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Confocal microscopic imaging of fast UV-laser photolysis of caged compounds

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Abstract

Using a pulsed UV laser in a confocal scanning microscope, we present a relatively cheap, accurate and efficient method for fast UV laser flash photolysis of caged molecules in two-dimensional cultured neurons. The laser light is introduced through the imaging optics, can be localized by a parallel red laser and can photolyse a sphere of less than 1 μm^2, and evoke local fluorescence changes in the imaged neurons. Caged glutamate and caged fluorescein are used to illustrate a disparity between spines and their parent dendrites at a sub-micron resolution.
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1. Introduction

Using high resolution imaging of calcium variations in individual dendritic spines of living neurons, studies in the past decade have demonstrated that the spine is a unique calcium compartment (Harris and Kater, 1994; Segal et al., 2000; Shepherd, 1996). Synaptic and chemical stimulation were used in order to raise $[Ca^{2+}]_i$ to higher levels in the spine head than in its parent dendrite. The small size of the dendritic spine requires the development of methods for local application of minute quantities of chemicals to its vicinity. The use of minimal electrical stimulation of afferent fibers has its obvious advantages, but its drawback is that the stimulation current can spread to adjacent presynaptic fibers and thus other spines, that are not necessarily within the field of view, will also be activated. Iontophoresis through a micropipette is another method for local drug delivery (Murnick et al., 2002). Using this method, the application pipette needs to be placed in close proximity to the studied dendrites to maintain its spatial selectivity, which is not always easy to achieve and standardize. An alternative method of local drug application, which has been used successfully in recent years, involves the local flash photolysis of caged molecules (Augustine, 2001; Canepari et al., 2001; Delaney and Zucker, 1990). It is based on the ability to chemically 'cage' the molecule of interest, and activate it by breaking the caging bonds with UV light, causing a local and rapid increase in the concentration of the 'uncaged', activated molecule. Several variations of methods for flash photolysis have been recently described (Brown et al., 1999; Callaway and Katz, 1993; Dodt et al., 2002; Parpura and Haydon, 1999; Pettit et al., 1997; Svoboda et al., 1996; Wang et al., 2000), and they are aimed at producing a highly localized photolysis of caged molecules. Obviously, flash photolysis of caged molecules has many advantages over the more conventional methods for drug delivery, and the ability to uncage molecules inside a small neuronal compartment is just one of them. With this method it is crucial to be able to localize, measure and minimize the sphere of photolysis so as to allow a precise topical uncaging of the active substance. We have developed an effective system for fast UV laser-induced flash photolysis of caged molecules in a restricted sphere of less than 1 μm in diameter, which can be used for studying calcium variations in individual dendritic spines of cultured neurons.

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2. Methods

2.1. Equipment

As an imaging platform, we use an upright Pascal scanning confocal microscope (Zeiss) equipped with standard Argon and HeNe lasers for excitation at wavelength of 488 and 543 nm, respectively. An air-cooled tripled ND:YAG laser (New Wave Research Inc.) (Fig. 1, 1), emitting 355 nm, 3.6 mJ, 4.0 ns single light pulses at a rate of 1 Hz are used for the photolysis of caged molecules. The UV laser light is coupled to the confocal microscope using a dichroic mirror with high reflection at 355 nm and high transmission of visible light (Locus, Optical Devices & Elements Ltd.) (8) and is focused on the image plane through a 63× 0.9NA Achroplan water immersion objective (9). Due to chromatic aberrations of the objective (i.e. different focal distances for the visible and ultra violet spectrum), the focal plane for 355 nm UV light is about 20 μm lower than for blue/green light. To compensate for this chromatic aberration, a focus-correcting lens (KPX073 EFL-250 mm, Newport) (7) is introduced at the entrance of the UV beam to the microscope. A second laser, Melles Griot red helium–neon (1 mW, 632 nm) (6) is used to align the UV laser in the field of view. The red beam is steered using Newport 10D20 ER2 dielectric mirrors (4) and the two beams are combined before coupling to the microscope using one of the UV attenuating filters (2) as a dichroic mirror. The focal depth of the red beam is further corrected to match that of the UV beam using a telescopic lens system (5). Both 355 mn UV light and 632 nm red light are directed into the confocal microscope using two high energy UV mirrors Y3-1025-45-P (from CVI Laser corporation) (3). Prior

