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Combining Uncaging Techniques with Patch-Clamp Recording and Optical Physiology

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1. Introduction

Patch-clamp recording is a powerful approach to monitoring membrane electrical activity with high temporal resolution. However, the spatial resolution of patch-clamp recording in a distributed structure such as a neuron or a brain slice is limited by the fact that each electrode records from just one point, making recording from more than a very small number of points impractical.

An approach that allows biological signals to be monitored and manipulated with high spatial resolution is the use of optical methods. For the monitoring of signals, a powerful approach is the use of activity-dependent fluorescent dyes. In this type of recording, one or more cells are loaded with a fluorescent dye that is sensitive to some change of interest, such as intracellular calcium or membrane voltage. Loading can be done by including the dye in the patch pipette, by bulk loading of many cells at once with acetoxymethyl (AM) ester dyes (Garaschuk et al., 2006; Sullivan et al., 2005; Tsien, 1999) or voltage-sensitive dyes (Djurisic et al., 2003; Grinvald and Hildesheim, 2004), or by expression of an activity-dependent fluorescent protein (Bozza et al., 2004; Wang et al., 2003). Regardless of the means of loading, biological signals lead to variations in fluorescence that can be detected by high-speed fluorescence microscopy. The combination of electrophysiological and fluorimetric recording thus allows monitoring of biochemical and electrical signals simultaneously with high temporal and spatial resolution.

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An additional optical approach, photolysis of caged compounds, can be used to manipulate cellular biochemistry. Uncaging techniques nicely supplement the ability to measure electrical and biochemical signals by providing a means to affect the signals with high spatial and temporal resolution. This chapter discusses the combination of uncaging with patch clamp and fluorescence recording, starting with an overview of caged compounds and applications, a description of the construction of several specific focal uncaging setups, and selected recent technical developments. For additional perspectives we refer readers to other recent reviews (Eder et al., 2004; Kramer et al., 2005; Thompson et al., 2005).

2. Principles of Caged Compounds

Before beginning, it is helpful to ask whether uncaging is needed at all. In easily accessible preparations such as isolated cells or ripped-off membrane patches, direct application by pressure ejection through a pipette can achieve rapid application on millisecond time scales (Isaacson and Nicoll, 1991). In semi-intact preparations such as brain slices, another approach is iontophoretic application, which can achieve submicrometer resolution. Uncaging is useful in applications where bringing in a physical electrode is impractical or inadvisable. Examples include the study of intracellular signaling (Adams and Tsien, 1993), dendritic spine physiology (Matsuzaki et al., 2001; Svoboda et al., 1996), and multisite activation of neural circuitry (Gasparini and Magee, 2006; Shoham et al., 2005).

2.1. Basics of Uncaging

Caged compounds are biologically active molecules that are made inactive by the addition of a light-sensitive "caging" group (Adams and Tsien, 1993). When illuminated by ultraviolet (UV) light, the cage group absorbs a photon, leading to the breakage of a covalent bond linking the cage group to the rest of the molecule. The molecule is then free to act on its biological target. When the uncaging light is provided at precise times and locations, activation can occur with high temporal and spatial resolution.

Most caged compounds are made by the synthetic addition of cage groups to neurotransmitters, second messengers, and peptides. A few exceptions exist: calcium has been caged by making a chelator whose affinity for calcium is altered by photolysis, and nitric oxide (NO) has been caged by making photolyzable small

molecules that release NO upon illumination (Makings and Tsien, 1994; Pavlos et al., 2005). Commercial caged compounds are available for many molecules, including adenosine triphosphate (ATP), glutamate, γ-aminobutyric acid (GABA), *N*-methyl-D-aspartate (NMDA), and carbachol, and for the second messengers inositol 1,4,5-triphosphate (IP_3), calcium, and NO.

In general, photolysis techniques have found great use in biology whenever precise temporal and spatial control is important (Gurney, 1994; Nerbonne, 1996). The first biologically useful caged compound, caged ATP (Engels and Schlaeger, 1977; Kaplan et al., 1978), has been used to study muscle contraction, ATP-dependent channels, and molecular motors. Caged fluorescent markers have been used to track movement of cellular components (Theriot and Mitchison, 1991) and the migration of cells during development (Li et al., 2003), and to measure diffusional coupling between dendrites and spines (Svoboda et al., 1996). The caged second messenger IP_3 has been applied to study signal transduction during fertilization (Jones and Nixon, 2000), muscle activation (McCarron et al., 2004), and neuronal signaling (Khodakhah and Armstrong, 1997). Caged compounds are also used in drug discovery (Dorman and Prestwich, 2000).

