Use of Luciferase Chimaera to Monitor PLCζ Expression in Mouse Eggs

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Abstract

The microinjection of cRNA encoding phospholipase Cζ (PLC zeta) causes Ca^{2+} oscillations and the activation of development in mouse eggs. The PLCζ protein that is expressed in eggs after injection of cRNA is effective in causing Ca^{2+} oscillations at very low concentrations. In order to measure the amount and timecourse of protein expression we have tagged PLCζ with firefly luciferase. The expression of the luciferase protein tag in eggs is then measured by incubation in luciferin combined with luminescence imaging, or by the lysis of eggs in the presence of Mg-ATP and luciferin in a luminometer. The use of luciferase to monitor protein expression after injection of cRNA is a sensitive and effective method that efficiently allows for sets of eggs to be used for PLCζ quantitation, Ca^{2+} imaging, and studies of embryo development.

Key words: Luminescence, luciferase, phospholipase, egg.

1. Introduction

Mammalian eggs are large cells (~100 μm in diameter) and readily amenable to microinjection. We have used pressure-based microinjection as a means of introducing molecules into mouse eggs for many years. Our particular interest over the last few years has been focused on the role of a sperm-specific phospholipase Cζ (PLC zeta) in causing the Ca^{2+} changes that lead to egg activation in mammals. This protein can trigger repetitive Ca^{2+} oscillations that are very similar to those seen at fertilization in mouse, pig, and human eggs (1). We have proposed that PLCζ is the “sperm factor” that is delivered by the sperm into the egg following
gamete fusion (2). It has been shown that recombinant PLCζ can cause Ca\(^{2+}\) release when it is injected into mouse eggs (3). We, and others, have also carried out biochemical studies of recombinant PLCζ (3,4). However, one of the major problems of working with recombinant PLCζ protein is that its Ca\(^{2+}\) oscillation-inducing activity is very labile, consistent with our observation that the PIP\(_2\) hydrolysis enzymatic activity is difficult to maintain. Consequently, we have used cRNA injection as a general means of introducing PLCζ into mouse eggs. The injection of cRNA for PLCζ also has the advantage that no protein contaminants are introduced into cells, in contrast to native protein isolation procedures. One of the disadvantages of injecting cRNA PLCζ is that the level of expressed protein cannot be readily determined in living cells. An effective way to measure how much protein is being expressed is to inject cRNA for PLCζ that has been tagged with firefly luciferase (4).

Using luciferase luminescence to measure protein expression is a highly sensitive technique. Luminescence has an advantage over the use of fluorescence-based methods in that it does not suffer from interference from auto-fluorescence which is quite considerable in mammalian eggs (5). The issue of sensitivity is particularly important since mouse PLCζ is active in mouse eggs at concentrations of 1–10 nM (3), and this very low level of protein is on the limit of detection for the most sensitive fluorescent proteins used in eggs (6). Furthermore, since human and monkey PLCζ appear to be more potent in causing Ca\(^{2+}\) oscillations in eggs than the mouse PLCζ (7), the physiological levels of PLCζ expression in many species may be undetectable using fluorescently tagged PLCζ. The chief disadvantage in using luciferase luminescence is that the localization of the expressed protein is poor compared to fluorescence probes, where high-resolution confocal imaging can be used. However, with photon imaging cameras it is certainly possible to identify which individual eggs, or cells, are expressing luciferase and it is possible to quantitatively estimate how much luciferase-tagged protein is being expressed.

In this chapter, we describe how we study the effects of PLCζ, and other PLC isoforms, or various mutant versions of PLCs, by injection of cRNA encoding luciferase-tagged versions of the proteins. In many experiments, we inject the cRNA and then measure Ca\(^{2+}\) signals from eggs for several hours after injection, before calibrating luciferase expression at a set time-point. In other cases, we monitor Ca\(^{2+}\) oscillations and luciferase expression from eggs and then place them in culture for further studies on their development. The methods we describe for PLCζ can be readily applied to other proteins and, for example, we have also injected and studied cyclin B levels in mouse eggs using a luciferase-tagged cRNA (unpublished data).
2. Materials

2.1. DNA and RNA Preparation

1. To produce sufficient quantities of DNA plasmid, we use Qia-gen’s Plasmid Maxi Kit. Restriction enzymes are routinely purchased from New England Biolabs. To produce polyadenylated cRNA, mMessage mMachine T7/T3/SP6 kits and Poly (A) Tailing Kits are used, along with SUPERase-In RNase Inhibitor (Ambion). Rabbit Reticulocyte Lysate (Promega) is used to assess RNA quality.

