Title: Investigating mitochondrial Ca^{2+} dynamics in isolated mitochondria and intact cells: Application of fluorescent dyes and genetic reporters

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Abstract

Mitochondrial Ca^{2+} buffering is a hallmark of eukaryotic cellular physiology, contributing to the spatiotemporal shaping of the cytosolic Ca^{2+} signals and regulation of mitochondrial bioenergetics. Often, this process is altered in a pathological context, therefore it can be scrutinised experimentally for therapeutic intervention. In this chapter, we describe fluorescence and bioluminescence measurement of mitochondrial Ca^{2+} in both isolated mitochondria and intact cells.

Keywords

Mitochondrial calcium, fluorescence calcium imaging, Calcium sensitive genetic probes, bioluminescence calcium sensing

Introduction

Cytosolic Ca^{2+} signals are shaped by mitochondrial Ca^{2+} buffering, linking an increase in bioenergetic demand to ATP production. When cytosolic Ca^{2+} concentrations rise above a certain threshold, energised mitochondria take up Ca^{2+} in an electrogenic manner, primarily through the MCU complex. The activity of three rate-limiting mitochondrial enzymes in the TCA cycle is in turn upregulated by Ca^{2+} ions, thus providing more reducing power in the form of NADH to the electron transport chain for ATP synthesis[1]. Mitochondrial Ca^{2+} levels are further modulated by efflux through the Na^{+}/Ca^{2+} exchanger at physiological levels, as a pathological increase in mitochondrial Ca^{2+} levels can induce the opening of the mitochondrial permeability transition (mPTP), ultimately resulting in bioenergetic collapse and cell death. Not surprisingly, perturbations in mitochondrial Ca^{2+} buffering, have been widely implicated in neurodegenerative disease (reviewed in [2]). Therefore, monitoring dynamic changes in

mitochondrial Ca^{2+} levels can illuminate pathological mechanisms underlying disease aetiology.

The techniques to monitor mitochondrial Ca^{2+} are continuously evolving; here we will briefly describe four different techniques that are routinely used in our laboratory.

i) Use of extramitochondrial Ca^{2+} -sensitive fluorescent dyes with isolated mitochondria in a fluorescence plate reader
ii) Use of intracellular AM-ester based Ca^{2+} - sensitive cationic fluorescent dyes in intact cells
iii) Use of fluorescence based genetic probe, 2mtGcamp6, in intact cells: Ideal for ratiometric single cell analysis to identify relative inter-population and intra-population differences
iv) Use of luminescence based genetic probe, mitoaequorin, in intact cells: Ideal for cell population mitochondrial Ca^{2+} analysis

Materials

Measuring mitochondrial Ca^{2+} in isolated mitochondria using a fluorescence plate reader

1. Isolated mitochondria
2. Fluo-5N, pentapotassium salt **Note 1**
3. MSK buffer: 75 mM Mannitol, 25 mM Sucrose, 5 mM Potassium phosphate monobasic, 20 mM Tris-HCl, 100 mM Potassium chloride, 0.1% (w/v) BSA, pH adjusted to 7.4
4. Succinate
5. Rotenone
6. Calcium Chloride
7. 96 well clear or black plastic plate
8. Fluorescence plate reader with the following optical requirements= Excitation:494 nm, Emission=516 nm, and integrated fluid addition function

Measuring mitochondrial Ca^{2+} in intact cells using Rhod-2, AM or 2mtGcaMP6 with fluorescence microscopy

1. Rhod-2, AM ester **Note 1**
2. MitoTracker Green
3. 2mtGcamp6 adenovirus or plasmid

4. MitoTracker Deep Red
5. Recording buffer: Any medium that is compatible with your cells, ideally the medium used to grow the cells without phenol red and with additional pH buffers such as HEPES
6. Glass coverslips/imaging cells
7. For Rhod-2: Fluorescence microscope with the following optical requirements: Excitation = ≈552 nm, Emission = ≈550-610 nm
   For 2mtGcaMP6: Fluorescence microscope with the following optical requirements: Isosbestic Ca^{2+} independent excitation= ≈410 nm, Ca^{2+} dependent excitation = ≈480 nm, Emission = ≈500-550 nm
8. Cytosolic Ca^{2+} agonist: for example, glutamate or ATP
9. Ionomycin

Measuring mitochondrial Ca^{2+} in intact cells using mitoaequorin using a luminescence plate reader

1. Mitoaequorin adenovirus or plasmid
2. Krebs Ringer Buffer: 125 mM NaCl, 5.5 mM D-Glucose, 5 mM KCl, 20 mM HEPES, 1 mM Na_3PO_4, 1 mM Glutamine, 100 mM Pyruvate, 1.2 mM $CaCl_2$, pH adjusted to 7.4
3. White 96 well polystyrene plate
4. Coelenterazine (Commercially available)
5. Luminescence plate reader with integrated fluid addition function
6. Cytosolic Ca^{2+} agonist: for example, glutamate or ATP
7. Digitonin

Methods

Measuring mitochondrial Ca^{2+} uptake in isolated mitochondria using an extra-mitochondrial fluorescence indicator

1. Add 10 mM succinate and 1 µM rotenone to the MSK buffer; this promotes mitochondrial respiration and therefore allows mitochondrial Ca^{2+} uptake by maintaining the mitochondrial membrane potential. Prepare a 1 µM Fluo-5N solution using the buffer (Fluo-5N buffer).
2. Make a 100 µM $CaCl_2$ solution in the Fluo-5N buffer, to be added by the integrated fluid injections of the plate reader.