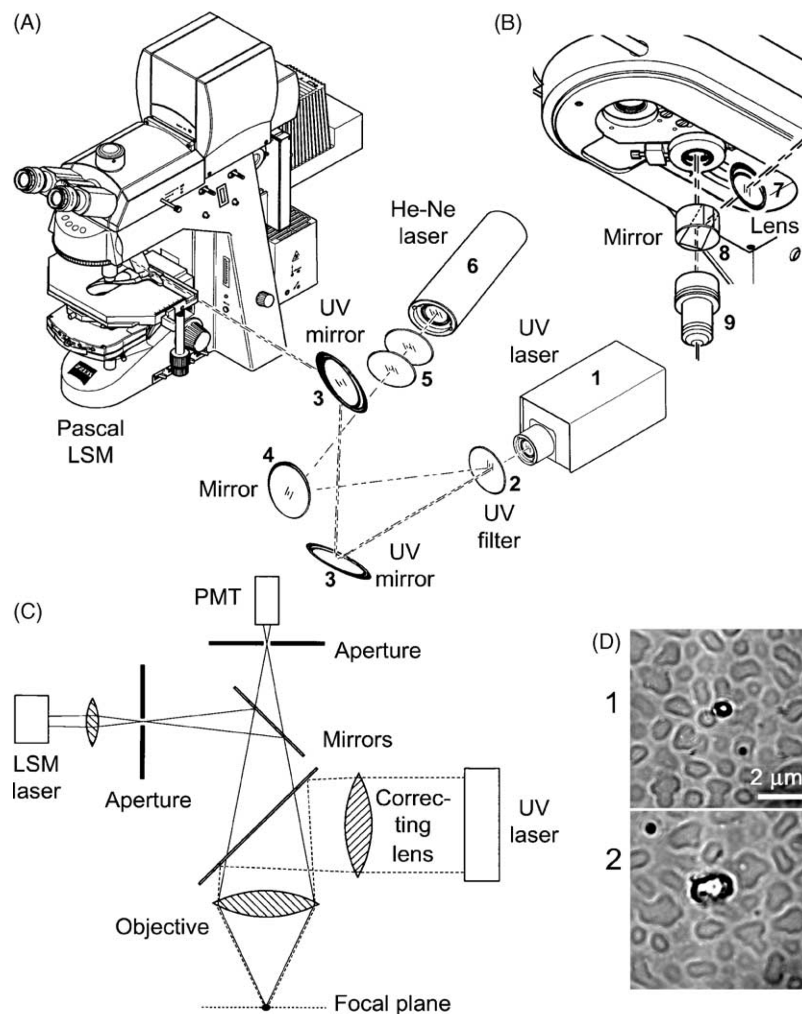


Fig. 1. Schematic diagram of the Pascal confocal microscope equipped for flash photolysis. (A) Low power image of the microscope, showing the two lasers, the UV laser, and the HeNe red laser used for alignment. The two light paths are merged with mirrors to enter the microscope through a mirror located above the objective. (B) A higher resolution image showing the light path deflected via a dichroic mirror above the objective lens. (C) Schematic diagram of the light paths, including the LSM lasers used for the imaging, and the UV laser for photolysis. A correction lens is placed in the light path to adjust the focal plane of the two lasers. (D) Two images showing the impact of different intensities of UV laser on a plane of focus, a glass slide coated with blue marker, visualized in phase microscopy. The light pulse creates a hole in the plane of the dye. The smaller hole, obtained with a full array of UV filters, above, creates a hole that is less than 1 μm in diameter. The larger hole was created with a higher power laser light pulse.

