# Chapter 12

# Relation Between Mitochondrial Membrane Potential and ROS Formation

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## Abstract

Mitochondria are considered as the main source of reactive oxygen species (ROS) in the cell. For this reason, they have been recognized as a source of various pathological conditions as well as aging. Chronic increase in the rate of ROS production is responsible for the accumulation of ROS-associated damages in DNA, proteins, and lipids, and may result in progressive cell dysfunctions and, in a consequence, apoptosis, increasing the overall probability of an organism's pathological conditions. The superoxide anion is the main undesired by-product of mitochondrial oxidative phosphorylation. Its production is triggered by a leak of electrons from the mitochondrial respiratory chain and the reaction of these electrons with $O_2$. Superoxide dismutase (MnSOD, SOD2) from the mitochondrial matrix as well as superoxide dismutase (Cu/ZnSOD, SOD1) present in small amounts in the mitochondrial intramembrane space, convert superoxide anion to hydrogen peroxide, which can be then converted by catalase to harmless $H_2O$. In this chapter, we describe a relation between mitochondrial membrane potential and the rate of ROS formation. We present different methods applicable for isolated mitochondria or intact cells. We also present experiments demonstrating that a magnitude and a direction (increase or decrease) of a change in mitochondrial ROS production depends on the metabolic state of this organelle.

**Key words:** Mitochondria, Membrane potential, ROS, Amplex Red, JC-1, Superoxide, Respiration, Confocal microscopy

## 1. Introduction

It has been repeatedly demonstrated, on different experimental models, that a strong positive correlation exists between mitochondrial membrane potential ($\Delta\Psi$) and reactive oxygen species (ROS) production (1, 2). At present, it is widely accepted that

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Carlos M. Palmeira and António J. Moreno (eds.), *Mitochondrial Bioenergetics: Methods and Protocols*, Methods in Molecular Biology, vol. 810, DOI 10.1007/978-1-61779-382-0_12, © Springer Science+Business Media, LLC 2012

mitochondria produce more ROS at high membrane potential. It has been shown that ROS production dramatically increases above 140 mV (2). Studies performed on mitochondria from *Drosophila melanogaster* showed that even a slight decrease in the ΔΨ (10 mV) can cause a significant decrease in ROS production (according to authors the decrease of ROS production diminished by approximately 70%) by complex I of the respiratory chain (3). In contrast, an increase in the ΔΨ produced either by a closure of the mitochondrial permeability transition pore or an inhibition of ATP synthase (4) is associated with increased ROS production. Interestingly, in certain pathological conditions, opposite correlations between ΔΨ and ROS production can also be observed. In the case of ATP synthase dysfunction (mutation T8993G in the mitochondrial ATPase-6 gene), higher ΔΨ and increased ROS production is observed (5). On the other hand, in the case of mitochondrial disorders associated with the dysfunctions of the respiratory chain components, lower ΔΨ and decreased activity of the respiratory chain is observed with a simultaneous increase in ROS production (6).

## 2. Materials

### 2.1. Isolation of Crude Mitochondria from Mouse Brain

1. Stirrer motor with electronic speed controller.
2. Motor-driven tightly fitting glass/Teflon Potter Elvehjem homogenizer.
3. Loose fitting glass/Teflon Potter Elvehjem homogenizer.
4. Homogenization buffer (320 mM sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4). Store at 4°C (see Note 1).
5. Mitochondria isolation buffer (75 mM sucrose, 225 mM mannitol, 5 mM Tris–HCl, pH 7.4). Store at 4°C (see Note 2).

### 2.2. Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (heat inactivated), 2 mM L-glutamine, and 1.2% antibiotic: penicillin/streptomycin.
2. KRB saline (135 mM NaCl, 5 mM KCl, 0.4 mM $KH_2PO_4$, 1 mM $MgSO_4$, 20 mM HEPES, bring to pH 7.4 with NaOH). Glucose 1 g/L and 1 mM $CaCl_2$ should be added the day of experiment.
3. Fibroblasts:
   (a) Primary culture of human skin fibroblasts grown from explants of skin biopsies of control individual and patient with mitochondrial disorder.
   (b) NHDF, neonatal dermal fibroblasts (Lonza).

4. Ehrlich ascites tumor cells were cultivated in Swiss albino mice and harvested as described in ref. (7).
5. 24-Well Cell Culture Cluster.

### 2.3. Measurement of Hydrogen Peroxide Production in Isolated Brain Mitochondria

1. Spectrofluorimeter.
2. Measurement medium (75 mM sucrose, 225 mM mannitol, 5 mM Tris–HCl, pH 7.4). Store at 4°C (see Note 2).
3. 0.5 M Succinate, pH 7.4 (adjusted with KOH).
4. 1 mM Antimycin A (ethanol solution).
5. 1 mM Carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP) (ethanol solution).
6. 5mM5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-$H_2$DCFDA), in DMSO.

### 2.4. Measurement of Superoxide Production by Peroxidase/Amplex Red Assay in Isolated Mitochondria

1. Multiwell plate reader.
2. 24-Well Cell Culture Cluster.
3. Reaction buffer (75 mM sucrose, 225 mM mannitol, 5 mM Tris–HCl, pH 7.4). Store at 4°C (see Note 2).
4. 5 μM Amplex Red.
5. 7 U/ml Peroxidase.
6. 0.5 M Glutamate, pH 7.4.
7. 0.5 M Malate, pH 7.4.
8. 1 mM Oligomycin (ethanol solution).
9. 1 mM CCCP (ethanol solution).
10. 1 mM Antimycin A (ethanol solution).

### 2.5. Measurement of the Mitochondrial Transmembrane Potential Using Safranine O in Isolated Mouse Brain Mitochondria

1. Spectrofluorimeter.
2. Measurement medium (75 mM sucrose, 225 mM mannitol, 5 mM Tris–HCl, pH 7.4). Store at 4°C (see Note 2).
3. 5 mM Safranine O.
4. 0.5 M Succinate, pH 7.4 (adjusted with KOH).
5. 1 mM Antimycin A (ethanol solution).
6. 1 mM Oligomycin (ethanol solution).
7. 1 mM CCCP (ethanol solution).

### 2.6. Measurement of the Mitochondrial Transmembrane Potential in Human Fibroblasts Using JC-1

1. Multiwell plate reader.
2. 24-Well Cell Culture Cluster.
3. 5 mM JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide); (Invitrogen/Molecular Probes) in DMSO. Store at –20°C.
4. KRB saline (see item 2 in Subheading 2.2).

5. 1 mM Antimycin A (ethanol solution).
6. 1 mM Oligomycin (ethanol solution).

### 2.7. Measurement of "Mitochondrial Matrix" Superoxide Production in Human Fibroblasts Using MitoSOX Red

1. Multiwell plate reader.
2. 24-Well Cell Culture Cluster.
3. 5 mM MitoSox Red in DMSO. Store at −20°C.
4. KRB saline (see item 2 in Subheading 2.2).
5. 1 mM Antimycin A (ethanol solution).
6. 1 mM Oligomycin (ethanol solution).

### 2.8. Measurement of the "Cytosolic" Superoxide Production in Human Fibroblasts Using DHE

1. Multiwell plate reader.
2. 24-Well Cell Culture Cluster.
3. 10 mM Dihydroethidium (hydroethidine) in DMSO. Store at −20°C.
4. KRB saline (see item 2 in Subheading 2.2).
5. 1 mM Antimycin A (ethanol solution).
6. 1 mM Oligomycin (ethanol solution).

### 2.9. Measurement of the Oxygen Consumption in Ehrlich Ascites Tumor Cells

1. Clark-type oxygen electrode (YSI, Yellow Springs, OH, USA), equipped with a unit calculating the equivalent to the rate of oxygen consumption (first derivative of the oxygen concentration trace).
2. KRB saline (see item 2 in Subheading 2.2).
3. 1 mM Oligomycin (ethanol solution).
4. 1 mM Cyclosporin A (ethanol solution).
5. 1 mM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (ethanol solution).

