Title: Monitoring mitochondrial membrane potential in live cells using time-lapse fluorescence imaging

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Abstract

The mitochondrial membrane potential (ΔΨm) generated by proton pumps (Complexes I, III and IV) is an essential component in the process of energy generation during oxidative phosphorylation. Tetramethylrhodamine, methyl ester, perchlorate (TMRM) is one of the most commonly used fluorescent reporters of ΔΨm. TMRM is routinely employed in a steady state for the measurement of membrane potential. However, it can also be utilised with time-lapse fluorescence imaging to effectively monitor the changes in membrane potential in response to a given stimulus by analysing the change in distribution of the dye with time.

Key Words: Mitochondria membrane potential, TMRM, fluorescence microscopy, uncoupler, primary skin fibroblasts

Introduction

The mitochondrial membrane potential (ΔΨm) is a semi-quantitative read-out for the full proton-motive force defined as the difference in electrical potential between the mitochondrial matrix and the cytosol [1]. Alteration in ΔΨm can indicate disruption in oxidative phosphorylation, ATP synthesis, or ionic exchange through the mitochondrial membrane [2, 3].

TMRM, tetramethylrhodamine, ethyl ester (TMRE), and JC1 are cationic, membrane permeant reporters that are commonly used to measure mitochondrial membrane potential [4-6]. These dyes are able to cross the cell membrane and partition between compartments depending on the electrochemical potential gradients [2]. The magnitude of the accumulation of these dyes in negatively charged compartments is described by the Nernst equation [2], but is also affected

by the mitochondrial binding of the probe. TMRM is widely used due to its low toxicity, rapid equilibration and ability to be excited on epifluorescence, confocal, or two-photon microscopes (Fig. 1).

In general, two types of approaches can be performed when using TMRM at low concentration (5-25 nM): (i) dynamic measurements to stablish differences in cell–cell signalling or pathophysiological condition, and (ii) a comparison of populations of cells that have been previously exposed to different conditions. It is also possible to quantify the change in membrane potential as a function of time in response to a given stimulus.

Using TMRM and a confocal microscope, bright pixels can be localised in mitochondria leaving black pixels as a background (Fig. 2). Therefore, when plotting the fluorescence distribution of the pixels along the cell, a high standard deviation will result, and these data can be divided by the mean fluorescence values to calculate changes over a period of time (Fig. 3) [7]. After applying an external variable, illustrated here with the response to an uncoupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), the fluorescence from mitochondria will decrease and that of the cytosol will increase as the dye redistributes between compartments.

Overall, the cell fluorescence will remain stable in the short term, due to the distribution of the dye between the mitochondria and the cytosol. In the longer term, the dye will start to leak from the cell. Thus, we have set up a simple experiment where we can interrogate the response of the mitochondrial membrane potential

Materials

Recording medium: Prepare recording medium containing phenol red-free DMEM (Gibco, A1443001) with 10 mM glucose, 1 mM glutamine, 10 mM HEPES, adjusted to pH 7.4.

TMRM stock solution: Prepare a 10 mM stock solution of TMRM by dissolving 5 mg of TMRM in 1 ml of dimethyl sulfoxide (DMSO; Sigma-Aldrich, D2650). Make aliquots and store them at -20°C protected from light.

Staining solution: Prepare a staining solution of the TMRM reagent by diluting to 25 nM in recording medium. Additionally, use Hoechst 33342 at 5 µM for visualising the nuclear structure in live cell imaging **(Note 1).** Prepare a fresh mixture when working different days and protect the solution from direct exposure to light.

Drugs: Prepare 1 µM of FCCP, following the manufacturer's instructions. This compound will depolarise the mitochondrial membrane potential so then, changes in fluorescence can be compared among conditions over time. This same approach can be used to explore potential mitochondrial toxicity of drugs, or of any biological reagents of interest.

Additional reagent: Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, 14190094), glass-bottom 24-well plates (Greiner Bio-One, 662892).

Methods

Seeding and staining skin fibroblasts

The entire procedure should be conducted at room temperature unless otherwise stated in the text.

