applications, does not possess the exceptionally high spatial resolution typical of confocal imaging, or the ability to optically section samples to discern details of their interior morphology (**Figure 15B**). CompuCyte's instrument systems have evolved to combine the technology's basic non-confocal LSC capability with a complementary confocal imaging module employing Nipkow disc technology and a CCD camera (in addition to the PMTs used by LSC). The resulting integrated system provides excellent rapid quantification and confocal visualisation of cellular or tissue biochemical constituents in a single platform. An example of this approach is shown in a study of the effects of mitochondria-targeting drugs on apoptosis induction. CHO cells were treated with test compounds and stained to simultaneously measure (by non-confocal laser scanning) DNA content, mitochondrial membrane potential, plasma membrane permeability, cell size, chromatin condensation and propidium iodide exclusion for cell viability. DNA content and chromatin condensation were then used to identify cells exhibiting drug-induced apoptosis (the yellow boxes in **Figure 16A**). Non-confocal laser scan images were used to visualise the gross morphology of the identified cells (**Figure 16B**), followed by confocal imaging to

further evaluate the morphological effect of the drugs on organelles. The confocal imaging revealed prominent vacuoles located around the nuclear membrane at low drug dosages (**Figure 16C**). This exceptionally high-content analysis, obtaining data for several thousand cells per well on 96-well plate, was accomplished in less than two hours. For tissue analysis, tumour cells within a tissue section may be identified by DNA content or immunological markers using traditional non-confocal laser scanning. Laser scanning is also excellent for total signal quantification of FISH probes. Subsequent confocal observation may be employed to separate overlapping spots and examine detailed morphology.

Microscope-based high-content instruments offer high optical resolution, however, the limited field of view afforded by their objective lenses can significantly affect performance of some assays. Key is the limited number of cells present in each image (typically around 100) as this represents only a small percentage of the total number of cells in each test well. For applications requiring the use of high magnification objectives, such as 40x, capturing only 15 cells per image is not uncommon. In these cases an LSC with a low resolution scan lens offers an alternative approach to capture non-confocal images and subsequently batch process them using image analysis software. **TTP LabTech**'s (www.ttplabtech.com) newly

High Content Screening

launched triple laser Acumen® eX3 microplate cytometer is such an LSC. It is equipped with up to three lasers at 405, 488 and 633nm, offering an excitation wavelength range that is similar to that of white light source instrumentation, significantly increasing the variety of fluorescent reagents compatible with the system. The new Acumen® eX3 performs cytometric analysis across entire wells at throughputs of up to 300,000 data points in 24 hours. Although regarded as low resolution devices, TIFF images (8 or 16-bit) generated from unprocessed PMT readings correlate well with those captured using a 20x microscope objective[1] (**Figure 17**). The whole well scanning capability of the Acumen® eX3 microplate cytometer allows high content analysis of every cell in every well at high throughput. This has many advantages over restricted reporting, which results from only capturing a few images from a small well area. First, whole well analysis reports data for every cell, thus generating statistically robust data from a truly representative cell population. This is key for rare event detection assays such as mitotic index and stem cell differentiation. Secondly, whole well analysis can overcome problems of variable stimulation and random cell distribution often observed in screening plates. Thirdly, it enables normalisation of biological responses to total cell number, offering a simple toxicity or proliferation readout with every test. Finally, whole well analysis removes the need for image stitching steps prior to image analysis on large objects such as C elegans or cell colonies.

In addition to dedicated high-content screening systems, it is also possible to use a confocal microscope imaging system to study samples in microplates, as well as the more traditional microscope slides. Confocal microscope imaging systems, while being of a lower throughput, do offer the user the chance to perform very sophisticated experiments, such as FRAP (Fluorescence Recovery After Photobleaching), which are capable of delivering the highest content for cellular analysis. The ability to perform FRAP and similar techniques on a spinning disc confocal system has been long awaited by the live cell imaging community and is highly desirable due to the low photo-damage seen with a spinning disc confocal. With its new PhotoKinesis™ Accessory, **PerkinElmer**'s (www.perkinelmer.com) Ultra*VIEW*™ confocal imaging system (**Figure 18**), introduces this technology to the market. The PhotoKinesis accessory supports FRAP, FLIP (Fluorescence Loss In Photobleaching), photoactivation and photobleaching techniques which are largely (but not exclusively) confined to studying live samples. In addition, the low levels of 'background' photobleaching seen with the Ultra*VIEW* during sample imaging means that the data generated will suffer less from artefacts caused by this effect and will make the system more sensitive to observing subtle effects during the bleaching process. By utilising any of the imaging wavelengths for bleaching applications, the PhotoKinesis Accessory offers a high degree of flexibility. In addition, the onboard 'Track-It' technology allows the user to dynamically interact with the sample during image capture. This means that interesting structures can be subjected to bleaching, photoswitching and photoactivation as they arise during an experiment, without the need to stop recording or come out of live image mode (and potentially miss rapid events).

