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Analysis of Signaling Pathways in Zebrafish Development by Microinjection

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Abstract

The zebrafish oocyte differs substantially from the zygote and cleavage-stage embryo with regard to the ease with which it can be microinjected with proteins or reagents that modify subsequent development. The objective of this chapter is to describe methods developed in this and other laboratories for microinjection and calcium imaging in the unfertilized zebrafish egg. Methods of immobilizing the oocyte include a holding chamber and a holding pipette. The holding chamber allows imaging of three or four oocytes simultaneously, while the holding pipette facilitates imaging of localized regions in the oocyte. Injection of calcium green dextran via holding chambers allowed detection of global changes in Ca^{2+} release following fertilization and development through early blastula stages. Injection and imaging with the holding pipette method allowed discrimination of calcium changes in the egg cortex from that in the central regions of the cell. The results demonstrate the highly localized nature of calcium signaling in the zebrafish zygote and the implications of this signaling for embryonic development.

Keywords

Zebrafish; egg; oocyte; zygote; fertilization; Fyn; Yes; Src

1. Introduction

The zebrafish has many advantages for the study of fertilization and early embryonic development due largely to the effectiveness of genetic and mutagenesis approaches in this model system. The optical clarity of the zebrafish egg and early embryo facilitates observation of dynamic signaling events by fluorescence microscopy and a considerable knowledge base has been developed on calcium signaling events in this species. The resting free calcium level in the zebrafish oocyte has been estimated at 60 nM (1) and the dynamics of calcium signaling following fertilization in this species have been studied with fluorescent reporters such as aequorin (2), calcium green (3), as well as ratiometric dyes such as Fura-2 (4). The benefits and limitations of these different reagents have been reviewed recently (5).

The calcium signaling events triggered by fertilization of the zebrafish oocyte include an initial activation wave that traverses the egg at a velocity of around 9 $\mu\text{m/s}$ in the egg cortex and 69 $\mu\text{m/s}$ in the central cytoplasm (1) followed by more localized events associated with

the streaming of cytoplasm toward the animal pole during blastodisc formation. At the first cell division, repeated calcium transients are associated with the cleavage furrow and are involved in furrow deepening (6). The cytokinesis-associated transients are repeated with subsequent cell cycles and are easily observed since these cell cycles remain synchronized for the first several cycles (1). While measurement of the total calcium-induced fluorescence in the zygote or embryo provides a certain amount of information, we have now used more detailed morphological analysis to quantitate calcium signaling in different subcellular regions of the zygote. The results demonstrate how localized changes in calcium signaling characterize the egg activation process. These studies have required further refinement of microinjection methods to overcome the physical difficulties inherent in the unfertilized zebrafish egg.

2. Materials

2.1. Buffers

1. *Hank's BSA*: 137-mM NaCl, 5.4-mM KCl, 0.25-mM Na₂HPO₄, 1.37-mM CaCl₂, 1.0-mM MgSO₄, 4.2-mM NaHCO₃, pH 7.2, 5 mg/ml BSA.
2. *Injection buffer*: KCl, 0.15 M; NaCl, 3 mM; KH₂PO₄, 10 mM (pH 7.2); glutathione, 1 mM; sucrose 80 mM; and 10-kDa calcium green dextran (50 µM) (Molecular Probes, Eugene, OR).
3. *Sperm extender buffer*: 10-mM HEPES; 80-mM KCl, 45-mM NaCl, 45-mM NaOAc, 0.4-mM CaCl₂, 0.2-mM MgCl, pH 7.2.

