Custom-made modification of a commercial confocal microscope to photolyze caged compounds using the conventional illumination module and its application to the observation of Inositol 1,4,5-trisphosphate-mediated calcium signals

Lorena Sigaut
Mariano Barella
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Lorena Sigaut, Mariano Barella, Rocío Espada, María Laura Ponce, and Silvina Ponce Dawson
Ciudad Universitaria, Departamento de Física, FCEN-UBA, and IFIBA, CONICET, Pabellón I, (1428) Buenos Aires, Argentina

Abstract. The flash photolysis of "caged" compounds is a powerful experimental technique for producing rapid changes in concentrations of bioactive signaling molecules. These caged compounds are inactive and become active when illuminated with ultraviolet light. This paper describes an inexpensive adaptation of an Olympus confocal microscope that uses as source of ultraviolet light the mercury lamp that comes with the microscope for conventional fluorescence microscopy. The ultraviolet illumination from the lamp ($350 - 400$ nm) enters through an optical fiber that is coupled to a nonconventional port of the microscope. The modification allows to perform the photolysis of caged compounds over wide areas ($\sim 200\ \mu$m) and obtain confocal fluorescence images simultaneously. By controlling the ultraviolet illumination exposure time and intensity it is possible to regulate the amount of photolyzed compounds. In the paper we characterize the properties of the system and show its capabilities with experiments done in aqueous solution and in *Xenopus Laevis* oocytes. The latter demonstrate its applicability for the study of Inositol 1,4,5-trisphosphate-mediated intracellular calcium signals. *© 2011 Society of Photo-Optical Instrumentation Engineers (SPIE)*. [DOI: 10.1117/1.3592497]

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1 Introduction

Flash photolysis is a technique which utilizes especially engineered molecules to generate sudden changes in the concentration of chemicals.[1–3] It is based on the use of biologically active molecules that are synthesized with a covalently attached photosensitive "masking" group, a cage, that makes them inactive. Illumination with light, usually in the ultraviolet (UV) range, transforms or cleaves the cage and the molecule becomes active. Therefore, flash photolysis generates controlled and reproducible stimuli with which to examine physiological phenomena that depend on fast concentration changes. Furthermore, it can be used in conjunction with indicator dyes that are excited by longer wavelength photons to observe the consequences of the stimuli. Among the various active biochemicals that are currently commercially available in caged form, Inositol 1,4,5-trisphosphate (InsP3) has proved very useful to elicit intracellular Ca^{2+} signals in a variety of cell types.

InsP3 is a second messenger that mediates Ca^{2+} release during many physiological processes, including development, gene regulation, secretion, contraction, synaptic transmission, and apoptotic cell death.[4] It functions by binding to InsP3 receptor/channels (IP3R's) that are mainly located on the membrane of the endoplasmic reticulum (ER).[5] IP3R's also need to bind Ca^{2+} to become open and allow the flow of Ca^{2+} ions into the cytosol.[6] In intact cells, InsP3 is made, as a response to certain stimuli, by the hydrolysis of a phospholipid that is located in the plasma membrane. It subsequently diffuses into the cell acting together with basal Ca^{2+} to induce the opening of one IP3R. The Ca^{2+} released through the first open channel then acts on other channels inducing their opening in a phenomenon known as Ca^{2+} induced Ca^{2+} release. The use of caged InsP3 and Ca^{2+} dyes that either permeate or are previously microinjected into the cells provide a controlled tool for the elicitation and observation of IP3R-mediated Ca^{2+} signals.[7] The technique allows rapid (millisecond) elevations in InsP3 avoiding the time delay and modulation associated to ligand-active InsP3 generation. It has been largely used showing a hierarchy of responses that go from very localized Ca^{2+} signals (blips in which one IP3R becomes open and puffs that involve the concerted opening of several IP3R's in a cluster) to waves that propagate throughout the cell.[8,9] The nonuniform distribution of the Ca^{2+} and the interchannel coupling that the released ions induce is a defining feature of the signals. Flash photolysis experiments with caged InsP3 allow to separate the effects of the spatio-temporal distribution of Ca^{2+} from those of InsP3. This is achieved by photolyzing the caged precursor as uniformly as possible inside the cell. Therefore, for this type of experiment it is desirable to have an even illumination in the near UV range over a relatively large

region. Previous experiments achieved this goal with the use of a mercury lamp.[7–9] We follow this approach in the modification of the confocal microscopy that we introduce in the present paper.