1. Seed cells at appropriate density ($1x10^4$/well) in glass-bottom 24-well plates or similar imaging plates 2-3 days before imaging. **Note 2**

2. Wash the cells twice with pre-warmed Recording Medium without Hoechst 33342 or DPBS.

3. Incubate the cells with recording medium containing 25 nM of TMRM and 5 µM Hoechst 33342 for 30 minutes at 37°C.

4. Replace the medium with Recording Medium without Hoechst 33342.

5. Set up the stage and focus.

6. To perform live imaging of cells incubated with TMRM and record dynamic changes, confocal laser scanning microscopy (LSM 880, Carl Zeiss Inc.), with the application of live time-series program, is used **(Note 4).** Apply low-resolution if needed and attenuated laser power. Excite TMRM with a 561 nm Argon laser with a output power

of 0.2 mW. Hoechst 33342 needs to be excited at 405 nm and it is recommended to start with low laser power when using this channel, as TMRM is very phototoxic. **Note 5**

7. Start acquiring images in basal condition during a few seconds.

8. Add FCCP into the imaging plate, according to previous determined concentration. Wait for the dissipation of the mitochondrial potential and the distribution of the TMRM.

9. Save the time-series and use an appropriate software to obtain the data. Fiji (image J) could be useful for this purpose.

Data analysis

1. Define a threshold for the images to exclude black pixels and then select and calculate the average fluorescence intensities from all ROIs of each cell for TMRM and its standard deviation of pixels over the arbitrary threshold.

2. Select regions next to the cells to calculate the background fluorescence intensity.

3. Subtract the average background fluorescence intensity from average fluorescence intensities of ROIs in each cell for each time point using Microsoft Excel.

4. Plot the standard deviation over the mean signal and analyse the results.

Notes

1. Hoechst 33342 concentration can be modified depending on cell type and it needs to be manipulated with caution since Hoechst strains are known mutagens. Staining intensity may increase with time if samples are imaged without washing.

2. Different cell densities can be used to determine the mitochondrial membrane potential. It is a common practice to plate cells and wait for them to grow until confluence before imaging.

3. A different microscope can be used depending on the availability.

4. Make sure of carefully adding the FCCP. Disturbing the plate will change the focus and the final analysis cannot be performed.

5. If needed, it is possible to use the same concentration of TMRM (25nM) and microscope settings to analyze the steady state measurements of pretreated cells. We recommend acquiring multiple Z-stacks of the cells and use Fiji (image J) to obtain the maximal intensity projection of the images. Then, compare the mean intensities.

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Figure legends:

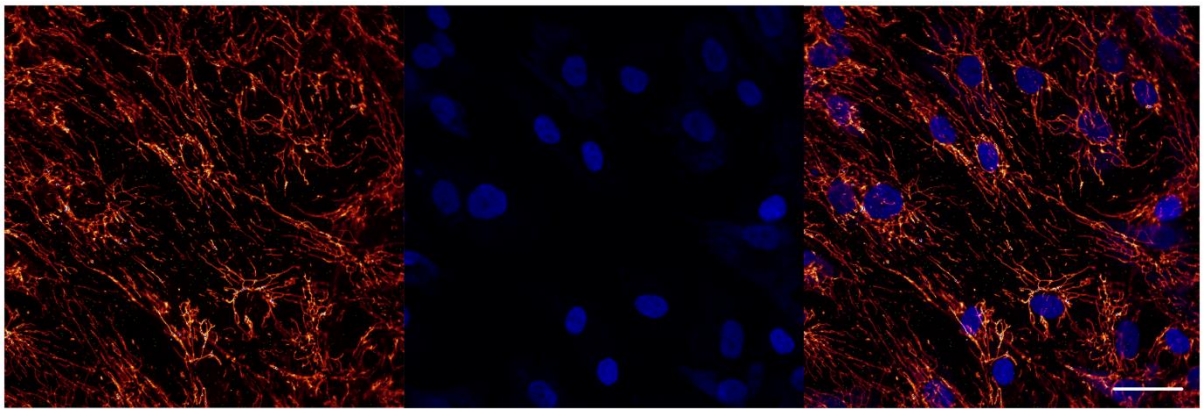

Figure 1: Equilibration of 25 nM TMRM in the mitochondria after 30 minutes of incubation in skin fibroblasts. Nuclei were labelled with Hoechst 33342.

