The 64th Symposium of the Society for General Physiologists: optogenetics and superresolution microscopy take center stage

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Novel optical technologies applicable in cellular physiology and biology were the focus of the 64th annual meeting of the Society of General Physiologists, held September 8–12th, 2010, at Woods Hole. This is the third time the Society has taken up this "nonbiological" theme as a subject for its annual meeting and symposium. The last time was in 1999, and the developments in the field of optical methods in the past dozen years have been breathtaking, so it was considered time to revisit this topic. The meeting, organized by Graham Ellis-Davies of the Mount Sinai School of Medicine, brought together many of the pioneers and leaders of this burgeoning field (http://www.sgpweb.org/symposium2010.html). The symposium had two overarching themes, photochemical sensing and photochemical actuation of processes in living cells and animals (Fig. 1). These two general themes were played out in four interacting subdisciplines: (1) two-photon (2P) imaging of living cells, in culture and in vivo; (2) novel microscope technologies that enable either high speed or spatial "superresolution" imaging of cellular structure and function; (3) 2P uncaging of signaling molecules in living cells; and (4) genetically encoded optical actuators and probes.

Roger Tsien delivered the first symposium keynote address on the evening of September 8th. Tsien occupies a unique place in the history of optical methods for cell physiology and is the only person who has been a speaker at all three SGP conferences on this subject. His visionary use of organic chemistry and molecular biology has transformed the science we do, including the development of an amazing array of optical sensors and actuators of cellular function, such as the first fluorescent Ca dyes, of membrane-permeant dyes, the first caged Ca, the first spectral variants of GFP, the first indicator for cAMP, circular permutations of GFP, the first genetically encoded indicator based on GFP, the first caged glutamate sensitive to 2P excitation, the first genetically encoded indicator of kinase activity, etc., etc. Tsien was co-recipient of the Nobel Prize in Chemistry in 2008 with Osamu Shimomura and Martin Chalfie for green fluorescent protein (GFP). In the first part of his talk, Tsien summarized the development of the abundant toolkit of fluorescent proteins (FPs) (Tsien, 2010), including recent infrared variants. He then turned to ongoing work developing contrast agents that would function on two radically different scales: for electron microscopy (EM), and for tumor cells. Correlation of structures seen in live cell fluorescence imaging with posthoc EM of fixed cells is extremely challenging. Tsien and co-workers have developed a new genetically encoded tag that can enable EM contrast. Like GFP, the new probe ("miniSOG") can be attached to a wide range of cellular proteins without disturbing their cellular distribution and function. However, unlike GFP, the small (106–amino acid) protein is also a very effective singlet oxygen generator. Thus, when exposed to blue light, miniSOG oxidizes nearby lipids and proteins, generating EM contrast in fixed tissue of previously imaged cellular structures. Only the addition of diaminobenzidene is required for this process, making miniSOG a potential alternative to much larger antibody-based nanoparticle probes. For surgeons to detect the margins of tumors during resection is a matter of subjective judgment, as there is no obvious visual contrast between healthy and cancer-infected tissue. Tsien realized that because certain polycationic peptides readily penetrate cells ("cell-penetrating peptides" [CPPs]; Jiang et al., 2004), they could be used for tumor cell tagging. Tsien's group has chemically synthesized CPPs with neutralizing polyanionic peptides connected by a peptide loop that is cleaved by a peptidase highly expressed in tumors, creating activatable CPPs (ACCPs). Tsien showed that dendrimeric nanoparticles (red dendrimers) coated with ACCPs can deliver fluorescent payloads to tumor cells (Olson et al., 2010). When complemented with a similarly effective blue contrast agent for nerves, this new visualization method enabled real-time fluorescence imaging in surgery that improves the completeness of tumor resection (Nguyen et al., 2010). Tsien commented that this work could be his most important contribution to science.

On September 9th, **Oliver Griesbeck** of the Max Planck Institute for Neurobiology in Martinsried reported recent research aimed at creating, optimizing,

