

The 53rd Annual Meeting and Symposium of the Society of General Physiologists *New Optical Methods in Cell Physiology*

(organized by Brian M. Salzberg and Jack H. Kaplan)

In his introductory remarks to open the symposium "New Optical Methods in Cell Physiology," held in Woods Hole, MA, September 9–11, 1999, Brian Salzberg noted that 15 years of technological innovation had intervened since the Society of General Physiologists had held its first symposium on this topic, entitled simply "Optical Methods in Cell Physiology" (organized by Paul De Weer and Brian M. Salzberg). That first meeting highlighted work with voltage-sensitive dyes (enabling noninvasive recording of electrical activity in cells, or groups of cells), and with photolabile (e.g., caged) compounds (permitting sudden release of, say, ATP or Ca^{2+}), and featured the introduction of the highly successful ratiometric fluorescent indicators, Fura-2 and Indo-1, which opened the door to routine measurement of the transient changes in intracellular $[\text{Ca}^{2+}]$ that underlie so many facets of cellular signal transduction. The latest symposium stood squarely on the shoulders of that previous one, and showcased several sparkling new methodologies, some of which have already yielded spectacular results.

One of these is the recent development of genetically encoded reporter molecules, which offers the possibility of temporally and spatially controlling the expression of optical probes in transgenic animals. Gero Miesenböck (Memorial-Sloan Kettering Cancer Center) reported on efforts to design genetically encoded probes for following synaptic activity in collections of neurons. Initial trials employed a secretory form of luciferase fused to the synaptic vesicle-associated membrane protein VAMP: externalization of the luciferase during synaptic activity did yield measurable flashes, but they were weak and depended on the presence of the exogenous substrate, luciferin. Far more successful are the second generation probes that comprise VAMP fused to pH-sensitive green fluorescent protein (GFP) mutants (pHluorins), whose emission varies with intravesicular pH. Because H^+ -ATPases rapidly acidify the lumen of synaptic vesicles upon endocytosis, real time measurements of pHluorin emission provide a convenient monitor of synaptic vesicle exocytosis and endocytosis and, hence, of synaptic activity (Miesenböck and Tim Ryan, Cornell University Medical College). Roger Y. Tsien (University of California, San Diego), who introduced the workhorse Ca^{2+} indicators Fura-2 and Indo-1 at the last Optical Techniques symposium, presented the

credentials of the next generation of Ca^{2+} indicators, dubbed cameleons. These genetically encoded indicators comprise a single fusion protein in which calmodulin (CaM) linked to cyan GFP is fused to a CaM-binding peptide (M13, the CaM-binding domain from MLCK), which is linked to yellow GFP. A rise in intracellular $[\text{Ca}^{2+}]$ enhances Ca^{2+} occupancy of CaM, which then binds to M13. The increased proximity of CaM and M13 is monitored by enhanced fluorescence resonance energy transfer (FRET; see below) from cyanGFP-CaM to M13-yellow GFP. It appears that the linked CaM and M13 moieties in the cameleons predominantly interact with each other, rather than with other CaM-binding proteins or free CaM, so they report Ca^{2+} concentration with little disruption of endogenous CaM-dependent signaling pathways. Because the cyan-GFP can be excited by single- or two-photon approaches, even at video rates (30 frames/s), by using near infrared wavelengths (~ 790 nm), cameleons can be used for imaging $[\text{Ca}^{2+}]$ changes in optically opaque tissues, including, eventually, neurons in brain slices.

Multiphoton techniques represent a powerful extension of the fluorescence imaging methodology introduced at the first symposium, and they were discussed by several speakers at this new meeting. Photodamage and bleaching are two major drawbacks of the high intensity ultraviolet illumination needed for obtaining bright images using single-photon methods. By permitting excitation of a fluorophore within a targeted femtoliter volume by the simultaneous absorption of two lower energy (infrared) photons, neither of which has sufficient energy to excite by itself, it is possible to collect fluorescent images while minimizing photobleaching and photodamage, both in and outside the targeted spot, usually at the focus point of the laser beam. The longer wavelengths that are needed to reduce photodamage also penetrate opaque material well, as they are much less affected by scattering. The useful working depth, however, is ultimately limited by the exponential decrease in light intensity with distance, which leaves the surface tissue vulnerable to photodamage when the targeted spot lies deep. As already mentioned, R.Y. Tsien and colleagues have used scanning two-photon excitation of cameleons to perform ratio imaging and optical sectioning of changes in cytoplasmic and nuclear (via cameleons bearing a nuclear targeting sequence) $[\text{Ca}^{2+}]$

