MULTIPHOTON IMAGING

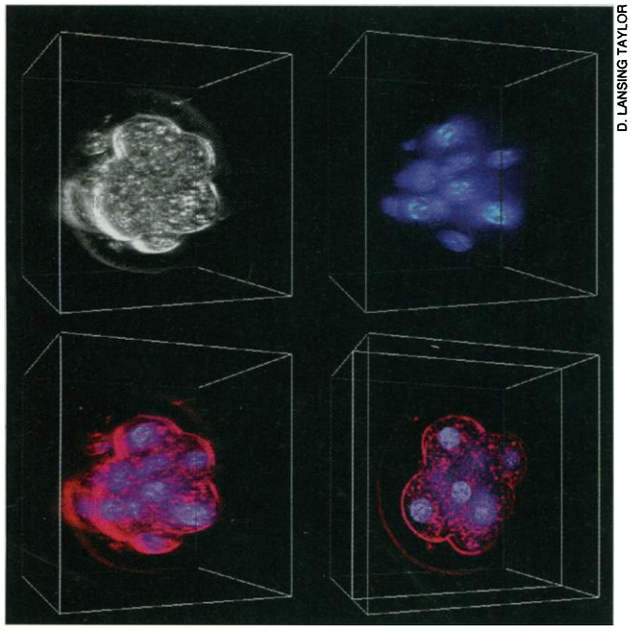
Biologists Get Up Close and Personal With Live Cells

When Dutch naturalist Anton van Leeuwenhoek constructed a simple microscope in 1683, he opened a window to the previously hidden universe of microorganisms. Although it took biologists 200 years to understand fully the true nature of the specks that van Leeuwenhoek saw for the first time, his invention forever changed scientists' perspectives on the natural world. Now, the naturalist's intellectual descendants are bringing into focus an even tinier biological world: the inner workings of single living cells. And their efforts are promising another fundamental shift in perspective as biologists view processes that, until now, have been seen only in the mind's eye.

Armed with a battery of lasers, photodetectors, and computers, plus a novel crew of powerful and adaptable fluorescent reagents (see sidebar), microscopists are literally bringing to light live cells, tissues, and even whole organisms. With a technique called multiphoton imaging, which gently excites fluorescence from cellular components without killing the cells, researchers are following fertilized zebrafish egg cells as they mature to larvae, seeing nerve cells exchange signals deep in the brains of live rats, and viewing organelles communicating in hard-to-image plant cells. "Every time we turn around, we find a new application," says multiphoton-imaging pioneer Watt Webb at Cornell University in Ithaca, New York.

Others are pushing the frontiers of inner space with a version of a technique that astronomers are using to probe outer space: interferometry. Interference microscopes use two interacting beams of light to tease apart tiny cellular structures with a resolution of less than 100 nanometers—sharp enough to see cytoskeletal proteins pushing forward migrant cells and clusters of genes lined up on separate chromosomes. And a few pioneers are now combining a variety of these cutting-edge technologies into a single instrument: a microscopist's dream machine that will allow researchers to pick and choose imaging modes, and even interact with their specimens, without moving their samples from under the instrument's lens.

"There is a small revolution going on in light microscopy," says Stefan W. Hell, a physicist at the Max Planck Institute for Biophysical Chemistry in Heidelberg, Germany, who recently patented and licensed his own version of an interference-based microscope called a 4Pi-confocal. "This will definitely change how biological imaging is being done."



D. LANSING TAYLOR

In-depth view. These 3D images of a mouse embryo were obtained with Carnegie Mellon's Automated Interactive Microscope. Images are processed in different ways to reveal different structural features and the spatial relationships between them.

Multiplier effect. The driving force behind this small revolution is molecular biology itself, says D. Lansing Taylor, director of the Center for Light Microscope Imaging and Biotechnology at Carnegie Mellon University in Pittsburgh. "Once you clone a gene, overexpress it, and knock it out, you still need data that can tell you when and where things are happening," he says.