and using troponin C (TnC)-based genetically encoded calcium indicators (GECIs). Although the small organic Ca dyes developed by Roger Tsien in the 1980s are extremely effective for 2P imaging of calcium in vivo, such dyes can only be used in acute experiments. If one wants to use longitudinal 2P imaging of activity in the CNS, GECIs are required. The first GECIs were developed in Tsien's laboratory and used calmodulin as the Ca sensor. Griesbeck (a former Tsien postdoc) reasoned that Ca sensors based on skeletal and cardiac muscle isoforms of TnC might be more effective for neuronal Ca imaging because CaM is abundant in the CNS. The goal of the sensor design has been to link a pair of TnC molecules with a select pair of FPs, such that calcium binding alters their intermolecular distance, thereby changing their FRET status. Many cycles of engineering have led to "TN-XXL," which links CFP and YFP to the ends of a tandem of TnCs whose first and second EF hands have been replaced with the third and fourth to yield an overall K_d for calcium of ~0.2 μM (Mank et al., 2008). Expression of TN-XXL in *Drosphila melanogaster* eyes has shown it to be a sensitive, reliable probe of neuronal activity (Reiff et al., 2010). Griesbeck's group has expressed TN-XXL in mice under the CAG promoter and used the mouse to measure the activity of visual cortex simple cells. The wide diversity of TnCs from different
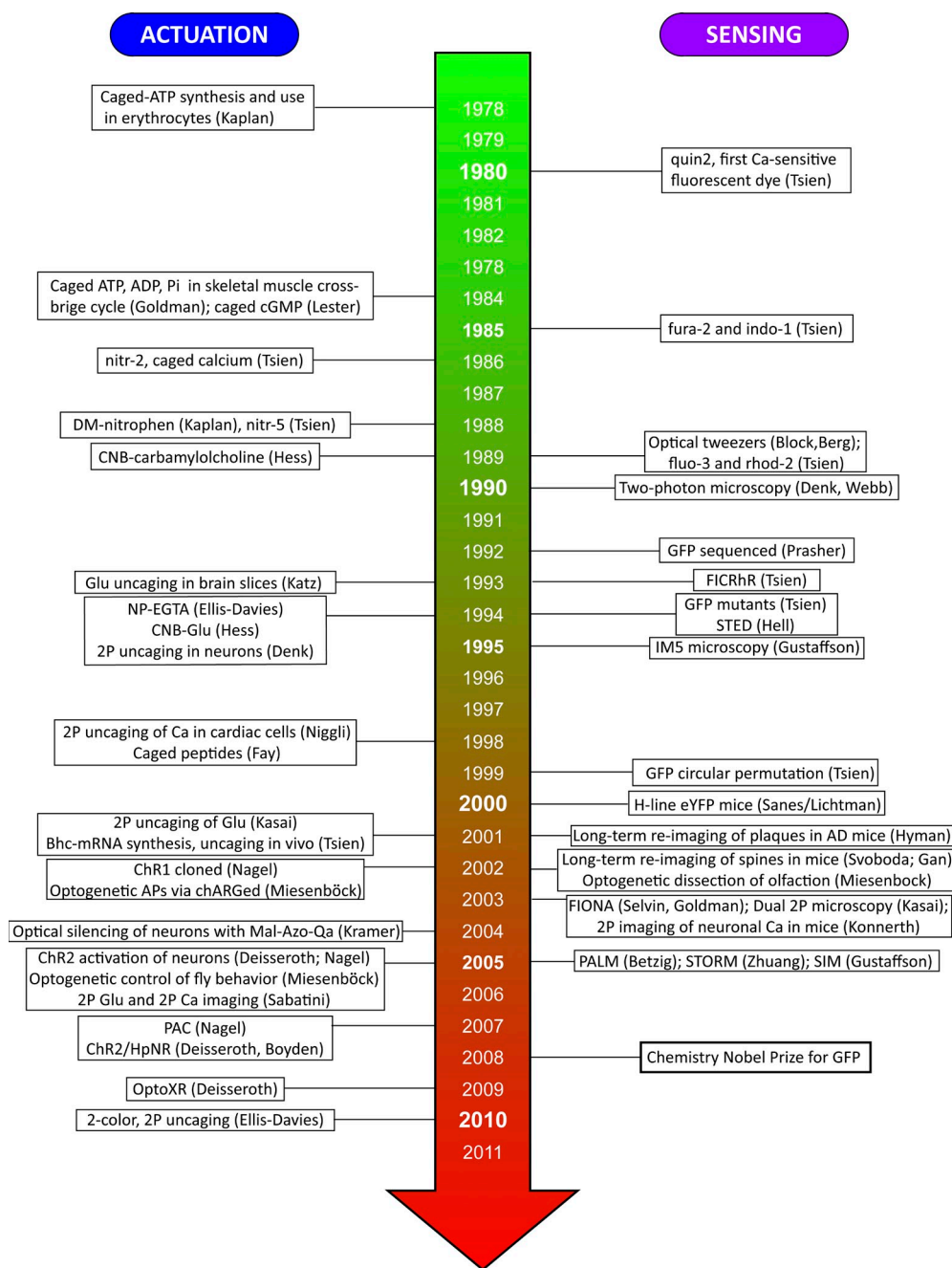

Figure 1. Timeline of appearance of optical methods for actuation and sensing of cellular processes from 1978 to the present. Each method has been tagged with the name (in brackets) of the corresponding author of the paper in which the method was introduced. We regret any inadvertent omissions.

species holds promise of creating similar calcium sensors with a range of binding affinities and other properties.

Fritjof Helmchen of the Brain Research Institute, University of Zurich, reported on efforts to optically measure the neurodynamics of populations of mouse whisker barrel neurons at high temporal bandwidth using calcium-induced fluorescence change of dyes or biosensors measured with in vivo 2P microscopy (Kerr et al., 2005, 2007). Traditionally, one has to live with a tradeoff if one wants to perform rapid imaging, loss of spatial information. Confocal microscopes use galvonometers to scan in x and y, and the inherent inertia of these mirrors impedes rapid wide-field scanning. Helmchen and coworkers have developed a 2P microscope using dual acousto-optical deflectors (AODs) that enables high speed, random-access scan patterns to be generated. Because scanning using AODs allows one to move the imaging beam to an arbitrarily defined set of points, Helmchen and his colleagues have been able to record the activity of 800 different cells at 200 Hz, 50 cells at 300 Hz, and 20 cells at 1 KHz (Grewe et al., 2010). Further advances in the works include genetically encoded calcium biosensors and the use of a chronic glass window in the skull, which allow high quality recording from the same neurons over many experimental sessions (Lütcke et al., 2010). This has enabled measurements of the plasticity of large populations of cortical neurons in response to whisker trimming. This plasticity is manifest as a shift in the responsivity to spared whiskers. A target for future work is achieving deeper tissue penetration, perhaps through the use of infrared-sensitive FPs.

