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Computer-assisted live cell analysis of mitochondrial membrane potential, morphology and calcium handling

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ABSTRACT

Mitochondria are crucial for many aspects of cellular homeostasis and a sufficiently negative membrane potential ($\Delta\psi$) across the mitochondrial inner membrane (MIM) is required to sustain most mitochondrial functions including ATP generation, MIM fusion, and calcium uptake and release. Here, we present a microscopy approach for automated quantification of $\Delta\psi$ and mitochondrial position, shape and calcium handling in individual living cells. In the base protocol, cells are stained with tetramethyl rhodamine methyl ester (TMRM), a fluorescent cation that accumulates in the mitochondrial matrix according to $\Delta\psi$, and visualized using video-microscopy. Next, the acquired images are processed to generate a mitochondria-specific binary image (mask) allowing simultaneous quantification of mitochondrial TMRM fluorescence intensity, shape and position. In a more advanced version of this protocol a mitochondria-targeted variant of green fluorescent protein (mitoAcGFP1) is expressed to allow mask making in TMRM-stained cells. The latter approach allows quantification of $\Delta\psi$ in cells with a substantially depolarized $\Delta\psi$. For automated quantification of mitochondrial calcium handling in space and time mitoAcGFP1-expressing cells are stained with rhod-2, a fluorescent calcium indicator that accumulates in the mitochondrial matrix. In this paper, a detailed step-by-step description of the above approaches and its pitfalls is provided.

1. Mitochondrial membrane potential

Important mitochondrial functions like ATP generation, protein import, organelle fusion and Ca^{2+} uptake/release depend on a sufficiently large electrochemical proton gradient across the mitochondrial inner membrane (MIM). This gradient is maintained by the action of four protein complexes (complex I to complex IV), which together constitute the electron transport chain (ETC). The energy generated by the electron transport is used to extrude protons from the mitochondrial matrix across the MIM (at complexes I, III and IV). The energy, or proton motive force (PMF), stored in the proton gradient consists of an electrical ($\Delta\psi$) and chemical component (ΔpH). At complex V, reentry of protons in the mitochondrial matrix is used to drive ATP production. The electrical and chemical contribution to the PMF is given by [1]:

$$PMF = \Delta\psi - \frac{2.303 \cdot RT \cdot \Delta pH}{F} \quad (1)$$

where F is Faraday's constant, R is the ideal gas constant and T is the absolute temperature. The PMF is dominated by the mitochondrial membrane potential ($\Delta\psi$; negative inside), with ΔpH only contributing ~15% to the total PMF [1,2]. Therefore, $\Delta\psi$ is regarded as a key-indicator of mitochondrial health and metabolic activity and its quantification in intact cells is important to address many research questions. Most assays for monitoring $\Delta\psi$ in living cells employ lipophilic fluorescent cations, which pass cellular and mitochondrial membranes and accumulate within the mitochondrial matrix in a $\Delta\psi$-dependent manner [3,4]). The extent of uptake of these cations in response to $\Delta\psi$ is described by the Nernst equation [5]:

$$\Delta\psi(mV) = \frac{2.303 \cdot RT}{zF}{}^{10}\log(C_m/C_{cyt}) \quad (2)$$

where z is the charge of the permeable cation, C_m is the mitochondrial concentration of the cation and C_{cyt} is the cytosolic concentration of the cation. At 37 °C the factor before the logarithmic term for

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monocations is 61 mV, therefore, as the mitochondrial membrane potential *in vivo* is approximately. 120–180 mV [6], lipophilic monocations accumulate 10^2–10^3-fold within mitochondria [5]. Importantly, this accumulation not only depends on $\Delta\psi$ but also on the potential difference across the plasma membrane (ΔV):

$$C_m = C_{ext} \exp[-(\Delta V + \Delta\psi)zF/RT] \quad (3)$$

where C_{ext} is the extracellular concentration of the cation [4,7]. This means that experimental quantification $\Delta\psi$ should always be accompanied by control experiments ruling out changes in ΔV. An approach allowing proper quantification of $\Delta\psi$ in the presence of ΔV alterations has been described elsewhere [8].

2. Mitochondrial potentiometric dyes

Rhodamine 123 (R123), the methylester (TMRM) and ethylester (TMRE) of tetramethylrhodamine, and the ratiometric indicator 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) have been most widely applied to monitor $\Delta\psi$ [9–15]. Of these cations, TMRM is best suited for $\Delta\psi$ quantification because it is least toxic to mitochondria, equilibrates fastest across membranes and displays lowest aspecific binding [3,4]. Here, it is important to emphasize that accumulation of this dye above a certain threshold concentration leads to autoquenching making $\Delta\psi$ to appear depolarized [4]. To test for autoquenching, cells are loaded with increasing concentrations of TMRM and subsequently treated with *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) to dissipate $\Delta\psi$. If the increase in extracellular TMRM concentration does not alter the intensity of the mitochondrial TMRM signal and if FCCP induces a decrease instead of an increase in TMRM fluorescence, autoquenching is absent [16]. While TMRM dequenching can also be used to monitor $\Delta\psi$, we here focus on the use of TMRM in the nonquenching mode. In this mode, a decrease in mitochondrial TMRM fluorescence intensity reflects a reduced accumulation of TMRM in the mitochondrial matrix and thus a more depolarized $\Delta\psi$. Although the different fluorescent cations preferentially accumulate in the mitochondrial matrix, a certain level of fluorescence is always present within the cytosol and nucleoplasm [10,17]. Because the phosphorylation state of the cytosolic ATP pool is very sensitive to $\Delta\psi$ it is important to exclude these nonmitochondrial signals from the analysis [18]. We first present a protocol for simultaneous quantification of mitochondrial TMRM intensity (Section 3) and shape (Sections 4 and 5) in living cells. Next, we extend this approach to quantify spatiotemporal mitochondrial Ca^{2+} handling (Section 6) and position (Section 7). Finally, we briefly discuss possible caveats of our approach and their solution (Section 8).