### 2.10. Fluorometric Measurement of the Mitochondrial Membrane Potential Using TMRM in Ehrlich Ascites Tumor Cells

1. Spectrofluorometer.
2. 100 μM TMRM in $H_2O$.
3. KRB saline (see item 2 in Subheading 2.2).
4. 1 mM Oligomycin (ethanol solution).
5. 1 mM Cyclosporin A (ethanol solution).
6. 1 mM FCCP (ethanol solution).

### 2.11. Fluorometric Measurement of Hydrogen Peroxide Production in Ehrlich Ascites Tumor Cells

1. Spectrofluorometer.
2. 5 mM CM-$H_2$DCFDA in DMSO.
3. KRB saline (see item 2 in Subheading 2.2).
4. 1 mM Oligomycin (ethanol solution).
5. 1 mM Cyclosporin A (ethanol solution).
6. 1 mM FCCP (ethanol solution).

***2.12. Measurement of Mitochondrial Membrane Potential in HeLa Cells Using a Confocal Microscope***

1. Laser scanning or Spinning disk confocal microscope equipped with 546 or 561 nm laser.
2. Microscope cover slips (24 mm diameter, 0.15 mm thickness).
3. KRB saline (see item 2 in Subheading 2.2).
4. 10 μM TMRM in absolute ethanol.

***2.13. Measurement of Hydrogen Peroxide Production in HeLa Cells Using a Confocal Microscope***

1. Laser scanning or Spinning disk confocal microscope equipped with 488nm laser.
2. Microscope cover slips (24 mm diameter, 0.15 mm thickness).
3. KRB saline (see item 2 in Subheading 2.2).
4. 5 mM CM-$H_2$DCFDA in DMSO.

***2.14. Measurement of Mitochondrial Superoxide Production in HeLa Cells Using a Confocal Microscope***

1. Laser scanning or Spinning disk confocal microscope equipped with 514nm laser.
2. Microscope cover slips (24 mm diameter, 0.15 mm thickness).
3. KRB saline (see item 2 in Subheading 2.2).
4. 5 mM MitoSOX Red in DMSO.

***2.15. Measurement of Cytosolic Calcium in HeLa Cells Using a Confocal Microscope***

1. Laser scanning or Spinning disk confocal microscope equipped with 488nm laser.
2. Microscope cover slips (24 mm diameter, 0.15 mm thickness).
3. KRB saline (see item 2 in Subheading 2.2).
4. 5 mM Cell-permeant fluo-3 AM in DMSO.

***2.16. Measurement of the Respiratory Chain Activity in Human Fibroblasts***

1. Multiwell plate reader.
2. 24-Well Cell Culture Cluster.
3. 1 mM Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one-10-oxide sodium salt) in $H_2O$. Store at –20°C.
4. KRB saline (see item 2 in Subheading 2.2).
5. 1 M KCN in $H_2O$.

***2.17. Fluorometric Measurement of Hydrogen Peroxide Production in Human Fibroblasts***

1. Multiwell plate reader.
2. 24-Well Cell Culture Cluster.
3. 5 mM CM-$H_2$DCFDA in DMSO. Store at –20°C.
4. KRB saline (see item 2 in Subheading 2.2).

***2.18. Determination of Protein Concentration***

1. Lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton-X, 0.1% SDS, and 1% sodium deoxycholate).
2. Bio-Rad Protein Assay.
3. Spectrophotometer.
4. Acryl Cuvettes 10 × 10 × 48 mm.

## 3. Methods

To study the relationship between mitochondrial ROS production and mitochondrial bioenergetic parameters (like membrane potential and respiration), a variety of methods can be used. Some of them, particularly those dedicated to human fibroblasts could potentially be adapted as diagnostic procedures in case of suspected mitochondrial disorders (5).

### 3.1. Isolation of Crude Mouse Brain Mitochondria for Measurement of $\Delta\Psi$ and ROS Production

1. Kill the mouse by decapitation, remove the brain immediately, and cool it down at 4°C in the homogenization medium (see Note 3).
2. Wash the brain with the homogenization medium (to remove blood). Add fresh homogenization medium in a proportion of 5 ml/g of brain.
3. Homogenize the brain in a glass Potter–Elvehjem homogenizer with a motor-driven Teflon pestle (see Note 4).
4. Centrifuge the homogenate for 3 min at 1,330 × *g* at 4°C.
5. Discard the pellet and centrifuge the supernatant for 10 min at 21,000 × *g* at 4°C.
6. Gently resuspend the resulting mitochondrial pellet in approximately 15 ml of the mitochondria isolation buffer and centrifuge it again for 10 min at 21,000 × *g* at 4°C.
7. Gently resuspend the final crude mitochondrial pellet in 1–2 ml (depending on the pellet volume) of the mitochondria isolation buffer using a loose Potter–Elvehjem homogenizer.
8. The material can now be used for further experiments (Such isolated mitochondria contain synaptosomes. If necessary additional steps in the isolation procedure can be undertaken to isolate the pure mitochondrial fraction).

### 3.2. Measurement of Hydrogen Peroxide Production with the Use of CM-$H_2$DCFDA in Isolated Mitochondria

1. Adjust the fluorometer: Excitation – 513 nm; Emission – 530 nm; Slits (excitation and emission) ~ 3.
2. Fill the fluorometer cuvette with 3 ml of the measurement medium (75 mM sucrose, 225 mM mannitol, 5 mM Tris–HCl, pH 7.4. Store at 4°C (see Note 2)) containing 1 mg of mitochondrial protein.
3. Start measurement.
4. Add CM-$H_2$DCFDA to the final 5 μM concentration and record changes in fluorescence.
5. Make traces in the presence of following additions:
   (a) First trace: 2 μM antimycin A.
   (b) Second trace: 2 μM FCCP.
6. An example of the results obtained is shown in Fig. 1a.