In general, commercial scanning confocal microscopes do not have the possibility of simultaneously acquiring confocal linescan images and performing UV flash photolysis, unless they possess a UV laser that can be commanded independently of the main scanner laser. The cost of a UV laser exceeds by far the cost of the system described here. Furthermore, illumination with a laser does not provide the uniformity properties mentioned before. Here we describe the adaptation of an Olympus confocal microscope FV1000 to photolyze caged compounds during confocal imaging, which can be easily adapted to other commercial scanning confocal microscopes. The system is flexible so that changes in the exposure time or intensity of the UV flash can be made during the experiment, and it can be synchronized with laser scanning acquisition. This relatively simple and inexpensive system uses an external mercury lamp (which can also be used for conventional fluorescence observation) as a UV source, connected to the microscope via fiber cable through a SIM port (SIMultaneous port). The total cost to add the photolysis system to a confocal scanning microscope is less than $10,000 U.S.

In Sec. 2 we describe the modification of the commercial microscope that we have introduced and the basics of the experiments that we have performed. In Sec. 3 we show the characterization of the UV illumination, in terms of the UV power that it delivers through different attenuators and of the spot size where it reaches. We also demonstrate some of the capabilities of the modified system with experiments in aqueous solution employing a sensitive Ca^{2+} indicator dye (Fluo 4 dextran) and the Ca^{2+} cage nitrophenyl-ethylene glycol tetraacetic acid (NP-EGTA) in Sec. 3. In Sec. 3.3 we present an application of the system: the study of InsP3 evoked calcium signals. We show different Ca^{2+} signals employing confocal linescan microscopy together with the Ca^{2+} indicator (Fluo 4 dextran) and caged InsP3 in *Xenopus Laevis* oocytes. The *Xenopus* oocyte has proved to be a highly advantageous model cell system to study elementary events of Ca^{2+} signaling by virtue of its large size and lack of ER Ca^{2+} release channels (e.g., ryanodine receptors and cADP-ribose receptors) other than IP3R's.[10,11] In this cell type IP3R's are grouped in clusters (release sites) that are $\sim$100 nm in side and that are separated by a few micrometers.[12] For this reason, the full hierarchy of Ca^{2+} signals involving the activation of one or more release sites (blips, puffs, and waves) has been observed in these oocytes[9]

2 Materials and Methods

2.1 *System Design*

The microscope that was modified is a spectral confocal scanning Olympus FluoView1000. It has a spectral scan unit connected to an inverted microscope IX81. A laser combiner module is used to deliver light from several lasers (multiline Argon, green He–Ne) for confocal illumination. An external mercury lamp (100 W Olympus) is used for conventional wide field fluorescence imaging. Both illumination systems are connected to the scan unit via fiber optic ports.

In our modification, the UV light is provided by the external mercury lamp that is used for conventional fluorescence mi-

