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[25] High-Content Screening: Emerging Hardware and Software Technologies

By SEUNGTAEK LEE and BONNIE J. HOWELL

Abstract

The field of high-content screening has flourished since 2000 with advancements in automated fluorescence microscopy technologies, fluorescent labeling techniques, and sophisticated image analysis software. Through the use of these technologies, researchers can now monitor cellular and molecular events in individual cells *in vitro* following drug treatment or RNAi and rapidly screen compound and siRNA libraries. This chapter discusses current and next-generation hardware and software features and capabilities.

Introduction

High-content screening (HCS) has gained tremendous popularity in the past few years in the drug discovery industry from advances in fluorescence microscopy and automated screening technologies. HCS provides an opportunity to rapidly screen chemical or siRNA libraries by imaging subcellular and molecular events of individual cells with an automated fluorescent microscope. HCS gets its name from the rich and diverse set of information provided by analyzing the phenotype of whole cells. The ability to multiplex with multiple markers or fluorescent probes and even multiple cell lines simultaneously allows for more efficient screens for target identification, target validation, lead identification, and lead optimization. Although fluorescence microscopy and digital image analysis have been around for decades, the complete integration and automation

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of cell-based assays, image capture, and image analysis exploded into the pharmaceutical scene in the late 1990s. The use of this technology has also been fueled by advances in molecular cloning, fluorescent proteins, for example, green fluorescent protein (GFP), and vast arrays of immunolabeling kits. Currently, more than 10 vendors worldwide provide sophisticated HCS systems, ranging from low-throughput assay development platforms to ultrahigh-throughput screening systems. These systems have become a rising trend in drug discovery as exploratory platforms as well as primary and secondary screening tools.

There are four main components to successful, reproducible HCS campaigns: cell preparation and labeling, image acquisition, image analysis, and data management. Each component has an important role and scientists must carefully design assays to ensure adequate image capture, quantification of biological events of interest, and discrimination between on-target and off-target effects. Important factors include use of validated reference/control molecules, choice of cell line(s), kinetic vs end-point readout, fluorescent labeling techniques, imaging approaches (wide-field fluorescence, confocal, bright field, etc.), image analysis modules, and data presentation. This chapter outlines the importance of these four HCS components, using the GPCR Transfluor assay as an example/case study.

Cellular Assay and Imaging Preparation

An important factor for HCS success is cell line choice and cell preparation. As with most cellular assays, adherent cells are ideal but HCS is also amendable to suspension or semisuspension formats. For adherent-based assays, cells are first dispensed into the wells of microtiter plates (96- or 384-well plates) and allowed to adhere to the bottom surface. Optimal cell attachment is not only important for proper biological response, but also for ease of capturing a biological process within a single imaging plane. Occasionally, plates are coated with poly-D-lysine, fibronectin, collagen, or other extracellular matrices to promote cell adherence and cell spreading. For semisuspension or suspension cells or to minimize cell loss due to reagent addition/wash steps, centrifugation steps can be added to the routine.

For a chemical library screen, compounds are transferred to the assay or cell plates and incubated at 37° for an appropriate duration. Depending on the assay, some reagents may be added before or after compound addition. For an end-point assay, cells are fixed after drug treatment to retain physiological state and antigen (protein) distribution. In some cases, permeabilization is performed to allow for antibodies to reach the antigens within the cell. Two of the most common fixation methods include paraformaldehyde

or ice-cold methanol. A typical immunofluorescence protocol involves numerous blocking and washing before, in between, and after primary and secondary binding steps. This conventional staining protocol is very time-consuming and difficult to miniaturize for robotic screening systems. Some of these steps have been combined and truncated to conserve time and to make the assay robotic friendly. Advantages of fixed-cell kinetic studies include multiplexing capabilities, stable signal for convenient imaging, flexibility in cell line choice, and large availability of antibodies. Some disadvantages include fixation and permeabilization artifacts, static information, and laborious labeling protocols.