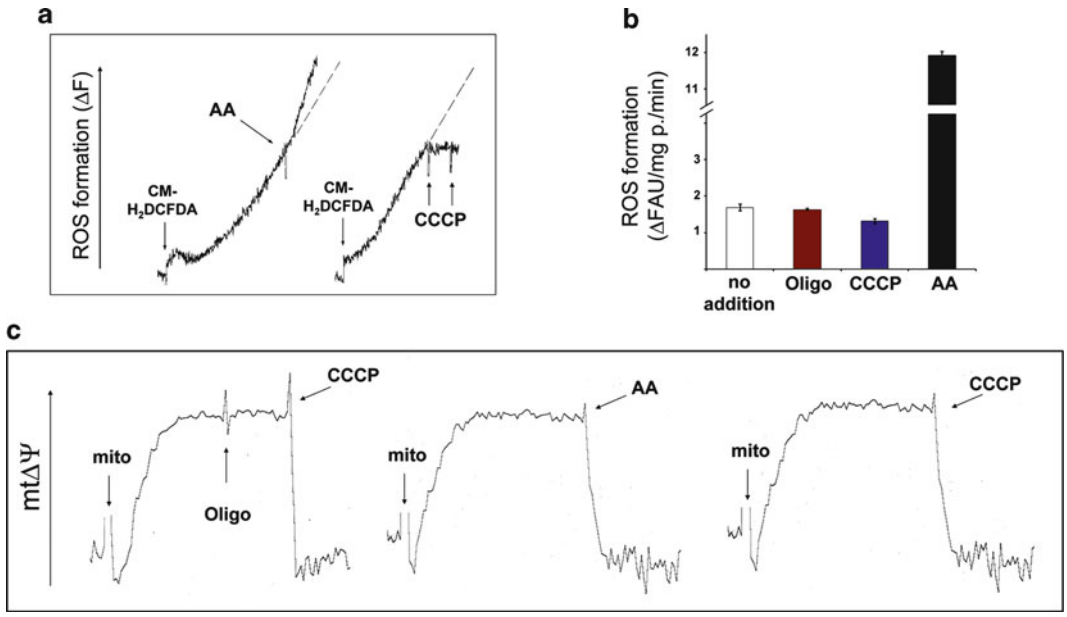


Fig. 1. Effect of antimycin A, CCCP, and oligomycin on mitochondrial membrane potential and ROS formation measured in isolated mitochondria. (**a**) Effect of antimycin A and CCCP on mitochondrial $H_2O_2$ production measured with the use of CM-$H_2$DCFDA (Subheading 3.2); (**b**) Effect of oligomycin, CCCP, and antimycin A on superoxide production measured by peroxidase/Amplex Red assay (Subheading 3.3); (**c**) Effect of oligomycin, antimycin A, and CCCP on $\Delta\Psi$ measured with the use of Safranine O (Subheading 3.4). Addition of antimycin A or CCCP leads to the collapse of the $\Delta\Psi$ (panel **c**); however, each compound results in contradictory effects in terms of ROS formation in isolated mitochondria. These effects can be observed with the use of different probes, such as CM-$H_2$DCFDA (panel **a**) and peroxidase/Amplex Red assay (panel **b**). Antimycin A is an inhibitor of complex III of the respiratory chain and causes the accumulation of reduced intermediates leading to the chain's blockage and an increased leakage of electrons. This in turn results in an increase in ROS formation. CCCP as a mitochondrial uncoupler decreases the amount of reduced respiratory chain intermediates and decreases ROS formation (panels **a** and **b**). In "coupled" mitochondria, in the absence of ADP, no significant effect of oligomycin (an inhibitor of the mitochondrial ATP synthase) neither on the $\Delta\Psi$ nor on the ROS production is observed (panels **b** and **c**). A significant effect of oligomycin on both parameters can be seen in the intact cell model (see Fig. 2).

### *3.3. Mitochondrial Superoxide Measurement by Peroxidase/Amplex Red Assay*

1. Prepare 13 ml of the reaction buffer (see Note 5).
2. Supplement the reaction buffer with 3 µl of Amplex Red, 13 µl of proxidase, 130 µl of glutamate, and 130 µl of malate.
3. Aliquot 0.5 ml of the reaction solution into the wells.
4. Supplement individual wells with oligomycin, FCCP, and antimycin A.
5. Start the reaction with the addition of 100 µg of mitochondrial protein.
6. Place the plate in the microplate reader and read the fluorescence at 510 nm excitation and 595 nm emission wavelengths. An example of the results obtained is shown in Fig. 1b.

### 3.4. Measurement of the Mitochondrial Membrane Potential with the Use of Safranine O

1. Adjust the fluorometer: Excitation – 495 nm; Emission – 586 nm; Slits (excitation and emission) ~ 3.
2. Fill the fluorometer cuvette with 3 ml of the measurement medium (see Note 5).
3. Add 3 μl of safranine O and 30 μl of succinate.
4. Start measuring fluorescence.
5. Add the mitochondrial suspension corresponding to about 1 mg of protein and observe changes in fluorescence.
6. Make traces with the following consecutive additions:
   (a) First trace: 5 μl oligomycin, 5 μl CCCP.
   (b) Second trace: 5 μl antimycin A.
   (c) Third trace: 5 μl CCCP.
7. An example of the results obtained is shown in Fig. 1c.

### 3.5. Measurement of Mitochondrial Membrane Potential in Human Fibroblasts with the Use of JC-1

1. Remove the culture medium and wash the cells gently with warm KRB before preincubation with effectors. Dilute stock solutions of antimycin A and oligomycin to final concentration 2 μM in KRB. Add 0.5 ml of KRB with particular effector to selected wells, and KRB alone to control wells. Preincubate the plate, in the incubator, for 15–30 min prior to the measurement (see Note 6).
2. Prepare 5 μM solution of JC-1 in KRB (see Note 7).
3. Remove the preincubation solution from wells and add the KRB solution containing JC-1. As the effectors must be present till the end of measurement, add adequate amount of each to selected wells (see Notes 5 and 8).
4. Incubate the plate for 10 min in the incubator (see Note 9).
5. Gently wash the cells twice with KRB.
6. Add 0.5 ml of KRB to each well (see Note 10).
7. Place the plate in reader and read the fluorescence. The read must be done twice, first at 485 nm excitation and 520 nm emission wavelengths for JC-1 green fluorescence detection and second at 535 nm excitation and 635 nm emission wavelengths for JC-1 red fluorescence detection. The value of mitochondrial potential is the ratio of red to green fluorescence (see Note 11). An example of the results obtained is shown in Figs. 2a, b and 8a.

### 3.6. Measurement of Mitochondrial Superoxide Production in Human Fibroblasts with the use of MitoSox Red

1. Remove the culture medium and wash the cells gently with warm KRB before preincubation with effectors. Dilute stock solutions of antimycin A and oligomycin to a final concentration of 2 μM in KRB. Add 0.5 ml of KRB with particular effector to selected wells and KRB alone to control wells. Preincubate the plate, in the incubator, for 15–30 min prior to the measurement (see Note 6).

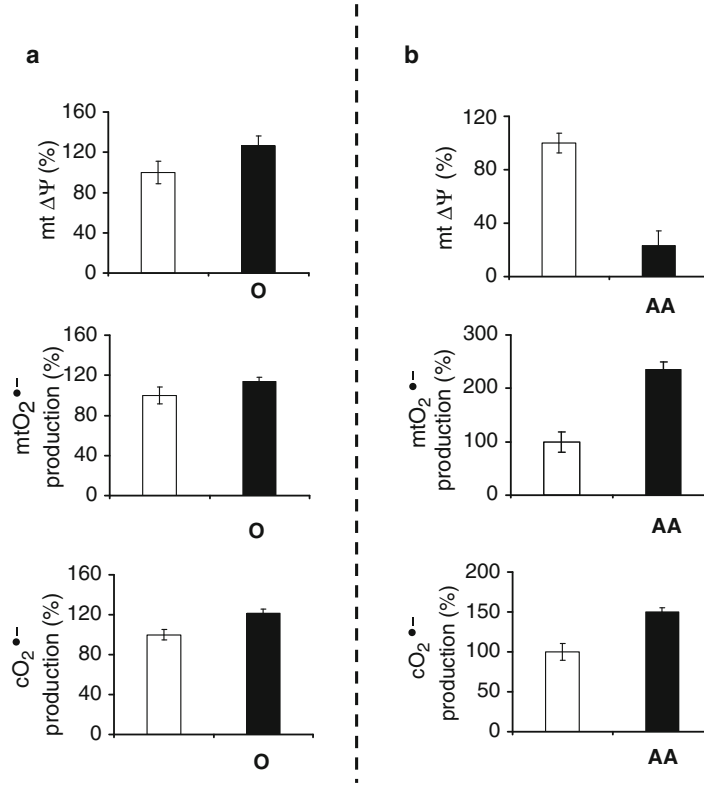


Fig. 2. Effect of oligomycin and antimycin A on mitochondrial membrane potential and superoxide production measured in intact human fibroblasts. Effect of (**a**) oligomycin (O) and (**b**) antimycin A (AA) on $\Delta\Psi$ measured with the use of JC-1 (Subheading 3.5); mitochondrial superoxide production measured with the use of MitoSOX Red (Subheading 3.6) and cytosolic superoxide production measured with the use of DHE (Subheading 3.7). All parameters were measured in a multiwell Plate Reader; O, oligomycin; AA, antimycin A; Oligomycin by the inhibition of ATP synthase causes an increase of $\Delta\Psi$. It is a result of a drift from mitochondrial respiratory state III to state IV. Mitochondrial coupling is connected with the increase of superoxide production by mitochondria determined either in mitochondrial matrix ($mtO_2^{\cdot-}$) or in cytosol ($cO_2^{\cdot-}$). Similarly to isolated mitochondria (Fig. 1.), in intact fibroblasts antimycin A increases superoxide formation with the simultaneous decrease of $\Delta\Psi$.

2. Prepare 5 μM solution of MitoSox in KRB. Protect the solution from light (see Notes 5 and 8).
3. Remove the preincubation solution from wells and add 0.5 ml of the MitoSox Red solution per well. Add proper amounts of antimycin A or oligomycin to the selected wells (see Notes 5, 8, and 10).
4. Incubate for 10 min in the incubator.
5. Gently wash the cells twice with warm KRB.
6. Add 0.5 ml KRB to each well (see Note 10).
7. Place the plate in the microplate reader and read the fluorescence at 510 nm excitation and 595 nm emission wavelengths (see Note 11). An example of the results obtained is shown in Figs. 2a, b and 8d.

