Monitoring Calcium Oscillations in Fertilized Mouse Eggs

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Summary

In mammalian species, including human, fertilization is characterized by the triggering of long-lasting calcium (Ca^{2+}) oscillations in the egg cytoplasm. The monitoring of these Ca^{2+} oscillations is a valuable technique to demonstrate that fertilization has occurred, to study egg activation events elicited downstream of the Ca^{2+} signal, as well as to evaluate sperm quality. This chapter describes our protocol to monitor sperm-induced Ca^{2+} oscillations in mouse eggs, using fluorescence microscopy techniques and the Fura-2-AM ratiometric Ca^{2+} indicator.

Key Words

oocyte; egg; sperm; fertilization; calcium; Ca^{2+}; fluorescence; imaging

1. Introduction

In the great majority of mammalian species, the ovulated egg is blocked at metaphase of the second meiotic division (Metaphase II, or MII). Only fertilization will allow for meiosis to resume, leading to the extrusion of one set of the segregated maternal chromatids, into the second polar body. This is immediately followed by meiotic exit, formation of male and female pronuclei, and progression to the first embryonic mitotic division (cleavage). In conjunction with these cell cycle events, fertilization also elicits the release of the egg's cortical granules in order to prevent polyspermy, and the recruitment of maternal mRNAs for translation of new proteins important for the early stages of embryo development. These events are collectively referred to as "egg activation" ***(1-4)***.

In all mammalian species studied to date, the earliest activation event that is detected in the fertilized egg is the triggering of cytoplasmic Ca^{2+} oscillations (Figure 1A) that last for several hours ***(1,4)***. Detailed description of the spatio-temporal dynamics of these Ca^{2+} oscillations, and the mechanism of their generation, have been provided using the mouse egg as a model ***(5-7)***. The importance of these Ca^{2+} oscillations is demonstrated by the fact that their inhibition, using Ca^{2+} chelators, results in a complete failure of meiotic resumption and egg activation ***(8)***. Interestingly, the initiation and completion of the various egg activation events are differentially sensitive to the number, amplitude,

and duration of the Ca^{2+} spikes, suggesting that the timing and temporal sequence of these events are encoded in the pattern of the fertilization-induced Ca^{2+} oscillations ***(9-12)***. Intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) also increases in fertilized eggs from non-mammalian species, such as amphibians, fish and marine invertebrates, where the sperm-induced Ca^{2+} signal takes the form of a single transient, or a series of oscillations ***(13)***.

The monitoring of Ca^{2+} oscillations in fertilized eggs can serve both research and clinical purposes. On the egg side, Ca^{2+} oscillations are recorded to investigate the dynamics and molecular aspects of egg activation events, such as the regulation of cell cycle resumption by Ca^{2+}-calmodulin-dependent protein kinase II, a downstream effector of the $[Ca^{2+}]i$ rise ***(14)***. There has been also considerable efforts to uncover the molecular identity of the sperm factor responsible for initiating these Ca^{2+} oscillations, with compelling evidence pointing to the sperm-specific phospholipase C zeta ***(4,7,15)***. Accordingly, the recording of $[Ca^{2+}]$ fluctuations in the egg cytoplasm represents a valuable assay to evaluate sperm "quality", i.e. its ability to induce long lasting Ca^{2+} oscillations that are required for full egg activation. In line with this, a number of studies have reported the use of mouse eggs as "reporter" of the fertilizing capacity of human sperm, via the monitoring of cytoplasmic Ca^{2+} oscillations elicited after ICSI, or injection of human sperm extract or of human recombinant PLCzeta ***(16-19)***.