Fluorescence microscopy, when applied to living cells, provides critical insight into how proteins behave in time and space and offers a clearer understanding of protein function and biological processes. Recent advances in molecular cloning, optical imaging, and the discovery of fluorescent proteins have fueled live-cell imaging and offer a nondestructive method for studying dynamic processes *in vivo*. An example is GFP-β-arrestin Transfluor technology from Norak Biosciences (now Molecular Devices) (Oakley *et al*., 2002). This assay can be used to detect and screen for compound activity against G-protein-coupled receptor (GPCR) targets. The assay is based on the desensitization and association of GPCRs with β-arrestin upon ligand binding. When a GPCR is activated, it binds to a cytoplasmic protein called β-arrestin. This interaction inactivates or desensitizes the receptor signaling, which is followed by receptor internalization for recycling. Tagging β-arrestin with GFP allows for live-cell monitoring of GPCR activation with fluorescence microscopy. Before GPCR activation, β-arrestin is distributed diffusely in the cytoplasm. Upon receptor activation, β-arrestin translocates to the plasma membrane and binds activated receptor where it can be visualized as punctate pits. Depending on the binding affinity between the receptor and β-arrestin, internalization of the receptor–β-arrestin complex via endocytic vesicles can be visualized as well. This assay can also be combined with conventional immunofluorescence microscopy as the fluorescence of GFP remains after fixation of cells with paraformaldehyde.

Image Acquisition

A second component to successful HCS campaigns is hardware choice and image acquisition. Most HCS instruments are based on an inverted fluorescent microscope; however, various components and peripherals make each machine unique. Users should consider the following features when purchasing an imaging system and defining image quality: wide field versus confocality, excitation source (lamp or laser), objectives for magnification and collection of emitted fluorescence, excitation/emission

filters and detectors for proper light separation, autofocus mechanism, stage precision, environmental control, robotic load, throughput, and software for controlling the hardware. Detailed discussions of these principal hardware features, as well as caveats to keep in mind when selecting a system to maximize image quality, are discussed next. Please refer to Table I for a summary of these features.

Wide-Field Versus Confocal Systems

There are several factors to consider when selecting an imaging platform for generating HCS data: sensitivity of detection, flexibility in wavelengths, speed of acquisition, and specimen viability. In wide-field microscopy, the entire specimen in the field of view is illuminated with light from a mercury or xenon lamp source, and the emitted fluorescence is captured by the objective (Fig. 1). All fluorescence emitted from the sample (from both the focal plane and the out-of-focus area below and above) is projected onto a charge-coupled device (CCD) chip for a set amount of exposure time. Hence, the throughput of the imager is somewhat dependent on the exposure time and the platform may not be suitable for thick specimens. These platforms, however, offer flexibility in wavelength choice, fast image acquisition, and low cost. Also, due to their wide depth of field, wide field has an advantage when imaging heterogeneous cell population with varying cell thickness. A number of wide-field systems are available commercially, including but not limited to the IN Cell Analyzer 1000 from GE (www.amersham.com) and the Arrayscan from Cellomics (www.cellomics.com).

In confocal microscope systems, blurry or out-of-focus light is removed by two changes to the conventional wide-field microscope: (1) laser illumination and (2) a confocal pinhole or a barrier to block out-of-focus light. The former provides better illumination of the specimen, whereas the latter prevents out-of-focus light from entering the hole/slit and reaching the detector. Because of these features, confocal microscopy provides sharper images and improved subcellular resolution through decreased background fluorescence and shallow depth of field but decreased throughput, as the field of view must be raster scanned with a single point of a laser and collected point by point. Fortunately, this issue has been improved through adjustments and/or additions to the microscope. For example, one variation is the use of a line-scanning technique as with IN Cell 3000 from GE (www.amersham.com). With this system, a horizontal laser line is used to excite the field in the Y direction and an adjustable slit is used to block out-of-focus light. Although some vertical or Z resolution is sacrificed (Cox, 2002), the increase in scanning rate is improved significantly

