Chapter 6

Photo-Activatable Probes for the Analysis of Receptor Function in Living Cells

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Abstract

Photo-activatable (caged) probes are powerful research tools for biological investigation. The superb maneuverability of a light beam allows researchers to activate caged probes with pinpoint accuracy. Recent developments in caging chemistry and two-photon excitation technique further enhance our capability to perform photo-uncaging with even higher spatial and temporal resolution, offering new photonic approaches to study cell signaling dynamics in greater detail. Here we present a sample method that combines the techniques of photo-activation and digital fluorescence microscopy to assay an important class of intracellular receptors for the second messenger D-*myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3, or IP_3). The imaging assay is performed in fully intact living cells using a caged and cell membrane permeable ester derivative of IP_3, cm-IP_3/PM.

Key words: IP_3, IP_3 receptors, IP_3R, cell permeable ester of IP_3, caged IP_3, two-photon uncaging, calcium signaling, calcium imaging.

1. Introduction

Caged probes are molecules whose biological, biochemical, or physicochemical activities are masked by light-sensitive protecting groups. The parent molecule of a caged compound usually contains functional groups (carboxylate, phosphate, amine, hydroxy, phenoxy, amide, etc.) that are crucial for its function. Caging these functionalities reduces or eliminates the activity of the parent molecule, yet its activity can be abruptly restored with a flash of light (typically ultraviolet or UV light). Because light beams can be precisely guided to targeted areas at the time of our choice,

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photo-uncaging offers the advantage of high spatiotemporal definition for controlling dynamics of cellular biochemistry. Since the pioneering work of Kaplan and Hoffman who first invented caged ATP to study cellular ATPase (1), a large number of caged probes have been developed and applied to biological and biochemical research (2, 3).

To assay receptor functions in cells, caged ligands for both cell surface and intracellular receptors have been developed. For example, caged glutamate (4) is widely used to control the activation of glutamate receptors on the plasma membrane of neurons; caged D-*myo*-inositol 1,4,5-trisphosphate (IP_3) (5, 6) and caged cyclic ADP ribose (7) were developed to study Ca^{2+} release from intracellular Ca^{2+} stores gated by the IP_3 receptors (IP_3Rs) or ryanodine receptors, respectively. Ideally, caged probes for the analysis of receptor functions in living cells need to meet a number of requirements: (1) be inert, i.e., neither activating nor inactivating cellular receptors or proteins; (2) be reasonably soluble in aqueous solution and biocompatible (non-toxic); (3) have high photolysis efficiency by UV or two-photon excitation so that only a small dose of light is needed for photo-activation, thus minimizing potential photo-damage to live cells; (4) have favorable photolysis kinetics so that the parent molecule can be rapidly released to fully activate its receptor prior to inducing desensitization; (5) non-invasive delivery of caged compounds into cells if their targets are located intracellularly.

cm-IP_3/PM (*see* **Fig. 6.1**), a caged and cell membrane permeable ester of IP_3, meets these requirements and is ideally suitable for studying dynamics and functions of Ca^{2+} release from intracellular Ca^{2+} stores gated by IP_3Rs. This compound is neutral, with three phosphates protected by six propionyloxymethyl (PM) esters. The PM ester masks the negative charge of phosphate and conveys lipophilicity to the molecule to allow cm-IP_3/PM diffuse passively across cell membranes. Once inside cells, the PM ester is hydrolyzed by ubiquitous cellular esterases to produce cm-IP_3, a caged IP_3 analogue that remains trapped inside cells. In cm-IP_3, the 6-hydroxy of *myo*-inositol is caged by 4,5-dimethoxy-2-nitrobenzyl group (DMNB). Because 6-hydroxy plays a crucial role in the interaction between IP_3 and its receptors, cm-IP_3 has negligible binding to IP_3Rs. Photolysis of the DMNB group frees 6-hydroxy and generates m-IP_3, a highly potent IP_3 analogue with 2- and 3-hydroxies protected by a methoxymethylene group (**Fig. 6.1**). M-IP_3 binds to IP_3Rs with an affinity about 75% of that of IP_3 (6), and it is rapidly metabolized in cells at a rate very close to that of natural IP_3 (8). These properties make m-IP_3 and its caged precursor cm-IP_3 ideal pharmacological reagents for controlling the activity of IP_3Rs and for studying the regulation and function of IP_3-Ca^{2+} signaling pathway in living cells.