croscopy (100 W Olympus) but instead of having it connected to the microscope via the conventional fluorescence mercury lamp fiber optic port, the light is introduced via the SIM port (see Fig. 1). The SIM port is supposed to be used to incorporate a second scanner unit dedicated for laser light stimulation, in addition to the primary scanner. In our modification we have added some optical elements to direct the light from the fiber optic to the SIM port. To that end, we have used the same optics as that of the conventional fiber optic port plus a fluorescence filter 377/50 (Brightlines) to select the short wavelengths that are necessary to perform the uncaging. All these elements are placed in a black tube inside a container that can be moved vertically so that the circular output aperture of the fiber cable can be positioned at the microscope's image plane, producing a disk of UV energy at the object plane of the microscope. The UV spot's size depends on the fiber optic diameter and the magnification of the microscope objective. One adjustment screw allows the axial location of the UV spot to be changed relative to the microscope focal plane. As the fiber cable is composed of several fiber optics, if the circular output aperture of the fiber cable is placed exactly at the microscope's image plane, the image of the different fibers can be seen at the scanned plane obtaining a nonuniform illumination. In order to prioritize homogeneity, we slightly defocus the image so that a more uniform UV illumination can be obtained at the scanning plane. Figure 1(a) shows a lateral view of the confocal scan unit, with the laser combiner fiber port, the conventional fluorescence mercury lamp fiber port and the adaptation tube connected to the SIM port. Figure 1(b) shows a more detailed photograph of the adaptation tube for the uncaging mode.
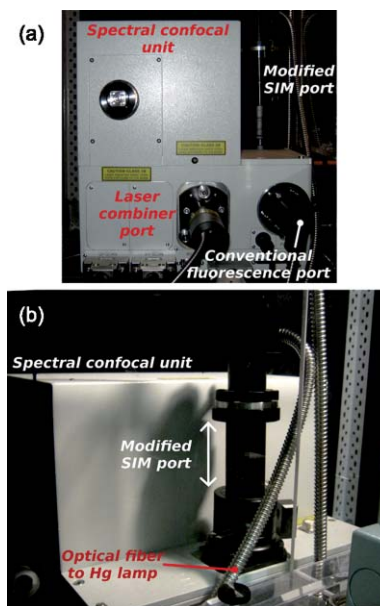

Fig. 1 Photograph of the spectral confocal unit with the modification. (a) Lateral view showing the three illumination ports: the conventional fluorescence port for the mercury lamp light, the laser combiner port for confocal imaging, and the modified SIM port in which the mercury lamp light enters for photolysis purposes allowing simultaneous uncaging and confocal imaging. (b) Detailed view of the modified SIM port showing the vertical displacement that allows the axial location of the UV spot to be changed relative to the microscope focal plane.
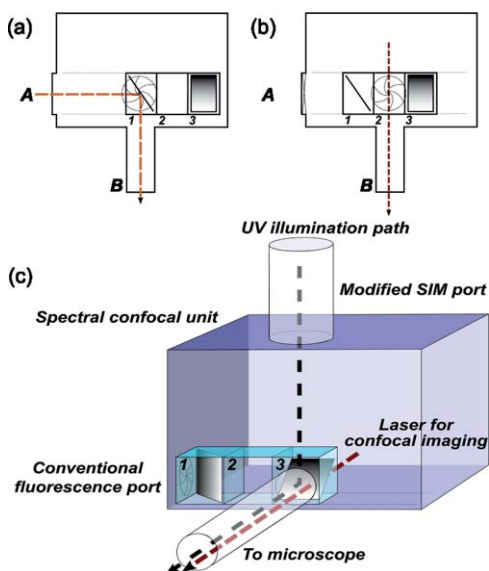
Fig. 2 Scheme of the different illumination paths that may be used by changing the rail position: (a) Position 1 has a mirror that redirects the light of the mercury lamp for conventional wide field fluorescence observation (A: conventional fluorescence port; B: to IX81 microscope). (b) Position 2 is empty so that the light from the laser combiner can reach the microscope for confocal imaging (c) Position 3 has a dichroic mirror that reflects the short wavelengths (< 450 nm) of the mercury lamp light to do the photolysis and allows the transmission of the lasers to do confocal imaging at the same time.

All the different illumination beams enter through the scan unit to the microscope IX81. The scan unit has a motorized three position rail (see Fig. 2). The first position has a mirror that redirects the light of the mercury lamp in the conventional wide field fluorescence observation mode (in this case light from the laser combiner cannot pass to the microscope). The second one is empty, so that the light from the laser combiner can reach the microscope (but the light from the mercury lamp cannot pass). In our modification we have used the third position for the uncaging placing a dichroic mirror to reflect the short wavelengths (< 450 nm) and transmit the long ones (> 450 nm). In this way the reflected beam can be used for the photolysis. This last configuration allows the transmission of the light from the laser combiner to occur simultaneously with the UV illumination from the mercury lamp. It also allows the transmission of the light from the sample to the confocal detectors, so that confocal imaging can be performed at the same time.

