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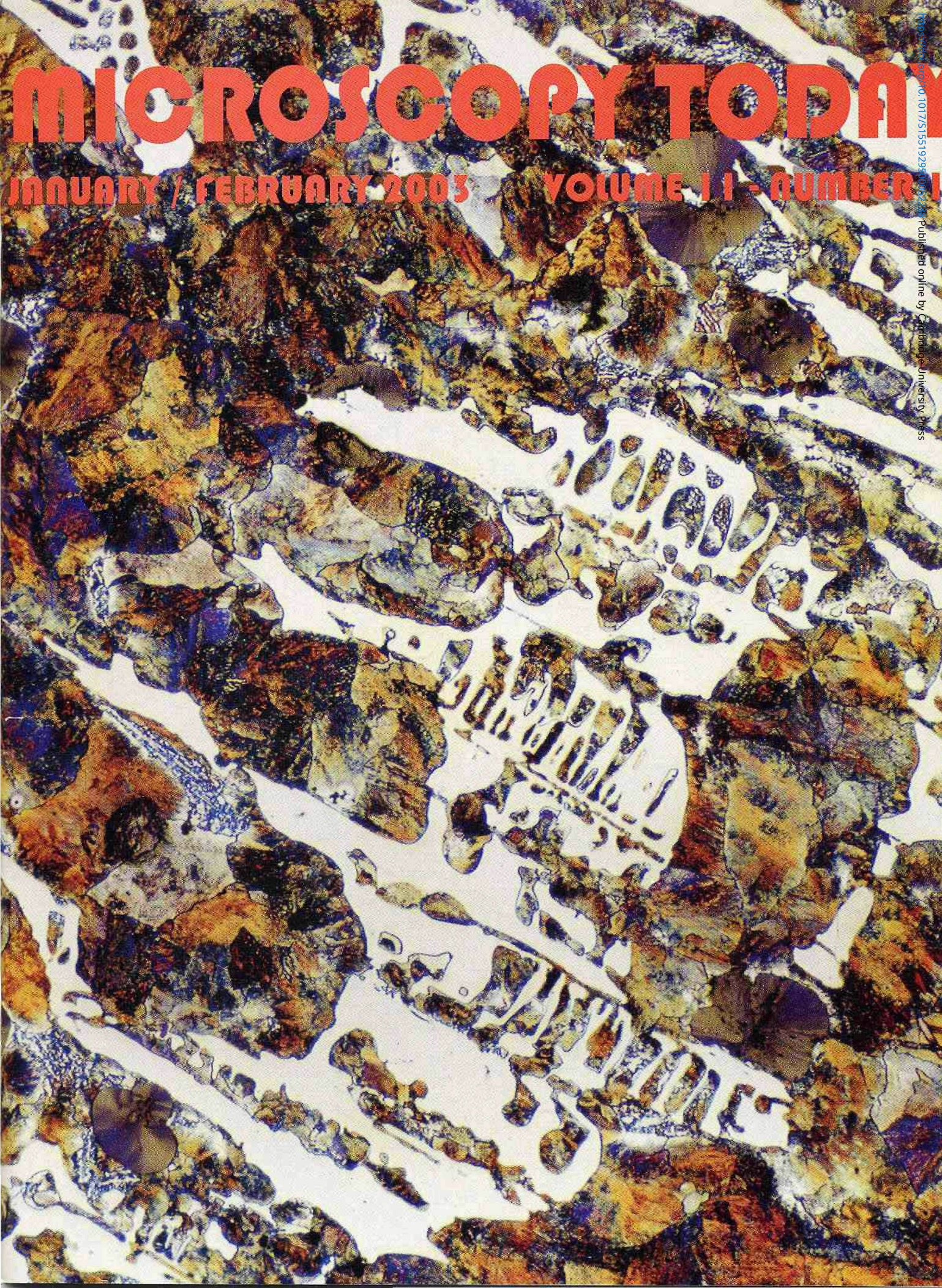

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Out with the Old, In with the New

