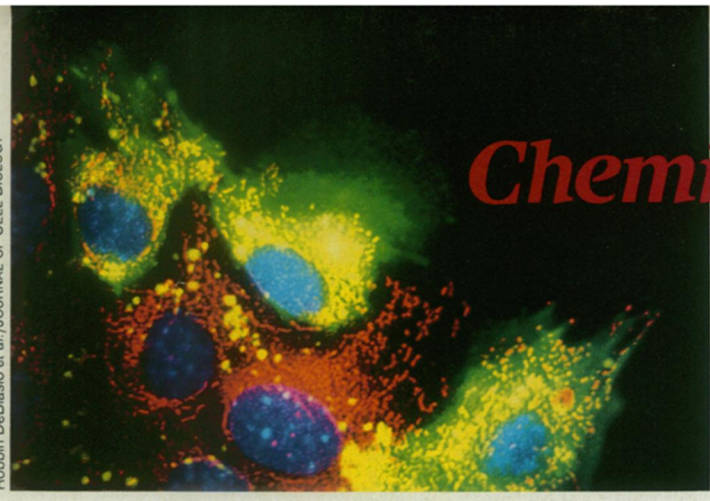


Robbin DeBiasio *et al.*/JOURNAL OF CELL BIOLOGY

Chemical Revelations

Tattletale molecules make cells glow

By ELIZABETH PENNISI

As an undergraduate, Roger Y. Tsien disliked his chemistry courses so much that he decided against becoming a chemist. Nevertheless, this biologist has made his mark on science because of his chemical concoctions.

With molecules that they build or remodel, Tsien and his colleagues at the University of California, San Diego, carry on intracellular espionage. In fact, they and scientists at Carnegie Mellon University in Pittsburgh have pioneered means of tapping into the internal message network that cells use to keep operations running smoothly.

Before these advances, scientists were like detectives at a stakeout: They could watch molecular messengers going in and out, but they could only guess at the details of what went on behind the closed doors of the cell membrane. Now researchers can "wire" molecules that sneak in and eavesdrop on a cell's chemical conversations. "[They] give us a readout of what the cell is doing inside," Tsien explains. "You can watch one part of the cell passing messages to another part."

Tsien does his watching in the dark — his undercover molecules tip him off to their locations by fluorescing. These spies represent an expanding cadre of glowing reagents designed to track key chemicals and reveal changes in a cell's interior. To decipher their messages, Tsien relies on sophisticated microscopy as well as special image-processing techniques. Thus, he can detect subtle changes in fluorescence and piece discrete bits of information into a comprehensive picture of cellular activity.

Likewise, researchers at the Center for Light Microscope Imaging and Biotechnology at Carnegie Mellon University develop their own fluorescing molecules and work to improve the technology for monitoring these agents. Together, the two research groups have provided tools that enable researchers to follow events inside the cell minute by minute and perhaps eventually to manipulate those activities.

Above: Four fluorescent probes label the nuclei (blue), mitochondria (orange), endosomes (yellow), and actin (green) of cells active in wound repair.

When Carnegie Mellon's D. Lansing Taylor first decided to use fluorescent dyes, he simply wanted to track the intracellular movements of a molecule called actin. So in 1978 he created double agents — copies of actin with a fluorescent dye attached. Once injected into the cell, these fluorescent analogs mix with the cell's natural actin. Because the analogs glow, they reveal to researchers the location of actin within a cell.

Tsien wanted his molecular spies to do more than just tail proteins, so he decided to build them from scratch. His first goal: an optical sensor that would let him know when calcium was released into the cell's interior. That molecule and others subsequently developed by Tsien and by Taylor's group represent the new generation of sensors — molecules able to monitor changes in the local internal environment of a cell.

Calcium is a key messenger in the body. It prompts nerve cells to fire and muscles to contract. It turns enzymes on and off and helps activate the immune system. When sperm and egg first meet, the egg releases a burst of calcium ions into its interior. Other cells use calcium to commit suicide: The calcium activates enzymes that cut up proteins and lipids vital to cell survival.

