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Imaging Ca^{2+} Signals in *Xenopus* Oocytes

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Summary

Xenopus oocytes have become a favored preparation in which to study the spatiotemporal dynamics of intracellular Ca^{2+} signaling. Advantages of the oocyte as a model cell system include its large size, lack of intracellular Ca^{2+} release channels other than the type 1 inositol trisphosphate receptor, and ease of expression of foreign receptors and channels. We describe the use of high-resolution fluorescence imaging techniques to visualize Ca^{2+} signals in *Xenopus* oocytes at levels ranging from global Ca^{2+} waves to single-channel Ca^{2+} microdomains.

Key Words: Ca^{2+}; caged IP_3; calcium; confocal; dye; flash photolysis; fluorescence; imaging; inositol; IP_3; linescan; microinjection; microscopy; receptor; signaling; TIR; video rate; *Xenopus* oocytes.

1. Introduction

Xenopus oocytes are a favored model cell system in which to image Ca^{2+} signals evoked by inositol 1,4,5-trisphosphate (IP_3) and possess distinct advantages over most other cell types. Intracellular Ca^{2+} liberation is mediated only by type 1 IP_3 receptors in the absence of ryanodine receptors ***(1)***, their large size greatly facilitates intracellular injections, and they are among the best-characterized cells for Ca^{2+} signaling ***(2,3)***. Moreover, the ability of *Xenopus* oocytes to express foreign receptors and ion channels ***(4)***, such as calcium channels, further enhances the versatility of this already-favorable model system.

The protocols described in this chapter are aimed at the beginner in the field, assuming no previous knowledge of the methods. We describe the use of confocal microscopy techniques, together with flash photolysis of caged IP_3, for imaging intracellular Ca^{2+} signals in *Xenopus* oocytes. We use "custom-built" confocal microscopes, but the methods are directly applicable to the use of commercial instruments. We also describe the use of total internal reflection fluorescence microscopy (TIRFM) for visualizing Ca^{2+} signals from individual voltage-gated channels.

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2. Materials

2.1. Preparation and Microinjection of Xenopus Oocytes

1. Female *Xenopus laevis* frogs (Nasco, Fort Atkinson, WI; *see* **Notes 1–3**).
2. MS-222 (Sigma, St. Louis, MO). Store at –20°C in a desiccator. This is a possible carcinogen; wear gloves.
3. Frog guillotine.
4. Gentamicin solution, 50 mg/mL (Sigma). Store at 4°C; toxic.
5. Modified Barth's solution (MBS): 88 m*M* NaCl, 1 m*M* KCl, 2.4 m*M* $NaHCO_3$, 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.14 m*M* $CaCl_2$, 5 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.4, containing 0.5 mg/mL gentamicin. Store at 17°C (*see* **Note 4**).
6. Two forceps (Dumont 5) (Fine Science Tools Inc., Foster City, CA).
7. Upright light stereo dissection microscope.
8. Regular Petri dishes and one lined with nylon netting (for oocyte injection).
9. Glass vials (~15-mL capacity).
10. Collagenase type 1 (Sigma). This is toxic.
11. Ringer's solution: 120 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 5 m*M* HEPES, pH 7.3. Store at 4°C (*see* **Note 4**).
12. Glass capillaries (7-in. Drummond 3-00-203-G/XL).
13. Horizontal pipet puller (Narishige, Tokyo, Japan).
14. Microinjector (Drummond Nanoject Drummond Scientific Co, Broomall, PA).

2.2. Imaging

2.2.1. Confocal Imaging

1. Caged IP_3 (Molecular Probes, Eugene, OR). Store at –20°C; light sensitive (but *see* **Note 5**).
2. Ca^{2+} indicator dyes (Molecular Probes). Store at –20°C; light sensitive.
3. Inverted confocal microscope with excitation laser (e.g., 488-nm argon ion laser).
4. 40× Oil objective (numerical aperture [NA] = 1.35) and low-viscosity immersion oil.
5. Oocyte recording chamber.
6. Glass cover slips (12-545C, 22 × 40-1, Fisher Scientific, Pittsburgh, PA).
7. Image acquisition and processing software. We use custom routines in *Labview* (National Instruments, Austin, TX) for acquisition and IDL (Interactive Data Language: RSI Inc., Boulder, CO) for processing.

2.2.2. Total Internal Reflection Fluorescence Microscopy

1. Stripping solution: 200 m*M* K-aspartate, 20 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* EGTA (ethyleneglycol *bis*-(2-aminoethylether)-*N,N, N' N'*-tetraaceticacid), 10 m*M* HEPES, pH 7.4.
2. High-Ca^{2+} Ringer's solution: 110 m*M* NaCl, 6 m*M* $CaCl_2$, 2 m*M* KCl, 5 m*M* HEPES, pH 7.4.
3. Calcium indicator dye (Fluo-4 dextran; Molecular Probes, Eugene, OR).
4. Complementary RNA (cRNA) encoding N-type Ca^{2+} channel $\alpha_{1B\text{-}d}$ and β_3 subunits (mixed 1/1 to a final concentration of 0.1–1 μg/μL in diethylpyrocarbonate [DEPC] water).
5. Inverted microscope equipped for TIRFM, including TIRF (total internal reflection microscopy) objective lens (e.g., Olympus 60× oil immersion, NA 1.45), laser (e.g., 100-mW argon ion laser), low-light level, high-speed camera (e.g., Cascade; Roper Scientific, Tucson, AZ). *See* **Subheading 3.2.8.** for more details.

6. Low-fluorescence immersion oil for TIRFM (type DF; Cargill Laboratories, Cedar Grove, NJ).
7. Image acquisition and analysis package (e.g., *MetaMorph*, Universal Imaging Corp., West Chester, PA).

2.2.3. Two-Electrode Voltage Clamp

1. Two manual micromanipulators (e.g., model MM-3; Narishige USA, East Meadow, NY) mounted on the microscope stage.
2. Glass capillaries (e.g., 1.5 mm × 0.86 mm, 6 in., with filament; A-M Systems Inc., Carlsborg, WA).
3. 3*M* KCl solution for filling electrodes.
4. Chlorided silver wires (chloride silver wire by dipping in solution of household bleach for a few minutes) for electrodes and ground wire for recording chamber.
5. Voltage clamp (e.g., Geneclamp 500, Axon Instruments, Foster City, CA).
6. Recording and analysis software (e.g., *pClamp*, Axon Instruments; or *Strathclyde WinWCP*, available as a free download to academic users at http://www.bio-logic.fr/electrophysiology/winwcpandwinedr.html).