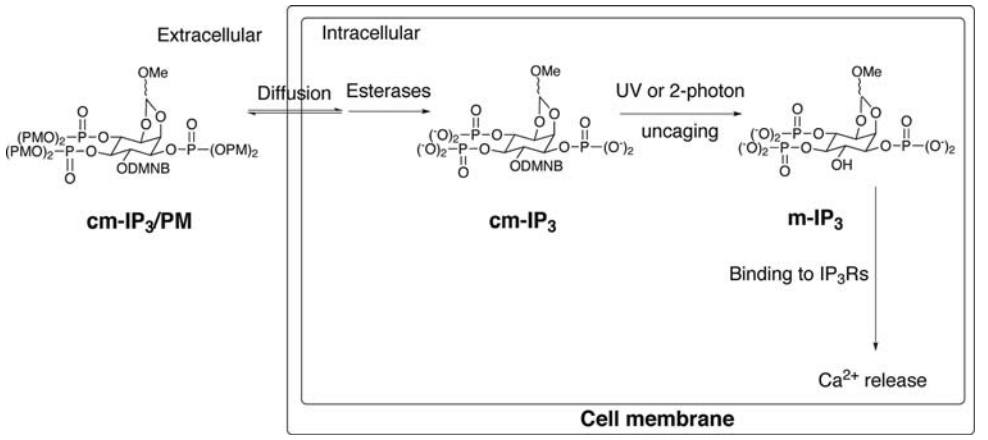

Fig. 6.1. Chemical structures of m-IP_3, cm-IP_3 (caged m-IP_3), and cm-IP_3/PM (neutral PM ester of cm-IP_3) and their mode of actions in living cells.

Intracellular Ca^{2+} activities ($[Ca^{2+}]_i$) control a variety of essential biological processes, and IP_3 is an important and ubiquitous second messenger that regulates Ca^{2+} homeostasis. IP_3 is hydrolyzed from phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C, which is activated by cell surface receptors upon ligand stimulation. PIP_2 also releases diacylglycerol that activates protein kinase C. Since methods relying on endogenous mechanisms to produce IP_3 also activate many branching signaling events, it is difficult to dissect the function of IP_3-Ca^{2+} signaling branch. Moreover, to examine the spatial heterogeneity of IP_3 sensitivity or Ca^{2+} release activity of IP_3Rs, it would require elevating IP_3 concentration selectively in subcellular areas.

Since two-photon excitation only occurs at the focal point of a focusing objective, two-photon uncaging achieves very high spatial selectivity of photo-activation with resolution of about 1 μm^3 (9). This technique is particularly useful for studying cell signaling dynamics in three dimensions because areas above or below the focus are not excited. Biological preparations including dissected tissues, organotypic cell cultures, or living model organisms are ideal subjects for the application of this technique. In addition, two-photon uncaging offers a unique and powerful experimental approach to the analysis of subcellular heterogeneity of receptor distribution, receptor activation, and functional consequences of localized signaling events.

To monitor $[Ca^{2+}]_i$ in living cells, a number of fluorescent indicators are available for Ca^{2+} imaging. These sensors vary in their chemical composition (small synthetic dyes and genetically encoded protein sensors), Ca^{2+} affinity (from sub-micromolar to over tens of micromolar), excitation and emission wavelengths (UV excitable to red emitting), dynamic range of signal change (from less than twofold to more than 100-fold), and cellular

localization (10, 11). A suitable Ca^{2+} indicator should be chosen based on the biological question to be addressed and the biological preparation to be used. In this protocol, I describe how to apply cm-IP_3/PM to activate IP_3Rs in living cells by photo-uncaging, using cultured HeLa cells as a model system. $[Ca^{2+}]_i$ is monitored with Fluo-3, a long wavelength and high affinity Ca^{2+} sensor (K_d = 0.39 μM) that exhibits a more than 100-fold fluorescence enhancement upon binding Ca^{2+}. Detailed procedures are provided for cell loading, imaging of Fluo-3 by digital wide-field and confocal laser scanning microscopy, UV and two-photon uncaging, data processing, and measuring the amount of released m-IP_3 using the IP_3 binding assay after photo-uncaging.

2. Materials

2.1. Reagents and Solutions

1. cm-IP_3/PM (synthesized from *myo*-inositol according to (6)) prepared as stock solution (1 or 2 mM) in dimethyl sulfoxide (DMSO). The stock solution is stable for at least 4 months if stored at –20°C. High purity anhydrous DMSO (Sigma-Aldrich) should be used to minimize the hydrolysis of the PM ester during storage. Vials containing the compound should be wrapped in aluminum foil to avoid light exposure, and kept on ice during usage.
2. Fluo-3/AM (Invitrogen), a membrane-permeable fluorescent calcium indicator with the excitation maximum near 490 nm, prepared as 1 mM stock solution in dry DMSO (stored at –20°C, stable for at least 6 months).
3. DMSO stock (10% w/v) of Pluronic F127 (BASF, cat. no. 583106). It is stable for at least several months if stored at room temperature.
4. Histamine (Sigma) (5 mM stock solution in water), a compound which stimulates Ca^{2+} release in HeLa cells by activating phospholipase C to produce endogenous IP_3, is used it as a control to check that cells are functional in IP_3-Ca^{2+} signaling.
5. Ionomycin (LC Laboratories, Woburn, MA), a Ca^{2+} ionophore which raises cellular Ca^{2+} to a high level (~1 μM), is used to check Ca^{2+} responsiveness of Fluo-3 at the end of an imaging experiment.
6. 2-Aminoethoxydiphenyl borate (Aldrich) and Xestospongin C (Sigma) are membrane permeable inhibitors of IP_3Rs.

