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Nuclear Microinjection to Assess How Heterologously Expressed Proteins Impact Ca^{2+} Signals in *Xenopus* Oocytes

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Abstract

The *Xenopus* oocyte is frequently used for heterologous expression and for studying the spatiotemporal patterning of Ca^{2+} signals. Here, we outline a protocol for nuclear microinjection of the *Xenopus* oocyte for the purpose of studying how subsequently expressed proteins impact intracellular Ca^{2+} signals evoked by inositol trisphosphate ($InsP_3$). Injected oocytes can easily be identified by reporter technologies and the impact of heterologously expressed proteins on the generation and properties of $InsP_3$-evoked Ca^{2+} signals can be resolved using caged $InsP_3$ and fluorescent Ca^{2+} indicators.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Caged $InsP_3$ (D-*myo*-inositol 1,4,5-triphosphate, *P*4[5]-[1-(2-nitrophenyl)ethyl] ester, tris[tri-ethylammonium] salt) (Life Technologies)

This compound is light sensitive.

Fluorescent Ca^{2+} indicators (Life Technologies)

These compounds are light sensitive.

Modified Barth's solution <R> containing gentamycin (50 μg/mL) (MBS)

Oocyte dissociation solution <R>

Progesterone (optional; see Step 10)

Solution of cDNA containing gene of interest

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Most eukaryotic promoters suffice for *Xenopus* expression. Because many useful Ca^{2+} indicators display enhanced green fluorescent emission in their Ca^{2+}-bound forms (conveniently detected with FITC filter sets), it is best to use longer wavelength (red) fluorescent reporter tags as expression markers (Kremers et al. 2011). Alternatively, co-injected secreted reporters can be used if a fusion protein is undesirable (Tate et al. 1990).

Xenopus oocytes

*Oocytes can be obtained by surgical extraction (*Sive et al. 2000; Dargan et al. 2005; Green 2010*) or directly sourced from commercial vendors (e.g., EcoCyte Bioscience and Nasco) or resource centers (*Pearl et al. 2012).

Equipment

Capillary glass (borosilicate; ~1 mm outside diameter and ~0.5 mm internal diameter without filament)

Dissecting microscope

Forceps

Incubator (set at 16°C–20°C)

Inverted confocal microscope

See Dargan et al. (2005) *for a discussion of equipment for confocal imaging and flash photolysis.*

Light box (with gooseneck illuminator)

Mesh (0.5–0.8 mm nylon or polypropylene)

Microinjector (with nanoliter precision and foot pedal; e.g., Drummond Nanoject II Auto-Nanoliter Injector)

Micromanipulator

Micropipette puller (e.g., P-97 or P-1000 from Sutter Instrument or Model 720 from Kopf Instruments)

Petri dishes

Pneumatic injector (e.g., PMI-100 from Dagan Corporation)

UV photolysis source (with shutter)

Watchmaker’s forceps (Dumont #5 or #55)

METHOD

1. Isolate and prepare the oocytes for microinjection. Remove the follicular cells that envelop the oocyte either enzymatically or manually. For enzymatic defolliculation of cells en masse, incubate small pieces of ovarian tissue in oocyte dissociation solution with gentle agitation for <1 h in an incubator set at 16°C–20°C. Wash the

dissociated cells several times in MBS and select stage V and VI oocytes (>1 mm in diameter) for microinjection. For manual defolliculation, use watchmaker's forceps and follow the procedure described by Sive et al. (2000). In either case, allow the cells to recover overnight in MBS before microinjection.

*Our preference is not to use large-scale enzymatic treatment owing to the risk of impaired oocyte viability and poorer in vitro maturation profiles after protracted collagenase exposure. Unless large numbers of oocytes (>1000) are required, we manually defolliculate the oocytes. This method takes practice, but we believe the resultant oocyte quality is better (*Sive et al. 2000*). The recovery period allows for identification and removal of damaged oocytes. Unless tight adherence of the oocytes to the chamber is required (e.g., for TIRF imaging) or patch clamping is to be performed, the vitelline membrane does not need to be removed. Defolliculated oocytes ready for microinjection can also be sourced commercially (e.g., EcoCyte Bioscience). See Troubleshooting.*

2. Align the oocytes for microinjection with the animal pole facing upward. Secure a restraining nylon or polypropylene mesh (0.5–0.8 mm) within a Petri dish and, using forceps, carefully orientate the oocytes so they sit vegetal side down in the mesh. Prepare ~100 oocytes at a time for microinjection.