TABLE I
ADVANTAGES AND DISADVANTAGES OF THE VARIOUS HCS SYSTEM FEATURES/PERIPHERALS

Features	Options	Advantages	Disadvantages
Fluorescent technology	GFP or other fluorescent proteins	Live-cell imaging, easy assay protocol, temporal and space information	Time to develop stable cell line, heterogeneous population and activity interference
	Immunofluorescence	Availability of antibodies, ability to multiplex, stable signal, flexibility in cell line choice	Difficult assays steps, more washes, non-specific staining or artifacts, no temporal information
Excitation source	Lasers	Stronger illumination for confocal imaging	Limited wavelengths
	Lamp (mercury/xenon)	Wide range of wavelengths available	Longer exposure time
Objectives	Air/dry	Can image plastic plates with various thickness	Lower NA (numerical aperture)
	Water/oil	Higher NA; images are brighter and sharper	Only thin, cover glass thickness plates can be used
Autofocus	Laser based	Very fast and independent of well content	Cannot change from well to well
	Image based	Optimizes focus on well content	Significantly lowers throughput when wells are empty
Microscopy	Wide field	Better imaging of heterogeneous cell population	Poor Z resolution, high background, long exposure times
	Line scanning	High throughput with much larger field of view	Semiconfocal
	Nipkow spinning disk	High throughput with true confocality	Longer exposure times
CCD camera	No binning (full resolution)	Best resolution	Longer acquisition time
	Binning (2×2, 4×4, etc.)	Faster acquisition with shorter exposure time	Loss in resolution
Plate type	Plastic	Great cell attachment and viability, less costly	Loss is image quality
	Glass/film	Optimal for bright and sharp images	Poor cell viability, expensive

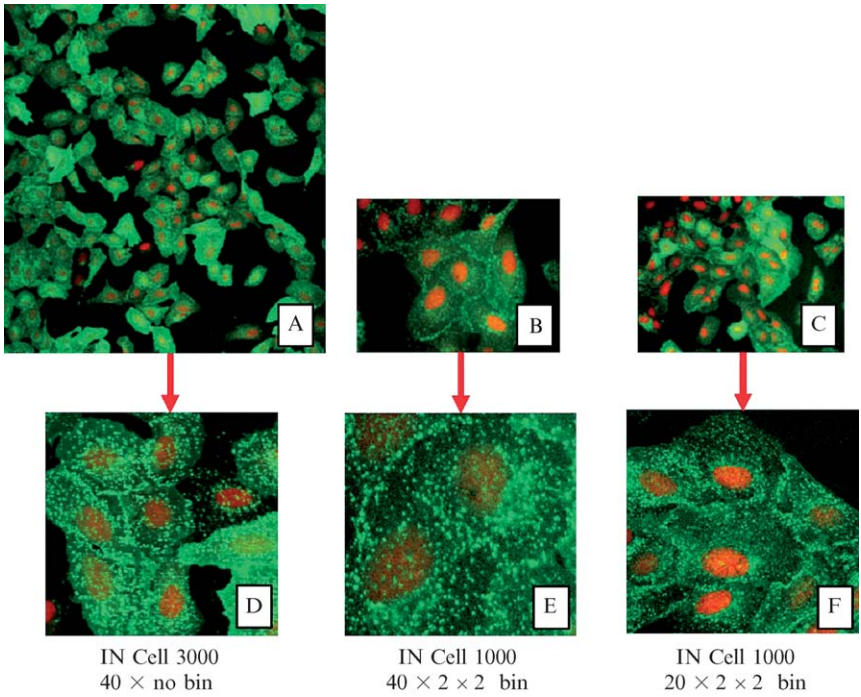


FIG. 1. Sample images of Transfluor technology taken from plastic bottom plates using wide-field and confocal (line-scanning) microscopes. Images A and D were captured with the IN Cell 3000 line-scanning confocal imaging system with its 40× objective with no binning. Image A is a full-field view (zoomed out), and image D is a cropped full-resolution image (100% zoomed). IN Cell 3000 can acquire around 200 cells per image with very fine resolution of 1-μm-sized clathrin-coated pits (shown in green). Images B and E were captured with the IN Cell 1000 wide-field fluorescent imaging system with a 40× object with binning of 2 × 2. Image B is a full-field view (zoomed out), and image E is a cropped full-resolution image (100% zoomed). With a 40× objective, only about 12 cells can be acquired per image but the pits can be resolved easily. Images C and F were also captured with the IN Cell 1000 but with a 20× objective with binning of 2 × 2. Image C is a full-field view, and image F is a cropped full-resolution image. With the 20× objective, around 50 cells can be acquired per image with sufficient resolution of the pits.