7. Hank's balanced salt solution (HBS) (Gibco, cat. no. 14065, diluted ten times with water) supplemented with 20 mM HEPES buffer, adjusted to pH 7.3 with NaOH or HCl and filtered through a 0.2-μm sterile filter.
8. Ca^{2+}-free DPBS buffer (Gibco cat no. 14190) supplemented with 5.5 mM glucose, 1 mM $MgCl_2$, and 20 mM HEPES, pH 7.3, and filtered through a 0.2-μm sterile filter.
9. Amersham IP_3 [^{3}H] Biotrak Assay kit (GE Healthcare Life Sciences).
10. 1 M aqueous solution of trichloroacetic acid (Aldrich).
11. Mixture of trichlorotrifluoroethane and trioctylamine (3 vol: 1 vol, both from Aldrich).

2.2. Cell Culture

1. HeLa cells (American Tissue Culture Collection) cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells are passed regularly when they reach high density (>80%). During passage, cells are detached from culture dishes by trypsinization (0.25% trypsin and 1 mM EDTA, Gibco/BRL) for several minutes at 37°C.
2. For imaging, cells are seeded on glass-bottom imaging dishes (MatTek, cat. no. P35G-0-10-C, 3.5 cm diameter) at low to medium density (< 50%).

2.3. Imaging and Uncaging Equipment

1. Axiovert 200 M inverted fluorescence microscope (Carl Zeiss) for wide-field Ca^{2+} imaging and UV uncaging. The scope is equipped with a cooled CCD camera (ORCA-ER, Hamamatsu), a 40× oil-immersion objective (Fluar, 1.3 NA, Carl Zeiss), and an excitation source for rapid wavelength switching (Lambda DG-4, with a 175 W Xenon lamp, Sutter Instrument). Equivalent imaging platforms and hardware from other manufacturers may also be used.
2. Optical filters for imaging Fluo-3: 480 nm ± 20 nm (excitation filter), 535 nm ± 25 nm (emission filter), and a long-pass 505 nm beamsplitter coated with UV reflection material to facilitate UV uncaging (Chroma Technology or Omega Optical).
3. Open-Lab imaging software (http://www.improvision.com/products/openlab/) for controlling image acquisition, uncaging, and post-acquisition analysis. Similar softwares from other vendors can be used.
4. Hand-held UV lamp B-100 AP (UVP, Upland, CA) for global uncaging of all cells in a culture dish.

5. IL 1700 Research Radiometer with SED033 detector (International Light Inc., Newburyport, MA) for measuring light intensity of the hand-held UV lamp.
6. Electronic timed mechanical shutter (Uniblitz, Model VMM-T1; Vincient Associates, Rochester, NY) for gating UV exposure.
7. Zeiss LSM510 imaging system (Carl Zeiss) equipped with a 30-mW Argon laser, a Chameleon-XR laser (Coherent), and a 40× oil-immersion objective (Fluar, 1.3 NA, Carl Zeiss) for laser scanning confocal imaging and two-photon uncaging. Other equivalent imaging platforms can be used.
8. Power meter with PM 10 sensor (FieldMate, Coherent) for measuring the average power of femtosecond pulsed laser for two-photon excitation.

3. Methods

All caged compounds including cm-IP_3/PM and its cellular hydrolysis product cm-IP_3 should be protected from room light throughout an experiment, and never be exposed to day light. Use a red safety light (available from local hardware stores) to provide illumination when handling caged compounds.

All the experiments in this protocol are carried out at room temperature (~25°C) unless specified otherwise.

3.1. Cellular Loading of cm-IP_3/PM and Fluo-3/AM

Since it takes 15 steps to synthesize cm-IP_3/PM, consumption of this valuable material should be minimized. The following procedure uses approximately 0.4 nmol or 0.5 μg of cm-IP_3/PM per loading, and it works well for a number of cultured cell lines, including HeLa, HEK293 human embryonic kidney cells, NIH3T3 fibroblasts, and 1321N1 astrocytoma cells. Optimal loading conditions should be empirically determined for each cell type.

