Bringing life to center stage

Microscopy and molecular tagging are allowing researchers to test the effects of new drugs on live specimens.

BY RANDALL C. WILLIS

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Contrary to the way it appears in many high-throughput screening laboratories, life does not occur in a vacuum, and drug developers must always be cautious about data that result from assays based on idealized two- or three-molecule interactions.

"With all this excitement with the genome project, all this proteome work going on right now, the importance of cell biology is really going to come to the forefront," said Kenneth Dunn, director of biological microscopy at the Indiana University School of Medicine, in a recent interview with *bio.com*. "People really need to understand the temporal and spatial regulation of where proteins are in cells and tissues and the body, when they're turned on, what proteins they're interacting with; and there's really not a better way of getting at that than with fluorescence microscopy."

Focus on fluorescence

Fluorescence microscopy has been applied to a variety of experimental systems and is commonly used by clinicians and scientists to study everything from chromosomal abnormalities to ion channel activity. In this technique, researchers use specific wavelengths of visible light to excite a fluorescently labeled molecule or fluorophore. The fluorophore then emits light at a longer wavelength, and this light is separated from reflected incident light by a beamsplitter before passing through an eyepiece to a camera. Images that result from fluorescence microscopy can be blurry, however, because of the diffuse nature of the fluorescent signal, which can severely compromise experiments that look for subtle changes in cell localization.

To address this problem, many researchers use laser scanning confocal microscopy (LSCM), which relies on the same fundamental principles as fluorescence microscopy. In LSCM, however, the fluorophore light is further defined by passing it through a small aperture, which removes extraneous light from outside the sample's focal plane. The resulting image is much sharper, and by digitally combining images from several planes, researchers can construct a 3D image of the sample.

But even with these advanced microscopy techniques, scientists might only be looking at a single moment in time, missing the dynamic aspects of drug–cell interactions.

"The major disadvantage to working with fixed specimens is that you get only a snapshot of what is really happening, without any cause-and-effect information, and you can miss a great deal," says Phil Vanek, director of strategic marketing at Atto Bioscience, Inc. "Also, you have really limited access to information about how fast things are happening, and many biological processes may not be dictated by whether they occur, but at what rate."

"In a biochemical assay, you lyse or otherwise fix the cells at a given time point," Vanek continues. "You are then averaging all your cellular measurements over the population of cells. This situation gets to the question of a drug screen showing a 10% calcium response at an IC_{50} dose of compound: Does this mean that you are seeing 10% of the cells responding at 100% or that 100% of the cells are responding at 10%? A big difference. On the other hand, by working with living cells with a system like the Pathway HT, which can do both kinetic and end-point measurements, you can