(Fig. 1). A second approach has been to include a Nipkow spinning disk as seen on the Opera from Evotec (www.evotec-technologies.com) and Pathway HT from BD Biosciences (www.atto.com). This technology utilizes a spinning disk with a large number of pinholes in which the light source passes through to excite the specimen (Cox, 2002). Multiple points are excited at once and multiple foci are generated and captured simultaneously with a CCD camera. The emitted light from the sample returns through the

same set of pinholes in which the out-of-focus light is blocked from reentering. Therefore, a large number of points are collected at a time, and the spinning property of the system allows fast scanning and acquisition of the field of view, resulting in near real-time image capture and decreased photobleaching. Unfortunately, illumination strength is sacrificed greatly when the excitation light is mostly hindered by the holes on the spinning disk (Cox, 2002). Therefore, longer exposure time may be necessary, thereby impacting throughput. In both the line-scanning system and the Nipkow spinning disk system, the acquisition rates are sufficient for live-cell imaging, as well as high-throughput fixed-cell applications.

Excitation Source and Filters

With a lamp, one could have a wide range of selection for their excitation wavelengths with the appropriate exciter filters. Xenon lamps release a spectrally uniform intensity profile from the UV range (300) to far red (>700). Mercury light sources releases energy of similar profile but is much stronger at the following wavelengths: 365, 400, 440, 546, and 580 nm (Herman, 1998). Exciter filters are used in front of a lamp to select the wavelength of choice for transmission while blocking the rest of the spectrum from passing the filter. HCS systems that utilize lamps for excitation will have multiple positions (in a filter wheel) for a range of exciter filters that can be moved into position automatically for sequential multicolor capture.

Laser-based excitation systems are bound to the specified wavelengths and intensities of the lasers. Lasers can be replaced and/or added but they are serious projects involving realignment of optics and other components as well as high cost. Also, due to the space restrictions, systems may only be installed with three or four laser lines. Each laser line outputs only a single wavelength, such as 488 argon ion and 633 Red-HeNe laser systems. However, because there are multiple excitation sources, multiple color fluorochromes may be excited at once, permitting simultaneous multicolor image acquisition (with appropriate dichroic, emission filters, and multiple detectors). Therefore, laser-based imagers have an advantage in throughput when it comes to multicolor imaging.

Objectives

Most HCS systems have objectives ranging from 4 to 60× magnification from various microscope manufacturers (i.e., Nikon and Olympus). The objective(s) one requires will depend on their particular application, but in general, 10× objective (0.25 NA) is a minimum in order to observe and monitor subcellular components and molecular interactions. Higher

objectives with a higher numerical aperture (NA) can be used to capture brighter and sharper images; however, throughput (number of cells captured per second) will be sacrificed greatly. Sufficient data points (cells) are required in order to correctly represent the response of the cells in the well, partly due to the heterogeneous cell population and diverse cell phenotype. Accordingly, number of cells captured per image is a significant factor in throughput. Some systems offer only a single objective, either 20 or 40×. Generally, assuming semiconfluent population of cells, around 50 cells are captured per image using a 20× objective (0.45 NA) while only around 12 cells are captured per image using a 40× (0.6 NA) objective. Differences in the full-field image and a fully zoomed image between 20 and 40× objectives are shown in Fig. 1. With higher objectives and/or underconfluent wells, more images or frames are captured per well to collect sufficient cell data.

Detector

The most commonly found detectors in HCS systems are highly light-sensitive, cooled CCD cameras. CCDs are basically an array of photodiodes (referred to as pixels or picture elements) that collect and convert the accumulated photons from emitted fluorescence from the sample to electronic charge. The converter translates the analog signal to digital values that are analyzed by the computer (optimal microscopy primer; http://www.micro.magnet.fsu.edu/primer/index.html). Twelve-bit CCD cameras will provide 4096 grayscale values at each pixel whereas 8 bit provides only 256 values. Therefore, a higher bit-depth implies greater range of image data so that the digital values can represent the actual image more accurately. Most HCS systems will offer a 12-bit camera(s). Also, most cameras have the option to bin the pixels, or combine signals from multiple pixels. Binning results in a higher signal-to-noise ratio, greater sensitivity to weak fluorescence, and faster acquisition rate, but with the sacrifice of reducing image resolution.

Autofocus Mechanisms

Autofocus of specimens is achieved either through contrast- or laser-based approaches. In the image-based approach, images of cells at different Z planes are acquired and analyzed quickly, and the optimal plane with the sharpest focus of the cells or features of interest is selected. This technique allows for very accurate focus at each well independent of the plate irregularity and unevenness. It is also able to sharply image heterogeneous population (cells with different thickness or focus plane) from well to well, as the focus is based on the content of each well. However, disadvantages

include slower throughput and inability to focus on wells with a low cell count. Cell Lab IC 100 from Beckman Coulter (www.beckman.com) utilizes this technology.