### 3.7. Measurement of Cytosolic Superoxide Production in Human Fibroblasts with the Use of DHE

1. Remove the culture medium and wash the cells gently with warm KRB before preincubation with effectors. Dilute stock solutions of antimycin A and oligomycin to a final concentration of 2 μM in KRB. Add 0.5 ml of KRB with particular effector to selected wells and KRB alone to control wells. Preincubate the plate, in the incubator, for 15–30 min prior to the measurement (see Note 6).
2. Prepare 5 μM DHE solution in KRB (see Notes 5, 8, and 10).
3. Remove the preincubation solution from wells and add 0.5 ml of DHE solution to each well of 24-wells plate. As the effectors must be present till the end of measurement, add proper amounts of antimycin A or oligomycin to the selected wells.
4. Incubate for 20 min in the incubator.
5. Gently wash the cells twice with KRB.
6. Add KRB to each well (see Note 10).
7. Place the plate in the microplate reader and read the fluorescence at 535 nm excitation and 635 nm emission wavelengths. This is a single read, no kinetic should be defined. An example of the results obtained is shown in Figs. 2a, b and 8c.

### 3.8. Measurement of Oxygen Consumption (Respiration) in Ehrlich Ascites Tumor Cells

1. Add cells (approximately 6 mg) to the chamber, fill the chamber with the measurement medium (NaCl 135 mM, KCl 5 mM, $KH_2PO_4$ 0.4 mM, $MgSO_4$ 1 mM, HEPES 20 mM, adjusted to pH 7.4 with NaOH. Glucose 1 g/L and 1 mM $CaCl_2$) and close the chamber.
2. Start the oxygen consumption measurement (first derivative of the oxygen concentration trace).
3. Wait for the stable signal (usually it takes about 1–3 min).
4. Make traces with the following additions:
   (a) First trace: 1 μl of oligomycin → 1 μl of CsA → 1 μl of FCCP.
   (b) Second trace: 1 μl of CsA → 1 μl of oligomycin → 1 μl of FCCP.
5. An example of the results obtained is shown in Fig. 3a, b.

### 3.9. Fluorometric Measurement of Mitochondrial Membrane Potential in Ehrlich Ascites Tumor Cells with the Use of TMRM

1. Adjust the fluorometer: Excitation – 556 nm; Emission – 576 nm; Slits (excitation and emission) ~ 3.
2. Fill the fluorometer cuvette with 3 ml of the measurement medium (NaCl 135 mM, KCl 5 mM, $KH_2PO_4$ 0.4 mM, $MgSO_4$ 1 mM, HEPES 20 mM, adjusted to pH 7.4 with NaOH. Glucose 1 g/L and 1 mM $CaCl_2$) containing $1 \times 10^7$ of EAT cells.
3. Add 5 μl of 200 μM TMRM.
4. Start the fluorescence measurement.

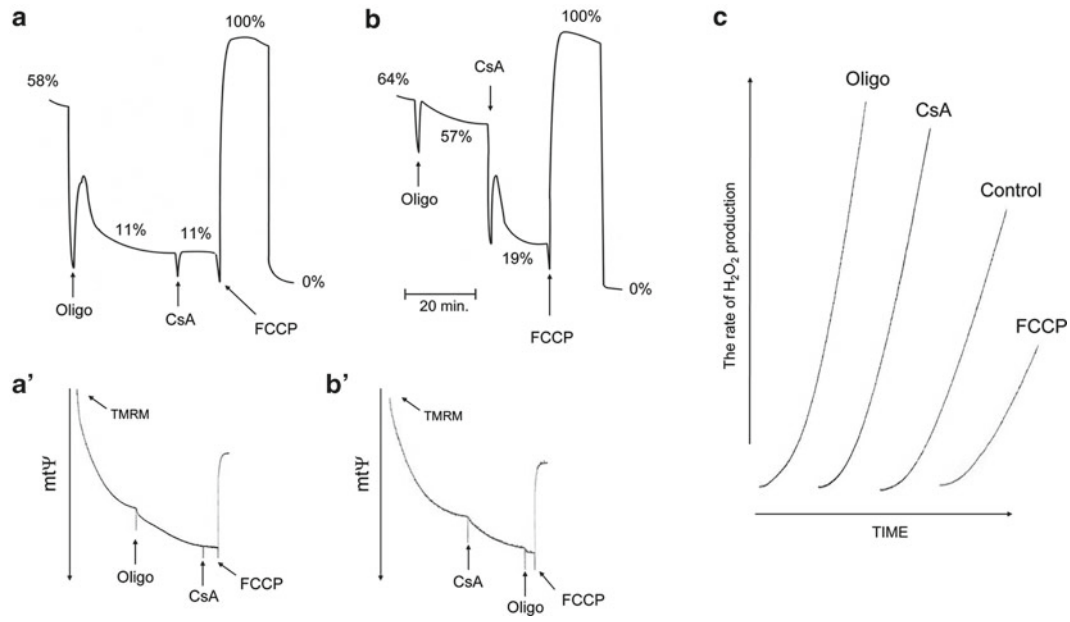


Fig. 3. Effect of oligomycin, cyclosporine A, and FCCP on oxygen consumption, mitochondrial membrane potential, and $H_2O_2$ production measured in Ehrlich ascites tumor cells. (**a, a′**) oxygen consumption was measured with the use of a Clark-type oxygen electrode (Subheading 3.8) (**b, b′**) $\Delta\Psi$ was measured with the use of TMRM in a Shimadzu Spectrofluorimeter RF 5000 (Subheading 3.9); (**c**) $H_2O_2$ production was measured with the use of CM-$H_2$DCFDA in Shimadzu Spectrofluorimeter RF 5000 (Subheading 3.10). The addition of oligomycin (oligo) to the Ehrlich ascites tumor cells caused a decrease in oxygen consumption (manifested as a decrease of first derivative value of the oxygen concentration trace) (Panels **a** and **b**) and an increase in mt $\Delta\Psi$ (Panels **a′** and **b′**), corresponding to the resting-state (respiratory State 4) level. The effect of oligomycin on mitochondrial bioenergetic parameters is manifested in an increased rate of $H_2O_2$ production (Panel **c**). Further addition of FCCP resulted in the acceleration of oxygen consumption, a rapid collapse of the $\Delta\Psi$, and decreased rate of $H_2O_2$ production. Cyclosporine A (CsA), an inhibitor of the permeability transition pore, added before oligomycin also partially decreased oxygen consumption (Panel **b**), increased the mt $\Delta\Psi$ (Panel **b′**), and increased the rate of $H_2O_2$ production (Panel **c**). Incompatibility of the amplitude changes in TMRM fluorescence with the alterations in the rate of oxygen consumption after oligomycin and cyclosporine A addition was evoked by the effect of both of these compounds on the multidrug resistant (MDR) proteins. Basing on the oxygen consumption data, it is recommended to use an inhibitor of MDR proteins as, e.g.: Sulfinpyrazone 100 μM.

5. Make traces with the following additions:
   (a) First trace: 5 μl oligomycin, 5 μl of CsA, and 5 μl FCCP.
   (b) Second trace: 5 μl CsA, 5 μl of oligomycin, and 5 μl FCCP.
6. An example of the results obtained is shown in Fig. 3a′, b′.

### *3.10. Fluorometric Measurement of $H_2O_2$ Production in Ehrlich Ascites Tumor Cells with the Use of CM-$H_2$DCFDA*

1. Adjust the fluorometer: Excitation – 513 nm; Emission – 530 nm; Slits (excitation and emission) ~ 3.
2. Fill the fluorometer cuvette with 3 ml of the measurement medium (NaCl 135 mM, KCl 5 mM, $KH_2PO_4$ 0.4 mM, $MgSO_4$ 1 mM, HEPES 20 mM, adjusted to pH 7.4 with NaOH. Glucose 1 g/L and 1 mM $CaCl_2$) containing $5 \times 10^6$ of EAT cells.
3. Start measurement.