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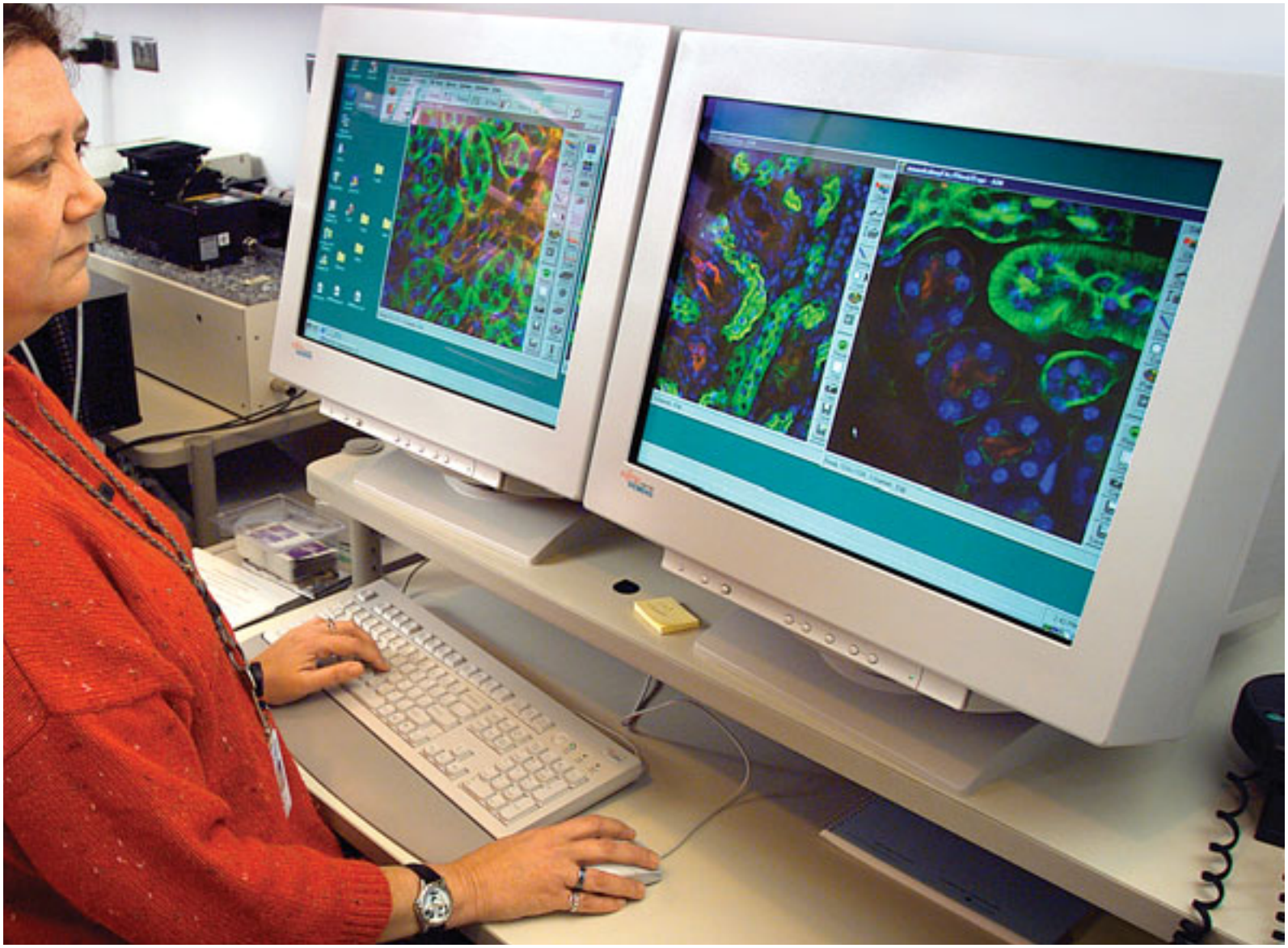

explore those biologically relevant time points and develop better end-point assays."

For this reason, the microscopy and drug discovery communities have collaborated to develop new techniques that allow scientists to examine live specimens in a fourth dimension: time. For example, researchers at Johnson & Johnson Pharmaceutical Research & Development used the fluorescent dye Nile red and the Pathway HT to monitor the cytotoxic effects of various drugs on hepatic cells. They found that the microscopic method—unlike standard plate readers—allowed them to detect dye uptake rapidly and at lower drug concentrations, and to distinguish between living and dead cells.

Vanek's comments were recently echoed by Daniel Gerlich and Jan Ellenberg of the European Molecular Biology Laboratory. "Four-dimensional imaging gives us access to new worlds of dynamic function in live cells," they said in a special supplement to *Nature Cell Biology*. "By taking both space and time into account, processes that involve, for example, changes in structure, compartmentalization, fluxes, directed transport, and signal-mediated localization can be studied quantitatively in real time."

Multifaceted multiphoton

A challenge of LSCM, however, is that the incident laser light can be too intense for living samples to withstand the long and frequent exposures required to perform these experiments. To address this problem, many researchers have come to rely on a modified version of LSCM called multiphoton microscopy which uses two or more lower-energy photons.

Scientists have used multiphoton microscopy to study everything from embryo development to angiogenesis and cancer progression. For example, Brian Backsai and colleagues at the Massachusetts General Hospital have used multiphoton methods to analyze Alzheimer's disease (AD) development in mouse models, literally watching through a window on the brain.

"Historically, AD research has depended a lot on fixed samples of AD brain," Backsai says. "It's a huge advantage to be able to see what's happening in a live brain over time. How do plaques form? What or when are the critical steps to prevent formation? What happens to the rest of the brain when the plaques are cleared? These questions can be supported with fixed-tissue analyses, but are much more directly answered in vivo."

One of the challenges of AD, according to Backsai, is that it cannot be diagnosed with certainty in living humans; diagnosis instead relies on postmortem detection of senile plaques. For this reason, researchers and clinicians have been somewhat hamstrung in their efforts to develop ways to test new drugs.