A laser-based autofocus uses an IR beam and a detector to identify the interface between the plate surface and the solution in the well where the reflective index changes. With this method, the autofocus quality is not determined by the content in the wells. In cases where there is a lack of cells in the wells due to toxicity or low cell count, image-based systems will fail while attempting to find cells to focus on. Hence, the laser-based autofocus has a significant advantage in this regard as cells are prone to detaching from the plate when they are undergoing mitosis or apoptosis. IN Cell Analyzer 1000, 3000, and Evotec Opera are some of the instruments that utilize this technology.

Environmental Control/Kinetic Imaging

Many systems offer environmental control on the instrument for live cell and kinetic imaging. The instruments are usually enclosed by a housing to regulate temperature and CO_2 levels. These systems emulate the environment of an incubator to allow for cells to grow and respond to treatments under optimal conditions while imaging simultaneously. Nontoxic, cell-permeable dyes or cell lines with fluorescently tagged protein (i.e., GFP) will be required for this application. Liquid dispensers are also available within the instrument to perform complete kinetic assays all within the system. Live cellular images can be acquired immediately before and after reagent dispense. These tools allow for live monitoring of molecular interactions within complex cellular systems under optimal conditions.

Image Analysis

A third component to successful HCS campaigns is image analysis. Each imager is supplied with software that includes image analysis capability in one way or another. In most cases, vendors supply a list of ''canned'' modules that perform analysis for a specific assay (receptor trafficking, object intensity, translocation, cell cycle analysis, etc.). An image analysis algorithm in an HCS context is a step-by-step procedure of analyzing the contents of the image for a particular event(s) in each cell. Canned modules are designed to be turnkey solutions for quantifying a particular biological event or changes in intensity.

As HCS assays become more complex, tools must be available for modifying existing modules and creating new algorithms that allow flexibility in measuring phenotypic results. Presently, some software packages have

an open environment where programmers can access the code and commands of the algorithms in a specified programming environment (C++, MATLAB, etc.). Also, many packages provide Application Programming Interface (API), which allows third-party software (i.e., database, image capture software) to access tools or parts of the software for further modification, addition, and automation. A few provide scripting (macro) abilities where nonprogrammers can create a new protocol/algorithm by using the available image analysis tools, e.g., Acapella by Evotec (www.evotec-technologies.com), Developer by GE Healthcare (www.amersham.com.), and Cellenger by Definiens (www.definiens.com). The latter option is preferred, as scientists without programming experience can modify or even create algorithms for their specific applications.

When the images are analyzed, numerous outputs, hence the high content, are generated for each image or well. Simple and common outputs include nuclei/cell count, size, and shape, average intensity at the region of interest, and other attributes from the segmented object are generated for each well. In addition to these data, more complex phenomenon can be represented by numerical outputs such as number of internalized vesicles per cell, cell toxicity, apoptosis, cell cycle phase, and many others. Therefore, each well can generate many numerical outputs that may be useful in a given assay.

Image analysis can be performed on the individual cell level or on the whole field level. Cell-by-cell or per cell analysis provides measurement and statistics on the feature of interest for each cell. Whole-field analysis simply calculates the average value of the cellular event in the entire field by dividing the total sum value of the event by the total number of cells. For example, when obtaining the average number of vesicles per cell, whole-field analysis will simply count the total number of vesicles in the image and divide by the total number of cells. In individual cellular analysis, the number of vesicles in each cell will be obtained before calculating the field average (Fig. 2). The cell-by-cell analysis provides numerous advantages, such as subpopulation evaluation, cell filtering or selection, individual cell statistics for detailed analysis, and many others. In the case of Fig. 2, cells expressing little or no GFP and cells with very small nucleus (dead or apoptotic cells) were excluded in the cell by cell analysis; therefore, only viable cells that were filtered through were analyzed for receptor activation. Analyzing only the relevant subpopulation in the well or image provides better numerical representation of the biological activity under investigation.