to the experiment, the size of the focused UV laser beam is verified to be of less than 1 μm^2 using blue marker paint on glass, as illustrated in Fig. 1. Various high energy UV filters (2) adjusted for 355 nm light (optical densities of 0.5, 1.0 or 2.0, corresponding to a reduction of 3, 10 or 100, respectively, all from CVI Laser Corporation) are used separately or in combination to control the intensity of the beam. When all three UV filters are used in series, the light intensity is reduced to 1/3000 of nominal intensity, about 1 μJ. Final adjustment of the UV laser power is done by inserting one to four UV-grade uncoated glass windows into the beam path. Each window reduces the UV light intensity by about 10%. When the additional reduction in light intensity due to filters, mirrors, glass lenses and passage through water are taken into account, the calculated UV light intensity amounts to 200–300 nJ, which produce a flux of $4–6 \times 10^{11}$ photons per pulse, focused on a less than femtoliter of medium containing from 1000 to 100,000 caged molecules.

2.2. Chemicals

Caged glutamate (*N*-(-carboxy-2-nitrobenzyl)-L-glutamic acid (*N*-(CNB-caged) L-glutamic acid) (molecular probes)) are prepared in the dark from stocks, and added to the recording medium at a final concentration of 0.1–0.5 mM. Caged fluorescein (CMNB-fluorecein, molecular probes, 5 mM) is back-filled into a micropipette tip, which is then filled with 3 M K-acetate, and injected into selected neurons. Ambient light is reduced to minimum throughout the recording session. The solutions are used within 2–3 h of preparation, to avoid spontaneous uncaging over time.

2.3. High resolution imaging of individual dendrites and spines

A coverslip is transferred from the 24 well plate where neurons grow for 2–4 weeks (Goldin et al., 2001; Papa et al., 1995) into the recording chamber, placed in an automated X–Y stage (Luig and Neuman, Germany) of the confocal microscope, where it is bathed with recording medium containing (in mM) NaCl 129, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, glucose 4.2, and HEPES 10, pH is adjusted to 7.4 with NaOH, and osmolarity to 320 mOsm with sucrose, at room temperature. Voltage gated sodium currents are blocked by TTX. Individual cells are impaled with a micropipette containing Alexa 555 (5 mM) for viewing cell morphology and Fluo-4 (5 mM), or 10 mM Oregon Green-1 for calcium imaging. The dyes are rapidly injected into the cell, and are allowed to equilibrate in the cell for at least 20 min before experiments are commenced. Alternatively, recording is made with patch pipettes having axial resistance of 4–6 MΩ, containing (in mM) K-acetate 120, Mg ATP 2, Hepes 20, KCl 10, Na_2 phosphocreatine 10, and Alexa 555 and/or Fluo-4, 0.02–0.1. Images of 512 × 512 pixels are taken with the 63× water immersion objective. 3D reconstruction of the dendrites is made from successive 0.2–0.5 μm optical sections. A line is scanned through the center of a dendrite/spine pair (0.7 ms per line) to detect fast changes in fluorescence during a response to drug application. The imaging laser lights (488 and 543 nm) are reduced to 3–5% of nominal intensity. Using this setting, we are able to continuously stimulate the same spine/dendrite pair with no significant loss of reactivity due to dye bleaching or photodynamic damage. Also, baseline fluorescence does not change across the 2–3 h observation time. The acquisition program of line scans in the confocal microscope triggers the UV laser flash using a Master 8 stimulator (AMPI, Israel). This also triggers the electrical stimulation and acquisition of membrane currents in the recorded cells. Signals are amplified with Axopatch 200 (Axon Instruments, California), stored and analyzed using PClamp software.

