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Time-resolved imaging of mitochondrial flavin fluorescence and its applications for evaluating the oxidative state in living cardiac cells.

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Running title: FLIM of mitochondrial flavins

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Summary

Time-resolved fluorescence spectrometry is a highly valuable technological tool to detect and characterize mitochondrial metabolic oxidative changes by means of endogenous fluorescence. Here, we describe detection and measurement of endogenous mitochondrial flavin fluorescence directly in living cardiac cells using fluorescence lifetime imaging microscopy (FLIM) after excitation with 473 nm picoseconds (ps) laser. Time-correlated single photon counting (TCSPC) method is employed.

Keywords

Mitochondrial oxidative state, endogenous flavin fluorescence, FLIM, TCSPC, energy metabolism

1. Introduction

Fluorescence lifetime imaging and spectroscopy has made an important impact in the field of monitoring of the mitochondrial metabolic processes implicated in energy production in living cells and tissues [1-4]. Naturally occurring endogenous fluorophores and their fluorescence (also called autofluorescence) offer the possibility to study non-invasively mitochondrial metabolic processes. Despite their lower fluorescence yield when compared to fluorescence probes or dyes, endogenous fluorophores are highly advantageous for non-invasive tracking of changes in mitochondrial metabolic state by natural means. To evaluate mitochondrial metabolism, NAD(P)H and flavoproteins were proposed to be the marker of the metabolic oxidative state thanks to their principal role in energy production [5-7]. Addition of the time resolution, fluorescence spectroscopy enabled a deeper insight into metabolic processes thanks to the ability to distinguish individual fluorophores involved in energy production [8, 9]. Fluorescence lifetime is an internal property of the fluorophore. In general, it depends on the ability of a fluorophore to transfer energy to its environment – and thus on the physico-chemical characteristics of such environment, while being independent on the fluorophore concentration.

Endogenous fluorescence is an intrinsic property of living cells and while NAD(P)H is stimulated after excitation of the sample by ultraviolet (UV) light, flavins and flavoproteins are excited by visible light [4, 6, 8, 9]. This is particularly true in energetically highly active cells, such as cardiac myocytes [10-12]. Endogenous fluorescence results predominantly from mitochondria [13-14] and it is therefore a good practice to verify the mitochondrial localisation of the recorded images of cellular autofluorescence and/or to compare them with AF distribution. Flavins are best excited in the range from 400 to 500 nm [13, 15] and their fluorescence occurs with maximum at 490-560 nm. After binding, flavins are reduced into FADH, a non fluorescing molecule [8, 9, 12, 15]. Time-resolved approach identified two fluorescence decay components: 1.8-2.0 ns for the “free” form of flavins and 0.4-0.6 ns for, most likely, flavins “bound” to enzymes of the mitochondrial ETC (electron transport chain), in other words, “in use flavins” [3, 8, 9]. At lower wavelengths, fluorescence of NAD(P)H (emitting up to 500 nm) can affect the recording of the flavin fluorescence [16-18]. For the best results it is crucial to darken the experimental setup as much as possible: enclose it into a light-impermeable box/cover, or prevent the outside light to enter the laboratory.

Different types of flavins can be distinguished by different metabolic modulation [4-6, 9]. Modulation of the processes of energy production enables to study their roles in the cell metabolic state and can also provide a “calibration” of the metabolic oxidative state. OXPHOS (oxidative phosphorylation) can be either inhibited to reduce the oxidation, leaving the cells in a fully reduced state; or uncoupled from energy production, thus inducing a fully oxidized state [4-6, 19].