2. RNase-free solutions and plasticware are prepared using treatment with diethyl pyrocarbonate (DEPC, Sigma).

3. Media for mouse eggs consists of M2 and acidified Tyrode’s solution (Sigma). We also use KSOM and a Hepes version of KSOM (HKSOM) which is made from stock using embryos-tested chemicals (Sigma) and clinical-grade water. The constituents of KSOM and HKSOM are given in reference (8) and (9). Hyaluronidase M2 and acid Tyrodes solution are stored in aliquots at −20°C. The M2 or HKSOM media are stored at 4°C and used for 2–3 weeks. For all imaging studies, firefly luciferin is added to HKSOM media. The luciferin (L6882 from Sigma) is made up at 100 mM in distilled water and stored in the −20°C freezer for ~1 month. It is diluted into HKSOM shortly before use to give a final concentration of 100 μM (see Note 1).

4. The injection buffer consists of KCl/Hepes (120-mM KCl, 20-mM HEPES, pH 7.2). The buffer is made up in plastic vessels and then mixed with ~1% Chelex 100 beads (Sigma) for ~1 h (to remove divalent cations) before being filter-sterilized. For experiments where intracellular Ca²⁺ is to be measured Oregon Green BAPTA dextran (OGBD) (Molecular Probes, www.probes.com) is added to the injection buffer. Aliquots of injection buffers are stored in the −20°C freezer.

2.2. Mouse Eggs

1. We regularly use the MF1 strain of mice, but have obtained similar results with other strains of mice such as CD-1, or with F1 hybrid cross strains. The hormones were purchased from Dunlops Veterinary Supplies (www.dunlops.com). The superovulation of mice and collection of eggs is described in laboratory manuals (10).

2. Eggs were manipulated in M2 media (Sigma) using fine-bore glass pipettes that were pulled in a flame to a diameter of approximately 80–100 μm.

2.3. Microinjection

1. For microinjection, borosilicate glass capillaries (GC150F, Harvard Apparatus Ltd., 1.5-mm outer diameter and 0.86-mm inner diameter) with an internal filament were pulled on a
vertical pipette puller (Model P-30; Sutter Instruments). The pipettes used for injection should be checked for appropriate tip size. This can be done by finding the minimal pressure required to blow bubbles in ethanol (11). Injection needles are backfilled with sterile microloader pipette tips (Eppendorf).

2. Injection needles are clamped in a holder with a silver wire and side port (World Precision Instruments Inc, www.wpi-europe.com). The holder is plugged into a preamplifier that is electrically connected to an intracellular amplifier (e.g., Electro 705 or Cyto 721; WPI). The preamplifier is held in the micromanipulator.

3. Pressure is applied to the back of the needle by pulses from a pressure injection system (Picopump, WPI) connected to the side port of the needle holder with stiff silicone tubing.

4. Mouse eggs are injected while being held by a “holding” pipette (Hunter Scientific) using suction via a syringe system (Narashige) containing embryo-tested mineral oil (Sigma).

5. The preamplifier and needle holder, and the ‘holding pipette’, are mounted on hydraulic manipulators (Narashige) that are fixed to the inverted microscope (TE2000, Nikon UK Ltd).

2.4. Imaging and Quantifying Luciferase

1. For imaging, the eggs are maintained in drops of media in a heated chamber (Intracel Ltd.) on the stage of an inverted fluorescence microscope (either a Nikon TE2000, or Zeiss Axiovert S100). Each microscope has the facility to direct 100% of the light from the eggs to the camera via either a side port or the base port.

2. The light collected from injected eggs is imaged using photon-counting imaging cameras. The cameras we currently use are cooled intensified CCD cameras (Photek Ltd.; www.photek.com). Photek’s software is used for data collection and analysis (see Note 1).

3. The imaging systems are contained in a lightproof dark box. In one case, we have the facility to direct bright field or fluorescent illumination to the eggs via fiber optical cables. The gating of these light sources is controlled via Photek’s software that controls electro-mechanical shutters (Uniblitz; www.uniblitz.com) that are integrated into the light box. In another case, the microscope light sources are inside the box and simply switched off during luminescence imaging (see Note 2).

