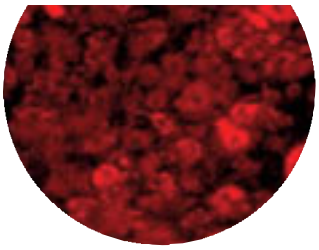
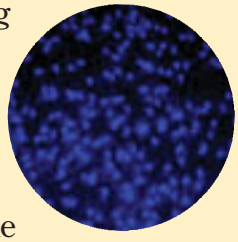


Physiomics and drug discovery

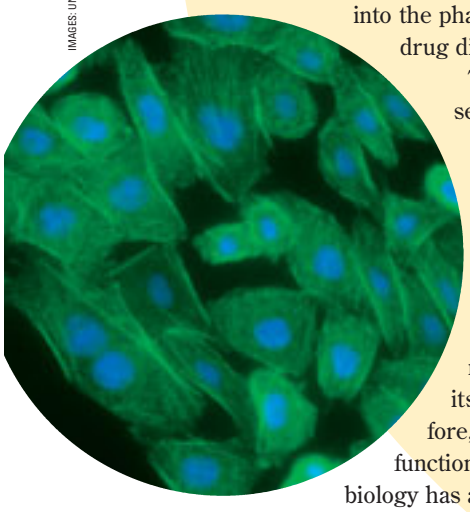
Using automated fluorescence microscopy, researchers are performing high-content cell-based screening assays.

BY JEFF STUCKEY AND ROBERT THOMAS



Unraveling the human genome will have a dramatic effect on the way drug companies discover and develop new drugs. It is also clear that studying the products of gene expression alone will not produce all the answers, because they do not indicate changes in protein expression, which are essential to a full understanding of disease mechanisms. For this reason, it is now accepted that both genomic and proteomic information will become critical to completely define the best targets for pharmaceutical research (*1, 2*).

As we move into the postgenomic period, the relationship between genomics and proteomics will become apparent and exploited. However, the enormous challenge still remains of interconnecting these molecular events in living cells to provide insight into the pharmacological pathways that drug discovery programs require.



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There is no question that to see the complete picture, it is critical to gain a perspective on drug discovery that is cell-based in addition to being focused on isolated genes or proteins. It is the information held in the cell that defines the temporal and spatial interactions of its components and, therefore, its normal and abnormal functions. This concept of systems biology has a growing following and has led to research areas that target the whole cell (cellomics) and physiological pathways (physiomics). Research in these new fields has led, in turn, to a dramatic increase in the use of cell-based assays as screening tools in both the research and drug discovery arenas. The knowledge base of the cellome or physiome will be built by connecting layers of these interactions into the pathways and networks that govern cellular life. Just as automated gene and protein analysis and their database tools are pushing the genomics and proteomics frontiers, automated cell analysis using rapid screening techniques and bioinformatics is going to be absolutely essential to understand cellular functions at the molecular level.

Cell-based assays

Ultimately, any potential drug therapy must be effective at the cell, tissue, and human level. During the process of lead compound evaluation, choices must be made as to which structural changes are beneficial, so that further modifications can be made. The use of cell-based assays is an important source of information driving this process.

