Title: Assessing the redox status of mitochondria through the NADH/FAD+ ratio in intact cells

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Abstract

This methods section aims to describe the measurement of NADH and FAD+ levels in intact cells using fluorescence microscopy. Both NADH and $FADH_2$ are major electron donors for the electron transport chain through shifting of their redox status. Furthermore, within their redox couples, only NADH and FAD^{2+} are fluorescent. Therefore, calibration of the NADH and FAD^{2+} fluorescence signal is a crucial factor in accurately assessing mitochondrial function and redox status.

Key Words

NADH, FAD+, autofluorescence, redox status, ETC

Introduction

Mitochondria are often referred to as the "powerhouse" of the cell, as they generate ATP through the function of the electron transport chain (ETC). The ETC is composed of several complexes which use energy derived from redox reactions to pump protons across the inner mitochondrial membrane to generate an electrochemical gradient. This proton motive force is subsequently harnessed to phosphorylate ADP to ATP by the F1F0-ATP synthase. Within the mitochondrial electron transport chain (ETC), Complex I (NADH: ubiquinone oxidoreductase) and Complex II (Succinate dehydrogenase) utilize NADH and $FADH_2$ respectively as substrates/coenzymes. Through oxidation, these molecules donate protons and electrons to the ETC, therefore contributing to the establishment of the mitochondrial membrane potential and ATP production[1].

Consequently, NADH (Nicotinamide adenine dinucleotide) and FAD^{2+} (flavin adenine dinucleotide) molecules are shifting constantly between the oxidized and reduced forms, due to the function of the different enzymes implicated in energy-producing pathways, for example,

matrix dehydrogenases in the citric acid cycle. The balance between the 2 distinct forms is crucial for determining redox status and ETC function. The reduced form of NAD^+ (NADH) and the oxidized form of FAD (FAD^{2+}) are both intrinsically fluorescent, with distinct spectral properties, while the redox pair is not fluorescent. Thus, we can measure both species simultaneously using a confocal microscope. This allows us to assess the redox status of the mitochondrial NADH and FAD^{2+} pools[2].

To determine the dynamic range of NADH and FAD^{2+} pools, drugs including NaCN and FCCP (Phospho-trifluoromethoxy carbonyl cyanide phenylhydrazone) are often used. NaCN is an inhibitor of complex IV (COX: cytochrome c oxidase), therefore, it prevents the reduction of oxygen to water. This in turn inhibits the transfer of electrons through the ETC. This downstream inhibition will inhibit complex I and II, resulting in the accumulation of NADH and $FADH_2$, thus, providing a measure for maximally reduced mitochondrial NADH and $FADH_2$ pools. Meanwhile, FCCP is an uncoupler of ETC, which allows protons to travel freely across the mitochondrial inner membrane independent of the ATP synthase, resulting in maximum activity of ETC complexes, which are normally constrained by the proton gradient. In this case, NAD^+ and FAD^{2+} accumulate in their oxidized form due to rapid oxidation by CI and CII, and therefore gives a measure of the maximally oxidised pool. Taken together, these drug treatments allow us to determine the dynamic range of the NADH and $FADH_2$ pools shifting between the extremes of their redox states[3, 4].

Overall, this approach is to investigate redox status of NADH and $FADH_2$ pools within their maximum and minimum dynamic range, thus, allows us to extrapolate backwards to define a resting ‘redox index’ as a measure which is defined by the balance of resting respiratory rate and resting generation of reduced cofactors by the TCA cycle. These measurements are taken using a confocal microscope and application of drugs (NaCN and FCCP).

Materials

Equipment:

1. Cells need to be seeded into a fluorescence imaging compatible coverslips, plates or dishes; it is very important to ensure that the imaging glass is UV-light transmissible for the NADH signal.

2. A fluorescence microscope with excitation in the UV-range (normally containing a 355 nm laser).

3. It is necessary to use UV compatible objectives.

Recording medium: Use a recording medium that is compatible with your cells, ideally the medium used to grow the cells without phenol red and additional pH buffers such as HEPES.

Drugs: NaCN and FCCP (Phospho-trifluoromethoxy carbonyl cyanide phenylhydrazone) are required to generate maximum and minimum pools of NADH or FAD+. NaCN solutions should be prepared fresh on the day of the assay and should be pH adjusted. NaCN is volatile, and concentrations decrease rapidly after preparation, and so it must be used fresh. 1 mM FCCP stock solutions can be prepared and stored at -20^{o}C.

