[26] An Infrastructure for High-Throughput Microscopy: Instrumentation, Informatics, and Integration

By EUGENI A. VAISBERG, DAVID LENZI, RICHARD L. HANSEN, BRIGITTE H. KEON, and JEFFREY T. FINER

Abstract

High-throughput, image-based cell assays are rapidly emerging as valuable tools for the pharmaceutical industry and academic laboratories for use in both drug discovery and basic cell biology research. Access to commercially available assay reagents and automated microscope systems has made it relatively straightforward for a laboratory to begin running assays and collecting image-based cell assay data, but doing so on a large scale can be more challenging. Challenges include process bottlenecks with sample preparation, image acquisition, and data analysis as well as day-to-day assay consistency, managing unprecedented quantities of image data, and fully extracting useful information from the primary assay data. This chapter considers many of the decisions needed to build a robust infrastructure that addresses these challenges. Infrastructure components described include integrated laboratory automation systems for sample preparation and imaging, as well as an informatics infrastructure for multi-level image and data analysis. Throughout the chapter we describe a variety of strategies that emphasize building processes that are scaleable, highly efficient, and rigorously quality controlled.

Introduction

The application of high-throughput image-based cell assays to early stage drug discovery began in the late 1990s, and their use in both industry and academia continues to steadily increase (Abraham, 2004; Mitchison, 2005; Taylor, 2001; Yarrow, 2003). This trend has been driven by the realization that image-based cell assays can play a valuable role throughout the drug discovery process from target validation to primary screening to late-stage lead optimization. Access to commercially available assay reagents and automated microscope systems has further fueled adoption of these technologies.

Although it can be relatively straightforward to begin running assays and collecting image-based cell data, obtaining controlled, reproducible data on a large scale can have numerous challenges and pitfalls. First-order

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challenges include process bottlenecks, such as those associated with sample preparation, image acquisition, and data analysis. Potential pitfalls include problems with assay consistency and day-to-day reproducibility that can result from either suboptimal data acquisition or insufficient quality control. As these challenges are overcome, second-order challenges arise in managing and digesting unprecedented quantities of data, including terabytes of image files and databases filled with extracted image features and biological readouts.

The aim of this chapter is to provide a framework for addressing these challenges and avoiding these pitfalls through the development of a robust scalable infrastructure and implementing processes aimed at maximizing assay quality. The components of this framework highlighted in this chapter include instrumentation and processes for assay plate creation and image acquisition, strategies for image analysis, and a database and software infrastructure built to allow data integration at multiple levels. The final component described is an approach to quality control (QC), which is important during each step of both assay processes and postassay data analysis. Cell culture (Freshney, 2005), compound handling (Chan and Hueso-Rodriguez, 2002; Gosnell *et al.*, 1997), and specific assay readouts are also critical to the success of image-based cell assays; however, these topics are not covered in depth in this chapter as they have been addressed by several others in this volume and the existing literature. The assay processes described throughout this chapter apply directly to end point (i.e., fixed cell) assays. Although live cell assays are not discussed, many of the same strategies, infrastructure components, and practices can be applied. This system has broad utility and has been used in a variety of contexts from primary phenotype-based screening, to compound classification and clustering (Adams *et al.*, 2006), to applications in compound profiling as part of drug discovery lead-optimization campaigns (see, e.g., Tanaka *et al.*, 2005). In order to put the assay and analysis processes and infrastructure components into context, an automated cell cycle assay is used as a common example throughout the sections of this chapter.

One of the keys to building a high-performance and flexible infrastructure for high-throughput image-based assays is attention to selection of components and details of process design. The systems that we describe have been built with careful attention to selection of the most appropriate hardware and software components and integration of them into a common framework. In most cases the selected components were available commercially; however, custom software and databases were built to optimize performance and process efficiency. A detailed description of the custom software is beyond the scope of this chapter, although several important design considerations are provided. For example, opportunities for parallel

processing have been utilized whenever possible. This design feature contributes to the scalability and flexibility of the system for running a variety of different assays either independently or concurrently, and it also allows for the development of efficient processes where the throughput of various process steps can be matched for optimal performance.

An overview of a generalized assay process is shown schematically in Fig. 1 and is referred to and elaborated on throughout the chapter. This process flow diagram illustrates both processes involving manipulations of assay plates, as well as data analysis and the interplay of multiple databases. At the point of the initiation of an experiment, plate streams are

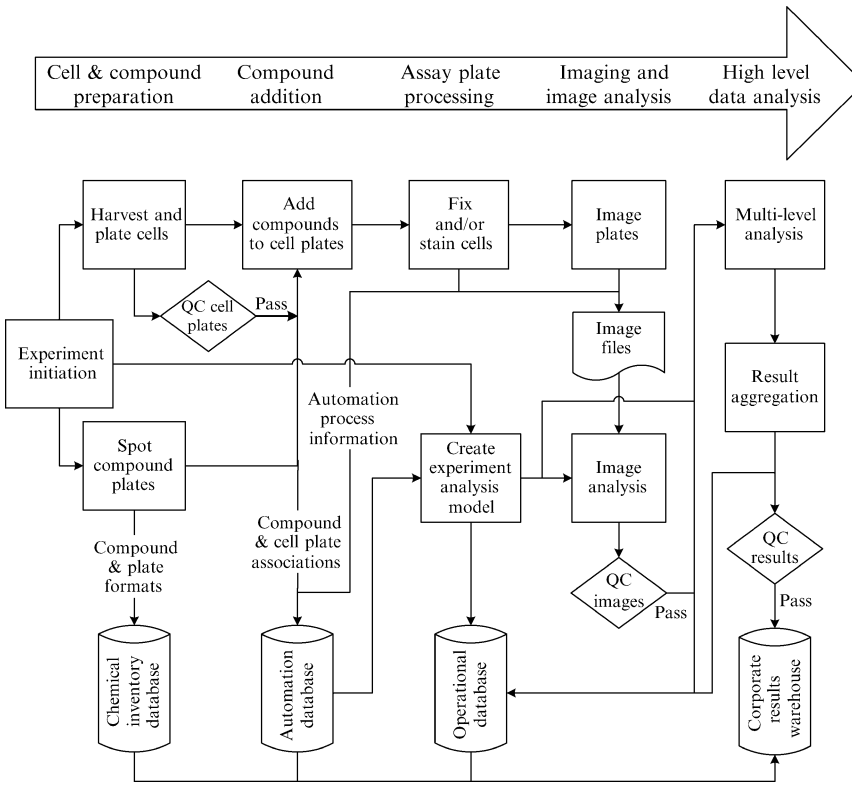

FIG. 1. Generalized assay process flow diagram illustrating steps from cell and compound plate preparation through assay processing, imaging, and data analysis. This is a simplified view of a complex process involving the coordination of assay plate manipulations, data analysis processes, quality control steps, and data flow between multiple databases. Several additional process steps, analysis processes, and quality control steps have been omitted for simplification. See text for additional description.

created for both cell plates and compound plates, and an analysis model is prepared to execute multilevel analysis steps specific to the experiment. The two plate streams are then brought together in a compound addition step. After cells are treated with compounds for a specific period of time, the assay plates are prepared for imaging through a series of steps that typically involve cell fixation and staining with fluorescent dyes for cellular components. Following plate imaging, which is used to collect raw image files, data analysis steps occur first at the level of individual cellular features followed by multiple levels of higher-order analysis. The results of an experiment are subsequently aggregated and stored in a data warehouse. Quality control processes are inserted at multiple points throughout the process flow of each experiment to ensure high-quality results and to provide opportunities to abort process streams when necessary without further investment of valuable reagents or resources. Process information is also collected and stored in a separate automation database to allow for additional quality checks and to simplify troubleshooting when problems arise.

Assay Processing

As detailed in Fig. 1, this section discusses infrastructure supporting creation of cell plates, treatment of the cells with compounds, and preparation of the cell plates for imaging. Each of these steps is generally amenable to and can benefit greatly from automation. Aside from the obvious time-saving benefit of automating routine and repetitive tasks, data quality and consistency can be improved greatly. Data consistency and process stability are important during prolonged primary screening campaigns where hundreds of thousands to millions of compounds are tested over weeks or months, but it is also important for assays that are routinely run over an extended period of time, such as those used in drug discovery lead-optimization campaigns, which may span years.