A number of fluorescent Ca^{2+} indicators have been used to investigate fluctuations in $[Ca^{2+}]i$ in mammalian eggs ***(20)***. In this chapter, I describe our protocol for the monitoring of Ca^{2+} oscillations in fertilized mouse eggs, using the popular ratiometric fluorescent Ca^{2+} indicator Fura-2. Fura-2 is a high affinity Ca^{2+} indicator (Kd = 145 nM, in vitro), which shows a shift in its excitation spectrum when bound to Ca^{2+}, while the fluorescence emission peak (510 nm) remains unchanged. It is therefore classified as a dual-excitation indicator. Free Fura-2 shows an excitation peak around 380 nm, while Ca^{2+}-bound Fura-2 shows an excitation peak around 340 nm. Fluctuations in $[Ca^{2+}]i$ are therefore translated into reciprocal fluctuations in Fura-2 emitted fluorescence (F) when excited at 340 or 380 nm [Figure 1 near here] : upon $[Ca^{2+}]i$ increase, the emitted fluorescence at 340-nm excitation (F340) will increase; conversely, the emitted fluorescence at 380-nm excitation (F380) will decrease. The

corresponding fluctuations in $[Ca^{2+}]i$ are displayed as the F340/F380 ratio (Figure 1B). The advantage of a ratiometric measurement is that the ratio signal is less affected by fluorescence bleaching, or by variations in Fura-2 loading among the egg population. Note that the F340/F380 signal is not a quantitative measurement of the actual Ca^{2+} concentration, which would require calibration of the signal ***(20)***. It provides however useful informations regarding the rate, duration, frequency and relative amplitude of the Ca^{2+} oscillations.

2. Materials

All solutions and culture media are prepared with ultrapure water with a resistivity of 18.2MΩ-cm.

2.1. PMSG and hCG hormones for superovulation

1. PMSG : Lyophilised pellet of Pregnant Mare Serum Gonadotrophin/PMSG (Intervet) is resuspended in filtered (0.20 µm) ice-cold PBS to a final concentration of 25 IU/ml. The solution is then distributed into 1-ml syringes, which are next fitted with 27-gauge needles and immediately frozen at -20°C.

2. hCG : Lyophilised pellet of human Chorionic Gonadotrophin/hCG (Intervet) is resuspended in filtered (0.20 µm) ice-cold PBS to a final concentration of 25 IU/ml, and aliquoted as ready-made syringes as described for PMSG.

2.2. T6 medium for sperm capacitation : 100 mM NaCl, 1.42 mM KCl, 0.47 mM MgCl2, 0.36 mM Na2HPO4, 1.78 mM CaCl2, 25 mM NaHCO3, 25 mM Na lactate, 0.47 mM Na pyruvate, 5.56 mM glucose, 10 mg/L phenol red. Store in fridge for 2 weeks. The day before the experiment, add 16 mg/ml BSA, filter (0.20 µm) and incubate overnight in a 5% CO2 incubator.

2.3. Egg handling media

1. M2 medium : for egg collection. We use commercial M2 medium (SIGMA)

2. M2-hyaluronidase : A 10x stock solution is prepared by dissolving hyaluronidase (from bovine testes, SIGMA) into M2 medium to a final concentration of 3 mg/ml, and immediately frozen into 250-µl aliquots.

3. H-KSOM medium for $[Ca^{2+}]i$ recordings : 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH2PO4, 0.2 mM Na pyruvate, 1 mM L-glutamine, 0.01 mM EDTA, 0.2 mM MgSO4, 10 mM Na lactate, 4 mM NaHCO3, 1.71 mM CaCl2, 0.2 mM glucose, 20 mM HEPES, 10 mg/L phenol red (*see* **Note 1**), pH 7.4. This medium does not contain BSA, so as to promote egg adhesion to the glass coverslip.

2.4. Acidic Tyrode for zona removal

We use commercial acidic Tyrode's solution (pH 2.5, SIGMA) that is stored at -20°C as 500-µl aliquots.

2.5. Keeping the eggs warm

Eggs must be kept warm for the whole duration of the isolation and dye-loading steps. To do so, culture media and dishes are stored on a hot block set to 37°C (*see* **Note 2**).

2.6. Egg manipulation using a mouth pipette

The manipulation and transfer of eggs from one dish to another, is facilitated using a "mouth pipette", consisting of a rubber aspiration tube connected to a glass Pasteur pipette. The Pasteur pipette needs to be narrowed at the tip to an inside diameter slightly bigger than the eggs diameter. This is achieved by heating the glass using a Bunsen burner followed by sharp pulling. Cut the pulled glass pipette to the desired length and allow for the pipette to cool down to room temperature before manipulating the eggs.