Na Ji of Eric Betzig's research group at Janelia Farms reported on the application of adaptive optics (AO) technology to improve optical resolution during deep tissue 2P imaging. A central problem for all forms of optical microscopy is that during the propagation of electromagnetic radiation from a "point source," the definition becomes blurred. AO is a technology first developed by astronomers to sharpen the quality of images of stars and other astronomical objects. A focusing mirror is subdivided into a large number of independently and rapidly adjustable refractile elements, allowing for local and real-time correction of wavefront aberrations across the entire telescope aperture. Na Ji showed design and development of a new, sophisticated AO 2P microscope for fluorescent imaging of muscle and brain. Images of tissue to depths of 450 μm with resolution approaching the diffraction limit were obtained, showing that the AO approach almost completely overcame the scattering of light that is normally unavoidable in such deep tissue imaging. The current systems using LCD panels for AO elements are limited to 10 Hz for corrections, but systems using deformable mirrors expected to reach 100 Hz are expected to be available soon.

The symposium next turned to talks given by the two Cranefield Award winners, postdoctoral fellow **Xiao-Dong Zhang** of Tsung-Yu Chen's group at UC Davis and recent PhD recipient Jill Jensen of Bertil Hille's group at the University of Washington.

Josh Sanes of Harvard University led off the evening session with a talk focused on the molecular determinants of the laminar specificity of the neuronal wiring of the retina and retinotectal system. Large molecular histochemical scans for candidates in the Ig superfamily in developing chick retina yielded four homologous homophilic adhesion molecules expressed in specific retinal sublaminas: Sdk1, Sdk2, Dscam1, Dscam2; these four molecules appear to completely specify the contacts between the presynaptic bipolar cells and postsynaptic ganglion cells (Yamagata and Sanes, 2008). Moving the project from chick to mouse and taking advantage of mouse genetic technology, molecular scans of the Ig superfamily have yielded new molecular determinants of retinal wiring, including the first marker for "off" retinal ganglion cells (Kim et al., 2010). Using "brainbow mice" (Livet et al., 2007; Lichtman et al., 2008), Sanes and colleagues are now pursuing the anatomical and functional characterization of mammalian retinal cell types that can be reliably identified optically.

Brian Bacskai (another former Tsien postdoc) of Harvard/MGH reported on the application of longitudinal 2P imaging of the cortex of a mouse model of familial Alzheimer disease (FAD), mice that transgenically overexpress human mutant amyloid precursor protein. Using the genetically encoded calcium sensor YC3.6 (Nagai et al., 2004) delivered by AAV2, calcium in the distal dendrites of layer 5 pyramidal cells was imaged at high resolution in the transgenic mice and wild-type (WT) controls. This revealed a subpopulation ($\sim$20%) of pyramidal cells in the FAD mouse that had stable resting calcium levels about fivefold higher than normal. The neurites of cells near amyloid plaques were more likely to have elevated calcium. Furthermore, in such cells, calcium concentrations in the dendrites were correlated with calcium levels in the spines, but no so in WT cells (Kuchibhotla et al., 2009). Bacskai and coworkers also examined the effect of plaques on astrocytic calcium homeostasis and signaling. They found similar effects on Ca using in vivo 2P imaging of Ca with synthetic organic dye Oregon-green BAPTA (OGB1) and also revealed intercellular "calcium waves" propagating in the astrocytes that were independent of neuronal activity (Kuchibhotla et al., 2008). These observations suggesting that amyloid plaques interfere with calcium processing in nearby astrocytes were further confirmed in primary cultures of astrocytes using highly sensitive 2P fluorescence lifetime imaging (2P-FLIM). 2P-FLIM of OGB1, which allows quantitative measurements of calcium concentration via imaging the Ca-bound and -unbound states, showed that resting calcium is elevated

in astrocytes near plaques. Collectively, the observations reveal that amyloid plaques give rise to pathological calcium metabolism and processing in a mouse model of FAD that has no cell death, a characteristic of the early stages of the human disease. Bacskai's work suggests that dysregulation of calcium homeostasis may be a key component of the first stages of Alzheimer's and potentially a new therapeutic target.

