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Imaging of Mitochondrial and Cytosolic Ca^{2+} Signals in Cultured Astrocytes

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Abstract

This unit provides a step-by-step protocol for constructing cell-type and mitochondria-targeted GCaMP genetically-encoded Ca^{2+} indicators (GECIs) for mitochondrial Ca^{2+} imaging in astrocytes. Mitochondrial Ca^{2+} plays a critical role in controlling cytosolic Ca^{2+} buffering, energy metabolism, and cellular signal transduction. Mitochondrial Ca^{2+} overload contributes to various pathological conditions including neurodegeneration and apoptotic cell death in neurological diseases. Live cell mitochondrial Ca^{2+} imaging is an important approach to understand mitochondrial Ca^{2+} dynamics and thus cell physiology and pathology. We implement astrocyte-specific mitochondrial targeting of GCaMP5G/6s (mito-GCaMP5G/6s). By loading X-Rhod-1 in astrocytes, we can simultaneously image mitochondrial and cytosolic Ca^{2+} signals. The current protocol provides a novel approach to image mitochondrial Ca^{2+} dynamics as well as Ca^{2+} interplay between the endoplasmic reticulum and mitochondria.

Keywords

Mitochondrial Ca^{2+} uptake; mitochondrial matrix; astrocyte; endoplasmic reticulum (ER); GCaMP5G/6s; X-Rhod-1; ATP

INTRODUCTION

It has been established that astrocytes participate in synaptic transmission as a part of tripartite synapse through Ca^{2+}-mediated gliostransmitter release. Mitochondria can be used as Ca^{2+} sinks to buffer local or cytosolic Ca^{2+} rises in astrocytes. Mitochondrial Ca^{2+} uptake into its matrix [see Figure 1] affects metabolic processes by regulating oxidative phosphorylation and stimulates ATP production under physiological conditions (Burkeen et al., 2011;Griffiths and Rutter, 2009;Llorente-Folch et al., 2013;Pizzo et al., 2012). Mitochondrial Ca^{2+} uptake is also a determinant for inducing necrotic and apoptotic cell death in brain disorders (Duchen, 2012;Gouriou et al., 2011;Qiu et al., 2013). Mitochondrial Ca^{2+} overloading opens mitochondrial permeability transition pores (mPTPs) which can initiate apoptotic cell death. Therefore, studying mitochondrial Ca^{2+} dynamics in living cells is important for understanding cellular (patho)physiological function. Under physiological conditions, mitochondria maintain matrix Ca^{2+} homeostasis through a balanced Ca^{2+} uptake and efflux. Mitochondrial Ca^{2+} uptake is mainly mediated by mitochondrial Ca^{2+} uniporters (MCUs) while mitochondrial Ca^{2+} efflux is mediated by Na^{+}-Ca^{2+}-Li^{+} exchangers

(NCLXs). The balance can be perturbed through the stimulation of GPCRs (Finkel et al., 2015) (Figure 1A).

Many synthetic organic Ca^{2+} indicators have been used for cellular Ca^{2+} imaging (for reviews see Contreras et al., 2010;Paredes et al., 2008); however, these indicators are partitioned between mitochondria, other organelles and the cytosol, making absolute measurements difficult. In contrast, genetically encoded Ca^{2+} indicators (GECIs) can be expressed in different cell types and subcellular compartments including mitochondria for cell- and compartment-specific Ca^{2+} imaging *in vitro* or *in vivo*. Fluorescence intensity-based GCaMP Ca^{2+} indicators have recently emerged as major GECIs (Akerboom et al., 2012;Chen et al., 2013;Tian et al., 2009;Yamada et al., 2011). In this unit, we provide protocols for astrocyte- and mitochondria-specific targeting of GCaMP5G and GCaMP6s (GCaMP5G/6s) to image mitochondrial Ca^{2+} dynamics in primary cultured astrocytes. Combined with organic Ca^{2+} indicator X-Rhod-1, we also provide a protocol for simultaneous imaging of mitochondrial and cytosolic Ca^{2+} signals. Using this novel approach, mitochondrial Ca^{2+} uptake in individual mitochondria in cultured astrocytes can be revealed after ATP stimulation. We demonstrate that mitochondrial Ca^{2+} signal is tightly coupled to IP_3R-mediated Ca^{2+} release from the ER in astrocytes, indicating the dependence of mitochondrial Ca^{2+} dynamics on cytosolic Ca^{2+} changes.

BASIC PROTOCOL 1

IMAGING MITOCHONDRIAL AND CYTOSOLIC Ca^{2+} IN ASTROCYTES USING GCAMP5/6—

To image mitochondrial matrix and cytosolic Ca^{2+} simultaneously in astrocytes, we constructed DNA plasmids with astrocyte-specific promoter combined with mitochondrial targeting sequence to encode GCaMP5G and GCaMP6s (GCaMP5G/6s) (Li et al., 2014). The DNA plasmids are transfected in cultured astrocytes to express GCaMP5G/6s. The transfected astrocytes are ready for imaging one day later. To simultaneously image mitochondrial matrix and cytosolic Ca^{2+} signaling, the astrocytes are then loaded with organic red fluorescence Ca^{2+} indicator X-Rhod-1. Time-lapse imaging is conducted using two-photon (2-P) or confocal microscopy. ATP will be applied to astrocytes through a solution perfusion system to stimulate Ca^{2+} signal in astrocytes. Both mitochondrial fluorescent GCaMP5G/6s signal and cytosolic X-Rhod-1 fluorescent signal will be acquired through an imaging/acquisition system.