Both flavins and NAD(P)H can serve as metabolic oxidative state modulators. Although for spectral region where the NAD(P)H emits there is also a marginal collagen fluorescence, in the spectral regions where the flavins emits there are not only several types and forms of flavins emitting in a wide spectral band from 480 to approximately 560 nm, but also several other absorbing and emitting fluorophores (lipofuscins, melanin, riboflavin, porphyrins, etc.) [8-10]. Ability to detect and to distinguish between different forms of flavins by spectrally and time-resolved fluorescence is therefore highly useful to gather closer insight into the mitochondrial metabolism of various tissues [3, 12, 19] and, consequently, to understand changes that occur during either physiological (aging, differentiation) or pathophysiological

conditions linked with modifications in mitochondrial metabolic oxidative state such as inflammations, transplanted tissue rejections, hypoxia and/or cancer [4, 15, 16, 20]. Here, we describe flavin fluorescence lifetime imaging microscopy in cardiac cells isolated from rat hearts.

2. Materials

2.1. Solutions

Prepare all solutions using ultrapure water (produced by purifying the deionized water to attain the resistivity of 18 MΩ.cm at 25 °C). Adjust pH in all solutions to 7.35 with 1 mol/l NaOH, osmolarity 330 mosm/l.

2.1.1. Solution 1Ca : 135 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 1.2 mM NaH₂PO₄, 10 mM HEPES; 10 mM glucose, 10 mM creatine, 10 mM taurine.

2.1.2. Solution 0Ca-EGTA: 135 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 0.1 mM EGTA, 10 mM HEPES; 10 mM glucose, 10 mM creatine, 10 mM taurine.

2.1.3. Solution 50μM Ca: 135 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 0.05 mM CaCl₂, 1.2 mM NaH₂PO₄, 10 mM HEPES; 10 mM glucose, 10 mM creatine, 10 mM taurine.

2.1.4. Enzymatic solution: 135 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 0.02 mM CaCl₂, 1.2 mM NaH₂PO₄, 10 mM HEPES; 10 mM glucose, 10 mM creatine, 10 mM taurine and 0.05–0.2 mg/ml Liberase TM (Roche, Cat. No. 05 401 127 001).

2.1.5. BSA-enzymatic solution equals to the Enzymatic solution with addition of 1% (w./v.) of BSA.

2.2. Drug preparation

2.2.1. Prepare rotenone at 1 mM stock solution in water and store at –20 °C. Use at final concentration of 1 μM.

2.2.2. Prepare 2, 4-dinitrophenol (DNP) at 1mM stock solution in DMSO and store at –20 °C. Use at final concentration of 50 μM.

3. Methods

3.1. Description of microscopy methods

- 3.1.1. All procedures were carried out at room temperature (see **Notes 4.2-4.3**).
- 3.1.2. Prepare cardiac cells (see Cell preparation for microscopy).
- 3.1.3. For image acquisition, keep myocytes in the solution 2.1.1 (see **Note 4.1**).
- 3.1.4. Based on the employed objective, choose the right thickness of the microscope cover slip. For example, for C-Apochromat 40x, we used the No.#1 (0.13-0.16 mm) glass cover slip bottoms to seed the cells onto.
- 3.1.5. FLIM images were recorded using time-correlated single photon counting (TCSPC) technique [2] (see **Note 4.4**), following excitation with a 473 nm picosecond laser diode (BDL-473, Becker&Hickl, Germany, see **Note 4.5**) Axiovert 200 LSM 510 Meta (Carl Zeiss, Germany) equipped with C-Apochromat 40x, 1.2W corr NA. The laser beam was reflected to the sample through an epifluorescence path of the laser scanning confocal microscope. The emitted fluorescence was separated from laser excitation using LP500 nm and detected by HPM 100-40 photomultiplier array employing SPC-830 TCSPC board (both Becker&Hickl, Germany). The setup was kept in the darkness.