2.7. Fura-2

Prepare a stock solution by resuspending Fura-2-AM in DMSO to a final concentration of 1 mM. Aliquote and store at -20°C. Avoid exposure of the aliquots to light.

2.8. Fluorescence microscopy

In brief, ratiometric Ca^{2+} imaging using Fura-2 is performed with an inverted epifluorescence microscope equipped with a 20x objective, a UV light source and a filter wheel with the relevant filters for Fura-2 dual excitation (peak transmission at 340-nm and 380-nm), a 510-nm dichroic mirror, and a 520-nm long-pass emission filter. Fluorescence images (F340 and F380) are captured using a cooled CCD camera, and stored on a computer running a fluorescence imaging software that controls the camera shutter and the excitation filter wheel. To prevent photo-toxicity, a neutral density filter is placed in the path of the excitation light. Importantly, the microscope must also be fitted with a stage incubator, in order to provide a warm (37°C) environment to the cells during imaging (*see* **Note 3**).

2.9. Fluorescence analysis

Fluorescence measurements (F340 and F380) are realized by drawing a circular region of interest on the fluorescence images, such as to collect fluorescence intensity from the bulk of the egg cytoplasm. The numerical values reporting the fluctuations in F340 and F380 intensities are exported towards Microsoft Excel to generate line graphs and for calculation of the F340/F380 ratio.

3. Methods

3.1. Superovulation of mice

We use mice from either the CD-1 or MF-1 strains. In order to obtain large amounts of ovulated eggs (*see* **Note 4**), inject female mice intraperitoneally with 5-7.5 units pregnant mare's serum gonadotrophin (PMSG), followed 44-48 hours later with 5-7.5 units human chorionic gonadotrophin (hCG) to induce ovulation. Ovulation occurs around 12h following hCG injection.

3.2. Preparation of mouse sperm

1. On the day preceding the experiment, prepare two 35-mm dishes each containing 0.5 ml of T6 medium with BSA, under a layer of mineral oil to prevent evaporation. Leave overnight in a 5% CO2 incubator at 37°C for equilibration.

2. On the day of the experiment, sacrify a male mouse of proven fertility, dissect out the epididymides and place them in the drop of T6-BSA under oil. With the help of the dissection

binocular and a sterile needle, puncture the epididymides in order to release sperm into the medium. Discard the epididymal tissue fragments and return the dish to the incubator. Allow 20 minutes for the sperm to disperse.

3. Add 100 µl of this sperm suspension into the second dish containing 0.5 ml T6-BSA. Make sure the drop remains covered with oil. Return the dish to the incubator and incubate for 3 hours to achieve sperm capacitation.

3.3. Egg collection

1. Ovulated MII eggs are collected 13-15h after hCG injection. Sacrifice the mice by cervical dislocation and collect the two oviducts in a small tube containing 1 ml warm M2 medium.

2. Thaw one aliquot (250 µl) of M2-hyaluronidase and dissolve into a final volume of 2.5 ml M2, to reach a working concentration of 0.3 mg/ml.

2. Using a dissection binocular, slice open the oviducts using a sterile needle, in order to release the cumulus masses (i.e., ovulated oocytes surrounded by a cloud of granulosa cells, *see* **Note 5**) into a 35-mm dish containing pre-warmed M2-hyaluronidase.

3. Following a 3-5 minutes incubation at 37°C on a hot-block, the cumulus cells are dispersed under the action of hyaluronidase and cumulus-free oocytes can be collected by mouth-pipetting (*see* **Note 6**). This is followed by two extensive washes in fresh M2 in order to rinse out hyaluronidase.

3.4. Preparation of the eggs for Ca^{2+} imaging

In the following procedures, it is advisable to protect the eggs from light, as Fura-2 is light-sensitive. During egg incubation on the hot block, an opaque plastic box is placed on top of the dish to reduce light exposure to a minimum.