It is clear that automating an assay requires instrumentation for plate and liquid handling and software to control and coordinate these devices. An automated imaging system is also of clear benefit as is a robust image analysis platform, both of which are discussed in detail later in the chapter. However, an informatics infrastructure to track and characterize processes, samples, and reagents is often overlooked. In order to create the most robust and informative results, care must be taken to capture the specific details about how each sample was generated, handled, and treated. The goal of process tracking is to enable results to be linked back to instrumentation, reagents, and protocols used during assay preparation. Additionally, process information should allow results to be linked to specific

cell samples and include lineage, culture, and passaging information. This level of information is valuable for understanding variation in large data sets and becomes invaluable during troubleshooting. Process-tracking databases are most effective when tied in with chemical inventory and image result databases. Typically the unique identifiers enabling all assay information to be tied together across multiple databases are assay plate barcodes. A robust informatics infrastructure facilitates assay processing and tracking greatly and becomes crucial when running multiple assays in parallel simultaneously. Throughout this section we highlight examples of relevant process information.

Cell and Compound Plate Preparation

At the initiation of an experiment, two plate streams are created. One contains compounds for testing and the other contains cultured cells. Consistent delivery large numbers of cell samples for large-scale assays can be challenging. Large-scale cell culture processes have been automated successfully, including cell passaging, cell harvesting, and cell plating. For example, the SelecT (The Automation Partnership) can maintain multiple cell lines using unique passaging protocols for each cell line. The device can also prepare assay plates at specified seeding densities in a variety of plate formats. Automation of routine cell culture activities ensures consistency in cell handling by establishing routine cell maintenance schedules, which minimize the effect of personnel and work-week schedule conflicts. Each passaging and plating event is recorded in a process database. In this way, robust tracking of sample lineage, passage number, growth rates, and viability can be achieved.

When cells are harvested from culture flasks, they can be plated efficiently from a stirred suspension with a simple manifold device such as the Multidrop (Thermo). We use such an instrument to dispense cells into microplates with minimal cell-shearing forces. During plating, a sufficient number of cell plates for each cell line should be generated to satisfy the experiment design and supply several additional sentinel plates for assessing plating density and uniformity and other process QC tests. Although immediate visual inspection of the plated cells provides a rough idea of plating quality, a short-term imaging experiment to count stained nuclei is more quantitative. Typically cells are fixed after the cells have adhered to the plate and stained with a simple nuclear marker such as Hoechst 33342. This short-term count can also serve as a baseline for growth rate measurements during the rest of the assay. A second cell count is performed just prior to adding compounds to the cell plates. This QC step serves as a pass/fail checkpoint; if the samples do not pass QC, valuable materials (i.e., compounds and staining reagents)

are not committed to the assay. In our cell cycle assay we have optimized the number of cells delivered per well for each human tumor cell line based on the cell size and growth rates so that cells are in exponential growth during the compound treatment period. As one example, for the ovarian tumor cell line SKOV3, we deliver approximately 1200 cells per well (in a 384-well plate), with the expectation that without compound, they will double approximately twice during the 48-h assay.

Compounds are typically dissolved and stored in dimethyl sulfoxide (DMSO), and compound plates are prepared in an assay-ready format with low-volume spots of high compound concentration. Compound plates are typically registered in a chemical inventory database that provides compound-to-well mappings for use during analysis. At assay time, cell growth medium is added to the compound plates to reduce the compound and DMSO concentration to acceptable levels (usually $<0.4\%$ DMSO in the final cell assay). A portion of this diluted compound solution is then transferred to one or more cell assay plates.

Assay formats can range from single concentration tests, as are often used in primary library screening, to complex multiconcentration dose–response formats. Compound-handling instrumentation can readily accommodate advantageous well groupings and dilution schemes. For example, we and others have determined that there are sufficient edge well effects to warrant skipping use of the outer perimeter wells of microtiter plates. Most importantly, any standardized plate format should accommodate control compounds.

Control or reference compounds can be used to qualify assay performance in several ways. During development and optimization, assays are characterized to determine if they can report on a specific cellular activity with statistical significance. Known reference compounds are especially valuable for assay validation. Once an assay is qualified and implemented for a high-throughput application, control compounds are used to monitor assay performance through comparison of control data with historically accumulated values. Controls are most informative if they are chosen to span the dynamic range of the readouts of the assay and should include solvent-only or null treatment wells. It is important to process controls alongside other test compounds to best gauge the effectiveness of plate-processing steps.

Another role for control compounds is to assist with data analysis. If an assay is inherently noisy or run over an extended period of time, it may be necessary to normalize or bound data on a per-plate or per-batch basis. For example, the dynamic range of the biological response for a given assay instance can be calibrated by reference compounds. Controls are also important, as discussed in a later section for building classification models in which individual cells are grouped into subpopulations. For example, in

our cell cycle assay, we use DMSO-only treatment as a negative control and dilution series of paclitaxel (peaking at 800 n*M*) as a positive control to arrest cells in mitosis. Test compounds are ranked relative to the assay bounds determined by these controls. This strategy can help minimize excess variance introduced by deterministic noise sources such as low cell growth rates, marginal assay staining, or other hard-to-identify environmental influences.

Cell plate and compound plate streams meet after the cells have had sufficient time to adhere to the plate surface, stabilize their morphology, and resume growth (often 24 h following cell plate preparation). One compound plate can usually supply compounds sufficient to treat multiple cell plates. This has the advantage of reducing assay variance because all replicates spanning multiple cell lines or even several different assays receive the same compound treatment. Similar to including reference compounds in an assay, reference cell lines may also be included to differentiate or categorize compound response. For example, control cell lines may be refractory to certain compound classes or display characteristic phenotypes or response ranges. As compounds are added to one or more cell plates, barcode associations are created in the process database, which will enable assay results to be linked to compounds.

Automated Assay Processing

Efficient processing of compound and cell plates requires at minimum automated pipetting and is typically best accomplished with integrated systems that operate on a static schedule. A number of fully integrated single vendor solutions for cell processing are currently on the market. For example, a BioCell (Velocity11) can be configured to prepare live cell assays. Vendor solutions provide complete systems with little need for automation expertise, although the choices of adorning instruments and configurations are limited to what the vendor can make or is willing to support. The advantage of custom integration is that systems with the best options can be created. The best-suited liquid-handling devices, storage devices, and readers or imagers can all be incorporated regardless of manufacturer. A second advantage is that the system can be tailored to quickly meet the needs of shifting assay requirements.

Assay processing steps can be abstracted to several classes. Plates must be introduced into a system, possibly from a device that can support live cells, they must be moved from device to device, and liquids must be added and removed from the plates. Ideally the processed plates leave the system sealed and stable for imaging. Figure 2 illustrates the layout of a custom-integrated cell processing system that meets these

