# Electron microscopy gets a multicolor makeover

Selective deposition of lanthanide elements picks out molecular features from the background.

PHOTO BY PAUL STEINBACH/UCSD

**FIGURE 1. NOBEL LAUREATE ROGER TSIEN** at a lab bench. The green stains on his gloves give away his activity: synthesizing fluorescent dye.

If spatial resolution were the only figure of merit for imaging instruments, the sub-angstrom performance of the transmission electron microscope (TEM) would leave it with few rivals. For visualizing subcellular structures in biological specimens, though, the ability to highlight specific proteins or other molecules can be just as important. Electron microscopists often present their images in a stunning array of colors. But those colors aren't intrinsic to the specimen being imaged; they're added during image processing to enhance contrast or for purely aesthetic reasons.

Color is intrinsic to light microscopy, which can exploit the plethora of fluorescent proteins whose glows come in a rainbow of hues. And cells can be genetically programmed to produce the colorful markers themselves. In fluorescence-microscope images, the proteins' telltale glows help biologists track the activation of genes and other molecular processes.

The late Roger Tsien (figure 1) of the University of California, San Diego (UCSD), shared the 2008 Nobel Prize in Chemistry for his work developing many of those fluorescent proteins (see PHYSICS TODAY, December 2008, page 20). For years, he and other researchers endeavored to invent an analogous trick for electron microscopes. In a posthumously published paper, Tsien and his UCSD colleagues have outlined just such a method.[1]

The group refined sophisticated biochemical methods to selectively deposit lanthanide elements on subcellular components of interest. Electron energy-loss spectroscopy (EELS), a familiar TEM technique for condensed-matter physicists and materials scientists (see the article by Yimei Zhu and Hermann Dürr, PHYSICS TODAY, April 2015, page 32), can then pick out the element-specific signature of each lanthanide. An overlay of false-color elemental maps on a conventional TEM image highlights subcellular features at a resolution that would be impossibly out of reach for optical microscopes.

## Contrast and color

A TEM image is formed by directing electrons through a thin specimen to a detector. In conventional bright-field imaging, dark areas of the image correspond to electron-dense regions of the sample, and light areas correspond to electron-sparse regions. To enhance contrast, biological specimens are typically stained with an electron-rich heavy metal such as osmium, uranium, or lead.

In 1982 Anthony Maranto at Harvard University suggested that injecting cells with a fluorescent dye that can photooxidize the organic compound diaminobenzidine (DAB) could improve the contrast even more.[2] The oxidation reaction polymerized the DAB into a thin coating that promoted the deposition of an osmium stain. The technique worked, but microscopists still had little control over where in the cell the osmium would land.

In the early 1990s, the UCSD group, led by Tsien and his longtime collaborator Mark Ellisman, attached fluorophores to specific antibodies to locally oxidize DAB, which in turn boosted localized osmium staining of subcellular structures. Furthermore, the researchers discovered that fluorescence photons mediated DAB oxidation indirectly by generating reactive excited states of oxygen.[3] Oxidation of DAB could therefore be optimized by using fluorophores that efficiently generated those states. In subsequent work, the researchers enlarged the palette of DAB-oxidizing fluorophores to include a dye that selectively binds to certain proteins and a fluorescent protein called miniSOG engineered to generate reactive oxygen.[4]

Stanford University's Alice Ting credits the UCSD group with inventing for electron microscopy what the green fluorescent protein was for light microscopy—a prototype genetically encodable tag to label specific proteins and organelles. But she points out that electron microscopy still had another drawback relative to fluorescence microscopy: "It has only one color—black. This second drawback is much more challenging."