1. To prepare loading solution, mix DMSO stock solutions of cm-IP_3/PM (1 mM, 0.4 μL), Fluo-3/AM (1 mM, 0.3 μL), and pluronic (10%, 0.3 μL) in a 0.6-mL centrifuge tube. Then add 0.1 mL of HBS solution and vortex it briefly (*see* **Notes 1**, **2**, **3**).
2. Remove culture medium from an imaging dish containing cultured cells using a disposable Pasteur pipette. Gently rinse cells twice with 1× HBS solution. After the second rinse, remove residual HBS solution from the dish and carefully wipe off cells from plastic surface with a piece of folded

Kimwipe, without touching cells on the central part of the dish. This leaves cells only on the glass surface (*see* **Note 4**).

3. Gently add 0.1 mL of HBS on top of the glass surface to cover the cells, then add 0.1 mL of loading solution from step 1. Cover the dish with a lid to minimize water evaporation (*see* **Note 5**).
4. Incubate cells in the loading solution in dark for 20–45 min. Incubation time should be adjusted based on the cell type and confluence: longer incubation loads more probe into cells, and higher cell confluence requires longer incubation time.
5. Remove the loading solution with a Pasteur pipette and rinse cells once with HBS. Add 1.5 mL HBS to the dish and incubate cells in the dark for another 10 min to allow complete hydrolysis of the AM and PM esters in cells.

3.2. Wide-Field Imaging of Fluo-3 and Global UV Uncaging of cm-IP_3

Fluo-3 and other Ca^{2+} indicators may gradually lose their Ca^{2+} responsiveness upon intense, prolonged excitation. When attempting Ca^{2+} imaging for the first time, acquisition parameters such as intensity of excitation light, exposure time, acquisition frequency, and the duration of imaging experiments should be optimized. To check the Ca^{2+} responsiveness of Ca^{2+} indicators, Ca^{2+} ionophores such as ionomycin (2–10 μM) are used to raise $[Ca^{2+}]_i$ to fairly high levels. If the indicator remains sensitive to Ca^{2+} at the end of an experiment, it should respond to ionomycin similarly as a freshly loaded indicator. However, if it loses Ca^{2+} sensitivity after excessive excitation, the amplitude of ionomycin-stimulated Ca^{2+} signal is seen to be reduced.

1. Place an imaging dish on the microscope stage. Bring cells into focus by observing cellular Fluo-3 signal. If loading is successful, fluorescence should be dim but visible, and uniformly distributed inside cells.
2. To perform global UV uncaging through a 40× objective, choose a field containing 5–15 cells. Adjust the exposure time of the CCD camera to obtain a Fluo-3 image. In resting or unstimulated cells, $[Ca^{2+}]_i$ is low and Fluo-3 signal is fairly weak. In our set-up, we typically set the exposure time at 50–200 ms so that the average cellular Fluo-3 intensity is 2–3 times above the background (signal in cells without Fluo-3). Since excess illumination of Fluo-3 diminishes its Ca^{2+} responsiveness, we do not recommend using long exposure time or high intensity excitation light to bring up image intensity.
3. Start image acquisition by acquiring Fluo-3 signal every 5–10 s for about a minute. Baseline signal should be stable. Just prior to uncaging, increase acquisition frequency

to ≥ 1 image every 2 s. Quickly switch the filter to UV excitation to photolyze cm-IP_3 then back to image acquisition immediately. A successful uncaging should produce enough m-IP_3 to stimulate Ca^{2+} release that is detectable by Fluo-3. The optimal uncaging duration can only be determined empirically, since it depends on a number of variables including UV light output from the excitation source, UV transmission efficiency of the imaging system, amount of cm-IP_3 loaded into cells, cellular expression level of IP_3Rs, IP_3 sensitivity of different IP_3R isoforms, etc. In our set-up, the uncaging duration ranges from tens of milliseconds to over a second depending on the magnitude of Ca^{2+} increase which we aim to generate (*see* **Note 6**).

4. Continue imaging $[Ca^{2+}]_i$ fluctuations until Fluo-3 signal drops back to its basal level. M-IP_3, once being generated from cm-IP_3 by photolysis, is rapidly metabolized in cells by cellular phosphatases (8), so it typically induces Ca^{2+} transients that last less than a minute. When $[Ca^{2+}]_i$ returns to the resting level, decrease the frame rate to ≤ 1 image every 5 s to minimize Fluo-3 excitation.
5. Steps 3 and 4 can be repeated multiple times to produce many Ca^{2+} spikes mimicking natural Ca^{2+} oscillations (6, 8). Higher doses of UV light should be used for subsequent episodes of uncaging to compensate for the consumption of cm-IP_3 in previous photolysis. To reliably generate repetitive Ca^{2+} spikes in the same cells, it is necessary to control the extent of UV photolysis and avoid producing too much m-IP_3 in any single uncaging event.
6. cm-IP_3 loaded into cells is metabolically stable for at least 8 h at the room temperature (6). This allows multiple uncaging experiments to be conducted on the same dish of loaded cells, by moving each time to a different imaging field. We usually use cells that have been kept in HBS for less than 3 h. To check the health of these cells and to confirm that they still maintain robust IP_3-Ca^{2+} signaling at the end of an experiment, a cell surface receptor agonist (histamine, for example) can be added to activate phospholipase C to produce endogenous IP_3 which raise $[Ca^{2+}]_i$. Healthy cells should respond to agonist stimulation like freshly loaded cells.
7. To confirm that photo-released m-IP_3 induces Ca^{2+} release from intracellular stores, replace HBS with Ca^{2+}-free DPBS solution. Similar Ca^{2+} transients should be observed in Ca^{2+}-free solutions after photolyzing cm-IP_3.
8. To confirm that m-IP_3 induced Ca^{2+} release is from intracellular stores gated by IP_3Rs, IP_3R antagonists including