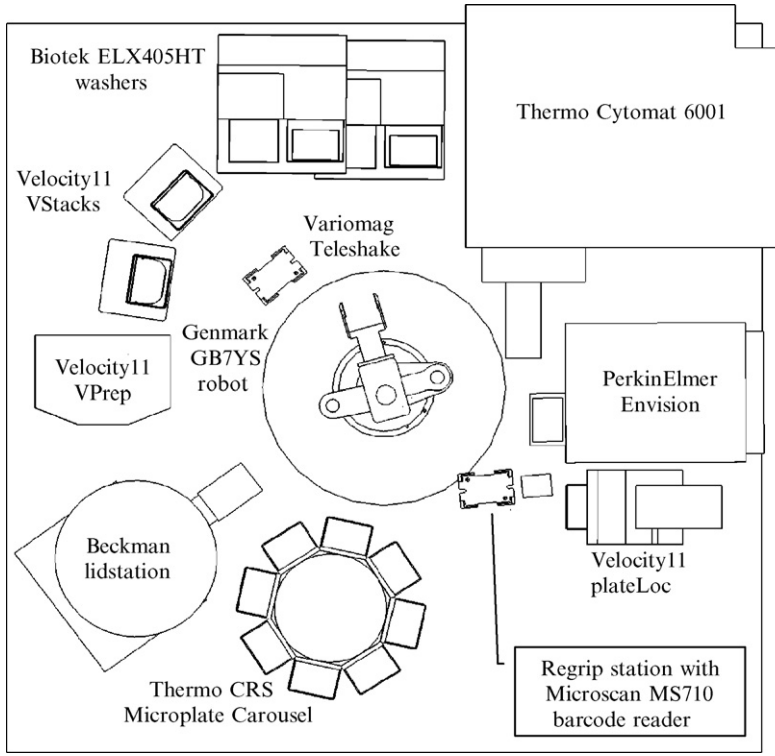

FIG. 2. Schematic of integrated cell processing system. This system is designed specifically for processing cell assay plates. In a typical assay, plates start in a random access CO_2 incubator Cytomat 6001 (Thermo) and move around the system by a robot adapted from the semiconductor industry, GB7 (Genmark Automation). Plates are fixed and receive staining reagents via a parallel pipetting device, VPrep (Velocity11). Plates can be washed on one of two plate washers, ELX405HT (BioTek), and incubated with reagent on an open random access device, CRS Microplate Carousel (Thermo). When finished, plates are sealed with a thermal plate sealer, PlateLoc (Velocity11), and moved to stackers, VStacks (Velocity11). Curtains (not shown) shield the system from room light to minimize bleaching of photo-labile reagents.

general requirements. The components have all been chosen for their speed, quality, and performance.

The central component of the system is the plate-handling robot. This system uses a robot adapted from the semiconductor industry, GB7 (Genmark Automation). This six-axis robot is fast and was designed to carry small silicon wafer payloads, similar in weight to microtiter plates. This type of robot is very easy to work with and will stall easily (and

appropriately) during a collision. A second option for robotics in this type of system is an industrial robot adapted from manufacturing applications. These robots are very reliable and typically have much higher working payloads as compared to the semiconductor robots. Examples of such robots include the CRS F3 (Thermo) and CRS CataLyst5 (Thermo). Additional care must be taken when working with the larger manufacturing robots to avoid collisions and situations that occur when the arm is commanded to move through a physically impossible trajectory in order to achieve a position. The result can be an unexpected and often rapid move, resulting in a potentially damaging collision with other system components.

The system allows plates to enter, exit, or incubate in one of several devices. Each has been purchased or configured to operate preferentially in landscape plate orientation. Two plate stackers, VStacks (Velocity11), are included, which are typically used to introduce empty labware or collect processed plates from the system. A random access CO_2 incubator, Cytomat 6001 (Thermo), holds live cell plates on the system, and a random access carousel, CRS Microplate Carousel (Thermo), provides 120 locations to park in-process plates. One advantage of this carousel is that the robot can place and retrieve plates directly from its nests, speeding plate access greatly. A lid-handling station, Sagian Lidding Station (Beckman Coulter), allows lid removal and replacement of up to five plates at a time. Any plates that enter the system have their barcodes read and captured in the process database.

An important piece of equipment from an assay quality and speed perspective is the parallel pipetting device. We chose the VPrep (Velocity11) for several reasons. First, it was designed for use in a radial system such as ours, with a small footprint and vertically integrated design. The system allows users to easily change between 96 and 384 pipetting heads as needed and can deliver good pipetting precision and accuracy with either head. The device has 8 pipetting shelves, which allow simultaneous delivery of plates to the device while the unit is performing pipetting tasks at another shelf position. Another important device for creating quality cell assay plates is the plate washer. We included two duplicate washers, ELX405HT (BioTek), in the system for added throughput. Care must be taken when establishing washer settings to aspirate and deliver wash buffer at the correct speed and height above the plate bottom in order to not affect the sample adversely. For example, in our cell cycle assay, washing too vigorously may remove loosely adhered mitotic cells, whereas insufficient washing or poor removal of wash buffer can lead to suboptimal staining.

The system is equipped with a plate sealer, PlateLoc (Velocity11), and homemade low-volume autofilling reservoirs to work on the VPrep. These devices allow processes to run into the night or over the weekend without

compromising assay quality. The system also contains a multilabel plate reader, Envision (Perkin Elmer), to accommodate nonimage-based assays such as end-point cell viability assays.

Process Control Software

The present system is controlled by custom-built software that allows users to model assay processes and generate static schedules for execution. A static schedule means that all steps have been choreographed based on sequence and timing constraints and scheduled prior to the assay. This is in contrast to a dynamic scheduling system, which processes plates as soon as the next instrument resource becomes available. Consider the following fix-and-stain protocol for compound-treated cell plates.

1. Remove live cell plates from incubator, read barcode, and add formaldehyde fixative.
2. Incubate 1.5 h.
3. Remove fixative and culture medium, wash, and add blocking solution.
4. Incubate 1.5 h.
5. Remove blocking solution, wash, and add primary antibody.
6. Incubate 4 h.
7. Remove primary antibody, wash, and add secondary antibody solution.
8. Incubate 1.5 h.
9. Remove secondary antibody and wash.
10. Seal plates.

Computing a static schedule for this process requires designing a process model (assay script) complete with timings for each step, including all robotic plate movements. A scheduling engine creates an ordered set of execution tasks to most efficiently process the plates and avoid resource conflicts. The static schedule order will be adhered to even if the process does not follow exact timings. Figure 3 shows the static schedule used for fixing and staining up to 112 plates for the aforementioned protocol, a process that takes over 10 h. When a process involves lengthy steps such as incubation with an antibody, care should be taken to match the timings of early and late incubations; this will allow the largest batch sizes to be achieved during which any plate in the system, regardless of number, will experience consistent timings. When a suitable schedule is developed allowing maximal batch sizes, the schedule is saved to a process database. The master schedule is adhered to for processing any number of plates up to the batch size limit.

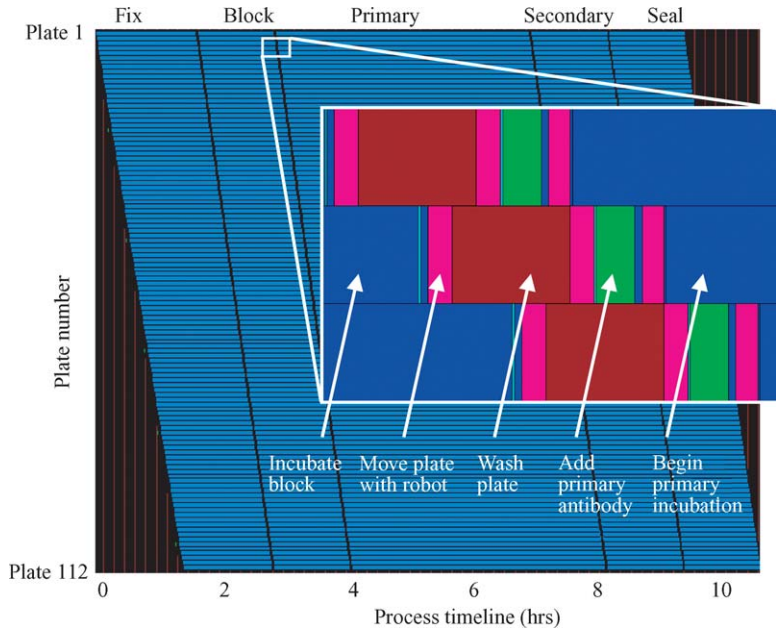

FIG. 3. Example of a static schedule for fixing and staining cell plates. Depicted is a schedule for processing plates on the system in Fig. 2 for the fixation and stain protocol given in the text. The schedule is compiled for 112 assay plates and runs over approximately 10.5 h. Each line on the *y* axis is a graphic representation of the instrument tasks required to process a single plate; the tasks are color coded by device. For example, all incubations utilizing the random access carousel are blue, robot moves are pink, plate washes are red, and pipetting steps are green. The entire schedule contains 7392 individual tasks or 66 for each plate. (Inset) A blowup of the processing steps for removal of blocking solution, washing, adding primary antibody, and starting incubation with primary antibody. In brief, the plate is removed from the carousel by the robot, washed on a plate washer, moved by a robot to the pipetting device, has primary antibody added, and is returned to the carousel by the robot for an extended incubation.