Until recently, biologists generally had to infer what is happening from electron micrographs of thin, specially prepared specimens or by tagging molecules with fluorescent dyes and flooding specimens with light. But traditional fluorescence imaging, like electron microscopy, often limits observations to dead tissue. Many dyes and cellular proteins fluoresce only when they are zapped by short-wavelength, high-energy photons, which can be highly damaging to living cells. And because the entire specimen is illuminated, photons bouncing off other cellular components can greatly reduce the contrast of the image. Researchers have partially solved the contrast problems with the so-called confocal microscope, a device that illuminates only one section of the specimen and has a pinhole in front of the photodetectors to block out much of the stray light. But multiphoton microscopy tackles both the contrast and the photodamage problems.

The key to this technique is the use of special pulsed lasers to fire precisely focused bursts of lower energy photons at the sample. If two or three photons strike the target molecule almost simultaneously, they produce the same effect as one photon with two or three times the energy. This double or triple punch lights up proteins that have been tagged with special dyes, and it can make some proteins fluoresce on their own. The lower energy of the individual photons cuts down collateral damage, allowing the cell to be kept alive for hours instead of minutes. And the tight focus of the laser beam—compared with bathing the entire specimen with light—greatly reduces effects of light scatter.

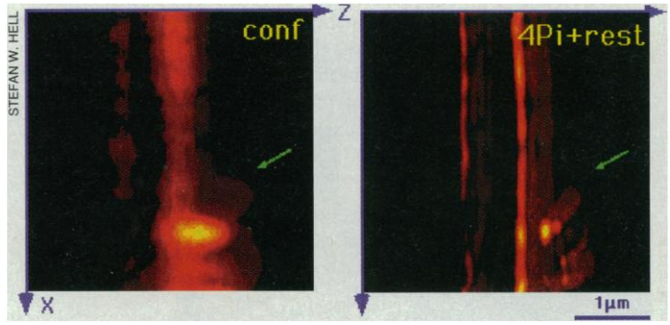
"The technology is catching on like mad," says Webb, who, with his colleagues Winfried Denk and James Strickler, reported the invention and first use of two-photon excitation in 1990 (*Science*, 6 April 1990, p. 73) and—along with several other groups—has since extended the technique to three-photon excitation (*Science*, 24 January, p. 530). Warren Zipfel, a biophysicist in Webb's lab, recently teamed up with plant biologist Maureen Hanson's group at Cornell to use multiphoton imaging to track communication signals between plant organelles called plastids (see sidebar on p. 1989). "This is an illustration of what you can do with multiphoton excitation," says Webb. "It's enabling plant biologists to see processes in living cells in ways never possible before."

Neuroscientists also are using multiphoton imaging to look at previously unseen processes. "If you want to go even 500 micrometers into brain tissue, it is impossible with confocal [microscopy]," says Denk, a physicist-turned-biologist now at Bell Laboratories in Murray Hill, New Jersey. "You lose so much of the excitation energy because of light scattering." What that means is fluorescence gets lost as the light bounces around inside tissues such as the brain. The situation is analogous to walking into a cave illuminated only by sunlight from the entrance—the farther inside you go, the darker and fuzzier the surroundings appear.

Denk and his colleagues have gotten

around the problem by using a multiphoton microscope to peer into the brains of live rats, homing in on the dendritic spines, spikey branchlike structures at the junctions between nerve cells. After filling individual neurons with a calcium-sensitive fluorescent dye and tickling the animals' whiskers, the researchers followed calcium ions as they traveled between the spines. The results provide information about the biochemistry of learning and memory formation that Denk says could not be obtained by studying neurons in petri dishes or in slices of brain mounted on slides. "There has been a big debate in neuroscience as to whether results in primary culture can really be generalized to live tissues," Denk says. "People want to know, 'How does a cell really work with other cells around it?' "