2.2. Microinjection Chambers

1. The holding insert based on that originally described (2) was used to immobilize unfertilized zebrafish oocytes for microinjection.
2. Cut insert (1.5–2.0 cm) from a sheet of opaque plastic approximately 0.75-mm thick.
3. Drill holes as a row along one edge of the plastic using a hand-held jewelers drill and a pin series bit #65 from Huot Corp. St. Paul MN, USA obtained at a local specialty tool retailer.
4. Cut slots to open these holes to the edge of the plastic sheet using a scalpel.
5. Assemble a sliding cover from sections cut from a plastic coverslip (Fig. 6.1).
6. Trim surfaces free of plastic fragments and attach the insert to the bottom of a plastic culture dish using two-part epoxy glue.

2.3. Microinjection Pipettes

1. Pull injection pipettes using borosilicate glass capillaries 1 × 350 mm of thick wall construction (World Precision Instruments, Sarasota, FL, USA).

2. Pipettes are beveled with a rotating disc pipette beveler (World Precision Instruments, Sarasota, FL, USA) at an angle of 30° to produce a tip diameter of approximately 2.5–3.5 µm.

2.4. Holder Pipettes

1-mm o.d. capillaries were flame-polished to reduce the opening to approximately 25% of the egg diameter, then bend over a flame or microforge to allow the tip to lay parallel to the bottom of the dish used for microinjection. The pipette was then filled with mineral oil and Hank's BSA was drawn into the tip before picking up an egg (Fig. 6.2).

2.5. Microinjector

We have used both a Picospritzer II pressure injector (United Valve Corp. Fairfield, NJ, USA) and a CellTram Vario syringe injector (Eppendorf Corp. Hamburg, Germany).

2.6. Confocal Microscopy

The eggs were imaged by confocal fluorescence microscopy on an inverted Nikon TE2000U microscope using a 4x or 20x super fluor objective. Long-working distance objectives were needed to focus through the plastic dish as well as the 700-µm egg. Illumination was provided with a 488-nm Spectra Physics (Mountain-view, CA) laser with pinhole settings set to obtain a theoretical 24-µm optical section through the equator of the embryo. Emitted fluorescence was recorded at 15 s intervals and separate images were collected using a transmission detector to obtain a "brightfield" image and a 515-nm/30-nm bandpass filter to obtain calcium green fluorescence.

3. Methods

3.1. Zebrafish Culture Conditions and Egg Collection

1. Wild-type zebrafish between 3 and 6 months of age were maintained at 28° C under wide-spectrum fluorescent lighting (Coralife Trichromatic, Energy Savers Unlimited, and Carson, CA) with a 13 h-on/11-h off light/dark cycle.
2. Diet consists of Tetramarine flake food (Tetra Sales, Blacksburg, VA) supplemented daily with freshly hatched, live brine shrimp (Aquatic Lifeline Inc., Salt Lake City, UT) (*see* Note 1).
3. For sperm and egg collection, fish were anesthetized with 0.02% tricane (Sigma-Aldrich, St. Louis, MO) and squeezed between gloved fingers to express eggs or sperm.
4. Sperm were suspended in sperm extension buffer (2), which maintains them in an immotile state, and stored on ice for up to 2 h.
5. Eggs were stored in Hank's BSA at 25°C and used within 30 min of collection (*see* Note 2).

¹*Fish Maintenance:* Fish are fed approximately 2 g of flake food plus brine shrimp hatched from 0.5 g of dried eggs each day. This high level of feeding results in improved egg production but also causes rapid acidification of the aquarium system. It is important to adjust the pH daily with Na₂CO₃ and wash the filter media each week.

3.2. In Vitro Fertilization, Microinjection Chamber Method

1. The microinjection chamber holes were filled with Hank's BSA and a droplet (200 μ l) of Hank's BSA was formed across the edge containing the pipette slots.
2. Freshly obtained unfertilized eggs were transferred to the microinjection chamber with a yellow pipette tip cut to accommodate the large-diameter egg. Every effort was made to position the micropyle at 90° to the pipette slot so that it would not be damaged by the injection pipette.
3. Then the clear camber cover was slid over the egg to prevent its escape (Fig. 6.1C).
4. Next the sperm suspension (2 μ l of 2 mg/ml sperm protein containing approximately 1.23×10^6 sperm) was added to the 200- μ l Hank's BSA.
5. The microscope field was then positioned to include three eggs (4x objective) or a single egg (20x objective) and three or four images were recorded to obtain pre-fertilization calcium levels.
6. Fertilization was triggered by adding 2 ml of aquarium water to activate sperm motility and allow fertilization to proceed.

