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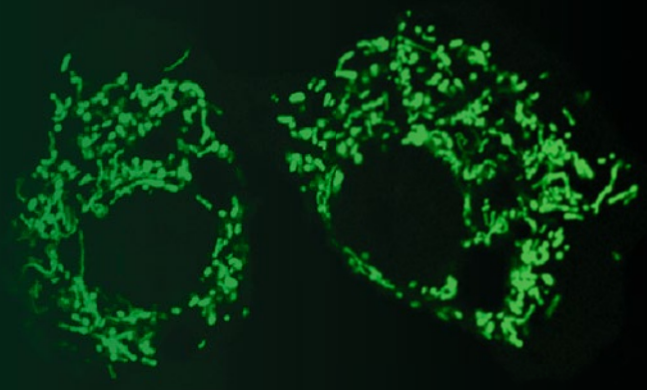

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Chapter 14

Assessment of Mitochondrial Membrane Potential and NADH Redox State in Acute Brain Slices

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Abstract

Brain is one of the most energy-demanding organs. Energy in the form of ATP is produced in brain cells predominantly in oxidative phosphorylation coupled to mitochondrial respiration. Any alteration of the mitochondrial metabolism or prolonged ischemic or anoxic conditions can lead to serious neurological conditions, including neurodegenerative disorders. Assessment of mitochondrial metabolism is important for understanding physiological and pathological processes in the brain. Bioenergetics in central nervous system is dependent on multiple parameters including neuron–glia interactions and considering this, in vivo or ex vivo, the measurements of mitochondrial metabolism should also be complimenting the experiments on isolated mitochondria or cell cultures. To assess the mitochondrial function, there are several key bioenergetic parameters which indicate mitochondrial health. One of the major characteristics of mitochondria is the mitochondrial membrane potential ($\Delta\Psi m$) which is used as a proton motive force for ATP production and generated by activity of the electron transport chain. Major donor of electrons for the mitochondrial respiratory chain is NADH. Here we demonstrate how to measure mitochondrial NADH/NAD(P)H autofluorescence and $\Delta\Psi m$ in acute brain slices in a time-dependent manner and provide information for the identification of NADH redox index, mitochondrial NADH pool, and the rate of NADH production in the Krebs cycle. Additionally, non-mitochondrial NADH/NADPH autofluorescence can signify the level of activity of the pentose phosphate pathway.

Key words Mitochondria, NADH, Mitochondrial membrane potential, Acute brain slices

1 Introduction

Functioning of the brain is one of the most energy-dependent processes in our organism. Maintenance of the resting membrane potential and ion homeostasis in neurons is regulated by utilization of ATP, which is directly dependent on the consumption of oxygen [1]. Oxygen is transported to the cells of CNS as a complex with Hb in the form of oxyhemoglobin (HbO_2) and is passing through the blood–brain barrier through the basement membranes of the

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microcirculatory vessels (brain capillaries). Oxygen is then used by the cells for the execution of the biochemical process of oxidative phosphorylation that is coupled with the functioning of electron transport chain (ETC) in mitochondria. The donors of electrons for complex I and complex II from the electron transport chain are NADH and $FADH_2$ (flavoprotein) which are produced in the tricarboxylic acid cycle (TCA cycle or Krebs cycle). NADH and the oxidized form of $FADH_2$—FAD—are fluorescent, and this helps to assess them in live cells and tissues [2–4]. However, NADH fluorescence cannot be separated from NADPH and it is also produced in the cytosol. Thus, to assess the autofluorescence of NADH in mitochondria, uncouplers in combination with inhibitors of the ETC should be used [5].

Electron transport chain of mitochondria generates the mitochondrial membrane potential ($\Delta\Psi m$), which is used as a proton motive force to produce ATP in the F0-F1-ATP synthase. Any change in mitochondrial metabolism reflects over to the $\Delta\Psi m$ and considering this, measurement of the mitochondrial membrane potential can be considered as an indicator of mitochondrial "health" [6].

Although mitochondrial function could be assessed in isolated mitochondria or in live cells in cell cultures, possibilities need to be developed for this measurement in in vivo or ex vivo models. This is specifically important for the brain where bioenergetics is complex and dependent on the multiple processes including neuron–glia interaction [7, 8].