3. Set up the fluorescence plate reader: excitation and emission settings, priming the injector and setting the temperature to 30°C. Adjust the gain using a well containing the 100 µM $CaCl_2$ as a maximum reading.
4. Make a 0.5 mg/ml mitochondrial suspension in the Fluo-5N buffer and add 100 µL of the suspension per well of the 96 well plate in triplicate for each condition.
5. Add a bolus of 10 µM $CaCl_2$ to each well and measure the response every 10 seconds. Addition of the $CaCl_2$ will cause an increase in fluorescence intensity, followed by a decay as the Ca^{2+} is buffered by the mitochondria. Fitting a slope to this decrease can provide a readout for rate of mitochondrial Ca^{2+} uptake.
6. The protocol can be further modified to include repeated 10 µM $CaCl_2$ additions to determine the calcium retention capacity of mitochondria, beyond which the mitochondria undergo permeability transition (mPTP). As a result, all the buffered Ca^{2+} is released causing a massive increase in fluorescence. The area under the curve can provide a surrogate for amount of Ca^{2+} buffered or the "calcium retention capacity", which can be compared between different conditions. Fluid additions should be set up at regular intervals. **Note 2**

Measuring mitochondrial Ca^{2+} in intact cells using Rhod-2, AM or 2mtGcaMP6 with fluorescence microscopy

1. The seeding density of cells will be determined by cell type, where the user should aim to have a healthy monolayer of cells on the day of imaging, allowing single cell resolution. **Note 3**
2. For Rhod-2: On the day of the assay, aspirate the growth medium and wash the cells. Replace the medium with recording buffer containing 5 µM Rhod-2, 100 nM Mitotracker Green and 0.002% pluronic acid. Incubate in the dark at room temperature for 30 min. Aspirate the dye solution, wash the cells and replace with pre-warmed recording buffer. **Note 4**
3. For 2mtGcaMP6: Transfect the cells with the 2mtGcamp6 with your method of choice, 48 hours before imaging. On the day of the assay, aspirate the growth medium, wash the cells and replace it with pre-warmed recording buffer. **Note 5**
4. If possible, maintain the temperature of the cells at 37°C during the course of the experiment.
5. Set up the microscope with the optical paths described:
   For Rhod-2: Excitation = ≈552 nm, Emission = ≈550-610 nm

For MitoTracker Green= ≈490 nm, Emission = ≈500-630 nm

For 2mtGcaMP6: Isosbestic Ca^{2+} independent excitation= ≈410 nm; Ca^{2+} dependent excitation = ≈480 nm; Emission = ≈500-550 nm.

For Mitotracker Deep Red = ≈644 nm, Emission = ≈665-700 nm

6. Adjust the parameters of your fluorescence system for both optical paths (i.e exposure/laser power of excitation, master gain, digital offset). If using a confocal, adjust the pinhole to ensure the best compromise between signal:ratio and resolution.

   For 2mtGcaMP6: The isosbestic excitation track will have a lower intensity and provides a measure for expression of the protein. This in turn offers an internal Ca^{2+} independent control for each cell, allowing relative comparisons between individual cells and populations. For the initial experiments, we recommend confirming mitochondrial localisation of the genetic probe by co-staining with commercially available live mitochondrial dyes like MitoTracker Deep Red.
7. For measuring basal mitochondrial Ca^{2+} levels, take multiple images at the desired magnification. We recommend imaging between 10-50 cells for relatively homogenous populations.
8. In order to look at kinetic responses to external stimuli, set up a time series with a relatively short time interval (≈1s). You might have to reduce resolution to speed up acquisition.
9. After capturing 3-5 baseline images, administer your calcium agonist carefully using a Gilson pipette or perfusion system if available. Please ensure that the z-plane is not disturbed.
10. Monitor the change in fluorescence in both optical tracks.

    For Rhod-2 and MitoTracker Green: Ideally you should see an increase in intensity in the Rhod-2 image.

    For 2mtGcaMP6: Ideally you should see an increase in intensity in the 488nm image, while the 410nm image remains unchanged.
11. Wait 3-5 minutes, for the fluorescence trace to plateau and/or return to baseline. Following this, add the ionomycin solution at a final concentration of 2 µM as a positive control. Ionomycin is an ionophore that should result cause an influx of Ca^{2+} into the mitochondria, causing a significant increase in the intensity of the Rhod-2 image and the 488nm image of 2mtGcaMP6.

12. Once the images have been obtained, they can be analysed using any image processing software (We recommend ImageJ). Subtract background from each image. Draw regions of interest around every cell in a field of view.

