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Automated Confocal Imaging and High-Content Screening for Cytomics

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INTRODUCTION

The vast amount of biological information emerging from large-scale high-volume genomics and proteomics has significantly changed biological research. The problem is, however, that we currently do not have the methods to analyze the enormous complexity of cells or cellular systems within reasonable time intervals using the traditional approach of hypothesis formulation followed by experimental verification. One challenge is that the data gained from observing a few cells is rarely suited for statistical evaluation. This limitation has stimulated development of new approaches to the comprehensive collection of information with more immediate biological importance at the cellular level (cytomics). This effort spans both basic biomedical research and drug development.

Recent advances in combinatorial chemistry, genomics, and **high throughput** screening (HTS), as they apply to drug discovery, have produced a deluge of potential therapeutic "hits." Conventional cell-based HTS assays rely on measuring single parameters from lysates of cell populations grown in multi-well high-density plates (whole-well average measurement), at single time points (end-point format) with very limited temporal resolution and no spatial resolution. Using standard or more advanced plate readers it is possible to reach the speed requirement of HT primary campaigns for drug discovery. Unfortunately, although advances in automation offer increasing rates of HTS throughput, the past few years have witnessed a dearth of newly approved drugs. At the same time, slowness in the identification and validation of cellular targets potentially affected by leads originally identified by HTS has created a bottleneck in the drug discovery process. Many promising compounds have failed in costly animal studies and in even costlier clinical trials farther down the drug discovery pipeline.

In response, new screening approaches have recently been proposed that are referred to as **high-content** screening (HCS). HCS relies on more sophisticated assays where the higher **quality** (as opposed to higher **quantity** in HTS) of the data being generated is believed both to increase the likelihood of discovery and to accelerate the profiling of successful drugs (Taylor *et al*., 2001; Dove, 2003).

HCS readouts can include simultaneous measurements of several physiological parameters from individual cells living within a population. HCS provides more complex but also more informative data than single, simpler HTS "presence-or-absence" readouts. Importantly, high-content data allow for a faster, comprehensive, mechanistic understanding of all the cellular functions that are affected by the signaling molecules/drug candidates under test and permit us to understand their physiological relevance, in the context of an intact biological system.

In recent years, single-cell imaging has been recognized as a revolutionary way to look at biology (Comley, 2005; Cole *et al*., 2003). As a result, imaging holds a great potential in HCS because of the dynamic and multi-dimensional (multiple targets over time and space) aspect of the data generated.

HTS technologies fail because they look at cell populations, averaging results over the entire well and wrongly assuming the cells to be homogeneous. Although such techniques have provided valuable cell biological insights, they necessarily miss or downplay subtle or rare cellular responses. As an example, if a small subset within a cell population exhibits a cytotoxic reaction to a test compound, such unwanted effect may go undetected if only the overall population response is considered. This could cause the compound not to pass the more stringent screenings in costly animal models later in the drug discovery process. Single-cell imaging on the other hand, allows one to appreciate the heterogeneous behavior of cells (presence/absence of response, magnitude of response, temporal/spatial dynamics) and to characterize it in the context of the cells' innate biological variability (e.g., different stages in the cell cycle, receptor expression, genetic makeup). Consequently, drug profiling can take place in a physiological background that is more representative of an *in vivo* scenario and, therefore, more likely to reflect the true individual global response.

To fulfill their purpose, HCS assays must be able to perform multiplexed analyses (preferably simultaneously and in real time) in the same cell population or, in a higher resolution imaging mode, within spatially distinct domains inside the same cells. A wealth of non-invasive fluorescent probes have been developed and are now available for monitoring cellular/subcellular targets in living specimens. In particular, fluorescence imaging of signalling pathways is a powerful new tool for understanding signal transduction cross-talks (DeBernardi and Brooker, 1998; Meyer and Truel, 2003), as well as the molecular architecture and functionality of cell systems conceived as integrated biological entities or cytomes (Valet, 2003). Rather than dissecting cellular pathways in order to study single components *in vitro,* it is becoming possible to watch

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Handbook of Biological Confocal Microscopy, Third Edition, edited by James B. Pawley, Springer Science+Business Media, LLC, New York, 2006.

important biochemical events unfold at the cellular and even organismal level. Confocal microscopy, with its high-content information yield, is the tool of choice that provides the required spatial and temporal imaging resolution. As is well documented in the other chapters of this book, confocal imaging techniques have the potential to generate phenotypic, functional, and molecular information via quantitative assessments of subcellular, cellular, and tissue constructs that have long proved capable of elucidating molecular mechanisms. Contextual biology (where/when/how it happens/what happens then) is indeed the current frontier of the life sciences.

PLATFORMS USED FOR AUTOMATED CONFOCAL IMAGING

With their larger pinhole size, lamp-based, spinning-disk confocal systems provide greater scanning speed and light transmission, but thicker optical sections than their laser-based, line-scanning, single-beam relatives. However, for researchers who must balance the need for thin sectioning against the requirement for full-frame, high-speed, real-time confocal imaging, spinning-disk–based confocal systems (see Chapter 10, *this volume*) are the tool of choice. They provide the ability to monitor rapidly occurring events within living cells without compromising *xy*-resolution, and record events that would be missed during the time required to perform a single-beam laser scan.

Besides reduced maintenance cost, other important advantages offered by spinning-disk–based systems include using white light sources rather than lasers permitting full spectrum excitation capability [from ultraviolet (UV) to near-infrared (IR)], and, because of the lower light intensity delivered to the sample (in photons/s/μm^2), reduced photobleaching of the probes and less phototoxicity to the specimen (see Chapters 38 and 39, *this volume*).

These features are of crucial importance for high-content imaging where multiple fluorescent probes (whose absorption peaks often do not match laser lines) can be efficiently excited throughout the spectrum or where prolonged monitoring of fluorescently labeled living cells is needed to follow real-time translocation events.