3. Results

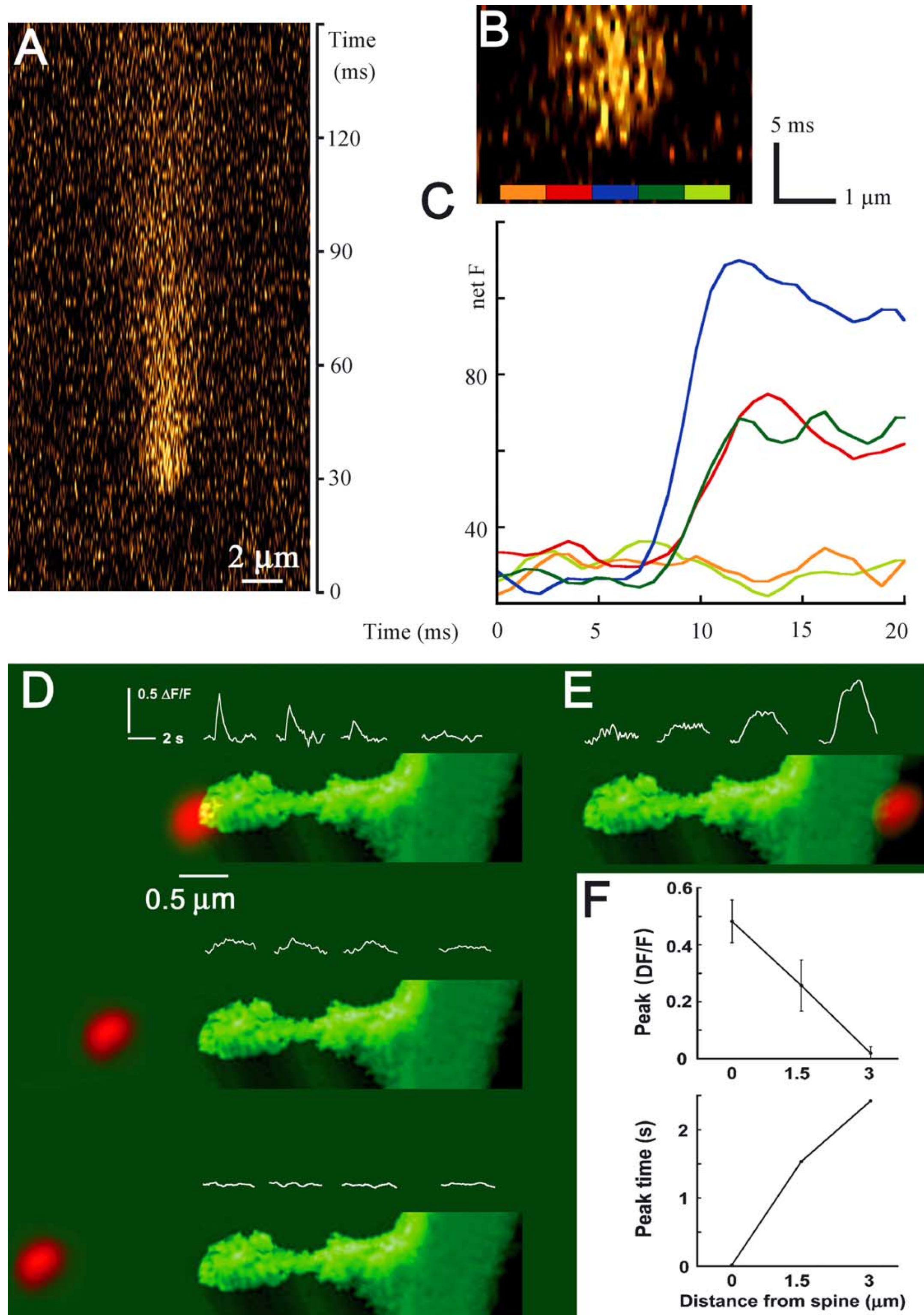
3.1. Determination of the location and size of the UV spot

Two methods were used to estimate the location and size of the UV laser light spot. In the first (Fig. 1D) a glass coverslip was painted with a blue marker pen, allowed to dry, immersed in water and placed in the microscope light path. Following focusing of the red light, the UV light pulse was applied, causing the formation of a hole in the paint. Fine adjustment was sometime needed to verify that the UV laser overlaps with the center of the red laser spot. The size of the hole varied as a function of the glass filter used, with minimum effective size being less than 1 μm. This method was used routinely to produce small adjustments in the alignment of the UV laser with the red laser spot in the light path.

The second method employed caged fluorescein. A thin layer of caged fluorescein was painted on a glass slide, allowed to dry, covered with a glass coverslip and sealed with varnish, to avoid exposure of the dye to water. The glass coverslip was then immersed in water and placed in the microscope. The size of the fluorescent spot could be measured through the microscope image using fast line scan (Fig. 2A–C). This method was used to measure directly the size of the UV laser spot. As can be seen in Fig. 2B and C, the width of the fluorescent spot is less than 1 μm, and the lateral diffusion of the fluorescent dye is minimal.