4. Add 10 μM of CM-$H_2$DCFDA and record changes in fluorescence.
5. Make similar traces in the presence of the following additions:
   (a) Second trace: 2 μM oligomycin.
   (b) Third trace: 2 μM antimycin A.
   (c) Fourth trace: 2 μM FCCP.
6. An example of the results obtained is shown in Fig. 3c.

### 3.11. Confocal Measurements of Mitochondrial Membrane Potential in HeLa Cells

1. Plate cells on 25 mm coverslips 2 days before the experiment, in a number determined for each cell type, in order to obtain not more than 90% confluent culture on the day of the experiment. Before plating cells, the coverslips must be sterilized by UV exposure (30 J) or by temperature (200°C for 2 h).
2. Prepare a 10 nM solution of TMRM in KRB saline supplemented with glucose (1 g/L) and 1 mM $CaCl_2$ just before loading the cells. The total volume should be calculated considering 1 ml for each coverslip and 100 μl for each drug or chemical addition executed during experiment.
3. Prepare a 10× solution of each compound required for the experiment with an exceeding volume of the TMRM solution (see Notes 12–14).
4. Wash cells twice in order to remove dead cells and cell debris, add 1 ml of TMRM solution (room temperature) to the coverslip then incubate for 20–40 min at 37°C. The correct loading time with TMRM may vary for different cell types.
5. After loading, the coverslip should be mounted in a metal cage, or a different appropriate support depending on the microscope model, and covered with 1 ml of the same TMRM solution as used for loading.
6. The coverslip and its support should be placed on the inverted confocal microscope equipped with a thermostated stage set at 37°C. Correct visualization of mitochondria should be performed using a 40–100× oil immersion objective. Optimal illumination is obtained using a 543 nm HeNe gas laser or a 561 nm solid state laser, while emission should be selected using a long pass 580 filter (see Note 15).
7. We suggest time laps with a delay of at least 10 s between each measurement step in order to avoid phototoxicity. To investigate the effect of the compound of interest (e.g., oligomycin), addition of 100 μl of the 10× concentrated solution is recommended. This is required in order to obtain a fast diffusion of the substance in the chamber. To obtain the basal fluorescence intensity level, terminate each experiment by adding 500 nM of FCCP.

8. After the experiment, fluorescence intensity can be measured in selected regions drawn around mitochondria. An example of the results obtained is shown in Fig. 4a.

### 3.12. Confocal Measurement of Hydrogen Peroxide Production in HeLa Cells

1. Plate cells on 25 mm coverslips 2 days before the experiment, in a number determined for each cell type, in order to obtain not more than 90% confluent culture on the day of the experiment. Before plating cells, the coverslips must be sterilized by UV exposure (30 J) or by temperature (200°C for 2 h).
2. Prepare a 5 μM solution of CM-$H_2$DCFDA in KRB saline supplemented with glucose (1 g/L) and 1 mM $CaCl_2$ just before loading the cells. The total volume should be calculated considering 1 ml for each coverslip (see Note 16).
3. Prepare a 10× solution of each compound used for the stimulation or inhibition of $H_2O_2$ production in complete KRB saline.
4. Wash cells twice in order to remove dead cells and cell debris, add 1 ml of the $H_2$DCFDA solution (room temperature) to the coverslip and incubate for 10 min at 37°C.
5. After loading, the coverslip should be mounted in a metal cage, or a different appropriate support depending on the microscope model, and covered with 1 ml of the same $H_2$DCFDA solution used for loading.
6. The coverslip and its support should be placed on the inverted confocal microscope equipped with a thermostated stage set at 37°C. Images should be recorded with a 40–100× oil immersion objective, illuminating with 488 Argon or solid state laser. Emitted light will be preferably selected with a 505–550 band pass filter (see Note 17).
7. We suggest time laps with a delay of at least 15–30 s to avoid photoactivation not related to $H_2O_2$ production. Stimulation with chemical is obtained by adding 100 μl of the 10× concentrated solution is recommended. This is required in order to obtain a fast diffusion of the substance in the chamber.
8. After the experiment, fluorescence intensity will be measured drawing small region around each cell without touching edges to avoid artifacts (see Note 18). An example of the results obtained is shown in Fig. 4b.

### 3.13. Confocal Measurement of Superoxide Production in HeLa Cell

1. Plate cells on 25 mm coverslips 2 days before the experiment, in a number determined for each cell type, in order to obtain not more than 90% confluent culture on the day of the experiment. Before plating cells, the coverslips must be sterilized by UV exposure (30 J) or by temperature (200°C for 2 h).