In order to control the exposure time and UV flash intensity, we have placed a fast shutter (Uniblitz) and a motorized filter wheel (Olympus) with neutral filters immediately after the mercury lamp and before the exit of the optical fiber (see Fig. 3). The motorized filter wheel, which can be commanded from the Olympus software, has six places where we have put five neutral density filters (New Focus) of optical density between 0.1 and 2, leaving one place empty to have maximum intensity. To control the flash duration, we have connected a home made pulse generator to the fast shutter driver unit (VCM-D1, Uniblitz) which sends 5 V transistor transistor logic (TTL) pulses of the desired duration starting at 10 ms with 10 ms steps. The Olympus microscope has a TTL output signal that is commanded with the Olympus software FW10-ASW (version 01.07.03) through the time controller window. This enables the
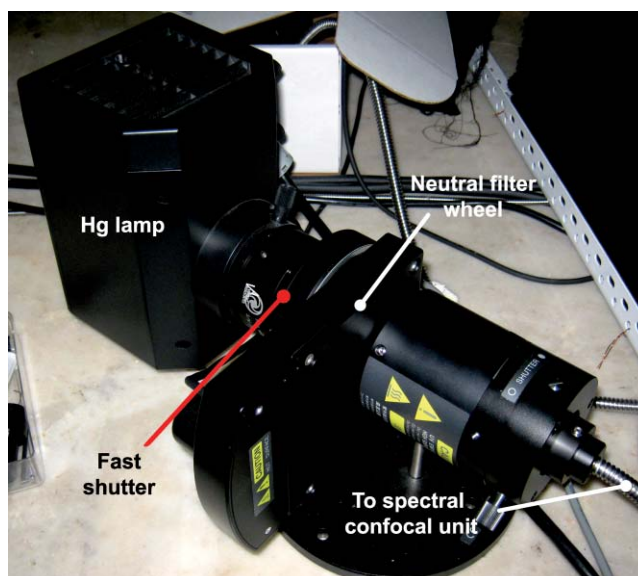

Fig. 3 Photograph of the external mercury lamp used for conventional fluorescence observation and uncaging. The mercury lamp house, fast shutter, and motorized neutral filter wheel, together with the fiber delivery system, are shown.

user to program the acquisition routine (scanning mode, laser intensity, etc.), together with the delivery of the output signal. To synchronize the UV flash with the imaging scanning, the outgoing TTL signal is connected to the pulse generator. Employing the time controller routine it is possible to choose the time at which the TTL output signal is sent and the UV flash is delivered.

2.2 Calcium Signaling Experimental Procedure

2.2.1 Oocyte preparation

Experiments were done using immature *Xenopus Laevis* oocytes previously microinjected with caged InsP3 and the calcium indicator Fluo 4 dextran (high affinity), to a final concentration of ~ 9 and ~ 40 μm, respectively. In order to see puffs, some oocytes were also loaded with the Ca^{2+} buffer EGTA (final concentration ~ 90 μm). Due to its slow binding rate (20 $\mu m^{-1}s^{-1}$), EGTA inhibits Ca^{2+} diffusion over micrometer distances between release sites, something that prevents the occurrence of Ca^{2+} waves. In this way, release sites are functionally uncoupled but their autonomous behavior remains unchanged so that puffs can be studied in detail.[13,14] All calcium indicators and caged compounds are from Invitrogen–molecular probes, other compounds are from Sigma.

