

# Alignment and calibration of a focal neurotransmitter uncaging system

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**Photolysis of caged compounds is a powerful tool for studying subcellular physiological functions. Here we describe protocols for the alignment and calibration of a focal uncaging system. We also report procedures for convenient quantitative calibration of uncaging. Using these methods, we can achieve submicron lateral resolution of photolysis and probe biological function in spines, the smallest signaling compartments of neurons. Initially, the entire alignment procedure takes 4–6 h to perform; periodic fine-tuning of the system takes 1–2 h.**

## INTRODUCTION

Caged ATP was the first biologically useful caged compound to be synthesized nearly 30 years ago<sup>1</sup>. Since then, a variety of caged groups have been developed and a large number of molecules have been caged<sup>2</sup>. Caged compounds have many applications, including manipulation of extracellular and intracellular biochemical species, tracing living cells during development, and monitoring the movement of cellular components. Caged second messengers are used to study intracellular signal transduction pathways during muscle activation, fertilization, secretion and other cellular functions<sup>3</sup>. In general, light-activated proteins have potential applicability to almost any aspect of cellular biology<sup>4</sup>. Photochemical tools have found great use in neuroscience, in which precise temporal and spatial control provided by the photolysis technique are particularly important<sup>5</sup>.

In parallel to the design of new caged probes, spatially resolved photolysis systems have been developed that bring the uncaging light to a submicron-diameter spot. Recently, we reported a system that can uncage in both spatially and temporally defined patterns at up to 20,000 locations per second<sup>6</sup>. In addition, during the past decade, novel approaches for photostimulation have come into use that allow not only lateral but also axial resolution: chemical two-photon uncaging<sup>7</sup>, which substantially decreases the out-of-focus production of active molecules; and true two-photon uncaging with two infra-red (IR) photons<sup>8</sup>, an approach for spatial localization that is particularly useful for samples in which conventional ultraviolet (UV) light is heavily scattered.

Here we describe a convenient protocol to align and calibrate our focal uncaging and microscopy system. We cover, in detail, how to achieve parfocality between the imaging and uncaging light, which is critical for measuring responses at the exact site of uncaging. We describe how to prepare a thin, immobile layer of caged dye, which, because it can be examined at leisure after an uncaging flash, greatly simplifies how to align the uncaging system. This sample is also indispensable for calibration of the patterned uncaging setup. Detailed instructions on assembling the uncaging

system itself are given in the supplementary note to our previous work<sup>6</sup>. Finally, we describe methods to calibrate the uncaging efficiency of the system by photolysis of caged fluorescein. Alternative calibration methods, not described here, are also possible based either on pH changes in a test solution of caged ATP (or another phosphate)<sup>9</sup> or on physiological responses<sup>10</sup> (D.V.S., S.E. Gelber, J.W. Walker and S.S.-H.W., manuscript in preparation).

In the alignment of our system, in which the fluorescence excitation light and the uncaging excitation light must converge on the same plane of focus, adjustments in the axial dimension are important because any given objective will bend light differently as a function of wavelength. This difference can be particularly pronounced in systems that are built on two-photon microscopes due to the large differences between IR- and UV-light properties of many objectives. In our specific system ( $\lambda_{\text{uncaging}} = 355 \text{ nm}$ ,  $\lambda_{\text{imaging}} = 830 \text{ nm}$ , Achroplan 63 $\times$ , 0.9 NA water-immersion infinity-corrected objective; Zeiss 440067), UV light needs to impinge on the back of the objective with an angle of convergence of 0.016 radians (0.9°). This slight difference from the collimation of the IR excitation beam compensates for the focal shift of 32  $\mu\text{m}$  between IR and UV light. Note that the beam expander will need to provide convergence or divergence, depending on the details of the objective design. In two-photon systems with sufficient power, and by using caged compounds of sufficiently high cross-section, uncaging can be achieved by gating the full intensity of the IR beam between imaging and uncaging using a Pockels cell, thus achieving parfocality automatically.