One advantage of a statically scheduled process is that other processes upstream or downstream can be timed to run at a similar rate. For example, if one plate can be fixed every minute then the rate at which compounds are added to plates the previous day should also be at one per minute. In this way if hundreds of plates are processed in the same order, a common compound exposure (e.g., 24-h exposure in our cell cycle assay) is achieved even across a large assay run. As plates are processed on the system, each processing task is recorded to the database, including information about which device instance was used. This process record creates an audit trail for the plates, which is useful during QC and troubleshooting activities.

Image Acquisition

Once cells have been treated with compounds, fixed, and stained, the next step is to image them to acquire data for subsequent analysis (Fig. 1). Imaging should have an appropriate throughput to match both upstream and downstream processing, reliable plate handling, and provide consistent, robust data. How can these requirements be met? Whether making a single biological measurement (such as translocation of a protein) or capturing many metrics per cell (morphology, intensity), imaging a single wavelength, or several channels per field of view, the same general considerations apply: choice of hardware, single vs multiple microscopes, the trade-off between image resolution and field of view, and developing strategies to make data comparable within and between experiments. This section discusses these instrument and imaging choices.

Imaging System Instrumentation

As with the cell processing system described earlier, we chose to custom integrate commercially available instrument components with custom-built control software to build our imaging system. Although the system includes robotics for plate handling and storage, the heart of the system is the automated microscope. Primary considerations for choosing an automated microscope system are speed, flexibility, and field of view size. Speed is important because imaging is typically rate limiting to the entire process flow, and flexibility is important because multiple assays are likely to be implemented on the system. Magnification at the image, or pixel resolution, is determined by the microscope objective, downstream optics, and the pixel dimensions of the detector (often a CCD camera). There is always a trade-off between field of view and resolution of features. Use of the lowest magnification objective that can resolve the subcellular features of interest for a given assay can provide a throughput benefit by enabling the largest number of cells to be imaged simultaneously, thus minimizing the need to acquire multiple fields per well.

There are many microscope choices, from motorized research instruments to specialized screening imagers, but the greater speed of the dedicated machines makes them the best choice, especially if data turnaround times need to be minimized, as often is the case in a drug discovery effort. Screening microscopes (Smith and Eisenstein, 2005) fall into two broad classes, wide-field imagers (including Cellomics ArrayScan, Molecular Devices ImagExpress and Discovery-1, and GE Healthcare IN Cell Analyzer 1000) and confocal imagers, either line scan (GE Healthcare IN Cell Analyzer 3000) or Nipkow disk (BD Pathway; Evotec Opera). Although improved z-axis resolution and contrast favor confocal microscopes in

some cases, their cost and complexity are not required for many immunocytochemistry assays. Wide-field imaging is often capable of capturing images with sufficient contrast for reliable automated image analysis.

We built our current system around three identical Molecular Devices Discovery-1s. Although distributing imaging across three instruments poses a challenge for data normalization, advantages include a tripling of throughput, the ability to swap parts for fault diagnosis, and the capability of running different experiments on different machines. This is particularly useful for imaging high-priority quality control plates such as those used to determine plating density or growth curves, interleaved with assay plates. Furthermore, because the system is modular, we can add additional microscopes easily with minimal effect to the control software.

Regardless of microscope selection, imaging large numbers of plates is generally time-consuming so automated plate handling must be reliable enough for unattended operation around the clock. Figure 4 shows the layout of our current imaging system. Again, as described for the cell processing system, we have favored the submillimeter precision and near-zero failure rates of industrial robots. Here, a CRS CataLyst-5 (Thermo) five-axis robot, which also carries a barcode reader, is mounted on a track and shuttles plates between a plate hotel and the microscopes. Upgrading the stages to a closed-loop design with rotary encoders also greatly improved the reliability of plate positioning during imaging. While each Discovery-1 has its own computer, a fourth master computer controls plate traffic and instructs each microscope on which imaging protocol to run for each plate. Unlike the static scheduling implemented on the cell processing system, the imaging system runs instead on a dynamic schedule, sending plates to microscopes as they become available. Application of dynamic scheduling is possible because of the end-point nature of fixed cell assays, and it enables on-the-fly addition of plates to the imaging queue.

Imaging Parameter Optimization

Images can be acquired in many ways, varying camera gain, the number of pixels, exposure and magnification to name a few variables. For many assays we find an advantage in acquiring images at low magnification in order to increase sample size. In our cell cycle assays, for example, where we describe the distribution of cells across the phases of the cell cycle, we require a sufficient sample of cells in each phase, including mitosis, which is short lived and therefore rare. Imaging with a $4\times/0.2$ NA objective captures 1000 to 2000 cells per image, sufficient to compute accurate DNA classification models and to accurately measure the mitotic proportion in the population without having to image multiple sites per well. Nevertheless, with a 1380×1024 pixel

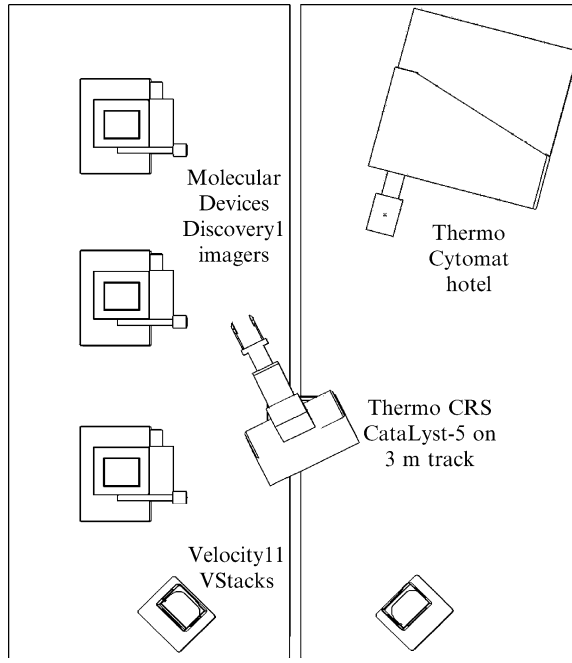

FIG. 4. Schematic of robotic imaging system. A CRS CataLyst-5 track robot system (Thermo) carrying a barcode reader shuttles plates among three Discovery-1 (Molecular Devices) microscopes, input and output stackers VStacks (Velocity11), and a plate hotel Cytomat (Thermo). Microscope controllers, powers supplies, and computers sit in the bays below each instrument, with the xenon lamps beneath the table connected to the imagers by liquid light guides. Control software maintains an inventory of plates in the hotel, interacts with the database to set imaging parameters, and feeds plates to the next available imager.

camera, CoolSnap HQ (Roper), pixel resolution is 1.6 μm/pixel (in x and y), which is sufficient to resolve subcellular morphology.