heparin, 2-aminoethoxydiphenyl borate, and Xestospongin C can be applied. These compounds are expected to block the effect of m-IP_3 (12). The later two drugs are membrane permeable, and their cellular application is straightforward, whereas heparin requires microinjection. Since these drugs affect multiple cellular targets including ryanodine receptors, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and various ion channels of plasma membranes, none of them should be considered as specific inhibitors of IP_3Rs.

3.3. Localized Two-Photon Uncaging of cm-IP_3 and $[Ca^{2+}]_i$ Imaging by Laser Scanning Microscopy

Caged probes based on the traditional 2-nitrobenzyl caging group typically are not very sensitive to two-photon photolysis, and there have been a fair amount of efforts devoted to developing new caging chemistries suitable for two-photon photolysis (13–18). In cm-IP_3/PM, the caging group (DMNB) has a relatively low two-photon uncaging cross-section, on the order of 0.01 Goeppert-Mayer (GM, 1 GM = 10^{-50} cm^4s/photon) (19). However, since cm-IP_3 is loaded into cells at sub-millimolar concentrations, and because IP_3 binds to IP_3Rs with nanomolar affinity (20, 21), only a small fraction (~0.1%) of loaded cm-IP_3 needs to be photolyzed within the two-photon excitation volume to locally activate IP_3Rs. This is an important consideration that eliminates the need of using high doses of laser light to uncage, thus avoiding cell damage. Even though two-photon excitation is restricted to the focal area, femtosecond laser pulses can still cause substantial photobleaching and cell injury at high power levels (22, 23).

For clarity and simplicity, we illustrate with cultured cells the procedure of two-photon uncaging of cm-IP_3 and confocal imaging of Fluo-3, though the protocol is applicable to studying IP_3-Ca^{2+} signaling in tissues or other biological preparations exhibiting three-dimensional architecture. In addition to confocal imaging, two-photon laser scanning microscopy can be combined with two-photon uncaging in the same experiment (*see* **Note 7**).

1. Load cells with cm-IP_3 and Fluo-3 using the procedure described in **Section 3.1**. Place the imaging dish on the microscope stage and bring the cells into focus by observing cellular Fluo-3 signal.
2. When using Zeiss LSM510 system, configure it as shown in **Fig. 6.2** and start with the settings given in **Table 6.1**. During two-photon uncaging of cm-IP_3, set laser power at the specimen at ~10 mW (determined with a power meter placed just above the objective).
3. Acquire a z-stack of confocal images of Fluo-3 using 488 nm excitation. Like in the wide-field imaging, baseline signal of Fluo-3 at resting $[Ca^{2+}]_i$ is fairly weak. Do not use high laser power to enhance image intensity.

4. Choose a cell to perform two-photon uncaging. Adjust the objective focus to a z-plane that cuts across the middle of the cell. Take a single confocal image at this height and use this image to define the area for two-photon uncaging.
5. Open the "Bleach Control" module in the LSM510 imaging software. Define the uncaging area using the "Define Region" function. Depending on the goal of experiments, the dimension of the uncaging area can be set from less than 1 μm^2 to larger than tens of μm^2. The uncaging area shown in **Fig. 6.3 c**orresponds to a circle of about 3.5 μm in diameter.
6. Using "Laser Control" function, set the wavelength of the Chameleon XR laser to 730 nm. Under the "Bleach Control," set the uncaging wavelength to 730 nm. During two-photon uncaging, the laser repeatedly scans through the defined uncaging area. The scanning repetition can be defined using the "Iterations" function. We typically set this value between 10 and 20 when the average power of laser input at the specimen is near 10 mW (*see* **Note 8**).
7. Use "Time Series" module of LSM510 imaging software to perform a time-lapse imaging experiment. Define acquisition frequency, timing of photo-activation and length of experiments in the "Time Series." The automation first acquires a number of confocal images of Fluo-3 at the defined frequency, then two-photon uncages cm-IP_3 at 730 nm, and then continues imaging Fluo-3 to follow m-IP_3-stimulated $[Ca^{2+}]_i$ elevation.
8. To follow $[Ca^{2+}]_i$ fluctuations in three dimensions in dissected tissues or in other physiological preparations after localized two-photon uncaging, set up a z-stack by centering the uncaged area along *z*. Use "Time Series" automation to acquire 4D images (*xyz-t*) of Fluo-3 before and after photoactivation.
9. Perform additional episodes of two-photon uncaging by repeating steps 7 or 8 to uncage the same or a different area in the same cell, or other areas in different cells.

