THE JOURNAL OF NIH Research

APRIL 1997 VOL. 9 NO. 4

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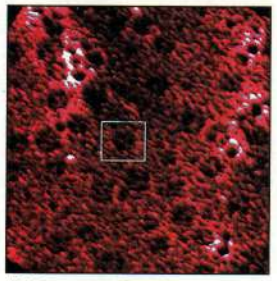

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THE JOURNAL OF NIH RESEARCH (ISSN 1043-609X) is published monthly by Medical Economics with editorial, advertising, circulation and administrative offices located at 1444 I Street, N.W., Suite 1000, Washington, DC 20005. *Periodical* postage paid at Montvale, NJ 07645, and at additional mailing offices.
SUBSCRIPTION/SINGLE COPY RATES: Individual subscription (12 issues): $79. Institutional subscription: $99. Elsewhere, $150 (air mail). Single copy cost is $10 (U.S.A.), $15 (elsewhere). Bulk rates are available upon request.
ADDRESS CHANGES: Address circulation and subscription related correspondence to Subscriber Services Dept, The Journal of NIH Research, P.O. Box 824, Winchester, MA 01890
POSTMASTER: Send address changes to THE JOURNAL OF NIH RESEARCH, P.O. Box 824, Winchester, MA 01890.

that alter specific features of the CED-9 structure decrease its ability both to bind CED-4 and to block the cell-death events that commence when CED-4 interacts with CED-3, according to both Dixit and Hengartner.

Gabriel Nunez, Dayang Wu, and Herschel Wallen of the University of Michigan Medical School came to the same conclusions independently working in yeast and in several mammalian cell lines. In the Feb. 21 issue of *Science*, they report that CED-9 is anchored to intracellular membranes such as the nuclear membrane, whereas CED-4 ordinarily is dispersed throughout the cytoplasm. However, CED-9 can bind to CED-4 and thereby sequester it along such membranes, Nunez says. Such behavior "may be important for the regulation of CED-4 activity"—preventing it from activating the killer protein, CED-3, he says.

When the gene encoding CED-9 is deliberately modified to remove this protein's hydrophobic carboxyl-terminal region, it can no longer anchor itself to membranes. In such cases, its ability to sequester the CED-4 protein to such sites is also lost. "Although we have no direct proof yet that membrane localization is crucial for their actions, we think it is," Nunez says.

The mammalian equivalent of CED-9 is Bcl-2, and CPP32, also known as caspase-3, is homologous to CED-3. To do its apoptotic work, the mammalian caspase-3 must first be modified, probably by a protease, after which it becomes an active protease in its own right, says Nunez. Bcl-2, the mammalian homologue of CED-9, apparently does not serve as a substrate for the protease homologue of CED-3.

Xiaodong Wang of the University of Texas Southwestern Medical Center at Dallas and colleagues there and at Emory University School of Medicine in Atlanta report that in mammalian cell lines, overexpression of Bcl-2 at the outer mitochondrial and nuclear membranes can block apoptosis, and that it may do so by blocking release of cytochrome c from mitochondria. The release of cytochrome c precedes any general loss of mitochondrial integrity that may occur later if apoptosis were to be triggered, they say.

Independently, Donald Newmeyer, Douglas Green, and their colleagues at the La Jolla Institute for Allergy and Immunology in San Diego find in *Xenopus* oocytes that Bcl-2 acts at the mitochondrial surface and that its ability to interfere with cytochrome c release may be specific. But Newmeyer and his colleagues found no significant change in membrane potential during the course of their experiments; any changes in membrane potential are apparently later than cytochrome c release, he says. In further experiments now being prepared for publication, Newmeyer's team finds that cytochrome c does not by itself process the precursor of caspase-3. "At least one other cytosolic factor is also required," he says.

Importantly, Newmeyer says, cytochrome c release from mitochondria is an "early event" in apoptosis. One possibility, he says, is that Bcl-2 plugs specific mitochondrial membrane pores, another is that it serves as a pore that somehow controls cytochrome c release, and still another is that its activity is subject to additional membrane-acting components. For the moment, whatever causes mitochondria to release cytochrome c and how cytochome c activates caspases are "both mysteries" reflecting basic gaps in understanding apoptosis, he says. "There may be a receptor or protein complex on the outer membrane of the mitochondria to signal pores there to release cytochrome c, but this process is really mysterious."

—JEFFREY L. FOX

New Microscopes See On The Nanoscale

Any botanist could tell you that cross-pollination produces the strongest hybrids. Cross-pollination of ideas between biologists and physicists has already given us the X-ray, magnetic resonance imaging, computed tomography, and positron emission tomography, among other techniques.