3. Methods

The methods described are aimed at the beginner in the field and outline the preparation and microinjection of *Xenopus* oocytes and techniques for imaging intracellular Ca^{2+} signals.

3.1. Preparation and Microinjection of Xenopus Oocytes

Because detailed protocols for preparing and microinjecting *Xenopus* oocytes are listed elsewhere in this book, we only give a brief summary covering the essential steps. The subheadings outline terminal and survival surgery; removal of epithelial layers, follicular cells, and the vitelline membrane; and oocyte microinjection.

3.1.1. Terminal and Survival Surgery

1. Place a female *X. laevis* frog (**Fig. 1A**) in a 0.17% solution of MS-222 (made up in water) until sufficiently anesthetized.
2. If all of the oocytes are to be used, terminal surgery is performed: the frog is anesthetized and decapitated prior to removal of ovaries.
3. If only a few lobes are required for experiments, survival surgery may be performed. Make a small incision (~5–10 mm long) into the abdominal cavity of a fully anesthetized frog and remove a few ovarian lobes (using presterilized instruments) and place them directly into MBS. Suture the incision, using separate stitches for the abdominal wall and skin. Allow the frog to recover in fresh water and monitor for a few hours before returning to the vivarium. Perform alternate surgeries on opposite sides of the frog, with intervals of longer than 1 mo between successive surgeries.

3.1.2. Removal of Epithelial Layers and Follicular Cells

1. Place individual ovarian lobes in MBS, cut them open, and view them under a stereomicroscope (**Fig. 1B**).
2. Carefully remove epithelial layers ***(5)*** from healthy (uniform pigmentation) stages V and VI oocytes ***(6)*** using two fine forceps (Dumont 5): one to hold the ovary membrane steady and the other to manually "peel" the epithelial layers from the oocyte. Repeat this procedure until enough oocytes have been obtained for experiments (typically 200 oocytes).

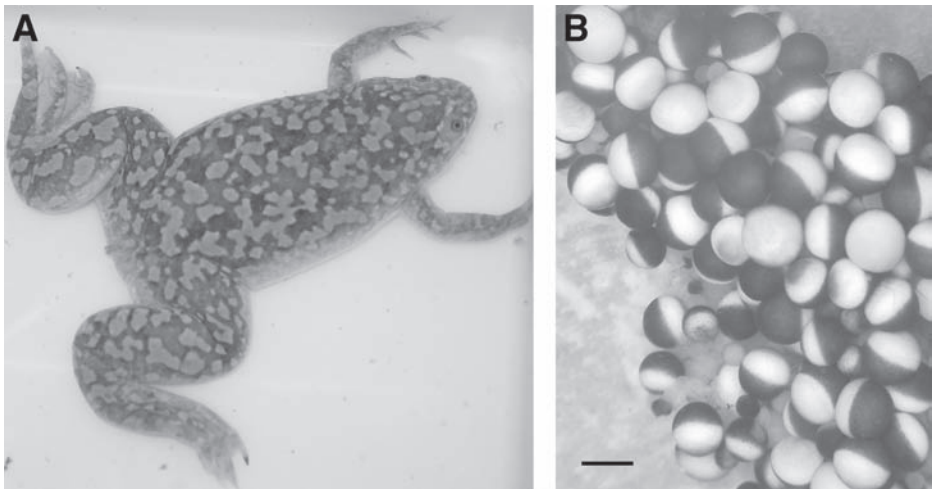

Fig. 1. (**A**) Female *Xenopus laevis* frog. (**B**) Ovarian lobe (cut open); scale bar = 1 mm. The pigmented (black) and unpigmented hemispheres, termed the animal and vegetal poles, respectively, can be seen.

3. Remove surrounding follicular cells, which are electrically coupled to the oocyte itself through gap junctions. Place oocytes (with the epithelial layers already removed) in approx 5 mL of 0.5 mg/mL collagenase type 1 (in Ringer's solution) with gentle agitation for approx 45 min. Wash repeatedly (about five washes) in MBS.
4. Store oocytes in MBS at 17°C until use. They can be stored in batches of approx 20 oocytes in individual vials filled with about 10 mL MBS. Rinse with fresh MBS twice daily and discard unhealthy oocytes. Alternatively, oocytes can be maintained individually in multiwell plates. Oocytes usually remain healthy for about 7 d. We do not remove the vitelline membrane for standard confocal imaging experiments, but this is stripped (as described in **Subheading 3.1.4.**) prior to TIRFM or patch-clamp recording.

3.1.3. Microinjection

1. After collagenase treatment, leave oocytes overnight in MBS at 17°C. Oocytes are easier to inject after overnight incubation because the membrane becomes firmer.
2. Pull a glass capillary (Drummond) (**Fig. 2A**) and examine under the microscope; using a fine pair of forceps, gently break off the end of the needle. For cytoplasmic injections, the needle width can be around 15 to 20 µm.
3. Backfill the needle with mineral oil, avoiding air bubbles, and assemble it onto the nanoinjector (**Fig. 2C**). Once the needle is mounted, press the "empty" button on the injector for a few seconds to dispel any air bubbles from the tip.
4. Prepare the solution to be injected. For calcium imaging and photolysis of caged IP_3, this is an aqueous solution containing Ca^{2+} indicator dye (1.5 m*M*) together with caged IP_3 (0.25 m*M*) D-*myo*-inositol 1,4,5-trisphosphate, $P_{4(5)}$-(1-(2-nitrophenyl)ethyl) ester). For injection of cRNAs, thaw the selected cRNA preparation (e.g., 1/1 mixture of $\alpha_{1B\text{-}d}$ and β3 cRNA N-type calcium channel clones) and spin at approx 9000*g* for 1 min to precipitate any salt crystals.

