CELL SIGNALING:
IN VIVO VERITAS

Innovative and increasingly sophisticated technologies are allowing scientists to visualize signaling pathways and cellular interactions with finer detail than ever before. New types of fluorescent tags and novel chemistries, in conjunction with technological improvements in microscopes, are driving research forward at speed. Pausing for a moment, this article looks under the hood at the science behind the technology—from quantum dots to microendoscopy, and beyond.

By Jeffrey M. Perkel

Imagine trying to understand the rules of football by taking a single photograph of a particular game at a specific moment in time, and comparing it with other photographs taken at other times, during different games. Because each game is unique, naturally, you would fail.

Yet according to Jeff Lichtman, professor of molecular and cellular biology at **Harvard University**, this is precisely the strategy most biologists take as they try to understand dynamic cellular events.

Consider cell shape, for instance. "If you see some object in different animals or different cells, the object has different shapes," he says. "That could mean that there are 20 different shapes, or it could mean that every object is constantly morphing. There's no way to know unless you watch the same object over time."

You have to sit in the bleachers for the whole game and study tapes of the play action, if you really want to understand the dynamics, he says.

That's where in vivo imaging comes in. Tools are now available to image cellular events in living animals at levels from cellular to organismal—events that either were inaccessible, or simply did not occur, on fixed slides and tissue culture dishes. By observing cells in their natural environments, in living animals rather than fixed on slides, it becomes possible to watch as neurons extend and retract, signal transduction pathways fire, and tumors invade new tissues—all without having to worry about animal-to-animal variation.

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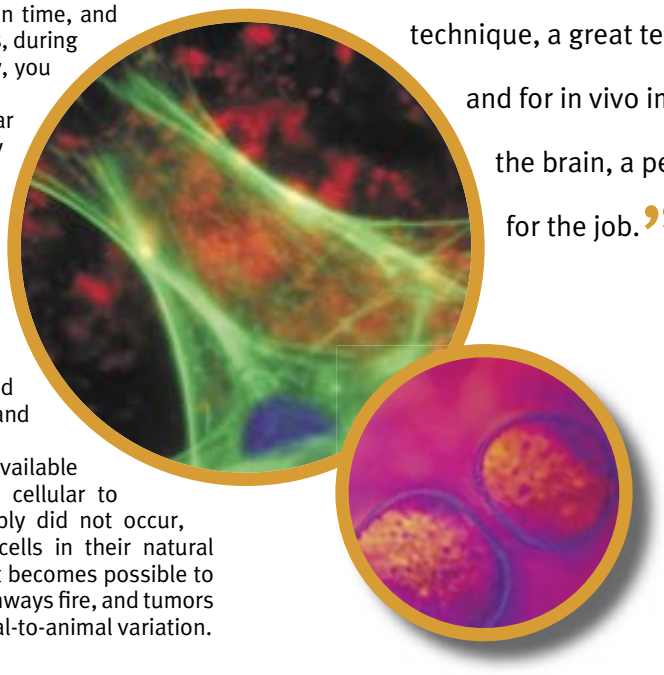

Two Photons Better Than One

Lichtman's lab focuses on how neural connections are established during development. Early in development, individual muscle cells are enervated by many neurons. But over time, most of these nerve fibers retract, until just one remains.

To figure out how this happens, and which is the last neuron standing, Lichtman and his team have effectively taken to camping out in the molecular bleachers and watching as these events unfold.

The two teams in this case are neurons, some yellow, and some blue—the result of mating mice that express either of two fluorescent protein variants. These colored cells are tagged as with team jerseys, Lichtman explains, and he can scan the nerve-muscle interface in the exposed tissue of an anesthetized, immobilized animal until he finds a blue and a yellow axon in close proximity. Then, he says, "We look at the blue and yellow axon on one day, and look the next day, and keep doing that until one of the two inputs disappears."

Lichtman's lab records these processes using both confocal and two-photon imaging. Confocal imaging uses a laser to sweep back and forth across a sample, and a small pinhole to block light emanating from above or below the plane of focus. The result is a sharp image of a single, virtual slice of the specimen. The problem is that as in-focus light exits the sample, it can get deflected by other cells and cell components, and thus not reach the pinhole. Less and less emitted light is visualized as depth increases, so confocal really is effective only for near-surface imaging.

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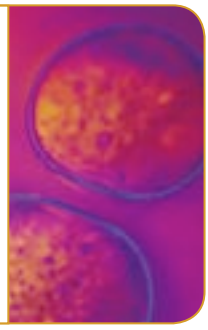
"Being able to go into the deep brain, where no microscope has ever been, period, and to make images with a resolution of even three micrometers is huge progress."