Although we are working with a UV-laser-based uncaging system that has been integrated with a two-photon microscope, the protocols described can be used to calibrate systems that are built on commercial one-photon confocal microscopes, as well as systems for two-photon uncaging. We recommend that readers become acquainted with protocols and reviews on imaging calcium in neurons<sup>11</sup>, two-photon uncaging<sup>8</sup> and other UV-light-based uncaging systems<sup>12–14</sup>.

## MATERIALS

### REAGENTS

- DMNB-caged fluorescein dextran, 3000 MW (D-3309; Invitrogen)
- Fluorescent beads (0.2  $\mu\text{m}$ , cat. no. 17151; and 0.06  $\mu\text{m}$ , cat. no. 17149) (Fluoresbrite; Polysciences Inc.)

- Fluorescent beads, excitable by UV light (1  $\mu\text{m}$ , cat. no. 17458, Fluoresbrite BB; Polysciences Inc.)
- Fluorescein sodium salt (cat. no. F-6377; Sigma)
- Bovine serum albumin (BSA) (cat. no. B-4287; Sigma)

**EQUIPMENT**

- ND filters designed for UV light (CVI Laser or Edmund Optics)
- Quarter order wave plate (cat. no. WPQ05M-355; Thorlabs) and polarizer (cat. no. GL5-A; Thorlabs)
- Dichroic mirror (cat. no. 390DRLP; Omega Optical)

- UV beam expander (cat. no. BXUV-4.0-5X-355; CVI Laser)
- Laser scanning microscope (conventional or two-photon)
- UV laser (Model 3501, DPSS Corp.)
- Micromanipulator (cat. no. MP-285; Sutter Instrument Company)
- Powermeter (Field Master, Coherent Inc.)

**PROCEDURE**

**Rough alignment of uncaging and imaging beams**

**1|** As a source of uncaging light, we use a Q-switched, frequency-tripled Nd:YVO<sub>4</sub> UV laser (50–60 ns pulses;  $\lambda = 355$  nm at a 100-kHz repetition rate). 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 particularly useful for a rapid beam-steering, patterned uncaging system<sup>6</sup>. To filter uncaging light, use reflective neutral-density filters that are designed to withstand high-energy pulses of UV light. An alternative is to use a polarizer combined with a beamsplitter. For focal uncaging, the UV laser beam is introduced to the optical path of the microscope (**Fig. 1a**) using a dichroic mirror. For best uncaging resolution, the introduced UV beam should be parallel to the optical axis of the microscope and should be expanded to fill up the back aperture of the objective. Overfilling the back aperture improves resolution by taking maximum advantage of the numerical aperture of the objective, but also potentially decreases the total amount of UV power delivered to the sample. To widen the beam, we use a 5× UV beam expander mounted to the pillar that supports the microscope. The same beam expander is used to converge or diverge the UV beam, which is necessary to achieve parfocality between the imaging and uncaging light in Step 4.

**! CAUTION** UV and IR laser emission are invisible to the human eye, presenting a particular hazard. When you are working with lasers, always use eyewear that blocks both UV and IR light, especially when using custom-made systems in which the beams are often more exposed (see [http://www.eh.doe.gov/paa/Laser\\_Safety\\_Report.pdf](http://www.eh.doe.gov/paa/Laser_Safety_Report.pdf)).

**2|** For initial alignment, pour a 10  $\mu$ M water solution of fluorescein into a chamber in front of the objective and illuminate it with UV light (less than 0.1 mW) (**Fig. 1b**) or IR light (**Fig. 1c**). Under these conditions, the cone of the excited fluorescein solution should be visible from the side (**Fig. 1d**). If the observed cone is not axially symmetric with respect to the optical axis of the system (**Fig. 1e**), or does not coincide with the point at which the excitation light is focused (**Fig. 1c**), adjust the angle and position of the incoming UV light by aligning the last mirrors in the beam path. Avoid developing bubbles under the objective.