The seeds of a solution came to Tsien and Ellisman 15 years ago. They imagined a two-part approach. First, they would paint different organelles, proteins, or other subcellular components with differ-

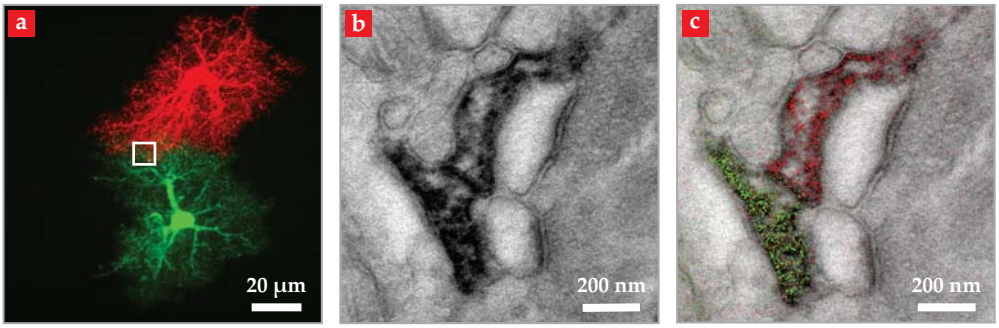


**FIGURE 2. SYNAPTIC ACTIVITY** in the brain is regulated with the help of nonneuronal cells called astrocytes. **(a)** This fluorescence-microscope image reveals two astrocytes that have been injected with different cell tracers. **(b)** A conventional transmission-electron-microscope image of the area inside the white square in panel a has far higher resolution, but cell boundaries and other details are obscured by insufficient contrast. **(c)** Those details are brought out by selectively depositing in each cell a molecular complex that carries cerium (green) or praseodymium (red). Then spatially resolved electron energy-loss spectroscopy maps the elements' locations. (Adapted from ref. 1.)

ent elements. Then they would use an analytical technique such as EELS or energy-dispersive x-ray spectroscopy to distinguish those components. "We started to reason about how much signal we would need, what sort of signal-to-noise we would need," recalls Ellisman. They chose lanthanide elements as the most promising elemental paints, and "Roger, being the brilliant chemist that he was, went to think about ways that we could deposit those by some action induced by light."

In 2003 Tsien tackled the problem as one of his annual Christmas projects. Stephen Adams, a research scientist in Tsien's group, explains that the holiday season was "the one quiet time of the year that he could get back in the lab and do some chemistry at the bench."

Within a year Tsien and his colleagues had designed a structure resembling a molecular cup with two DAB handles, into which they could insert a lanthanide atom. Because the methods for oxidizing DAB also worked for their designer lanthanide–DAB complex, the biochemists had access to various deposition paths. They could thus hope to sequentially deposit different lanthanides on different specific targets.

## Method must serve science

Ellisman explains that the technique showed promise from the beginning: "We knew it would work after the first few times we tried it. We just needed to