A rarely discussed issue in image-based screening is exposure setting. Although it is obviously important when comparing data from multiple microscopes, developing an exposure setting strategy is helpful even with a single imager in order to compare data across experiments. While perhaps less important when the goal of the assay is to measure the presence or absence of a signal, it becomes more significant when signal intensity contains information such as DNA content for cell-cycle analysis, or in a phenotype assay where many metrics, including intensity distributions of markers, are measured in each cell (Adams *et al.*, 2006). The goal of the exposure setting is to compensate for sources of variation not related to the biology of interest, including variability of staining reagents lots, fluidics

processing, and time-dependent changes in microscope lamps and filters. Several approaches are possible, but all require imaging a control sample, which often consists of stained, untreated cells. The simplest approach is to use the pixel intensity histogram and assume that some statistic is a reliable index of upstream processing variation. For example, the mode of the distribution could be assumed to represent the background signal, or the 99th percentile of the distribution to represent the cell pixels. While this approach is the simplest, it is indirect. A more robust method is to measure the features of interest directly. We have therefore developed an exposure setting method that identifies the cells in the image and measures their brightness using the same image analysis methods used to compute assay results. At the beginning of an imaging run, we acquire 72 test images, three exposures at each of four wavelengths in six replicate wells. After analysis, including background flattening and object segmentation, object intensity is plotted against exposure and a linear regression is computed for each wavelength (or marker). Finally, the exposure required to achieve a given target intensity is calculated for each wavelength (Fig. 5A). Target intensities are determined during assay development by examining cell intensities and contrast (Fig. 5B) in positive and negative control conditions to choose the lowest target intensity where sufficient contrast is achieved in a negative control, and few pixels are saturated in the positive control. In this way exposures are as short as possible, which speeds throughput, but images remain in the linear region of the dynamic range of the camera. Exposures can be computed this way for each plate, although for many assays, plate-to-plate cell feature intensity variations are sufficiently small if exposures are determined from the first plate only, even with multiple cell lines included in the batch.

Quality control metrics monitored during imaging are principally exposure lengths and differences between microscopes. Extended exposures typically indicate the need for replacement of the main wear items, such as the lamps and, more rarely, excitation filters and light guides. Xenon arc lamps gradually decay in brightness until exposures become impractically long, typically at around 1000 h of use.

Imaging Speed Optimization

Typically, the microscope spends a considerable amount of time focusing, so minimizing the focus range is important for throughput. However, the focus range must be broad enough to compensate for plate curvature, stage leveling, and variation in plate positioning by the robot. Additional time can also be saved if refocusing is not required between wavelengths. At 4× magnification on our system, focusing is only performed on the first

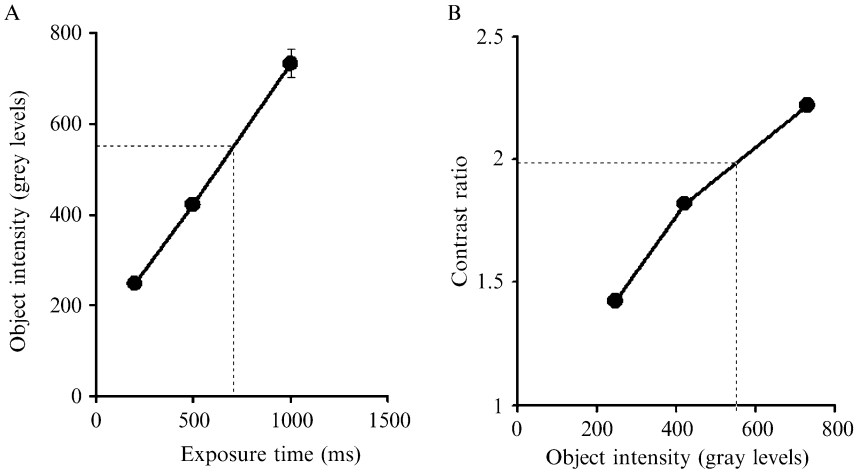

FIG. 5. Exposure calculation for Hoechst-stained nuclei. Six wells were each imaged at three exposures and nuclei were segmented using a custom algorithm. After correction for uneven illumination, object (nuclei) and background (mode of the pixel intensity histogram) intensities were computed for each image. (A) Average object intensity for the three test exposures. An exposure of 708 ms, computed by linear regression, was required to achieve a target object intensity of 550 (dotted lines). (B) Average contrast (object intensity divided by the background) is approximately linear with object intensity. During assay development, a target object gray level of 550 (of 4095) was chosen as providing sufficient contrast without saturation under most assay conditions. A similar computation is used for each fluorescence channel. Error bars show standard deviation, but are only visible if larger than the plotted symbol.

of four wavelengths. If focus is not identical for all channels, sometimes a subset of the wavelengths can still be imaged at the same focus or, alternatively, a fixed z offset defined between wavelengths. Furthermore, only a small focus range usually needs to be explored within a field of view so microscope software that can fine-focus within a well but have a coarser focus range between wells will realize a benefit in speed. With these parameters optimized, our imaging system typically averages one 384-well plate imaged at four wavelengths every 20 min (60 min per plate per imager), with a batch size of up to 120 plates. The system routinely runs overnight and almost never mishandles a plate.

Image and Data Analysis

With a robust infrastructure for assay processing and image acquisition, it is important to have an equally robust and flexible infrastructure for image and data analysis. A productive high-throughput image analysis

system for a drug discovery environment ideally has several important features. It should be flexible enough to adapt to a variety of assays and assay formats, which are rapidly emerging and changing, it should produce consistent and reliable results, despite inevitable variations in image inputs, and it should be built to have a scalable throughput. The large scale of experiments makes user intervention to adjust processing parameters difficult, if not impossible, thus analyses should be robust and capable of automatically adjusting to these variations. Finally, and most importantly, the analysis system should produce measurements that adequately represent the complex biological phenomena of interest.

We designed our analysis system based on several general principles:

- Modularity of analyses
- Multiple levels of analyses
- Importance of detailed analysis of individual cells and characterization of cell subpopulations
- Rigorous quality control of analysis results
- Robustness of analysis results vis-à-vis variations in cell populations, cell processing, and image acquisition.

This section focuses on the application of these principles to building a robust and flexible image and data analysis infrastructure, as well as describing the analysis software components that underlie the capabilities of the system.

Analysis Modularity

For our purposes, a modular analysis system implies that analyses are implemented as a loosely coupled library of image and data analysis algorithms. Multiple algorithms are implemented for image segmentation, feature extraction, and aggregation of results. Individual algorithms can be assembled into a variety of ''programs'' for use in a specific assay or experiment. Most of the algorithms can be tuned to better adapt to specifics of a particular assay, and an algorithm with an established set of tuning parameters can be reused in different programs. Analysis programs can also be adjusted and expanded by the addition of new algorithms and by adjusting parameters of existing algorithms. Outputs of each algorithm are stored in a database and can be used for subsequent analyses at a later time without the need to rerun the entire analysis program.

Basic Feature Extraction/Cell-Level Analysis

Data processing includes analyses at multiple levels of data aggregation. Typically, the first stage of an analysis pipeline is aimed at image

preparation for subsequent quantitative analyses. This stage might include correction of uneven field illumination, image smoothing, noise reduction, and similar types of image preprocessing. This image preparation step is highly dependent on specific types of imaging equipment and assay conditions. In some cases, if very high-quality images are acquired consistently, this step may be omitted.

The next stage of analysis is to identify individual cells and subcellular compartments. Our library of segmentation algorithms includes edge detection-based segmentation and a modified version of a watershed algorithm that is used for segmentation of low-contrast objects. These algorithms are used to identify cells and subcellular compartments, such as cell nuclei and peripheral areas of the cytoplasm. Results of the segmentation analysis (''segmentation masks''), as well as relationships between objects (''belongs to,'' ''is neighbor of,'' etc.), are stored in a database and are available for use by subsequent feature extraction algorithms and for review by investigators.

Feature extraction algorithms are applied to segmentation masks (possibly, in conjunction with preprocessed images) to produce a number of shape, intensity, and texture features. A broad review of methods and techniques for image preparation and analysis has been presented in Russ (2002) and Soille (1999). An important characteristic of our system is that segmentation algorithms are uncoupled from feature extraction algorithms so that intensity and texture-related features for multiple markers can be extracted for each of the segmented cellular components.

Cell Classification/Well-Level Population Analysis

The analysis steps described so far are similar to those used in a number of image analysis systems used in high-throughput and high-content screening: individual objects are identified in images and multiple measurements of these objects are collected. A typical subsequent step in analysis is to generate aggregated metrics for an image or a well, for example, average and standard deviation values for each of the features. Our experience shows that there is rich biological information reflected in changes of individual subpopulations of cells and objects rather than global changes of cell populations. This information is rarely fully exploited. We utilize this information by classifying objects identified in images into groups of interest. To that end, our system contains a number of binary and multiclass classifiers that use individual object features and their combinations as inputs. Most of the classifiers are implemented as two algorithms: one for model building and one for classifying objects based on a model. Classification models are built automatically for each assay plate using information from positive and negative controls on the plate. This approach allows

us to design classification algorithms that can adjust to and compensate for inevitable plate-to-plate variations in image properties due to variations in cell staining and imaging.