   The germinal vesicle rests within the animal hemisphere of the oocyte (darkly pigmented half).

3. Prepare needles for microinjection using capillary glass and a micropipette puller. Ensure that the needle has a reasonable shank (up to 900 μm from the tip) and a final needle tip of <20 μm (outer diameter) after breakage under a microscope.

   A good needle is crucial for oocyte viability, especially with nuclear injection.

4. Load the needle with a solution containing cDNA (<3 ng; ~50–200 μg/mL) following the manufacturer's instructions for the microinjector.

   A variety of different microinjectors are suitable for oocyte nuclear microinjection. The large volume of the oocyte nucleus (~40 nL) can tolerate a surprisingly large injection volume (<15 nL). Any stably mounted microinjector that reproducibly dispenses nanoliter volumes is suitable. Depending on the equipment used, this step commonly involves backfilling the needle with oil, breaking the tip, and then frontfilling with injection solution.

5. Align the oocyte to be microinjected under the injection needle loaded with cDNA and move the needle down. Allow the oocyte surface to dimple under pressure from the needle before penetration; the needle should now be far enough into the cell that further movement is not necessary. Inject once (using the foot pedal), pause, and then gently withdraw needle. Move to the next oocyte either by moving the micromanipulator or the microinjection dish. Periodically withdraw the tip of

the needle from the solution to check the needle has not clogged. Repeat for other constructs. Inject a cohort of oocytes with a vector control.

We secure the Petri dish containing the oocytes on a manipulatable stage that can be ratcheted to position cells for injection. This maximizes throughput and minimizes risk of needle damage.

6. After microinjection, carefully separate the oocytes into individual vials containing MBS (<20 cells per vial). Return the oocytes to the incubator set at 16°C–20°C. Monitor the oocytes for viability at the start and end of each day, performing fresh solution changes at those times.

   It is important to remove apoptotic cells to prevent deleterious effects on healthy oocytes in the same vials. Some investigators maintain oocytes individually in multiwell plates. See Troubleshooting.

7. Screen for the expression of fluorescent-tagged protein constructs.

   *Expression is most commonly assessed visually by epifluorescence or confocal microscopy. Alternatively, fluorescence emission can be quantified by a plate reader or scanning imager (*Musa-Aziz et al. 2010*). Owing to the optical turbidity of the oocyte, signals are stronger for cell-surface proteins than for intracellularly targeted constructs. For the latter, individual cell screening on a confocal microscope is recommended. Expression of fluorescent proteins can be observed 24 h after injection and peak levels are attained after ~48–72 h. For fluorescent-tagged protein constructs, we place oocytes' animal pole down on a glass-bottomed Petri dish and screen using lasers interfaced through the side-port of an inverted microscope (*Dargan et al. 2005; Boulware and Marchant 2008). *See Troubleshooting.*

8. To elicit and resolve Ca^{2+} signals, microinject the oocytes with a mixture of a fluorescent Ca^{2+} indicator (e.g., the high-affinity dyes, Fluo-4 and Oregon Green 488 BAPTA-1; see **Ca^{2+}-Sensitive Fluorescent Dyes and Intracellular Ca^{2+} Imaging** [Bootman et al. 2013]) and caged $InsP_3$ (see **Photolysis of Caged Compounds: Studying Ca^{2+} Signaling and Activation of Ca^{2+}-Dependent Ion Channels** [Almassy and Yule 2013]). Inject the mixture cytoplasmically using a simple pneumatic injector and ensure that the Ca^{2+} indicator and caged $InsP_3$ attain final intracellular concentrations of ~20–40 and 5 μM, respectively. Choose an injection site distinct from the area to be imaged. After injection, return the oocytes to the incubator for ~30 min before imaging.

   Nonratiometric long-wavelength Ca^{2+} indicators are well suited for this application. In our laboratory, we typically use dextran-linked indicators owing to lower compartmentalization and larger fluorescence ratio changes. However, these conjugates take longer to diffuse throughout the cell following injection.