Now, physicists and biologists are homing in on the nanoscale. Almost ready for prime lab time in biomedical research laboratories are two new microscopy techniques: atomic force microscopy, which allows researchers to watch nanometer-scale structures such as pores and ion channels work on the surface of living cells in real time, and nonlinear fluorescence microscopy, in which a pulsed-beam laser produces a long-wavelength, infrared beam that is gentle on living cells and allows researchers to delve more deeply into tissue than does the current standard, confocal microscopy.

Physicists have been using atomic force microscopy and nonlinear fluorescence microscopy for half a dozen years. But neither technique has been widely used to study biological systems. That may be about to change.

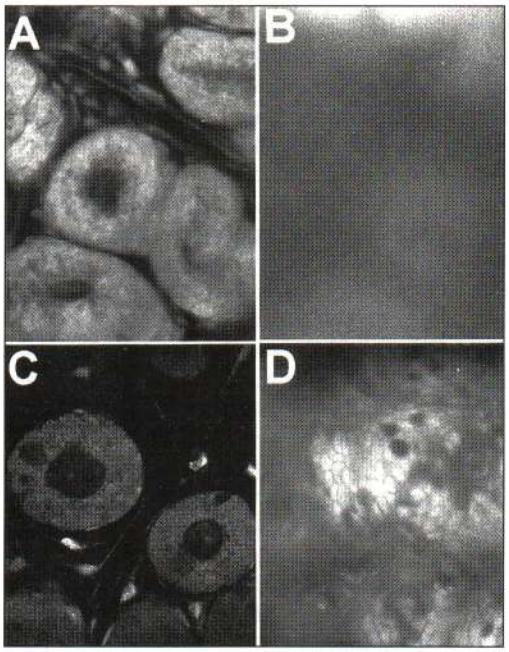
A flurry of recently published papers demonstrates that nonlinear fluorescence microscopy, which is also known as multi-photon excitation, is becoming a practical tool for three-dimensional fluorescence of tissue sections more than 100 microns thick, two to three times thicker than is possible with confocal microscopy.

In May, at the American Gastroenterological Association meeting in Washington, D.C., Marshall Montrose of Johns Hopkins University in Baltimore is scheduled to present data showing a clear advantage of two-photon excitation over confocal microscopy for imaging living intestinal tissue.

"Confocal microscopy was the best thing to come down the pike several years ago," Montrose says. "But in living tissue, at 40 microns depth you hit the wall and can't see any deeper. With two-photon excitation, the difference was striking. We were able to go in threefold deeper, get a clear fluorescence signal, and maintain subcellular resolution."

Many biologically important molecules produce fluorescent light when they are excited by a series of two or three low-energy infrared wavelength photons, according to a review article in the Oct. 1, 1996, issue of the *Proceedings of the National Academy of Sciences* (*PNAS*) by Watt Webb, Rebecca Williams, Chris Xu, Warren Zipfel, and their colleagues at Cornell University's Developmental Resource for Biophysical Imaging and Opto-Electronics in Ithaca, N.Y. The energy of a photon, produced by the device's pulsed titanium-sapphire laser, is absorbed by a single molecule, called a fluorophore. Then, as additional photons are fired in one hundred-femtosecond (10^{-13} second) pulses, the energy accumulates and the molecule emits a high-energy fluorescent signal.

This multi-photon approach has significant advantages over current fluorescence imaging techniques, such as confocal microscopy, says Zipfel. In confocal microscopy, researchers tag molecules of interest with dyes that produce fluorescent light when excited by short-wavelength, usually blue to ultra-



Two-photon microscopy vs. confocal microscopy for deep-tissue imaging. Fluorescent images are of living mouse colonic mucosa. Panels A and B are by confocal microscopy focused at 15 micrometers (μm) (A) and 80 μm (B) into the mucosa—resolution deteriorates at the deep focal plane. Panels C and D are by two-photon microscopy at 20 μm and 100 μm. The image in D still shows junctions between individual cells (approximately 200 nanometers wide) at the surface of the colonic epithelium. [Courtesy Shaoyou Chu and Marshall Montrose, Johns Hopkins University, Baltimore.]

violet (UV) light. The fluorescent signal produced by the dye passes through a pinhole aperture in only one focal plane. Any out-of-plane light is blocked by the aperture. A computer then assembles the image for display on a video monitor.

By contrast, the pulsed laser light used in multi-photon excitation is in the red to infrared range—700–900 nanometers (nm)—which doesn't bounce around inside a living cell and cause as much photodamage as does the short-wavelength UV light (280–300 nm) that is used to excite fluorescent dyes in confocal microscopy.