Examples of implemented classifiers for our automated cell cycle assay include:

- Identification of dust particles and segmentation artifacts (based on size and intensity)
- Identification of cell debris (based on DNA content)
- DNA content classifier (based on DNA stain)
- Interphase/mitotic classifiers (based on cell cycle marker staining, or shape and DNA content)
- Identification of live and dead cells
- Identification of out-of-focus cells
- Identification of cells with multiple projections

Most of these classifiers utilize just two algorithms: Expectation Maximization algorithms with mixture model (Dempster *et al*., 1977; McLachlan and Krishnan, 1997) and Cytokinetics' proprietary heuristic thresholding algorithm.

Identification of multiple subclasses of cells allows aggregation of object features to be performed for specific subpopulations of cells. For example, DNA content classification allows us to compute the average size of nuclei at each of the stages of the cell cycle. Such ''group-specific'' aggregation is performed for all subclasses and all features of objects. Classification results produced by each of these algorithms can be used not only on their own, but in any combination. For example, after completion of a DNA content classifier, an interphase/mitotic classifier (see Fig. 6), and a classifier that identifies cells with long projections, a user can then automatically define derivative classes, such as ''interphase cells without projections and with 4*N* DNA content,'' for further analysis.

Multiwell and Plate-Level Analysis

Our analysis system includes a number of algorithms that can integrate data across multiple sets of images or wells on a plate. Different groupings of wells are defined as a part of a plate format definition. This feature allows for a flexible use of plate controls. For example, algorithms that generate models for object classification require a large number of objects of each class as a training set. In some cases the required size of a training set can be achieved by pooling object data from multiple control wells. Plate-level analysis is also used extensively for quality control purposes. QC algorithms pool data from control wells on each plate and compute

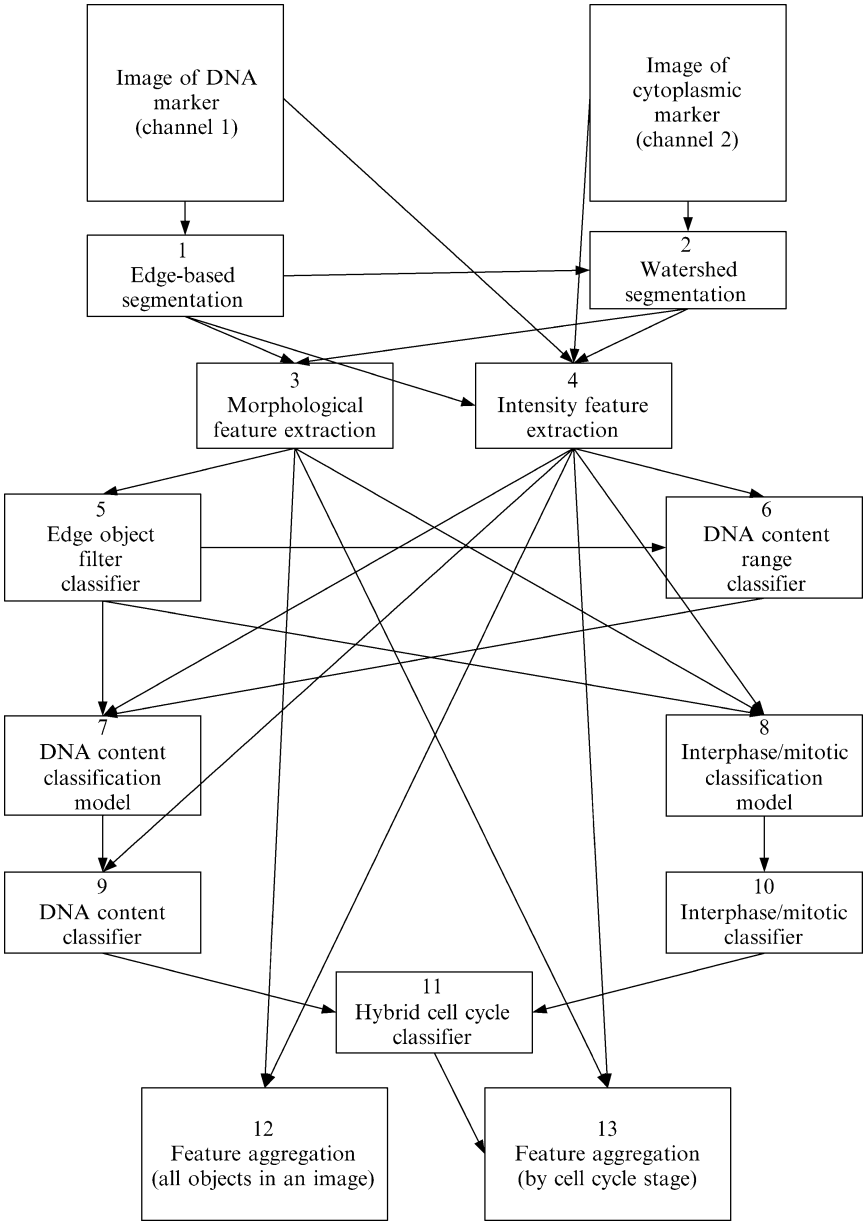

FIG. 6. Program for analysis of two-channel assays with basic feature extraction, artifact removal, cell cycle classification, and feature aggregation. Each box represents an individual algorithm. Arrows show data dependencies for algorithms. See text for details.

statistics, such as average and trimmed average attribute values for each control type and variation in values in control wells.

Experiment-Level Analysis

The next level of analyses are at the experiment level and include many different data aggregation and summarization methods—from aggregation of replicate data to advanced data mining. Typical processing in a high-throughput setting includes automatic aggregation of data for replicate wells, which are run on different plates, and, when appropriate, characterization of dose–response relationships for test compounds. An example of a more elaborate high-content compound profiling application of experiment-level analyses using our system is presented in Adams *et al*. (2006).

Analysis Program Example

Figure 6 shows an example of a simple analysis program for a two-marker assay where cell nuclei and cytoplasm are identified by distinct fluorophores. Each box in the diagram represents an individual algorithm, and arrows indicate data dependencies for algorithms. Numbers in each box indicate the possible order of the algorithms' execution within the program.

Analysis starts with edge detection-based segmentation applied to nuclei images (1). Results of this segmentation are then used by a watershed segmentation algorithm that identifies cells (2). Resulting object masks are used to produce a number of morphological object features (3) and, in conjunction with images, are used to generate intensity and texture-related features for each object (4). Objects that are too close to or intersect with image edges are identified by an edge object filter (5). Cell debris and segmentation artifacts are identified by a DNA content range classifier (6). Objects detected at steps 5 and 6 are excluded from subsequent analyses. Next, a classification model for discrimination of cells with sub-2*N*, 2*N*, 4*N*, and greater than 4*N* DNA is built based on controls on the plate (7). This model is used for DNA content classification of each cell in every image (9). Similarly, an interphase/mitotic classification model is built based on the morphology and intensity of a DNA marker in control wells (8) and applied to every cell in every image (10). The hybrid cell cycle classifier combines classifications from steps 9 and 10 to characterize each cell as being in G1, S, G2, or M phase of the cell cycle (11). Feature aggregation algorithms (12,13) compute average, median, standard deviation, and quantiles for each of the object features extracted at steps 3 and 4. These statistics are computed for all cells in an image (12), as well as for subpopulations at each stage of the cell cycle (13). Results of this aggregation can be used for higher-level analyses, such as those described in Adams *et al*. (2006).

Analysis Software Components

Our analysis infrastructure was designed to support analysis and integration of a very large number of images and extracted features and classifiers. It is a modular, distributed system, where each component is responsible for a specific task. Figure 7 shows the major software and hardware components of the infrastructure.