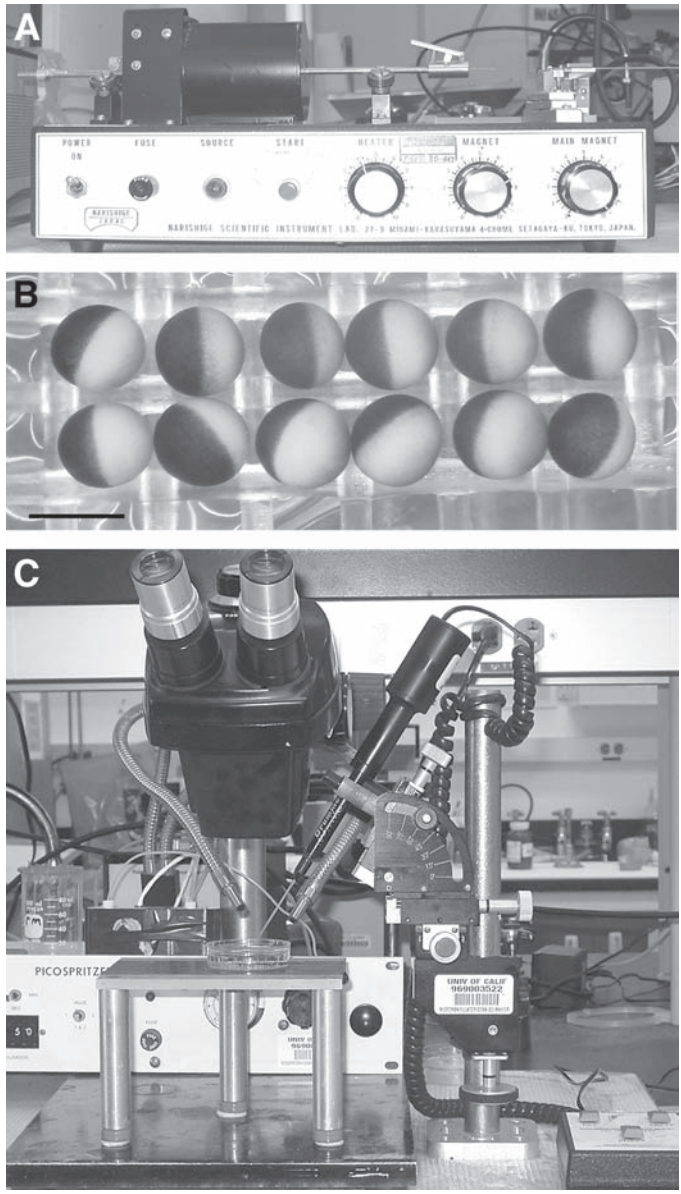

Fig. 2. Equipment for microinjection. (**A**) Pipet puller (Narishige, Tokyo, Japan); (**B**) oocytes aligned for microinjection; and (**C**) Drummond microinjector.

5. Pipet 1 to 2 µL of the injection solution onto a piece of fresh Parafilm positioned under the microscope. When injecting cRNA, wear clean gloves and use fresh pipet tips to avoid degradation by RNAases.
6. Using a stereomicroscope, focus on the drop under the microscope and position the micropipet over it; make sure you can see the micropipet enter the drop, then press the "fill" button on the Drummond nanoinjector. After the sample has been loaded, immediately position the tip of the micropipet in the injecting chamber containing MBS to avoid drying solution at the tip.
7. Transfer 10 to 30 oocytes into an injection chamber made by cementing a plastic net into the bottom of a Petri dish to obtain individual "wells" in the bottom of the Petri dish. In this situation, the oocytes can be steadily positioned in each mesh in any required orientation. Line the oocytes up with the equatorial band facing upward toward the injecting needle to avoid penetrating the nucleus (**Fig. 2B**).
8. Position the micropipet as vertically as possible with respect to the oocyte (angle ~75°) and gently lower the pipet until the tip penetrates the oocyte. The oocyte will at first dimple and then "spring back" as the pipet penetrates the membrane.
9. Inject the required volume of solution (*see* **Note 7**).
10. After all oocytes have been injected, place them into a glass vial containing approx 5 mL MBS and store at 17°C for about 30 min (*see* **Note 5**) to allow for intracellular distribution of the dye and caged IP_3 prior to imaging Ca^{2+} responses.

3.1.4. Stripping the Vitelline Membrane for TIR Imaging

For TIRFM imaging, the plasma membrane of the oocyte must approach within less than 100 nm of a glass cover slip forming the base of the imaging chamber. It is therefore necessary to first remove the vitelline envelope, a connective tissue layer that remains even after collagenase treatment and removal of follicular cells.

1. After injecting collagenase-treated oocytes with calcium indicator dye, place a few oocytes in cooled hypertonic stripping solution at 10°C.
2. Wait for 10 to 20 min and then observe oocytes under a stereomicroscope using a fiberoptic illuminator with the light directed from the side. The oocyte should have shrunk, revealing the vitelline membrane as a surrounding translucent membrane. Grip the membrane using two pairs of fine forceps (Dumont 5), taking care not to pinch the oocyte, and gently tear open the membrane.
3. Use a dropper pipet to transfer the oocyte briefly to wash in a Petri dish filled with normal Ringer's solution, then transfer again to the imaging chamber. Once the vitelline membrane is removed, oocytes are extremely fragile and quickly adhere to clean glass and plastic surfaces.

3.2. Imaging Techniques

We describe the basic principles of epifluorescence microscopy, video rate and linescan confocal laser scanning microscopy (CLSM), and TIRFM with respect to imaging intracellular Ca^{2+} signals in *Xenopus* oocytes.

3.2.1. Ca^{2+} Indicator Dyes

The most commonly used Ca^{2+} indicators are comprised of a chromophore conjugated to a Ca^{2+} chelator (BAPTA (1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N' N'*-tetracetic acid))) backbone ***(7,8)***. Ratiometric Ca^{2+} indicators demonstrate a spectral

shift in either their excitation (e.g., Fura-2) or their emission (e.g., Indo-1) spectra on Ca^{2+} binding, whereas the nonratiometric dyes do not. The most obvious advantage of using ratiometric dyes is that they provide an intrinsic normalization, eliminating variations in factors such as probe loading, dye retention, photobleaching, and cell thickness. Moreover, measurements obtained using ratiometric dyes can be converted into absolute free [Ca^{2+}]. However, ratio imaging is slow and complex and, because currently available ratiometric dyes (e.g., Fura-2 and Indo-1) are excited in the ultraviolet (UV) spectrum, they cannot readily be used for confocal imaging and cannot be used in conjunction with photolysis of caged compounds.