1. Thaw a Fura-2-AM aliquot and incubate the MII eggs in M2 medium containing 2 µM Fura-2-AM (*see* **Note 7**), for 10 minutes at 37°C on a hot block. Next, transfer the eggs in a new dish

filled with fresh M2 in order to wash off the residual Fura-2-AM. Leave the eggs on the hot block for an additional 15 minutes, to allow for cytoplasmic esterases to cleave off the AM groups.

2. Remove the zona pellucida, in order to accelerate the fusion of the fertilizing sperm with the egg. Place a drop (500 µl) of acidic Tyrode medium in the center of a culture dish, on the hot block at 37°C for warming. Using a pipette filled with acidic Tyrode, transfer the eggs into the drop of acidic Tyrode, then return the dish on the hot block. The acidic medium will induce a progressive dissolving of the zona pellucida (*see* **Note 8**). Once the zona has fully dissolved, return the eggs to M2 medium on the hot block.

3. Prepare the imaging chamber. We use 35-mm glass-bottom dishes (MatTek) with a 20-mm glass diameter. A 500-µl drop of H-KSOM without BSA is deposited on the glass coverslip and covered with mineral oil to prevent evaporation, avoiding flattening of the drop. Place the imaging dish on the pre-warmed stage of the inverted microscope inside the incubation system (*see* **Note 3**).

4. Using a mouth pipette filled with H-KSOM, transfer the zona-free eggs to the center of the imaging dish. This is accomplished by careful mouth pipetting, while looking down the microscope eyepieces to adjust the positioning of the pipette close to the surface of the glass coverslip. Slowly release the eggs so that they remain grouped in the field of view.

3.5. Setting up fluorescence recordings

Start aquiring F340 and F380 fluorescence in order to adjust the exposure time for each wavelength (in the order of 100-300 ms) to obtain a good fluorescence signal, and to set the wavelength-switching time and the frequency of acquisition (such as one image every 5 sec, see Figure 1). Using the ROI (Region Of Interest) function of the imaging software, draw a large circle in the cytoplasmic area of each egg to collect F340 and F380 fluorescence values to be exported in Excel. Background fluorescence should be subtracted from each excitation wavelength before creating the ratio image.

3.6. Fertilization

Add 5-10 µl of the capacitated sperm suspension to the imaging dish containing the eggs loaded with Fura-2 (*see* **Note 9**). Add the sperm slowly and away from the egg area, to avoid creating a drift that could lead to the eggs detaching from the coverslip. Look down the eyepieces to check for sperm binding to the eggs surface, before starting the actual experimental recording.

4. Notes

1. The use of phenol red as an indicator of pH changes in the culture medium is not an absolute requirement. Actually, it can be omitted in the medium used for performing the actual Ca^{2+} imaging experiment (H-KSOM), as the dye generates background fluorescence at the wavelengths used to excite Fura-2.

2. It is recommended to adjust the hot block temperature slightly above 37°C, such that a temperature of 37°C is effectively delivered in the culture dish where the eggs are seated, and which is exposed to ambiant air. This calibration is accomplished by using a wire temperature probe that is positioned against the bottom of a test dish filled with M2.

3. To ensure eggs and sperm will be kept warm for the duration of the Ca^{2+} recording, we use a multiple temperature control scheme : in addition to the large size incubator covering the entire microscope, an additional mini-incubator (Tokai Hit) is fitted on the stage of the microscope, while the 20x objective is fitted with a lens heater (Tokai Hit). Temperature controls are set to generate 37°C inside the drop of medium in the imaging dish, as verified with a wire temperature probe.

4. Superovulation is not an absolute requirement to obtain mouse eggs, i.e. injection of hCG alone can be envisaged. However, superovulation (PMSG + hCG) will substantially increase the egg yield, allowing performing the experiment using a minimal number of animals (one or two mice). The efficiency of superovulation may vary with the mouse strain, the age of the females, and the dose of hormone administered. Using the common CD-1 strain, young females (4-5 week old) will be efficiently stimulated with 5 units of PMSG and hCG. Older females may require up to 7.5 units of

each hormone. In addition to the increased overall body weight, the increased amount of fat in the peritoneal cavity of older females may act as a sink for the injected hormones. Using the CD-1 strain, we routinely obtain 30-40 healthy MII eggs from one mouse.