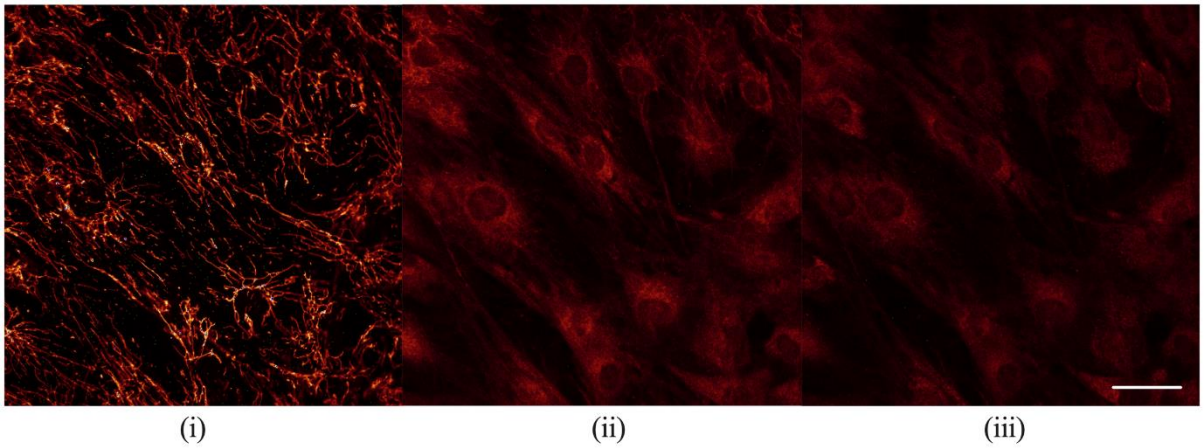

Figure 2: Distribution of TMRM in skin fibroblasts before (i), during (ii) and after (iii) the FCCP treatment.

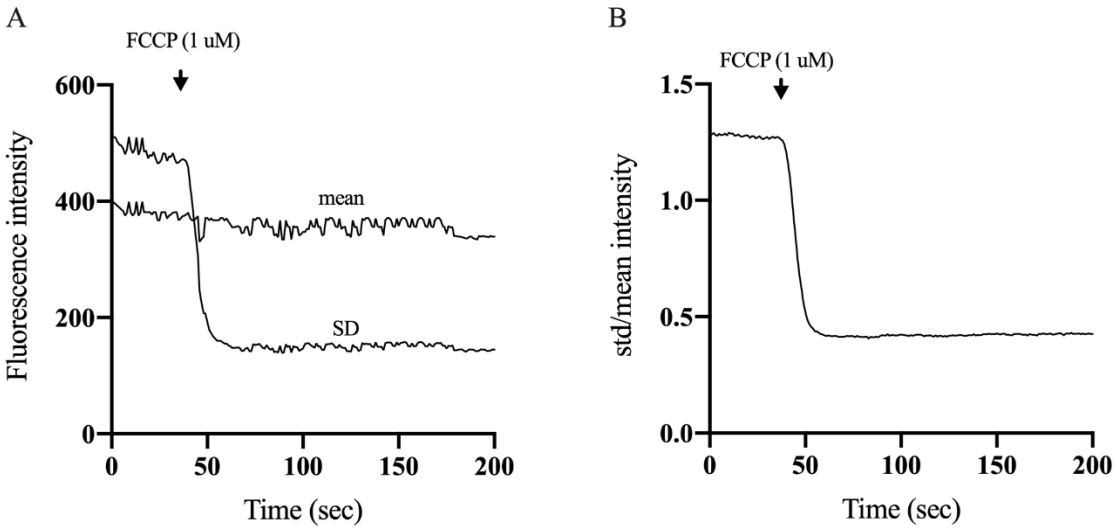

Figure 3: A) Mean intensity and its SD over a region of interest, and B) shows the ratio of SD/mean fluorescence signal.