Elizabeth Hillman of Columbia University closed out the session with a talk on in vivo imaging that showed the many ways that contrast could be obtained and enhanced in the imaging of live tissue by exploiting high speed multispectral imaging systems. Guided by the motto "Go fast, go widefield, change scale, and seek contrast," her group has used 2P excitation to investigate many targets, including the vascular and hemodynamic responses of rodent cortex (Hillman et al., 2007; Mahadevan-Jansen et al., 2010; Chen et al., 2011; McCaslin et al., 2011). A remarkable conclusion of their work is that extraordinary in vivo histology can be achieved, and a great variety of structural/dynamic relations such as neurovascular coupling and glucose uptake can be measured with relatively inexpensive technology that exploits natural fluorochromes and quantitative extraction of natural spectral basis sets.

Eduardo Rios of Rush University kicked off the second day of the symposium on September 10th with a talk on the control of calcium in excitation–contraction coupling in skeletal muscle using localized 2P uncaging of calcium, a method that was in its infancy when the society had its last symposium on optical methods (DelPrincipe et al., 1999). Rios dealt with the question of whether calcium-induced calcium release (CICR) from the SR makes a material contribution in amphibian and mammalian muscle. The spatiotemporal profile of SR calcium release from a brief RyR opening was estimated by uncaging NDBF-EGTA (Momotake et al., 2006; Ellis-Davies, 2008) with 2P excitation, creating a calibrated, brief diffraction-limited "point source" in the T-tubules and rapid confocal line-scanning of fluo-4FF fluorescence. Comparison of the results with electrically induced SR calcium release showed that amphibian muscle fibers (which have both RyR1 and RyR3 isoforms) have CICR, whereas mammalian muscle fibers (which have only RyR1s) do not. The analysis also revealed an interesting anisotropic propagation of the wave of calcium increase.

Gero Miesenbock of Oxford next spoke on efforts to use uncaging and optogenetics to (re)write memories in vivo in fruit flies. Initially, the Pavlovian association of an aversive unconditioned stimulus (a mild electric shock) with a neutral stimulus (odorant) was present in one of two available sites in an elongated chamber that the flies traversed. This classical approach was mimicked by selective photostimulation of a small set of dopaminergic neurons containing genetically targeted ATP-gated P2X$_2$ channels (Lima and Miesenböck, 2005). ATP was uncaged only when the fly experienced the initially preferred odor, and thereby the memory was rewritten such that place preference became associated with the other odor. This approach led to definitive identification of a cluster of 12 dopaminergic neurons in the PPL1 neuronal cluster as the source of aversive reinforcement (Claridge-Chang et al., 2009). High resolution mapping of the projection of the PPL1 neurons onto targets in the mushroom body can be expected to yield information that will show how the memory is organized, written, rewritten, and read out in the flies' behavior.

Bernardo Sabatini of Harvard presented work characterizing changes in local synaptic currents in striatal and cortical cell dendrites. He began his talk by summarizing optical tools that have been used for analysis and neuromodulation of the strength of synapses in dendritic spines of cortical neurons. These tools include caged nucleotides, caged glutamate, caged calcium chelators, and calcium-sensing dyes, along with uncaging and calcium sensing performed with high temporal and subcellular spatial resolution using 2P methodology (Ellis-Davies, 2007; Higley and Sabatini, 2008). Combining these tools with classical electrophysiology, his group has addressed such issues as how cholinergic signaling modulates cortical synapses. It has been established for some time that cholinergic stimulation, acting through M1 muscarinic receptors in dendritic spines, leads to a slow synaptic potentiation resulting from suppression of K conductance, but the precise mechanism remained elusive. Normally, depolarization of a spine, by activating Ca$_V$ channels and/or NMDA receptors, gives rise to an increase in Ca^{2+} that activates slow K (SK) channels, an effect of opposite sign to that caused by cholinergic stimulation. SK channels are expressed in the postsynaptic density of dendritic spines, where they are normally activated by Ca^{2+} entering through NMDA receptors and activating SK channels via calmodulin bound to each of its four subunits. Giessel and Sabatini (2010) showed that M1 stimulation has no direct effect on either AMPA or NMDA receptors, but rather acts through a Gq cascade on PKC to down-regulate the SK channels by decreasing their sensitivity to calcium. These and other elegant experiments cited—such as the use of photoactivatable opiod peptides—powerfully illustrated the wave of advances unleashed by precision timing and spatial localization of neuronal stimulation of neurons with 2P techniques.

Ryohei Yasuda of Duke University presented research that addressed the protein-signaling cascades in the development and maintenance long-term potentiation (LTP) of synapses in the spines of hippocampal dendrites. Yasuda has pioneered the application of 2P uncaging and imaging methods to study the real-time biochemistry in single dendritic spines in organotypic