4. After imaging, the eggs can be lysed in order to quantify luciferase expression. This is done in a lysis buffer using a custom-made luminometer (see reference (12)). This essentially consists of a dark-proof tube holder that is adjacent to a cooled photomultiplier tube (S20 type tube, with a
cooled housing and amplifiers from Electron Tubes Ltd.; www.electrontubes.com). The light output is measured using photon-counting discriminators and amplifiers with software supplied by Electron Tubes Ltd. Commercially available tube luminometers would also be suitable.

5. The lysis buffer consists of phosphate-buffered saline with 1-mM MgATP and 100-μM luciferin. Eggs are lysed with Triton X-100 and the amount of luciferase calibrated with recombinant luciferase protein. All these reagents are purchased from Sigma.

3. Methods

3.1. Synthesis of cRNA

3.1.1. Preparation of DNA

1. RNAse-free solutions and plasticware are prepared by incubation in a solution of 0.1% DEPC overnight in a fume hood. Following this incubation, residual DEPC is removed by autoclaving. We routinely generate microgram quantities of the required DNA plasmid using Qiagen’s Plasmid Maxi Kit, following the manufacturer’s instructions (a standard molecular biology/microbiology textbook (13) provides further information on Escherichia coli strains, transformation, and handling). Depending on the plasmid copy number, 500 μl of DNA with a concentration of 0.5–2 mg/ml can be harvested from 250 ml of culture.

2. Due to the high processivity of RNA polymerases, it is necessary to linearize the circular plasmid DNA to prevent the production of extremely long RNA molecules. This can be achieved by digestion with a suitable restriction enzyme, obviously avoiding those which cut between the promoter and gene of interest. A 100-μl restriction digest containing 10 μg of DNA and 20 U of enzyme is incubated at a suitable temperature overnight. Complete linearization can be confirmed by running 2 μl on an agarose gel if necessary.

3. The linearized DNA is cleaned up by phenol/chloroform extraction. Essentially, an equal volume of TRIS-buffered phenol:chloroform:isoamylalcohol (25:24:1 v/v/v) is added and mixed by vigorous inversion for 30–60 s. The phases are separated by microcentrifugation at 14000 g for 3 min and the top, aqueous phase is transferred to a DEPC-treated microfuge tube using a DEPC-treated tip, taking care not to transfer any of the proteinaceous, white interphase. This extraction is repeated twice more.
4. The DNA is precipitated by addition of 80 μl of isopropanol and 18 μl of 3-M sodium acetate, pH 5.2 and, following an incubation at −80°C for 1 h, is pelleted by microcentrifugation at 14000 g for 20 min at 4°C. The supernatant is removed and the pellet washed with 80% ethanol. Following a further 20-min spin, the pellet is left to air-dry for 10–15 min.

3.1.2. Translation and Polyadenylation of RNA

1. The DNA pellet is resuspended in 6 μl of nuclease-free water containing 20 U of SUPERase-In RNase Inhibitor. This is then transferred to a fresh microcentrifuge tube, and a transcription reaction assembled at room temperature by adding 10 μl of ice-cold 2xNTP/CAP mix, 2 μl of room-temperature 10X reaction buffer, along with 2 μl of enzyme.

2. The reaction is then incubated at 37°C for 2 h. Addition of a poly-A tail, to enhance RNA longevity, is achieved by addition of 36-μl nuclease-free water, 20-μl E-PAP buffer, 10-μl 25-mM MgCl₂, 10-μl 10-mM ATP, and 4-μl E-PAP to the 20 μl transcription reaction. The reaction is again incubated at 37°C for a further 2 h.

3. The polyadenylated RNA is precipitated by addition of 150-μl lithium chloride precipitation solution, and incubated at −80°C for >1 h. The RNA is pelleted by 4°C microcentrifugation at 14000 g for 30 min. The supernatant is discarded, the pellet washed with 80% ethanol, and further centrifuged for 15 min. Following air-drying, the RNA is resuspended by addition of 9 μl of nuclease-free water and 1 μl (20 U) of SUPERase-In RNase Inhibitor.