In addition to the existing approaches to making caged compounds, several recent innovations are worth noting. Molecules have been modified with two groups instead of one to allow chemical two-photon uncaging, a nonlinear two-photon-like effect that especially improves axial resolution (Pettit et al., 1997). Uncaging can be combined with molecular biology-driven approaches, including the expression of light-sensitive ion channels or of non-native channels that can then be selectively activated using caged ligands (Banghart et al., 2004; Chambers et al., 2006; Tan et al., 2006), opening the possibility of light-based activation of specific cells or cell types. Finally, the recent advent of light-activated inhibitors of protein synthesis or caged proteins opens the possibility of optically probing the role of any protein (Goard et al., 2005; Lawrence, 2005).

2.2. Properties of Caged Compounds

Good caged compounds must have several important properties. First, in the inactive state, caged molecules should have minimal interaction with the biological system of interest. Possible interactions with the system are not limited to the receptor of interest,

since a caged molecule may be inert at one receptor but still have residual activity at others; for instance, caged molecules with no agonist activity can still act as inhibitors for target receptors (Nerbonne, 1996; Sarkisov et al., 2006). Second, products of the photolysis reaction should not affect the system. Since it seems impossible to test caged compounds for agonist as well as antagonist activity on every putative target, uncaging experiments should be designed with proper controls in mind. A critical third property of caged compounds is that they must release ligands efficiently and quickly in response to illumination, and not at other times. To characterize the ability of a compound to be uncaged, a useful parameter is the *uncaging index*. The uncaging index of a cage group is defined as $\varepsilon^*\varphi$, where φ is the quantum yield, or the probability of a group to be photolyzed after it absorbs a photon, and ε is the extinction coefficient. To estimate the uncaging index, the extinction coefficient ε needs to be measured at the right wavelength; for a given caged group, ε is constant. Quantum yield φ does not change over a range of wavelengths as long as other absorption bands are avoided, but does vary as a function of the identity of the caged molecule and the caging position. The uncaging index is important because the higher the index, the lower the amount of light needed to achieve uncaging. Light levels used to photolyze caged compounds should not damage or interact in other ways with the biological system. This issue is especially important since most cage groups absorb high-energy UV photons that are more likely to cause damage to the sample than visible or infrared light. Uncaging parameters for some commonly used or otherwise important compounds are shown in Table 1.

2.3. Handling of Caged Compounds

In uncaging experiments relatively high concentrations of the caged compound are applied to the system for long periods of time. Under such conditions, even if a small fraction of compound is uncaged, either spontaneously or by stray light, the system will be perturbed. For this reason we recommend storing caged compounds under conditions that minimize their degradation. While exact guidelines vary depending on the stability of the used drug, taking extra precautions is appropriate. Solutions of the caged compound should be kept in the freezer between experiments. A point at which care must be taken is the storage of unused compounds

Table 1
Uncaging Parameters for Selected Compounds

Compound	ε in $M^{-1}cm^{-1}$ (wavelength)	φ	Uncaging index, ε*φ
CNB-caged glutamate	~500 (350 nm)	0.15	75
NPE-caged IP_3	500 (350 nm)	0.65	325
MNI-caged glutamate	4,300 (350 nm)	0.085	366
NPE-caged ATP	660 (347 nm)	0.63	416
CNB-caged carbachol	~600 (350 nm)	0.8	480
DMNB-caged fluorescein dextran	4,000 (338 nm)	0.13*	520*
NDBF-EGTA	15,300 (350 nm)	0.7	10,710

*Estimated.
CNB, α-carboxy-2-nitrobenzyl; NPE, 1-(2-nitrophenyl)ethyl; MNI, 4-methoxy-7-nitroindolinyl; ATP, adenosine triphosphate; DMNB, 4,5-dimethoxy-2-nitrobenzyl; DMNP, 4,5-dimethoxy-2-nitrophenyl; EDTA, ethylenediaminetetraacetic acid; NDBF-EGTA, nitrodibenzofuran-ethyleneglycoltetraacetic acid (Momotake et al., 2006).

between experiments. Large volumes of solution should be divided into aliquots to minimize freezing/defreezing cycles. If aqueous solutions are unstable (for instance with carboxy-2-nitrobenzyl (CNB)-caged glutamate; see Rossi et al., 1997) or if a compound will be stored for prolonged periods of time, aliquots can be dried in a lyophilizer or SpeedVac™ (Thermo Savant, Holbrook, NY) before freezing.