3. Quantification of mitochondrial TMRM intensity by live cell microscopy

3.1. Cell culture

In our research, which focuses on the cytopathology of ETC disorders (see also the articles by Calvaruso et al. and Aiyar et al., this issue), we use primary skin fibroblasts of healthy individuals and patients with isolated ETC deficiency [19–21]. We have learned that, because of their flat appearance, these cells are perfectly suited for imaging mitochondria-specific fluorescence and subsequent computer-assisted quantification of the morphological and functional parameters of these organelles [17]. Alternatively, for less flat cells/specimens, other techniques are available (*Song* et al. and Tanji and Bonilla, this issue). Three days prior to microscopy analysis, cells are seeded on 24 mm circular coverslips (Omnilabo International, Breda, The Netherlands) placed in 35 mm Cell Star® tissue culture dishes (Sigma). During culture, a fibroblast-optimized medium is used (medium 199 with Earle's salt) supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin (Invitrogen, Breda, The Netherlands). Culture dishes are kept in a humidified atmosphere (95% air, 5% CO_2, 37 °C) until ~70% cell confluence is reached.

3.2. Digital imaging microscopy

To make a 'master' stock solution, TMRM (Invitrogen) is dissolved in dry DMSO (dimethylsulfoxide; Sigma) to a final concentration of 1 mM, subdivided into aliquots and stored at −20 °C. On the day of the experiments, one vial of 'master' stock solution is thawed to create a 'working' stock solution (10 μM TMRM in DMSO). At the end of the day the thawed vial with 'master' stock solution is discarded. Immediately before use, 10 μl of 10 μM 'working' stock solution is pipetted into 1 ml of medium 199 and thoroughly mixed. This 'loading solution' (containing 100 nM TMRM) is added to the culture dish and cells are incubated for 25 min at 37 °C in the dark (incubator). After loading, cells are thoroughly washed with phosphate-buffered saline. Next, coverslips are mounted in a temperature-controlled incubation chamber (37 °C) placed on the stage of an inverted microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany). Although we use custom manufactured incubation chambers, the latter are also commercially available (e.g., Invitrogen, Carl Zeiss). During fluorescence recordings, cells are maintained in a colorless HEPES-Tris (HT) solution (132 mM NaCl, 4.2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM D-glucose, 10 mM HEPES, pH 7.4). For acquiring TMRM fluorescence emission images, the dye is excited with 540 nm light (bandwidth: 8–15 nm) during 100 ms. Excitation light originates from a monochromator (Polychrome IV, TILL Photonics, Gräfelfing, Germany) and is delivered to the cells via a quartz fiber and a ×63, 1.25 NA Plan NeoFluar oil-immersion objective (Carl Zeiss). TMRM fluorescence is directed by a 560DRLP dichroic mirror (Omega Optical Inc., Brattleboro, VT, USA) through a 565ALP emission filter (Omega) onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific Photometrics, Vianen, The Netherlands). This cooled CCD camera system provides a 1392 × 1040 imaging array, has a pixel size of 6.45 × 6.45 μm and displays maximal quantum efficiency (50–60%) between 400 and 700 nm. The latter property makes this camera particularly well suited for the fluorescent reporter molecules (TMRM, Rhod-2, AcGFP1) described in this paper. Emission filters are placed in a motorized filter wheel (Sutter Instrument Company, Novato, CA, USA). Selection of excitation wavelengths, dichroic mirrors and emission filters, as well as image acquisition and microscopy functions, are controlled using Metafluor 6.0 software (Molecular Devices Corporation, Downingtown, PA, USA) running on a PC with Windows XP (Microsoft Corporation, Redmond, WA, USA).

3.3. Computer-assisted quantification of mitochondrial TMRM fluorescence

We demonstrated that when the above protocol is used, TMRM autoquenching, dye leakage and/or extrusion, photobleaching or light-induced opening of the mitochondrial permeability transition pore (PTP) are absent [16]. Routinely, 20–25 FOVs (fields of view) are recorded (containing ~20–40 cells) from a cross-shaped area transecting the center of the coverslip. For each cell line and/or experimental condition, measurements are at least performed in triplicate on three different days. Each acquired image (Fig. 1A; 'RAW') is background corrected ('BC') to yield a 'COR' image (Fig. 1B). This is performed by subtracting an image acquired from a region of the coverslip devoid of cells. To determine mitochondrial position, the COR image is converted into a binary image