3.4. Post-acquisition Data Analysis

To analyze $[Ca^{2+}]_i$ fluctuations before and after uncaging cm-IP_3, quantify Fluo-3 intensity in cells using data generated from the digital microscopy. Changes in Fluo-3 intensity are normalized against baseline signal at resting $[Ca^{2+}]_i$. This analysis can be applied to data obtained by both wide-field and laser scanning imaging.

1. Open a time-lapse Fluo-3 image sequence. Draw regions of interest (ROI) in selected cells.

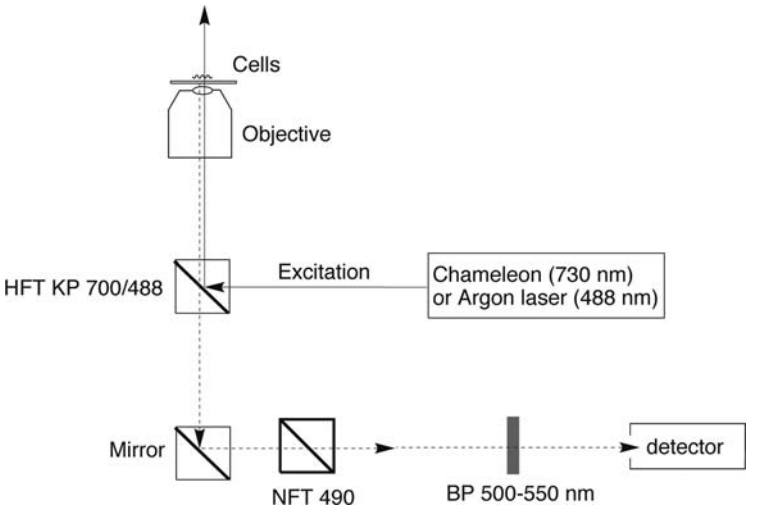

Fig. 6.2. Optical configuration of LSM 510 for confocal laser scanning imaging of Fluo-3 and two-photon uncaging. *Solid* and *dashed lines* represent excitation and emission light paths, respectively.

Table 6.1
Example settings of Zeiss LSM510 for confocal imaging of Fluo-3 and two-photon uncaging

Variable	Setting
Objective	40 × 1.3 NA (Plan-Neofluar)
Digital zoom	2.5
Laser	Argon laser emitting at 458, 477, 488, and 514 nm
Laser power	≤ 0.5% of total laser output (30 mW)
Image dimension (XY)	256 × 256
Z stepping size	1 μm
Scan speed	9 (3.2 μs pixel time)
Scan mode	Unidirectional
Amplifier offset	0.1
Amplifier gain	2.5
Averaging	1
Detector gain	≤ 650
Pinhole diameter	200

2. Measure the time course of the average fluorescence intensity (F_t) of these ROI using OpenLab, ImageJ, or other equivalent imaging software. Also measure the fluorescence intensity of an area that contains no cells as the background signal (F_b).

3. Subtract the background signal from the fluorescence signals of all ROI. Plot $(F_t–F_b)/(F_0–F_b)$ against time, where F_t is the fluorescence intensity of a ROI at time t, and F_0 is the fluorescence intensity of the same ROI at the start (time 0).

An example of such an analysis is shown in **Fig. 6.3**. The experiment involved two-photon uncaging of a small region in a HeLa cell loaded with cm-IP_3. Ca^{2+} activity rose immediately at the uncaging area, then quickly propagated throughout the cell, and gradually returned to the basal level in about a minute.

3.5. Quantification of Cellular IP_3 Mass After Loading and Uncaging cm-IP_3

To quantify the total amount of cm-IP_3 loaded into cells, or to evaluate how much m-IP_3 is released after a UV flash, we measure the amount of IP_3 in cells using the competitive IP_3 binding assay. Since the IP_3 binding assay typically detects IP_3 mass with nanomolar sensitivity, it is necessary to culture cells at high density.