During experiments, unwanted uncaging may result from exposure to ambient light. Stray uncaging from room light and microscope-transmitted light can be reduced by using UV and yellow filters; the latter can be made from material used for filtering theater lighting. For viewing specimens a good way to minimize uncaging is to visualize the specimen with infrared differential interference contrast (IR-DIC) imaging, which is popular for viewing brain slices. Yet another means of minimizing production of agonist is to use double-caged compounds (Pettit et al., 1997), for which small amounts of light generate products that are predominantly still single-caged.

The expense of caged neurotransmitters dictates conservation of the amount of material used. Conservation is usually achieved by using a recirculating bath with a peristaltic pump. One problem of recirculation is the cumulative buildup of photolysis by-products; unwanted uncaging can be reduced by applying caged neurotransmitters locally through capillary tubing (Furuta et al., 1999).

Commercially available compounds may contain impurities or be partially photolyzed. If doubt exists about the quality of the compound, it may be repurified on a column. Fortunately, commonly used caged compounds are now available from different manufacturers such as EMD Biosciences (San Diego, CA), Invitrogen (Carlsbad, CA), Sigma-Aldrich (St. Louis, MO), and Tocris Bioscience, (Ellisville, MO), so switching to another supplier is sometimes an effective solution. Finally, during experiments, caged compound solutions should be protected from light and kept on ice when not in use.

3. Designing an Uncaging Setup

3.1. Types of Uncaging Systems

Uncaging systems differ in the way that the uncaging light is delivered to the sample. The particular design affects spatial and temporal resolution of photolysis, cost of construction, and simplicity of construction and maintenance. Ideally, the choice of design depends on the needs posed by studying the biological preparation of interest.

One of the simplest systems delivers a brief pulse of UV light to a whole region of the specimen, such as the full field of view on an epi-illumination microscope (e.g., Brasnjo and Otis, 2004). Among the advantages of such a configuration are low cost and simplicity. A UV flashlamp or even an intense arc lamp can simply be mounted to the optical port of the microscope with appropriate coupling and focusing optics. Although with the use of flash lamps systems configured in full-field mode do not provide high spatial resolution (usually >50 μm), temporal resolution can be submillisecond and spatial resolution can be improved by positioning an aperture in the UV path (Xu et al., 1997).

One means of improving spatial resolution is to deliver uncaging light through a fiber optic light guide introduced into the optical path (Bagal et al., 2005; Diamond, 2005; Dodt et al., 2002; Wang and Augustine, 1995; Yang et al., 2006). Focusing assemblies are available commercially, for instance, from Oz Optics (Carp, Canada), or Rapp OptoElectronic (Hamburg, Germany). Fiber optics can be brought up directly to the sample (Kandler et al., 1998) or even inserted into it (Godwin et al., 1997). With direct introduction of a fiber, resolution of tens of micrometers can be achieved depending

on the shape of the fiber ending. Micrometer resolution is possible if tapered quartz fiber is used (Eberius and Schild, 2001).

Resolution of the uncaging system can be improved down to the diffraction limit of light by focusing a laser beam into the specimen through the objective using a system of mirrors (Katz and Dalva, 1994; Sarkisov and Wang, 2006). In both fiber optic and mirror-based systems, power losses are a principal design consideration because of limited acceptance cones and absorption in fibers, and because of losses by mirrors and filters. In both cases, if resolution in all three dimensions is desired, the emerging UV beam should fill as much of the back aperture of the objective as possible. The discussion below focuses on considerations in designing and building a focal uncaging system using a laser and mirrors.

3.2. Light Source

Different light sources are used in different types of uncaging systems. The UV light source should be powerful enough to provide sufficient energy for photolysis of caged compounds. The key parameter that determines maximal uncaging efficiency is the amount of light energy delivered per unit area in the specimen. As a rule of thumb, a light density of ~0.5 $\mu J/\mu m^2$ will be sufficient. In a focused uncaging system, possible widening of the uncaging spot due to scattering by the tissue should be taken into account.

Xenon lamps, flash lamps (Rapp, 1998), and mercury arc lamps (Denk, 1997) can all be used for whole-field uncaging. However, since UV lamps are not intrinsically collimated, significant loss of light energy is unavoidable. For fast uncaging, flash lamps deliver the most light but also generate an electrical discharge that can cause large electrophysiological artifacts.