While Denk's work and that of others, including Steve Potter at the California Institute of Technology (Caltech) in Pasadena, are beginning to answer that question for neurobiologists, developmental biologists are also hot on the multiphoton trail. Developing embryos are hard to image because egg yolk tends to scatter light, says cell biologist Victoria Centonze, deputy director of the Integrated Microscopy Resource Center at the University of Wisconsin, Madison. But multiphoton microscopy can cut through the scatter, and its relatively gentle probing can provide up to 20 hours of observation of a live embryo. This allows researchers to follow egg cells as they shape into embryos or track a gene that controls limb development.



Revolution in resolution. Stefan Hell's 4Pi microscope, combined with computerized image restoration *(right)*, yields 10- to 15-fold improvement in 3D resolution of actin filaments of a mouse fibroblast, compared with confocal image *(left)*.

Interfering lasers. While multiphoton imaging is opening a window into cellular interiors, interference microscopy is beginning to sharpen biologists' view of individual structural components. The technique involves splitting laser light into two beams and focusing them on a sample, so that their light waves interact with each other as they pass through the specimen. The resulting pattern of light-dark interference fringes, in essence, illuminates the sample in layers instead of lighting up the whole sample at once. "Where there are regions of brightness, it stimulates fluorescence, and where there is a null, there is no excitation," says Taylor of Carnegie Mellon. That pattern also improves contrast, says Taylor, because neighboring zones will not fluoresce and blur the one being imaged. And the layers can be moved up and down to provide three-dimensional information by shifting the angle of the laser beams and thus the interference pattern.

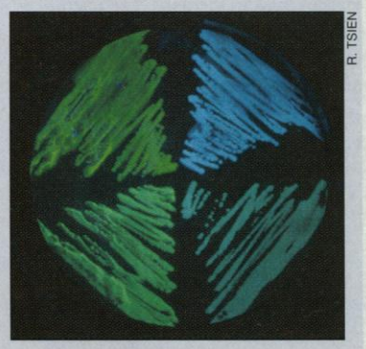
Biophysicist Fred Lanni and his colleagues at Carnegie Mellon have used a simple version of an interference microscope to follow fibroblasts as they crawl into a healing wound. At the same time, several groups in Germany, including Hell's team at the Max Planck Institute, are modifying interference principles in other ways: to monitor cell-scaffolding proteins during mitotic division or to look at aberrations, tagged with fluorescent dyes, in DNA from patients with genetic illnesses such as Prader-Wille Syndrome and certain types of leukemia. Hell says that when computers are used to correct blurring of the image, the three-dimensional (3D) resolution of cellular components such as F-actin proteins can be improved up to 15 times over that of confocal or multiphoton imaging alone.

In another modification of interference microscopy, researchers at the Max Planck Institute for Psychiatry in Munich have devised a system to peer at the neuronal networks in rats. German physician Hans-Ulrich Dodt, who had an avid interest in astronomy, teamed up with Walter Zieglgänsberger, a pharmacologist and physiologist, and created an instrument that uses an infrared-imaging technique similar to one used by astronomers. "We went from light-

Jellyfish Proteins Light Up Cells

The power of multiphoton imaging to illuminate components deep within living cells and tissues rests almost as much on improving fluorescent reagents as it does on high-tech lasers (see main text). Although some proteins, such as serotonin, fluoresce naturally when zapped with multiple photons, others must be tagged with fluorescent dyes in order to become visible.

The dyes themselves, or the procedures to get them into cells, tend to kill everything before a laser beam ever touches the sample. However, a new type of marker, based on a protein from a jellyfish that glows brilliantly when hit with 500-nanometer-wavelength light, is helping to light up living cells. Called green fluorescent protein (GFP), it was first cloned in 1992 by Doug Prasher's group at Woods Hole Oceanographic Institution in Massachusetts, and can be genetically engineered into cells—eliminating the need to apply toxic stains to the specimen.