A number of automated imaging platforms have been introduced to the market. Each instrument solves the problems related to imaging and specimen handling, fluidics/kinetics capabilities, sample throughput, and data analysis in different ways to accomplish HCS. Some of these imaging workstations are confocal (either laser line based or spinning disk based) while some are not (see Table 46.1). It is clear this is a growing new segment of the imaging community that will evolve extensively over the next decade.

One class of commercially available confocal imaging workstations is represented by an automated bioimager that combines full spectrum, Nipkow-based confocal microscopy with kinetic and end-point single-cell imaging capabilities in a compact, flexible benchtop unit (PathwayHT, Bioimaging Systems, BD Bioscience, Rockville, MD). The system is suitable for both academic basic research and pharmaceutical applications in assay development and secondary screening (Vanek and Tunon, 2002).

When the goal of the experiment is to measure multiple cellular events at once, it is advantageous to employ multiple filter sets and multiple light sources. For example, two mercury arcs allow full-spectrum illumination, from 350 nm to near IR, while 16 excitation filters, 10 dichroic mirrors, and 8 emission filters (housed in software-controlled filter wheels) enable the use of most available dyes, both single wavelength [e.g., green fluorescent protein (GFP), Fluo4, rhodamine, Alexa dyes) and ratiometric (e.g., fura-2 and JC-1). Multiple filter sets provide flexibility both in assay development, where fluorescent probes with a wide range of excitation requirements must be tested, and in secondary screening, where multiple cellular events must be measured in a single assay to provide improved speed and more relevant biological information.

The spinning-disk confocal module (readily interchangeable with widefield mode) provides real-time confocal imaging of fast events in live cells with high signal-to-noise ratio and minimal photobleaching and phototoxicity. High-resolution multi-color confocal *z*-stacks and three-dimensional (3D) analysis capabilities are available for localization/redistribution studies of multiple fluorescently tagged biomolecules within subcellular compartments.

Real-time confocal imaging requires sensitive cameras, such as high quantum efficiency charge-coupled device (CCD) cameras that provide high resolution and optimal signal-to-noise ratio. Once the new electron multiplier (EM)-CCD cameras (with exceptional sensitivity, greater speed, and minimal readout noise) become more widely available, it is expected that automated imaging platforms will greatly benefit from their use (see Chapters 2 and 12).

Other required features include integrated temperature and CO_2 control to ensure the consistency of physiological data. On-stage liquid handling for automated drug delivery to living cells provides an image-while-you-add capability and allows one to follow fast-onset kinetic responses (Fig. 46.1).

To permit imaging of adjacent fields while the stage and the sample remain stationary, proprietary optical designs allow objectives mounted below the specimen to move in x-y-z directions while both the stage and the sample remain stationary. This enables suspended or poorly adherent cells to be imaged without mechanical disruption. Motorized *x*-, *y*- , and *z*-positioning of the objective with 100 nm (*xy*) and 50 nm (*z*) precision permits revisiting previously imaged fields. This feature allows (a) prolonged time-lapse experiments to be carried out simultaneously on multiple fields by imaging each field sequentially at different time points, and (b) capture of montaged images of samples larger than the microscopic field of view. Montaging is useful when imaging tissue arrays or whole organisms, such as embryos of *Caenorhabditis elegans*, *Arabidopsis,* zebrafish, and *Drosophila*, and also when dealing with highly heterogeneous populations with rare responders, where a larger sample size is needed to improve statistical significance.

Depending upon the resolution required by a specific application, the type of imaging substrate used (glass/plastic) and the throughput desired, different magnification objectives (2×, 4×, 10×, 20×, 40× dry and 60× oil) can be employed. These optics can be optimized for the demands of the assay. Low power, low NA dry lenses are used for high speed in non-confocal applications. High NA immersion lenses matched to the dish in which the cells are cultured are used for slower studies where higher spatial resolution is needed.

As with other automated bioimagers, the system is amenable to robotic integration with industry standard plate handling and batch processing devices and can be operated in a fully automated mode under software control. A Windows-based software system seamlessly integrates image capture, sample navigation (for multi-well plates; chamber slides, standard microscope slides, culture dishes, etc.), liquid addition, and on-the-fly image analysis (see Chapter 14, *this volume*). A key requirement of the system software is the ability to drive a broad range of endpoints (biomarker identification, localization, translocation, redistribution) and kinetics (ion/second messenger real-time measurements).

TABLE 46.1. Non-Confocal HCS Systems

	Amersham IN Cell Analyzer 1000	Axon ImageXpress	Beckmann-Coulter/Q3DM	Cellomics Arrayscan Vti	Cellomics KineticScan	Universal Imaging Discovery 1
URL		www.axon.com	www.beckmann.com	www.cellomics.com		www.universal-imaging.com
Imaging system	CCD camera–based Non-confocal Nikon objectives	CCD camera–based Non-confocal Nikon objectives	CCD camera–based Non-confocal system	CCD camera–based Non-confocal ZeissApotome Grating optional Zeiss objectives	CCD camera–based Non-confocal Zeiss objectives	CCD camera–based Non-confocal Nikon objectives
Light source	Xenon lamp and filter wheel	Xenon lamp and filter wheel	Mercury lamp	Mercury lamp and filter wheel	Mercury-xenon lamp	Lamp-based and filter wheels
Excitation/ emmission filter #	6/6	10/10	10/10	10/8	8/8	10/10
Binocular eyepiece	No	No	No	No	No	No
Objective choices	4×, 10×, 20×, 40×	4×, 10×, 20×, 40×	4× to 40×	5×, 10×, 20×, 40× & 60×	5×, 10×, 20×, 40×	2×, 4×, 10, 20×, 40×
Plates	96, 384 well	96, 384, 1536 well	96–1536 well & slides	96–384 well	96–384 well	96, 384, 1536 well
Environmental controls	Temp 37 or ambient	Temp CO_2	No	No	Temp CO_2 Humidity	No
Liquid dispensing	Yes	Yes	No	No	Yes	No