Stephen W. Carmichael,[1] Mayo Clinic

Temporal resolution has long been a challenge to microscopists. Certainly, spatial resolution has occupied center stage, but we're all concerned about what happens over time in a biologic system, for example, a cell. Tags such as green fluorescent protein (GFP) have been used with confocal microscopy and other light microscopic techniques to achieve outstanding temporal resolution, but good spatial and temporal resolution have proven to be difficult to achieve simultaneously. This has been accomplished in a remarkable study by Guido Gaietta, Thomas Deerinck, Stephen Adams, James Bouwer, Oded Tour, Dale Laird, Gina Sosinsky, Roger Tsien, and Mark Ellisman, who demonstrated a pulse-chase technique that correlates with both fluorescence and electron microscopy.[2]

The keys to the technique developed by Gaietta *et al.* are two dyes referred to as FlAsH (Fluorescein Arsenical Hairpin binder) and ReAsH (Resorufin Arsenical Hairpin binder). FlAsH fluoresces green, ReAsH fluoresces red, and both molecules are much smaller than GFP and similar dyes, allowing them to be active intercellularly without interfering with cell functions. Both dyes contain two atoms of arsenic, but application of micromolar amounts of an antidote avoids toxicity. These dyes have a high specificity for a sequence of amino acids that contains four cysteine molecules in a certain order. The protein of interest is modified by insertion of this tetracysteine tag and the fluorescent dyes then specifically label the protein.

Connexin molecules are the protein constituents of gap junctions. Six connexins form a hemichannel (or connexon) that docks with a connexon from an adjacent cell to constitute a gap junction. Each junction contains a central pore to allow ions, signaling molecules, and metabolites smaller than one kiloDalton in size to pass between the two communicating cells. Hundreds to tens of thousands of gap junctions assemble into plaques. The number of gap junctions changes constantly in plaques as the cells dynamically modulate this connectivity. Connexins are known to have a half-life of less than 5 hours. But how are connexins added to and taken out of the plaques?

Gaietta *et al.* engineered a recombinant connexin so that the tetracysteine tag was on one end of the molecule. Several different methods were used to demonstrate that the tagged connexins functioned properly in cultured cells. A modification of the commonly used pulse-chase technique was then applied to label the engineered connexins with the green dye, wash out the unbound dye, then stained with the red dye 4 or 8 hours later. At 4 hours, the oldest connexins, stained green, formed circular cores, surrounded by a ring of red, with remarkably little mixing. By 8 hours, the red periphery gradually replaced the central green core, with small green intracellular punctae (presumably endocytosed connexins). Reversing the order of green and red dye application reversed the color pattern. This lead to the conclusion that newly-synthesized connexins assemble into channels at the edges of a gap junction plaque and move in an orderly radial flow toward the center of the plaque, where they are removed by endocytosis.

ReAsH not only fluoresces, it can also be used for the photoconversion of diaminobenzidine (DAB), allowing direct correlation of images in living cells with high resolution electron microscopy. The ReAsH labeling was visualized in cultured cells which were then fixed with glutaraldehyde, treated with DAB, exposed to bright light, then treated with osmium tetroxide which binds to the converted DAB. The osmium could be localized in electron micrographs. The results suggested that connexins move from the Golgi to nonjunctional sites of the plasma membrane, then flow to the plaque, later to be removed into lysosomal-like bodies.

The approach developed by Gaietta *et al.* allows the study of the life cycle of proteins within cells. This correlated light and electron microscopic technique has considerable applicability to many systems since the tag (a tetracysteine motif) is tiny, genetically encoded, and versatile. These impressive studies will surely open avenues to observe the complexity and sophistication of membrane protein assembly and turnover! ■

References:

[1] The author gratefully acknowledges Drs. Guido Gaietta and Mark Ellisman for reviewing this article.

[2] Gaietta, G., T.J. Deerinck, S.R. Adams, J. Bouwer, O. Tour, D.W. Laird, G.E. Sosinsky, R.Y. Tsien, and M.H. Ellisman, Multicolor and Electron Microsc. Imaging of Connexin Trafficking, *Science* 296:503-507, 2002.

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ABOUT THE COVER

By Janina M. Radzikowska
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Ledeburite in nodular cast iron. This image won "Third in Class" in the International Metallographic Society, International Metallogrphic contest, "Artistic Microscopy" category at the Microscopy and Microanalysis - 2002 meeting in Quebec, Canada.