1. Culture cells in 35–mm-diameter tissue culture dishes until cells become nearly confluent. Wash cells twice with HBS and incubate them in 0.4 mL of HBS. cm-IP_3/PM (2 mM × 1 μL) and pluronic (10%, 1 μL) mixed in 0.1 mL HBS is then added to the cells.
2. Incubate cells on a shaker at room temperature for 1 h. Remove the loading solution by aspiration. Wash cells once with HBS and add 0.5 mL of fresh HBS to each dish. Incubate cells on the shaker for another 40 min to allow complete digestion of PM esters.
3. To measure the total amount of cm-IP_3 loaded into cells, add ice cold trichloroacetic acid (TCA, 0.1 mL of 1 M aqueous solution) to quench cells. Place the dish on ice or in a cold room.
4. Photolyze cm-IP_3 in the cells with a hand-held UV lamp by placing the front of the light bulb approximately 5 cm above the dish. The light intensity reaching the dish surface is typically on the order of 1×10^{-8} E (cm^2 s). This can be measured by ferrioxalate actinometry (24) or using a light power meter. Under these settings, cm-IP_3 is photolyzed almost completely after 10 min of illumination.
5. Alternatively, to quantify how much IP_3 is released after a UV uncaging, cells loaded with cm-IP_3 from step 2 are illuminated with the UV lamp. We typically expose cells to UV light for 6 s, delivered either in one episode or in three episodes (2 s/episode) spaced 1 s apart. To ensure that the same amount of UV light is delivered from run to run, we use a mechanical shutter (Uniblitz) to gate UV exposure.
6. Immediately after UV uncaging, add 0.1 mL of ice-cold TCA (1 M) to cells. Shake the dish briefly to ensure