Lasers are more expensive than lamps but can deliver up to several watts of collimated light. Different types of lasers used for uncaging include nitrogen, frequency-doubled ruby, argon, and neodymium-doped yttrium-aluminium-garnet (Nd:YAG). A convenient and economical solution is the type of laser used in our laboratory, a Q-switched, frequency-tripled neodymium-doped yttrium-vanadate (Nd:YVO_4) laser (series 3500, DPSS Lasers Inc, Santa Clara, CA) that provides up to 5 W of 355 nm light at a 20 to 150 kHz repetition rate. The flash duration, 50 to 60 ns, is long enough that multiple excitations of a caged molecule are possible during a single pulse, which is desirable for maximizing the

likelihood of uncaging per flash of a given energy. The high power output of Q-switched lasers and the ability to deliver a precise number of flashes (down to a single flash) are important for many applications, and are especially useful for a rapid patterned uncaging system (Shoham et al., 2005) that we will describe later in this chapter.

3.3. Ultraviolet Optics

For work using UV lasers, several important issues should be considered. Always use protective eyewear, especially on custom-made systems where the beams are often more exposed. UV and infrared (IR) emission are invisible to the human eye, thus presenting a special hazard. The process of troubleshooting and aligning a system is especially prone to risk.

The concentration of light from pulsed lasers in brief pulses leads to peak power levels that can damage optical components. High-energy pulses can be attenuated using a polarizer, a beamsplitter, and a beam stop (Fig. 1A). An alternate approach is to use reflective neutral density filters that are designed to withstand high-energy UV pulses. A more expensive but popular option is to use a Pockels cell, which allows light to be controlled quickly, within tens of microseconds (Thompson et al., 2005). Finally, to reduce per-area light density, a beam expander can be used to widen the laser beam just after it leaves the laser head.

Measurement of UV power at the sample is very useful and important, but some care must be taken to avoid technical errors. Semiconductor sensors have high sensitivity, but because silicon has a high index of refraction, such sensors require incident light to come in at a near-perpendicular angle, and thus cannot be used to measure a converging beam, as occurs in front of a microscope objective. Thermal sensors do not require collimated light, but may not be sensitive enough to detect light transmitted through the objective. Attempting to increase laser power output to a detectable level may cause permanent damage to the objective. One solution is to measure power at the back of the objective and adjust it by the transmission coefficient of the objective. The transmission coefficient can be estimated from the manufacturer's specification or, better yet, measured directly by passing the converging beam telescopically through a second objective positioned backward.

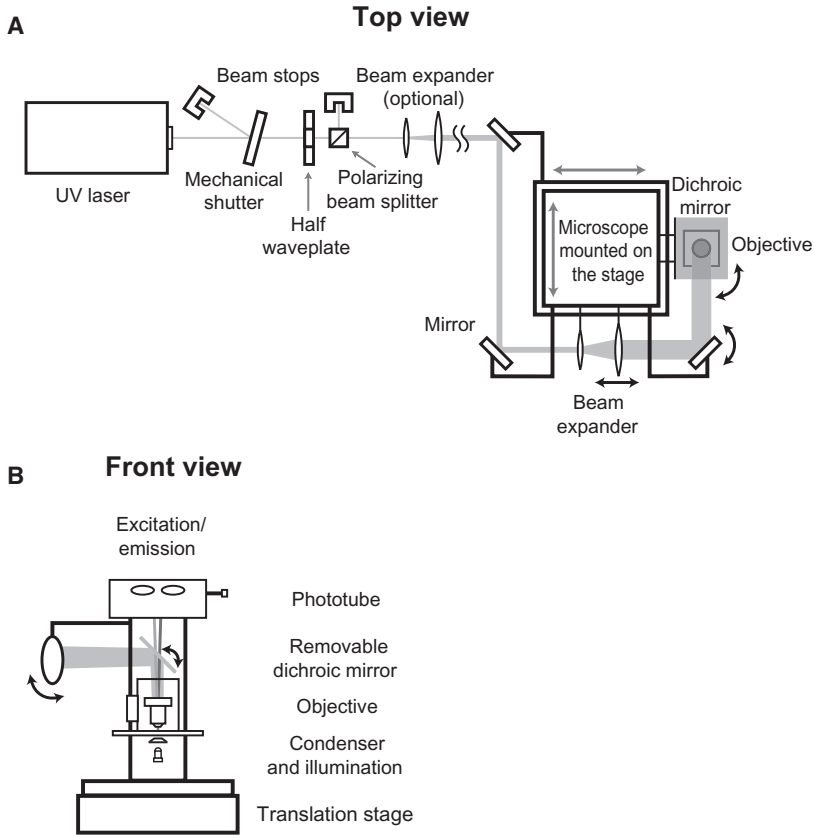

Fig. 1. Optical path of the ultraviolet (UV) uncaging system. After passing through the mechanical shutter and attenuator, the laser beam is directed by two mirrors mounted to the translation stage to the beam expander (**A**). The expanded beam is introduced to the optical path of the system be the dichroic mirror. Black arrows show the optical components used for precise alignment. (Adapted from Sarkisov and Wang, 2006.)