Nonratiometric indicator dyes (such as Oregon Green 488 BAPTA, Fluo-3, Fluo-4, Calcium Green, and Calcium Orange) are more popular for imaging Ca^{2+} signals in *Xenopus* oocytes for several reasons. These long-wavelength indicators generally demonstrate a large increase in fluorescence emission intensity on Ca^{2+} binding (except for Fura red, which shows a decrease) and are optimally excited at wavelengths compatible with those produced by laser illumination (e.g., the 488-nm line of the argon ion laser). The problems commonly associated with dye (am-ester) loading and retention in small cells ***(9)*** are obviated in oocytes because the free indicator dye can be directly injected into these "giant" cells. Because the most commonly used Ca^{2+} indicator dyes do not show a spectral shift on binding Ca^{2+}, fluorescence signals are generally expressed as "pseudoratios" (F/F_o or $\Delta F/F_o$) of the fluorescence F at each pixel relative to the mean resting fluorescence F_o at that pixel prior to stimulation.

To select the most appropriate indicator dye for the application at hand, the parameters of the indicator (*in situ* binding affinity, dynamic range, rate of compartmentalization, and sensitivity to photo-damage) need to be taken into consideration ***(9)***. For example, small Ca^{2+} signals are best monitored using an indicator that binds Ca^{2+} with high affinity (e.g., Oregon Green 488 BAPTA-1, OG-1), whereas if the main goal is to track high-amplitude signals, then a low-affinity dye (e.g., Oregon Green BAPTA-5N) should be used to avoid saturation. Because the "apparent" binding affinities of indicator dyes *in situ* can vary significantly from those measured in vitro and affinities can even vary between different intracellular compartments, you may wish to calibrate the properties of your dye of choice in the oocyte ***(9)***. A relatively recent report demonstrating that the Ca^{2+} indicator dye OG-1 can evoke elementary Ca^{2+} signals in *Xenopus* oocytes ***(10)*** attributed this action to the mobility of the indicator. If dye mobility is important for your application, you may wish to use a "low-mobility" (dextran-conjugated) Ca^{2+} indicator dye (*see* **Note 6**), available from Molecular Probes.

3.2.2. Basic Principles of Ca^{2+} Imaging and Epifluorescence Microscopy

Epifluorescence microscopy involves the use of short-wavelength (e.g., blue) light to excite fluorophore molecules within a specimen (e.g., an oocyte). The light source is directed to the specimen through an objective lens. As fluorophore molecules absorb high-energy incident photons, provided by the light source, they rapidly (nanoseconds) emit photons of lower energy (longer wavelength; e.g., green light). Emitted fluorescence is imaged through the same objective lens. This is made possible by a filter "cube" (containing a dichroic mirror together with excitation and barrier filters)

that is positioned directly behind the objective to enable satisfactory separation of excitatory and emitted light. Because the objective lens also functions as the condenser in this arrangement, the NA is a very important consideration. Overall, the brightness of the fluorescence image increases as the fourth power of the NA. Attaining high numerical apertures requires use of immersion objectives. In principle, a water immersion objective should be optimal to avoid spherical aberration caused by refractive index mismatch, but in practice we achieve best results using a 40× oil immersion objective (NA 1.35).

3.2.3. Confocal Laser Scanning Microscopy

Confocal microscopy differs from regular wide-field epifluorescence in that the excitation light is focused as a diffraction-limited spot in the specimen, and emitted light is detected through a small aperture (pinhole) to reject out-of-focus fluorescence. This has the great advantage of providing a thin optical "slice" within a thick specimen but involves additional complexity as the confocal spot must be raster scanned, point by point, to build up an image. It is not possible to cover all of the principles of confocal microscopy in this short review; thus, we refer the reader to the *Handbook of Biological Confocal Microscopy* ***(11)*** for a more extensive description of the technique.

3.2.4. Commercial vs Homemade CLSMs

Commercial CLSMs, such as the Nikon (Melville, NY) RCM 8000, Noran Odyssey (Middleton, WI), and Bio-Rad (Hercules, CA) RTS2000, are expensive, generally surpassing the budget of individual investigators. Most research institutions therefore have shared optical imaging facilities, and a handful of researchers have even taken to building their own confocal instruments (e.g., ***12–14***). Many commercially available CLSMs (and associated software) were not designed with dynamic studies in mind, which can present many problems for users wishing to image fast events, such as Ca^{2+} transients, and can thus constrain the types of recordings that can be made. Moreover, the light paths in such instruments are generally complex, and adjusting the alignment can be difficult. To obviate such problems, in our laboratory we image intracellular Ca^{2+} signals using homemade CLSMs (**Figs. 3**,**4**; **refs. *12–15***).

3.2.5. Framescan vs Linescan Confocal Microscopy

Most commercial confocal microscopes can acquire full-frame images at rates of only one or a few frames per second. This is too slow to resolve dynamic changes in intracellular [Ca^{2+}], which typically occur on a time-scale of tens of milliseconds. However, it is still possible to obtain good temporal resolution by imaging in linescan mode. Here, spatial information is traded for temporal information, and the laser spot is repeatedly scanned along a line to image a single spatial dimension with a time resolution as good as 1 or 2 ms per line (**Fig. 3**). As well as the improved temporal resolution, advantages of this technique are that the resulting data files are relatively compact, and that the spatiotemporal profile of Ca^{2+} signals is readily apparent from single images (**Fig. 3C**). Disadvantages include the limited spatial information, sampling from only a very small region of the oocyte, and uncertainty regarding whether the observed local Ca^{2+} signals originated directly on the scan line.