3.1.3. Quantification and Dilution of RNA

1. The RNA is quantified by measuring its absorbance at 260 nm in a 500-μl quartz cuvette. We routinely use 1 μl of sample, giving a dilution factor of 1 in 500. The amount of RNA is then calculated using the standard equation:

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\text{RNA conc (μg/ml)} = 40 \times A_{260} \times 500
\]

2. The RNA can stored at −80°C in 1-μl aliquots. We commonly store RNA as either undiluted stock aliquots, or working aliquots diluted to 2, 0.2, or 0.02 mg/ml. When diluting, we commonly add 20U SUPERase-In to the RNA prior to aliquotting.

3. Just before injection, the RNA is mixed with other components, such as OGBD, and then kept on ice for the period over which injections are carried out (<1 h). If there is any remaining solution in the aliquots after injection, it is discarded and a fresh aliquot is used for each injection session.
3.1.4. Assessment of RNA Quality

Initially, it may be necessary to check batches of RNA for signs of RNase contamination, which leads to degradation of the RNA. Commonly this can be achieved by running an aliquot of RNA on a denaturing agarose gel, checking for the presence of a single species of defined size without a smear of lower-molecular-weight fragments, indicative of RNA degradation. However, due to the heterogeneity in size of the poly-A tail, a single species is rarely seen, leading to some degree of uncertainty about the quality of the RNA. Instead, we check that the RNA can be translated into a protein of the predicted molecular weight. This is achieved using 1–2 μg of RNA in a Rabbit Reticulocyte Lysate reaction (Promega). We label the protein with [³⁵S]-Pro-Mix, and, following separation on SDS-PAGE, use autoradiography to determine its molecular weight. Alternatively, we have also satisfactorily used “cold” methionine in the reaction, and then used antibodies to detect the protein of interest on a western blot following the SDS-PAGE.

3.2. Microinjection of Eggs

1. Zona intact mouse eggs are placed in a shallow drop (~1 ml) of media covered in oil, in the lid of a petri dish. The dish is placed on the microscope stage without heating. A silver wire is placed in the injection drop and this wire is held in place via a small manipulator. This wire is connected to a longer standard copper wire to the chassis ground of the electrical amplifier. This wire allows for an electrical circuit to form between the ground and the tip of the pipette once it is place in the media (a circuit is indicated by the amplifier). If this does not occur, then the pipette should be replaced because the tip is probably blocked.

2. The RNA solution to be injected should be spun (13,000 rpm in a benchtop microcentrifuge) for several minutes before injection. For RNA injection, we generally use tips of 0.75–0.9 μm in diameter. The injection pipettes are backfilled with <1 μl of injection solution containing the RNA.

3. This pipette is then held in the specialized holder (containing a silver wire) that is then fitted onto the preamplifier that is itself clamped into the micromanipulator.

4. For experiments where fluorescence is also measured, to look at changes in Ca²⁺ dynamics within the egg, the luciferase-tagged cRNA is mixed with an equal volume of 1-mM OGBD prepared in KCl Hepes pH 7.2. Even if a Ca²⁺ dye is not to be used, it is useful to mix the RNA solution 1:1 with KCL Hepes buffer so as to have some salts present in the injection buffer.

5. For injection, each egg is held by suction with the holding pipette and then the tip of the injection pipette is manipulated so that it will touch the plasma membrane. The injection pipette is then pushed into egg in a way that deforms the zona pellucida. At some point, the zona will jump back into
shape, which is a sign that the zona pellucida is penetrated. At this point, the negative capacitance is applied to the amplifier that is connected to the back of the specialized pipette holder. This causes the pipette tip to enter the cytoplasm. The operator should then make sure that the tip of the injection pipette is in focus and a pressure pulse is applied from the picopump to push a bolus of solution into the egg.

6. The pressure pulses we use are typically 100 ms–1 s long, at a pressure of ~20 psi. The volume of solution injected is estimated by the diameter of cytoplasmic displacement caused by the injection and should correspond to 3–5% of the egg volume. In practice, the first egg can be used for a test with the pulse duration and pressure being adjusted to suit the amount of solution injected. Once familiar with this system, it is possible to inject >30 eggs in 20 min. However, tips often become blocked during injection and need to be replaced.