Materials

Papain (20 IU/ml; Sigma)

EBSS - Earle's Balanced Salt Solution (Invitrogen)

Glass coverslips (12 mm in diameter; Fisher Scientific)

Dulbecco’s Modified Eagle Medium (DMEM) medium (Invitrogen)

Fetal bovine serum (FBS) (Hyclone)

Cultured astrocytes grown on glass coverslips in 24-well plate

Endofree plasmid maxi kit (Qiagen)

DNA plasmids with astrocyte-specific promoter and mitochondrial targeting sequence to express GCaMP5G/6s in the mitochondria in astrocytes. DNA construction is outlined below but if additional details are needed see Li et al., 2014.

Transfection reagent: Lipofectamine® 2000 Transfection Reagent (Thermo Fisher)

Artificial cerebrospinal fluid (ACSF) at room temperature, see recipe in reagents and solutions

ATP stock solution (10 mM) prepared with distilled water or ACSF kept at −20°C

Perfusion chamber (PH1, Warner Instruments, MA)

Perfusion system (ALA-VM8, ALA Scientific, NJ) which can control solution change and drug application using pinch valves

Two-photon (2-P) or confocal fluorescence microscope with imaging and acquisition system

Weigh out sodium bicarbonate and place it into a 100mL (200 mL) bottle. Add the α–MEM, L-glutamine, D-glucose, sodium pyruvate, and penn/strep. Mix until sodium bicarbonate goes into solution. Sterile filter the solution through a 0.2μm cellulose acetate bottle top filter into a sterile media bottle. Add the serum. Store the modified media at 4°C. Do not leave media in an incubator for more than a day.

Construction of DNA plasmid for mitochondrial expression of GCaMP5G/6s

1. Insert mitochondrial matrix (MM)-targeting sequence (mito-) ATGT CCGTCCTGAC GCCGCTGCTG CTGCGGGGCT TGACAGGCTC GGCCCGGCGG CTCCCAGTGC CGCGCGCCAA GATCCATTCG TTG (Rizzuto et al., 1995) in the backbone of AAV plasmid pZac2.1 containing astrocytic gfaABC$_1$D promoter (Lee et al., 2008;Li et al., 2014;Xie et al., 2010).
2. Subclone GCaMP5G/6s or monomeric red fluorescence protein (mRFP) as a control into the cloning sites BamH I and Not 1 using PCR to obtain new expressing plasmids that can target transgene expression in mitochondria of astrocytes (Li et al., 2014) (Figure 2).
3. Plasmids are purified for transfection using Endofree plasmid maxi kit.

Primary cultured astrocytes: Astrocytic cell cultures were prepared as previously described (Gottipati et al., 2012). All procedures were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Missouri Animal Care Quality Assurance Committee.

4. Remove brain from 0–2 day old C57BL/6J mouse pups and place into a small culture dish containing 1 ml cold EBSS (4 °C). [Note: one pup is enough for culturing one 25 cm^2 flask of astrocytes. The gender of pups is not considered].
5. Isolate cortices (bilateral) and place into a 15 ml centrifuge tube containing 10 ml of cold EBSS.

   Mince cortices with a scissor.

6 Decant EBSS and replace with 3 ml papain (20 IU/ml; Sigma) in the presence of L-cysteine (0.2 mg/ml).

7 Incubate at 37°C for 1 h.

Gently mix with finger tapping for about 1 min/15 min or with continuous shaking with an orbital shaker at low speed for 1 h during the papain treatment.

8 Neutralize with 5 ml trypsin inhibitor (type II-O, 10 mg/ml; Sigma) for 5 min at room temperature (22–25°C).

Triturating the tissue gently to avoid disrupting the cells or injury.

9 Decant trypsin inhibitor and replace with 2 ml DMEM medium.

10 Gently triturate the tissue using a glass seriological pipet.

11 Plate 1 ml of the resulting cell suspension into a 25 cm^2 tissue culture flasks containing 15 ml α-MMEM (see Table 1) and place the flask in an incubator maintained at 37°C in a 95% air/ 5% CO_2 atmosphere.

12 One hour post plating, replace with fresh α-MMEM.

Be careful when replacing medium since the cells do not attach to the bottom of plate very strongly at this time point.

13 Put back in the tissue flask in the incubator and add 1ml of fresh MMEM every 5 days to maintain astrocyte cultures.

It should take about 10–14 days to obtain cell growth and proliferation to ~60% confluency.

14 Shake the flasks twice on an orbital shaker, first for 1.5 h followed by two exchanges with culture media and again for 18 hours, at 37°C and 260 rpm to remove microglia.