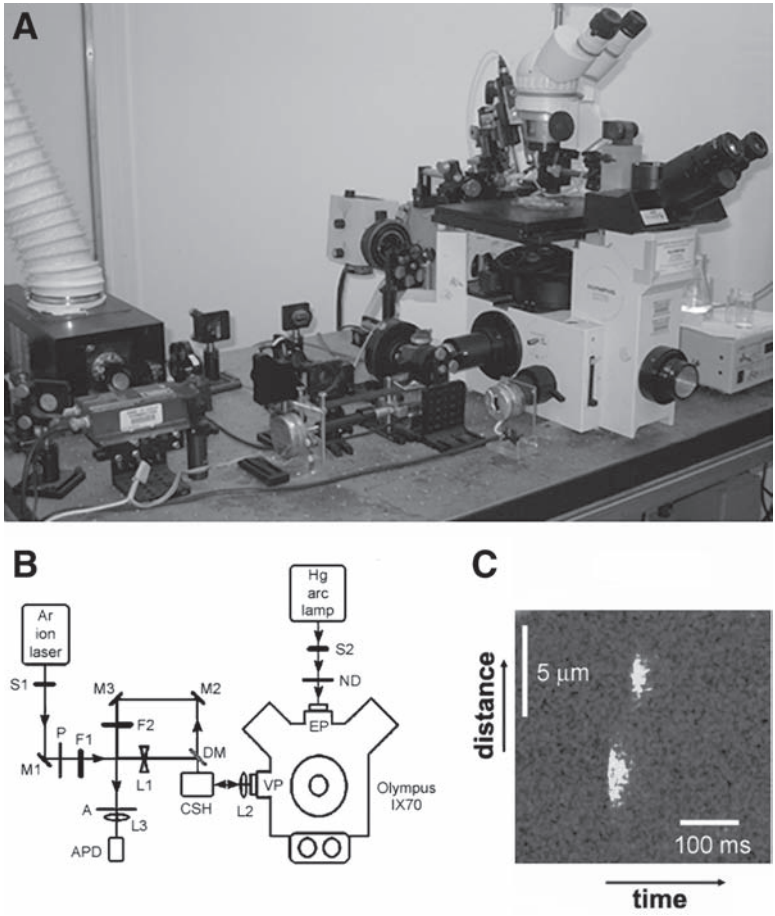

Fig. 3. Linescan CLSM. (**A**) Photograph; (**B**) schematic diagram of the optical layout of our homemade linescan CLSM. The CLSM is interfaced through the video port (VP) of an inverted microscope (Olympus IX70), and the photolysis system (light path of the mercury arc lamp) is interfaced through the epifluorescence port (EP). Electronic shutters (S1 and S2); mirrors (M1–M3 are fully reflecting front surface mirrors and DM is a dichroic mirror, λ = 500 nm); a rotating polarizer (P); filters (F1 is a 488 narrowband interference filter, and F2 is a barrier filter); lenses (L1 is a diverging lens with focal length of –10 cm, L2 is a scan lens made from a Zeiss 10× wide-field eyepiece, and L3 is a converging lens with a focal length of 5 cm); and a confocal aperture (A) were placed in the laser path as stated. The intensity of the UV photolysis light was attenuated using neutral density filter wheels (ND), and an avalanche photodiode module (APD) was used to detect photons. The scan mirror in the confocal scan head (CSH) is positioned 2 cm from the front surface of L2; the distance from L2 to A is 80 cm, from L2 to L1 is 70 cm, and from L3 to APD is 5 cm. (**C**) A representative image of IP_3-evoked Ca^{2+} puffs.

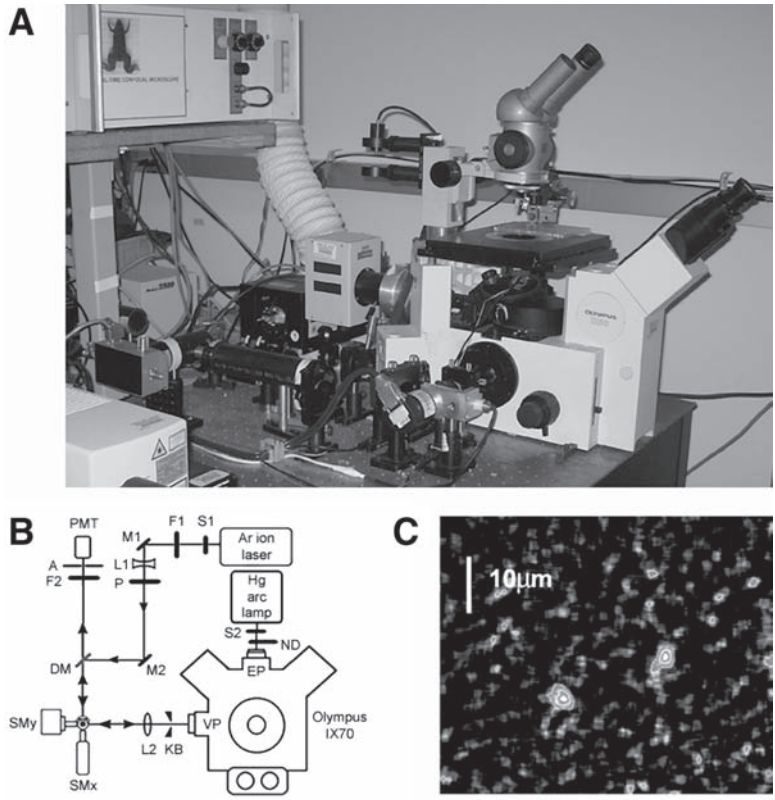

Fig. 4. (**A**) Homemade videorate CLSM. (**B**) Schematic of optical system. (**C**) A representative image of IP_3-evoked Ca^{2+} puffs. The optical components of the laser scanning system are interfaced through the video port (VP) of the Olympus IX70 microscope, and the UV light path generated by the mercury arc lamp is directed through the epifluorescence port (EP) at the rear of the microscope. Electronic shutters (S1 and S2); mirrors (M1–M4 are fully reflecting, broadband coated mirrors, and DM is a dichroic mirror, $\lambda = 500$ nm); lenses (L1 is a plano-concave lens [$f = -50$ cm], and L2 is a scan lens [10× Olympus eyepiece lens]); and a knife blade (KB) aperture are situated in the light path of the laser beam as illustrated. The scan head comprises two galvanometer-driven mirrors: a *y*-scan off-axis scan mirror (SMy) and a resonant scanning mirror (SMx).

A few confocal microscopes are available that permit full-frame imaging at video rate (30 frames per second) or even higher based on rapid scanning systems utilizing a resonant mirror, acousto-optic deflector, or spinning microlens array. These provide good two-dimensional spatial information and thus offer an intuitive view of intracellular Ca^{2+} signaling events (**Fig. 4**). However, the temporal resolution still does not approach that of linescan imaging, and the signal-to-noise ratio is not as good because of shot noise constraints arising from the brief dwell time at each pixel.

3.2.6. Setting Up an Inverted CLSM for Oocyte Imaging

Whichever technique you chose to use, the basic principles of setting up the microscope to obtain good images are the same.