Mitochondrial metabolism is vitally important in central nervous system and any alteration in function of mitochondria leads to the development of pathology, i.e., neurodegeneration [9–13] and other neurological conditions, including ataxias and epilepsy [14–16].

Here, we describe our methods for measurement of mitochondrial membrane potential and mitochondrial NADH and FAD in acute brain slices using fluorescent imaging. This method helps to visualize and measure the dynamics of brain metabolic changes and biochemical processes that occur in the living brain cells.

2 Materials

All manipulations must be carried out in accordance to the ethical standards for humane treatment of animals. Follow all waste disposal regulations when disposing waste materials.

2.1 Equipment

1. Large scissors or guillotine.
2. Vibratome.
3. Fluorescent imaging setup (*see* **Note 1**).

2.2 Reagents for NADH Determination

1. HBSS with 10 mM HEPES (two types: with pH 7.4/RT and with pH 7.4/4 °C) (*see* **Notes 2** and **3**).
2. 100 mM FCCP stock solution in DMSO (close the tube with foil and store at −20 °C) (*see* **Note 4**).
3. 100 μM FCCP working solution in HBSS (pH 7.4/RT).
4. 100 mM NaCN working solution in double-distilled water. Use freshly prepared solution (*see* **Note 5**).

2.3 Reagents for Mitochondrial Membrane Potential Determination

1. HBSS with 10 mM HEPES (two types: with pH 7.4/RT and with pH 7.4/4 °C).
2. 1 mM of rhodamine 123 stock solution in DMSO (should be stored in a dark place (cover the tube with tin foil) and store at −20 °C).
3. 100 mM FCCP stock solution in DMSO (cover the tube with tin foil and store at −20 °C).
4. 100 μM FCCP working solution in HBSS (pH 7.4/RT).
5. 1 μM solution of rhodamine 123 in HBSS (pH 7.4/RT).

3 Methods

NADH possesses intrinsic fluorescence with an absorption maximum at 360 nm and an emission maximum at 460 nm. The fluorescence of the slices is determined by the physiological content of NADH, all over the cell (in the cytosol and in cellular compartments). When protonophore (FCCP) is added to the slice under measurement, cell respiration reaches its maximum due to the ability of mitochondria to correct for the electrochemical gradient and, accordingly, for the mitochondrial membrane potential. At the same time, the NADH fluorescence intensity reaches a minimum, determined by the reduced form of coenzyme content exclusively in the cytosol, while in mitochondria, complete oxidation of NADH occurs with the formation of nonfluorescent NAD+ (Fig. 1). With the subsequent addition of cyanides, blocking the complex IV of the respiratory chain, and the oxidation of NADH (Fig. 1), the fluorescence level reaches the maximum level determined by the reduced form of coenzyme content in the cytosol and in mitochondria.

NADH is an electron donor for complex I, and therefore NADH levels are inversely correlated with respiratory chain activity. To measure the redox index, FCCP (1 μM) is used to maximize respiration and therefore minimize the NADH pool, then NaCN (1 mM) is added to block mitochondrial respiration and thus maximize the NADH pool. Initial autofluorescence is calculating as a percentage of this range (Fig. 1). Besides, the total mitochondrial pool of NADH can take as an indicator of the presence of substrate for complex I.