3.3. Imaging of Luciferase Luminescence

1. After injection, the eggs are placed in drops of media for imaging. In most experiments, the eggs are left zona intact and placed in a small (50 μl) drop of HKSOM media, which is under mineral oil in a heated (37°C) chamber with a glass coverslip that sits on the inverted microscope. The HKSOM media contains BSA (4 mg/ml) and 100-μM luciferin (see Note 3). Figure 2.1 shows the luminescence from a single mouse egg injected with cRNA for luciferase alone. The time-course of luciferase expression lasts at least 10 h with a peak at around 4–5 h post-injection.

2. For some experiments where we want to add extra compounds, or sperm, during the course of imaging, the eggs have to be stuck down. We do this by briefly treating the eggs with acid Tyrode’s solution to remove the zona pellucidas and then immediately placing the eggs in 1-ml drop of the HKSOM in a chamber that has a polylysine-coated (1 μg/ml) coverslip.

![Fig. 2.1. Luminescence (in photon counts per minute) from a mouse egg injected with luciferase cRNA. The egg was injected with ~10 pl of 2 μg/μl of cRNA and imaged in media in the presence of 100-μM luciferin 10–20 min after injection.](image-url)
3. To image intracellular $\text{Ca}^{2+}$ in eggs (injected with OGBD), we monitor fluorescence for the period during which $\text{Ca}^{2+}$ signals occur (5 h). At the end of this period, the luminescence is then measured on the same set of eggs (see Note 4).

4. Fluorescence or luminescence is imaged in the eggs using either 20x 0.65 NA or 10x 0.5 NA Fluor objectives. The light (100%) is directed via either a sideport or baseport to the ICCD camera. We use the same ICCD camera to monitor both fluorescence and luminescence. The main difference is that during fluorescence measurements, the eggs are exposed to excitation light. This means that a standard fluorescence filter block is in place to enable epifluorescence illumination. For OGBD we use a FITC block or else a modified block with a 500-nm longpass filter. Figure 2.2a shows the relative changes in fluorescence from eggs injected with OGBD and PLCζ-luc cRNA. The spike-like increases indicate intracellular $\text{Ca}^{2+}$ oscillations, as described previously.

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**Fig. 2.2.** Mouse eggs injected with PLCζ-luc cRNA. Eggs were injected with $\sim$10 pl of 0.2 $\mu$g/µl cRNA. In (a) the fluorescence (in arbitrary units, a.u.) of Oregon Green BAPTA dextran is shown. The oscillations in fluorescence indicate intracellular $\text{Ca}^{2+}$ oscillations are occurring. In (b) the luminescence from two other eggs injected with PLCζ-luc cRNA and incubated in 100-µM luciferin is shown. The luminescence is recorded continuously with this experiment. In (c) an image of the group of eggs injected with PLCζ-luc cRNA is shown for different time periods. The arrows point to eggs 1 and 2 that are shown in (b).
5. The excitation light source is from a halogen lamp (see Note 5). Fluorescence is monitored in injected eggs for 4 h or 5 h, and then by measuring the OGBD fluorescence with low-level excitation light, the luminescence is measured from the same set of eggs by recording the light from eggs with the excitation light turned off. During the fluorescence recording, the luminescence signal may increase slightly and so the recorded fluorescence signal actually contains a small component of luminescence. However, the fluorescence signals are typically more than 100 times greater than the luminescence signals, so can be ignored in practice. During fluorescence recording, the camera’s sensitivity can be reduced to 10%.

6. At the end of the fluorescence measurements, the same set of eggs are then monitored for luminescence by integrating light emission (in the absence of fluorescence excitation) for 20 min using the same ICCD camera. The cameras we use, typically have a very low background count such that the background noise from an area the size of one egg is about 1 photon per minute. The luminescence signal starts to increase above background within 10 min of the start of recording (Fig. 2.2). The signal then continues to increase for several hours and does not start to decrease until about 8–10 h. The level of signal depends upon the amount of cRNA injected and the particular construct used (see Note 6). Figure 2B and C shows the images and luminescence integrated from different time periods, as well as the timecourse of expression from two of the eggs in the group that illustrate the range of variation in luminescence.

7. If the experiment only requires a measure of the relative amounts of luciferase expression, then zona intact eggs can be removed from the imaging drop and placed in KSOM media in drops under oil in a 37°C 5% CO₂ incubator. If an absolute calibration of expression is required then the eggs are lysed in a luminometer.