1. Place a small drop of immersion oil on the 40× oil objective lens.
2. Using the coarse focus knob, lower the objective as far as possible.
3. Prepare the recording chamber: mount a clean, new cover slip in the recording chamber and secure the chamber to the stage of the inverted CLSM. Be aware that the cover slips tend to stick together. If two are inadvertently mounted, it will not be possible to focus on the oocyte because the working distance of oil immersion objectives is very short.
4. Using the coarse focus knob, raise the objective slowly until the oil makes contact with the cover slip of the recording chamber.
5. Fill the chamber with Ringer's solution and place an oocyte into the chamber.
6. Manipulate the position of the oocyte such that it is centered directly over the objective lens with the animal pole (dark side) facing down.
7. Focus in the layer of pigment granules in the animal hemisphere.
8. Open the laser shutter and focus the laser scan at the pigment granules of the oocyte (at a depth of ~5 µm into the animal hemisphere of the oocyte; this is where the Ca^{2+} release sites are most concentrated).
9. Acquire images (linescan or framescan) using software provided by the microscope manufacturer, third-party image acquisition software (e.g., *MetaMorph*, Universal Imaging, West Chester, PA), or custom-written software (we have written linescan acquisition and analysis routines using the IDL programming environment of Research Systems Inc., Boulder, CO).
10. The nanoinjector can be mounted on the microscope stage to make successive injections while imaging (*see* **Note 8**).

3.2.7. Flash Photolysis of Caged IP_3

Caged IP_3 (*see* **Note 5**) injected into oocytes cannot bind to IP_3 receptors and is therefore inactive. Irradiation with a short pulse of UV light (~360 nm) generates a concentration jump in IP_3, by releasing it from its caged precursor. This photo-released IP_3 can bind to, and open, IP_3 receptors (IP_3Rs) on the endoplasmic reticulum (ER), liberating Ca^{2+} from this intracellular store. Advantages of this technique for studying IP_3-mediated Ca^{2+} signaling include the facts that complexities resulting from upstream agonist activation of the phosphoinositide pathway are circumvented, and that precise control is achieved of the relative amount of IP_3 formed, its timing, and (by restricting where the UV light is focused on the oocyte) its spatial distribution.

A mercury arc lamp (50 or 100 W) provides a good source of UV light, and a regular microscope epifluorescence illuminator can be readily modified for flash photolysis by incorporating an electronic shutter (e.g., Uniblitz, Vincent Associates, Rochester, NY) and a filter cube with a dichroic mirror and excitation filter designed to reflect light less than 400 nm toward the objective lens (**Figs. 3B** and **4B**; also *see* **Note 9**).

1. Prefocus the UV light beam of a xenon or mercury arc lamp as a 200-µm spot surrounding the image scan line ***(16)***. This can be visualized using a test slide made by rubbing a yellow highlighter pen on the upper side (i.e., the side away from the objective lens) of a cover slip.

2. Mount the recording chamber and focus the microscope at the depth of the pigment granules in an oocyte as above. Photorelease IP_3 from its caged precursor by briefly opening the electronic shutter in the UV light path to deliver a very brief UV flash (in the order of milliseconds). This will result in a uniform release of IP_3 throughout the 200-µm region.
3. The amount of photoreleased IP_3 can be controlled (in a linear manner) by varying the flash duration (by altering the open duration of the electronic shutter). Because each flash consumes only a negligible fraction of the caged IP_3 ***(16)***, it is possible to acquire numerous consistent responses from the same oocyte using repeated flashes. Neutral density filters can be inserted into the UV light path to evoke responses over an appropriate range of flash durations (we typically use 5–100 ms). Mechanical inertia in the shutter limits the shortest flashes to about 5 ms, whereas flashes longer than a few hundred milliseconds may outlast the Ca^{2+} response itself.
4. Intervals of longer than 90 s should be respected between recordings to allow IP_3Rs to recover from desensitization, and for cytosolic [Ca^{2+}] to recover to basal levels.

3.2.8. Total Internal Reflection Fluorescence Microscopy

Although confocal imaging provides an optical slice within the oocyte and can thus localize Ca^{2+} signals in the axial (*z*) dimension much more effectively than wide-field epifluorescence microscopy, the axial resolution is nevertheless limited to about 700 nm. Evanescent wave imaging, or TIRFM, provides a way to obtain a much thinner section, which we have used to visualize Ca^{2+} signals arising from activation of individual voltage-operated Ca^{2+} channels expressed in the plasma membrane of oocytes ***(17)***.

The principle of this technique is that light undergoing total internal reflection at an interface from high to low refractive index (e.g., from glass to aqueous solution) penetrates a short distance into the low refractive index medium. This creates an evanescent wave that can be used to excite fluorescence confined within the thin plane (30–100 nm) immediately adjacent to the interface ***(18)***. Thus, if a cell containing a fluorescent Ca^{2+} indicator is made to adhere closely to a glass cover slip, fluorescence signals are recorded only from a region immediately adjacent to the cell membrane.

Advantages are that the TIRFM section is much thinner than can be achieved with confocal microscopy, and because no scanning is required, images can be acquired with regular or intensified charge-coupled device (CCD) cameras. The major disadvantage is that imaging is restricted to objects immediately adjacent to the interface, and unlike confocal microscopy, it is not possible to focus more deeply into the specimen.

Our homemade TIRFM system is based around an Olympus IX7I microscope (**Fig. 5B**,**C**). Similar systems are now commercially available from Olympus and other microscope manufacturers. An Olympus 60× TIRFM objective with high NA (NA = 1.45) allows the excitation light from an argon-ion laser to be directed through the extreme edge of the objective aperture for TIRF excitation or to be directed more centrally for conventional wide-field excitation. In this epi-illumination configuration, both the excitation and emission light paths travel through the same objective lens. Thus, it is possible to obtain TIRFM images from thick, opaque specimens (such as the *Xenopus* oocyte), and space above the specimen is freely accessible for positioning microelectrodes for electrophysiological recording. The emitted fluorescence is collected after passage

through barrier filters (>510 nm to block 488-nm laser excitation and > 650 nm to block infrared emission from the laser tube) and is imaged using a fast, cooled CCD camera.

The generation of cameras (such as the Roper Scientific Cascade) employing on-chip electron multiplication provide high sensitivity (quantum efficiency as good as 90%), low noise, and fast frame rates (500 fps or higher with subregion binning).