3.3. In Vitro Fertilization, Holder Pipette Method

1. Unfertilized eggs were placed in a droplet (200 μ l) of Hank's BSA in the center of a 35- or 50-mm culture dish in the view field of an inverted microscope. The holder pipette was then used to rotate one oocyte so that the micropyle could be identified. The oocyte was then held by applying suction to the holder pipette with a screw-driven syringe pump (Fig. 6.2).
2. Once immobilized, the oocyte was injected with calcium green dextran or other reagents and the injection pipette withdrawn.
3. After a recovery period of approximately 5 min, sperm were added to the droplet as above and image acquisition was begun.
4. Fertilization was induced by adding aquarium water (3 ml) to the dish which reduced the ionic strength and allowed sperm to become motile (*see* Note 3).

3.4. Image Acquisition and Analysis

1. An example of calcium release during activation of the zebrafish egg in a holding chamber is shown in Fig. 6.3. Within 60 s of fertilization, calcium release is evident in the cortical cytoplasm, but little change has occurred in the central cytoplasm.
2. Calcium green fluorescence (*see* Note 4) intensity can be measured simultaneously in several zygotes (*see* Note 5).

²*Handling gametes:* Unfertilized eggs are susceptible to aging and to rough handling and can undergo parthenogenic activation if they are exposed to too much agitation. Aging causes the chorion to harden and become even more resistant to injection pipettes. Sperm can be activated by exposure to aquarium water, so we rinse the anesthetized male fish with a few drops of sperm extender buffer before collecting sperm.

³Full activation of sperm requires that the Hank's BSA be diluted at least 10–15-fold by aquarium water. Therefore, a 200- μ l droplet of Hank's BSA would be diluted with 3 ml of aquarium water to initiate fertilization.

3. Once data collections were complete, calcium green fluorescence of an optical section through the entire oocyte was quantitated by Metamorph software and is presented graphically in Fig. 6.4 (upper panel). These measurements made from confocal image sections revealed that fertilization is followed by an initial low-amplitude calcium transient beginning as early as 15 s after sperm addition (black arrows) followed by two high-amplitude transients, one reaching a maximum at 2–3 mpf (grey arrows) and the second reaching a maximum at 8 mpf. These three initial transients are followed by later calcium signaling events associated with blastodisc expansion and cytokinesis as reported elsewhere (6,7).
4. To more accurately study the localized calcium changes in the cortex and central cytoplasm, these regions were quantitated separately by measuring pixel intensity in different regions of the image. The cortical region was drawn as an arc to include approximately 20% of the egg cortex centered over the initial site of calcium release. The central cytoplasm region was drawn as a circle encompassing approximately 25% of the cross-sectional area of the oocyte. Both regions had to be relocated during the time series as the zygote moved during chorion elevation, and during cortical contractions; however, this analysis allowed detection of small, localized changes that were poorly represented in measurements from the entire oocyte.
5. Quantitation of the cortical and central cytoplasmic fluorescence demonstrated the difference between calcium signaling in these two compartments of the egg. For example, the initial low-amplitude spike at 15–30 s post-insemination was detected only in the cortical region (Fig. 6.4, bottom panel, black arrow) which is reminiscent of the cortical “hot spots” observed in *Xenopus* oocyte (8,9). The first high-amplitude transient (grey arrow), which reached a maximum between 2 min and 3 min post-insemination, was also more prominent in the cortical region than in the central cytoplasm. Later cortical transients did not occur in a consistent pattern, but did indicate that the cortical cytoplasm remained highly active. In contrast, the central cytoplasm exhibited only moderate changes until 5–6 min post-insemination when the second high-amplitude transient began, primarily involving the central cytoplasm rather than the cortical region (Fig. 6.4, middle panel).
6. Analysis of more localized changes in calcium signaling required that the egg be totally immobilized in a holding pipette to prevent movements from taking a given region out of the focal plane. This method allows exact positioning of the oocyte so that localized features such as the micropyle can be observed. At fertilization in fish, sperm penetrate the chorion through an opening (the micropyle) so the investigator can visualize the actual point of sperm–egg contact. As seen in Fig. 6.5 the cortical region under the micropyle responds to fertilization with increased cytoplasmic calcium within 30 s of sperm activation (arrows).