3.2. Cell preparation for microscopy

- 3.2.1. Isolate left ventricular myocytes based on retrograde Langendorff perfusion with the solution 2.1.4. See [7, 22] for details. The protocol is optimized for a rat weighting 200-400 g.
- 3.2.2. After removing the heart with sufficiently long ascending aorta, mount the aorta immediately to the cannula of the Langendorff system. Secure the aorta with a thread.
- 3.2.3. First perfuse the heart with the solution 2.1.1. to clean the heart from the blood (usually up to 5 minutes). At the end the dripping solution should be completely transparent.
- 3.2.4. Then perfuse with the solution 2.1.2 for 5 minutes to stop the contractions of the heart.
- 3.2.5. Consequently, perfuse the heart with solution 2.1.4 driven by a peristaltic pump at a rate of 3 ml/min until the tissue become soft (usually ~9 min).

- 3.2.6. Remove the right ventricle and cut the left ventricle gently into small pieces.
- 3.2.7. Filter the suspension through 100 μm nylon mesh.
- 3.2.8. Centrifuge at 50g for 1 min and replace the supernatant with the Solution 2.1.3.
- 3.2.9. Digest the remaining tissue with the Solution 2.1.5. for 5 min.
- 3.2.10. Repeat the steps 10-12 until the tissue is dissolved completely (usually 4-5 batches of myocytes).
- 3.2.11. Use the batch with highest yield of rod-shaped cardiac cells for further experiments. Centrifuge the batch at 50g for 1 min and replace the supernatant with the fresh solution 2.1.3. to completely remove the enzyme.
- 3.2.12. Increase gradually the concentration of Ca^{2+} in this batch to 100, 250, 500 and 1000 μM in 5 minutes intervals to keep the myocytes calcium tolerant (see also **Note 4.1** for other cell solutions). Use the myocytes up to 8 hours after isolation, or until degradation of the cells quality (observed namely by the presence of hypercontracted cell edges).

3.3. Fluorescence lifetime imaging of flavin fluorescence in living cells

- 3.3.1. Put cover slips with cells onto a microscopy stage.
- 3.3.2. Prepare recording setup: for flavin measurements, based on its excitation/emission characteristics (as detailed in the Introduction), use excitation at 473nm (or below, see **Note 4.5**) with an appropriate emission filter. To collect maximum fluorescence, use LP 500nm or below. See **Note 4.6** on how to gather spectrally-resolved data.
- 3.3.3. Create recording protocol: collect high resolution time-resolved images of cells for 120s with ADC (analog-to-digital converter) resolution 256 to record image size of 256x256 pixels (see Fig. 1 for illustration). To improve data resolution, see **Note 4.7**. For recording of fast processes, see **Note 4.8**.
- 3.3.4. When the recording is finished, save data in a corresponding file.
- 3.3.5. Verify the mitochondrial localisation of the endogenous fluorescence and absence of the nuclear one from the recorded images.

Figure 1 here

3.4. Testing mitochondrial presence

- 3.4.1. Fluorescence probe Rhodamine 123 can be employed to evaluate the presence of mitochondria in living cells (see **Note 4.9**). Apply Rhodamine 123 in external medium at final concentration of 1 μ M to the cells. Leave for 10 minutes and then replace with clean external medium.
- 3.4.2. Put cover slips with cells onto a microscopy stage.
- 3.4.3. Prepare recording setup: based on excitation/emission properties of the probes (500/530 nm for Rhodamine 123 [23]), we have used excitation with 473 nm picoseconds laser and LP 500nm.
- 3.4.4. Create recording protocol: collect high resolution time-resolved images of cells for 120s with ADC resolution 256 (see Fig. 2 for illustration; same recording setup and protocol can be employed for the measurement of flavin endogenous fluorescence). Verify the mitochondrial localisation of the Rhodamine 123 fluorescence from the recorded confocal images.

Figure 2 here

3.5. Reducing mitochondrial oxidation by OXPHOS inhibition

- 3.5.1. Prepare rotenone at 1-5 μ M in water.
- 3.5.2. Apply rotenone to unstained cells by gently replacing external medium with external medium + rotenone, or by adding higher concentration of rotenone to the solution with cells and shaking gently.
- 3.5.3. Leave rotenone to act for 10 minutes at room temperature (see **Note 4.2**): cells are now in a fully reduced state.
- 3.5.4. Verify that the fluorescence intensity has decreased and fluorescence lifetime shifted towards shorter lifetimes (shifting towards yellow colours, see example at Fig. 3; however with significantly decreased fluorescence verify the precision of the lifetime estimation, namely by checking the goodness of the fit with χ^2 value). See **Notes 4.10-4.12** for testing of other metabolic modulators.