Procedures for TIRFM imaging of Ca^{2+} flux through individual N-type channels expressed in the oocyte membrane are described next.

1. Before beginning experiments, ensure that the microscope is properly adjusted for TIRF operation. Place a small drop of low-fluorescence immersion oil on the objective and raise this to contact a clean glass cover slip on the microscope stage. Pipet a small (~10 µL) droplet of aqueous suspension of fluorescent microspheres (0.2 µm diameter fluospheres, Molecular Probes Inc.) on the cover slip. Focus the microscope on beads that have adhered to the cover slip. Adjust the position of lens L3 (**Fig. 5C**) so that the laser light leaves the objective lens at an angle greater than the critical angle for total internal reflection. If adjusted correctly, stationary beads adhered to the cover glass will appear bright, whereas beads moving by Brownian motion deeper in the droplet will not be visible.
2. Load oocytes previously injected with cRNAs to express N-type Ca^{2+} channels with Fluo-4 dextran and strip the vitelline membrane.
3. Mount a fresh cover slip in the TIRFM imaging chamber (**Fig. 6**), place the chamber on the microscope, fill with Ringer's solution containing 6 m*M* [Ca^{2+}], and introduce an oocyte with the animal hemisphere facing the objective. An elevated Ca^{2+} concentration is used to enhance Ca^{2+} flux through plasma membrane channels.
4. Penetrate the oocyte with two microelectrodes to allow voltage clamp control of the membrane potential. Clamp at a resting potential of –80 mV.
5. View the resting fluorescence of the indicator dye by TIRFM. Move the microscope stage (and attached micromanipulators) to find a region of the oocyte showing roughly uniform fluorescence throughout the imaging field (indicative of a "footprint" in which the membrane lies at about the same depth in the evanescent field).
6. Apply voltage clamp depolarizations (to about 0 mV for a few seconds). Look for the appearance of localized flashes of increased fluorescence ("sparklets") arising from Ca^{2+} flux through individual N-type channels (**Fig. 5D**). The distribution of channels in the oocyte is very "patchy," and it will probably be necessary to search throughout different regions of the oocyte.
7. Stream image TIRFM sequences to computer memory for subsequent analysis. In favorable cases, more than 100 separate channels can be captured in the image frame (80 × 80 µm). Process records by dividing each frame by an average image of resting fluorescence before stimulation, so that measurements reflect fractional increases in fluorescence ($\Delta F/F_o$), thus correcting for differences in fluorescence resulting from factors including uneven laser illumination and variation in distance of the membrane from the cover glass (**Fig. 5A**).
8. Measure fluorescence ratio signals from small (<1 µm) regions of interest centered on sparklet sites to resolve the gating of individual channels (**Fig. 5E**).

4. Notes

1. Alternative suppliers of *X. laevis* frogs include *Xenopus* express (www.xenopus.com), *Xenopus* 1 (Dexter, MI; www.xenopusone.com), and Carolina Biological Supply (Burlington, NC; www.carolina.com).

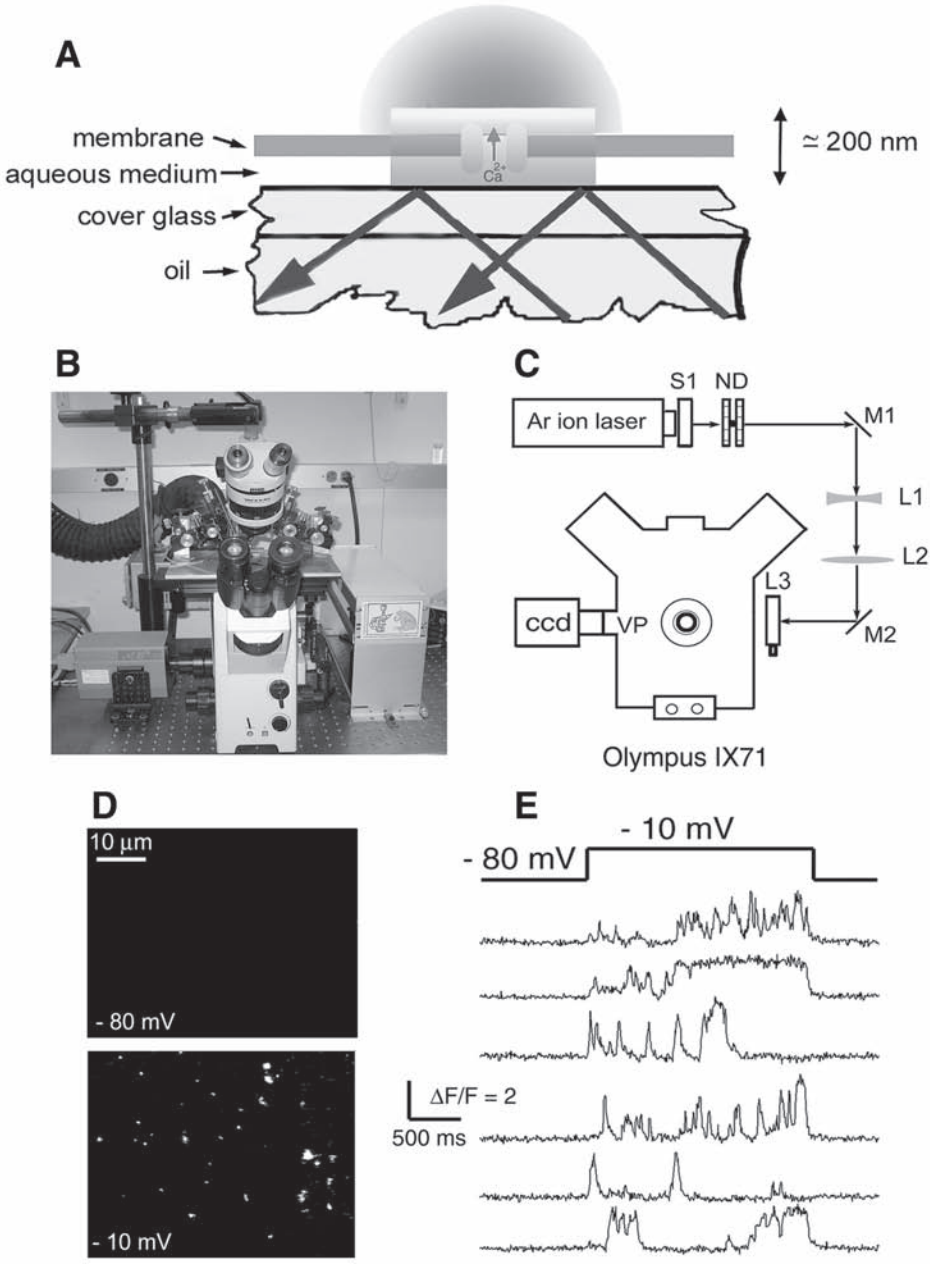
A
membrane
aqueous medium
cover glass
oil
Ca2+
≃ 200 nm
B
C
S1
ND
Ar ion laser
M1
L1
L2
L3
ccd
VP
M2
Olympus IX71
D
10 µm
- 80 mV
- 10 mV
E
- 10 mV
- 80 mV
ΔF/F = 2
500 ms

Fig. 5.