Confocal HCS Systems

	Amersham IN Cell Analyzer 3000	BD Pathway HT	Evotec. Opera
URL	www.amersham.com	www.bdbiosciences.com	www.evotec-technologies.com
Imaging system	CCD camera–based confocal line scanning Nikon objectives	CCD camera–based confocal system Nipkow spinning disk Olympus objectives	CCD camera–based confocal spinning disk (Yokogawa)
Light source	2 lasers Krypton and argon 3 lines	Mercury lamp and filter wheel	4 lasers and xenon lamp for UV (non confocal)
Excitation/emmission filter #	3/8	16/8	4/6
Binocular eyepiece	No	Yes	No
Objective choices	40×	2×, 4×, 10×, 20×, 40×, 60×,	10×, 20×, 40×, 60×
Plates	96–384 well	96–384 well & slides; slide holder provided	96, 384, 1536 well
Environmental controls	Temp CO_2 Humidity	Temp CO_2	Temp CO_2
Liquid dispensing	Yes, limited	Yes	Yes, optional

TYPES OF ASSAYS

Selected automated confocal imaging platforms support current kinetics (e.g., calcium and sodium fluxes, mitochondria and plasma membrane potential changes, etc.) and endpoint cell assays (e.g., cytotoxicity, apoptosis, cell cycle, translocations) using common commercially available fluorescent probes (Table 46.2) Multiplexed assays are only limited by the optical compatibility of the fluorescent probes used. In particular, selected applications amenable to automated high-content imaging will benefit from the advantages provided by confocal microscopy including, but not limited to, the following:

- Live/fixed cell assay where fluorescent probes photobleach rapidly, either because of the chemical nature of the fluorophore or because of high sampling frequency (fast kinetics imaging with no or minimal delay between two illumination cycles) [Fig. 46.2(A), left panel].
- Live-cell assays where the fluorescent probes used to label cells are not removed during the imaging phase. This might happen for several reasons:

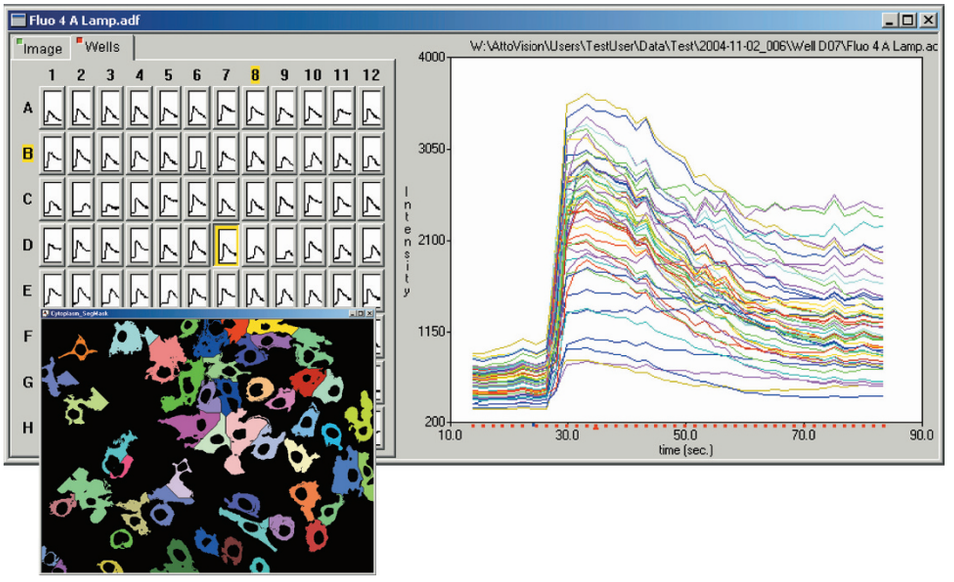

FIGURE 46.1. Automated single-cell kinetic calcium imaging. Screenshot from PathwayHT showing single-cell, real-time calcium fluxes in HeLa cells. Cells were grown on a 96-well plastic plate overnight, labeled with 2 u*M* Fluo-4 and imaged (20×, 0.75NA, at 37°C and 5% CO_2) in resting conditions and during/after automated addition of ATP (50 u*M*), a purinergic receptor agonist that mobilizes intracellular Ca^{2+}. On-the-fly visualization of single-cell-derived kinetic traces reveals profound heterogeneity in the magnitude/dynamic of cell responses to ATP. After completing a user-defined time-lapse, average responses are plotted in each well enabling ready visualization of overall cells' responsiveness. Color-coded single-cell traces identify corresponding cells (cell segmentation mask shown) allowing ready comparison of cell morphological and biochemical patterns.