cultured brain slices. Yasuda's work uses the uncaging of MNI-glutamate (Matsuzaki et al., 2001, 2004) to stimulate single, visually designated spines on the dendritic tree of hippocampal neurons, and the 2P version of FRET-FLIM (introduced to the society by Philippe Bastiaens at the 1999 symposium) to study Ras, a member of a family of small GTPases (Ras, Rho, Rab, Arf, and Rcn) with a wide range of regulatory functions. Ras in particular activates the extracellular signal–related kinase (ERK), whose activity plays a critical role in NMDA receptor–induced LTP. ERK is also important for the gene transcription and protein synthesis involved in the maintenance of synaptic modification. Initial work in the laboratory was directed at developing a genetically encoded FRET-based fluorescent sensor of Ras activation. This was achieved by tagging H-Ras with EGFP and the Ras-binding domain (RBD^{R59A}) with two monomeric RFPs: the binding of RBD-RFP to H-Ras-EGFP enables FRET between the rapidly reversible binding partners that is quantifiable with 2P-FLIM (Yasuda et al., 2006). Using 2P release of caged glutamate on individual spines to induce LTP, and quantifying local Ras activation as the fraction of Ras-RFP molecules binding to RBD, it was established that (a) Ras activation is a consequence of the local calcium that entered the spine through the NMDA-Rs; (b) several calcium-sensitive downstream factors were involved (e.g., CaMKII, PKC); and (c) activation of the ERK pathway was necessary for the persistent increase in spine volume that accompanied LTP and lasted tens of minutes (Harvey et al., 2008). The investigation also showed that, unlike the calcium increase, which is largely localized to an individual spine and rapidly dissipates after the stimulation, Ras signaling spreads ∼10 µm along the dendrite, altering the synaptic plasticity of other spines for tens of minutes. The work powerfully illustrates the wealth of information extractable from live, subcellular compartments with the combination of genetically encoded FP sensors and 2P microscopy. Yasuda presented work in his group that was recently published using similar techniques to address the variation in time and spatial spread of signaling by different GTPases, RhoA, Cdc42, Pak, and Rock, which has allowed them to define a spatio-temporal cascade for long-term LTP at single, visually indentified spines (Murakoshi et al., 2011).

Ernst Bamberg, director of the Max Planck Institute for Biophysics in Frankfurt, Germany, spoke in the afternoon of the third day of the conference on the discovery and characterization of Channelrhodopsin-1 and -2 (ChR1 and ChR2) in the green alga, *Chlamydomonas reinhardtii* in Bamberg's laboratory by Georg Nagel (Nagel et al., 2002, 2003). The channelrhodopsins are light-driven cation channels, members of a growing family of microbial light-gated seven-transmembrane helix proteins that also include the well-studied bacteriorhodopsin (a light-driven proton pump) and halorhodopsin

(a light-driven chloride pump). The light sensitivity of ChR1 and ChR2 is conferred by an obligatory all-trans retinal chromophore, but the λ_{max}'s are bathochromically shifted from that of all-trans retinal (380 nm) to ∼500 and ∼470 nm, respectively. The ChR2 photocycle can be described in terms of a four-state model comprising two closed and two open states, with transition rates from each closed to its corresponding open state dependent on light intensity, that is, the rate of photon absorption (Nagel et al., 2003; Hegemann et al., 2005; Nikolic et al., 2009) . Channelrhodopsin-2 is proving to be of particular value to the neuroscience community, because it is an inwardly rectifying, cation-selective channel with a unitary conductance of ∼60 fS under physiological conditions, a relatively large pore (based on its permeability to organic cations), and ready expressibility in mammalian cells (Feldbauer et al., 2009). Moreover, Bamberg and colleagues have developed a modified version of ChR2 (L132C), "CatCh," that has an approximately twofold increased permeability to Ca^{2+}, and increased open-channel lifetime (Kleinlogel et al., 2011). The net effect of these modifications is the ability of CatCh to function as a highly sensitive, fast-depolarizing light switch in neurons. One fundamental problem of other variants of ChR2 is the extremely low single-channel conductance, necessitating high photon fluxes and high plasma membrane protein expression for effective depolarization. In contrast, the light sensitivity of CatCh currents in hippocampal neurons is ∼70-fold increased over those of comparably expressed WT ChR2. Bamberg closed his talk with a description of a tandem construct comprising ChR2 and NpHR, the light-activated halorhodopsin chloride pump of *Natronomas pharamonis*, linked by a (BK channel β subunit) (Lanyi, 1990; Lanyi et al., 1990), and tagged with YFP as a reporter. Because the spectral absorbance of NpHR is maximal near 600 nm, expression of this tandem construct produces "on/off" electrical switch, with short wavelength light depolarizing, and long wavelength light hyperpolarizing, the target cell.

Karl Deisseroth of Stanford began his eponymous talk by defining "optogenetics" as "the combination of optics and genetics to achieve gain or loss of function of well-defined events within specific cells of living tissue" (Deisseroth, 2011). In principle, genetically programmable elements for optically manipulating the membrane potential of cells have been available since the discovery of the light-driven chloride and proton pumps of halorhodopsin by Matsuno-Yagi and Mukohata (1977) and bacteriorhodopsin by Stoeckenius and colleagues (Stoeckenius, 1979; Stoeckenius et al., 1979). The characteristic feature of optogenetics as a distinct discipline is the explicit understanding that a growing array of genetically programmable "actuators" and sensors (Fig. 1; Miesenböck, 2009) enables spatially and temporally resolved manipulation and interrogation of many aspects

