Video Article

Live-imaging of PKC Translocation in Sf9 Cells and in Aplysia Sensory Neurons

Carole A. Farah[1], Wayne S. Sossin[1]

[1]Neurology and Neurosurgery, McGill University

Correspondence to: Carole A. Farah at carole.abifarah@mcgill.ca

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Abstract

Protein kinase Cs (PKCs) are serine threonine kinases that play a central role in regulating a wide variety of cellular processes such as cell growth and learning and memory. There are four known families of PKC isoforms in vertebrates: classical PKCs (α, βI, βII and γ), novel type I PKCs (ε and η), novel type II PKCs (δ and θ), and atypical PKCs (ζ and ι). The classical PKCs are activated by Ca^{2+} and diacylclycerol (DAG), while the novel PKCs are activated by DAG, but are Ca^{2+}-independent. The atypical PKCs are activated by neither Ca^{2+} nor DAG. In *Aplysia californica*, our model system to study memory formation, there are three nervous system specific PKC isoforms one from each major class, namely the conventional PKC Apl I, the novel type I PKC Apl II and the atypical PKC Apl III. PKCs are lipid-activated kinases and thus activation of classical and novel PKCs in response to extracellular signals has been frequently correlated with PKC translocation from the cytoplasm to the plasma membrane. Therefore, visualizing PKC translocation in real time in live cells has become an invaluable tool for elucidating the signal transduction pathways that lead to PKC activation. For instance, this technique has allowed for us to establish that different isoforms of PKC translocate under different conditions to mediate distinct types of synaptic plasticity and that serotonin (5HT) activation of PKC Apl II requires production of both DAG and phosphatidic acid (PA) for translocation [1-2]. Importantly, the ability to visualize the same neuron repeatedly has allowed us, for example, to measure desensitization of the PKC response in exquisite detail [3]. In this video, we demonstrate each step of preparing Sf9 cell cultures, cultures of *Aplysia* sensory neurons have been described in another video article [4], expressing fluorescently tagged PKCs in Sf9 cells and in *Aplysia* sensory neurons and live-imaging of PKC translocation in response to different activators using laser-scanning microscopy.

Video Link

The video component of this article can be found at https://www.jove.com/video/2516/

Protocol

1. Preparation and Maintenance of Sf9 Cell Monolayer Cultures

1. Sf9 cell culture and maintenance is performed in a tissue culture hood.
2. Sf9 cells are derived from *Spodoptera frugiperda* ovarian cells (Sf21 cells) and can be purchased as frozen cells in Grace's media from Invitrogen.
3. Place 8 mL of Grace's insect medium, supplemented (1X), to which 30% fetal bovine serum (FBS) has been added in a 75 cm^2 cell culture flask with a canted neck.
4. Thaw the frozen tube of Sf9 cells rapidly in a 37°C water bath by moving the tube back and forth (about 1-2 minutes).
5. Transfer Sf9 cells to the 75 cm^2 cell culture flask and rock plate gently by hand to distribute the cells evenly.
6. Incubate 1-2 hours at 27°C until cells have attached.
7. Remove old medium and replace with 10 mL of freshGrace's insect medium with 30% FBS.
8. Incubate at 27°C until the cells are confluent.
9. To maintain monolayer cultures, remove old medium from the confluent flask of Sf9 cells and add 5 mL of Grace's insect medium with 10% FBS.
10. Lightly tap on the side of the flask several times to detach Sf9 cells.
11. Using a 10 mL pipette and a pipettor, pipette the media up and down once gently, spraying flask wall while pipetting down. Transfer the cells to a sterile 15 mL Polysterene tube.
12. Add 2 mL of Sf9 cell suspension to 8 mL of Grace's insect medium with 10% FBS (1:5 dilution to maintain log phase growth) in new 75 cm^2 cell culture flask and rock plate gently by hand.
13. Incubate at 27°C until the cells are confluent.