TABLE 46.2. Methods Suitable for HCS Fluorescence Analysis of Cells

Application	Parameter Measured	Dyes	Information	Areas of Study
Cytotoxicity	Cell viability Cell proliferation	Calcein AM — Live cells Propidium Iodide — Dead cells Hoechst/DAPI nuclear dyes	Number/percentage of live/dead cells over a population	ADME/Tox Cancer biology
Apoptosis	Mitochondria potential changes Phosphatidylserine externalization Caspase activation Chromatin condensation/ fragmentation DNA strand breaks	JC-1, TMRE Annexin V Caspase sensors (cytoplasmic and nuclear) Hoechst/DAPI TUNEL	Early/intermediate/late apoptosis stage Apoptosis/Necrosis DNA damages	Pharmacology Toxicology Cell/cancer biology Genotoxicity
Translocation	Stimulus-induced target redistribution, e.g.: NF-kB (cytoplasm to nucleus) PKCα (cytosol to membrane) STATs (cytosol to nucleus) MAPKAk2 (nucleus to cytoplasm) Receptor internalization and fate	Detection is provided by antibody-based reagents for endogenously expressed targets. Alternatively, targets are expressed in cells as fusion protein with GFP, its variants or other fluorescent protein (biosensors). GFP-βarrestin, CypHer[a]	Organelle changes in fluorescence intensity/ patterns are indicators of specific target activation	Cell signalling Pathway cross-talk Immunology Receptor activation and desensitization
Kinetics	Calcium Sodium Membrane potential (MP) Mitochondria potential Multiplexed cAMP	Fluo-4, fura-2 SBFI Various MP Dyes JC-1 See below[b] ACT:One[c]	Kinetic changes in fluorescence intensity (or ratio) report live cells ion/second messengers response to stimuli. Multiplexing reveals real-time signaling cross-talk	Cell signaling Pharmacology Receptor/ion channel Activation Signaling, (cross-talk)
Cell cycle	DNA content and replication status Stage-specific target expression and/or phosphorylation	Hoechst/DAPI/Anti-nucleotide (e.g., BrdU) antibodies Anti-Histone/cyclins/cyclin-dependent kinase antibodies Cyclin-fusion protein	DNA content (2n/4n) in G1, G2, M phase. Cell cycle stages (e.g., phosphoH3 histone indicates M phase)	Cancer biology Genomics
Neurite outgrowth	Neurite extensions	Neuron specific antibody-based reagents	Number/length/branching upon neurite inducing stimuli	Neuroscience Regeneration Differentiation

Examples of cell-based assays suitable for automated imaging workstations. Listed are representative assays and fluorescent probes; more specifics on dyes can be found in Table 16.1, *this volume*. Kits, cell lines, and fluorescent agents are available from independent suppliers or imaging platform vendors that offer a variety of protocols. Examples include [a]GFP-βarrestin = Transfluor technology (by Norak Biosciences Inc); CypHer, pH sensitive dye (Amersham GE Healthcare); [b]calcium and sodium (Fluo-4 and SBFI); calcium and membrane potential (fura-2 and various MP probes); calcium and mitochondria membrane potential (Fura-2 and JC-1) [c]ACT:One cell lines and dyes for real-time cAMP measurement in live cells (Bioimaging Systems, BD Bioscience).

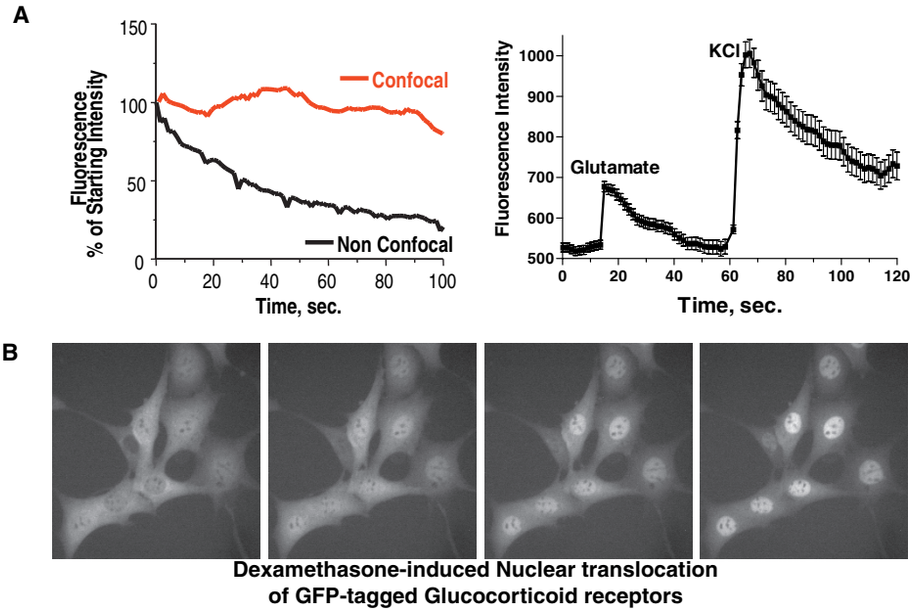

FIGURE 46.2. Confocal imaging of living cells. (A) Confocal imaging of primary rat cerebellar granule neurons. Cells were labeled with a membrane-potential dye and imaged confocally on PathwayHT (20×, 0.75NA) at 37°C and 5% CO_2 without dye removal. Left panel: cells were imaged under continuous illumination either in widefield or confocal mode. By 100 sec, a 75% loss in the probe-generated fluorescence signal was detected in cells imaged in widefield mode while in confocal mode, only ~25% of loss was detected. The reduced photobleaching provided by lamp-based confocal imaging platforms allows monitoring responses whose fast onset might require high-frequency sample illumination. Right panel: Membrane-potential depolarization induced by glutamate (10 u*M*) and KCl (50 m*M*) in primary neurons. Trace represents mean ± SD. (B) Real-time glucocorticoid receptor-nuclear translocation. Living MMTV mouse cells stably expressing GFP-tagged glucocorticoid receptors (GR) were imaged confocally on PathwayHT (40×, 0.9NA) at 37°C and 5% CO_2. GR nuclear translocation was induced by dexamethasone (1 n*M*) and followed over time; images were captured every 20 sec. From left to right: images at 0, 5, 10, 15 min after dexamethasone addition. Cells were kindly provided by Dr. G. Hager, NCI, NIH, Bethesda, MD.