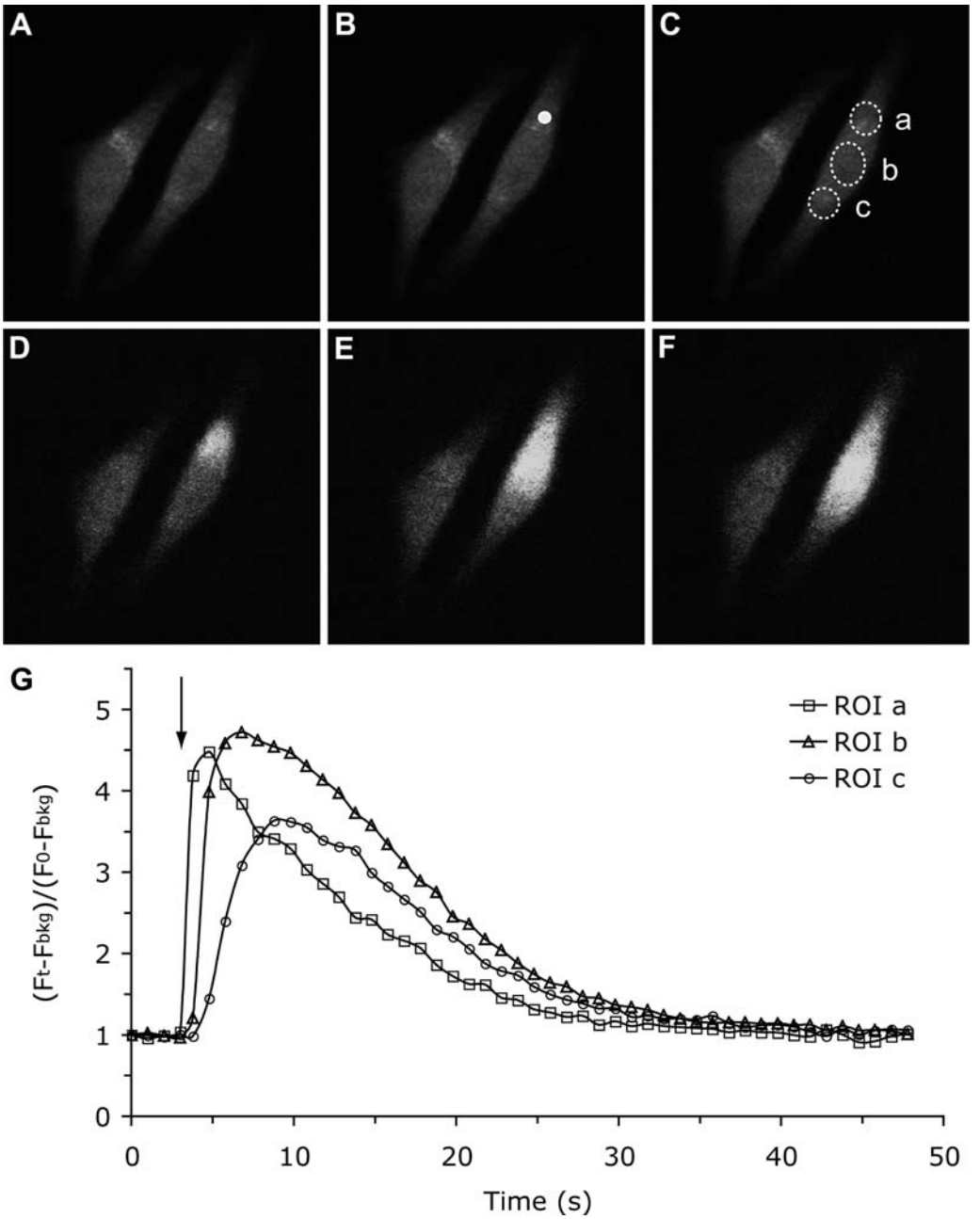

Fig. 6.3. Two-photon uncaging of cm-IP_3 and confocal laser scanning imaging of Fluo-3 in cultured HeLa cells. (**a**–**c**) CLSM images (Ex 488 nm, Em 500–550 nm) of HeLa cells. The uncaging area is outlined by the *white circle* in **b**. Three ROI (a, b, and c) are indicated by *dashed circles* in **c**. (**d**–**f**) Fluo-3 images approximately 0.5 s (**d**), 1.5 s (**e**), and 2.5 s (**f**) after the two-photon uncaging (730 nm) of cm-IP_3 in the area shown in **b**. (**g**) Time course of Fluo-3 signal normalized against its initial intensity in three ROI shown in **c**. The *arrow* indicates the time of two-photon uncaging. Modified from Figure 6 of (8) with permission from Elsevier.

thorough mixing and place it immediately on ice or in a cold room (*see* **Note 9**).

7. Leave petri dishes from step 4 or step 6 on a shaker in the cold room. After shaking the dish gently for 5 min, collect ~0.45 mL supernatant from each dish and transfer it to a 1.5-mL centrifuge tube. Spin the tubes at 4000 rpm on a bench-top centrifuge for 10 min at 4°C.
8. Transfer 0.4 mL of the supernatant to a new centrifuge tube (1.5 mL or larger). To each tube, add 0.8 mL of trichlorotrifluoroethane-trioctylamine (3 vol:1 vol) solution to neutralize and to extract TCA from the cell lysate. Vortex the tube on high speed for at least 15 s, then spin it at 4000 rpm for 1 min.
9. After centrifugation, carefully collect 0.3 mL of the top aqueous layer and transfer it to a new vial kept on ice. The pH of the solution should be around 4.5.
10. Assay the IP_3 mass in the neutralized cell lysate using the IP_3 binding assay kit, following the instruction from the manufacturer.

4. Notes

1. Pluronic is a non-ionic, mild detergent that helps to solubilize hydrophobic compounds in aqueous solutions and significantly improves the loading efficiency of cm-IP_3/PM and Fluo-3/AM.
2. If the pluronic stock solution turns cloudy during storage, warm it at ~37°C for several minutes.
3. The amount of DMSO in the loading solution should be kept below 0.5% (v/v); therefore, concentrated stock solutions are used.
4. Removal of cells from plastic should be done in less than a minute so that cells on the glass surface remain hydrated.
5. Make sure that peripheral plastic surface is dry before adding the loading solution. The surface tension keeps the solution within the glass area. Otherwise, spilling to the surrounding plastic can decrease the loading efficiency.
6. In addition to a Xenon or mercury lamp, low-cost UV light emitting diodes (25) can also be used to perform photolysis. For applications demanding rapid uncaging of less than 1 ms, strong excitation sources such as pulsed UV lasers or capacitor charged Xe flash lamps (26) should be used.
7. Two-photon laser scanning microscopy has been widely used to image $[Ca^{2+}]_i$ with Fluo-3, Fluo-4, Calcium Green-1, or Oregon Green 488 BAPTA at excitation wavelengths

of 800 nm and above. Since the efficiency of two-photon uncaging of DMNB group decreases rapidly above 750 nm (19), it should be possible to perform two-photon imaging exciting at 800 nm or above without photolyzing cm-IP_3. This would allow integration of two-photon uncaging of cm-IP_3 (near 730 nm) and two-photon imaging of $[Ca^{2+}]_i$ in the same experiment, using two Chameleon lasers set at different wavelengths. We have developed a similar approach to study cell–cell junctional coupling using a caged coumarin dye (15, 27).

8. The total amount of light energy input during two-photon uncaging can be estimated from the average laser power at the specimen and the summed laser exposure time. Using the settings in **Table 6.1** (1 pixel = 0.36 μm × 0.36 μm), the summed laser exposure time for a 3.6 μm square is about 4.8 ms (100 pixels × 3.2 μs pixel dwell time × 15 repetitions), so the total amount of the average light input is 48 μjoule. The actual uncaging duration, however, is much longer and is on the order of seconds. This is due to the mode of operation of laser line scanning in the LSM510 system. Improvements in the newer version of the hardware and software of the LSM510 imaging system makes it possible to shorten the uncaging duration.
9. M-IP_3, once generated from cm-IP_3 by photolysis, is rapidly metabolized in cells within 10 s (8). It is therefore crucial to quench cells with cold TCA solution immediately in order to measure the amount of m-IP_3.

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