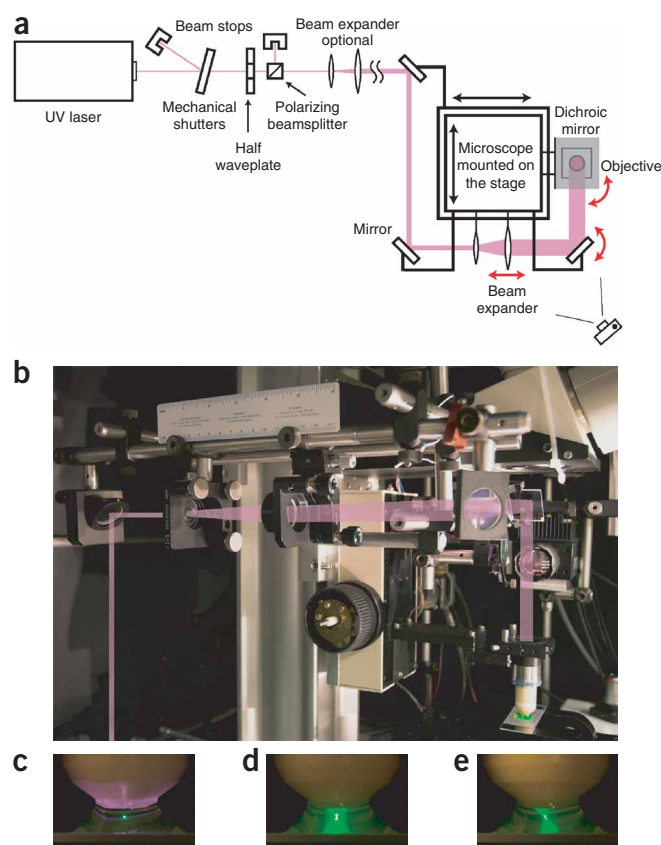
**3|** Observe fluorescein excitation through the eyepieces or camera. Make fine adjustments to the mirrors to move the fluorescent region to the middle of the field of view.

**! CAUTION** Only low levels of UV-light energy are needed for excitation of fluorescein solution. Do not increase UV-light energy above 1 mW if fluorescence is not visible. The increased intensities will damage your eyes or the detector. Instead, try making fresh fluorescein solution and darken the room.

**4|** Make slight changes to the distance between the lenses of the beam expander to minimize the size of the fluorescent spot, as observed in Step 3. The beam expander should be firmly mounted and properly aligned so that the changes in distance between lenses do not change the angle or position of the beam as it hits the back aperture of the objective. If the position of the fluorescent spot moves laterally, readjust the beam as in Step 3.

**Fine alignment of the uncaging and imaging beams**

**5|** There are two methods for fine alignment of the uncaging system: by uncaging in a thin layer of caged dye and by detection of fluorescence from UV-light-excitable fluorescent beads. The method using caged dye is fast and allows direct



**Figure 1 |** Design and alignment of a system for focal uncaging. **(a)** Sketch of the system (top view). Red arrows show components that are adjusted in the minimization of uncaging spot size. **(b)** Ultraviolet-light beam path is shown on a photograph of the integrated uncaging and two-photon microscopy system. In the properly aligned system, excitation light **(c)** and uncaging **(d)** and are focused on the same spot. Misalignment causes asymmetry in the excitation pattern **(e)**.

## PROTOCOL

observation of the size and position of the uncaging spot. The method using beads is slower but does not require the use of a coverslip, which may introduce aberrations. We recommend using both of these methods and comparing performance.

6| To make a sample of caged dye, prepare a 10  $\mu\text{l}$  water solution of 1% BSA and 2  $\text{mg ml}^{-1}$  caged fluorescein dextran.

7| Evenly spread the solution on the coverslip. Let it dry on a horizontal surface for about 1 h, until the water evaporates.