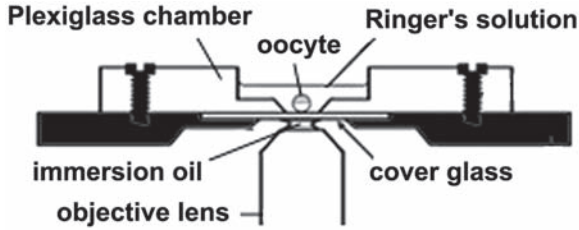

Fig. 6. Oocyte imaging chamber. The chamber is machined from two pieces of Plexiglas, which are held together to clamp a glass cover slip that forms the base of the recording chamber. A thin film of Vaseline applied to the upper section provides a watertight seal to retain Ringer's solution in the chamber. The chamber mounts onto the stage of an inverted microscope, and the oocyte is visualized through the cover glass by an oil immersion objective.

2. For experiments in which light absorption by pigment may lead to artifactually high thresholds in the animal hemisphere, ooctyes from albino frogs may be preferable ***(19)***.
3. *Xenopus tropicalis* oocytes may be suitable for certain experiments: the spatiotemporal characteristics of Ca^{2+} signals have been shown to be similar those in *X. laevis* oocytes, and *X. tropicalis* oocytes may offer further practical advantages with respect to imaging depth, Ca^{2+} signal magnitude, and electrical properties ***(20)***.
4. Barth's (no gentamicin) and Ringer's solutions can be made up as 10X stocks (1 L), stored in the refrigerator, and diluted as needed. Gentamicin needs to be added to 1X Barth's dilutions, pH 7.4, then stored at 17°C. The 1X Ringer's, pH 7.3, can be stored at room temperature or in the refrigerator (but bring to room temperature prior to recording).
5. Caged IP_3 can also be purchased from Calbiochem (La Jolla, CA), but in our experience the caged IP_3 from Molecular Probes is preferable because it has lower contamination levels of active IP_3. Caged IP_3 is highly charged and thus does not permeate through cell membranes. Although by-products of photoreleasing IP_3 (H^+ and nitroacetophenone) are toxic at high concentrations (1 mmol/L), this toxicity does not appear to be problematic in the oocyte (and other cells) because the concentrations of IP_3 required to evoke even maximal Ca^{2+} release are very small ***(16)***. We have not found it necessary to take any

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Fig. 5. *(previous page)* Total internal reflection fluorescence microscope (TIRFM). (**A**) Principle of through-the-lens TIRFM. Laser light directed through the periphery of a high-NA objective lens (NA = 1.45) passes through the immersion oil and cover glass at a shallow angle and undergoes total internal reflection at the interface between the glass and the aqueous medium, resulting in an evanescent wave propagating about 200 nm into the aqueous phase. The surface membrane of a vitelline-stripped oocyte lies within the evanescent field, allowing the imaging of near-membrane fluorescence from a Ca^{2+} indicator dye (Fluo-4 dextran) previously loaded into the oocyte. (**B**, **C**) Photograph and schematic diagram of the TIRFM imaging system. (**D**) Fluorescence images (single video frames) showing Ca^{2+}-dependent fluorescence at a negative holding potential and after depolarizing to −10 mV to cause openings of voltage-gated N-type Ca^{2+} channels in the oocyte membrane. Note the appearance of bright spots (sparklets) resulting from Ca^{2+} influx through individual channels. (**E**) Fluorescence traces monitored from small (1 μm^2) regions of interest centered on sparklet sites show pulsatile changes reflecting stochastic channel gating.

special precautions when handling caged IP_3 because standard room lights and even halogen fiber optics cause surprisingly little photolysis of the compound. Photolysis of caged IP_3 has advantages over injection of IP_3 in that homogeneous elevations in [IP_3] can be induced (by focusing a broad light spot), thus circumventing problems of diffusion delays.

6. If you choose to use a dextran-conjugated Ca^{2+} indicator dye, then the equilibration time (time between injection and confocal recording) must be extended to allow for the lower mobility inferred by its higher molecular weight (as a rough guide, we find that 2 h is usually sufficient for equilibration of dextran-conjugated Fluo-4, and compartmentalization does not occur until approx 5 h after injection).
7. As a rough guide, the cytosolic volume of the oocyte is usually estimated as approx 1 μL; therefore, to obtain final intracellular concentrations of 48 μ*M* dye and 8 μ*M* caged IP_3 you would inject 32 nL of a solution comprised of 1.5 m*M* dye and 0.25 m*M* caged IP_3. To minimize the possibility of accidentally "pinching" the nucleus, try to inject as close to the equator as possible.
8. When performing successive "experimental" injections (e.g., via an injector mounted on the confocal microscope) to obtain concentration–response relationships, we would recommend removing the pipet between successive injections to prevent any possible excess leakage of the experimental solution into the oocyte. When injecting large volumes (>50 nL) of solution, we usually perform multiple small-volume injections to try to minimize trauma to the oocyte.
9. The filter cube introducing UV photolysis light into the microscope light path should be positioned close to the objective lens. The dichroic mirror reflects UV light, but transmits visible light, so that both the laser excitation and emitted fluorescence light pass through without attenuation to the confocal scanner. Depending on the microscope design, engineering changes may be required to add an extra port for UV photolysis to an existing confocal microscope.
10. *The Axon Guide* ***(21)*** is a useful starting point for newcomers to the field of electrophysiology and includes a chapter entirely devoted to electrophysiological recordings from *Xenopus* oocytes.

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