The proper image analysis algorithm must be applied to measure the biological activity of interest correctly. The appropriately applied algorithm can provide improved statistics (i.e., lower coefficient of variance

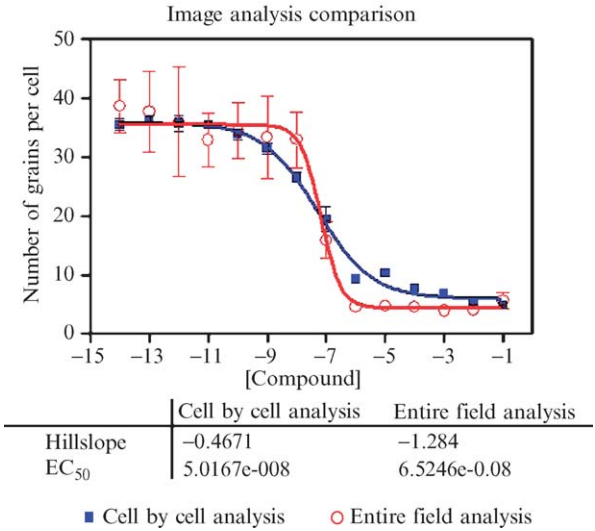
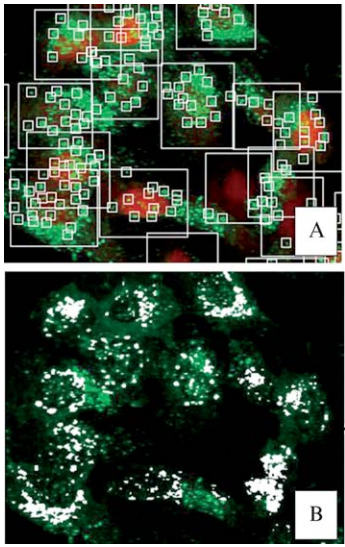


FIG. 2. Individual cell vs whole-field image analysis comparison. IN Cell 3000 software was used to perform the individual cell analysis, and Opera software was used to perform the entire field spot-counting analysis. (Opera software has the flexibility to perform both individual and whole-field analysis.) Curve represents a dose response of an inhibitor in the presence of ligand. The *Y* axis represents the average number of grains per cell, and the *X* axis represents the concentration of a compound. Whole-field analysis resulted in a much steeper hillslope and wider error bars. Both instruments acquired two images per well with Opera capturing 90 cells/well and the IN Cell 3000 capturing 290 cells/well.

and higher Z factor and S/N) and better assessment of compound potency using EC_{50} or IC_{50} values (Fig. 3). The algorithm may also be applied to a kinetic data set or stacks of images. A movie of receptor internalization (Transfluor assay) can be analyzed to quantify the temporal activity (Fig. 4).

Image Database and Data Visualization Tools

After completing the first three stages of HCS—cellular assay, image acquisition, and image analysis—one is left with a plethora of data to mine and explore. As with any other screens, there is much assay information that needs to be kept, such as cell type(s), protocols, and reagents (antibodies, fluorescent markers, nuclear stain, etc.). During a screen, numerous metadata are produced as with any typical HTS campaign, including, but not limited to, compound or siRNA ID, well ID, plate bar code ID, and the location of controls within each plate. The number of compounds screened,



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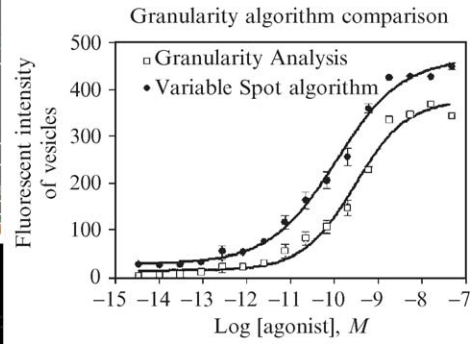

	Granularity analysis	Variable Spot algorithm
Hillslope	0.7323	0.5786
EC_{50}	2.9963e-010	1.1201e-010

FIG. 3. Comparison of two spot/vesicle counting algorithms. Images of activated receptor with Transfluor technology were captured and analyzed by the IN Cell 3000 software, Raven. (A) A canned algorithm called Granularity Analysis was used to identify the internalized vesicles and measure their average fluorescent intensity per cell. The Granularity Analysis captures vesicles of specified size and intensity; therefore, aggregated vesicles or smaller vesicles were not identified. This issue can be resolved using the modified Varible Spot algorithm (B) where vesicles of any size can be identified as long as their intensity level is above the specified threshold value. (C) EC_{50} values were determined by analyzing the same set of images from a 16-point agonist titration using both algorithms. The Variable Spot algorithm provided a larger window and a slightly left-shifted EC_{50} value.

number of images (frames) taken per well, and number of color channels per frame determine the amount of raw image data taken for the screen. Each raw image must be associated with the proper compound ID and control ID. These IDs must also be associated with the numerical outputs from image analysis. Linking all of the results and metadata not only requires a comprehensive data and image management solution, but also a large and upgradeable storage capacity.