Two-photon microscopy eliminates the pinhole, relying instead on an optical trick to create virtual sections. Consider a fluorophore that normally fluoresces when stimulated by a single 400-nm photon. In two-photon microscopy, photons of light at half the energy and twice the wavelength—that is, at 800 nm—are directed in a cone-shaped beam toward the sample in intense bursts. These photons arrive so closely together that the fluorophore acts as if it has captured a single 400 nm photon, and so fluoresces.

Only fluorophores in the focal plane (at the tip of the cone) will respond in this way, because that is where the density of photons is greatest. Those outside the focal plane, which are unlikely to capture two photons in time, remain silent. "That means you can look through very thick tissue and only see fluorescence from the point of focus," Lichtman says. "It's a remarkable technique, a great technology, and for in vivo imaging of the brain, a perfect tool for the job."

Adding to two-photon's appeal is the system's use of near-infrared (NIR) laser excitation light (700–1,100 nm). Unlike shortwave illumination, NIR light passes through living tissues relatively unimpeded. As a result, the technique can be used to probe deep tissue, up to 600 microns or so. It also is less damaging than shorter wavelength light, and is thus compatible with longitudinal studies.

"There's an awful lot of brain processing on the surface of the cortex," says R. Clay Reid, professor of neurobiology at **Harvard Medical School**, "so there's a lot of in vivo neuroscience that one can do and be limited to 600 microns."

An electrophysiologist by training, Reid says he has "a new lease on life" thanks to two-photon microscopy and cell-permeable calcium indicators like Oregon Green, which he "spritzes" into the surface of the brain to light up neurons as they fire in response to different visual stimuli.

"Electrophysiology is the gold standard for understanding how things work," Reid says, "but electrophysiology is one cell at a time or, with multiple electrodes, a small fraction of a circuit." Now, "We can see and therefore record the activity of literally every single cell, and that's the exciting part. We are never going to understand the brain one cell at a time."

Going Deeper

All the major microscope manufacturers—**Nikon, Leica, Zeiss**, and **Olympus**—address two-photon imaging in one way or another, providing either complete systems or the specialized optics needed to upgrade existing microscopes.

Yet the technique's power is generally restricted to those cells within 600 to 700 microns of the tissue surface at best. "Most of the mouse brain is out of reach of a traditional two-photon microscope," says Mark Schnitzer, a **Stanford University** researcher who has developed techniques using microendoscopic objective lenses as small as 350 microns in diameter to bring both one- and two-photon imaging deeper into the brain.

Also delving deep into tissue is Olympus' IV100 intravital imaging system, which features slim MicroProbe "stick" lenses, 1.3- or 3.5-mm wide and mounted on a tilting scanhead, to enable deep tissue and internal organ laser-scanning imaging, at angles from +10 to +70 degrees, through a small incision.

Mauna Kea Technologies in Paris, France, has commercialized its own deep-body laser-scanning system, the Cellvision-LAB, distributed by Leica Microsystems. Instead of glass lenses, "the objective of the [Cellvizio-LAB] microscope is a bundle of tens of thousands of optical fibers," says Sacha Loiseau, Mauna Kea's president and chief executive officer. About the thickness of three human hairs, this objective can be threaded through the animal to image just about anywhere in the body.

Harvard's Reid, who used the Cellvizio to recapitulate his two-photon work in portions of the brain that otherwise were inaccessible, says the system offers greater depth, but lacks the resolution and versatility of regular two-photon imaging.

According to Loiseau, the resolution of the Cellvizio-LAB is 1.1 microns or so, about a fifth of the 0.2-micron theoretical resolution limit of traditional optical microscopes.

"It's a tradeoff," he says. "Being able to go into the deep brain, where no microscope has ever been, period, and to make images with a resolution of even three micrometers is huge progress."

The Wide-Angle View

Still other companies offer a loftier, whole-animal view.

Whole-animal optical imaging enables researchers to visualize light-emitting cells or processes (such as tumorigenesis) through the skin of living animals. One of the challenges, says William McLaughlin, director of research and development at **Molecular Imaging Systems, Carestream Health** (previously an Eastman Kodak company), is making an imaging chamber that is dark enough, and with sufficiently low autofluorescence, that it becomes possible to keep a camera shutter open for minutes at a time while providing a range of excitation light and without creating significant background artifacts.

Astronomy-grade, low-noise, cooled CCD cameras provided the solution to the other technical problem with whole-body imaging: having a camera with sufficiently low background to reliably detect the faint light signals from within animals.