3.4. Quantifying Luciferase Expression in a Luminometer

1. Imaging the luciferase luminescence from eggs can ensure that all eggs counted in an experimental group express the luciferase-tagged PLC. To measure the amount of luciferase protein expressed, groups of eggs are collected from the imaging drop and then lysed in a buffer in a luminometer (see Note 7). For each experiment, groups of eggs, verified as being luminous, are collected and placed in a test tube containing phosphate-buffered saline with 1-mM Mg²⁺ ATP and 100-μM luciferin.

2. The eggs are then lysed with 0.5% Triton X-100 and the steady-state light compared to that emitted from serial dilutions of recombinant firefly luciferase (Sigma) in the same buffer. The amount of luciferase activity measured for each group of eggs is then divided by the number of luminous eggs to obtain the
mean value for protein expression of each type of PLC-luciferase. We have found that injection of mouse PLCζ-luciferase into mouse eggs can lead to Ca$^{2+}$ oscillations and the expression of 0.1–0.2 pg of protein in a 4-h period following injection (4).

4. Notes

1. We have previously used an imaging photon detector (IPD) system which was set up by, and used software from, Sciencewares (www.sciencewares.com). The IPD camera (Photek Ltd.) uses a different principal for light collection from the ICCD cameras. We have not noticed any significant difference in sensitivity of these two types of photon-counting detectors.

2. It is essential that some form of dark box is constructed around the microscope. The light level in a typical darkened room, where standard fluorescence microscopy is carried out, is usually much too high and causes considerable interference when imaging luminescence. Any light sources within the darkbox should be removed or covered with black tape. If the microscope used is motorized in any way, it will probably be necessary to switch it off completely during luminescence imaging since internal LEDs will cause an elevated background light.

3. We use 100 μM for mouse eggs, but a range of luciferin concentrations (1 μM to 1 mM) are cited for use in luciferase-imaging of cells in general. The higher concentrations are not always the most effective, because the luciferin luciferase reaction shows “flash kinetics” which means that the reaction rate can decrease with time due to inhibition from the reaction product, oxyluciferin (12). The best concentration to use can depend upon a range of factors that are specific to a cell type, and it is best to test different concentrations. The luciferase also depends upon ATP and can be used to monitor ATP concentrations in eggs (5, 14). For monitoring the timecourse of expression this is not a major issue because the changes in luminescence caused by ATP increases in activating eggs accounts for a 10–20% change in the total light output, and this is hardly detectable in studies using effective amounts of PLCζ-luc, where the luminescence signals are about 1–10 photons per second for each egg. In order to effectively measure the ATP change at fertilization, we have to inject high concentrations of recombinant luciferase protein, which results in luminescence values of 100–1000 photons per second per egg (14).

4. We use OGBD to measure Ca$^{2+}$ because the imaging systems we use can only monitor fluorescence at a single wavelength.
Consequently, a dye such as OGBD is used, since it undergoes an increase in fluorescence intensity with an increase in \( \text{Ca}^{2+} \), and being dextran-linked it does not undergo compartmentalization which can be a problem in mouse eggs (15). Other dyes, such as fura 2, which permits ratio excitation, could be used in conjunction with luciferase monitoring. However, it is worth noting that luciferin is fluorescent when excited with UV light, so if fura 2 is to be used, the luciferin should not be added to the media until the fluorescence imaging is finished.

5. A halogen light source with a stabilized power supply is used because this can easily be reduced to the minimum level required. The excitation light used with photon-counting cameras is generally much lower than with standard cooled CCD cameras and so the use of Xenon lamp (for example) with multiple neutral density filters creates unnecessary light and heat.

6. It is important to optimize the length of the spacer residues present between the protein of interest and luciferase, as we have found that this can alter the expression level of the protein. This may be related to the potential for changes in the protein secondary structure, consequent to tagging with luciferase, and will vary with each protein. For PLCs, we find that a spacer of four residues works well.

7. The relative expression of a PLC can be easily assessed and this can be used for studies on egg activation, or for studies on the effects of PLC\(\zeta\) on later embryo development. However, it is not simple to calibrate the absolute amount of protein expressed in single eggs using the luminescence from living eggs on the microscope. This is partly because the light emitted from firefly luciferase depends upon ATP and pH. Consequently, the precise level of free ATP and pH in an egg would have to be known to calibrate the absolute amount of luciferase.

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