in preparations such as beating myocytes and stimulated HeLa cells. A bold extension of this approach was described by Winfried Denk (Lucent Technologies, Bell Labs), who is developing a miniaturized two-photon scanning microscope, small enough to be attached to the head of a freely moving rat. This system is already capable of imaging dendrites and dendritic spines of somatosensory cortical pyramidal neurons filled with fluorescent Ca^{2+} indicator, as well as dynamic Ca^{2+} signals in dendrites by use of a line-scan technique. When the cerebral blood capillaries are stained with rhodamine-dextran, individual blood cells cast readily observed shadows, whereby line-scan measurements allow determination of capillary blood flow, one blood cell at a time, in an awake, behaving rat. Britton Chance (University of Pennsylvania) and Enrico Gratton (University of Illinois) also discussed imaging and spectroscopic techniques that border on multiphoton approaches. Their methods combine near infrared wavelengths, for tissue penetration depth, with fast scanners and detectors to follow changes in blood flow noninvasively, through absorption measurements of deoxy- and oxyhemoglobin. Both speakers described observations of blood flow changes associated with mental activity (e.g., backward spelling and long division) in the human brain, and both emphasized the advantages optical methods offer for enhanced detection of tumors in muscle and brain (Chance), and of mammary tumors (Gratton).

The two-photon effect can also be harnessed to minimize the photodamage associated with photolytic reactions used to uncage biologically active molecules, since the quadratic dependence on light intensity restricts photolysis to the focus point of the laser. Two-photon photochemical uncaging allows rapid, specific, localized release of compounds at will. For example, two-photon infrared excitation can yield high-speed ($45,000 \text{ s}^{-1}$) tightly spatially restricted release of Ca^{2+} from its DMNPE-4 (nitrophenyl-based) cage, as described by Graham Ellis-Davies (MCP-Hahneman University), who showed that such release in individual dendrites of cerebellar Purkinje cells causes long-term depression, and that two-photon release of caged Ca^{2+} in cardiac myocytes can generate signals mimicking SPARKs, the natural unitary Ca^{2+} release signal. Ellis-Davies also reported on the use of two-photon release (rate $\sim 100,000 \text{ s}^{-1}$) of caged glutamate from DM-CNB-glu (nityrobenzyl-based cage) to map the distribution of glutamate receptors on cultured hippocampal neurons by locally eliciting excitatory postsynaptic currents. Another improved caged glutamate, based on bromo-hydroxycoumarin, with larger two-photon action cross section, was discussed by R.Y. Tsien, who showed that it permits high-resolution three-dimensional mapping of glutamate sensitivity even of neurons deep in the light-scattering environment of intact brain slices. Jeffery W. Walker (University of Wisconsin) described efforts to extend the range of molecules amenable to photo-modification to include

caged peptides and lipids involved in signaling pathways. Photocleavage can be used to generate active peptides by detaching a fused inhibitory peptide, by uncyclizing an inactive cyclic peptide, or by removing a photolabile moiety added to protect key amino acid side chains. And peptides can be inactivated by release of a photolabile modifier of key residues. As an example, Walker found that photoinactivation of CaM-dependent kinase (CaMK) in eosinophils stopped their motility within 1–2 s, implying that CaMK's target, MLCK, normally is subject to continuous, rapid phosphorylation and dephosphorylation. Walker also reported on a bromo-hydroxycoumarin-based caged analogue of diacylglycerol, an important second messenger in signal pathways impinging on protein kinase C. By exploiting the sharp localization of the two-photon effect, he found qualitatively different responses following photorelease of caged dioctanoylglycerol near the surface membrane, as opposed to deep in the sarcoplasm, of rat ventricular myocytes, though PKC inhibitors prevented both kinds of response. The differing responses may be indicative of differences in the localization of specific signaling components, such as PKC isoforms.

Much of biology can be reduced to studies of the rich repertoire of protein-protein interactions, whether between different proteins, between proteins of the same kind, or between different parts of the same protein. Several speakers described application of powerful FRET methods for probing those interactions. The methods all depend on the ability of fluorescent light emitted by an excited donor fluorophore to excite a nearby acceptor fluorophore, when the two fluorophores have appropriate excitation and emission spectra and are sufficiently close to each other (usually within a few tens of angstroms). Given appropriate spectra, recorded emission from the acceptor due to energy transfer provides information on the distance between the two fluorophores. Pancho Bezanilla (UCLA) presented recent results obtained using one of these methods, called lanthanide-based resonance energy transfer (LRET), to examine the movements in a voltage-gated K^+ channel associated with opening and closing of its pore. Bezanilla and colleagues engineered cysteine residues into voltage-gated *Shaker* K^+ channel subunits, labeled them with a 4:1 mixture of maleimide-based donor and acceptor fluorophores, and measured gating-mediated changes in the distance between residues labeled with donor and those labeled with acceptor fluorophores, in the same tetrameric channel. Because of the fourfold symmetric subunit arrangement around the pore, labeling a single cysteine gave two distances reflecting energy transfer either between adjacent subunits or across the pore. Labeled cysteines at some sites reported increases in distance, at some other sites decreases in distance, and yet at others no change, when voltage was altered to open the channel. Together with other data, these measurements suggest that the highly