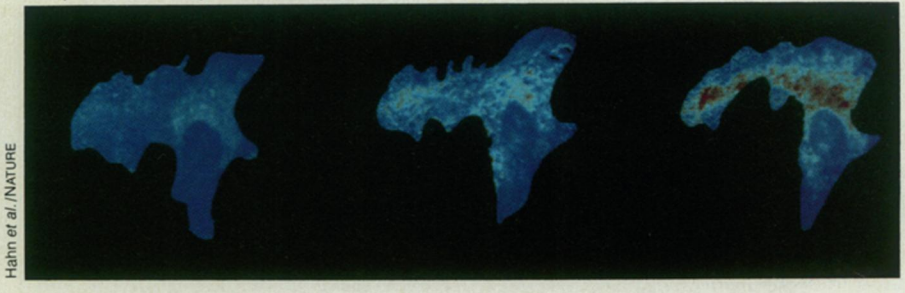
But as a nondescript chemical entity, calcium is also an elusive target. Designing a sensor therefore proved a tough job that spanned almost seven years. For one thing, the sensor needed a fluorescent tag, a dye that would respond strongly, yet selectively when the spy molecule encountered calcium.

The molecule also needed negative charges to attract the positive calcium ion. The sensor had to be small enough to squeeze into all the nooks and crannies of a cell where calcium might lurk and picky enough to bind to calcium ions but not to magnesium, another positively charged ion of similar size. "And we needed it to be able to reject [magnesium] by a factor of 100,000," says Tsien. In addition, the molecule had to work efficiently; that way, Tsien wouldn't need to add so many spy molecules that they would disturb the cell's natural chemical balance.

Finally, he and his colleagues had to figure out a way to smuggle these sensors into a cell. Injecting them one by one seemed too tedious, yet the molecule's negative charges rendered it incompatible with the cell membrane and unlikely that this spy could just wriggle its way inside.

Tsien first surveyed the chemical literature and discovered one chelator mole-

Sequence at 0, 8, and 16 minutes shows a cell pulling away from an injury. Calmodulin activity varies from low (blue) to medium (yellow) to high (red) as calcium activates this protein to help induce contractions.



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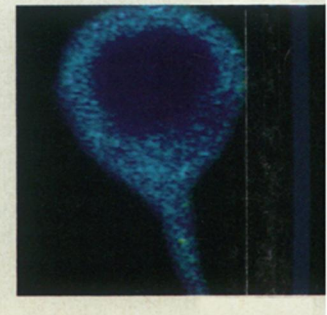
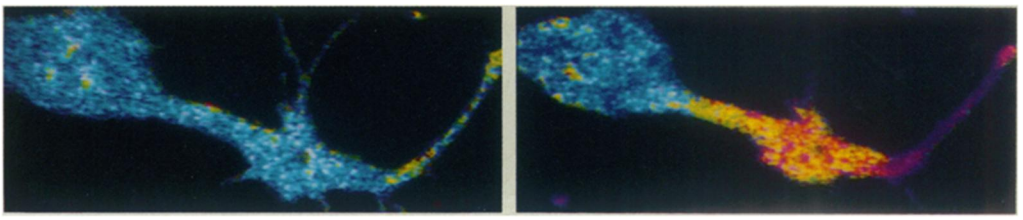


In these processed images of a nerve cell in an intact sensory cluster, cAMP varies in response to serotonin and other chemicals (magenta equals the highest; blue, the lowest; and yellow, moderate). Eventually, an enzyme subunit fills the nucleus (far right).

These colors (magenta equals the highest amounts of cAMP; blue, the least; yellow, a little) reveal that serotonin causes this isolated nerve cell to make cAMP mostly at the tip.



Hochner, Bacskai, Tsien, & Martyn Mahaut-Smith

cule that snagged calcium preferentially over magnesium in alkaline conditions. To make that molecule fluoresce and work in a cell's more acidic environment, he attached ever more complex side groups, eventually finding some that emitted enough fluorescence that even a single cell's worth could be detected.

To solve the access problem, Tsien borrowed chemical technology developed by pharmaceutical companies to help the gut take up antibiotics. By adding water-repelling side groups called esters to his sensors, he effectively covered up the negatively charged parts of these molecules so they could get through the lipid layers of the cell membrane. "After they get inside, the cell cuts [the esters] off," he explains.

These molecular intruders helped decipher many secrets. They revealed that individual liver cells respond differently to adrenaline. Under the microscope, these cells pulse about once a minute as they release calcium. Other types of cells show a sharp rise in calcium or seem to release calcium in spurts.