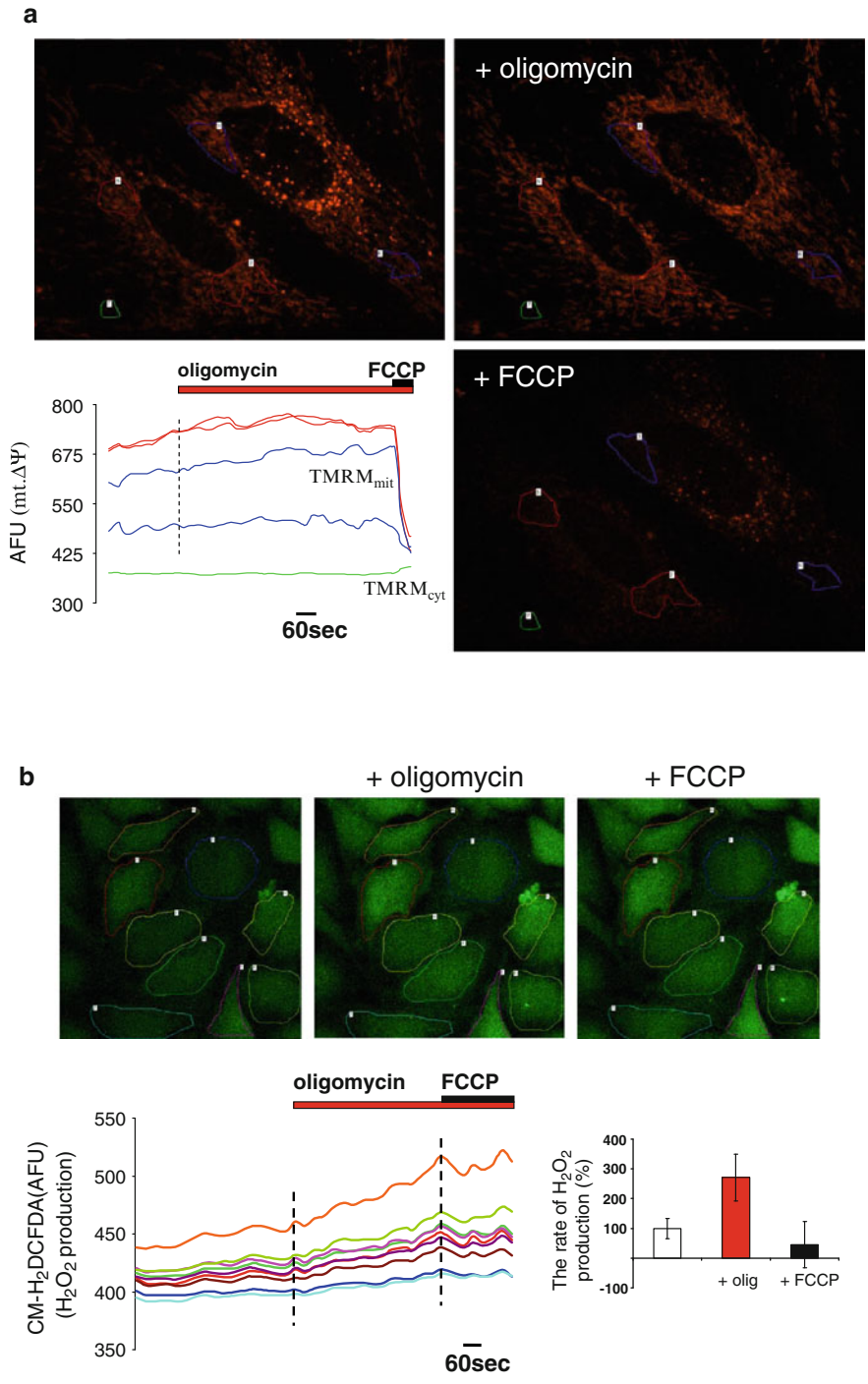


Fig. 4. Effect of oligomycin and FCCP on mitochondrial membrane potential and $H_2O_2$ production in HeLa cells. (**a**) $\Delta\Psi$ was measured with the use of TMRM in a confocal microscope (Subheading 3.11); (**b**) $H_2O_2$ production was measured with the use of CM-$H_2$DCFDA in a confocal microscope (Subheading 3.12). The addition of oligomycin to the intact cells led to hyperpolarization of mitochondria (drift from mitochondrial respiratory state III to state IV) (Panel **a**) and an increase of the rate of $H_2O_2$ production (Panel **b**). Further addition of FCCP resulted in a rapid collapse of the $\Delta\Psi$ (represented as a decrease of the TMRM fluorescence) (Panel **a**) and a decreased rate of $H_2O_2$ production to a level lower than the initial threshold (Panel **b**).

2. Apply the selected treatment to the sample before loading with MitoSOX Red. In the presented example, cells were incubated with 5 μM oligomycin for 15 min.
3. Prepare a 5 μM solution of MitoSOX Red in KRB saline supplemented with glucose (1 g/L) and 1 mM $CaCl_2$ just before loading the cells. The total volume should be calculated considering 1 ml for each coverslip and should be protected from light and high temperature.
4. Wash cells twice in order to remove dead cells and cell debris, add 1 ml of MitoSOX Red solution (room temperature) to the coverslip and incubate for 15 min at 37°C (see Note 19).
5. After loading, the coverslips should be washed three times with complete KRB saline and mounted in a metal cage or in a different appropriated support depending on the microscope model.
6. The coverslip and its support should be placed on the inverted confocal microscope. Recorded images with a 40–100× oil immersion objective, illuminating with 514 Argon or 488 solid state laser. Emitted light should be selected with a 580 nm longpass filter. A thermostated stage is optional (see Note 20).
7. In our experience, MitSOX Red is not able to perform kinetics of superoxide production. After images collection, mean fluorescence intensity should be measured by drawing small regions around the bright objects for each cell. Cytosolic area should be excluded to avoid artifacts. An example of the results obtained is shown in Fig. 5.

### *3.14. Simultaneous Measurement of the Mitochondrial Membrane Potential and Cytosolic Calcium in HeLa Cells Using Confocal Microscope*

1. Plate cells on 25 mm coverslips 2 days before the experiment, in a number determined for each cell type, in order to obtain not more than 90% confluent culture on the day of the experiment.
2. Prepare a KRB saline supplemented with 1 μM Fluo-3 AM, 10 nM TMRM, glucose (1 g/L). The total volume should be calculated considering 1 ml for each coverslip and should be protected from light and high temperature.
3. Just before loading the cells, add $CaCl_2$ with the final 1 mM concentration to the KRB saline prepared in the previous step.
4. Wash cells twice in order to remove dead cells and cell debris, add 1 ml of room temperature KRB solution containing 1 μM Fluo-3 AM and 10 nM TMRM to the coverslip and incubate for 30 min, 37°C.
5. After loading, the coverslip should be washed with KRB/$Ca^{2+}$, mounted in a metal cage or in a different appropriate support depending on the microscope model, and covered with 1 ml of the KRB saline supplemented with glucose (1 g/L) and 1 mM $CaCl_2$.

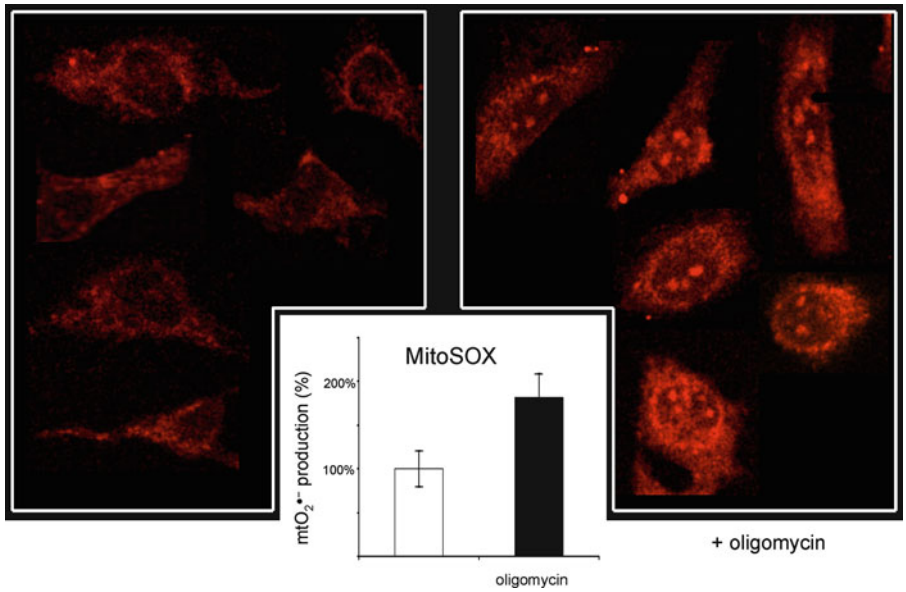


Fig. 5. Effect of oligomycin on mitochondrial superoxide production in HeLa cells. The mitochondrial superoxide production was measured with the use of MitoSOX Red in a confocal microscope (Subheading 3.13). Addition of oligomycin (*right panel*) to the HeLa cells augmented mitochondrial superoxide production represented by increased fluorescence. These data are in line with the previous observations that in intact cells, the hyperpolarization of the inner mitochondrial membrane accelerates ROS formation.

6. The coverslip and its support should be placed on the inverted Zeiss LSM510 Confocal Microscope equipped with a thermostated stage set at 37°C.
7. Start recording the images sequentially with a 40–100× oil immersion objective, illuminating with 488 Argon or solid state laser for Fluo-3 illumination and 543 HeNe or 561 solid state laser for TMRM. Collect emitted light for the Fluo-3 in the range ≥505–≥535 nm and for the TMRM as total emission ≥570 nm.
8. Stimulate the cells by the addition of 100 μl of the 1 mM histamine solution.
9. After the experiment, analyze the changes in fluorescence intensity with Zeiss LSM510 software. An example of the results obtained is shown in Fig. 6.

### *3.15. Simultaneous Measurement of the Mitochondrial Membrane Potential and $H_2O_2$ Production in HeLa Cells Using Confocal Microscope*

1. Plate cells on 25 mm coverslips 2 days before the experiment, in a number determined for each cell type, in order to obtain not more than 90% confluent culture on the day of the experiment.
2. Prepare the KRB saline supplemented with 5 μM CM-$H_2$ DCFDA, 10 nM TMRM, glucose (1 g/L), and 1 mM $CaCl_2$. The total volume should be calculated considering 1 ml for each coverslip and should be protected from light and high temperature.