Experiment manager: A client application that allows users to perform a number of the following functions.

1. Create/update/query experiments. Plates can be configured and registered in the database and later associated with or removed from an experiment.
2. Create and manage assay protocols that include information about cell lines, sets of markers, staining procedures, incubation times, etc.
3. Monitor analysis progression. Users can restart and abandon analysis tasks, and manage allocation of analysis servers for specific tasks.
4. Create/update/query assay meta-data elements, such as markers, cell lines, and plate types, as well as protocols for plating, staining, imaging, and analyzing data.
5. Define and associate plate formats with assay plates.
6. Create/update/query analysis programs composed of analysis algorithms and their parameters.
7. Export/import selected meta-data items.

Image loader: A service that registers images appearing on a temporary storage file server and moves them into a permanent storage location for subsequent analysis and review.

Analysis servers: Several individual servers dedicated to run analysis algorithms. These are conventional Windows-based computers with specifications common for modern desktop machines. The system is scalable to allow for additional servers to be added for increased analysis throughput. The last analysis step typically starts the warehouse builder, which transfers operational aggregated data to a data warehouse.

S-Plus analysis server/report generator: An analysis server dedicated to experiment-level statistical analysis. This server connects to an Oracle database via Open Database Connectivity protocol. Computation results are stored either in a database or as files in a file system and are available for review and further analysis in Spotfire via a Decision Site server.

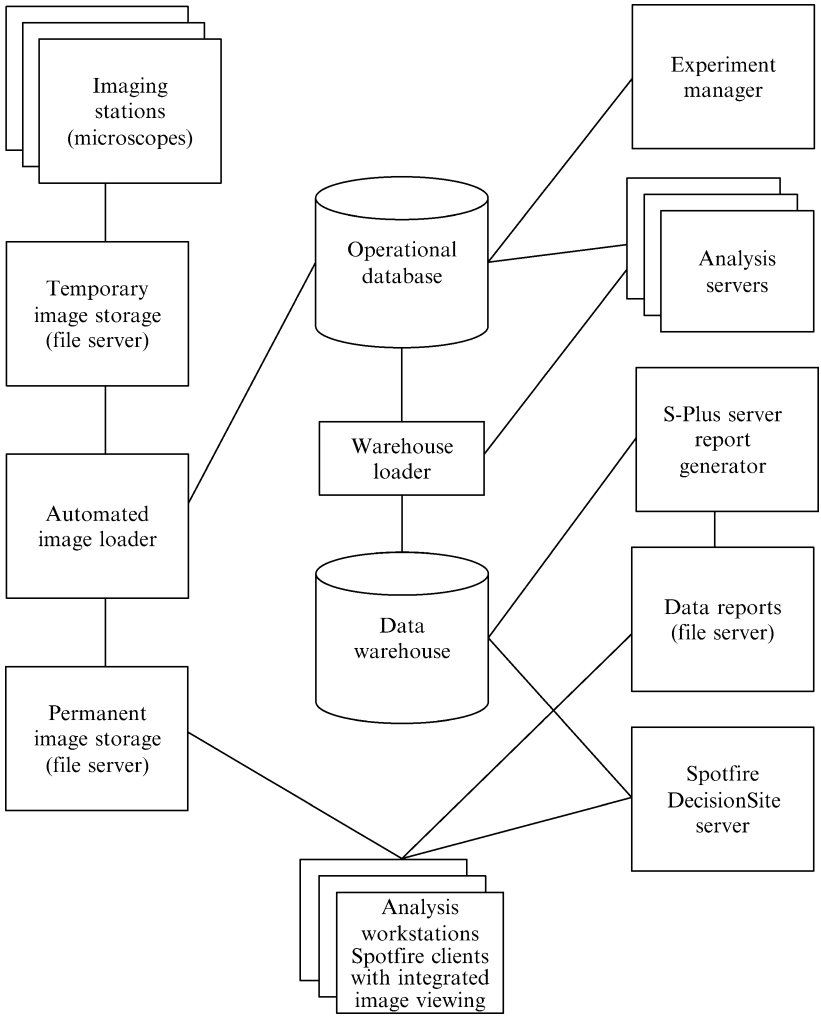

FIG. 7. Image analysis infrastructure. Boxes represent components of the analysis system responsible for image registration, analysis, and presentation of results. Lines indicate network connections between components. See text for a description of each of the components.

Operational database: Stores assay and analysis data (such as cell line and marker information, analysis programs and their parameters, execution status) and intermediate analysis results.

Data warehouse: Stores results of analyses, assay information, and compound information in a format optimized for data retrieval and report generation.

Spotfire DecisionSite server and Spotfire clients: Automated Spotfire (www.spotfire.com) guides provide access to QC metrics and analysis results (see next section).

These individual software components are loosely coupled and interact primarily through the database. The modularity of the system allows us to ''mix and match'' custom and commercial software components.

Analysis algorithms are implemented in Java and C++. Computations are performed in parallel in a distributed system on multiple analysis servers. This distributed and parallel analysis strategy removes a restriction that some commercial systems put on analysis performance, namely requiring image processing speeds to match image acquisition speeds. This allows us to use complex and time-consuming algorithms and ensures that the system is scalable so that any desired throughput can be achieved by simply using an appropriate number of analysis computers. Typically a single analysis server running one analysis session processes images from one assay plate at a time.

Data Review and Quality Control

Automated microscopy systems such as the one described here are capable of generating terabytes of data, and the scalable nature of the infrastructure will enable the creation of even larger data sets in the future. It is important to have in place an equally scalable data review process that allows distillation of complex data sets into meaningful information. There are two types of information to extract: information about the integrity of the data (data QC) and information about the biological activity of interest (results). As we have emphasized throughout this chapter, building high-quality processes is critical, and this section describes an approach to ensuring the integrity of data that will ultimately be used to drive future experiments and decisions.

Key concepts associated with an efficient data review process include keeping the intended use of the results in perspective, understanding factors that can affect the quality of data, defining metrics that report on problems associated with data quality, and assembling tools to aid in the filtering and visualization of the results. The development of a QC process needs to occur in concert with the development of the assay; this will ensure that controls are appropriately positioned throughout the process and that the image and data analysis approach provides metrics to detect

problems. Data QC processes can vary tremendously in their complexity, their stringency, their cost, and their effectiveness. It is therefore important to identify an appropriate level of scrutiny to apply to a particular data set and acceptable failure rates when designing a QC process. For example, in a primary screening application an appropriate QC process may be aimed at minimizing false-positive and false-negative rates while putting much less emphasis on the absolute accuracy of results. In contrast, for assays used to rank compounds as part of a lead-optimization campaign, the absolute accuracy of the results is much more important. Because applications can vary significantly, our discussion here is restricted to a general approach to identifying potential problems, building appropriate QC metrics, and a description of software tools that allow the data review process to be completed efficiently.

Identifying Potential Problems That May Affect Data Integrity

Sources of potential assay problems include the biology (cell health and behavior), cell handling, liquid handling and reagents, plate handling, and imaging. Problems can be inherent to a particular cell line or assay reagent or they can be process based, caused by a particular instrument or process. In addition, no matter how well controlled a process is, the risk of human error will always exist. By generating data and gaining experience with a given assay, knowledge of its vulnerabilities can be determined and used to design strategic control steps to robustly report on problems. Effective use of controls should isolate variables and report potential problems as quickly as possible. In our automated cell cycle assay, for example, we use both negative controls (DMSO-only treatment) and positive controls (paclitaxel) on each plate to flag a variety of problems with cells, reagents, and fluidics. In-process QC steps, such as the cell counts on sentinel plates described in the assay processing section, should be used whenever possible; however, the majority of assay problems will not be detected until postprocessing at the data review and QC stage.