  - — the probe needs to be in contact with the cells for functional measurements to be made (e.g., membrane potential dye) [Fig. 46.2(A), right panel];
  - — a homogeneous assay format with no washing steps is needed to optimize cell responsiveness and improve speed;
  - — active, postloading dye leakage from cells. Drug-resistant cancer cells are notorious for their active extrusion of small fluorescent probes because they over-expressed drug efflux pumps. The ability of the confocal microscope to image a single plane reduces the effect of the background generated by this excess probe, increasing signal-to-noise ratio and assay sensitivity.
- Live-cell assays in which phototoxicity might interfere with biological functions (e.g., prolonged time courses where small organisms expressing GFP or other fluorescent fusion proteins are followed through multiple cellular divisions or where cell lineage is being investigated) [Fig. 46.2(B)].
- Assays employing *in vitro* systems consisting of two-dimensional (2D), mixed-cell populations (e.g., primary neuron cultures with supporting glial cells, or special-purpose feeder layers for differentiating embryonic stem cells). Confocal microscopy will allow imaging at specified focal planes to emphasize the response of the desired cell population.
- Assays where high-resolution spatial discrimination is required for localizing fluorescent markers to specific subcellular compartments. This process could be as simple as assigning a given probe to a given structure (target identification or confirmation of organelle specificity for novel biomarkers) or as sophisticated as generating complete confocal image database towards a systematics for protein subcellular localization (Boland *et al.*, 2001).
- Cell surface targeting assays such as those aimed at identifying biomolecules that either are constitutively present on the outside of the plasma membrane (e.g., surface antigens and receptors) [Fig. 46.3(A)] or become newly exposed on cell surfaces in response to a stimulus (e.g., phosphatidylserine, normally found on the inner side of the plasma membrane, gets externalized during drug-induced apoptosis and its identification by fluorescent annexin V provides a robust assay for early apoptosis classification).
- Multi-color applications where different cell types within a tissue or different organelles or proteins within a cell are labeled with specific probes whose spatial localization or colocalization requires the high spatial resolution provided by confocal imaging [Fig. 46.3(B)]. This scenario might include fixed samples fluorescently labeled with multiple antibodies conjugated to either conventional organic dyes or recently developed semiconductor nanocrystals (quantum dots; Watson *et al.*, 2003; see Chapters 16 and 17, *this volume*) as wells as live/fixed cells expressing multiple GFPs, or other fluorescent fusion proteins, and targeting different subcellular compartments. Phenotypic profiling of either tissues or cell populations labeled with multiple, organelle-specific probes represents a recently developed approach to screen molecules that alter specific cellular parameters, allowing the identification of compounds (or siRNAs) that interact with different systems in the cell such as the cytoskeleton or various signal transduction pathways (Yarrow *et al.*, 2003).
- Pharmacological screening of the hepatoxic potential of compounds in primary liver cells or liver cells lines, using confocal imaging, was reported to provide visual discrimination

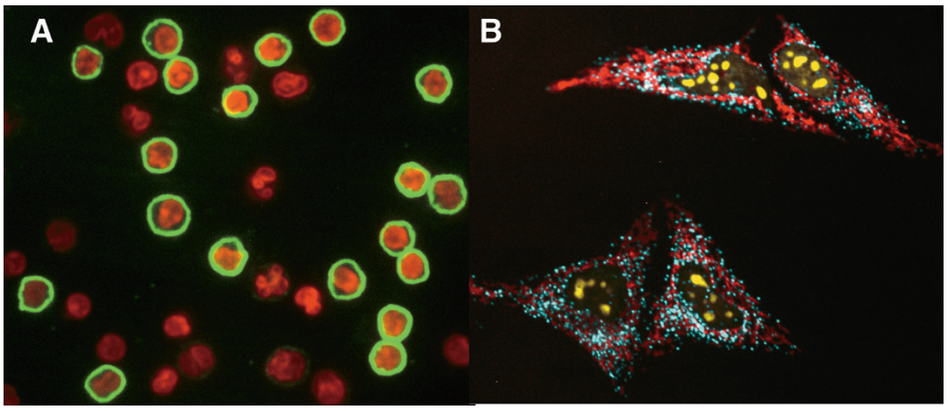

FIGURE 46.3. High resolution confocal imaging of surface and intracellular target. (A) Peripheral Blood Mononuclear Cells (PBMC) labeled with an APC-tagged, anti-CD4 monoclonal antibody (BD Biosciences) (pseudocolored in green) and counterstained with the nuclear dye, Hoechst 33324 (pseudocolored in red). Confocal imaging (60× oil, 1.4NA) clearly shows plasma-membrane-specific localization of cell surface CD4 receptors on a selected subset of PBMC. (B) HeLa cells expressing three organelle-targeted fluorescent fusion proteins: AmCyan-peroxisomes, ZsYellow-nucleus, HcRed-Mitochondria (Clontech, BD Biosciences) were fixed and imaged on PathwayHT (40×, 0.9NA) in confocal mode.

of compound-specific intracellular fat deposition patterns (McMillian *et al*., 2001).

- Endpoint translocation/redistribution assays where, following a relevant stimulus, the labeled target moves from one subcellular compartment to another (e.g., cytoplasm-to-nucleus translocation of NF-kB transcription factor or cytoplasm-to plasma-membrane translocation of protein kinase Cα) or undergoes changes in fluorescence pattern [e.g., redistribution assays where the fluorescence pattern of receptor-bound GFP-β arrestin changes from an evenly diffuse cytosolic pattern to a more punctate one (pits/vesicles formation) upon agonist-induced internalization of the receptor] (Fig. 46.4). Confocal microscopy provides high-resolution images of the fluorescence redistribution patterns that allow more sophisticated image analysis algorithms (such as granularity) to be optimally applied. These in turn lead to greater assay sensitivity.
- Assays based on 3D biological structures (micro-organs) where confocal *z*-stacks captured over time can provide physiologically relevant insights on cell proliferation, differentiation, drug responsiveness within a miniaturized *ex vivo* environment.
- Assays using quantum dots *in vivo* or on fixed cells (Chapters 16 and 17, *this volume*), which, because of their narrow emission bands, allow one to monitor the dynamics and location of several different proteins at once.