2.2.2 Data acquisition and imaging processing

Ca^{2+} dependent fluorescence line scan images were obtained along a 45 μm line focused in the animal hemisphere at a depth of 3 to 10 μm below the surface, employing light at 488 nm and detecting the fluorescence in the range (500 to 600) nm. The UV flash was delivered ~ 0.5 s after having started the data acquisition. Confocal measurements of fluorescence are expressed in units of $(F - F_o)/F_o$, where F_o is the minimum fluorescence, which is estimated from the experiments by averaging over time the intensity at each point before the UV flash is delivered. The

intensity profiles showing puffs were obtained by averaging 7 consecutive pixels along the scanning line. Choosing the number of pixels to average is a compromise between reducing noise levels and not modifying signal's shape. We found that averaging 7 consecutive pixels is the best way to reduce noise level of the signal without changing its shape.

3 Results

3.1 Characterization of the UV Illumination

3.1.1 UV spot size

One of the most common scanning aperture techniques to determine beam width is the scanning-slit profiler that measures the transmitted intensity as a thin slit cuts through the beam.[15] As the intensity is integrated over the slit width, the resulting measurement is equivalent to the original cross section convolved with the profile of the slit. To estimate the spot size of the UV light in our case, instead of measuring the transmitted intensity, we have measured the reflected intensity as a thin strand passed through the beam. By scanning the strand through the beam the profile of the reflected intensity can be obtained as a function of position. Assuming a Gaussian field distribution, the beam waist can be estimated by fitting the profile with a function of the form $E(x, y, 0) = E_0 \, e^{-(x^2+y^2)/\omega_0^2}$, where E_0 is a constant field vector in the transverse (x, y) plane, $z = 0$ at the beam waist and ω_0 denotes the beam waist radius.

In our setup, the strand was mounted onto a coverslip and was illuminated only by the UV spot. Employing a motorized stage (Scientific Roper) the strand was scanned with 10 μm steps, while the intensity was registered employing the confocal detectors (highly sensitive photomultipliers, Hamamatsu). Figure 4 shows the plot of the reflected intensity as a function of position together with the fitting curve. The experiments displayed here were done using a 60×, 1.35 N.A. oil-immersion objective (UPlanSAPO, Olympus) and a pinhole aperture of 105 μm. This lens produces a UV spot with a 212 ± 7 μm diameter.

3.1.2 UV power and attenuators

The mercury lamp power is 100 W, distributed over a continuous spectrum; it has an important peak at 365/366 nm which is useful for photolysis purposes. The optical components of the uncaging
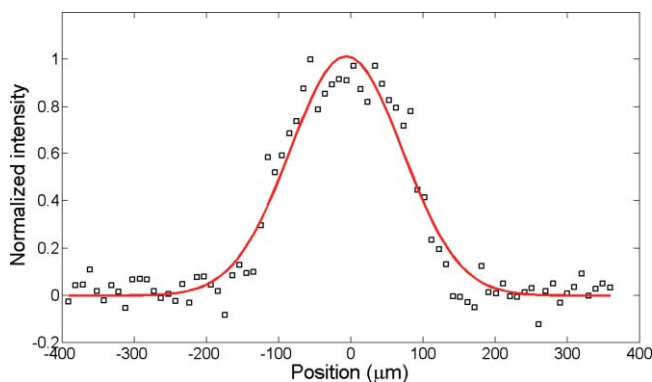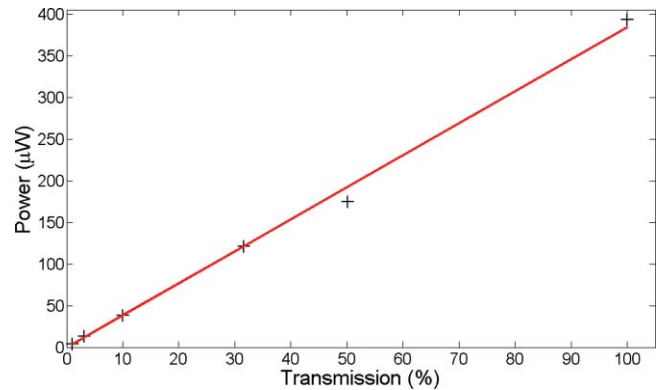

Fig. 5 UV power as a function of the neutral filter transmission specified by the manufacturer. Solid curve: linear fit of the data forced to pass through zero.