2. Expression of Fluorescently Tagged PKCs in Sf9 Cells

1. For live-imaging experiment, culture Sf9 cells on 35 mm MatTek glass bottom culture disheswith a glass surface of 14 mm and a coverslip thickness of 0.16 to 0.19 mm.
2. PKCs tagged with fluorescent proteins are expressed in Sf9 cells using Cellfectin II transfection reagent following recommendation of the manufacturer with the modifications detailed below.
3. Day 1: Prior to plating the cells, treat each MatTek dish with Grace's insect medium with 10% FBS for at least 30 minutes.
4. Count Sf9 cells from step 11 (above) using a hemacytometer.
5. Plate 0.05 x 10^6 cells on each MatTek glass coverslip in a total volume of 150 µL. Allow the cells to attach for 30 minutes at room temperature.
6. Add 2 mL of Grace's supplemented with 10% FBS to each dish 1 mL at a time slowly to avoid detaching the cells.
7. Incubate at 27°C overnight.
8. From this point on, use manufacturer's recommendation for transfection of Sf9 cells with plasmid DNA.
9. Day 2: Transfect Sf9 cells with fluorescently tagged PKCs using Cellfectin II transfection reagent. We have often co-expressed in this system PKCs tagged with enhanced Green Fluorescent Protein (eGFP) and monomeric Red Fluorescent Protein (mRFP) (details for plasmid construction have been previously described [2,5]). Expression of one fluorescent protein at a time yields about 70-80% expressing cells 72 hours post-transfection. Co-expression of two fluorescent proteins decreases transfection efficiency to about 30% co-expressing cells. When co-expressing eGFP-tagged PKC and mRFP-tagged PKC, use 2x more plasmid DNA for the mRFP-tagged protein for optimal protein expression.
10. Perform live-imaging 48-72 hours post-transfection (for optimal protein expression, wait 72 hours).

3. Expression of Fluorescently Tagged PKCs in *Aplysia* Sensory Neurons

1. Preparation of *Aplysia* neuronal cell cultures has been described in a video article by Zhao and colleagues [4].
2. PKCs tagged with fluorescent proteins are expressed in *Aplysia* sensory neurons using microinjection procedure detailed below.
3. Prepare a stock solution of 1% Fast Green (FCF) in distilled water, filter using a syringe and a 28 mm syringe filter (0.20 µm). Aliquot and store at -20°C.
4. Microinjection of the sensory neurons can be performed on day 1 or day 2 after isolating the neurons but we find that waiting 2 days allows for better cell adherence to the coverslip.
5. On day 2 after isolation of the sensory neurons, thaw an aliquot of Fast Green and filter it again using a syringe and a 28 mm syringe filter (0.20 µm).
6. Prepare a solution of plasmid DNA in distilled water containing 0.5% Fast Green. Concentrations of plasmid DNA as high as 0.4 µg/µL can be used but it is not recommended to go above these values.
7. Filter the plasmid DNA / Fast Green solution using a 1 mL syringe and a 4 mm syringe filter (0.20 µm).
8. Centrifuge the plasmid DNA / Fast Green solution at 16110 x g for 15 minutes using a microcentrifuge.
9. Prepare sharp electrodes for microinjection of neurons using a microelectrode puller (Sutter Flaming/Brown Micropipette Puller, Model P-97). Using a box filament, pull glass microelectrodes with 1 mm outer diameter, 0.75 mm inside diameter and 100 mm length using thin wall glass capillaries with a filament inside (World Precision Instruments TW100F-4). For more information about pulling microinjection electrodes please refer to Sutter electrode cookbook.
10. Fill each electrode with 2 µL of plasmid DNA / Fast Green solution using microloader pipette tip (eppendorf CA32950-050).
11. Transfer the MatTek glass bottom culture dish containing *Aplysia* neuronal cultures to the microinjection station.
12. The microinjection station consists of the following: a home-made large glass stage to hold the culture dishes on a vibration isolation table (Kinetic Systems vibration isolation workstation), a stereo-scope with external halogen illumination, a micromanipulator (Sutter Huxley Wall Type Micromanipulator which allows both coarse and ultra-fine positioning in the three-axis), a microelectrode holder connected to a pneumatic pump (World Precision Instruments PV820 Pneumatic Pico-Pump). The pressure Input of the pneumatic pump is connected to a compressed Nitrogen tank.
13. Insert a microelectrode into the microelectrode holder.
14. Viewing under a stereo-microscope, insert the tip of the micropipette into the cell nucleus.
15. Deliver short pressure pulses of Nitrogen (10 to 100 ms in duration; 20 lb/in^2) until the nucleus becomes uniformely green.
16. Incubate the cells for 4 hours at room temperature and keep at 4°C until use. For optimal PKC translocation, perform live-imaging experiment 20 - 24 hours post-injection.