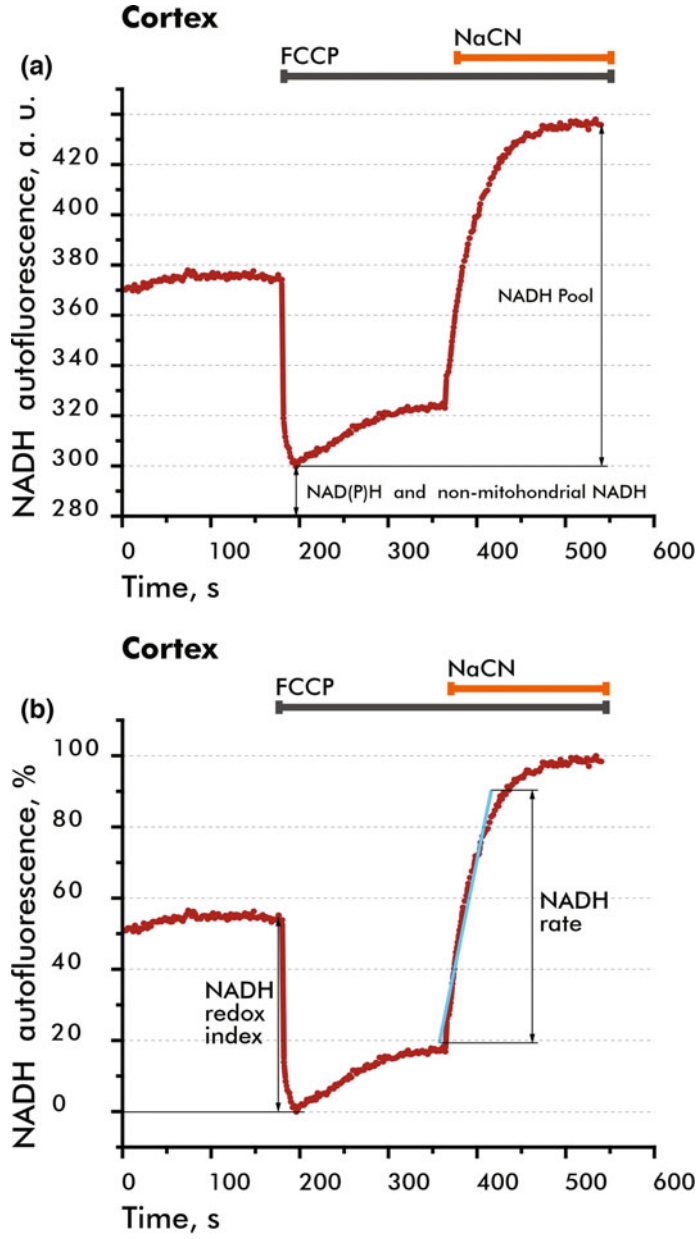

Fig. 1 Typical NADH change curves in healthy acute brain slices: calculation of (**a**) NADH pool and the level of non-mitochondrial NADH/NAD(P)H and (**b**) NADH redox index and NADH production rate

As soon as the baseline autofluorescence levels are obtained using data collection (2 min), the maximum possible signal stimulating respiration is achieved (defined as the reaction to the addition of 1 μM FCCP (the uncoupler)) and the maximum signal reduced. At the same time, respiration is completely inhibited as a reaction to the addition of 1 mM NaCN.

Finally, "redox indices of NADH" are generated by expressing the baseline levels of NADH, as a percentage of the difference between the most oxidized and maximally reduced signals. The NADH redox indices along the NADH pool provide information on the activity of the electron transport chain, as well as any disturbances in the function of the mitochondrial respiratory complexes. Using this method, it is also possible to assess the levels of NAD(P)H through an autofluorescence analysis of NADH. This allows the activity of the pentose phosphate pathway (PPP) to be evaluated, as a place where glucose can be alternatively oxidized.

The NADH pool is calculated by subtracting the lowest fluorescence value (after addition of FCCP) from the highest (after addition of NaCN) (Fig. 1a). The NAD(P)H value is calculated by subtracting the background from the minimum fluorescence (*see* **Note 6**). The NADH redox index is calculated after the recorded trace is normalized from 0% to 100% (Fig. 1b). The minimum NADH autofluorescence (after FCCP) is 0%, and the maximum autofluorescence (NaCN) is 100%. The NADH redox index is represented as the basal autofluorescence before any inhibitors are added and expressed as a percentage from the 0% to 100% scale (48% in Fig. 1b). The NADH growth rate (slope, Fig. 1b) is a direct reflection of the effectiveness of the tricarboxylic acid cycle (TCA cycle), as NaCN blocks all mitochondrial respiration. The NADH growth rate is then calculated using linear fit to determine the slope of the fluorescence spectrum after NaCN administration. An example of the calculation is shown in Fig. 1b [1].

The method of mitochondrial membrane potential determination is based on the ability of a lipophilic fluorescent cation rhodamine 123 to accumulate in mitochondria. The degree of accumulation is determined by the value of the mitochondrial membrane potential—the higher it is, the higher the ratio of the concentration of the cation in the mitochondrial interior to its concentration in the cytoplasm. Examined brain slices are kept in a rhodamine 123 solution for a certain time and after that the measurement of the fluorescence (excitation—505 nm, emission $>$ 540 nm) level is done in the following mode: first 3 min (basic fluorescence level), FCCP introduction, and subsequent measurement to the moment of fluorescence level stabilization. In the experiment, an increase in the level of fluorescence should occur because of the effect of concentration quenching after the subsequent introduction of FCCP (Fig. 2), a decrease in the mitochondrial potential occurs, and the subsequent exit of the cation into the cytoplasm. The degree of increase in fluorescence is an indicator of the level of membrane mitochondrial potential and can be used to compare different types of brain.