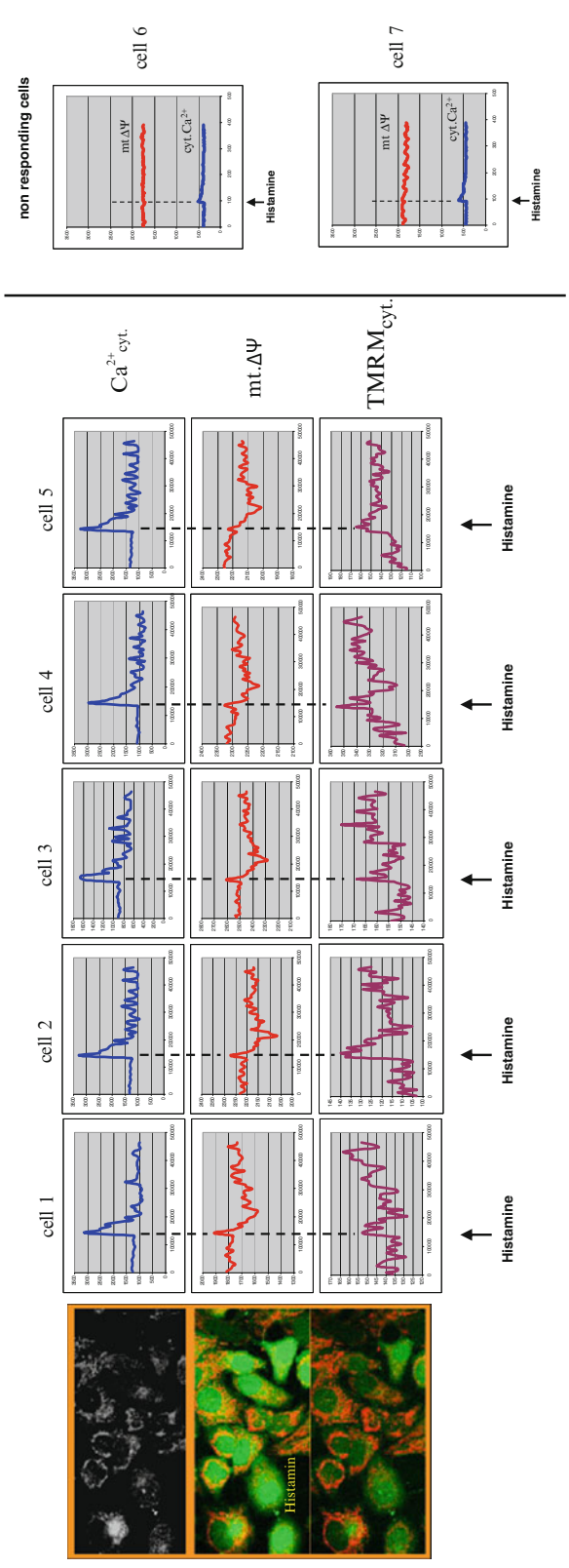


Fig. 6. Effect of histamine on cytosolic calcium and mitochondrial membrane potential in HeLa cells. Simultaneous measurements of cytosolic calcium and $\Delta\Psi$ with the use of Fluo-3 and TMRM in confocal microscope (Subheading 3.14). The addition of histamine to the intact HeLa cells induced a transient cytosolic $Ca^{2+}$ signal recorded as an increase of Fluo-3 fluorescence. Simultaneously, a transient partial mitochondrial depolarization (decrease of mitochondrial TMRM fluorescence and increased cytosolic TMRM signal) was observed. Decrease of $\Delta\Psi$ occurs due to the calcium uptake by mitochondria. Cells, not responding to the histamine stimulation, have unchanged $\Delta\Psi$.

3. Wash cells twice in order to remove dead cells and cell debris, add 1 ml of the KRB solution (room temperature) containing 5 μM CM-$H_2$DCFDA and 10 nM TMRM to the coverslip and incubate for 20 min, 37°C.
4. After loading, the coverslip should be washed with KRB/$Ca^{2+}$, mounted in a metal cage or in a different appropriate support depending on the microscope model, and covered with 1 ml of the KRB saline supplemented with glucose (1 g/L) and 1 mM $CaCl_2$.
5. The coverslip and its support should be placed on the inverted Zeiss LSM510 Confocal Microscope equipped with a thermostated stage set at 37°C.
6. Start recording the images sequentially with a 40–100× oil immersion objective, illuminating with 488 Argon or solid state laser for CM-$H_2$DCFDA illumination and 543 HeNe or 561 solid state laser for TMRM. Collect the emitted light for the CM-$H_2$DCFDA in the range ≥505–≥535 nm and for the TMRM as total emission ≥570 nm.
7. Stimulate the cells by adding 100 μl of the 1 mM histamine solution.
8. After a few minutes, when the rate of free radical production is constant, add 50 μl of 12 μM FCCP.
9. After the experiment, analyze the changes in fluorescence intensity with Zeiss LSM510 software. An example of the results obtained is shown in Fig. 7.

### *3.16. Measurement of Mitochondrial Respiratory Chain Activity in Human Fibroblasts with the Use of Resazurin*

1. Prepare a 6 μM resazurin solution in KRB.
2. Remove the culture medium and wash the cells gently twice with warm KRB (see Note 6).
3. Add 0.5 ml of the resazurin solution to each well of the 24-well plate.
4. Immediately after addition, start the measurement in the kinetic mode at 510 nm excitation and 595 nm emission wavelengths (see Note 21). An example of the results obtained is shown in Fig. 8b.

### *3.17. Measurement of Hydrogen Peroxide Production in Human Fibroblasts with the Use of CM-$H_2$DCFDA*

1. Prepare a 2 μM CM-$H_2$DCFDA solution in KRB.
2. Remove the culture medium and wash the cells gently twice with warm KRB (see Note 6).
3. Add 0.5 ml of the CM-$H_2$DCFDA solution to each well of the 24-well plate. Immediately after addition, start the measurement in the kinetic mode at 495 nm excitation and 520 nm emission wavelengths. An example of the results obtained is shown in Fig. 8e.

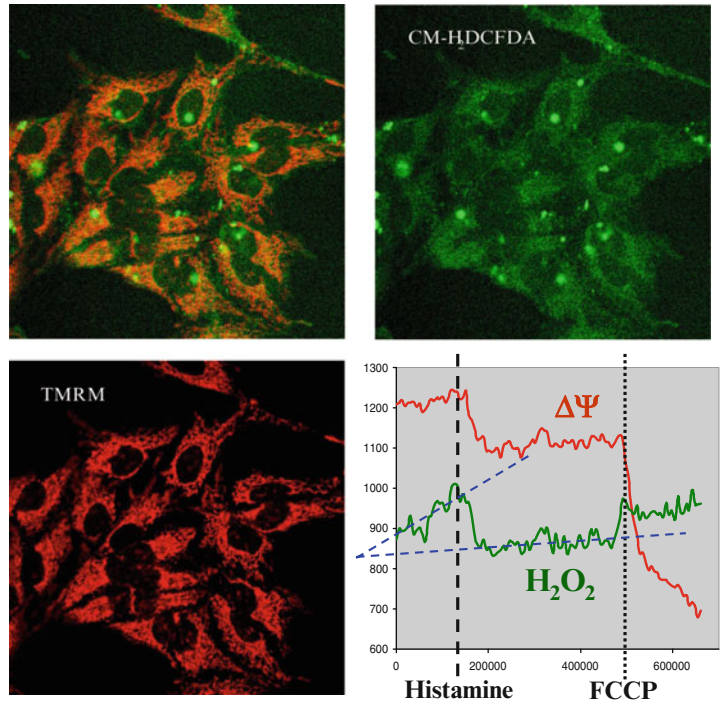


Fig. 7. Effect of histamine on mitochondrial membrane potential and $H_2O_2$ production in HeLa cells. Simultaneous measurement of $\Delta\Psi$ and $H_2O_2$ production with the use of TMRM and CM-$H_2$DCFDA in confocal microscope (Subheading 3.15). Also presented in Fig. 6, the addition of histamine to the intact HeLa cells induced a transient, partial mitochondrial depolarization (decrease of mitochondrial TMRM fluorescence) which is accompanied by the reduction of the rate of $H_2O_2$ production. These data are in line with the previous observations that in intact cells, partial depolarization of the inner mitochondrial membrane is manifested by a decrease in ROS production.

### *3.18. Measurement of Protein Concentration in the Plate Wells After Measurement of ROS and Mitochondrial Respiratory Chain Activity in Human Fibroblasts*

1. Usually cells are plated and grow in equal density at each well; however, addition of different chemical compounds may induce cell death. Thus, protein concentration on each well should be determined after measurement to calculate appropriate values of bioenergetic parameters.
2. For spectrophotometric measurement of protein concentration, cells grown on multiwell plates must be suspended in the lysis buffer, about 500 µl per well. Protein assay is based on Bradford method. Add 2.4 ml of $H_2O_2$ to the 3 ml spectrophotometer cuvette. Depending on the cell density, add the amount of sample (that the absorbance should be between 100 and 600 spectrophotometric units) from each well to separate cuvette. Then add 600 µl of room temperature Bio-Rad Protein Assay and shake the sample. Measure the absorbance at 595 nm(8).