An automatic confocal imaging workstation that provides control of temperature and CO_2, precise *xy*-position field revisiting, and automated multiple color *z*-sectioning capabilities with minimal photobleaching and phototoxicity will be the tool of choice for 3D model imaging with applications in cancer biology, toxicology, therapeutics, and regeneration.

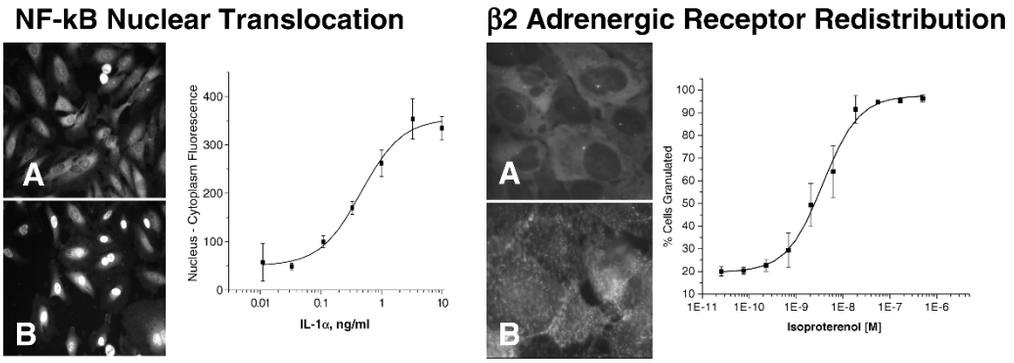

FIGURE 46.4. Automated fluorescence imaging applied to endpoint translocation assays. Left panel: Nuclear translocation of the transcription factor, NF-kB. HeLa cells were grown overnight on 96-well plastic plates and treated with increasing concentrations of IL-1α for 30 min. Cells were fixed, permeabilized and labeled with an anti-NF-kB Alexa488-IgG and the nuclear dye Hoechst 33342. Cells were imaged on PathwayHT in an automated mode (20×, 0.75NA) with fluorescence intensity being measured in the nucleus and the cytoplasm. Images show basal fluorescence signal (A) and IL-1-stimulated increase in nuclear NF-kB fluorescence (B). Cell-by-cell data analysis was performed and a dose-response curve generated using BD Image Data Explorer. Right panel: γ2-adrenergic receptor trafficking visualized by GFP-arrestin fluorescence redistribution. Human osteosarcoma cells (Transfluor technology, Xsira Bioscience, Research Triangle, NC) stably expressing GFP-arrestin were challenged with increasing concentrations of isoproterenol, a γ-adrenergic receptor agonist, fixed and nuclei counterstained with DAPI. Cells were imaged on Pathway HT (40×, 0.9NA) and GFP-β arrestin fluorescence measurements were taken in cytoplasmic regions. (A) basal fluorescence, (B) after isoproterenol. Granularity algorithms (variation in pixel intensity and distribution) were applied for image analysis and data were expressed as % of cells exhibiting a granularity value above a user-set threshold. Dose response curves were generated using BD Image Data Explorer.

3D CELL MICROARRAY ASSAYS

The cell microarray (CMA) is one embodiment of the tissue microarray (TMA) that is a particularly useful type of prepared specimen for confocal high-content screening.

A cell or tissue microarray is a microscope slide containing between 10 and 1000 individual samples arranged in a grid fashion (Braunschweig *et al*., 2004) (Fig. 46.5). This approach converts imaging from a descriptive endeavor to a high-throughput methodology producing databases and requiring statistical analysis. The cell-line array can be an array of different cell lines, or an array of cells grown under different conditions (or both) that can be used to address a variety of questions. At the simplest level, a cell microarray can be considered a "Western blot on a slide," but such a characterization covers only a small portion of the information that can be obtained from such specimens.

CMA can be used with confocal microscopy as a tool to understand the human cytome. Utilizing the 60 cell lines the NCI employs in drug testing, the Tissue Array Research Program (TARP Lab), at the National Cancer Institute, has developed a unique platform, the NC160 CMA, which is studied with a HCS instrument for protein expression, localization, and interaction.

Most CMA cells are obtained from cell-line cultures of transformed cells, however, primary cultures are also sometimes adequate. Current protocols require less than 500,000 cells as starting material. The cells can be from clinical samples such as leukophoresis or flow-sorted specimens. Cells grown *in vitro* must be harvested by scraping. The cells are then embedded in a low melt agarose plug, which is then fixed and processed as if it were tissue to produce a paraffin-embedded block (Hewitt, 2004). Unlike most tissue specimens, formalin is not the only choice of fixation (see Chapter 18, *this volume*). Ten percent formalin, although an excellent fixative for tissue, cross-links the proteins and nicks nucleic acids, as well as producing a significant amount of background autofluorescence. As an alternative, 70% ethanol combines coagulative fixation with dehydration, and does so without cross-linking proteins or nicking nucleic acids (Gillespie *et al*., 2002). An additional advantage of using 70% ethanol as a fixative is that it is significantly easier to translate an antibody used in Western blots or for the immunostaining of frozen tissue to ethanol fixed material. Tissues (and cell–agarose plugs) can be fixed in 70% ethanol exactly as if fixed in formalin, using the same times and volumes. When it is time to process the tissue (dehydration and replacement by paraffin), only the first stage on the processor (10% formalin) need be skipped, as the second stage is 70% ethanol. Although other alcohols and different concentrations are associated with excessive shrinkage and distortion of cellular morphology, 70% ethanol results in shrinkage comparable with 10% formalin (Gillespie *et al*., 2002; Chapter 18, *this volume*).