Methods

Imaging

1. Prepare the drugs: Prepare a 10X solution of NaCN in recording medium and a 10X FCCP stock in recording medium, vortex to dissolve and keep on ice. **Note 1**

2. Set up the microscope. The two optical paths required are:

1) NADH: excitation at 355 nm, Emission: 450 nm ± 30 nm

2) FAD+ signal: excitation at 458 nm (if you do not have a line at 458, it is possible to use the 488 nm line of an Argon laser), Emission: 550 nm ± 50 nm. **Note 2**

3. Aspirate the growth medium from the cells and wash once with pre-warmed recording medium before replacing with recording medium. Proceed to the microscope.

4. Set up the stage and focus. Find and save optimum image positions to image an appropriate number of cells. N**ote 3**

5. Adjust microscope settings. **Note 4**

6. We recommend trying time series at first on the microscope to record the change in fluorescence signal throughout the experiment, also helps with identifying time required to equilibrate after drug additions. **Note 5**

7. Start the acquisition to take images of cells under basal conditions with no drugs (we recommend 3 - 5 timepoints). **Note 6**

8. Add NaCN, according to previously determined concentrations. Allow to equilibrate and start acquisition. **Note 7**

9. Aspirate the recording medium after the acquisition has finished and carefully wash the dish/plate to remove the NaCN. **Note 8**

10. Add fresh recording medium. Add the FCCP into the Fluoro-dish or imaging plate, again wait for the drug to work and start the acquisition. **Note 9**

Data analysis

1. Quantification: Use an imaging processing software such as ImageJ to quantify the NADH/FAD^{2+} signal from each cell under the different conditions. **Note 10 and 11**

2. Determine the status of NADH/FAD^{+} within their respective dynamic range and Calculate the pool index of NADH (reduced states) /FAD+ (oxidized states) using this formula:

$$Pool\ Index = \frac{F_{basal} - F_{min}}{F_{max} - F_{min}}$$

Notes

1. For most cells, the final concentration of NaCN required to maximally inhibit respiration is between 0.1-10 mM, and the final effective concentration of FCCP is 1-5µM. Drug concentrations need to be optimised for individual cell lines.

2. If possible, set up a bright field path as well to confirm the outline of the cells.

3. If available, record the position of several fields on the microscope software to increase sampling from each condition, this would maximise the number of cells imaged.

4. Set the laser power or exposure as low as possible to limit photo-toxicity or bleaching, while ensuring you obtain a good signal: noise ratio. Adjust the master gain and digital offset accordingly if available to obtain an optimum signal output. These signals are dim – on most microscope systems we find we need to maintain a wide

pinhole on a confocal microscope to maximise signal detection. We also recommend setting the incubation of either the microscope chamber or the stage at 37^{o}C beforehand, to keep the temperature constant during the imaging process.

5. We recommend taking one time series at least once per cell line/type, to determine exactly how long it takes for the signal to reach plateau (maximum and minimum) after the drug addition.

6. Number of images depends on the confluency and homogeneity of cell population, generally at least 3-5 fields per dish/plate cells.

7. We would advise to try a range of drug concentrations, utilizing information from the time series to ensure you are capturing the maximum/minimum intensity post drug application.

8. A Pasteur Pipette with a thin tip is recommended for washing and aspirating the medium after the NaCN addition. It is very crucial to keep the dish/plate steady on the stage because we need to be able to find the previously saved positions.

9. For the drug additions, there are several things worth considering. Apart from the duration and dosage of drugs, the order of drug additions can also be adjusted with different cell types. Some cells are more sensitive to NACN and do not exhibit a robust NADH reduction post FCCP addition. Therefore, it is possible to first add FCCP, followed by NaCN without a wash step.

10. We recommend using Image J FIJI. Draw a region of interest around each cell, apply appropriate thresholding and record the signal intensity, subtract the background signal, obtain the basal (F_{basal}), maximum (F_{max}) and minimum signal (F_{min}). Note that the intensity of NADH and FAD^{+} should be inversely correlated with each drug treatments, for example, NADH pool reach the maximum under NaCN addition, whereas FAD+ reaches minimum here (Figure 1).

11. It is important to note that the autofluorescence signal will originate from both NADH and NADPH pools within the cell, which can only be spectrally distinguished using fluorescence lifetime imaging. Therefore, often NADH is referred to as NAD(P)H when quantifying autofluorescence.

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Reference

[1] A.L. Lehninger, D.L. Nelson, M.M. Cox, Lehninger principles of biochemistry, 6th ed., W.H. Freeman, New York, 2013.

[2] T.S. Blacker, M.R. Duchen, Investigating mitochondrial redox state using NADH and NADPH autofluorescence, Free Radic Biol Med 100 (2016) 53-65.