File size of a single high-resolution image can range from 1 to 5 megabytes, depending on resolution and size (height and width) of the image. Imaging an entire 384-well plate would produce anywhere from 384 to nearly 2000 megabytes or 2 gigabytes, and accordingly a 10,000 compound screen would produce at least 10 to 50 gigabytes of data. Thus, a typical computer hard drive would not have the capacity to hold images for a screening campaign.

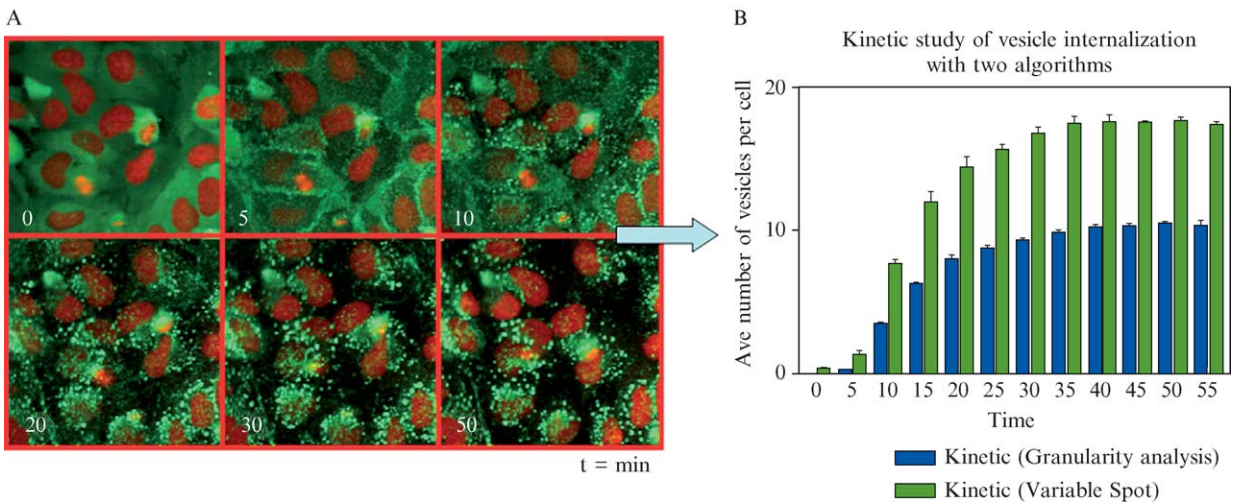

FIG. 4. Time-lapse image capture and analysis of receptor internalization (Transfluor technology) after agonist addition. The movie was captured with IN Cell 3000 upon activation of receptor with a known agonist for 55 min. The movie was converted into individual frames (A) using the IN Cell Movie Conversion tool and analyzed with the Granularity Analysis and the Variable Spot algorithm. The average number of vesicles per cell was measured for selected time points and plotted (B). The Variable Spot algorithm provided a larger signal window.

There are numerous options for storing terabytes of data. One option is to keep the images locally, on the computer(s). Media storage (i.e., DVD jukebox and tape) is also a possibility, but slow speed and management of hundreds of media are some of the issues associated with this option. A more popular solution is the storage area network (SAN). These storage devices have multiterabyte capacity and can be scalable to tens of terabytes. Commonly, data on the SAN are backed up on tape as well. Next, a database, such as Oracle, with an image management application would be required to organize and retrieve data efficiently. Cellomics, Inc. has developed a software platform called High Content Informatics (HCi) that provides a complete solution for image and data storage, retrieval, and interpretation. Bioimagene (www.bioimagene.com) has also released a product called CellMine HCS, which is a Scientific Image Management System tailored for HCS.

Conclusion

Four components to an HCS campaign include the following: assay design (cell line, probes, kinetic, or end point), image acquisition (confocal vs wide-field, hardware specifications), image analysis (canned or custom algorithms), and data interpretation and image management (storage space, database, and visualization). Technology associated with each component must be selected and assessed properly in order to carry out a successful HCS campaign. With the proper tools, HCS can be an invaluable tool to exploit the complexity of cellular signaling pathways at a single cell level in a high-throughput manner.