"Mouse models of AD, however, that develop amyloid pathology as they age, have been around since 1995–1996," Backsai says.

"We started soon after to try and image plaques in these living animals and selected multiphoton imaging as the way to go."

After labeling plaques with well-characterized histochemical stains, researchers use multiphoton methods to image deep—up to about 500 µm—into the brain to detect fluorescence with submicrometer resolution. In one study, Backsai and his colleagues were able to follow plaques in living animals for as long as six months. By directly imaging the plaques, researchers can use just a few mice—which are expensive—to test a drug, as opposed to using large cohorts, Backsai says.

"Once we established a detection platform, it was a natural progression to test therapeutics aimed at making the plaques go away," Backsai says. "We could do this by imaging individual, established plaques in old mice and watching how they changed or disappeared with treatment."

"Most recently, we helped characterize a novel and exciting PET ligand for diagnostic imaging in humans, that happens to be fluorescent," he explains, in reference to his work on the contrast agent PIB. "We showed in the mice that the compound PIB does exactly what it was designed to do."

Backsai and his colleagues have trained several pharmaceutical firms in their multiphoton techniques so that they could use them as an assay to test new drugs. Similarly, they've also trained other academic labs and are collaborating with some to evaluate new therapeutic approaches. Multiphoton microscopy is being used for a variety of purposes in other labs, according to Backsai, but he is also aware of its limitations in drug discovery endeavors.

"I honestly don't see multiphoton microscopy playing a huge role in drug discovery, because it's quite time-consuming compared with multiwell plate assays and other in vitro screens," he admits. "Ultimately, in vivo characterization may be necessary and will depend on an imaging technique like multiphoton, but except for AD, where the end points and biomarkers are scarce, I don't see it being a lead assay system."

Beyond cells

For all of their benefits, high-content screening systems that use live cells to characterize compound responses still suffer from a significant limitation: A living organism is more than just a collection of cells. Model systems of various human diseases—whether in mice, flies, or worms—offer one solution to this problem, but even here, the effects of potential drugs on mechanisms such as gene expression can remain invisible and must be deduced from secondary information such as animal behavior or limb formation.

To address this problem, several researchers have developed model organisms where genes of interest are hooked up to various fluorescent or luminescent markers (Figure 1). These "glow-in-the-dark" organisms allow investigators to watch the effects of drug treatment on the specimen at the molecular level both in real time and without the need to sacrifice the test subjects.

One company that has specialized in the use of fluorescent protein markers for whole-body imaging is AntiCancer, Inc., which uses the technique to study cancer growth, metastasis, angiogenesis, gene expression, and stem cell function, according to Robert Hoffman,

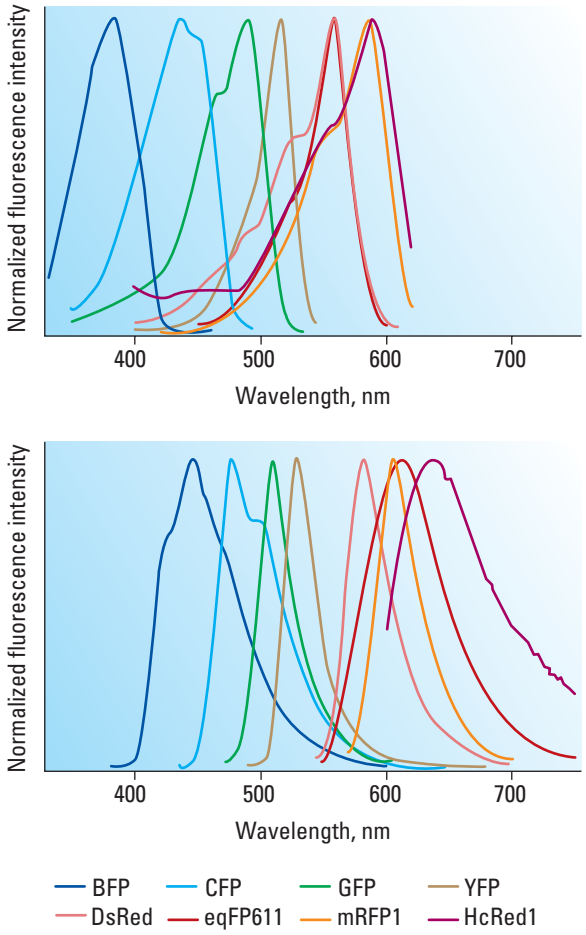

Figure 1. One photon, two photon; green photon, blue photon. The distinct excitation (top) and emission spectra (bottom) of the various fluorescent proteins allow researchers to examine multiple targets simultaneously, in both standard and multiphoton microscopy. (Adapted with permission from Miyawaki, A.; et al. *Nat. Cell Biol.* **2003,** *5,* S1–S7.)

company president. "AntiCancer has carried out contract work in the pharmaceutical development world for almost 20 years," he says. "Its customers now often request the use of the models with the fluorescent marker systems for new-drug testing."