Figure 18
PerkinElmer's Ultra*VIEW*™ confocal imaging system with new PhotoKinesis™ Accessory

New imaging system for QC prior to HCS

Genetix's (www.genetix.com) CloneSelecT Imager offers a unique, optical solution to rapidly assess cells in microplates. It is a bright field imager which utilises oblique illumination around each well of a microplate to enable rapid visualisation of unstained, live cells (**Figure 19**). As the system is 'dye free' and its speed so great, the Genetix CloneSelect Imager can be used to quality control each well of every 96 well plate before further high content analysis. For example, cell viability of thawed cells can be measured; cell number can be determined before GPCR measurements; and the system can be used for screening compounds for kinase inhibition or toxicity. In a typical conformation the central area ($14mm^2$) of each well of a 96-well plate can be imaged and analysed in approximately 1min per plate. The data (**Figure 20**) shows confluence measured by the area of the well on which the cells are attached and from this the cell number can be deduced using an algorithm in the data analysis software. The data can be stored and added to over time so that a time course of cell growth can be automatically generated. Data outputs include on- screen pie charts showing the confluence of each well in plate format, a histogram indicating the number of wells that have obtained a particular confluence and a precise value of confluence for each well in Excel or a .csv format. More information can be obtained by measuring the confluence of each whole well (four sectors/well), this increases the read and analysis time to 5min per plate. Using the zoom function each well can be imaged at high resolution in order to confirm the phenotype of the cells being imaged. Again the data can be stored and presented as growth curves for the whole well. In order to achieve maximum throughput the CloneSelecT Imager can be integrated with robotic arms from most leading manufacturers.

Conclusions

Judging by the number of new confocal imaging solutions launched in 2006 it seems reasonable to conclude that many vendors believe they need to offer a range of alternative cell imaging options if they are to maximise the chance that their platforms will be accepted as total solutions and implemented across discovery applications. These options include a variety of different hardware and software solutions all aimed a generating either 'true' confocal or the equivalent of a confocal image, by rejecting out-of-focus background fluorescence to improve the signal to noise. However, the jury still appears to be out on the importance of 'true' confocality if this year's meeting of the Data and Image Analysis Special Interest Group of

High Content Screening

Reference
1 Bowen, WP and Wylie, PG.
Application of Laser-Scanning
Fluorescence Microplate
Cytometry in High Content
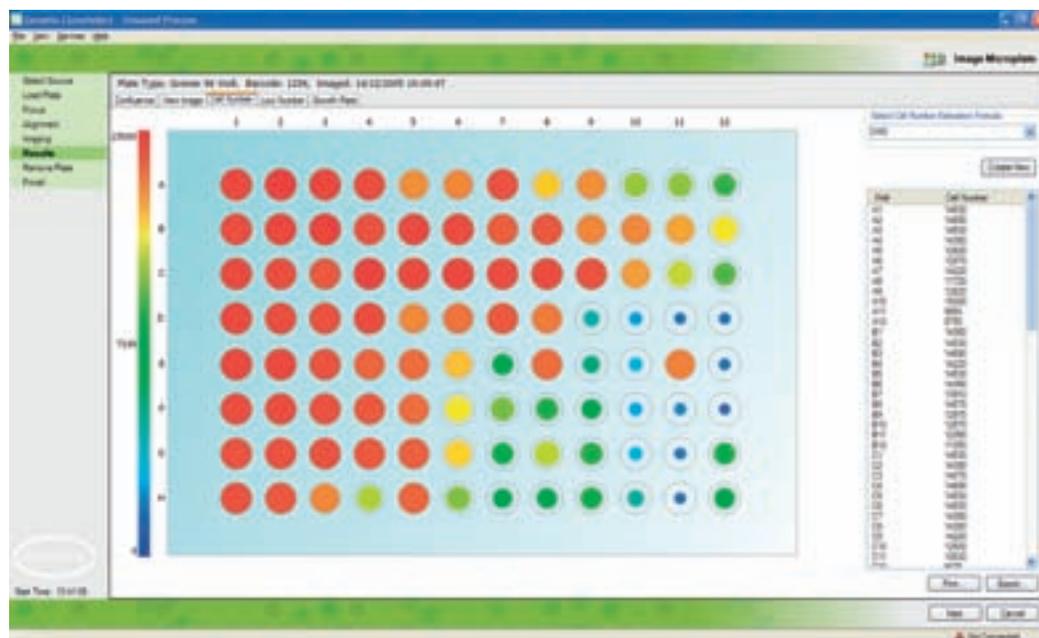Screening. Assay and Drug
Development Technologies 4,
209-221 (2006).

Figure 20: Genetix CloneSelecT Imager enables interrogation of the confluence results to provide an estimation of cell number in each well. The red wells indicate more cells and the blue wells less cells per well. An estimate of cell number is included in the data grid

the Society of Biomolecular Sciences Meeting (September 2006) is a guide, as the topic of comparing confocal with widefield image-derived results was at the centre of their agenda (http://www.ravkin.net/SBS/D&IA_SIG2006.htm). Perhaps this group will in the future provide the first comparative study on the influence of image acquisition modalities (confocal, widefield or LSC) on the quality of screen data subsequently derived from images.

Increasingly the use of hybrid approaches are being discussed, wherein a faster widefield run or LSC whole-well analysis would be undertaken to prescreen or filter plates. The results of this prescreen then might activate a trigger for the rescan of selected wells using a confocal solution. This approach seems to offer the best of both worlds. So which is better, widefield or confocal? The answer appears to lie with what researchers require to accurately and cost-effectively capture in the data they need. 'True' confocal optics and laser illumination certainly have their place but the user pays a significant premium, both financially and technologically, for that choice. What is evident is that users need to choose wisely for their actual rather than perceived needs. Finally, it is clear that the availability of new and improved confocal hardware and software solutions alone will not be enough. Confocal technology must be seamlessly linked with appropriate data management, analy-

sis and visualisation tools supporting validated applications if there is to be successful exploitation of those technically challenging targets that require optical sectioning, higher contrast and/or enhanced throughput. **DDW**

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