4. Construction of a Focal Uncaging Setup

4.1. Beam Path

A diagram of a UV laser-based focal uncaging system is shown in Fig. 1. We give specific catalog numbers for the components we use, as well as the general functional criteria for choosing them. Most components have analogous substitutes from other manufacturers.

A beam from a frequency-tripled Q-switched UV laser Nd:YVO_4 (model 3501, DPSS Lasers Inc.) is controlled by a mechanical shutter (LS6ZM2 shutter, VMM-D1 driver, Vincent Associates, Rochester, NY) that in the closed state reflects light into a beam trap (BT500, Thorlabs, Newton, NJ). The coating of the shutter's blades as well as the beam trap should be chosen to withstand high power densities. A combination of a zero-order quarter waveplate (WPQ05M-355, Thorlabs) and a calcite polarizer (GL5-A, Thorlabs) is used to attenuate laser power. An optional beam expander widens the beam several times to ensure even illumination over the back-aperture of the objective. After passing through the expander, the beam is directed by two mirrors (UV MAXBRIte, Melles Griot, Irvine, CA) aligned to match the directions of movement of an XY translation stage (XYR-8080, Danaher Corp., Washington, DC) that positions the microscope. Before the beam reaches the objective it is widened by a second, 5× beam expander (BXUV-4.0-5X-355, CVI Laser, Albuquerque, NM) and then introduced to the optical path of the microscope (Fig. 1B) using a dichroic mirror (390DRLP, Omega Optical, Brattleboro, VT). The same beam expander is used to converge the beam to compensate for the focal shift between fluorescence excitation and uncaging light (Sarkisov and Wang, 2006).

For best uncaging resolution, the final UV beam should be flat and approximately fill the back-aperture of the objective. If the back-aperture is overfilled, resolution is improved but the total amount of UV power delivered to the sample is decreased. For instance, when the incoming beam is gaussian with the diameter equal to the size of the back-aperture, 86% of the total incoming light enters the objective, and spatial resolution is made worse by somewhat less than 10%, compared with optimal (Helmchen and Denk, 2005). Diffraction-limited lateral resolution is approximately 0.23 μm full-width half-maximum (Thompson et al., 2005) using a purely mirror-based system. Two-photon uncaging leads to improvements in axial resolution, but because of the longer wavelengths used the lateral resolution is actually worse. A possible solution in the absence of strong scattering is the use of chemical two-photon uncaging.

4.2. Aligning the Setup

To achieve the optimal resolution of the uncaging system, the UV beam should follow the optical axis of the system. This is achieved by manual adjustment of the two last mirrors of the UV

beam path. Another important requirement is that uncaging light must converge to the same plane of focus as the imaging light. Since objectives bend light differently as a function of wavelength, two collimated beams uncaging and imaging light would usually focus to slightly different planes. To compensate for this focal shift, we make uncaging light slightly diverging (or converging, depending on the objective used) by adjusting the distance between lenses of the beam expander.

For initial alignment a water solution of fluorescein can be used. When illuminated with UV light, the excited fluorescein solution should be visible from the side (Fig. 2A). If the observed cone is not axially symmetric with respect to the optical axis of the system (Fig. 2B) or does not coincide with the point where the excitation light is focused (Fig. 2C), adjustments to the mirrors are necessary. Once the excitation cone appears symmetrical from the side, fluorescein excitation can be observed through the eyepieces and slight adjustments made to the mirror to put the fluorescent spot in the center of the field of view. Then the size of the uncaging spot is minimized by changing the distance between lenses of the beam expander.

Once the size and position of the uncaging spot are optimized under visual control, fine-tuning is then possible using a thin sample of dried caged fluorescein. A dried layer of caged dye greatly simplifies alignment of the uncaging system because it is immobile and can be examined at leisure after an uncaging flash. In our test samples, 10 μL water solution of the 1% bovine serum albumin is mixed with 2 mg/mL caged fluorescein dextran and dried on a coverslip. Then the coverslip is positioned under the microscope sample side down as shown in Fig. 2D, and an uncaging flash is delivered. The shape and position of the spot of photolyzed fluorescein are visualized after the flash, and the distance between lenses of the beam expander is adjusted to achieve optimal resolution (Fig. 2E). For a fixed distance between lenses we can measure the focal shift between imaging and uncaging light by finding the axial specimen position at which a light pulse gives a sharply focused uncaging spot. A plot of the focal shift as a function of distance between the lenses of the beam expander is shown in Fig. 2F; zero position is defined as a configuration that does not diverge or converge a collimated UV beam. Detailed instructions on alignment and calibration of the focal photolysis system are given in our recent work (Sarkisov and Wang, 2006).