This is a necessary step to obtain purified astrocytes (McCarthy and de Vellis, 1980). The purity of astrocyte can be quantified by immunostaining with anti-glial fibrillary acidic protein (GFAP) and nuclear counterstaining with Dapi. A purity of 95% should be obtained with this procedure.

15 Plate these astrocytes on the glass coverslips (12 mm in diameter; Fisher Scientific) (Xie et al., 2010) in 24-well plates and culture the plates in the incubator.

16 Feed the astrocytes every 48 h with fresh Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS as described previously.

Expression of mito-GCaMP5G/6s in astrocytes by DNA transfection

17 Change the medium of cultured astrocytes (up to 60% confluence) right before transfection.

18 For transfecting 4 wells in 24 well-plate, prepare two tubes: 1st tube: 100 μl DMEM (serum free)+2 μg DNA; 2nd tube: 100 μl DMEM (serum free)+ 4–6 μl lipofectamine 2000, gentle mixing for 5 min.

19 Add 2nd tube mixture to 1st tube dropwise while gently tapping the tube, and incubate the mixture at room temperature for 20 min (it remains stable for up to 6 h).

In the mixture, the ratio of DNA (μg):Lipofectamine 2000 (μl)=1:2–3.

20 Gently add 50 μl mixtures dropwise to astrocytes in each well for transfection.

21 Change culture medium 6 h later.

22 The cells are ready to use for imaging 24 h later. Transfected astrocytes can be visualized using a fluorescent microscope (Figure 1B, 2B and 3A).

Loading astrocytes with organic Ca^{2+} indicator X-Rhode-1 dye for simultaneous mitochondrial and cytoplasmic Ca^{2+} imaging

23 Make a X-Rhode-1 working solution of 1 μg/ml by diluting 1 μl stock solution with ACSF.

24 Transfer a glass coverslip grown with astrocytes to a well on a 24-well plate, and gently wash away the residue medium with 0.5 ml ACSF.

25 Add 0.5 ml X-Rhode-1 working solution to the astrocytes grown on the glass coverslip in the 24-well plate and incubate at room temperature for 45 min.

26 Wash away excess X-Rhode-1 with ACSF and incubate in ACSF for 30 min for the de-esterification of the dye.

Under a fluorescent microscope, both astrocytes expressing and not expressing mito-GCaMP5G/6s will be labeled with X-Rhod-1 (Figure 3A-C).

Imaging mitochondrial and cytosolic Ca^{2+} signaling (setup)

27 Make 100 μM ATP in 200 ml ACSF as working solution for stimulation of astrocytes.

28 Set the perfusion system and put 100 ml ACSF solution in one barrel and 100 ml 100 μM ATP-containing ACSF in the other barrel.

29 Test the perfusion system to make sure the solution flow into chamber freely with gravity and can be removed from chamber with vacuum pump (see Figure 4 for setup).

Two important issues: 1) Make sure there are no bubbles in the tube; and 2) make sure that the solution can be removed so that there is no overflow in the chamber.

30 Transfer the glass coverslip grown with astrocytes to a perfusion chamber for imaging.

31 Perfuse the cells with ACSF.

Note: the speed should be ~ 1–2 ml/min and make sure that ACSF does not overflow and the glass coverslip does not move.

32 Normally after a few minutes of ACSF perfusion, time-lapse Ca^{2+} imaging can be performed. After 1–2 minutes of data acquisition with ACSF perfusion, switch to ACSF with 100 μM ATP for 1–2 min to stimulate astrocytes.

Usually the imaging acquisition speed is 1 frame/second

33 Switch back to ACSF solution and image for 2 minutes.

The mito-GCaMP5G and X-Rhode-1 fluorescence should go back to the baseline when imaging is finished.

The 2-P microscope (Prairie Technologies, WI) should be turned on 30 min before step 18. We use a 40×/NA0.8 or 60×/NA0.95 Olympus water immersion objective. Excitation (850 nm) for x-Rhod-1 and mito-GCaMP5G/6s was generated by a pulse laser beam from a Chameleon Ti:Sapphire Ultra I laser (Coherent, CA). Emission was detected using two photo-multiplier tubes (PMTs). Images were acquired with a speed of one frame per second. All the raw fluorescence images should have pixel intensities without saturation and within the PMT's dynamic range (0–4095). Depending on the availability, a confocal microscope is also ideal for imaging.

Analysis of mitochondrial and cytosolic Ca^{2+} signal

34 Define the regions of interest (ROI) around the mitochondria or cytoplasm, or a blank region without any cultured astrocytes as a background on the acquired time-lapse images using of free software ImageJ or Metamorph software (Molecular Device, CA).

Figure 4 is the procedure for analysis of a single mitochondrion in region 2 in Figure 1B. The same procedure is used for the analysis cytosolic Ca^{2+} signals.

35 Using software, measure the mean pixel fluorescent intensities of each ROI as overall fluorescence signal (F_s (t)) meanwhile measure background intensity (F_b) of a blank region where no cell is grown (Figure 5A).

36 Export F_b(t) from F_s(t) to other software such as Origin software (OriginLab Corporation, MA) and subtract F_b(t) from F_s(t), i.e., F_s(t)-F_b(t). We define this as background subtracted fluorescence F(t) (Figure 5B).