"You'd never be able to catch this with traditional biochemistry," Tsien says. "We [now] have the ability to see individual cells and recognize their individual personalities."

Taylor, too, decided to follow calcium activity, but from a different perspective. To do so, he made copies of a protein called calmodulin. When it binds to calcium, it starts activating enzymes and thus serves as an intermediate messenger that amplifies chemical cascades inside a cell. Taylor and Carnegie colleagues Alan S. Waggoner and Klaus M. Hahn linked a fluorescent dye to calmodulin, creating a "biosensor" called MeroCaM, he explains. When MeroCaM encounters calcium, it undergoes a three-fold change in excitation ratio, a comparative measurement of fluorescence that compensates for distortions that arise during imaging.

"This was the first example of engineering a protein with a fluorescent probe to create a biosensor for living cells," says Taylor. "We can now track where in the cell [calmodulin] is binding calcium."

By injecting MeroCaM into fibroblast cells grown in a petri dish, then stimulating those cells with certain growth factors, the researchers confirmed that higher concentrations of free calcium correlated with increased activity by this protein. They also determined that this activity varied from cell to cell.

When the scientists trained their microscope on single cells, they could also discern, by means of a technique called ratio imaging, that calcium-carrying calmodulin shows up first just inside the cell membrane. Calmodulin then appears sporadically ever closer to the nucleus, the Carnegie Mellon group reported in the Oct. 22, 1992 NATURE.

The researchers then examined activity in cells involved in wound healing. They injured a layer of fibroblast cells with a razor blade and injected both MeroCaM and a calcium indicator. In cells that crept toward the wound, they observed that calcium concentrations and calmodulin activity were highest at the rear of the cell and lowest at the leading edge. Another fluorescent marker revealed that, at the site of all this activity, fibers stretching from one side of the cell to the other contracted and helped pull the cell away from neighboring cells.

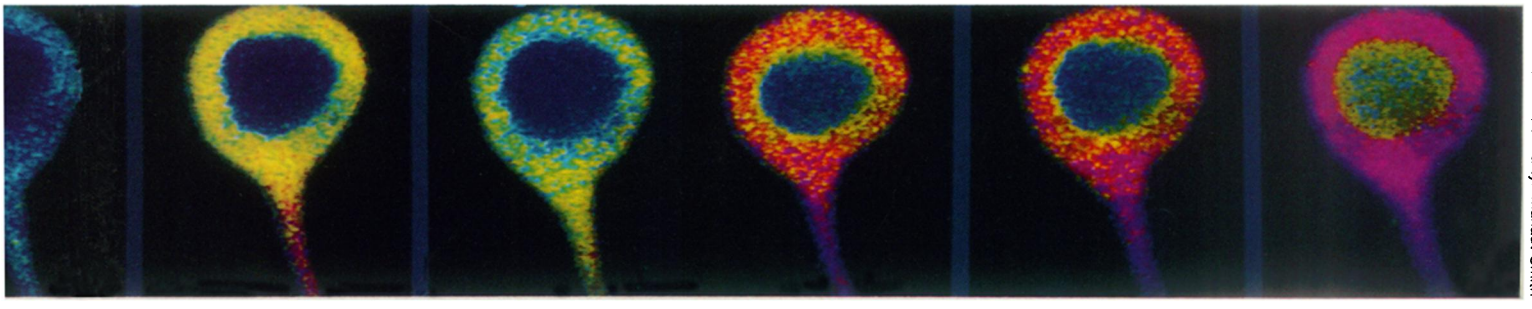
With calcium and, more recently, hydrogen, sodium, and acidity now under chemical surveillance by physiologists all over the world, Tsien and Taylor turned their attention to spying on more complex cellular messengers. Their recent successes attest to the power of combining protein engineering with fluorescence chemistry and of harnessing molecular biology to churn out lots of promising biosensors.

About a decade ago, Tsien targeted cyclic adenosine monophosphate (cAMP), a key molecule that can activate enzymes in a cell or its nucleus. After several years of sizing up and rejecting different proteins as markers for cAMP, he settled on a protein kinase enzyme. Two "catalytic" subunits and two "regulatory" subunits make up the inactive form. When the enzyme links up with cAMP, however, it splits and frees the catalytic units to chop apart other proteins.