5. The cumulus mass is easy to spot as it induces a distinct dilation of the oviduct. The size of the cumulus mass is generally proportional to the number of MII eggs that have been ovulated, and which are buried into the mass.

6. Healthy MII eggs should fill most of the space within the zona pellucida, except in the region where the first polar body was emitted. In contrast, eggs that appear shrunk and that make no contact with the ZP must be discarded. Occasionally, some eggs will be found already separated from the rest of the mass, before hyaluronidase had started to disperse the cumulus cells. These eggs generally display a shrunk morphology and must be discarded.

7. The “AM” form of Fura-2 means it is modified by addition of acetoxymethyl groups via an ester bond, which renders the dye membrane-permeant (passive diffusion), thus avoiding invasive loading techniques. Once the indicator has penetrated the egg, cytosolic esterases cleave the ester bond to release anionic Fura-2, which remains trapped inside the cell. However, a fraction of Fura-2-AM may also penetrate and remain sequestered into cytoplasmic organelles (compartmentalization), and generate a contaminating fluorescent signal that is unaffected by changes in $[Ca^{2+}]i$. To avoid this, Fura-2 salt (such as pentapotassium salt) can be used instead of Fura-2-AM, however it has to be micro-injected into the egg cytoplasm.

8. It is important to manipulate the eggs with a pipette filled with warm acidic Tyrode and not M2 medium, as the latter would contaminate the drop of acidic Tyrode and slow down the zona dissolving process. In addition, occasional gentle pipetting of the eggs using a pipette filled with acidic Tyrode, will accelerate zona removal, by promoting the rupture of the thinned zona pellucida. Note that the eggs are more fragile and prone to damage and lysis when denuded of their protective zona pellucida, and must therefore be manipulated with extra care.

9. Zona-free eggs have lost their barrier against polyspermy, which is provided primarily by the zona pellucida. It is therefore frequent to observe several sperm fusing with the same egg. This can be an issue when the aim of the experiment is to investigate Ca^{2+} oscillations parameters, because polyspermy notoriously increases Ca^{2+} oscillation frequency ***(21)***. For this reason, it is recommended to start by adding little amount of the sperm suspension at first, to increase the probability to obtain monospermic fertilization. This also means that the time required to obtain sperm-egg binding may be longer, only a fraction of the eggs layered on the imaging dish may be fertilized, and fertilization events in the egg pool may happen asynchronously. If no fertilization event happens during a prolonged period, add a second sample of the sperm suspension.