Glutamate is one of the more popular caged molecules, as it is being used for mapping of glutamate responses in culture and brain slices (Kandler et al., 1998; Matsuzaki et al., 2001; Pettit et al., 1997; Rossi et al., 1997). The use of caged glutamate highlights another major issue, i.e. the diffusion of the uncaged molecule, to activate high affinity membrane receptors remote from the site of photolysis. Obviously, the effective sphere is dependent on a number of factors, including the concentration of the photoreleased compound, the chemical stability of 'caging' bonds, the affinity of the studied ligand to the receptor, and the size, duration and intensity of

the UV flash. We estimated the effect of caged glutamate on postsynaptic glutamate receptors in our testing conditions, by varying systematically the distance between the center of the UV flash and the dendritic spine head, where the receptors are presumably located (Fig. 2D). Calcium transients were detected in the spine head when the UV laser beam was focused no further than 2 μm away from the spine head. In fact, activation of calcium changes in the spine head were not followed by calcium changes in the parent dendrite, and vice versa, when the laser light was focused on the parent dendrite the spine head did not respond (Fig. 2E), indicating that photoreleased glutamate acted locally in our testing conditions.



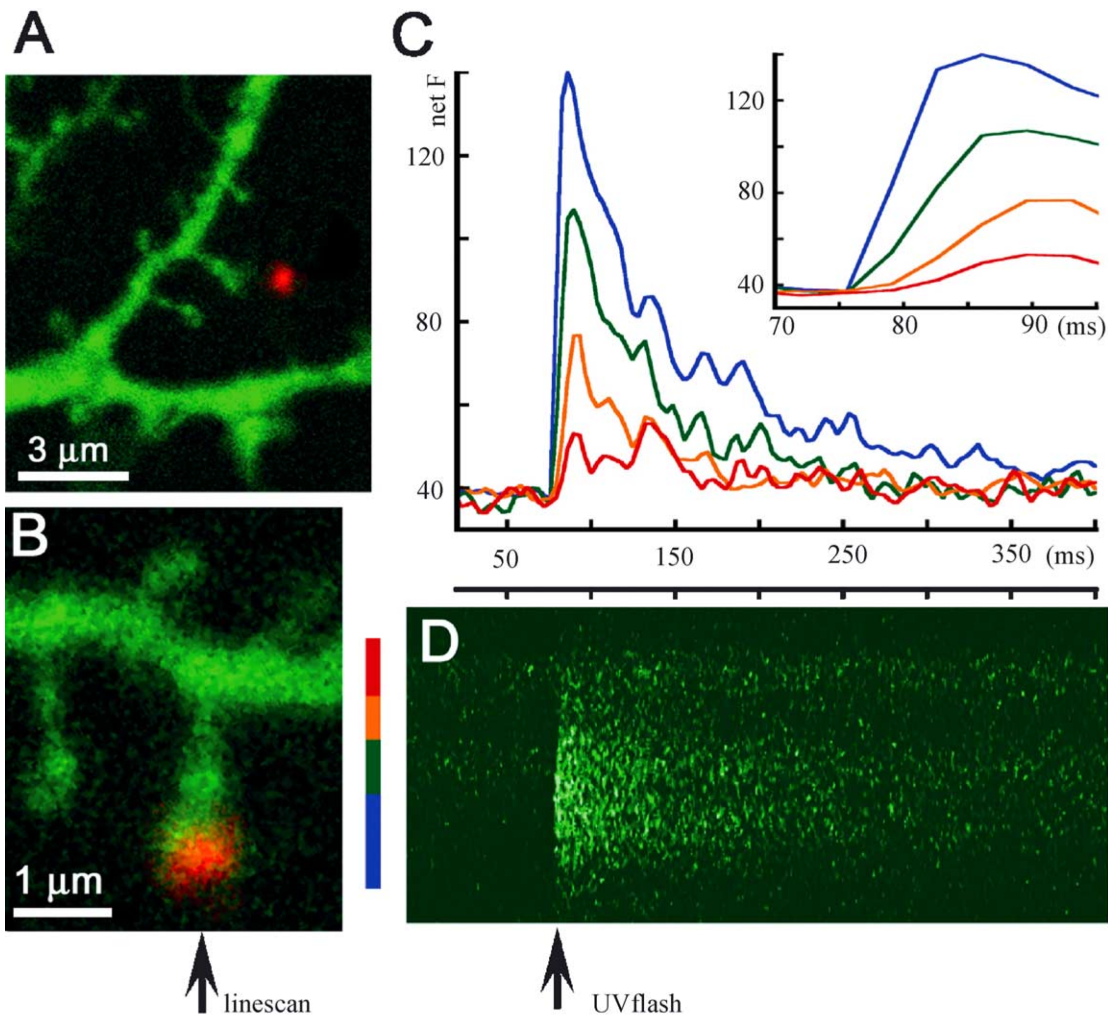


Fig. 3. Flash photolysis of caged fluorescein. (A) A dendritic field of a cell that was previously loaded with Alexa 555 and caged fluorescein, imaged with the PASCAL HeNe laser for the Alexa dye. A red dot in the center of the field indicates the red laser marking spot. (B) Higher power image of a section of the dendrite containing a spine, marked with the red laser dot. A line is scanned at the arrowhead, between the spine head and the parent dendrite. (C and D) The line scans of the spine/dendrite segment shown in (B), are running from left to right, at the time scale shown above (D). A UV flash is applied at the arrowhead, to produce a local rise in fluorescence, which spreads into the parent dendrite and decays exponentially. A color bar to the left indicates regions of interest, which are analyzed in (C). (C) Kinetics of the rise time and decay of fluorescence following flash photolysis of caged fluorescein, illustrated in (B) and (D). The color code is for the different compartments shown to the left of (D). Insert in C, a faster time base to show the rise time of fluorescence in the different compartments. The fluorescence rise in the parent dendrite lags by about 5 msec after the rise in the spine head.