system and the microscope optics are expected to attenuate the light intensity. For this reason we have measured the power of the uncaging light at the output of the 60× objective after it has gone through different neutral filters. Measurements were performed with an Advantest TQ8210 power meter, at 364 nm showing that the maximum power was $\sim$400 μW. In Fig. 5 we show the plot of power as a function of transmission. Given that optical density (OD) and transmission (T) follow the relation $T = 10^{OD}$, it can be inferred from the linear dependence in Fig. 5 that the filters are attenuating equally all wavelengths in the 350 to 400 nm range, a positive feature for uncaging that had not been informed by the manufacturer.

3.2 Calcium Caged Uncaging

As a first check of the uncaging capabilities of our setup we used NP-EGTA,[16,17] a photolabile chelator that exhibits a high selectivity for Ca^{2+} and which dissociation constant increases from 80 nm to more than 1 mm upon UV illumination. Therefore, starting with a solution that mainly contains Ca^{2+}-bound NP-EGTA, the application of a photolysis pulse induces a rapid delivery of Ca^{2+}. Employing the calcium indicator Fluo 4 dextran it is possible to monitor this calcium release and verify the effectiveness of the uncaging system.

For this purpose we have used a solution containing [NP − EGTA] = 1.5 mm, Ca = 8.5 μm and the calcium indicator Fluo 4 dextran, [F4] = 0.1 mm. Single point scanning measurements at a 100 kHz sampling rate were performed employing a 488 nm line with a 60× objective. No neutral filter was used for the UV illumination. An increase in the fluorescence signal was observed after the UV flash delivery in response to the release of Ca^{2+} from the caged NP-EGTA. We repeated the experiment using UV flashes of different durations that ranged between 100 and 400 ms. We computed the fluorescence amplitude (ΔF) as the difference between the maximun fluorescence obtained during the time course of each experiment and the basal fluorescence before the UV flash delivery.

We show the plot of maximum fluorescence amplitude as a function of UV flash duration in Fig. 6. In this figure it is possible to observe that the longer the duration of the UV flash, the greater the amount of Ca^{2+} that is released. Furthermore, both quantities are linearly related as shown by the linear regression
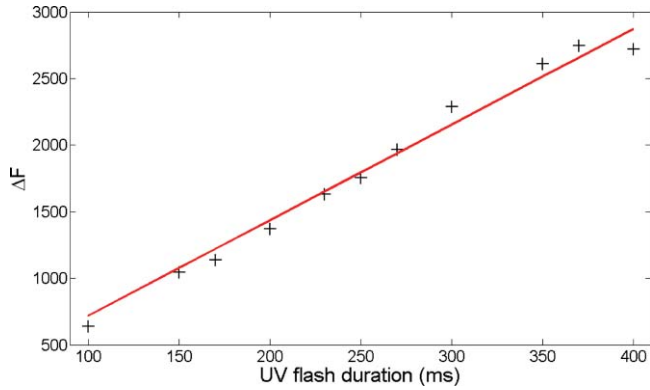

Fig. 4 Normalized intensity profile as a function of the strand position. Squares: experimental data points. Solid line: Gaussian fit.

Fig. 6 Maximum fluorescence amplitude (ΔF) as a function of UV flash duration. Solid line: linear regression forced to pass through the origin.

that is superimposed on the experimental data points. This is the expected behavior: the amount of uncaged compound is linearly proportional to the concentration of caged compound, flash intensity, and duration. This first set of experiments shows that

the modification that we have introduced allows caged components to be photolyzed having full control of the time duration of the UV flash that is delivered producing results that follow the expected dependence with this duration.