charged S4 segment (the proposed voltage sensor) twists within the plane of the membrane, rather than plunging through it, during channel opening. FRET measurements can also provide a convenient way to follow protein folding reactions, as illustrated by work from William DeGrado's lab (University of Pennsylvania) on a model protein labeled with both rhodamine 6G (donor) and Texas red (acceptor) fluorophores, and attached by the negative charge at its COOH terminus to an NH_2^+ -silanized glass coverslip. The degree of folding of the protein was assayed by FRET as folding was manipulated by varying the concentrations of urea or by varying pH. Philippe Bastiaens (Imperial Cancer Research Fund) reported on the use of FRET to assay changes in the proximity of proteins bearing different fluorescent labels during activation of signal transduction pathways within live cells. In this application, changes in cell geometry can distort the results, because the intensity of the measured signal depends on probe concentration and on the path length of the light. Bastiaens avoided this distortion by determining the nanosecond fluorescence excited state lifetimes, which are independent of concentration and light path length, using frequency domain measurements. The resulting images of live cells allow pixel-by-pixel assay of interactions between signaling proteins tagged with fluorescent probes, such as GFP-tagged PKC and its substrate ErbB1. The location of activated PKC in cells can be assayed, after fixing, by FRET between PKC and an antibody to phosphothreonine 250 in PKC, which is subject to autophosphorylation when PKC becomes activated. As already mentioned, FRET underlies the activity of cameleons, the new genetically encoded Ca^{2+} indicators. Tsien also described the use of FRET as the basis for a novel transcriptional reporter system in which expression of β lactamase is monitored by its cleavage of a membrane-permeant green fluorescent substrate that disrupts FRET and yields a blue fluorescent product. The enhanced sensitivity provided by the enzymatic amplification makes this reporter system suitable for clonal selection and separation of cell lines using flow cytometry, or for analysis of gene expression patterns in transparent zebra fish embryos.

Near-field methods allow collection of optical images with spatial resolution below the wavelength of light by exploiting the evanescent field generated either when light is passed through a subwavelength aperture or when light undergoes total internal reflection upon striking a reflective surface at a shallow angle. Robert C. Dunn (University of Kansas) and Jay K. Trautman (Pralux) both described the state-of-the-art in near-field scanning optical microscopy (now with video-rate scanning) in which light is passed through the 30–100-nm aperture at the tip of a drawn-out, etched light guide with sides made opaque with a thin layer of deposited aluminum. All surfaces of the microprobe are smoothed to minimize loss from light scattering, and feedback is used

to hold the probe tip very close to the object surface to keep it within the exponentially decaying evanescent field. The feedback signal can be force, as in atomic force microscopy, or light reflected from the surface of the object (by comparing the signal source and its reflection), allowing simultaneous measurements of object topography and fluorescence. A recent extension of the technique is near-field FRET, in which molecules labeled with a donor fluorophore (e.g., fluorescein) are excited via light passed through the probe tip, and transfer energy to an acceptor fluorophore, such as rhodamine, coated onto the probe surface: the emission from the acceptor is then collected. The inverse sixth power dependence of this energy transfer on distance between donor and acceptor molecules makes this a promising avenue for further enhancing the spatial resolution of fluorescence imaging. The other near-field technique, total internal reflection fluorescence microscopy (TIRFM), permits imaging of an optical section only ~ 100 -nm thick (as opposed to the 500–600-nm sections imaged in confocal microscopy) in which fluorophores are excited by the evanescent field, as discussed by Daniel Axelrod (University of Michigan). One benefit of the narrow optical section (which can be reduced to $1/20$ of the wavelength of light for very low incident angles) is the exclusion of background signals and consequent increased signal-to-noise ratio. Axelrod described the application of the technique to the study of z-axis motions of single adrenaline-containing secretory granules near the surface membrane of chromaffin cells: the granules move three orders of magnitude more slowly than appropriate for free diffusion, and with low autocorrelation of velocity, providing little evidence for persistent motion. By exciting a highly oriented membrane-embedded fluorophore (such as carbocyanin) with light polarized alternately perpendicular to, and parallel to, the reflective surface, the curvature of the object membrane can be followed in time, so that processes like exo- and endocytosis may be followed in time-lapse images. A third application of TIRFM presented by Axelrod was use of the evanescent wave to cause local photobleaching, after which the kinetics of fluorescence recovery provide information on rates of movement of the target fluorophore-tagged molecules.