After processing and embedding, a 3D cell pellet is obtained. This can both be sectioned and viewed as such, or the cell pellet blocks can be utilized for the construction of a CMA. The methods of arraying are straightforward, and can be fabricated manually or using different levels of automatic instrumentation (Braunschweig *et al*., 2004). The recipient array block (CMA block) can then be sectioned into slides for staining (Fig. 46.4). Unlike tissue sections where 4 or 5 µm sections are preferred, CMAs are optimally cut at between 7 and 10 µm to allow more complete 3D representation of the cells on the array.

For confocal imaging of the arrays, appropriate staining protocols must be worked out. It is essential that one use an excellent deparaffinization routine as residual paraffin will reduce the affinity of the antibodies, and will produce increased autofluorescence. Most normal staining protocols are inadequate, and three changes in xylene for 5 min each should be considered a starting point for deparaffinization. Depending on the targets and their cellular localization, the use of 4′,6-diamidino-2-phenylindole (DAPI) may or may not aid in imaging.

In its first embodiment, the NCI60 CMA consisted of 58 cell lines arrayed with 1.00 mm diameter cores of agarose plugs in triplicate cut as 7 µm sections on tape slides from Instrumedics [Fig. 46.5(A)]. The array was designed to present ~300 cells/core for analysis [Fig. 46.5(B)]. Cells were prepared at low density in an agarose plug, and cut as thick sections (7 µm) so that they would have a more homogeneous shape distribution in three dimensions, and had only a few artificial cell–cell contacts. This CMA is optimized for the investigation of cell signaling and protein localization, and their translocation. It is not designed for, and should not

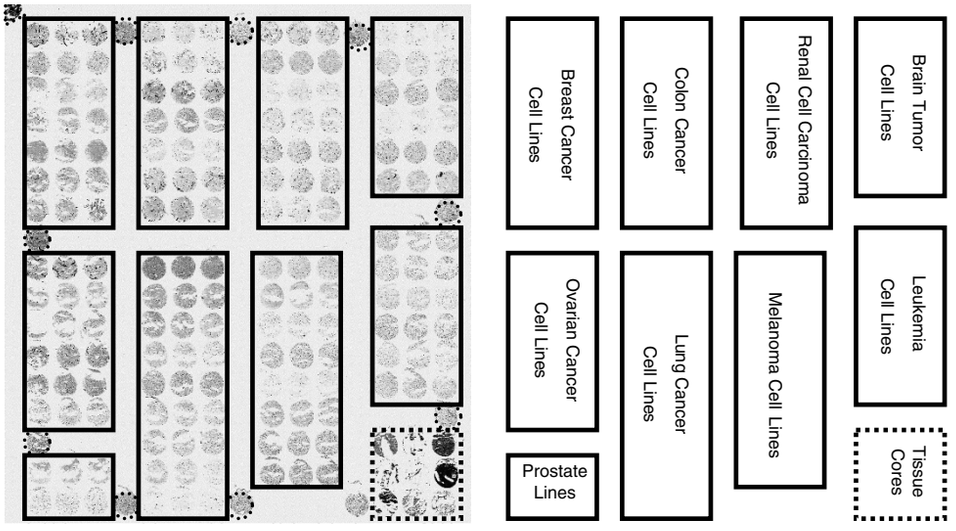

FIGURE 46.5. Cell microassays (Such as NCI160) exhibit different cell lines that allow rapid screening of cellular responses.

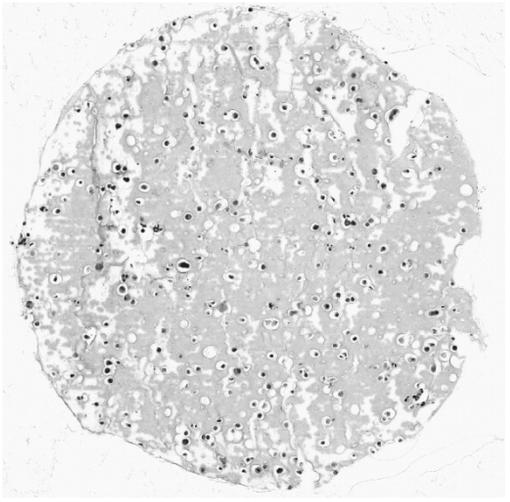

FIGURE 46.6. A single, 1.00 mm core of *in vitro* cultured cells embedded in agarose present on the NC160 CMA. This is a core of NCI-H23 cells, a cell line derived from human lung cancer.

be used for, studies of cell adhesion, communication, or most cytoskeletal studies. The dispersion of the cells within the cores is designed to make analysis simpler. This array is available on a limited basis from through the Developmental Therapeutics Program (DTP) of the NCI.

Confocal imaging of a complete CMA slide with 58 individual cell lines (in triplicate) requires an almost heroic effort and is impractical without an automated stage. Identification of the individual cores requires a "scout image" of the slide. Depending on the method being applied, a low-magnification image of the slide can be made to identify and locate the cores. Alternatively, a high-magnification, low-resolution image of the slide can be made for the same purpose. After the cores have been identified and located, the instrument is then programmed to image the cores individually based on the low-magnification map.

DATA MANAGEMENT AND IMAGE INFORMATICS

High-content imaging generates a vast amount of data that must be analyzed using software analysis programs, either those provided with the commercial instrumentation or custom written for very specific applications. For example, if two proteins are stained, and DAPI is used (especially useful for the low-resolution imaging to locate individual cells) on an NCI160 slide, the imaging demands on the platform are significant. One could anticipate requiring $58 \times 10 \times 3 = 1740$ images (58 cell lines $\times$ 10 z-steps $\times$ 3 fluorophores) for a first-pass imaging run. Assuming that the field imaged approximately 30 cells per core and did not take advantage of the triplicate over-sampling on the array, this experimental design would require $58 \times 10 \times 3 \times 3 \times 5 = 26{,}100$ images even if it attempted to analyze only half of cells presented. Although this approach sounds overwhelming, it is what must be done to deliver useful information.