Problems with cell health, such as abnormal morphology and growth performance, can be detected by monitoring negative controls (DMSO-only treatment) that are included on every plate to assess effects of the process on the cells. In our automated cell cycle assay, we assess changes in cell cycle and cell morphology metrics in these controls. For example, we have detected drift in nuclear size and variance, which traced back to a cell source. Abnormal values for these and other metrics, however, may result from other problems, including instrumentation or processing issues. For example, a clogged washer tip can alter image brightness by preventing stain removal. An integrated process tracking database links the results to

instrumentation and process components, making it possible to look for correlations between the problematic results and specific events in the assay process. For example, if an aberrant value is identified in association with a subset of the plates, we can query the database to determine if a specific plate washer unit or a specific imager correlates with the problem. If a problem with a new assay cannot be associated with cell health, reagent formulation, or a process or instrument malfunction, then a problem with an analysis algorithm should be considered. Manual inspection of images and object masks may provide some insight into potential problems, as algorithm failures can stem from errors in object classification.

Not all problems that can occur are easily categorized or anticipated. It is therefore important to complement strategic QC approaches with an unbiased approach to identifying problems. This can be achieved by looking for changes in patterns across a broad set of image analysis metrics. A clear understanding of the relevant metrics and features will then allow for a logical mapping to potential sources of variation.

Identifying and Validating QC Metrics

Once we have defined what could go wrong with an assay we need to determine what image analysis metrics are the most sensitive indicators of specific problems and define their acceptance ranges for data QC. During assay development, this can be determined experimentally by staging problems and assessing whether the chosen metrics flag the problem by crossing a QC threshold. Baseline values and variances should be established for each cell line within the context of the assay. Acceptable levels of variation can be determined by threshold or tolerance testing to establish performance criteria. These metrics can be reviewed and updated periodically by examining historical data.

As an example, to monitor cell health we assess nuclear and cell morphology descriptors, cell number and derived growth rates, and cell cycle distribution metrics. To monitor problems associated with liquid handling we assess background fluorescence levels and metrics that report on the spatial distribution of cell subpopulations within each image. For monitoring staining and image quality we assess contrast and intensity, as well as saturation levels associated with each imaging channel.

Software Tools

As with other aspects of our infrastructure the software applications used for our data review and QC processes are a hybrid of commercially available components and custom-built tools. We have chosen to build our data review process around Spotfire DecisionSite (Fig. 8). Customized

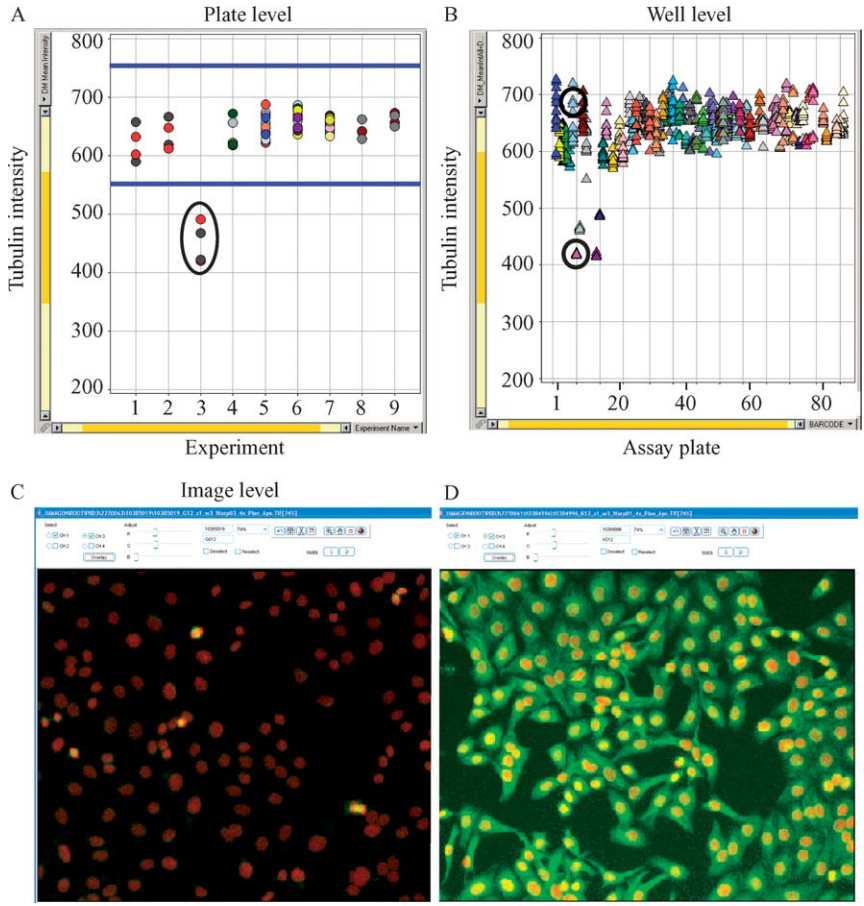

FIG. 8. Spotfire analysis of immunofluorescence data quality at multiple levels. (A) Experiment-level analysis of antitubulin intensity (gray level; range 0–4095). Points plot the mean cell intensity for each plate, computed from 14 DMSO-treated control wells per plate. Points are colored by barcode, grouped in columns by experiment date, and shown in relation to predetermined QC limits (blue lines). Circled points show plates in an outlier experiment where antitubulin staining was dim. (B) Well-level analysis of antitubulin intensity. Symbols show the mean intensity for all cells in one well. Points are colored and also grouped in columns by plate bar code. The custom-built image visualization tool enables the interactive selection of points (circled) and the display of images associated with those wells (C and D). (C and D) Two images, displayed at the same contrast, reveal noticeably different antitubulin intensity, confirming the difference seen in B. Images associated with lower (C) and upper (D) points circled in B, respectively. Only a subregion of each image is displayed. The nuclear channel (Hoechst, in red), is overlaid with the antitubulin stain channel (green). Plots show screenshots from an interactive Spotfire session; similar analyses can be performed for other channels and metrics.

Spotfire guides (automated workflows) have been created specifically for the data QC process. For example, we have generated an assay plate QC guide that loads plate-level summarization data, creates multiple visualizations for a number of metrics that report on marker intensity, cell number, image contrast, and so on, and allows querying on a preselected list of variables. Thresholds for each metric have been built into the graphical presentations to allow easy identification of outlier data. These data can be flagged automatically and marked for follow-up review. The querying tools aid troubleshooting efforts by establishing potential correlations to various experimental variables. For example, if a pattern of outlier values for multiple plates was detected, it would be possible to query on the microscope used to generate the images to determine if a particular instrument correlated with the problem.

We have also integrated custom tools with the Spotfire DecisionSite application. For example, we have integrated a custom image visualization tool that retrieves images and displays them within the Spotfire data review session. Individual wells or entire plates of images can be rejected at any point in the process before experiment-level results are finalized. In some cases, automated data filters eliminate poor-quality data, such as rejection of wells with dust particles or large cell clumps. In other cases, data are rejected as part of the semimanual data QC process using Spotfire and our custom-built image viewer.

The image analysis process reduces data at several levels, as described in detail in the previous section. Ideally, data should be reviewed at the highest level that allows direct access to the source of the problem. With increasing levels of summarization the granularity of data decreases. Figure 8 illustrates a typical set of data views encountered when progressing from plate-level review to image review. In this example we used the assay plate QC guide to assess immunofluorescence quality for a tubulin marker. As illustrated by the example described in Fig. 8, we start our review process at the plate level and work our way down to the image level leading to a triaged list of images for manual review. We typically only review data at the individual cell level if a problem cannot be resolved at any other level.

Summary

This chapter has outlined many of the issues encountered in building an infrastructure to support high-throughput microscopy. Our guiding strategy has been integration so that all steps in cell-based assays are linked by both informatics and process, including compound and cell handling, image acquisition, image analysis, and data storage and reporting. The ability to query all steps in the process at multiple levels of data analysis has made it

possible to build powerful quality control tools and to generate robust assay data. Integration also has been one of the keys to building a highly efficient system, capable of running large numbers of assays weekly. Our preference for modular and scalable hardware and software will ensure that our infrastructure will adapt and scale to our future needs.

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