⁴The calcium reporter calcium green dextran reports only the relative calcium changes and does not allow calculation of actual free calcium levels. It does have the major advantage that it can be excited with a 488-nm laser which causes much less damage to the cell than a shorter-wavelength ultraviolet source such as used for the fura series of ratiometric dyes.

⁵The spacing of the holes in the egg holding chamber can be spaced so that three or four oocytes can be imaged simultaneously with a 4x objective.

7. In summary, the unfertilized zebrafish oocyte can be effectively microinjected if methods are employed to immobilize the egg and allow the injection pipette to penetrate the very tough chorion. In addition, the method chosen to immobilize the egg can allow precise orientation so that specific subcellular regions can be observed. These techniques have shown the extent to which calcium signaling is compartmentalized in the zygote.

Acknowledgments

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Fig. 6.1.

Design of an oocyte holding chamber. The row of holes approximately 800 μm in diameter were drilled along one edge of the plastic and openings were cut with a scalpel to admit the injection pipette (**A**). The cover was cut from a plastic coverslip as were guides to hold the cover on the insert but still allow it to move (**B**). Panel (**C**) shows the cover slid over the oocyte chambers to prevent their escape upon addition of aquarium water to initiate fertilization.

Fig. 6.2. Holding pipette used to immobilize a zebrafish oocyte. A typical holding pipette produced by flame-polishing of a glass capillary is used to pick up and immobilize an unfertilized oocyte. Magnification is indicated by the bar which represents 100 μm .

Fig. 6.3.

Fertilization-induced changes in calcium green fluorescence. Zebrafish oocytes were injected with calcium green dextran and an inactive protein (glutathione-S-transferase) then imaged by confocal fluorescence microscopy before fertilization (*left*), or at 60 s post-insemination (*right*). The increase in calcium green is most apparent in the egg cortex at 60 s post-insemination. Magnification is indicated by the bar which represents 100 μm .

Fig. 6.4.

Quantitation of fertilization-induced changes in calcium green fluorescence. Oocytes were injected with calcium green dextran and GST, then images were recorded after a 10-min recovery period. Fertilization was initiated by addition of a mixture of sperm and water at time 0 and images were recorded every 15 s. Fluorescence was quantitated by pixel intensity quantitation using Metamorph 6.1 software. The top panel represents global fluorescence measured from a cross-section of the entire zygote. The middle panel represent fluorescence measured within a circular region in the center of the zygote (central cytoplasm) comprising approximately 25% of the total cross-sectional area. The bottom panel represents fluorescence measured within an arc traced over the cortex (cortical cytoplasm) as near as possible to the micropyle. This arc comprised approximately 20% of the perimeter of the zygote.

Fig. 6.5.

Calcium green fluorescence in the region of the micropyle. An unfertilized oocyte was picked up with a holding pipette and rotated until the micropyle was visible. The egg was then injected with calcium green dextran and fertilized as above. The images shown here were obtained at 50 s post-insemination and demonstrate the localized calcium green fluorescence in the region of the micropyle (*arrows*). Magnification is indicated by the bar which represents 100 μm .