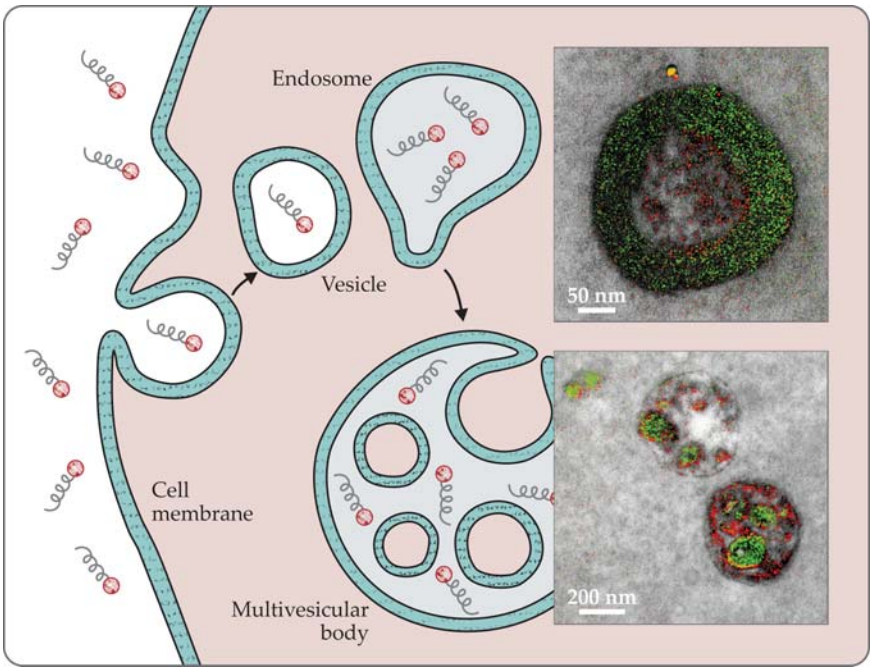


**FIGURE 3. ENDOCYTOSIS** is one process by which cells (shaded area) take in material from the extracellular environment. A small section of the cell membrane folds in on itself and envelops material outside the cell. The section pinches off to form a vesicle that then carries the internalized cargo to an endosome. Through a similar process, the endosome matures into a body that contains multiple vesicles. In the example illustrated here, the molecules brought into the cell are IR-fluorescent dyes (red spheres) attached to small amino acid chains called cell-penetrating peptides. The insets are lanthanide-resolved colorized transmission-electron-microscope images that highlight cell-penetrating peptides (red) inside an endosome (top) and two multivesicular bodies (bottom). (Insets adapted from ref. 1.)

do it better." In addition, Tsien and Ellisman insisted that the technique should be second to the science. Says Ellisman, "We really don't want to see work that comes from our labs be a technical tour de force without a scientifically meaningful conclusion."

With time and labor, those meaningful conclusions did come. In 2012 the UCSD researchers used the lanthanide elements cerium and praseodymium to differentiate the boundary between neighboring astrocytes—multibranched brain cells that provide structural support, produce fuel for neurons, and modulate synaptic activity.

In a slice of mouse brain tissue, they separately injected two astrocytes with different fluorescent markers. In the first cell, the fluorescent dye photooxidized Ce-DAB. Then Pr-DAB was deposited on the second astrocyte via an enzyme-catalyzed oxidation reaction. Figure 2 shows three renditions of the astrocyte pair: a fluorescence-microscope image, a conventional TEM image, and a false-color overlay of EELS elemental maps on the TEM image.

The fine arms of the two astrocytes, separately painted with Ce (green) and Pr (red), can be seen contacting two spines—the light blobs immediately to the right of the astrocytes—of a single synapse. Previously, neurobiologists hadn't known whether two astrocytes can share a single neuronal synapse.

Also in 2012, another experiment investigated the properties of short amino acid chains called cell-penetrating peptides (CPPs), which hold promise for transporting drugs and other membrane-impermeable cargo into cells. Many researchers suspect that CPPs enter the cell through a process called endocytosis, illustrated in figure 3.

In the first experimental step, the researchers programmed HeLa cells (a human cancer-cell line that's commonly used in research labs) to express a fusion of the fluorescent miniSOG protein with another protein, RAB5A. The RAB5A protein is known to attach to the surface of organelles called endosomes. Next, the researchers incubated the mutant HeLa cells in a bath containing CPPs attached to an IR-fluorescent dye.

After two hours, fluorescence microscopy showed that the CPPs had been taken up by the cells, and the IR-dye and miniSOG fluorescence appeared collocated. The researchers then irradiated the sample with 480 nm light to activate the miniSOG and oxidize Ce-DAB. After washing away unreacted Ce-DAB, they added Pr-DAB and irradiated the sample with 680 nm light to turn on the IR dye. The colorized EM images in the two insets of figure 3 show endosomes with Ce (green) and Pr (red) signatures that label RAB5A and CPP, respectively.

The upper inset, in particular, shows that RAB5A is localized to the endosome's membrane and the CPPs are contained inside. The peptide-containing endosome confirms that the cells internalized the CPPs through endocytosis. As endosomes mature into multivesicular bodies (lower inset), RAB5A migrates to vesicles formed by the inward budding of the endosomes' membranes.

## More colors to come

Having added two colors to transmission electron microscopy, the researchers are on the hunt to add more—that is, to deposit more elements. The problem is that once deposited, the polymerized DAB itself becomes a nucleation site, so subsequently deposited elements can end up in unwanted locations. The UCSD researchers are working to develop chemical means to prevent such cross talk. If they succeed, the researchers should be able to paint cell components with three or more colors.

At the same time, Ellisman is looking into adding on other analytical microscopy techniques such as energy-dispersive x-ray spectroscopy. "It might give us an opportunity to see additional elements," he explains. "I think this is just the beginning of a strategy that is going to bear more fruit."

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## References

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