Fig. 2. Imaging of flash photolysis of caged molecules. (A)–(C) Line scan imaging of caged fluorescein. The dye was smeared on a glass slide, allowed to dry, covered with a glass coverslip and placed in the confocal microscope field of view. (A) A 0.7 ms line sweeps from bottom to top, and a single laser flash is applied to produce a rise in fluorescence that is localized to the spot of UV flash. (B) A higher resolution image of the line scan shown in (A), to illustrate that the locus of initial uncaging is smaller than 1 µm in diameter. Bars of different colors are placed below the line scan to indicate regions of interests summarized in (C). (C) Changes in fluorescence intensity over time in five regions of interest, the center, two flanking regions and the more remote regions, color coded in (B), indicating that the initial response is limited to the center of the light spot, while the flanking regions lag behind, and the more remote regions, 1.5 µm away from the center, do not respond at all. (D)–(F) Responses to caged glutamate. (D) High power Alexa 555 image of a dendritic segment contains the spine, which protrudes to the left. Red laser light is directed to the tip of the spine (D, top), where it evokes the largest response, shown on the top left traces, as the DF/F of change in fluorescence of the calcium dye Oregon Green-1. While the response at the tip of the spine is largest, concurrent responses of decreasing amplitudes are recorded in the spine neck, and the parent dendrite, as shown on the top traces. Below, virtual lack of fluorescence changes when the laser is directed 1.5 or 3 µm away from the spine tip. (E) Aiming the laser light to the right side of the dendrite evokes a large response in the dendrite, and progressively smaller responses into the spine head. (F) Spatial resolution of the glutamate response. The largest response and the fastest ones are seen when the laser light is aimed at the spine head. Nearly no response is seen when the light is aimed 3 µm away from the spine head.