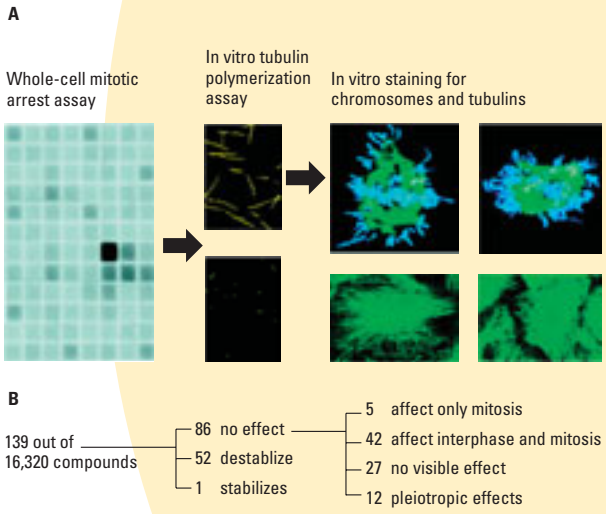
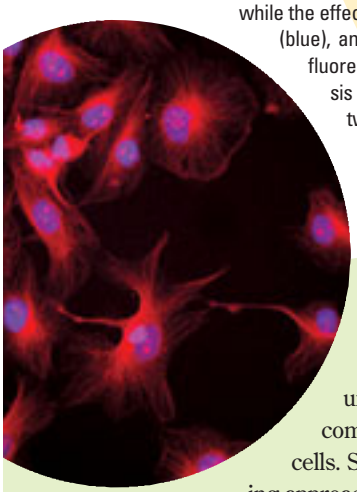


Figure 1. Five compounds that affect mitosis. (A) In a whole-cell immunodetection assay, 139 compounds that increased in phosphonucleolin staining were identified. Of these, 53 targeted pure tubulin and were eliminated, while the effects of the other 86 on microtubule (green), chromatin (blue), and actin (not shown) distribution were tested using fluorescence microscopy. Five compounds affected mitosis alone, and the four images (right) show the effects of two compounds on mitotic (upper) and interphase cells. (B) The screening results. (Reproduced with permission from Reference 6.)



Cell functions comprise many interconnecting signaling and feedback pathways. Screening compounds against isolated targets or in vitro preparations is not ideally suited for this process. A better understanding of the effectiveness of certain compounds requires the testing of whole living cells. Some of the many advantages of this screening approach include the following:

- compound usefulness is often best predicted by measuring biological behavior in intact cells,
- molecular interactions can be evaluated within the context of the cellular environment,
- toxicity and nonspecific effects can be identified,
- cell-type-specific drug effects can be distinguished,
- drug penetration can be evaluated, and
- the approach meets the requirements of some specific targets that need cell-based functional assays.

Many cell-based assays have found a niche in evaluating lead compounds and predictive toxicology to confirm positive results from primary screens. These include viral titer assays; second messenger assays; and cell proliferation assays using isotopic labeling, colorimetry, chemiluminescence, fluorescence, or spectroscopy methods.

Fluorescence detection

Of all these approaches, fluorescence detection, where a target biomolecule is tagged with a fluorescent label and made to fluoresce with wavelength-specific light, is probably the most versatile of the cell-based labeling techniques (*3*). Its ability to target individual molecules, structures, or cell types and measure the resulting fluorescent signal offers many benefits, including

- high sensitivity (transient events inside a living cell can be detected very rapidly and from a single fluorescent molecule);
- minimal interference with normal cell functions, because probes are used in such low concentrations;
- specific and customized labeling, which reduces nonanalyte background levels, improving signal-to-noise;
- specificity, which facilitates the simultaneous use of many different fluorescent labels (multiplexing);
- well-characterized results under a variety of conditions;
- superior dynamic range and linearity over other labeling methods; and
- an abundance of commercial dyes, labels, and probes.

For these reasons, fluorescence labeling and measurement of biomolecules have become the technique of choice for the vast majority of biochemical assays and cell-based screening techniques.

Fluorescence microscopy

One screening tool that is getting a great deal of attention today is fluorescence microscopy, which combines the benefits of fluorescence detection with high-powered digital microscopy. Recent advances in imaging technology and live-cell fluorescent probes like green fluorescent protein have allowed cell biologists to quantitatively examine cell structure and function at higher spatial and temporal resolutions than ever before. Thomas Mayer and his colleagues at Harvard University, Harvard Medical School, and Howard Hughes Medical Institute (Cambridge, MA) used fluorescence-based imaging to search for compounds in the study of complex mitotic mechanisms (*4*). They were faced with the problem that most small molecules used to perturb dynamic cellular processes inside a cell predominantly targeted the protein tubulin, a subunit of microtubules found in the mitotic spindle (*5*). However, they were interested in identifying cell-permeable small molecules that targeted other proteins involved in mitosis.