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5. References

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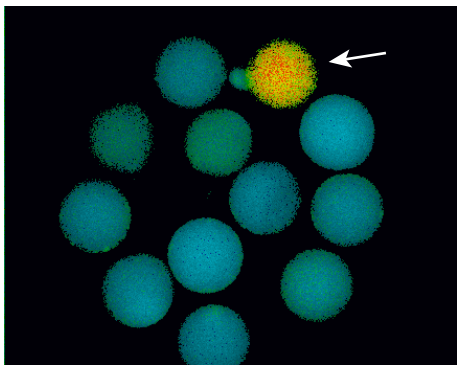
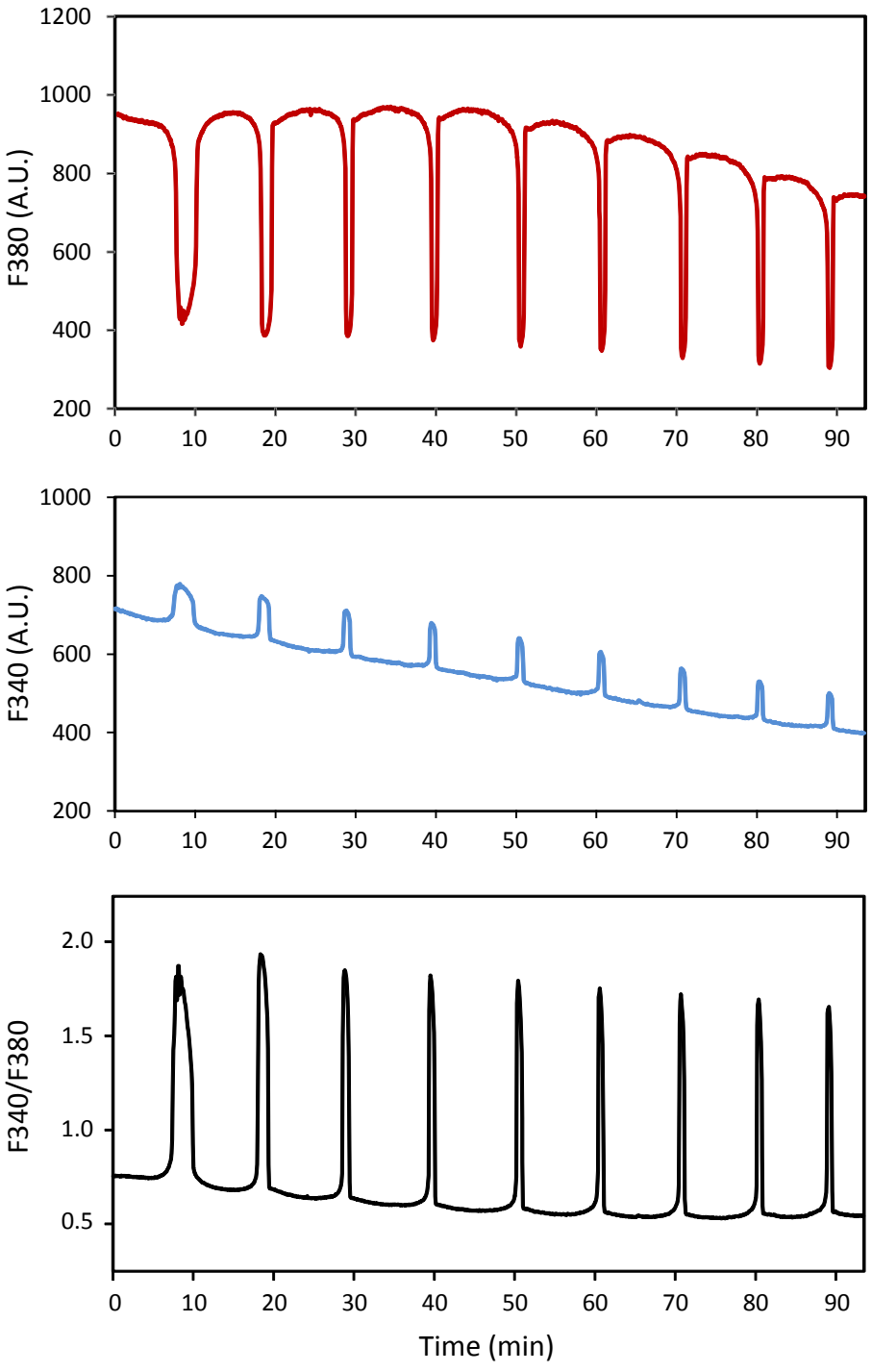
Figure caption

Figure 1. Monitoring of Ca^{2+} oscillations in mouse eggs using Fura-2. (A) Pseudocolor ratio image of a field of mouse eggs loaded with Fura-2-AM and undergoing fertilization. The arrow points to an egg in which cytosolic [Ca^{2+}] is rising (Ca^{2+} oscillation). (B) Line graphs showing the fluctuations in F340 and F380 (one acquisition every 5 sec), and the corresponding ratio trace, during fertilization-induced Ca^{2+} oscillations in a mouse egg.

A


high [Ca2+]i
low [Ca2+]i

B


F380 (A.U.)
1200
1000
800
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400
200
F340 (A.U.)
1000
800
600
400
200
F340/F380
2.0
1.5
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0.5
0 10 20 30 40 50 60 70 80 90
Time (min)