Xenogen, now part of **Caliper Life Sciences**, has traditionally focused on whole-body bioluminescence imaging, though its newest entry in this area, the IVIS (in vivo imaging system) Spectrum, can also record fluorescent signals.

In fluorescence imaging, a fluorophore—whether continued ›

TO RED AND BEYOND

When it comes to in vivo imaging, you need a dark box, and a good probe. Lots of companies can provide the box; some are listed in the main text. Others include **Advanced Research Technologies'** eXplore Optix and **LI-COR Biosciences'** Odyssey.

Thanks to the work of Roger Tsien at the **University of California at San Diego**, among others, researchers have literally dozens of fluorescent proteins to play with. No fluorescent protein yet reaches into the near infrared, but a few, such as Tsien's mPlum (emission maximum, 655 nm), and **Clontech's** Living Colors HcRed1 (618 nm), come close.

Several companies sell fluorescent imaging agents at or near the near-infrared, including LI-COR's IRDyes, **Invitrogen's** AlexaFluor-based SAIVI (small animal in vitro imaging) kits and QDot nanocrystal reagents, and **VisEn Medical's** VivoTags and NanoSPARKS.

Some companies also offer probes specifically for detecting signaling events. VisEn's ProSense and MMPSense NIR sensors light up when cathepsin and matrix metalloproteinases are activated, respectively, for instance, while **Immunochemistry Technology's** SR-FLIVO probe marks apoptotic cells red.

an organic dye, quantum dot, or fluorescent protein—is irradiated with light of a specific wavelength, inducing it to emit light at a different wavelength, that is, to fluoresce. Bioluminescence imaging, in contrast, requires no excitation; the emitted light comes instead from the enzymatic turnover of exogenously introduced substrate molecules like luciferin.

Where luminescence is basically monochromatic (though Promega, for instance, does offer two luciferase variants that differ in their substrate requirements and emission spectra), there exists a veritable palette of fluorescent proteins and fluorophores to enable multiplexed detection of different proteins or events simultaneously. Oftentimes, these colors can appear identical. That's the problem **Cambridge Research & Instrumentation** (CRI) addresses with its fluorescence-based Maestro in vivo imaging system.

Suppose you shine a red light and a green light on a wall, says James Mansfield, CRI's director of multispectral imaging systems. "Where you only see red, it's red, where you only see green, it's green, and where they overlap they would appear yellow."

That yellow is different, however, from the color you would see if you also shined a yellow laser on the wall. "Both appear yellow to us, when spectrally they are very different."

Multispectral imaging allows researchers to distinguish such differences, which become important biologically when, say, trying to distinguish fluorescein (which glows green) from green fluorescent protein, or more important, from the tissue's inherent autofluorescence, long the bugaboo of in vivo fluorescence imaging.

Carestream Health brings yet another imaging modality to the table: X-ray.

According to Shahram Hejazi, president of Molecular Imaging Systems, Carestream Health, X-ray imaging provides anatomic landmarks so that the precise source of optical signals can be identified. "When you capture an optical image from inside an animal, there is no anatomical image in the background. You see a blip on a dark background, so you have no idea where it comes from."

By superimposing a high-resolution X-ray on that image, however, "you can see that the 'blip' is on the third joint in the finger, or between two vertebrae." The company's newest product is the Kodak In-Vivo Multispectral Imaging System FX, which combines optical imaging with spectral unmixing, radioisotopic imaging, and digital X-ray imaging in a single instrument.

Despite their differences, many whole-body systems image three-dimensional animals in two dimensions, like paper on a photocopier. But not all do. The Pan-A-See-Ya imaging system from **Lightools Research** simultaneously images the top, left, and right sides of an animal under identical illumination and without moving the animal, while VisEn Medical's Fluorescence Molecular Tomography system uses a raster-scanning excitation source to capture and render the animal's fluorescence signal in three dimensions. Xenogen also offers a 3D version of its IVIS instrument, which reconstructs its image from eight individual bioluminescence or fluorescence frames.

Imaging Signaling Events In Vivo

According to Sam Gambhir, director of the Molecular Imaging Program at Stanford University, what distinguishes optical imaging from traditional medical imaging modalities like X-ray, PET (positron emission tomography), MRI (magnetic resonance imaging), and CT (computed tomography) is merely that part of the physical spectrum the system is tuned to hear.

"When you have an X-ray, they shoot radiation through you to see how much comes out the other side," Gambhir says. "So an X-ray is a map of density. That says nothing about what's going on in the cells in your lungs or bones. To do that you need a technology that lets you listen in on molecular events."