37 Calculate the baseline fluorescence Fo, and then subtract Fo from F(t), i.e., F(t)-Fo=ΔF (t), which is defined as the baseline subtracted fluorescence (Figure 5C).

Fo is the average fluorescence before ATP stimulation.

38 Ca^{2+} changes were calculated and expressed as ΔF(t)/Fo values vs. time (Figure 5D).

REAGENTS AND SOLUTIONS

Artificial cerebrospinal fluid (ACSF): 120 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 10 mM glucose, 10 mM Hepes, pH 7.4). ACSF should be made fresh for imaging experiments and remain at room temperature.

ATP stock solution (10 mM) and working solution (100 μM): Stock solution (10 mM) is made with ACSF or water and stored at −20 °C up to one year. Working solution (100 μM) is made by diluting stock solution with ACSF and used fresh.

X-Rhode-1 stock solution (1 μg/μl): Dissolve 50 μg X-Rhode-1 (X14210, Thermo Fisher) in 50 μl DMSO and mix well.

Aliquot into 25 tubes with 2 μl in each tube and store them at −20 °C up to one year.

Use distilled and deionized water in all protocol steps during imaging.

COMMENTARY

Background Information: Many synthetic organic Ca^{2+} probes have been used for cellular Ca^{2+} imaging (for reviews see Contreras et al., 2010;Paredes et al., 2008); the major disadvantage of these probes is the partitioning between mitochondria, other organelles and the cytosol, making measurements in specific regions difficult. Rhod-2 is the most widely used organic dye to image mitochondrial Ca^{2+} uptake, which preferentially locates to the mitochondrial matrix (for review see Contreras et al., 2010;Davidson and Duchen, 2012), but it also shows partial localization in the cytosol which causes errors in measurements. There are other problems generally associated with synthetic organic Ca^{2+} probes; these include cytotoxicity resulting from the de-esterification of reaction products and photodamage due to a prolonged exposure to high energy excitation light (Thomas et al., 2000).

Genetically encoded Ca^{2+} indicators (GECIs) provide substantial advantages over synthetic organic Ca^{2+} probes due to their high sensitivity, brightness and dynamic range of fluorescence. For these reasons, fluorescence intensity-based GCaMP Ca^{2+} indicators have recently emerged as major GECIs (Akerboom et al., 2012;Chen et al., 2013;Tian et al., 2009;Yamada et al., 2011). For instance, GCaMP5G and GCaMP6s can readily detect cytosolic Ca^{2+} increases in a neuron after triggering a single action potential (Akerboom et al., 2012;Chen et al., 2013). On the other hand, using molecular biology, these GECIs can be targeted to subcellular compartments such as the cytoplasm, mitochondria and nuclei. For *in vivo* studies, they can be expressed in different cell type using cell-type specific promotors (Xie et al., 2010). In this protocol, we employed astrocyte- and mitochondria-specific targeting of GCaMP5G/6s to assess mitochondrial Ca^{2+} dynamics in primary cultured astrocytes. ATP stimulates astrocytic Ca^{2+} increase through Ca^{2+} release from ER via activation of IP_3Rs (Figure 1)(Ding et al., 2007;Ding, 2013;Li et al., 2014). Large increases in Ca^{2+} signals can be observed in individual mitochondria in astrocytes with stimulation of ATP. Combined with cytoplasmic loading of red fluorescence Ca^{2+} probe X-Rhod-1, we can study the coupling between mitochondrial and cytosolic Ca^{2+} signals in astrocytes. Our results demonstrate that GCaMP5G/6s are suitable probes for detecting Ca^{2+} uptake in

individual mitochondria in astrocytes. This protocol can be used to study mitochondrial Ca^{2+} uptake and efflux for other cell types such as neurons and *in vivo* with slight modifications.

Critical Parameters

Microscope: Fluorescence imaging with multiple channel acquisition usually uses confocal and 2-P laser scanning microscope equipped with two or more PMTs. Either inverted or upright microscopes are suitable for imaging cultured astrocyte. A 40x or 60x high numerical aperture (NA) objective with high light transmission is desirable so that the maximal amount of fluorescence can be collected.

Image acquisition speed: Compared with neuronal Ca^{2+} signaling, Ca^{2+} signaling in astrocytes is relative slow. It is usually fast enough to acquire one image per second.

Perfusion system: For live cell imaging, it is important to have a good perfusion system for exchanging solutions with different drugs to stimulate or inhibit Ca^{2+} signals in astrocytes. Solution changes can be controlled with pinch values either manually or by computer. The speed of solution change will affect rising and decay time of fluorescence signals. If experiments focus on studying steady-state Ca^{2+} signals, the speed of solution exchange is less important.

Temperature: For Ca^{2+} imaging, the experiments are usually performed at room temperature.

Photobleaching: Theoretically all fluorescent indicators are subject to photobleaching. GCaMP5G/6s and X-Rhod-1 are quite stable but they will bleach under continual exposure to excitation light. For time-lapse imaging, it usually takes several minutes with acquisition rates of one image per second. Thus, profound photobleaching could take place. A general practice to reduce photobleaching is to avoid over-exposure of cells to laser light while enough fluorescence is collected. Exposure time can be reduced if high sensitivity PMTs and high light transmission objective are used. Photobleaching can also be reduced by closing shutter between images.