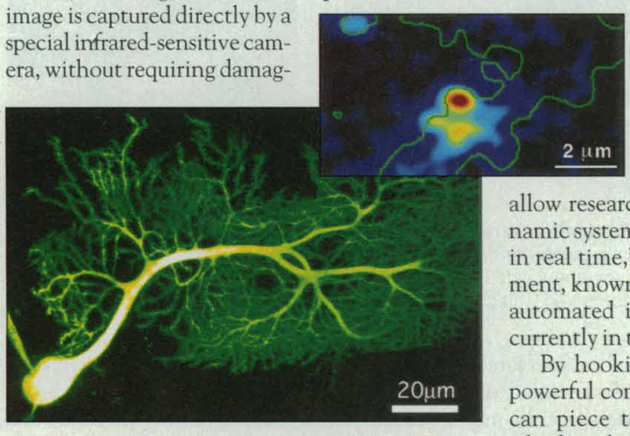
Colorful mutants. Green fluorescent protein comes in different hues.

The cDNA of this protein can be hooked up to any gene and expressed along with that gene's protein product. The only thing needed to make GFP fluoresce is a single dose of high-energy ultraviolet light or its lower energy multiphoton equivalent. "The crucial difference between GFP and [earlier, widely used dyes] is that it works [better] in live cells or animals," says GFP pioneer Roger Tsien of the University of California, San Diego. What's more, mutant forms of the protein glow in different colors—from yellow-green to bright blue—which enables researchers to follow the workings of several molecules simultaneously.

Tsien and others are now working frantically to make mutants that glow brighter, fluoresce in more colors, or hook onto calcium ions and phosphate groups in cells and tissues. A recent map of the protein's physical structure is aiding the task (*Science*, 6 September 1996, p. 1392), and already multiphoton-imaging pioneer Watt Webb's group at Cornell University has found that the excitation of one of the GFP mutants is much brighter than rhodamine, the brightest synthetic dye. "It's becoming a sort of mutual synergy," says Winfried Denk, of Bell Laboratories in Murray Hill, New Jersey. "Once you can see effectively, then there is the incentive to develop new [fluorescent markers]." –T.G.

years to micrometers," Zieglgänsberger says.

Their instrument illuminates the sample with obliquely angled beams of infrared light whose wavelengths are out of phase. The image is captured directly by a special infrared-sensitive camera, without requiring damaging dyes or fluorescent markers. Dodt and Zieglgänsberger have used the technique to visualize living brain structures down to the resolution of single spines, which allows them to perform a variety of observations with far greater precision. "It's like fishing in a pond blindly and then having the lake clear so that I can see all the fish," Zieglgänsberger says.



DENK AND SVOBODA, *NEURON* 18, 351 (1997)

Nervous action. Multiphoton image of living Purkinje cell filled with fluorescent dye; calcium ions released by dendritic spine *(inset)* of stimulated cell.

While infrared video microscopy has combined several existing techniques into one device, Taylor and his colleagues at Carnegie Mellon, along with others, are working on bringing together several existing microscopes into one instrument. "The goal is to allow researchers to interact with a dynamic system, like a developing embryo, in real time," says Taylor, whose instrument, known by the acronym AIM—for automated interactive microscope—is currently in the late development stages.

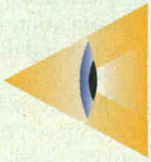
By hooking up the instrument to a powerful computing system, researchers can piece together the 3D images—which otherwise take hours to sort through—very rapidly, Taylor says. That way, researchers could watch a fertilized egg divide, say with multiphoton imaging, then add a drug or reagent and switch to interference microscopy to see how the drug takes effect. "The whole purpose of the AIM is that the data are collected, processed, and displayed during the time of a biological event," Taylor says. "That way a researcher can change the course of an experiment all in the time frame of a biological process such as cell division or locomotion."