Several fluorescent tags now widely used by researchers, such as green fluorescent protein, can be excited using the technique, says Zipfel, opening the possibility of tracking multiple molecules in a single cell under a variety of conditions.

Montrose used two-photon excitation to measure, in living mouse colon tissue, the uptake and metabolism of 5-aminosalicylic acid (5-ASA), an aspirin derivative used to treat irritable bowel syndrome. The intact drug fluoresces at 500 nm when excited by two-photon pulses at 720 nm, but once metabolized it fluoresces at a slightly different wavelength, 440 nm, allowing the researchers to monitor drug metabolism deep into the tissue.

In addition, says Montrose, because the laser beam can be focused on one plane, he and his colleagues were able to measure metabolism of therapeutic levels of drug. This had never been possible before, because in confocal microscopy fluorescence the whole sample is illuminated, and the high density of drug molecules along the optical path obscures the resulting image.

Similarly, the Cornell group used simultaneous three-photon excitation and fluorescence to visualize serotonin concentration in living rat basophilic leukemia cells—the first direct measurement of neurotransmitter concentration in living cells. Zipfel, Webb, and their colleagues report in the Jan. 24 issue of *Science* that they were also able to excite fluorescence in the neurotransmitters dopamine and tryptophan.

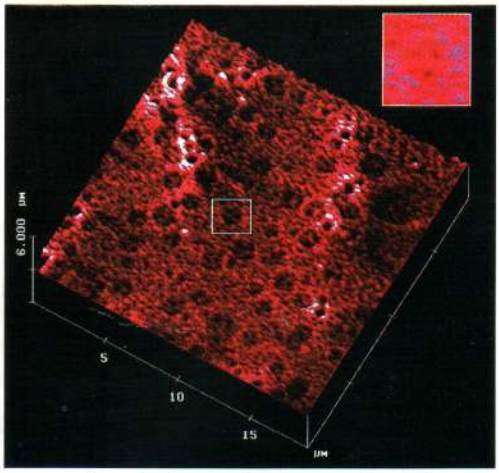
Joshua Zimmerberg, chief of the Laboratory of Cellular and Molecular Biophysics at the National Institute of Child Health and Human Development in Bethesda, Md., says multi-photon excitation is "the most promising technology" he's seen for imaging complex tissue. Zimmerberg is planning to purchase a nonlinear microscope that will be a shared resource for intramural NIH scientists studying complex tissue sections.

Despite promising preliminary results from the use of multi-photon excitation to observe biological processes such as neurotransmitter release in living cells, the Cornell group has encountered some limitations. The technique is so new that little is known about how many natural fluorophores exist in various tissues. If too many such molecules exist, it will be difficult to distinguish the various signals. For example, says Zipfel, structural fibers such as extracellular matrix proteins, found in many intact tissues, fluoresce brightly when excited using the multi-photon technique. This may limit the technique's usefulness in seeing events in connective tissue and in other tissues with extensive extracellular fiber networks.

"We don't see this background fluorescence in tissue culture cells," says Zipfel. "Cultured neurons work fine."

Right now, the major impediment to widespread use of the system is the cost, says Zipfel. The microscopes that Cornell's developmental group has been assembling on a custom basis cost about $250,000, and the titanium-sapphire lasers run about $140,000. Zipfel estimates that only about 20 nonlinear, multi-photon systems are in operation around the world.

But laser technology is advancing so quickly that Zipfel says the set-up cost should be comparable to that of a confocal microscope—roughly $150,000 to $300,000—within a few years. Cornell,



*Atomic force microscopy identifies never-before imaged pits ranging from 0.5 to 2 micrometers (μm) in diameter at the apical region of rat pancreatic acinar cells. Inset shows one pit with four depressions in it. Several pore-like structures of unknown function are also identified. [Reprinted with permission from S.W. Schneider et al., Proc. Natl. Acad. Sci. USA **94**, 316 (1997). ©1997 National Academy of Sciences, U.S.A.]*

which holds a patent on the technique, has licensed the rights to the laser technology to Bio-Rad Laboratories of Hercules, Calif., which is developing a commercial system, although none has yet been shipped. Neither has a price been

set. But workshops to demonstrate the technique are being scheduled this summer at Cold Spring Harbor Laboratory in Cold Spring Harbor, N.Y., and at the University of Wisconsin at Madison.

In contrast to the multi-photon laser, which probes deeply into cells and tissues, the atomic force microscope (AFM) has emerged as a powerful technique for scanning the surface of living cells.