of cellular function in living tissues, and indeed in living, behaving organisms. A milestone in this new discipline was passed when a team, led by Deisseroth and comprising the co-discoverers of ChR2 from Frankfurt, presented evidence for millisecond timescale optogenetic control of neuronal spiking (Boyden et al., 2005), soon followed by optical control of both neuronal excitation and inhibition with joint expression of ChR2 and NpHR in cultured neurons (Han and Boyden, 2007; Zhang et al., 2007). These proof-of-principle findings have been followed by a series of improvements in the subcellular targeting of the channels (with an ER export motif) to the membrane, the addition of a red-sensitive cation channel from *Volvox carteri* to the toolbox (Zhang et al., 2008), further enhancements in particular of NpHR ("eNpHR3.0") (Gradinaru et al., 2008), and an optogenetic toolbox specific for primate neurophysiology (Zhang et al., 2010). Moreover, the translational potential of optogenetics was unveiled with the demonstration that the expression of eNpHR3.0 could restore the hyperpolarizing light responses of cone photoreceptor cells in a mouse model of the blinding eye disease, retinitis pigmentosa (Busskamp et al., 2010).

Ehud Isacoff of Berkeley, in the final talk of the session, presented work on the "third way" of using light to modulate neuronal membrane potential, the development of an ionotropic glutamate receptor channel (iGluR) with photoreversible switches. In contrast to the use of caged compounds, this method (Banghart et al., 2004) is reversible and shows little photochemical fatigue, and in contrast to the use of ChRs, it modulates native membrane receptors with high single-channel conductivities. The chemical construct involved, called MAG, links a cysteine-reactive maleimide ("M") to an isomerizable azobenzene ("A") bound to a glutamate analogue ("G"). Cysteine scanning of iGluR6 guided by the crystal structure (Mayer, 2005) led to discovery of an optimum linkage locus, L439, and so MAG was tethered at Cys439 to form a light-activatable channel, LiGluR6 (Volgraf et al., 2006; Szobota et al., 2007). LiGluR6 is maximally activated by 380 nm light, which drives the azobenzene moiety to the all-trans form; illumination with 500 nm of light drives the chromphore back to the cis-form, and the LiGluR6 returns to the closed state. LiGluR6 is thus an "on/off" switch for cationic current and, because iGluR6 is highly permeant to calcium, a switch for calcium delivery to cells (Volgraf et al., 2006; Szobota et al., 2007). Isacoff then presented several published applications. In one application, LiGluR6 was expressed in pattern-generating motor neurons of the zebrafish spinal cord and 380-nm illumination shown to activate behavior previously linking to the targeted cells (Szobota et al., 2007). More recently, a MAG-activated K-selective channel has been added to the toolkit, enabling light-switchable neuronal inhibition (Janovjak et al., 2010).

Stefan Hell, Director of the Department of NanoBio-photonics at the MPI for Biophysical Chemistry in Gottingen, opened a session on superresolution imaging on Saturday morning, September 11th. Light has been used to study cells since the 17th century. But the finest details of subcellular structure were always out of reach, because traditional light microscopy is limited by the diffraction barrier, and so such subcellular details have been considered the preserve of EM. Hell described with great clarity the two fundamentally different approaches that have been taken recently to bypass the optical diffraction barrier, both by his own laboratory (stimulated emission depletion [STED], or "localized excitation") and others (PALM and STORM, or "localized emission"), to create a new era of optical far-field superresolution imaging. Superresolution microscopy is optical imaging that achieves a spatial resolution Δr of two objects lower than the diffraction limit. The latter, as formulated by Abbe, is $\Delta r = \lambda / 2n \sin\alpha$, where λ is the wavelength of the light in a vaccum, n is the refractive index, and α is the angular semiaperture of the objective. Abbe's limit is effectively the full-width, half-maximum of the point-spread function (PSF) of a diffraction-limited system. In the general case, super-resolution imaging relies on optical control of the spatial distribution of a population of molecules that can be in one of two states, one of which is brightly fluorescent (A) and the other (B) not, with a saturable A→B transition rate constant k_{AB} proportional to the local photon flux density I for subsaturating intensities ($k_{AB} = \sigma I$). Defining $I_{sat} = k_{AB}/\sigma$, one can see that the population of A molecules can be driven arbitrarily low for $I \gg I_{sat}$ (Hell, 2003, 2007). In the best-investigated example of such systems, STED, state A is a fluorescent excited state and state B its ground state, reachable by stimulated emission from A (Hell and Wichmann, 1994; Westphal and Hell, 2005). In STED, one laser is used to excite molecules into state A in a standard diffraction-limited PSF, while a second laser of a longer wavelength is focused in a concentric annular pattern, but with a nominal zero intensity at the center of the PSF of the first laser. The second laser depletes molecules from state A in an annular pattern, shrinking the center circle of emitters to a spot smaller than the PSF of the first laser. The relation governing resolution with STED is $\Delta r \approx \lambda / [2n \sin\alpha\sqrt{1 + (I_{max} / I_{sat})}]$, where I_{max} is the maximal intensity of the annular pattern, and I_{sat} is as just described. Resolutions as low as $1/50\lambda$ have been achieved with STED (Hell and Wichmann, 1994; Westphal and Hell, 2005), showing that Abbe's diffraction barrier, which seemed be set in stone, is now completely shattered. Several two-state systems with distinct photophysical or photochemical mechanisms that can support superresolution have been described theoretically and implemented, including ground-state depletion (Hell, 1995), saturated pattern excitation microscopy, and

photoswitching reversible saturable optically linear fluorescence transitions (Hell, 2007). Numerous applications in cell biology have been published (http://www.mpibpc.mpg.de/groups/hell/), for example, in characterizing the movement of synaptic vesicles (Willig et al., 2006; Westphal et al., 2008; Hoopmann et al., 2010; Kamin et al., 2010). In his summary, Hell gave the audience this advice: in superresolution work you "forget about waves and focus on states; separating molecules into 'on' and 'off' states in a predictable manner is what matters." A striking feature of Hell's talk was the passion with which he spoke not only about his own work, but also of that of the "competition."