Troubleshooting: Astrocytes sometimes have low fluorescent intensity of X-Rhode-1. This can happen for a couple reasons. First, it may result from the decay of X-Rhode-1 in the working or stock solutions. One should remember, the working solution can be used for only a few hours after it is made when kept at room temperature. To eliminate the possibility of decayed stock solution, make new stock solution and then new working solution. Normally, stock solution can be used for one year when it is kept in a −20 °C freezer. Second, the astrocyte culture is not healthy or too old. Ideally cultured astrocytes should be used in 7–10 days after plating. To make sure astrocytes are healthy, one should examine their morphology under phase-contrast microscopy before the experiment. Unhealthy astrocytes may have vacuoles and affected membrane integrity. Unhealthy astrocytes will also have poor expression of mito-GCaMP5G and consequently low mito-GCaMP5G fluorescence.

If fluorescence does not increase for a long time (such as 2 minutes) after switching to ATP solution during the live experiments, check the perfusion system to make sure solution is flowing normally and no bubble in the tubing.

If the fluorescence of mito-GCaMP5G/6s is poor due to low expression, the signal/noise will be low and this will in turn cause a low sensitivity to ATP stimulations.

Understanding Results: Typical results for the Basic Protocol are shown in Figure 1B and Figure 3A-C. The GCaMP5G/6s fluorescence should show mitochondrial structure since they are expressed in the mitochondria. When stimulated with ATP, both GCaMP5G/6s and X-Rhod-1 fluorescence will increase at similar time (Figure 3D). If accurate time courses of mitochondrial and cytosolic Ca^{2+} increases are required, one would use line-scan imaging with fast solution perfusion system.

Time Considerations: If the DNA plasmid and cultured astrocytes are ready, the astrocytes can be used one day after transfection. For imaging, the basic protocol should take about 1 h per experiment. It will take ~3 weeks from the generation of astrocyte cultures to conduct imaging experiments.

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Significance Statement

Astrocytes are the predominant glial cell type in the central nervous system. Although astrocytes are electrically inexcitable, their excitability is manifested by Ca^{2+} signaling. Mitochondria can uptake cytosolic Ca^{2+}, thus regulating the function of astrocytes. On the other hand, mitochondrial Ca^{2+} overloading could induce cellular death. Here we developed novel imaging technologies that express genetically encoded Ca^{2+} indicator (GECI) GCaMP5G/6s in the mitochondria of cultured astrocytes. By loading red fluorescence organic Ca^{2+} dye X-Rhod-1 in the cytoplasm, we can simultaneously image mitochondrial and cytosolic Ca^{2+} signals. This protocol demonstrates that GCaMP5G/6s are suitable probes for detecting Ca^{2+} uptake in individual mitochondria in astrocytes and could help to understand astrocytic function.

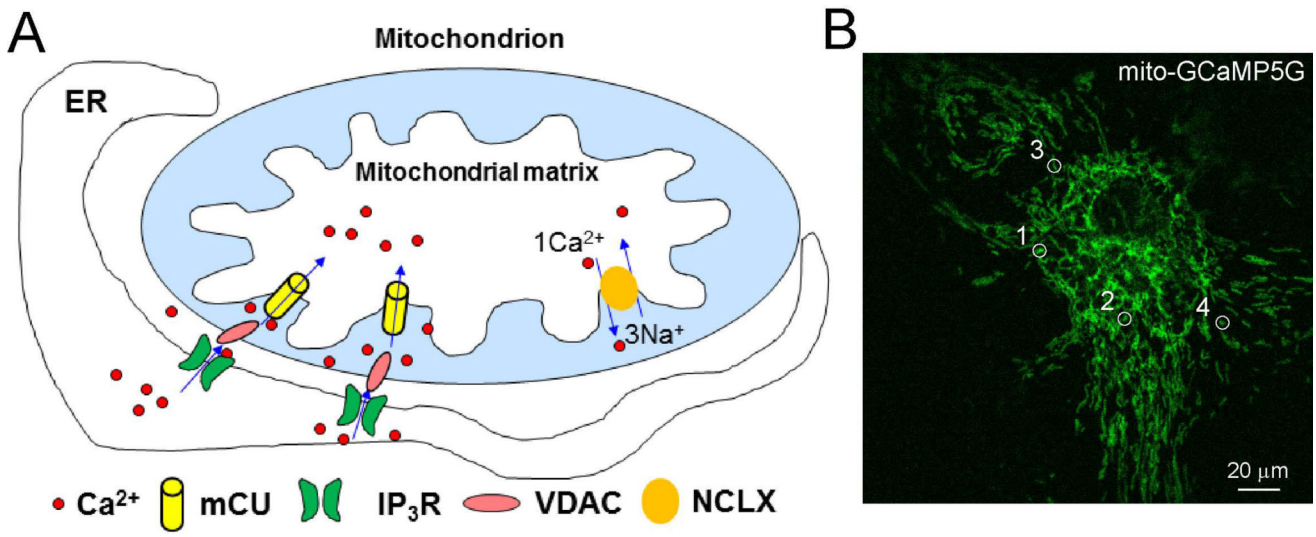

Figure 1. Brief illustration of mitochondrial Ca^{2+} signalling

A) Mitochondrial Ca^{2+} uptake basically is mediated by MCUs while mitochondrial efflux is primarily attributed to the function of the Na^{+}-Ca^{2+}-Li^{+} exchangers NCLXs IR_3R functions to release Ca^{2+} from intracellular store of endoplasmic reticulum (ER). B) A cultured astrocyte expressed with GCaMP5G in the mitochondria. Figure 1B is adapted from Li et al (Li et al., 2014).