    For Rhod-2 and MitoTracker Green: Rhod-2 is non-specific and will be visible in both the cytosol and the mitochondria. In order to isolate the mitochondrial signal, use the MitoTracker Green image to create a mask (threshold the image and apply a binary mask) and use this mask to measure the mitochondrial-specific Rhod-2 signal. For quantitative comparisons, the Rhod-2 signal can be calibrated using the following equation, where R_f (the dynamic range of the indicator) can be determined using commercially available calibration kits. F_{max} is the maximum signal obtained after application of ionomycin [3]

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$$[Ca^{2+}] = K_D \times \left(\frac{F}{F_{max}} - \frac{1}{R_F}\right) \Big/ \left(1 - \frac{F}{F_{max}}\right)$$

    For 2mtGcaMP6: Apply a threshold to only include mitochondrial pixels, before measuring mean intensity for both the 488 nm image and the 410 nm image. A ratio of 488 nm/410 nm intensities should provide a relative measure of mitochondrial Ca^{2+} concentrations and can be used to compare across conditions. Exclude cells that appear to be very bright (implying overexpression of the plasmid), as it might provide inaccurate results.

Measuring mitochondrial Ca^{2+} in intact cells using the bioluminescent probe, mitoaequorin

1. Plate 30-50k cells per well in the 96 well plate, in triplicate for each condition.
2. Transduce the cells with the mitoaequorin adenovirus 48 hours prior to the assay. **Note 5**
3. On the day of the assay, aspirate media from wells and replace it with Krebs Ringer buffer containing 5 µM coelenterazine. Incubate the plate in the dark for 2 hours at 37°C to reconstitute the mitoaequorin with its prosthetic group, coelenterazine. **Note 6**
4. While the plate is being incubated, prepare the following solutions:
   - 5 ml of Ca^{2+} agonist (10X stock) in Krebs ringer buffer (Suggested final concentrations: 10µM glutamate, 100µM ATP)
   - 5 ml of 1mM digitonin and 100 mM $CaCl_2$ in H_2O.

5. Set up the luminescence plate reader to add the Ca^{2+} agonist solution after 5 seconds and the digitonin solution after 30 seconds. Measure every second for a minute. The digitonin addition provides the luminescence value for aequorin in saturating Ca^{2+} conditions. **Note 7**
6. Run the assay at 37°C and collect the data for analysis.
7. Accurate mitochondrial [Ca^{2+}] for each timepoint can be calculated as follows:

L= (luminescence (counts per second))– (minimum luminescence count)

L_{max}= (Sum of luminescence counts from the given timepoint till completion of experiment)

Ratio =$\left(\frac{L}{L_{max}}\right)^{1/n}$

Equation:

$$Ca^{2+}(M)=\frac{\text{ratio}+(\text{ratio}\cdot K_{TR})-1}{K_R-(ratio.K_R)}$$

Where K_R= 10366185, K_{TR} = 120 , n= 2.99 for wild-type aequorin at 37°C. **Note 8[4]**

The parameters that can be measured in the above techniques include and are not limited to, rate of mitochondrial Ca^{2+} uptake, maximum [Ca^{2+}] peak, quantity of Ca^{2+} buffered, mitochondrial Ca^{2+} efflux rate to name a few. Resting mitochondrial [Ca^{2+}] can also be determined by the fluorescence microscopy techniques, provided appropriate calibrations are carried out. **Note 9**

Notes

1. For all methods described, the Kd of the chosen probe or dye should be compatible with the expected changes in [Ca^{2+}], to avoid erroneous results.

2. The number of cycles needs to be determined for your setup. Ideally mitochondrial Ca^{2+} uptake should be complete, i.e., the fluorescence should return to baseline, before the next addition of Ca^{2+}.

3. For certain cell types like neurons, low seeding density can have a negative impact on viability, whereas for other cell types, higher levels of confluency can cause crowding and cell death. The seeding density therefore needs to be optimised for your experimental setup.

4. The AM ester derivative of Rhod-2 is crucial as it is cell permeable. Furthermore, we have observed better mitochondrial loading of the dye at room temperature, due to decreased activity of the esterases allowing the dye to cross the mitochondrial membrane before being cleaved and trapped in the cytosol. Pluronic acid is a mild detergent which facilitates dispersal of the AM ester dyes, and therefore promotes cell loading.

5. The method of transfection will be dependent on cell type and the available technology. In our laboratory, we routinely use adenovirus to transduce primary cell cultures for higher transfection efficiency, which is essential for the mitoaequorin protocol.

6. If possible, always use Kreb's Ringer Buffer as coelenterazine is hydrophobic and serum containing medium can reduce bioavailability. If using serum containing medium for sensitive cultures, you might have to use higher concentrations of coelenterazine

7. Due to the nature of fast kinetics, we recommend running the assay in well mode (i.e., measure the entire time series in one well at a time). Automated fluid additions will further eliminate any loss of data due to delayed readings.

8. The constants for calibrating the aequorin measurements vary based on the type of aequorin and the temperature. These values can be found online.

9. Mitochondrial Ca^{2+} uptake or mitochondrial Ca^{2+} efflux can be pharmacologically targeted to examine the effects on mitochondrial Ca^{2+} dynamics using these techniques.

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