### ? TROUBLESHOOTING

8| Firmly attach a layer of Parafilm to a glass slide. To allow better contact, the glass slide may be slightly heated. Remove a patch of Parafilm from the center of the slide to make a hole that exposes some of the dried solution. Position the coverslip on top of the hole with the caged dye layer facing down towards the slide (**Fig. 2a**). To prevent water leaking into the dried sample, the coverslip can be attached to the Parafilm with cyanoacrylate glue (such as Krazy Glue).

9| Put the glass slide under the microscope and start imaging. Focus on the caged dye layer. Deliver an uncaging flash of 1–10  $\mu\text{J}$  energy. A spot of photolyzed dye should become visible. Make adjustments of the beam expander as in Steps 3 and 4 to obtain the best shape and position of the uncaging spot (**Fig. 2c**), moving the slide to expose fresh regions of dye as necessary.

**▲ CRITICAL STEP** It may be hard to focus on the layer of caged dye, which usually has very low fluorescence. To help, you can put a mark on the coverslip during Step 7 and then focus on it using visible light. Alternatively, on a system based on a two-photon microscope, you may add a small concentration of fluorescein to the water between the objective and the coverslip. Focus above the coverslip and observe fluorescence. Move the objective down until the focus reaches the coverslip and the fluorescence disappears. Increase the IR laser power. Once you move down for another 0.17 mm (the thickness of a standard coverslip), the excitation focal point will reach the layer of caged dye on the other side of the coverslip. This layer can be detected as a slight increase of the fluorescence signal.

**! CAUTION** Stray light from the uncaging flash could saturate or damage the detector in the scanning microscope. Depending on the type of detector, consider placing a UV-light filter in front of it or shutting it off during flash presentation. However, we have not observed severe deterioration in the performance of our Hamamatsu R3896 photomultiplier tube over time. The method described in Steps 6–9 can be used to produce thin layers of other materials. For example, the resolution power of the microscope can be measured by imaging an immobilized fluorescent bead that is substantially smaller than the theoretical resolution of the microscope. Such a sample can be made by replacing the caged dye used in Step 6 with a dilute solution of fluorescent beads.

### ? TROUBLESHOOTING

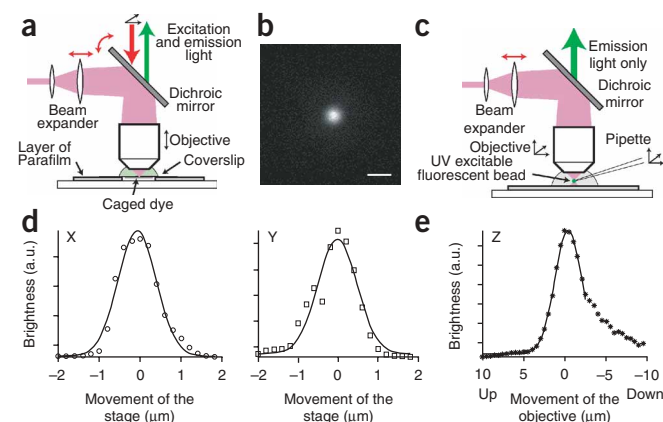
#### Aligning the system by imaging of fluorescent beads

10| Aberrations introduced by the cover glass may decrease the precision of calibration, especially in the axial direction. For this reason, for the final calibration of the system, we recommend using the method of imaging a single bead (**Fig. 2b**). In this protocol, a 1  $\mu\text{m}$  fluorescent bead, attached to the tip of a pulled glass microelectrode pipette, is illuminated by a low level of UV light (less than 1  $\mu\text{W}$ ). Emitted light is detected by the microscope detector. By changing the position of the UV spot with respect to the bead, we can measure the position and size of the uncaging spot.

11| Dilute the solution of beads by a factor of 10,000. Pour this dilute solution into the imaging chamber or onto a glass slide. Observe the beads using bright-field illumination.