Caged fluorescein was injected into neurons together with Alexa 555, which was used to image the cell morphology (Fig. 3), and detect local fluorescence changes following application of the laser pulse. With this application, the actual sphere of activation is restricted to the structure being studied, the spine head or the dendritic shaft, since there is no fluorescent dye outside the cell membrane. It is important to verify that the sphere of activation is smaller than the distance between the spine head and the parent dendrite, such that a direct measurement of the diffusion of the photoreleased dye can be made. This method was found to be highly reliable for the study of spread of fluorescent molecules between adjacent cellular compartments. In a similar series of experiments, flash photolysis of caged calcium was studied in small cellular compartments (Korkotian and Segal, unpublished observations, see also Brown et al., 1999).

The efficiency of uncaging can only be estimated at the present time. We made two pertinent observations on this issue with the caged fluorescein. First, in ideal conditions, we obtain a near maximal uncaging following a single UV flash. When we remove one of the interference filters, we are unable to increase the fluorescence response of caged fluorescein by much. This is not due to the saturation of the detectors, as the PMT can respond to higher fluorescence at a different site on the dendrite. Second, following one or two flashes aimed at a spine head, it will not respond to a third pulse at the same location for another minute or more, indicating that the caged molecule had been depleted. Under such conditions we can detect some bleaching of the already fluorescent dye (unpublished observations).

4. Discussion

The current system provides two main advantages for flash photolysis of caged molecules over existing systems. First, it allows easy and precise localization of the laser spot, within less than 1 μm sphere. This is done with a parallel red laser, corrected for chromatic aberrations. The size of the UV laser spot can be estimated using two independent methods, and its intensity can be controlled with neutral density filters. While the more expensive two-photon uncaging methods (Brown et al., 1999, Matsuzaki et al., 2001) allow a better control of the depth of the stimulated area, this is not a crucial dimension with cultured neurons, which are primarily two-dimensional structures having a depth of 3–6 μm.

The fast, 4 ns pulse, provides another advantage over existing systems in that it has a point source of a momentary rise in active molecules, with little diffusion away from the release site during the time of uncaging. This fast time scale contrasts with the slow uncaging produced by other sources (3 ms, Dodt et al., 2002; 5 ms, Pettit et al., 1997; 2–10 ms, Wang et al., 2000), where uncaged molecules are formed and diffuse away from the site of photolysis, causing an increase in the sphere of activated compounds to 3–5 μm (Wang et al., 2000). The sphere of photolysis is thus more restricted than is produced by other sources.

In earlier studies, we experimented with 0.2 mm dia. Shear-force NSOM quartz Fiber Probes (Nanonics, Jerusalem, Israel) pulled to diameter of 0.5–1 μm and coated with Cr/Al to minimize light loss as a means of bringing the UV light into the field of view (as in Kandler et al., 1998; Parpura and Haydon, 1999). UV laser flash was focused with additional 40× Zeiss objective and introduced into fiber. While there are some intuitive advantages in having an independent means for bringing the light into the sample, not via the optical path, the results were not satisfactory, as the UV light coming out of the fiber spreads to a large sphere, and the light intensity was reduced drastically in the fiber (about 50–70% loss per 1 μm) so that it was ineffective for photolysis in our testing conditions.

The co-localization of the UV light spot with the red laser is done in the same light path and optics. This enables an easy and reliable alignment and focusing of the laser, unlike other systems where the red imaging is done from another direction, and the location of the UV has to be estimated (Dodt et al., 2002).

Another means of local drug application is via microiontophoresis (Murnick et al., 2002). This method has been applied successfully to cultured neurons, and is both efficient and accurate, and certainly cheaper than flash photolysis. Indeed, for application of charged molecules to the vicinity of the neuron this may be a superior method, but when dealing with less electro-mobile molecules, or molecules that are released inside cells (e.g. Korkotian and Segal, 1998), this method is not useful.

The method of estimation of the effective diffusion of the flash-released molecule varies among different studies, and can affect the estimated size of activated molecules. One common method used in the present study, involves imaging of fluorescence of either the caged molecule or a calcium reporter molecule, Fluo-4. The use of line scan prohibits the detection of regions around the dendrite that are not actually scanned. In fact, even the estimation of the size of flash photolysis can be erroneous, if the line is not scanned in the middle of the flashed area. Another method for evaluation of the efficacy of the uncaging involves recording of electrical signals from the soma of the neuron which is exposed to the caged molecule (e.g. glutamate, Matsuzaki et al., 2001). With this method, the outcome of the uncaging is recorded remotely, and there is no certainty that the imaged organelle, e.g. spine, is the only one to be activated by the 'uncaged' glutamate. Undoubtedly, a combined recording of local fluorescence changes and remote electrophysiological changes should be used to control for possible inadvertent expansion of the sphere of photolysis.