David DeRosier (Brandeis University) presented electron cryomicroscopy as the method of choice for determining three-dimensional structures of macromolecules, or even of large complexes ($<10^7$ D), at molecular (and occasionally atomic) resolution. Because the electron dose has to be low enough to avoid destroying the object, a single molecule cannot be imaged, and so information from a large number of identical molecules must be averaged. To increase resolution, corrections can be applied for slight displacements of individual molecules and the average image then reinterpolated. DeRosier showed how electron cryomicroscopy can be

used to assemble images of organelles $>10^7$ D by a “divide and conquer” approach that first obtains images of the component parts, an approach he illustrated with studies of the rotary bacterial flagellar motor. It is anticipated that much novel information about the organization and function of complex macromolecules will come from such efforts to dock smaller structures, many eventually at atomic resolution, into larger outline maps.

Several speakers discussed dynamic microscopies, which can provide information about molecular order and/or motion. Shinya Inoué (Marine Biological Laboratory) provided an historical perspective on the development of instruments such as the polarizing microscope, the speckle confocal scanning microscope, the centrifuge polarizing microscope, and the dynamic aperture modulation microscope—instruments designed to permit observation and measurement of submicroscopic fine structures, like microtubules and actin filaments, inside living unstained cells, so bridging the gap between optical and electron microscopy. His colleague, Rudolf Oldenbourg (Marine Biological Laboratory) described a new polarized light microscope in which the orientation-dependent contrast of birefringence images is overcome by online adjustment of the retardance of liquid crystal filters, allowing measurement of local molecular and atomic order. Oldenbourg has used the new microscope to show that radial actin filaments are polymerized at the tips of filopodia in growth cones (in *Aplysia* bag cell neurons), and that the whole actin carpet then undergoes retrograde transport. The latest versions of commercially available laser-scanning confocal microscopes, and their application to studies of molecular motion using fluorescence recovery after photobleaching (FRAP) or fluorescence correlation spectroscopy, were discussed by Ulrich Simon (Carl Zeiss Jena). Fluorescence lifetime measurements were exploited by Arthur E. Johnson (Texas A&M University) to see whether fluorescent probes incorporated into nascent peptide chains sense a hydrophilic or a hydrophobic environment during their interaction with the translocon, which is the site of ribosome attachment, of insertion of transmembrane proteins, and of translocation of secreted proteins. The incorporated probes were found to sense an aqueous environment while in this translocation pathway across the endoplasmic reticulum (ER) bilayer, and collisional quenching measurements suggest that these translocon pores are 40–60 Å wide, and are closed at the cytoplasmic side by the ribosome and at the other side by the ER luminal protein BIP in the presence of MgATP.

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The final optical technique, discussed by Justin E. Molloy (The University of York), was the use of laser tweezers to make microscopic measurements of displacement and force in functional assays of single-molecule interactions between actin and various myosins coupled at low density to polystyrene microspheres. For some myosin isoforms, two phases of movement (each with an amplitude in the nanometer range) can be discerned during each interaction with an actin, and the duration of the second phase was found to vary with [ATP]. Molloy suggested that the two phases represent separate mechanical states that contribute to the working stroke of the acto-myosin motor. Correlation of biochemical and mechanical cycles is presently being pursued by combining the laser tweezer measurements with fluorescence microscopy using fluorescent ATP analogues.

The now traditional New Ideas, New Faces session was organized a little differently for this symposium, and comprised the two talks on uncaging by young investigators Graham Ellis-Davies and Jeffery Walker, together with a third talk by a speaker selected by the organizers from the free abstracts submitted to the meeting. This HOT Abstract talk was presented by Jurgen Klingauf (University of Colorado), who described the use of fluorescence correlation spectroscopy to examine movements of synaptic vesicles, whose membranes were labeled with the fluorescent styryl dye FM1-43, into and out of an ~ 0.05 fL cigar-shaped detection volume ($\sim 1/10$ bouton volume) in boutons of cultured hippocampal neurons. By recording for long periods (requiring switching the laser on and off to avoid photobleaching), Klingauf was able to observe a slow autocorrelation in the motion of vesicles, with a characteristic time of ~ 11 s, too slow for free diffusion. And because the myosin light chain kinase blocker ML-7 almost arrested the vesicles, and cytochalasin D slowed the vesicle movements, whereas colchicin had no effect, Klingauf inferred that actin-like molecules are involved in moving the vesicles.

In his keynote address, Watt W. Webb (Cornell University), a pioneer in the application of new optical techniques, presented a broad overview of past and current developments in the field. His status as technological pioneer was amply confirmed not only by his prescient early involvement in several fluorescence microscopies and by the fact that many of the symposium speakers were his former students or postdoctoral collaborators, but also by his highlighting of his talk with a futuristic green laser pointer!