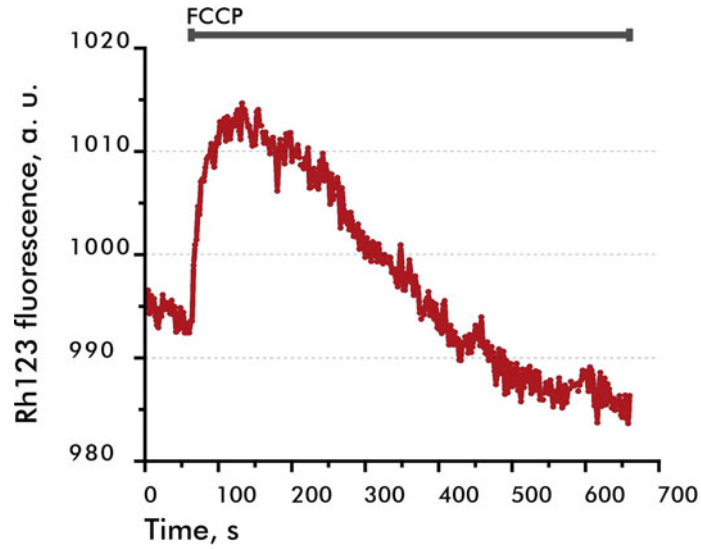

Fig. 2 Measurement of rhodamine 123 fluorescence in acute brain slices with complete depolarization of mitochondria upon application of FCCP

3.1 Acute Brain Slices Preparation Procedure

1. Cervical dislocation of the animal.
2. Separation of the head from the trunk in the cervical region of spinal cord by using large scissors or guillotine.
3. Dissection of the scalp along the sagittal line and opening of the cranium (generally starting in the areas of the nasal, temporal, and parietal bones).
4. Quick extraction of the brain with cerebellum, followed by periodic washing by cold HBSS (pH 7.4/4 °C). It is important to avoid surface drying of the sample.
5. Performing a sagittal section of the brain in an ice-cold glass petri dish.
6. Excision of the brain regions of interest with subsequent preparation of slices with a thickness of 100–200 μm. Acute brain slices were cut on a vibratome, according to a standard brain tissue cutting procedure [17, 18], in ice-cold HBSS (pH 7.4/4 °C).

3.2 NADH Determination Procedure

1. Prepared slices were maintained in the well of a glass slide, in 150 μl HBSS (pH 7.4/RT) for a minimum of 1 h before the measurement.
2. Then the level of NADH fluorescence is determined in a time-dependent manner:
   (a) Record the basic level: 0–180 s.
   (b) Addition of 5 μl of FCCP.

(c) Record of fluorescence level: 181–360 s.

(d) Addition of 5 μl of NaCN.

(e) Record of fluorescence level: 361–540 s.

3.3 Mitochondrial Membrane Potential Determination Procedure

1. One slice is transferred to a working solution of rhodamine 123 and incubated for 15 min.
2. The slice is washed using a large amount of HBSS (pH 7.4/RT), placed in clean HBSS (pH 7.4/RT) and incubated for 5 min. Then the washing procedure is repeated two more times.
3. The prepared slice is placed in the well of a glass slide, to which 150 μl of HBSS (pH = 7.4/RT) is added.
4. Then the level of fluorescence is determined at the settings of the laboratory setup closest to the fluorescence parameters of rhodamine 123:
   (a) Record of basic level: 0–180 s.
   (b) Addition of 5 μl of FCCP working solution.
   (c) Record of fluorescence level before reaching a plateau.

4 Notes

1. Measurements of $\Delta\Psi m$ and NADH level in brain slices can be done using various systems including confocal microscopy but can be dome in less sophisticated systems. Here, we suggest an easy-to-implement system for measuring endogenous NADH and NAD(P)H content.