[3] M.R. Duchen, A. Surin, J. Jacobson, Imaging mitochondrial function in intact cells, Methods Enzymol 361 (2003) 353-89.

[4] F. Bartolome, A.Y. Abramov, Measurement of mitochondrial NADH and FAD autofluorescence in live cells, Methods Mol Biol 1264 (2015) 263-70.

Figure legends:

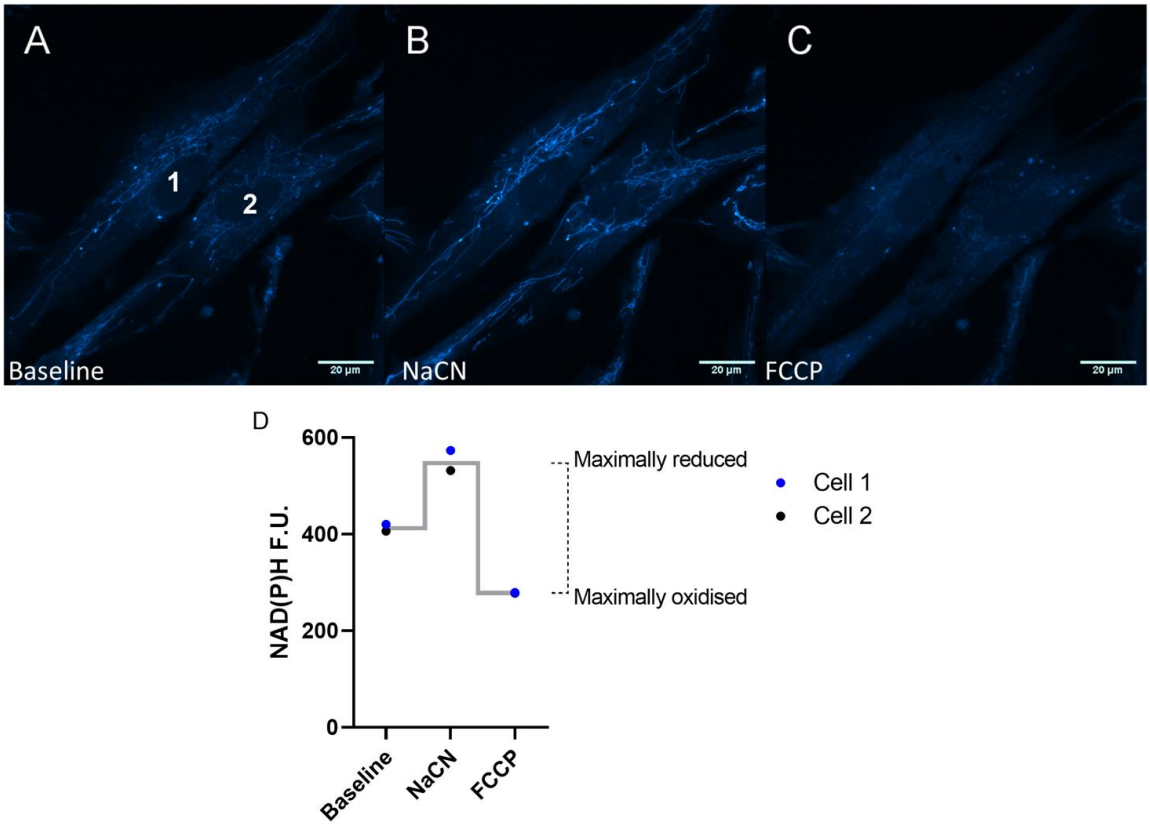

Figure 1: NAD(P)H autofluorescence in human fetal fibroblasts.

A) At baseline, NAD(P)H autofluorescence intensity is primarily observed in the mitochondrial network.

B) After the addition of NaCN, the mitochondrial NAD(P)H autofluorescence should increase as the NAD(P)H pool is maximally reduced due to inhibition of Complex IV.

C) In the presence of the uncoupler FCCP, NAD(P)H is maximally oxidised due to increased activity of the ETC, resulting in a significant reduction in fluorescence intensity.

D) Quantification of NAD(P)H fluorescence units (F.U.) where baseline NAD(P)H pool is calculated relative to the maximally reduced and maximally oxidised fluorescence values.

Scale bar: 20µm

The calculation of pool index of the two cells:

$$\text{Pool Index} = \frac{F_{basal}\text{-}F_{min}}{F_{max}\text{-}F_{min}} = \frac{420\text{-}279}{573\text{-}279} = 0.48$$

$$\text{Pool Index} = \frac{F_{basal}\text{-}F_{min}}{F_{max}\text{-}F_{min}} = \frac{406\text{-}278}{523\text{-}278} = 0.50$$