Xioawei Zhuang of Harvard University spoke next, on superresolution microscopy achieved with stochastic optical reconstruction microscopy (STORM) and its sibling, photoactivatable localization microscopy (PALM). STORM was first described by Zhuang and colleagues (Bates et al., 2005; Rust et al., 2006) at about the same time that the first report of PALM was published by Betzig et al. (2006). The essential feature of these techniques is that individual molecules in a tissue sample are switched "on" (to a fluorescent state) at a spacing well exceeding the PSF of the microscope, and then the "on" molecules are repeatedly excited until enough photons (N) are captured by the recording system so that the coordinates of the emitting molecules can be determined by localization of the center of the corresponding PSF with a precision $\sim \lambda / 2n \sin\alpha \sqrt{N}$; after adequate sampling, the "on" molecules are switched off by bleaching. That the localization of individual molecules can be achieved with a precision far exceeding the resolution of the microscope had been realized earlier (Thompson et al., 2002; Yildiz et al., 2003), but the first applications involved instances in which only one molecule was being recorded from Yildiz et al. (2003). In contrast, PALM/STORM introduced the novel insight that by switching molecules into an "on" state, nanometer scale determination of position could be applied to an ensemble of molecules such as found in a tissue, and thus give ultrastructural details that previously could only be had with EM. In the initial realization, an individual Cy5 cyanine dye molecule was tethered by to a nearby Cy3 molecule. The Cy5 (λ_{max}, $\sim$650 nm) molecule was continually probed with a weak (5 W cm^{-2}, 638 nm) red laser and switched to an "off" state with a relatively intense red laser (70 W cm^{-2}) exposure in $\sim$1 s; exposure to a green (532 nm, 0.1 W cm^{-2}) laser pulse switched the molecule back to the red fluorescent state (Bates et al., 2005). This switching was highly efficient and required the Cy3 (λ_{max}, $\sim$550 nm) to be within $\sim$1 nm of its Cy5 partner, suggesting FRET. Subsequent work revealed that the Cy5 photoswitching mechanism arises from a disruption of the π-electron cloud of the polymethine-conjugated chain by formation of reversible adduct (Dempsey et al., 2009). Of course, many different potential switching mechanisms exist for "on/off" switching, such as that of the FP Eos (Wiedenmann et al., 2004; Nienhaus et al., 2005), and the advantages and disadvantages of different selections—such as the number of photons per "on" cycle—will guide the evolution of PALM/STORM. Meanwhile, there is much development, such as multicolor STORM (Bates et al., 2007), and many applications to biology (Huang et al., 2008, 2009).

Yale Goldman of the University of Pennsylvania spoke next, on the topic of single-molecule biophysical imaging of various molecular motors. Goldman is one of the pioneers of new optical methods in physiology. His work in the early 1980s established the power of laser uncaging for the detailed study of the kinetics of the muscle cross-bridge cycle (Hibberd et al., 1984). Over the past few years, he has used the same detailed rigor and creativity, and has focused on other molecular motors. The critical advantage of single-molecule imaging is that, as in single-channel recording, it can potentially reveal distinct state transitions, fluctuations, and other events in a molecule's trajectory that are hopelessly lost in methods involving ensemble averaging. Among the disadvantages are the inherent noisiness and low throughput. One such effort in Goldman's laboratory has been the imaging of the movement of unconventional myosin motor proteins (Dantzig et al., 2006). Myosin V is a cargo-carrying processive motor that steps along actin filaments (Mehta et al., 1999). Goldman and colleagues tagged myosin V motors with bifunctional rhodamine or Cy3 on the light chain of the head and used total internal reflectance microscopy (TIRF) to image an individual myosin as it "walked" along an F-actin filament attached to the coverslip. Their "fluorescence imaging with 1-nm resolution" (FIONA) methodology relies on the same principle as PALM/STORM, namely that the location of the emission from a molecular point source can be specified with a precision far greater than two point sources can be discriminated from one. FIONA has allowed them to precisely quantify the details of the processive 37-nm steps (Yildiz et al., 2003). By measuring the polarization of the emission ("Pol-TIRF"), they have also been able to resolve the 3-D orientation of the light chains ("legs and feet") of the myosin V molecule as it wobbles between steps (Forkey et al., 2003, 2005; Quinlan et al., 2005). Applying the same method to kinesin and dynein motors walking on microtubules, Goldman and colleagues have characterized the behavior of the motors as they reach microtubule intersections, showing that they often switch tracks depending on the motor density (Ross et al., 2006, 2008). Goldman closed his talk with a summary of work investigating protein synthesis by single ribosomes with single-molecule FRET (Vanzi et al., 2003, 2005; Wang et al., 2007).