   The optical scheme is a standard scheme of reflected light microscopy. In this scheme, the microscope objective works both as a condenser and as an image-forming system. The key element for such optical schemes is a vertical illuminator. The main function of a vertical illuminator is to form a collimated light beam, direct it to the rear aperture of the lens, and then to the surface of the sample. The main component of a vertical illuminator in fluorescent studies is a dichroic mirror. This mirror deflects the exciting radiation coming from the horizontal illuminator by 90° to the vertical optical image-forming system. In addition, the dichroic mirror, located at an angle of 45° to the optical axes of the illuminator and the image-forming channel, passes fluorescent radiation coming out of the lens (*see* Fig. 3).
2. Acute brain slices are placed on glass coverslips just before the experiment. While imaging, tissue should be buffered using HEPES-buffered salt solution (HBSS medium).

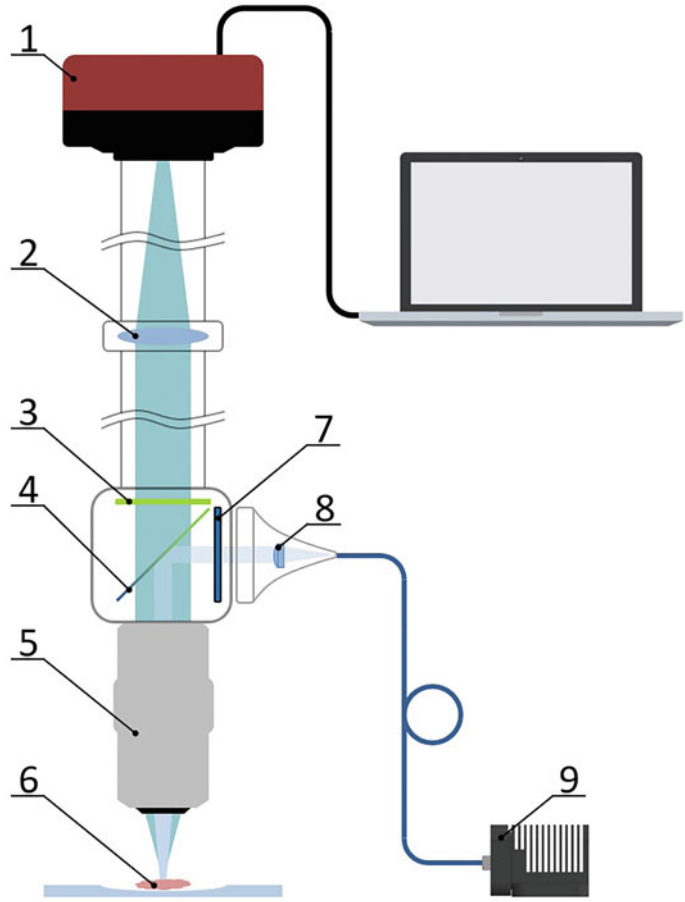

Fig. 3 Scheme of the experimental setup for measurement of endogenous fluorescence in acute brain slices. Excitation radiation (9) from an optical fiber passes through a collimator (8) and an extinction band filter (7) to cut out a narrow excitation band, and further through a dichroic mirror (4) and a planar apochromatic lens (5) is directed to the study area (6). In the image-forming channel, the back-reflected radiation from the source is filtered by a dichroic mirror (4) and an emission filter (3), and the fluorescent radiation through a long-focus lens (2) is registered by a highly sensitive cooled CCD camera (1). The field view of the system is a rectangular area of about 1 mm^2 with a resolution of about 2 μm

3. This buffer allows the imaging of cells or slices in a static chamber avoiding the need for continuous CO_2-equilibrated buffering.
4. FCCP is a lipid-soluble weak acid used as a mitochondrial uncoupling agent. FCCP is negatively charged allowing the anions to diffuse freely through nonpolar media, such as phospholipid membranes. It abolishes the obligatory linkage between the respiratory chain and the oxidative phosphorylation system which occurs in intact mitochondria.

5. NaCN is a respiratory chain inhibitor and it blocks respiration in the presence of either ADP or uncouplers such as FCCP. It specifically blocks the cytochrome oxidase (complex IV) and prevents both coupled and uncoupled respirations despite the presence of substrates, including NADH, and succinate.
6. The registered NADH autofluorescence upon addition of FCCP represents both the non-mitochondrial NADH autofluorescence and the NAD(P)H autofluorescence. To ensure that only NAD(P)H autofluorescence is recorded and analyzed, background fluorescence is deducted from the total fluorescence output.

Acknowledgments

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