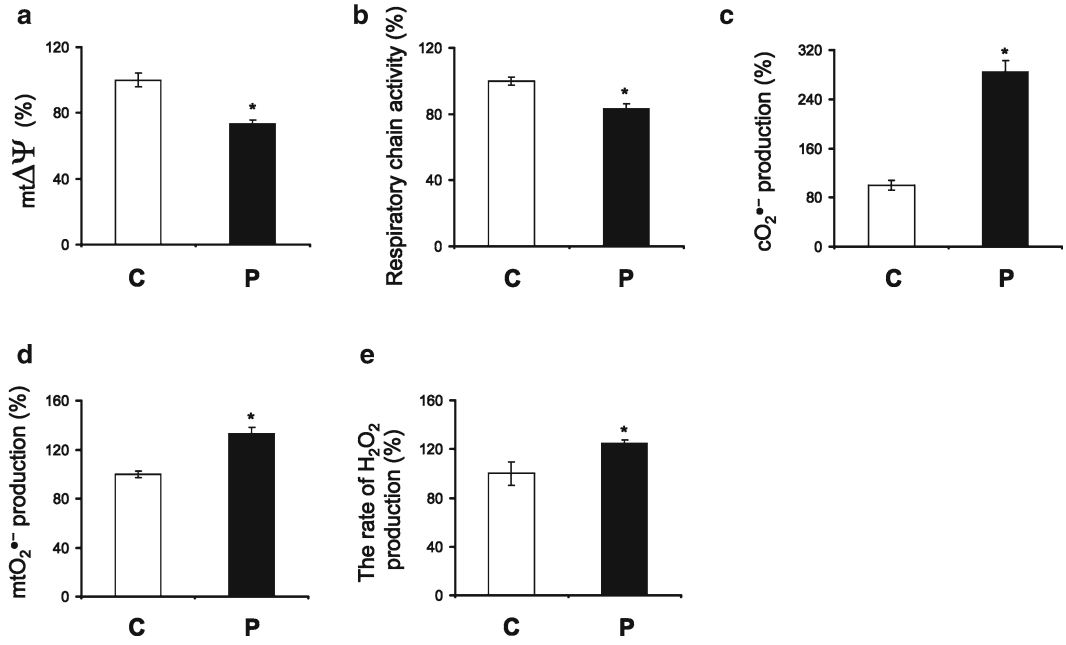


Fig. 8. Relation between mitochondrial membrane potential, respiratory chain activity, and ROS production in primary culture of fibroblasts from a healthy individual and a child with a mitochondrial disorder. C – control fibroblasts, P – patient fibroblasts. The patient used in these studies demonstrated a clinical phenotype of OXPHOS abnormality (mitochondrial encephalopathy) in muscle biopsies and in fibroblast culture. (**a**) $\Delta\Psi$ measured with the use of JC-1 in a multiwell Plate Reader (Subheading 3.5); (**b**) Respiratory chain activity measured with the use of resazurin in a multiwell Plate Reader (Subheading 3.16); (**c**) Cytosolic superoxide production measured with the use of DHE in a multiwell Plate Reader (Subheading 3.7); (**d**) Mitochondrial superoxide production measured with the use of MitoSOX Red in a multiwell Plate Reader (Subheading 3.6); (**e**) $H_2O_2$ production measured with the use of CM-$H_2$DCFDA in a multiwell Plate Reader (Subheading 3.17). Dysfunction of the respiratory chain in the patients' fibroblasts was represented as a decreased respiratory chain activity and lower mitochondrial potential compared to the healthy fibroblasts. A defect in the mitochondrial respiratory chain results in higher cytosolic (**c**) and mitochondrial (**d**) superoxide production. In such cells, the rate of $H_2O_2$ production is also increased (**e**).

## 4. Notes

1. To isolate intact mitochondria, it is necessary to use low-calcium sucrose.
2. The medium can be prepared in advance and stored in 4°C for approximately 2 weeks.
3. All solutions should be at 4°C and all equipment precooled.
4. Extreme care should be taken to avoid contamination with the ice and tap water.
5. For the assay, the reaction buffer should have room temperature.
6. Fibroblasts, plated with equal density, are grown on 24-well plates until they reach confluence, in conditions of 5% (v/v)

$CO_2$ in air at 37°C. The medium is changed every 2 days including the day before experiment.

7. Avoid higher JC-1 concentration as its precipitates are hard to wash out.
8. Avoid light exposure of the fluorescent probe solution and the loaded cells.
9. The time of incubation should not be longer than 10 min.
10. Keep in mind that antimycin A and oligomycin must be present during incubation with the fluorescent probe and during the measurement.
11. For the final calculation of the measured parameter, the background (basal fluorescence) should be subtracted.
12. TMRM solution may be kept for a few hours at 4°C; however, it is preferable not to add cold solution to the cells. Any changes of temperature during experiments should be avoided because it can cause alterations in TMRM distribution independently to $\Delta\Psi$.
13. In all experiments, TMRM concentration must always be the same in order to avoid differences in the redistribution of the dye in the cell.
14. Correct TMRM loading can be confirmed by a short time acquisition (i.e., 10 min). If stable fluorescence intensity is recorded, the cells are loaded correctly. If the signal is increasing during acquisition, the loading time should be increased.
15. The laser power should be kept low in order to avoid photoactivation and bleaching of the dye. With a HeNe laser, it is recommended to set the transmission lower than 10%, while with the solid state laser the power should not be higher than 30%.
16. CM-$H_2$DCFDA solution should be protected from light and high temperature.
17. The laser power should be kept low in order to avoid photoactivation and bleaching of the dye. With a Neon laser, it is recommended to set the transmission lower than 5%, while with the solid state laser the power should not be higher than 20%. A short recording can be executed before the actual experiment at basal conditions. If fast increase in fluorescence is observed probably, photoactivation of the CM-$H_2$DCFDA occurs. In this case, it is necessary to reduce the laser power and/or the number of the readings of the same frame (typical parameter for laser scanning confocal microscopes) and/or increase reading speed.
18. If a fast confocal system is available (i.e., spinning disk or swept field) Z stack acquisition is recommended. In conventional laser scanning confocal microscopy, Z stack acquisition will be too slow with the risk of recording artifacts.

19. No kinetic is recorded with MitoSOX Red dye, so loading time and washing must be carefully respected in order to avoid experimental artifacts.
20. Optimal excitation wavelength declared by the manufacturer is 510 nm, but we observed that even with the 488 nm solid state laser a good signal is recordable. In this case, short exposure time is essential to avoid phototoxic stress and photoactivation.
21. For the background fluorescence subtraction, selected wells on the plate can be incubated with 1 μl of 1 M KCN stock solution before (about 15 min) and during measurement.

## 5. Ethics

The studies with the use of human fibroblasts were carried out in accordance with the Declaration of Helsinki of the World Medical Association and were approved by the Committee of Bioethics at the Children's Memorial Health Institute. Informed consent was obtained from the parents before any biopsy or molecular analysis was performed.

## Acknowledgments

This work was supported by the Polish Ministry of Science and Higher Education grants N301 092 32/3407 and NN407 075 137 for M.R.W., J.D., M.L., and J.S. J.S. was also supported by a PhD fellowship from The Foundation for Polish Science (FNP), UE, European Regional Development Fund and Operational Programme "Innovative economy." ML is recipient of a fellowship from the Foundation for Polish Science (Program Start) and the L'Oreal fellowship (For Women in Science). P.P. and M.B. are supported by: AIRC, Telethon (GGP09128), local funds from the University of Ferrara, the PRRIITT program of the Emilia Romagna Region, the Italian Multiple Sclerosis Foundation (FISM Cod.2008/R/18), the Italian Ministry of Education, University and Research and the Italian Ministry of Health.

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