Liang Guo of Eric Betzig's laboratory at Janelia Farms next presented a talk on the recent development of

imaging with plane illumination (Huisken et al., 2004). Achieving simultaneously broad spatial and temporal bandwidth in live cell and tissue imaging is an important goal. The line-scanning approaches of confocal/ multiphoton microscopes are limited by the scan speed, and plane illumination synchronized with widefield (camera) imaging can overcome such limitations. Implementation of Bessel plane illumination has enabled acquisition speeds of up 200 image planes/s (33 Mvoxels/s), and can support very high temporal resolution 3-D particle tracking (Planchon et al., 2011). This new microscopic method readily incorporates and benefits from multiphoton excitation.

Karel Svoboda of HHMI, Janelia Farms Research Campus, gave the second keynote address and final talk of the meeting. Over the past 10 years, Svoboda has pioneered the application of optical methods to fundamental problems in neuroscience. Svoboda's talk described recent work in his laboratory on the mapping of the cortical circuitry in behaving animals and characterization of the mechanisms underlying the cortical plasticity that accompanies learning. The well-studied whisker barrel sensory and motor cortex (Petersen, 2007) provides an ideal preparation. The group developed and characterized a sensitive tactile position discrimination task that a restrained, awake mouse performs with a selected set of whiskers (O'Connor et al., 2010). In this "go/no-go" paradigm, whisker movements are recorded with high speed video, and their trial-by-trial movements are analyzed with respect to success (hit) or failure (false alarm). In work reminiscent of Meisenbock's in fruit flies, Svoboda's team presented two odors to mice during lick reward (water) or nonreward. Subsequently, the activity of whisker motor cortical neurons in L2/3, L4, and L5 was recorded with electrodes and with 4-Hz frame-rate 2P imaging of fluorescence of calcium using OGB1 or with the GECI GCaMP3 in L2/3 neurons (Akerboom et al., 2009; Tian et al., 2009). Activity of nearly half of the neurons in layers L2/3, L4, and L5 in the relevant barrel field correlated with object discrimination, with up to 13,000 total spikes in a barrel column available on a "go" trial to support the behavioral decision. In work with an odorant discrimination task, Svoboda and colleagues mapped the pyramidal cells in the mouse cortical tongue motor field controlling the licking response. Again, the activity of roughly half of the neurons in layer 2/3 showed task-related activity that was distinctive on reward ("go") and no-reward ("no-go") trials, with considerable activity preceding the tongue response (Komiyama et al., 2010). Neurons with different response profiles were spatially intermingled, and the temporal correlation of their activity with the behavior sharpened over trials, likely manifesting a strengthening of connections between the sensory and motor cortices during learning, and perhaps the construction of functional neuronal ensembles.

Optical methods in physiology: lessons from the past, predicting the optogenetic future

Optical methods are essential for modern physiology. It is interesting to see how techniques that were in their infancy at the previous SGP symposia in 1984 and 1999 have gone on to mature into commercially available methods that are very widely used (Fig. 1). Presented in the first meeting, fluorescent calcium imaging and confocal microscopy are two of the most important "non-electrophysiological" techniques now used by physiologists, and their widespread adoption owes much to the commercialization of the fluorescent dyes and confocal technology. From the second meeting, techniques introduced in talks by Denk and Webb (2P imaging), Bastiaens (FRET-FLIM), and Ellis-Davies (2P uncaging) have gone on to have significant scientific impact. In particular, the 2P imaging method has been very widely used, for all of the reasons outlined (Denk et al., 1990). However, the widespread adoption of 2P microscopy would not have occurred if solid-state, computer-controlled lasers had not been developed commercially. One of the generous symposium sponsors (Coherent) has been at the forefront of this commercialization.

So what of the future? It requires no imagination to predict that superresolution imaging of living cells will become a widely used optical method; indeed, several approaches have already been commercialized. Channelrhodopsins are starting to be exploited by neuroscientists, but what of the wider scientific community of "non-neuro" physiologists and cell biologists? The development of optogenetic methods for actuation of gene transcription in vivo seems to be the logical next addition to this growing family of methods, and there can be little doubt that it will have the ubiquity and impact of GFP and its siblings. It is hoped that optogenetic sensing of calcium will finally become as reliable as traditional synthetic organic dyes (transgenic GECI mice are in development; see Jackson Laboratories stock no. 014538). For cellular physiologists, the dominant methods of actuation (uncaging) and sensing (confocal and 2P microscopy of organic dyes and GFP) over the past 35 years have set the standard (Fig. 1). Will the new approaches introduced at the 64th SGP Symposium become as widely used as these methods? The physiology user community is unsparing in its critical assessment of new methods. Thus, once developed, they are tested according to two simple criteria: does it really work as advertised, and it is easy enough for more than a few specialist laboratories to use? In short, "The proof of the pudding is in the eating," and so the answer is only time will tell which methods achieve ascendancy in the decade ahead.

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