12| By applying negative pressure to the pipette (the tip of which is the diameter of a 15–20  $\text{M}\Omega$  patch-clamp recording electrode), suck one bead onto the end of the pipette. Carefully replace the bead solution with water to reduce background fluorescence. Maintain negative pressure in the pipette to keep the bead attached.

13| Image the bead with IR or visible light. Position the bead by moving the pipette or objective exactly where the uncaging spot was observed at the end of Step 9.



**Figure 2** | Two methods for the fine adjustment of the size and position of the uncaging spot. **(a)** First method: a flash of ultraviolet (UV) light uncages dye in a small region. Fluorescence is detected by a scanning excitation beam. **(b)** Image of the uncaging spot produced by 0.7  $\mu\text{J}$  flash. Scale bar, 1  $\mu\text{m}$ . **(c)** Second method: UV light causes fluorescence in a 1  $\mu\text{m}$  bead that is attached to a glass pipette. Scanning is performed by moving either the microscope or the pipette. **(d,e)** Fluorescent signal from the bead as the microscope moves laterally in the  $xy$ -plane **(d)** or the objective moves vertically along the  $z$ -axis **(e)**.

**14** Stop imaging the bead with IR or visible light and start to illuminate the bead with UV light, detecting the fluorescence of the bead by the microscope's light detector. As no scanning is performed at this step, you should see a uniformly bright image. It is important to choose the right level of UV-light excitation to give detectable fluorescence from the bead but no bleaching or high background.

**15** Move the bead laterally with respect to the UV-light spot by moving either the pipette or the objective and observe the change in total brightness. If the bead was positioned in the center of the uncaging spot, then the brightness should decrease (**Fig. 2d**). Adjust as needed.

**16** Move the bead or objective vertically back and forth (**Fig. 2e**). If total brightness increases as you move in either direction, it means that maximum axial intensity of UV light does not coincide with the bead position. Change the axial position of the uncaging spot by changing the divergence angle of UV light, as in Step 4.

**17** If the bead is positioned exactly in the center of the uncaging spot, movement of the bead in any direction should decrease total brightness. If you see a mismatch in lateral or axial direction, repeat Steps 15 or 16.

### Estimation of uncaging efficiency of the system

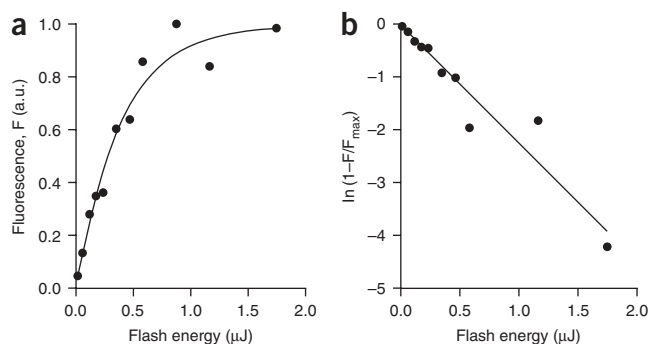
**18** Caged fluorescein dextran (or another caged fluorophore<sup>15</sup>) can be used to estimate the efficiency of the system. This measurement can also be used to extrapolate the expected uncaging efficiency of other compounds by considering the principle that uncaging efficiency is proportional to the uncaging index  $\epsilon^*\phi$ , where  $\epsilon$  is the extinction coefficient of a cage group and  $\phi$  is its quantum yield, the probability of uncaging after absorption of a photon.

**▲ CRITICAL STEP** In systems using UV-light pulses of a few nanoseconds, such as a pulsed N<sub>2</sub> laser, the fraction of the messenger that can be produced is limited to  $\phi$  or less due to the inability of a cage group to be excited more than once by a pulse of light. In these cases, the uncaging index is still calculated in the same way but the maximal possible concentration change per flash is reduced.