The AFM works basically like a record player. A sharpened, needle-like tip attached to a cantilever arm scans the cell and is deflected by the peaks and valleys of the cell surface. A computer then reconstructs a topographical map of the cell surface to 10-nm resolution.

"What you do with the AFM is more related to our sense of touching, when you close your eyes and feel with your fingers and with the help of your brain make images out of these experiences," says Heinrich Hoerber of the European Molecular Biology Laboratory in Heidelberg, Germany. "Our sense of touch can tell something about the geometry of an object, but also whether it is hard or soft, sticky or polished, warm or cold."

For biologists, the biggest advantage of AFM is that it works under normal physiological conditions, usually in an aqueous environment, which allows researchers to observe and measure physiological processes such as exocytosis, or secretion, at atomic resolution. Its sensitivity also allows researchers to measure exquisitely small forces, such as hydrogen bonding and electrostatic force, simultaneously with structural data.

The technique recently enabled a research team led by Bhanu Jena of Yale University in New Haven, Conn., to see 100–180 nm structures that he named "depressions" on the apical, secretory end of a rat pancreas cell. In the Jan. 7 issue of *PNAS*, Jena, Stefan Schneider, Kumudesh Sritharan, and their colleagues suggest that the structures are secretory valves for the digestive enzyme amylase during exocytosis. Such structures had been described but had never been imaged before by electron microscopy or other nanoscale imaging techniques. Jena thinks the structures are probably very delicate and sensitive to the fixatives used to prepare tissues for electron microscopy.

Although Jena and his colleagues did not actually see amylase secretion, they observed the depressions' diameters increasing during induced amylase secretion and decreasing as secretion stopped. The researchers are now trying to combine AFM with videomicroscopy to determine whether the structures are actually interacting with secretory vesicles. Jena expects AFM to become a useful technology for study of membrane-bound ion channels, receptors, and other surface features.

Using an AFM is not difficult to master, says Jena. But because measurements are nanometer-scale, any room vibrations can disrupt the measurements. His advice: "Set it up in the basement and do experiments in the middle of the night."

—KARYN HEDE GEORGE

Don't Make Me Laugh: Scientists Tackle Tickling

Imagine yourself pinned down, being tickled relentlessly, wiggling fingers in your armpits and along your sides, feathers brushing against the soles of your feet. You are laughing uncontrollably, convulsing with giggles, smiling the most delightful smiles. It's pure torture. So why do you look so happy?

MARY WICKLINE, UCSD

Tickle researcher Christine Harris of the University of California at San Diego displays her "tickle machine."

Perhaps humanity's silliest and most charmingly childish of behaviors, the guffaws produced by tickling are also among the most enigmatic forms of laughter. Yet although tickling has been pondered for centuries, it has remained little more than a cackle-filled curiosity far from the purview of modern science.

Approaching cautiously and with due scientific gravity, a handful of researchers have now begun entering the terra incognita of tickle. Attempting to answer questions pondered by Socrates, Galileo, and Darwin—as well as many a preschooler—tickle research leads to questions as amusing as why you can't tickle yourself and as profound as why humans have evolved to laugh at all.

"It's actually quite bizarre that someone rubbing their fingers up and down your sides or foot makes you laugh," says Christine Harris. Harris, along with fellow social psychologist Nicholas Christenfeld at the University of California at San Diego (UCSD), published a new tickle study in the January issue of the British journal *Cognition and Emotion*. In a field in which amusing speculation still heavily outweighs data, the Harris-Christenfeld study found that ticklish laughter is not the happy phenomenon that many have assumed it to be.

In the late 1800s, Charles Darwin and Ewald Hecker independently suggested that humor and tickling share deep underlying similarities. Both produce laughter, goosebumps, convulsive muscle contractions, and both, Darwin and Hecker suggested, appear to require a pleasant state of mind.

To test the Darwin-Hecker hypothesis, Harris and Christenfeld enlisted 72 UCSD undergraduates. Students in one group were tickled by female research assistants for 10 seconds (or until the tickling became intolerable) on both sides of the torso from underarm to waist, and then were shown videotapes of stand-up comedy routines and clips from "Saturday Night Live." Students in the second group watched the comedy first and then were tickled. Students in a control group watched a patently unfunny nature video, then were tickled.

The experiment relied on what's known as the warm-up effect, the phenomenon that provides work for warm-up comedians. If you've found something funny, researchers have previously shown, the next thing you encounter will seem that much funnier.

So if tickling and humor both produce laughter through a kind of mirthful mood, the amount of time spent laughing while being tickled or humored should increase if you've just been tickled or humored. It didn't. Tickling, Harris and Christenfeld suggest, does not cre-