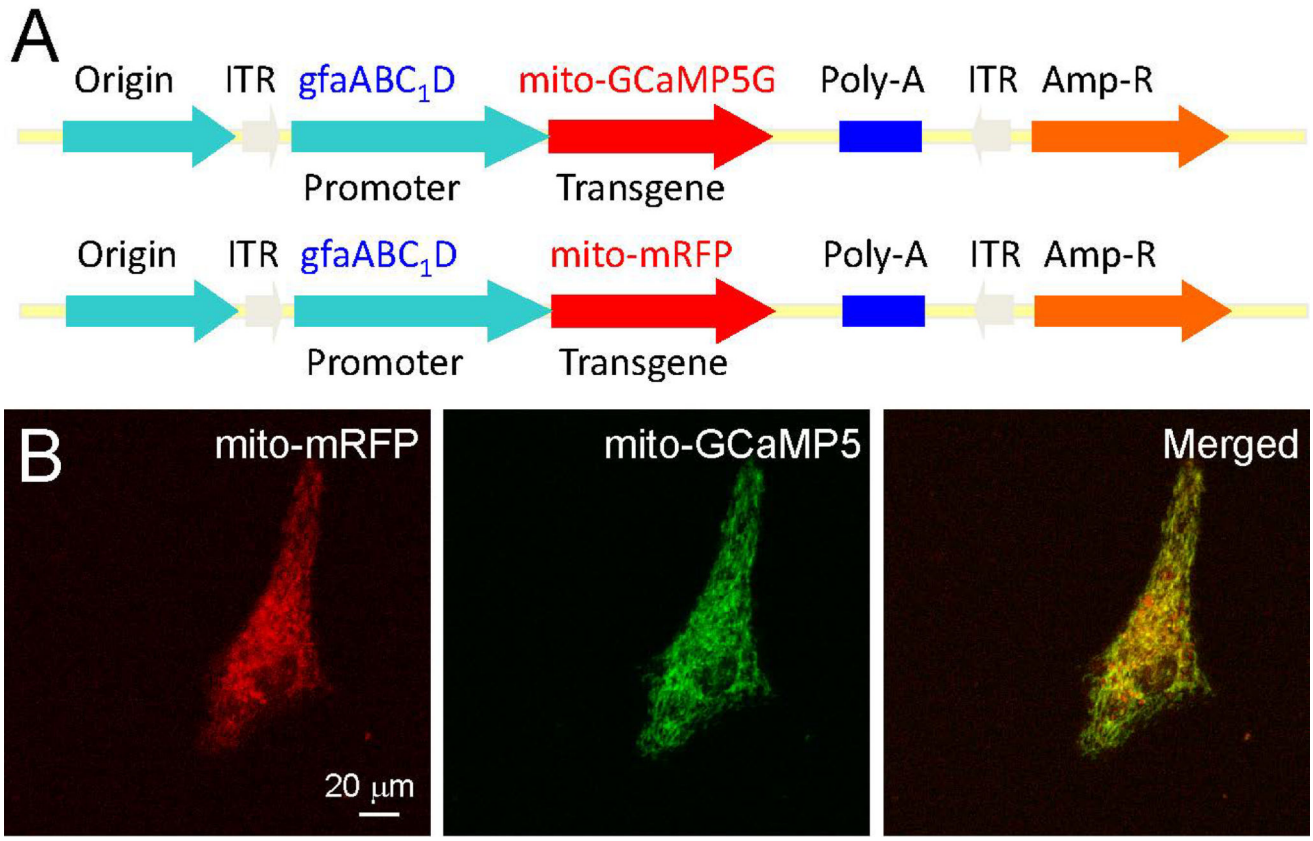

Figure 2. DNA constructs for astrocyte-specific and mitochondria-targeted transgene expression

A) Construction maps of genetically encoded Ca^{2+} indicator GCaMP5G (or GCaMP6s) and mRFP in pZac2.1 plasmid under gfaABC$_1$D promoter for delivering transgenes to astrocytic and mitochondrial matrix. Organelle-specific targeting is achieved using a mitochondrial matrix specific sequence (mito) appended to the N-terminus of the fluorescent proteins. B) Two-photon images showing the coexpression of mito-mRFP (left) and mito-GCaMP5G (right) in primary cultured astrocytes after DNA transfection. Notice exquisite organelle-specific colocalization of mito-mRFP (left) and mito-GCaMP5G. Data is adapted from Li et al (Li et al., 2014). *Note*: While this protocol focuses on astrocytes, using neuron-specific promotor CaMKII, GCaMP5G or GCaMP6s can also be expressed specifically in the mitochondrial in neurons.