9. Transfer the oocytes individually to a confocal microscope. Photolyze the 1-(2-nitrophenyl)ethyl (NPE) caging moiety of caged $InsP_3$ at wavelengths <360 nm using a light source such as a wide-field UV flash lamp, a light-emitting diode, or a laser (see **Photolysis of Caged Compounds: Studying Ca^{2+} Signaling and Activation of Ca^{2+}-Dependent Ion Channels** [Almassy and Yule 2013]). Monitor the emitted fluorescence (>510 nm for Fluo-4 and Oregon Green 488 BAPTA-1) before (instrument background) and during laser illumination. Flash photolysis (millisecond exposures) should release sufficient $InsP_3$ to trigger Ca^{2+} release and increase the fluorescence signal. Compare responses by measuring the magnitude and kinetics of Ca^{2+}-dependent changes in fluorescence (ΔF) relative to the resting fluorescence (F_0) of the Ca^{2+} indicator over consistently defined regions. Regulate the degree of stimulation by controlling the duration (shutter) and/or intensity of photolysis (neutral density). Increasing levels of photolysis will progressively stimulate Ca^{2+} release to yield first responses from clusters of $InsP_3$ receptors (Ca^{2+} puffs) at low levels of stimulation, and then abortive and repetitive propagating Ca^{2+} waves (Ca^{2+} oscillations). High-affinity Ca^{2+} indicators are needed to resolve Ca^{2+} puffs. Experiments with both low-(e.g., Oregon Green 488 BAPTA-5N) and high-affinity Ca^{2+} indicators may be needed to resolve the effects of heterologously expressed proteins. Perform identical Ca^{2+} imaging assays in control oocytes.

   *Alternatively, $InsP_3$ can be elevated by microinjection/ionophoresis or by stimulation of endogenous cell surface receptors (*Lechleiter et al. 1991a*). See Troubleshooting.*

10. (Optional) To monitor Ca^{2+} signals and/or ER dynamics in *Xenopus* eggs, add progesterone (1 μg/mL) to expressing oocytes to initiate in vitro maturation. Owing to variations in maturation efficiency, take care to mature a cohort of cells to ensure a sufficient supply of cells for imaging experiments. Changes in Ca^{2+} dynamics and ER structure will occur in <5 h (vegetal hemisphere) and complete within ~10–12 h (Boulware and Marchant 2008). Identify eggs by the appearance of a large white spot in the animal hemisphere following germinal vesicle breakdown.

   *Dextran-linked indicators must be used if Ca^{2+} indicators are injected before maturation. Alternatively, carefully microinject eggs with caged $InsP_3$ and Ca^{2+} indicator (*Busa and Nuccitelli 1985*), if the goal is to compare endpoints (oocyte versus egg). For resolving Ca^{2+} signals and ER morphology throughout maturation, imaging approaches that minimize photodamage and preserve oocyte viability must be used (see Discussion). See Troubleshooting.*

TROUBLESHOOTING

Problem (Step 1): Oocyte viability is poor.

Solution: Frog husbandry issues (water and nutritional quality, frog density, infections) can impact oocyte yield and health (Sive et al. 2000; Green 2010). Compare viability with oocytes sourced directly from vendors. Careful in vitro culture of oocytes is important. If the

cells show excessive pigment marbling or black spots appear on their surface, microbial contamination is likely and antibiotic supplementation is required (O'Connell et al. 2011).

Problem (Step 6): The viability of microinjected cells is poor.

Solution: Careful evaluate the microinjection needle and technique. If there is evidence of damage to the oocyte on microinjection (a persistent white wound), then the needle is likely too broad or blunt. Inclusion of Ca^{2+} in the bathing solution aids wound closure. Alternatively, confirm that expression of the construct is not toxic to the oocyte by comparing viability with the control, injected cohort. Given variability between donor animals, simply repeating the experiment may confer better outcomes.

Problem (Step 7): There is a low proportion of expressing cells.

Solution: Ensure the nucleus is visualized better for microinjection. If the efficiency of "blind" injections is limiting (typically ~30%–80% of oocytes express constructs), methods for better targeting the nucleus are available. These include centrifugation to raise the nucleus toward the surface or the use of albino oocytes to better reveal the location of the nucleus. Alternatively, low expression efficiency may result from a clogged microinjection needle (Step 5). Ensure there are no precipitates in the injected solution by centrifugation before loading the needle. If necessary, clean DNA plasmid preparations with a PCR purification kit (e.g., Qiagen 28106).