Gambhir has spent years developing sensors targeting such molecular events as protein-protein interactions, phosphorylation, and protein folding.

"We have a toolbox that lets you go from the surface of the cell, to pathways, into the nucleus, and we can monitor all of these in intact cells while the cells are within living subjects," he says.

Signaling sensors cannot rely on antibodies, Gambhir says, because there is no easy way to get antibodies into living cells. Instead, he relies on signaling event–mediated activation of otherwise silent biosensors. In one example, a functional, light-emitting protein is split in two, rendering it nonfunctional. "If those segments are fused to the two proteins you are interested in, the proteins are brought together and you reconstitute their ability to make light," a procedure known as protein-assisted complementation, or PAC.

Ryohei Yasuda, assistant professor of neurobiology at **Duke University Medical Center** in Durham, North Carolina, uses another approach, called two-photon fluorescence lifetime imaging microscopy, to detect signaling events as synaptic connections change in thick, living tissue sections.

Yasuda detects interactions between the signaling protein Ras and its interaction partners by coupling the molecules to different fluorescent proteins and using fluorescence lifetime measurements to monitor changes in fluorescence resonance energy transfer between them. Though he has not yet migrated this technique into living animals, Yasuda says he sees "no real barrier" to doing so.

"All the techniques to do in vivo signaling imaging already exist," he says, even if they mostly have only been used in vitro.

The resulting data will surely provoke reevaluation of old assumptions, as Jan Schnitzer, Scientific Director of the **Sidney Kimmel Cancer Center** in San Diego, can attest.

Using a battery of in vivo and ex vivo techniques, Schnitzer and his team reported recently that caveolae—tiny membrane invaginations that play a role in endocytosis and macromolecular trafficking—are far more efficient at moving molecules across endothelia than previously recognized. It's an observation that could not have been made in cell culture, he says, and one that could transform the way drugs and imaging agents are delivered to patients.

"That's why it's important to go in vivo," he says. "In vivo veritas, and in medical technology, that's the only thing that matters."

Jeffrey Perkel is a freelance science writer based in Pocatello, Idaho.

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Living Cell Research

The PALM DuplexDish 35 culture dishes, PALM DishHolder 35 with CapCheck, and PALM DishHolder 6/35 are accessories for the PALM MicroBeam system, a laser microdissection system capable of simultaneous visualization and microdissection under multichannel fluorescence illumination and extended focus. The accessories are designed to open the door to more flexible experiments in the isolation and micromanipulation of living cells. The DuplexDish 35 is a 35-mm diameter culture dish in which cells can be isolated, collected, and further cultivated with no need for an additional trypsinization step. The small diameter of the dish lessens reagent requirements. The base offers optimal gas exchange and exhibits low autofluorescence. For processing under the microscope, the culture dish is inserted in a holder on the microscope stage. An integrated aperture can be used to check the success of the specimen isolation. With the CapCheck function, after the microdissection is completed, the specimen can be examined in the cap without any need to remove the dish from the holder. The DishHolder 6/35 can accommodate up to six DuplexDish 35 culture dishes. It can be inserted in the microscope, but also can be used for preparation under the clean bench.

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Transporter Uptake Assay Kit

The live cell Neurotransmitter Transporter Uptake Assay Kit offers researchers a single tool to screen for live cell kinetic uptake of the three key neurotransmitters—dopamine, norepinephrine, and serotonin—in a homogeneous, fluorescence-based, high throughput screening procedure that eliminates the use of radioactive tags or labels. The kit improves on current procedures that make use of radiolabeled neurotransmitter or transporter inhibitors, both of which require specialized instrumentation, detect only the assay endpoints, and are encumbered by the disposal of radioactive materials. This assay can be used in both kinetic and endpoint modes and is a simple, mix-and-read protocol performed in 96-well or 384-well microplates. Applications include high throughput screening and lead optimization. Neurotransmitter targets have become important pharmaceutical targets because of their key role in depression and neurodegenerative diseases.

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Filter Sets for Fluorescent Proteins

Fifteen new filter sets for fluorescent proteins are now available, including filter sets for Invitrogen's Vivid colors, Clontech's Living Colors, and MBL's Coral Hues. Because live cell experiments are typically light starved, optimized filter sets must deliver high signal-to-noise ratio. These filter sets are manufactured using proprietary technology that produces steep slopes and accurate band placement, for maximizing excitation and emission energy and for minimizing background. With the addition of these new sets, Omega now has optimized sets for a total of 35 proteins, including proteins from the reef coral *Anthozoa* and the jellyfish *Aequoria victoria*.

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