4. Visualization of PKC Translocation in Living Sf9 Cells and in *Aplysia* Sensory Neurons

1. For imaging, we use a Zeiss LSM510 inverted confocal microscope with an Axiovert 200.
2. Imaging protocol is the same for Sf9 cells and for *Aplysia* sensory neurons except for the following: Sf9 cells are imaged with a x63 oil immersion objective whereas *Aplysia* sensory neurons are imaged with a x40 oil immersion objective. Sf9 cells are imaged in a temperature-controlled chamber maintained at 26-27°C whereas *Aplysia* sensory neurons are imaged at room temperature maintained around 18-20°C.
3. We use a 30 mW Argon laser (excitation at 488nm) with 50% laser output to image PKC tagged with eGFP protein and a HeNe laser (excitation at 543nm) to image PKC tagged with mRFP protein. The Argon and HeNe laser lines are attenuated to 4% and 50% transmission output respectively prior to imaging and the pinhole is adjusted to one.
4. It is important to have the culture dish stabilized on the imaging stage and to avoid movement or vibration.
5. Remove 1 mL of culture media from the dish gently using a 10 mL pipette leaving 1 mL total volume in the dish.
6. Take pictures in the middle section of the cells where the nucleus can be seen.
7. Set up a time series of 10-20 confocal images taking a picture every 30 seconds.
8. Start the time series.
9. After 2 pictures are taken (after the 30 seconds time point), add 1 mL of the drug (2X concentration) gently drop-by-drop on top of the cells using a P1000 pipette. The drug is added continuously for about 30 seconds.

10. Some drugs induce a fast translocation which can be visible immediately following drug treatment (at the 60 seconds time point) while others induce a slow translocation which is only optimal 5-10 minutes post-treatment (Movie 1 and Movie 2).
11. If the drug needs to be washed away, use 2 x 10 mL pipets to do the wash, pipetting the wash buffer down gently with one pipette on one side of the dish and pipeting up the media on the other side.
12. If the wash has been effective and if the effect of the drug is reversible, PKC reverts back to the cytosol within 30-60 seconds.
13. Save the movie as an LSM file.
14. Use NIH Image J software to open the LSM files and quantify the images Pre and Post drug treatment. Quantification has been described elsewhere [1].

Discussion

We have described a technique to image translocation of fluorescently tagged PKCs in real time in Sf9 cells and in *Aplysia* sensory neurons. Sf9 cells provide a simple system to image PKC translocation since culturing and transfecting them is pretty straight-forward. In contrast, microinjection of *Aplysia* sensory neurons takes some time to master; up to a few months. Difficulties related to this technique include electrode clogging. Filtering the injection solution and centrifuging it usually helps but sometimes air bubbles can form in the electrode while filling it up. We find that using the microloader pipette tips to fill up the electrode instead of capillarity helps to minimize the formation of air bubbles. Regarding live-imaging, two factors need to be tightly controlled during the imaging session: temperature and movement. Temperature fluctuations might affect translocation and the use of a temperature-controlled chamber should be considered. To avoid movement during imaging, a vibration isolation table can be used.

Disclosures

No conflicts of interest declared.

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