While interference and multimodal imaging devices are still in the developmental stages, multiphoton technology is now commercially available. But it comes at a high price. The Cornell Research Foundation has patented the technology and licensed it exclusively to BioRad Microscience Ltd., in Hemel Hempstead, U.K., which is selling the complete instruments for anywhere from $300,000 to $450,000. About $100,000 of that stems from the cost of the laser, while the rest of the major costs are based on meeting European codes for the instrument, according to Webb. Even with that price tag and BioRad's decision not to sublicense, many researchers are optimistic that multiphoton imaging will become widespread. "Its advantages are so clear," says biochemist Steve Potter, who has set up a multiphoton instrument in Scott Frasier's lab at Caltech. "Once the lasers become mass-produced, I predict every confocal [microscope] will become multiphoton."

–Trisha Gura

Trisha Gura is a science writer in Cleveland.

MEDICINE



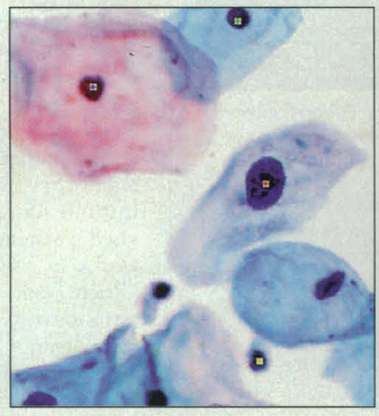
Spectral Technique Paints Cells in Vivid New Colors

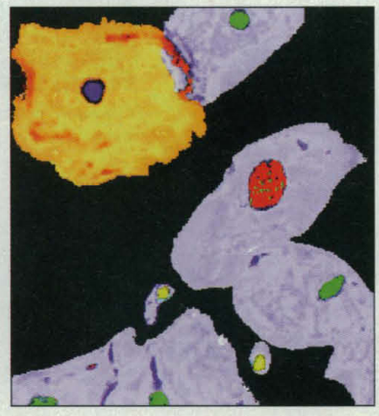
You can think of image enhancement as the art of helping the eye do what it does naturally. Take the two images below. On the left is a conventional micrograph of cells from a pap smear. The cells have been stained to bring out the contrast between different types: Mature epithelial cells are pink-orange, while younger cells stain blue-green, as does the precancerous dysplastic cell in the middle. A pathologist would identify it by its abnormally large nucleus, but it wouldn't be hard to miss.

On the right is the same image, spectrally classified. Richard Levenson and Daniel Farkas of Carnegie Mellon University in Pittsburgh created individual spectra for each pixel in the image with the help of a microscope called the SpectraCube. The microscope divides light from each pixel into beams that travel along paths of varying lengths, then are recombined and allowed to interfere. Mathematical analysis of the resulting interference patterns yields a spectrum.

By comparing each pixel's spectrum to those of reference pixels (boxes on original micrograph), Levenson and Farkas's system identifies groups of pixels with similar spectra and assigns them distinctive colors, making them much easier to tell apart than they are in the original stained micrograph. The nucleus of the dysplastic cell, only subtly different in color from that of a normal cell in the traditional micrograph, is here colored a unique and fiery red, befitting its threatening nature.

The technique had already been applied to cytogenetics by Thomas Ried and Evelin Schröck of the National Center for Human Genome Research in Bethesda, Maryland. They color-coded and differentiated the 24 pairs of human chromosomes after labeling them with tracers that endowed each one with a slightly different spectrum (*Science*, 26 July 1996, p. 494). Having extended the technique to pathology, Levenson and Farkas say it could be used throughout biology to increase the differentiation power of stains, dyes, and fluorescent molecules. "It divides the spectrum into a whole slew of new colors that otherwise couldn't be appreciated by the eye," says Levenson. "It's our belief that important information resides in those colors, and spectral classification can bring it out."





R. M. LEVENSON

–Gary Taubes