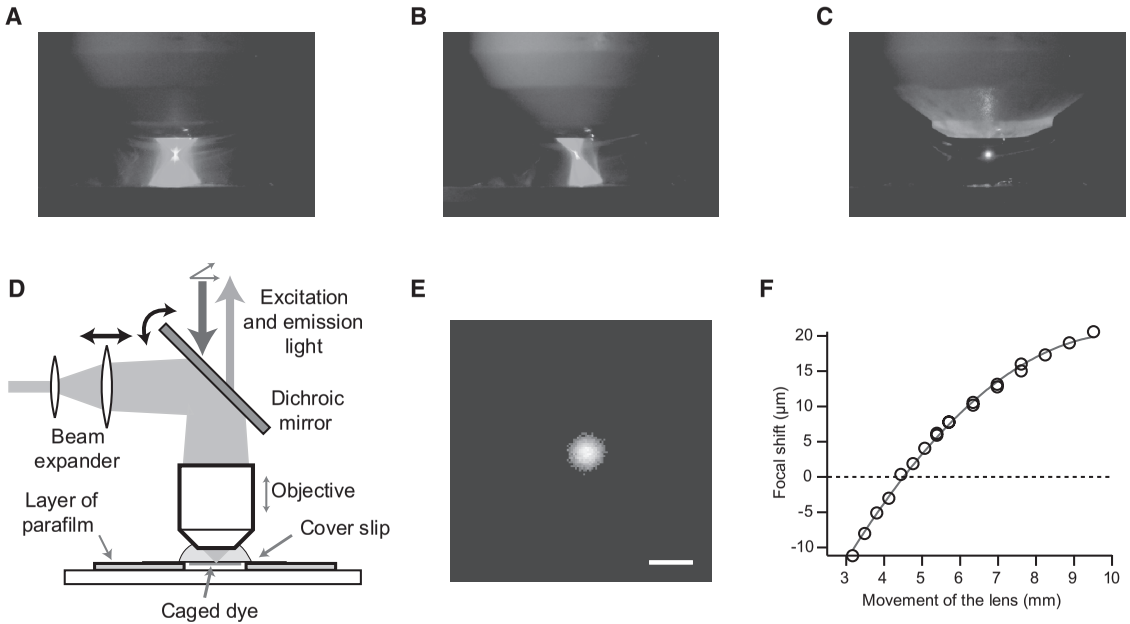

Fig. 2. Optimization of the geometry of the uncaging spot. The shape of the uncaging beam is visualized by the excitation of fluorescein. Optimal resolution is achieved when the cone of excited fluorescein is symmetrical (**A**). If light is asymmetrical (**B**) or does not focus to the location of two-photon excitation (**C**), the shape of the uncaging spot will be suboptimal. (**D**) The focal shift between imaging and the uncaging light is compensated for by changing the distance between the lenses of the beam expander and visualizing the shape of the uncaging spot on the sample of fried caged dye. (**E**) When the focal shift is fully compensated, submicrometer resolution of uncaging is achieved. Image of uncaged fluorescein that was immobilized on the coverslip. Scale bar 1 µm. (**F**) Change of the focal shift between IR and UV light as the distance between lenses increases. (Adapted from Sarkisov and Wang, 2006.)

4.3. Control of the System

Uncaging systems can be easily controlled by equipment commonly found in most physiology laboratories. In the simplest case a single transistor-transistor logic (TTL) pulse can be used to trigger a flash lamp or open a shutter. In a system based on a Q-switched laser two signals are necessary: one to open a mechanical shutter, and one to start lasing after a small delay to allow the shutter to open fully. Trigger signals can easily be generated by pulse stimulators (e.g., Master-8, AMPI, Jerusalem, Israel), digitizers (e.g., DigiData, Molecular Devices, Menlo Park, CA), or by a data acquisition board (e.g., National Instruments, Austin, TX) connected to the computer.