Tsien took advantage of this splitting behavior in making his cAMP biosensor. He attached one dye, fluorescein, to the catalytic subunits and a second dye, rhodamine, to the regulatory subunits. In blue light, fluorescein typically glows yellow-green; when it gets close to rhodamine, however, it transfers its energy, causing the rhodamine to fluoresce bright orange. Thus as long as the kinase remains intact, it glows orange. When cAMP breaks the enzyme apart, the glow changes to yellow-green and reveals that the enzyme has been activated. Tsien called this new biosensor FlCRhR.

Tsien put this signaling system to the test. In one experiment, he and Brian J. Bacskai, also at San Diego, injected the tagged kinase enzyme into nerve cells from a sea hare. Working with Beni Hochner of Columbia University in New York City, they then bathed the nerve cells in a neurotransmitter called serotonin, which stimulates the production of cAMP.

The color changes surprised them. Scientists had not known where nerve cells make cAMP. In this experiment, where the neurotransmitter washed over the entire cell, Tsien expected that cAMP



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would appear all over. Instead, "the cyclic AMP goes up quite locally," Tsien reported at the November 1992 meeting of the Council for the Advancement of Science Writing, held in San Diego.

Even in this neurotransmitter bath, the yellow-green indicated that the cell made cAMP first at the nerve-cell tips, where it cleaved the inactive kinase into its active subunits. The migration of the yellow-green glow then indicated that over several hours the catalytic subunits diffused into the nucleus, presumably to activate genes that might then dictate longer term changes in the cell's chemistry.

"These [observations] are of extremely great value," says Eric Kandel, a neurobiologist at Columbia University in New York City. He uses sea hares to study the molecular basis of memory. The results are "giving us initial ideas about how short-term processes can lead to long-term processes," he adds.

In addition, Tsien and his San Diego researchers noticed that when cAMP levels declined, the catalytic subunits gradually vacated the nucleus and eventually relinked with their regulatory counterparts, says Tsien. The fluorescence shifted back to orange.

More recently, Taylor's group has co-opted a different protein, myosin. He and colleagues at Brandeis University in Waltham, Mass., used genetic engineering to alter one amino acid in myosin. They inserted a cysteine near where myosin takes on a phosphate. This phosphate activates the protein. The added cysteine readily latches onto a fluorescent dye that changes color when the myosin links up with phosphate, Taylor explains.

With this modified myosin, Taylor hopes to examine how free-form cells such as white blood cells move. Other research suggests that contractile fibers containing these myosin molecules form at the front of the cell, move toward the nucleus, and then contract. Thus it seems that phosphate-bearing myosin at the rear of the cell may help propel the cell forward. "We're beginning to test that," he says. The new biosensor will indicate whether a gradient of phosphorylation correlates with increased fiber activity.

Taylor and his colleagues have already demonstrated that they can sneak several simple fluorescent analogs into a cell at once. Now they hope to insert four or five kinds of molecular optical biosensors. The multicolored images that result should document in detail how the cell receives, processes, and responds to chemical cues from its environment.

"The theory is that if you can map these changes in time and space as the cell functions, then you can define the mechanism of the cell's processes," Taylor says. That approach contrasts with studies in which biochemists have to grind up the cell before they can measure concentrations of certain chemicals.

As more scientists become aware of the potential of these techniques, use of them should escalate. "You can design and synthesize fluorescent probes [sensitive to] many environments," Taylor says. "By combining the probes with proteins, you take advantage of that chemistry and of molecular biology. This is a major [new] direction for this technology."

He also expects that these research tools will prove useful for screening new drugs, testing the toxicity of substances, and possibly diagnosing disease.

Already, these glowing molecules have proven quite popular. Thousands of scientific reports involving Tsien's calcium marker have been published. Both he and Taylor have spent a lot of time providing their colleagues with molecules and with help in using them.

Nevertheless, such demands have their rewards.

"Our ultimate motivation is [to understand] the biology," says Tsien. "But to some extent, our major contribution is making these molecules. Very few biologists want to build these molecules, but lots want to use them.

"Their aggregate contribution is greater than what we ourselves could ever do." □