Protocols

The Transfluor assay is used as a case study to examine the protocols involved in an HCS campaign. Protocols in cell preparation, hardware selection, and image analysis are outlined in this section. Note that the GFP-β-arrestin Transfluor technology for GPCRs described here is offered by Molecular Devices.

GFP-β-Arrestin Fixed-Cell Assay

Protocol for the Transfluor Assay to Screen for Small Molecule Inhibitors

1. Plate Transfluor cell line (U2OS cells) at 3000 cells/well (30 μl) in 384-well plates.
2. Incubate overnight to allow cell adherence.

3. Transfer 150 nl of 2 m*M* compound stock from compound source plates.
4. Incubate for 10 min at 37°.
5. Dispense 5 μl of agonist from 7× stock concentration.
6. Incubate at 37° for 60 min.
7. Dispense 35 μl of the staining solution. Staining solution contains 4% paraformaldehyde and 1 μM DRAQ5 [DNA labeling dye from www.biostatus.com, catalog number DRAQ5 (5 m*M*) HTS] in phosphate-buffered saline (PBS).
8. Incubate for 10 min at room temperature.
9. Remove staining solution and wash three times with PBS.
10. Perform image acquisition.

Protocol for Image Acquisition (Transfluor Assay) Using Confocal Microscopy

1. Select the appropriate plate format and type from the software and set the correction collar on the objective to the plate thickness.
2. The pinhole diameter or the slit width is usually fixed on these imaging systems.
3. Select the appropriate lasers and emission filters:
   a. For DRAQ5, use the 633-nm laser with 695/55-nm emission filter.
   b. For GFP, use the 488-nm laser with 535/45-nm emission filter.
4. Set the exposure time (in milliseconds), laser power, neutral density filters, gain, and other signal intensity settings for each channel. Ensure that the image is not overexposed (resulting in saturation) or underexposed (resulting in insufficient range of intensity values).
5. Set the correct autofocus Z offset (offset distance in um in the Z direction from the plate/liquid interface).
6. Assign the appropriate cameras to each channel (multiple cameras for simultaneous multicolor acquisition).
7. Enter the number of frames or images to be captured per well.
8. Begin image acquisition.

Protocol for Image Analysis Routine (Granularity Analysis Algorithm from IN Cell Analyzer 3000) on the Transfluor Assay (Fig. 5)

1. Select the Granularity Analysis algorithm from the list of analysis modules.
2. Assign the red channel (nuclear stain or DRAQ5) as the marker channel and assign the green channel (GFP-β-arrestin) as the signal channel.

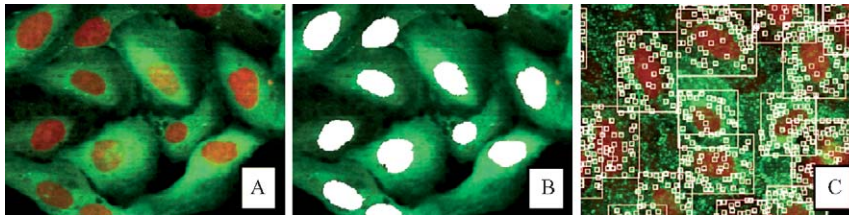

FIG. 5. Image analysis of Transfluor technology (GFP-labeled β-arrestin). (A) Arrestin (green) is distributed evenly in the cytoplasm before treatment with an agonist. The nuclear marker DRAQ5 is shown in red. (B) Nuclei are identified or segmented (labeled by a white bit map) on the red channel by selecting pixel intensity levels above the background. The nuclei bit map can be used to identify individual cells as well as to measure nuclear size and fluorescent intensity. (C) A bounding box of specified size can be drawn around the cell body to measure grains or spots of specified intensity and size within a single cell, shown by the smaller spot boxes.

3. Open a sample image and adjust the intensity levels (brightness enhancement) to clearly see the DRAQ5 and GFP signals.
4. Activate the marker bit map (white overlay on top of the image) showing the thresholding result of the DRAQ5 channel. Adjust the threshold accordingly to accurately identify the full nuclei.
5. Activate the granules bit map (large boxes surrounding the cells and small boxes surrounding the granules). Adjust the size of the bounding box around the cell to outline the cell appropriately. Adjust the parameters defining the granules within a cell (spot intensity and size).
6. Begin batch image analysis with the aforementioned settings.

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