3.3 *InsP3 Evoked Calcium Signals*

We illustrate in this section the capabilities of the modification that we describe in this paper to obtain confocal images of InsP3 evoked calcium signals. To this end experiments were performed in *Xenopus Laevis* oocytes that were previously microinjected with a mix of Fluo 4 dextran (high affinity) and caged InsP3 as explained in Sec. 2. Figure 7 shows confocal linescan images of representative signals obtained after the delivery of a 10 ms UV photolysis flash. In the case of Fig. 7(a), EGTA was added to the mix to prevent Ca^{2+}-wave propagation and increase the probability of observing localized signals such as puffs. Two different release sites can be identified in Fig. 7, denoted by the letters A and B. The time dependence of the intensity fluorescence profiles at these two sites are also displayed in Fig. 7. It
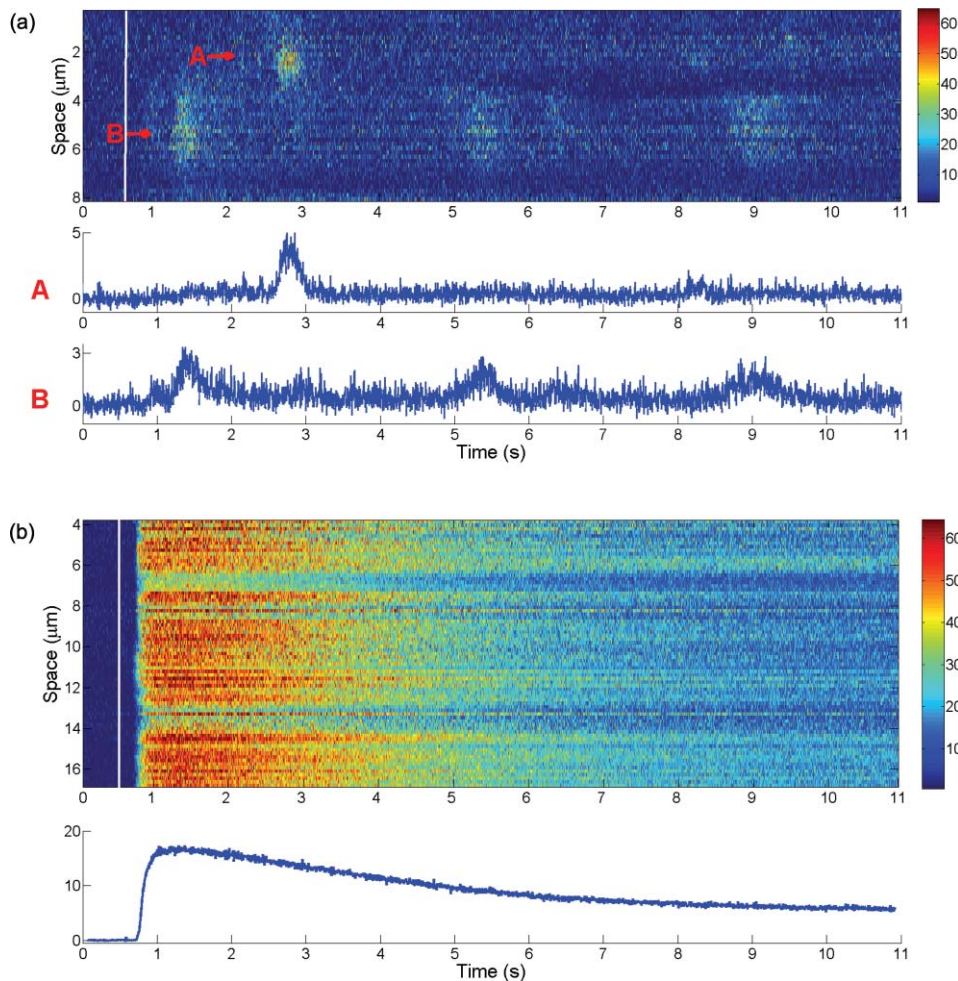