They started with the cytoblot immunoassay (*6*), which is based on increased phosphorylation of the protein nucleolin, to identify 139 compounds from an initial library of 16,320 small molecules (Figure 1). The list was narrowed down to 86 compounds that showed negative results in an in vitro tubulin polymerization assay. Using fluorescence microscopy, the researchers found that only five compounds specifically altered the mitotic spindles with no effect on the microtubules, actin filaments, or chromatin in undivided (interphase) cells.

These compounds were further screened using fluorescence microscopy for the selection of the 1,4-dihydropyrimidine compound monastrol that inhibited a significant fraction of the cells during mitosis. The screen for antimitotic small molecules was recently repeated using fluorescence microscopy as the initial screening assay, in place of the immunoassay. Mayer's group found that the fluorescence microscopy assay picked up all the plate reader hits and a number of real hits that the plate reader missed. Thus, library screening by fluorescence imaging of cells (high-content screening) was significantly more sensitive and reliable

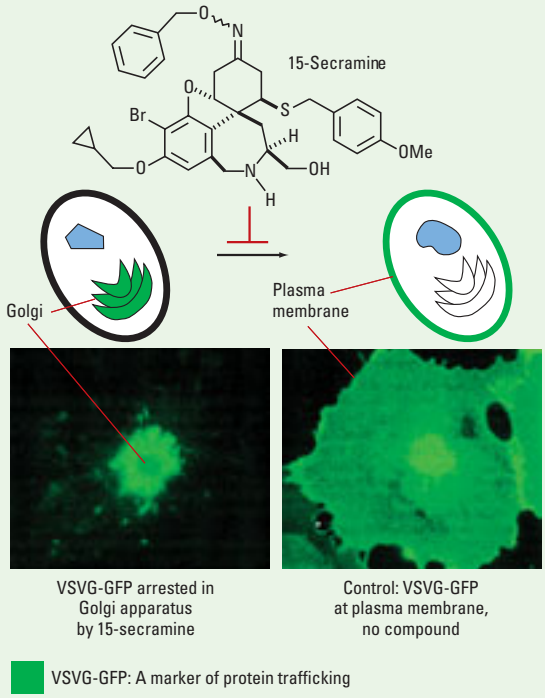
The benefits of collaboration

In their desire to market products that meet the real-world demands of high-content screening applications, commercial instrumentation manufacturers have sought the help of academic institutions and research organizations. For example, a collaboration was formed between Harvard Medical School's Institute of Chemistry and Cell Biology (ICCB, Cambridge, MA) and Universal Imaging Corp. (UIC, Downingtown, PA, www.universal-imaging.com). With input from Tim Mitchison, a Harvard professor and ICCB director, UIC developed a fluorescence-based imaging system for cell-based screening assays—the Discovery-1 Cell-Based Screening System. The instrument is fully automated and optimized for the demands of analyzing cell-based fluorescent assays in microwell plates.

Mitchison and his group at ICCB routinely use the technology to carry out multiple screens. In each case, the cell has been engineered to express a protein whose localization would indicate whether a particular pathway or process was perturbed in the desired way. An expressed protein was tagged with green fluorescent protein so that its localization could be determined by direct fluorescence imaging. Using this approach, small molecules were discovered that block the secretory pathway and prevent activation of an anti-inflammatory pathway, accumulation of a toxic protein, or replication of centrosomes. The figure shows images from the secretion study of a control cell and a cell treated with one of the hits from screening a combinational library (*J. Am. Chem. Soc.* **2001,** *123,* 6740–6741). It should be noted that in the distribution of a GFP-tagged membrane, the small molecule drastically alters the cell. This effect was used to identify the small molecule in the high-content screen.