Recently, Hoffman and other researchers from AntiCancer joined with scientists from the University of California at San Diego and the Massachusetts Institute of Technology to develop a dual-color fluorescence imaging system to examine tumor formation in mice. The scientists labeled various transplanted tumor cells with red fluorescent protein (RFP) and host mouse cells with green fluorescent protein (GFP). They then used fluorescence imaging and microscopy to follow interactions of healthy and cancerous cells. In some samples, the investigators noted that the foreign tissue induced the host tissues to form angiogenic blood vessels, whereas in other experiments, they witnessed an immunological attack on the foreign tissue by the host.

Although this investigation was largely a proof-of-principle experiment to determine whether they could visualize the events surrounding angiogenesis, the researchers suggest that "the model can also be used to develop specific therapeutics that attack or support host cells that affect tumor growth and progression."

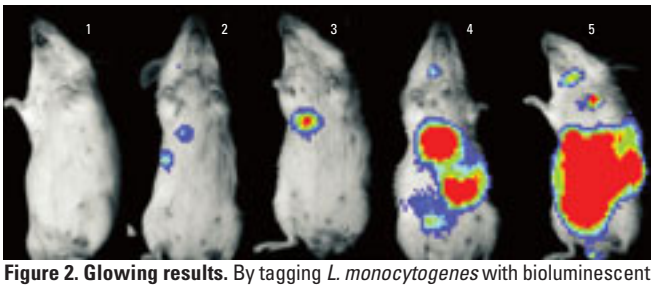

Figure 2. Glowing results. By tagging *L. monocytogenes* with bioluminescent molecules, researchers followed the spread of infection in mice over a five-day period. (Adapted with permission from Hardy, J.; et al. *Science* **2004,** *303,* 851–852.)

Alternatively, rather than rely on genetically modified model organisms, researchers can follow the progression of external compounds or organisms as they race through the body of test subjects. For example, scientists at the Stanford University School of Medicine and Xenogen Corp. recently examined the proliferation of *Listeria monocytogenes* infections in mice (Figure 2) using whole-body imaging.

Although *Listeria* is a significant cause of foodborne illness and has a high mortality rate, little is known about its pathophysiology. By tagging the bacterium with a bioluminescent molecule, the researchers determined that even in asymptomatic subjects, the microbe replicates in the gall bladder, where it might escape the host immune system. The bacterium then passes into the intestine, where it can reinfect the same host or be transmitted to infect others. By identifying specific virulence factors and some components of the infectious pathway, the researchers hope to better understand how to fight these infections.

The next stage

Recent efforts to expand upon early success in the various forms of live-cell and whole-body imaging are coming from multiple directions. Engineers are designing microscopes and cameras to handle an ever-widening array of sample types and sizes, and chemists and biologists are developing ever-more-versatile fluorescent and luminescent compounds to use in assays. Ultimately, however, the success of these new developments will hinge on whether they provide information in the context of life.

"The key requirement of the new systems biology is the ability to understand cellular events in context," says Atto Bioscience's Vanek. "It is no longer good enough to try to explain the workings of an engine after studying spark plugs for five years. New assays are being developed, for example, Norak Transfluor, that can only be explored using imaging techniques. Imaging opens up the opportunity to explore both kinetic and spatial events within cells."

"The Holy Grail of this technology is to build a technology that can image cells over time, in 3D, and monitor a multitude of channels—fluorescence, morphological, physiological—all at the same time," he continues. "Image quality is already there; speed is getting there; and data analysis has a ways to go, but progress is being made rapidly. Data storage and mining with cross-platform sharing are going to be a challenge. Fifteen years ago, however, no one would have thought to sequence the human genome, but that was doable. This is doable, too. The technology just needs to evolve." ■