Fig. 7 Representative confocal linescan images showing Ca^{2+} signals after a 10 ms UV flash employing Fluo 4 dextran and caged InsP3. In the panels, $(F - F_o)/F_o$ is plotted using a color code, with distance along the scanned line in the vertical axis and time running from left to right in the horizontal axis. Increasing fluorescence ratios (increasing free Ca^{2+}) are denoted by increasingly "warmer" colors (as indicated by the color bar to the right of the figure). The white vertical lines superimposed on the images indicate the UV flash delivery time. (a) Top: representative localized Ca^{2+} signals (puffs). Two release sites are indicated by the letters A and B. In this case EGTA was also microinjected in the cell. Bottom: Temporal profile of the puffs that occurred at sites A and B. (b) More global signal (Ca^{2+} wave) Bottom: Temporal profile of the wave.
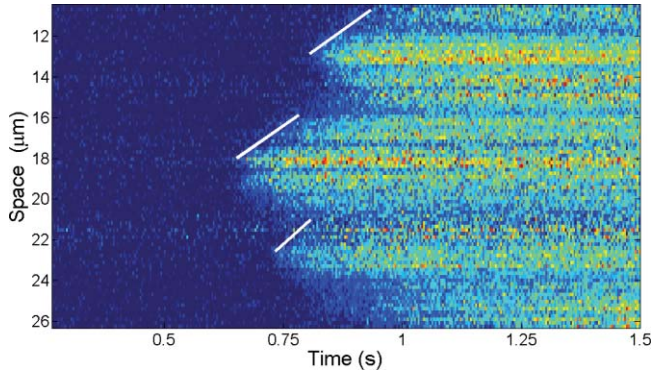
Fig. 8 Confocal linescan image showing Ca^{2+} waves after a 10 ms UV flash. White lines are aligned to the wavefronts in order to estimate the wave velocity from their slopes.

is clear that both sites act independently of one another in this record with Ca^{2+} release starting at disparate times. Site B, on the other hand, shows the occurrence of three consecutive Ca^{2+} puffs. It is evident, particularly in the intensity profile plot, that the first event has a greater amplitude than the others. The time elapsed between events is ~ 3.5 s, which is consistent with the interpuff time estimates presented in Ref. 18 and 19. A more global signal is shown in Fig. 7(b) which corresponds to an experiment in which no EGTA was added to the mix. In this case, all release sites become active almost simultaneously and liberate Ca^{2+} during a longer time. The intensity profile is also displayed in this case showing that the average Ca^{2+} concentration decays with a time scale that is consistent with previous observations.[20]

In Fig. 8 a typical calcium wave is presented. As it can be observed, after one release site is activated the Ca^{2+} that enters the cytosol diffuses and induces the opening of IP3R's in neighboring sites, generating the wave. It is known that the velocity of Ca^{2+} waves varies with InsP3 concentration and ranges between ~ 10 μm/s at low concentrations and ~ 50 μm/s at larger ones.[19] In order to estimate the wave velocity for the experiments of Fig. 8 we aligned a straight line with the apparent wavefront, as shown in the figure. The velocity obtained in this way is 18 ± 2 μm/s which is within the expected range of values.

The behaviors that we observe in Figs. 7 and 8 and the quantitative estimates that we can draw from them are in full agreement with previous observations of IP3R-mediated Ca^{2+}-signals. There is no doubt then that the system is effectively photolyzing the caged InsP3 that was previously injected in the cells and is simultaneously acquiring confocal images of the signals evoked.

4 Conclusions

The modification described in this work, implemented in the Olympus FV1000 confocal microscope, has succesfully been tested. The modification is simple and inexpensive and allows the simultaneous obtention of confocal fluorescence images with the photolysis of caged compounds. The UV illumination is relatively uniform over a wide spot of radius ~ 200 μm for wavelengths in the range 350 to 400 nm. The system enables to control the light intensity by means of neutral filters that we

have proved introduce the expected attenuation in this range of wavelengths also. The exposure duration can also be controlled in 10 ms steps and be synchronized with the confocal scanning acquisition process. We have proved the capabilities of the system in experiments done in aqueous solutions in which Ca^{2+} bound NP-EGTA was photolyzed to release Ca^{2+} and in *Xenopus Laevis* oocytes in which caged InsP3 was photoreleased to evoke intracellular Ca^{2+} signals. This last application opens up the possibility of exploiting the advantages of the Olympus FV1000 microscope for the study of the signals.

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