Finally, the combined use of the Pascal confocal microscope with the laser photolysis in the present configuration allows a relatively fast scanning of a field, without losing the focal plane of the flash laser, or the ability to record

simultaneously from the cell that is being activated. This allows a rather rapid and accurate mapping of hot spots of responses to caged molecules, down to the level of single synapses.

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References

Augustine GJ. Illuminating the location of brain glutamate receptors. Nat Neurosci 2001;4:1051–2.

Brown EB, Shear JB, Adams SR, Tsien RY, Webb WW. Photolysis of caged calcium in femtoliter volumes using two photon excitation. Biophys J 1999;76:489–99.

Callaway EM, Katz LC. Photostimulation using caged glutamate reveals functional circuitry in living brain slices. Proc Natl Acad Sci USA 1993;88:7661–5.

Canepari M, Nelson L, Papageorgiou G, Corrie JE, Ogden D. Photochemical and pharmacological evaluation of 7-nitroindolinyl- and 4-methoxy-7-nitroindolinyl-amino acids as novel, fast caged neurotransmitters. J Neurosci Methods 2001;112:29–42.

Delaney KR, Zucker RS. Calcium released by photolysis of DM-nitrophen stimulates transmitter release at squid giant synapse. J Physiol 1990; 426:473–98.

Dodt HU, Eder M, Schierloh A, Zieglgansberger W. Infrared-guided laser stimulation of neurons in brain slices. Sci STKE 2002;120(2): 1–11.

Goldin M, Segal M, Avignone E. Functional plasticity triggers formation and pruning of dendritic spines in cultured hippocampal networks. J Neurosci 2001;21:186–93.

Harris KM, Kater SB. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. Ann Rev Neurosci 1994;17:341–71.

Kandler K, Katz LC, Kauer JA. Focal photolysis of caged glutamate produces long term depression of hippocampal glutamate receptors. Nat Neurosci 1998;1:119–23.

Korkotian E, Segal M. Fast confocal imaging of calcium released from stores in dendritic spines. Eur J Neurosci 1998;10:2076–84.

Matsuzaki M, Ellis-Davies GCR, Nemoto T, Miyashita Y, Iino M, Kasai H. Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. Nat Neurosci 2001;4:1086–92.

Murnick GJ, Dube G, Krupa B, Liu G. High resolution iontophoresis for single synapse stimulation. J Neurosci Methods 2002;116:65–75.

Papa M, Bundman MC, Greenberger V, Segal M. Morphological analysis of the development of dendritic spines in primary cultures of hippocampal neurons. J Neurosci 1995;15:1–11.

Parpura V, Haydon PG. 'Uncaging' using optical fibers to deliver UV light directly to the sample. Croat Med J 1999;40:340–5.

Pettit DL, Wang SS, Gee KR, Augustine GJ. Chemical two-photon uncaging: a novel approach to mapping glutamate receptors. Neuron 1997;19:465–71.

Rossi FM, Margulis M, Tang CM, Kao JP. *N*-Nmoc-L-glutamate, a new caged glutamate with high chemical stability and low pre-photolysis activity. J Biol Chem 1997;272:32933–9.

Segal M, Korkotian E, Murphy DD. Dendritic spine induction and pruning—common cellular mechanisms? Trends Neurosci 2000; 23:53–7.

Shepherd GM. The dendritic spine: a multifunctional integrative unit. J Neurophysiol 1996;75:2197–210.

Svoboda K, Tank DW, Denk W. Direct measurement of coupling between dendritic spines and shafts. Science 1996;272:716–9.

Wang SS, Khiroug L, Augustine GJ. Quantification of spread of cerebellar long-term depression with chemical two-photon uncaging of glutamate. Proc Natl Acad Sci USA 2000;97:8635–40.