**19** Fill a sharp glass pipette with a water solution of caged fluorescein or prepare a sample of dried caged dye (see Steps 5–9). Glycerol may be added to slow diffusion, without affecting uncaging efficiency. Note that dried caged dye, although convenient for handling and measurement, leads to large variability in the calibration procedure.

**20** Focus on the sample. Deliver a series of UV-light flashes of different energies, up to the level that saturates the fluorescent response ( $F_{\max}$ ). Avoid using excessively high uncaging energies, as fluorescein bleaches easily.

**21** Measure the fluorescence at the center of the uncaging region as a function of flash energy (**Fig. 3**). If you are using a sharp pipette filled with water solution, acquire images at the fastest frame rate or in the line scan mode to decrease the level of mixing with surrounding regions. This mixing effect is less of a concern in systems that are not focused to or near the diffraction limit. Each fluorescence measurement should be made after sufficient time from the previous flash to avoid an increasing pedestal of fluorescence or depletion of caged compounds. Uncaging in dried samples can occur slowly, so wait several seconds between flash presentation and image acquisition.



**Figure 3** | Estimating the uncaging efficiency of the system. **(a)** Plot of the fluorescence at the center of the uncaging spot as a function of uncaging energy. A least squares fit with the function  $F = F_{\max} [1 - \exp(-E/E_f)]$  gives  $E_f = 0.39 \pm 0.05 \mu\text{J}$ . **(b)** Log-transformed data from panel **a**.

**TABLE 1** | Uncaging parameters for some commonly used caged compounds.

	$\epsilon$ in $\text{M}^{-1}\text{cm}^{-1}$ (wavelength)	$\phi$	Uncaging index, $\epsilon^*\phi$
CNB-caged glutamate	~ 500 (350 nm)	0.15	75
NPE-caged IP <sub>3</sub>	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*
DMNP-EDTA	4,330 (350 nm)	0.18	780

\*Estimated based on the value for DMNB-phenylephrine<sup>16</sup> and in the midrange found for DMNB-modified proteins<sup>4</sup>. CNB,  $\alpha$ -carboxy-2-nitrobenzyl; DMNB, 4,5-dimethoxy-2-nitrobenzyl; EDTA, ethylenediaminetetraacetic acid; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; MNI, 4-methoxy-7-nitroindolinyl; NPE, 1-(2-nitrophenyl)ethyl.

**22** Plot the fluorescence as a function of flash energy and fit it with:  $f = f_{\max} [1 - \exp(-E/E_f)]$ .  $E_f$  is inversely proportional to the uncaging index of a caged compound, so the fitted value for it can be used to estimate the amount of uncaging of other caged compounds. Uncaging parameters for commonly used probes are listed in **Table 1**.

**23** To estimate the uncaging index, the extinction coefficient  $\epsilon$  needs to be measured at the right wavelength;  $\epsilon$  is constant for a given cage group. Quantum yield  $\phi$  does not change over a range of wavelengths, as long as other absorption bands are avoided, but does vary as a function of what molecule is caged and where.

**? TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 2**.

**TABLE 2** | Troubleshooting table.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
Step 7	Impossible to spread solution on the coverslip evenly	Glass surface is hydrophobic	1. Increase the concentration of buffer protein (BSA) 2. Use coated coverslips 3. Treat coverslip to make it hydrophilic
Step 9	Layer of dried dye is not even; cracks are visible	Concentration of carrier protein (BSA) is too high	Decrease the concentration of BSA
Step 9	Fast diffusion of the uncaging spot	High water concentration in the sample	1. Let sample dry for a longer time period in Step 6 2. Use high-molecular-mass dextran conjugate 3. Make measurement in the linescan mode
Step 9	Uncaging spot shows up slowly	Low concentration of water in the sample	Decrease drying time in Step 6
Step 9	Uncaging spot is not bright	Low uncaging power; low dye concentration	Increase ultraviolet-light power, or make a new sample with a higher dye concentration
Step 9	High background fluorescence of caged sample	High concentration of uncaged dye	Make fresh sample

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