Time synchronization among the imaging system, the uncaging system, and the electrophysiological recording system is not hard to accomplish. If the camera or photomultiplier tube (PMT) used for image acquisition is not turned off during the uncaging flash to protect it from high light levels, a flash artifact will be visible on the image. We have not observed deterioration in the performance due to UV flashes in our conventional PMT (R3896, Hamamatsu, Hamamatsu City, Japan) over time. Power supplies of more sensitive gallium-arsenic PMT modules (e.g., H7422P-40, Hamamatsu) are equipped with protective circuits that shut off detectors when they are exposed to high light levels. A time mark that corresponds to the moment of uncaging can be recorded to the unused channel on the physiological system. In many custom microscopy control systems, CfNT (M. Müller, Max Planck Institute for Medical Research, Heidelberg, Germany) and MPScope (Nguyen et al., 2006) marks can also be embedded in the recorded optical signal.

5. Recent Developments in Uncaging

5.1. Two-Photon Uncaging

Optical two-photon uncaging provides a very effective way to achieve high spatial resolution of uncaging. In this technique a pulsed IR laser is used as the uncaging light source. If a wavelength is chosen such that the cage group can absorb two photons of IR light of similar energy to one UV uncaging photon, the probability of an uncaging event becomes proportional to the second power of light density, thus limiting uncaging to the focal volume where light density is maximal. In recent years cage groups have come into more common use with sufficiently high optical cross-section

to allow two-photon uncaging without major specimen damage (Fedoryak et al., 2005).

Aside from confining uncaging to the focal volume, the use of IR photons in true two-photon uncaging allows deeper penetration into the specimen. Since IR light is scattered less than UV light, increases in focal volume are less pronounced as light is focused more deeply into the sample. This advantage of true two-photon uncaging is especially important when working with highly scattering samples as brain slices. Two-photon uncaging is attractive in systems built upon a two-photon microscope since the imaging beam could be used for uncaging and the main necessary additional component is a Pockels cell to gate beam intensity. Use of infrared light for uncaging also simplifies alignment of such a system, since compensation for the focal shift between uncaging and imaging light becomes unnecessary. At present the main disadvantages of two-photon uncaging are the high cost of mode-locked infrared lasers and the limited number of caged compounds with sufficient two-photon uncaging index, a quantity that is closely related to the conventional uncaging index (see Table 1).

5.2. Chemical Two-Photon Uncaging

Chemical two-photon uncaging is a simple way to improve characteristics of an existing photolysis system. Improvement is achieved by adding a second inactivating caged group to the molecule of interest. Uncaging a double-caged compound requires absorption of two UV photons, making the probability of photolysis proportional to the second power of light density. Adding a second cage greatly reduces spurious out-of-focus uncaging, since at nonsaturating levels of light most double uncaging events occur in the focal volume where light density is maximal (Pettit et al., 1997). Confinement of photolysis volume is especially important when uncaging of a neurotransmitter is performed in the brain slices, since neurotransmitter action is limited to the cells located above and below the focal volume.

Double caging can also improve the chemical properties of the compound by making it more dissimilar in structure to the native agonist. Dissimilarity reduces the risk of undesired interaction with biological targets (Sarkisov et al., 2007). A third advantage comes from ease of handling; the requirement of two uncaging events makes the production of free agonists due to uncaging by room light or spontaneous degradation less likely.

5.3. Patterned Uncaging

Uncaging at multiple locations can be achieved by steering the uncaging beam (Gasparini and Magee, 2006; Matsuzaki et al., 2001; Shepherd et al., 2003; Shoham et al., 2005). Rapid beam steering is usually done using mirrors mounted on scanning galvanometers, as on a confocal or two-photon microscope. A faster means of scanning is the diversion of a UV uncaging beam with acousto-optical deflectors (AODs), which have fast switching times. In an AOD-based system we have achieved stimulation with submicrometer resolution at over 20,000 locations per second (Shoham, O'Connor et al., 2005). Our patterned uncaging system is capable of uncaging in an area of 170 by 170 μm (Fig. 3A), and is integrated with two-photon fluorescence microscopy and patch-clamp recording.

We have used this system to measure scattering in brain tissue of UV light. To perform these measurements fluorescent beads were embedded in different depths in molecular layer of a sagittal cerebellar slice (Wistar rats, P17–21), and the UV beam scanned over the tissue to form an image. Dependence on the size of the reconstructed bead as a function of the depth is shown on Fig. 3B. We found that the focal properties of the UV beam are approximately preserved for the first 25 μm of the slice.

We used this patterned uncaging system to study neuronal and circuit functionality in brain slices. Figure 3C illustrates an experiment in which connections between cerebellar granule cells and Purkinje cells were identified. Caged glutamate was photolyzed at over 20 locations in the granule layer, while electrophysiological responses from the Purkinje cell were recorded by whole-cell patch clamp recording. Out of 20 uncaging locations, seven responding regions were identified (Fig. 3D).