Problem (Step 7): Expression of construct is weak.

Solution: Because of the large oocyte volume, constructs that target to the cytoplasm may prove difficult to visualize. Increase the DNA concentration or microinjection volume (<15 nL). The smaller *Xenopus tropicalis* oocytes may confer advantages (Marchant and Parker 2001).

Problem (Step 9): Ca^{2+} signals in injected cells are weak.

Solution: Check the alignment of the photolysis system. $InsP_3$-evoked Ca^{2+} signals should trigger immediately and propagate rapidly after photolysis of caged $InsP_3$ (Callamaras et al. 1998; Marchant et al. 1999). Alternatively, if the spatial distribution of the Ca^{2+} indicator appears punctate, the dye has become sequestered within intracellular organelles (compartmentalized), impeding resolution of Ca^{2+} signals. Perform measurements sooner after microinjection or use dextran-conjugated indicators.

Problem (Step 10): There is poor oocyte maturation.

Solution: Maturation efficiency is variable between frogs. Run a control experiment with different progesterone concentrations to optimize maturation for each donor. Omit HEPES from MBS to improve maturation efficiencies.

DISCUSSION

The use of *Xenopus* oocytes for screening the impact of proteins on Ca^{2+} signals has yielded important insight for understanding physiological Ca^{2+} homeostasis (Lechleiter et al. 1991a,b; Camacho and Lechleiter 1993), as well as information regarding the pathogenic mechanisms of disease-causing proteins (Leissring et al. 1999; Osman et al. 2003; Demuro et al. 2011). Compared with cytoplasmic injection of cRNA constructs, nuclear injection is a more exacting method that does not yield invariant expression. However, it confers the key advantage that constructs are processed via the endogenous transcription, translational, and targeting pathways in the polarized oocyte in contrast to localized and often unnaturally targeted expression of proteins following cytoplasmic RNA injection. Although this may not be an important concern for electrophysiological recordings integrated across the entire cell surface, it is an important consideration for intracellular Ca^{2+} imaging.

For correlating Ca^{2+} signals with ER morphology over longer time periods—e.g., during *Xenopus* oocyte maturation—more specialized imaging equipment is required. Requirements are a capacity for multi-wavelength imaging and use of imaging modalities that maintain oocyte viability over the timeframe of maturation (≤12 h). For this purpose, our laboratory uses a dual, integrated confocal platform (Boulware and Marchant 2008), using a swept-field confocal approach (for time-lapse imaging of ER morphology) and a raster-scanning laser spot system well suited for localizing Ca^{2+} puff sites within restricted regions of the ER over short periods. Use of both approaches in the same cell revealed how *Xenopus* $InsP_3$ receptor sensitivity is regulated with high spatial acuity even between contiguous ER regions (Boulware and Marchant 2005, 2008).

RELATED INFORMATION

For a discussion of the utilization of the *Xenopus* oocyte as a model for investigating Ca^{2+}-permeable channels and transporters, see **The *Xenopus* Oocyte: A Single-Cell Model for Studying Ca^{2+} Signaling** (Lin-Moshier and Marchant 2013).

RECIPES

Modified Barth's Saline (MBS) (1X, pH 7.8)

Reagent	Quantity (for 1 L)	Final concentration (1×)
NaCl	5.143 g	88 mM
KCl	0.075 g	1 mM
$MgSO_4$	0.120 g	1 mM
HEPES	1.192 g	5 mM
$NaHCO_3$	0.210 g	2.5 mM
$CaCl_2$, dihydrate	0.103 g	0.7 mM
H_2O	to 1 L	

Adjust the pH to 7.8 with 10 M NaOH and sterilize by autoclaving. Store at room temperature indefinitely.

Oocyte Dissociation Solution

Reagent	Final concentration
NaCl	88 mM
KCl	2.5 mM
Na_2HPO_4	10 mM
HEPES	5 mM

Supplement with collagenase (~1–2 mg/mL; Type 1A, Sigma-Aldrich) when ready to use. Oocyte viability may be better preserved with lower collagenase concentrations and longer incubation periods at lower temperature.

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