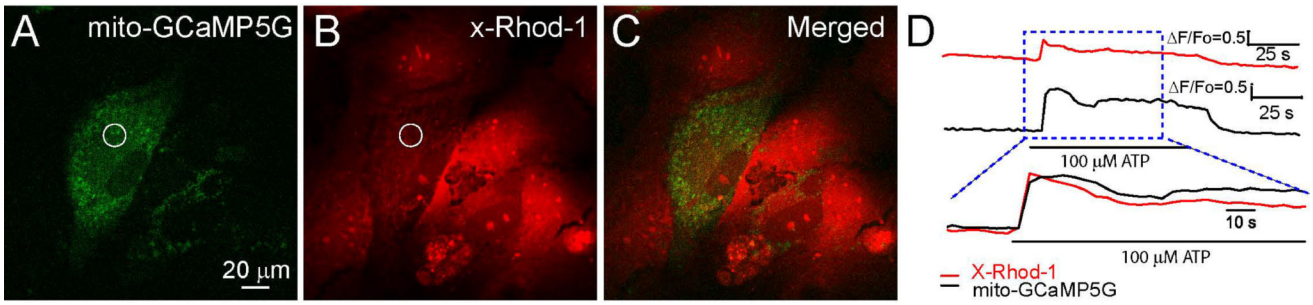

Figure 3. Simultaneous cytosolic and mitochondrial Ca^{2+} imaging in cultured astrocytes

A-C) Two-photon images of astrocytes expressing mito-GCaMP5G (A) and loaded with x-Rhod-1 (B) and the merged images (C) for simultaneous mitochondrial and cytosolic Ca^{2+} imaging. D) The time courses (ΔF/Fo, see Figure 4) of Ca^{2+} changes in cytosol (upper) and mitochondria (middle) in a region marked by a circle in (A-B). The bottom panel is normalized peak ΔF/Fo of mitochondrial and cytosolic Ca^{2+} signals showing the matching kinetics of mitochondrial and cytosolic Ca^{2+} increase after ATP stimulation. Data is adapted from Li et al (Li et al., 2014).

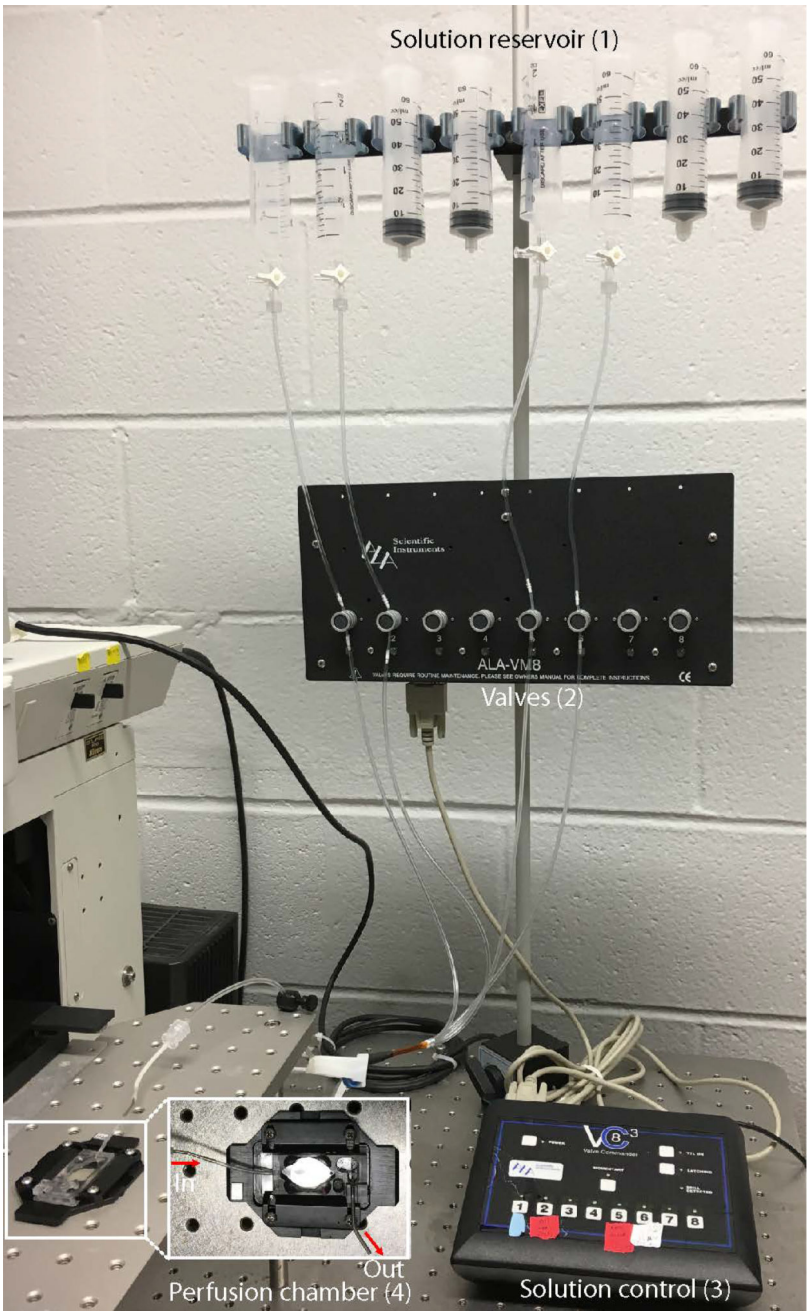

Figure 4. The setup of solution perfusion system

The system consists of 4 parts, i.e., reservoirs (1), pinch valve (2), solution control unit (3) and perfusion chamber (4). The vacuum pump that removes solution is not indicated.