5.4. Light-Activatable Proteins and Ectopic Caged Neurotransmitter Receptors

Recent developments in the use of genetically encodable probes provide an approach to controlling cell function that holds great promise (Lawrence, 2005; Miesenböck and Kevrekidis, 2005). Though these methods still require patch-clamp or optical recording, they are useful not only because they are less invasive but also because they have the potential to be cell type–specific. One approach is to express a light-sensitive channel that is normally not

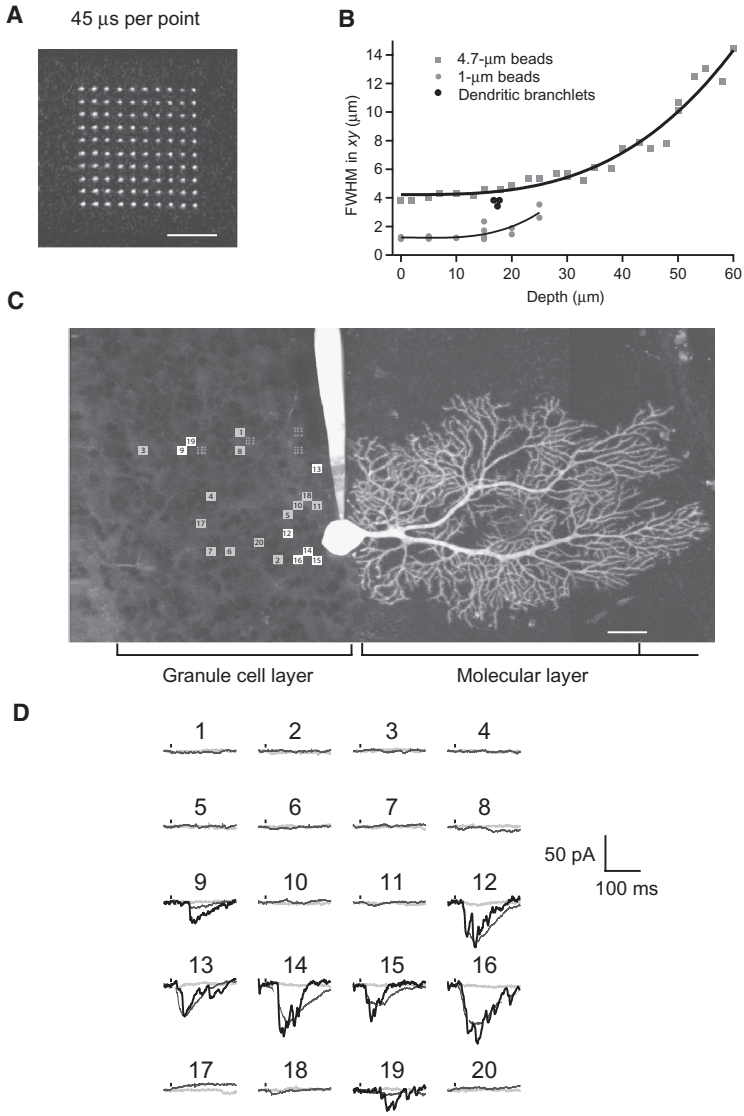

Fig. 3. Patterned uncaging with UV light. In this setup an uncaging beam can be focused laterally to different locations in the focal plane by means of acousto-optical devices. (**A**) Caged fluorescein was photolyzed in 100 locations with 45-µs interpulse time. Scale bar, 50 µm. (**B**) Measurement of the effect of UV scattering on resolution in a cerebellar brain slice. Images of fluorescent beads at different depths were taken by scanning with UV light. (**C**) Cerebellar Purkinje cell filled via patch-clamp recording electrode with fluorescent dye. The squares indicate the positions of the uncaging locations in the granule cell layer. Scale bar, 25 µm. (**D**) Postsynaptic responses to photolysis in the locations shown in **C**. (Adapted from Shoham et al., 2005.)

present in the neurons under a cell type–specific promoter (Boyden et al., 2005; Chambers et al., 2006). When illuminated by the light, the channels open, thus activating a cell of interest. A second approach is to ectopically express a receptor for a transmitter that is not native to the experimental species. Expression in mammals of receptors for the insect transmitter allatostatin has been used to inactivate subpopulations of neurons (Tan et al., 2006). These strategies exemplify the future power of combining protein-based probe design and optical methods.

Acknowledgments

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