It would have been possible to perform a small molecule screen with a more conventional assay system employing a plate reader. However, the high-content readout from cell imaging provided much more information during the primary screen, speeding up subsequent characterization of the hits. In the example shown in the figure, it was possible to identify toxic small molecules and to distinguish compounds that blocked the pathway at different steps from the primary screening data. In the case of protein accumulation and centrosome replication, it would not have been possible to design a conventional plate reader assay. Fluorescence imaging of cells was the only way to find small molecules with the desired effects. In each of these studies, the Discovery-1 system was used not only for the primary screen of many thousands of small molecules but also in follow-up dose–response and structure–activity experiments.

Another important consideration in high-content screening is the flexibility of the software tools for converting fluorescent images of cells into numbers that reflect the strength of a desired effect or undesired toxic side effect. For example, in the anti-inflammatory screen, the desired effect was to prevent a transcription factor from entering the nucleus, while the undesired effect was rounding up of the cell due to toxicity. These effects could be evaluated by visual inspection of the fluorescence image. However, for comparing the strength of different hits, and for dose–response studies, quantitative information was absolutely essential. The Discovery-1 system contains customizable image analysis routines for common types of measurement such as counting particles or measuring intensity. More importantly, it allows the users to build their own measurement methods by combining routines from a complete library of analysis and processing algorithms that have been developed by UIC. This flexibility in analysis software is key to accessing the full power of high-content screening, because it allows the users to convert information they see with their eyes into data that report the effect of the small molecules in the screen.

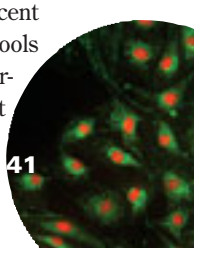


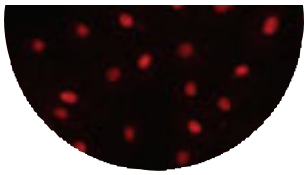
Studying secretion. Images from a control cell (right) and a cell treated with a screening hit (left). (Reproduced from *J. Am. Chem. Soc.* **2001,** *123,* 6740–6741.)

than the conventional technology.

High-content screening

As a result of the success of fluorescent microscopy, screening tools based on high-resolution imaging of multiple targets within a cell are now commercially available. These have become known as high-content screening systems because of their ability to present information on multiple spatial and temporal events in cells. Their design has been drawn from expertise in the fields of fluorescent microscopy and cell biology to create a new set of screening tools aimed at the bottlenecks found in the drug discovery and pharmacological research environments. They offer significant





advantages over fluorescent plate readers used in conventional cell-based assays because they measure biological variability of individual cells within a well as opposed to a single intensity per well.

A typical high-content screening system, which consists of imaging instrumentation (microscope), fluorescent reagents and probes, and software, can be fully automated using multiwell and robotic sampling systems. This approach can offer better efficiency in the validation of cellular targets, higher capacity for predictive toxicology, and more effective lead optimization compared to manual imaging methods. This makes it an ideal tool for the pharmacological research community and pharmaceutical companies because it decreases cycle times and increases the probability of clinical success. Some of the fields being studied using this technology include

- cell viability and counting,
- protein expression and targeting,
- probe localization,
- ratio analysis for physiological probes such as Ca^{2+} and pH,
- wound healing,
- study of metabolic activity during apoptosis,
- transcription factor activation,
- blood vessel and tissue formation and growth, and
- cell invasion/migration and neurite outgrowth.

There is no doubt that any potential new drug therapy must be effective at the cellular level. For this reason, the use of accurate, sensitive, and specific cell-based assays will become a critical tool to reach this goal. Combining fluorescence labeling with automated imaging for high-content cell-based screening has the potential to dramatically change the way pharmacological research and drug discovery programs are performed. By working very closely with instrument manufacturers, well-established experts in this field are helping to develop commercial equipment to guarantee that the right tools are available to do this work. There is no question that collaborations like these will take us to the next level in understanding the complex molecular mechanisms inside the human cell.

Acknowledgment

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