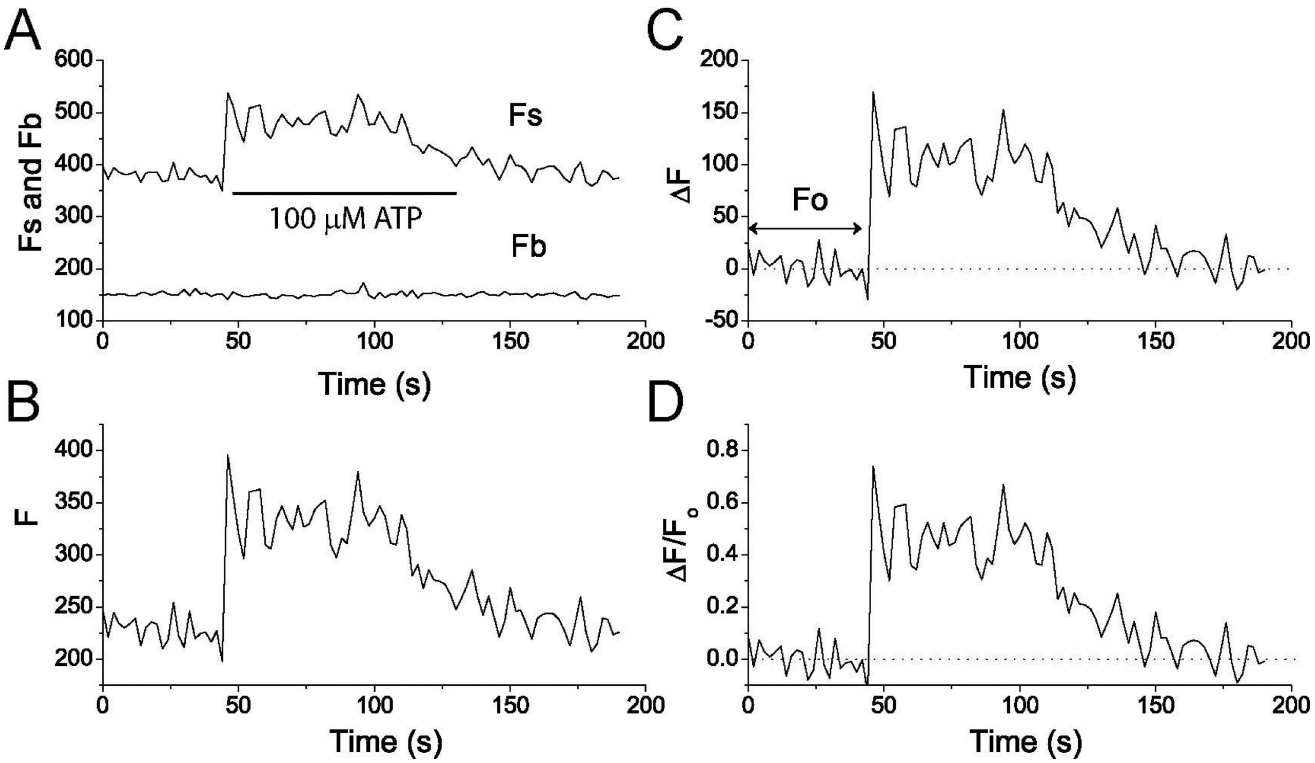

Figure 5. Analysis of mitochondrial Ca^{2+} signal based on mito-GCaMP5G fluorescence

A) Raw data of $F(t)_s$ and $F(t)_b$. B) background subtracted fluorescence $F(t)=F(t)_s-F(t)_b$. C) Baseline subtracted fluorescence change $\Delta F\ (t)=F(t)-Fo$. D) Ca^{2+} changes expressed as $\Delta F(t)/Fo$.

Table 1

α-Modified Minimal Essential Media (α-MMEM)

Ingredient	Supplier	Caltalog #	Concentration	Amount/100 ml
α-MEM	Gibco	41061-019	86%	86 ml
Serum	Hyclone	SV30014.03	10%	10 ml
Glutamine (200 mM)	Gibco	25030-081	2 mM	1 ml
D-glucose (2M)	Sigma	G7021	20 mM	1 ml
Na-pyruvate (100 mM)	Gibco	11360-070	1 mM	1 ml
Penn/Strep	Gibco	15140-122	1%	1 ml
Na-bicarbonate	Sigma	S5761	14 mM	117.6 mg