Automated high-content imaging offers the ability to interrogate thousands of cells, often for multiple responses, to collect a significant volume of data about them, and finally to derive meaning from this biological information The data generated by this class of microscopical scanners and applications, is in the terabyte (TB) range for many industrial applications and is stored in image databases, along with the resulting analytical data. Oracle/Web-object-based relational databases management systems (ORDMS) are the preferred solution for image data management, search, and analysis. Secondary image representations, made by subsampling or data compression, mainly serve Web-based viewing or control purposes and have been developed as part of solutions for the adequate handling and mining of such large datasets. The goal is automated analysis of assays without user intervention. Given the variations in staining and morphology inherent in cell populations, this is a more challenging task for HCS than for more simple endpoint assays in HTS.

The first step in automated imaging is to physically find the specimen, as well as the appropriate image planes for the sample, to identify targets of interest (tissue, cells, subcellular structures). No single slide (or plate) is absolutely flat and it should be expected that some adjustment will be required core to core/plate to plate to determine the imaging planes. This is followed by image capture in one/multiple fluorescence channels. The next step involves using a number of computational algorithms to segment the image into user-defined regions of interest (ROIs) such that each ROI represents a single cell or a compartment (e.g., nucleus, cytoplasm) within a cell from which the relevant biological information (changes in fluorescence intensity/pattern or spatial redistribution) can be extracted and quantified, either on-the-fly or offline. Real-time feedback provides information on the quality of the results, especially in kinetics assays.

Because individual cells are viewed, it is possible to separate (or gate) cells into different response categories (Boolean classification). Cells are grouped (cell classification) based on a number of cellular features (from one or multiple probes) such as overall fluorescence intensity, rate of rise/fall (for kinetics), area, object pixel statistics (average intensity, minimum, and maximum), and variation of pixel intensity within ROIs (e.g., granulation algorithm). Class of cells can be color-coded for easy visualization, and average measurements over a subset of cells can be taken. A well classification is then applied based on the number of cells that, in each well, meet a user-defined threshold. In turn, a response heat plate map that readily highlights cellular trends or hit compounds can be generated. This process enables to identify features that best reflect specific biological responses and, therefore, are good screenable parameters.

Typically, high-content, automated image analysis involves several user-defined, software-driven steps that are compiled into a template (or protocol) that is specific for each assay type (e.g., kinetics or endpoint, single dye or multiple dyes). Easy access to the templates allows users to modify preset parameters that might need day-to-day adjustment, such as camera exposure time, number/volume of drugs being delivered, time-course duration, image capture frequency, number/position of wells to be tested, and type of plates (96-, 384-well plate). Optimized imaging protocols and assay templates are stored (always in a re-settable format) for use in larger volume screening applications. Such protocols can be transferred to other imaging workstations ensuring coherent, standardization of image capture and analysis processes among multiple instruments. Robust analysis procedures that rely on the topology or pattern of cell organelles (see Chapters 15 and 47) and cells, and new object-oriented approaches are preferred solutions that have distinct advantageous over the prevailing pixel-oriented methods.

The kinetics and endpoint data generated by automated bioimagers are typically analyzed with proprietary software packages

that are either integrated with the system or are third-party analysis packages (e.g., ImagePro and Metamorph). Some packages, such as the BD Image Data Explorer (BD Bioscience) use customized database engines to store and access large, multi-dimensional data sets and apply sophisticated statistical routines to allow data visualization and analysis. Analysis programs allow one to quickly import raw data files from imaging workstations and associate them with drug treatment plate maps, containing information on the drug treatment that each well has received. In order to uncover artifacts or highlight rare events, software must be able to provide true **cell-by-cell** analysis rather than averaging cell response characteristics up front using **well-by-well** data.

Powerful data analysis programs can reduce large kinetics or endpoint image datasets into relevant pharmacological data. Data can then be compiled into informative reports that are archived with all the analysis steps included to maintain data integrity. Bar/line graphs, dose-response curves, EC_{50} , signal-to-noise ratio, *z*-scores, and other analysis tools useful to the assay developer and the cellular biologist/pharmacologist can be derived.

CONCLUSION

Automated HCS imaging instruments are hybrid instruments that bridge between low-throughput manual microscopes and high-throughput plate readers. At present, automated confocal imaging technologies mainly find their niche in target identification/validation and secondary drug screenings where the higher information content provided by single-cell imaging balances the limited volume throughput (relatively to HTS biochemical assay). Further refinement in hardware and software is expected to narrow the gap to produce an HTS imaging system capable to deal with primary screening efficiently.

The high-content, high-speed cytomics information yield of the disk-scanning confocal microscope makes it the imaging tool of choice to resolve multiple, distinctly labeled molecular targets in space and time, as they undergo quantitative (e.g., concentration) as well as qualitative changes (redistribution within cellular compartments) in single cells. With new automation, liquid handling, real-time capabilities, and the ability to manage multiple-well plates or cell-line–based tissue microarrays, confocal HCS systems are finding uses not only within the domain of the individual microscopist but in higher throughput (automated) imaging environments where thousands of cells are being quantitatively interrogated for multiple functional responses (Price *et al*., 2002; Giuliano *et al*., 2004; Perlman *et al*., 2004). As such, advanced confocal imaging technologies, amenable to scaling and automation, are opening new avenues in drug discovery and development, toxicology, and also in functional genomics and proteomics.

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