- 3.5.5. Record corresponding images using exactly the same protocol that was employed in control conditions to insure comparability.
- 3.5.6. Use cells for up to 20-25 min, or until the deterioration of their viability. See **Note 4.7**.

Figure 3 here

3.6. Increasing mitochondrial oxidation by uncoupling of respiratory chain

- 3.6.1. Prepare DNP at 50 μ M in the external medium.
- 3.6.2. Apply DNP to cells by gently replacing external medium with external medium + DNP.
- 3.6.3. Leave DNP to act for 10-15 minutes at room temperature (see **Note 4.2**): cells are now in a fully oxidized state following uncoupling of ATP synthesis.
- 3.6.4. Verify that fluorescence intensity has increased and fluorescence lifetime shifted towards longer lifetimes (shifting towards blue colours, see example at Fig. 4). See **Note 4.10** for use of other metabolic uncouplers.
- 3.6.5. Record corresponding images using exactly the same protocol that was employed in control conditions to insure comparability.
- 3.6.6. Use cells for up to 20-25min, or until the deterioration of their viability. See **Note 4.7**.

Figure 4 here

3.7. Analysis

Use SPCImage software to perform first estimation of intensity and fluorescence decays in the recorded images.

- 3.7.1. Send data to SPCImage, or import the recorded image.
- 3.7.2. Calculate decay matrix (for a selected channel or the whole image).
- 3.7.3. Use the pre-defined instrument response function (IRF) (or see **Note 4.13** to use the recorded IRF).
- 3.7.4. To improve image, use export color-coded images.
- 3.7.5. If the result is not good (e.g. bright specks are present in the image), go to “Options/Intensity” tab and turn the “Autoscale” off.

- 3.7.6. Keep an eye on the goodness of the fit represented by chi squared value (χ^2): usually, it should not be higher than 1.2 (see **Note 4.14** on how to improve χ^2).
- 3.7.7. For advanced analysis, refer to linear unmixing of individual components [11], or phasor approach [24].

4. Notes

- 4.1. Self-designed external solution can be replaced by PBS, depending on the type of the isolated and/or cultured cells used. Remember to check that the applied solution does not contain molecules fluorescing in the detected spectral range, most commonly the Phenol red, and that the medium is able to maintain the cells alive for the necessary measurement time, without affecting their metabolism and/or viability. For example, D-MEM without phenol red, FluoroBrite™ D-MEM medium, can be successfully used for cultured cells.
- 4.2. Procedures can be carried out at 35-36 °C using an on-stage incubator for tracking temperature-dependent changes. In such case, do not forget to use pre-heated solutions for cell preparation and verify that the level of photobleaching [25] did not increase.
- 4.3. Oxygenation of the solution by different O₂/N₂ mixture can be insured by an on-stage incubator/controller.
- 4.4. Alternative to TCSPC, frequency-modulated (amplitude domain) approach can be employed to evaluate fluorescence lifetimes [1].
- 4.5. Excitation by 473 nm ps laser can be replaced by shorter wavelength, namely 445 nm ps laser. Use an appropriate LP (long pass) or BP (band pass) filter.
- 4.6. To gather spectrally-resolved data, specific narrow BP filters can be employed accordingly. For the most effective detection of the flavin fluorescence, we recommend to use either BP filter to record wavelengths in the range 490-590 nm, or LP filter to detect wavelengths longer than 500 nm to cut off the excitation pulse and its reflections. For example, to record spectrally-resolved flavin fluorescence, BP 490 ± 10 nm and BP 540 ± 10 nm can be used to evaluate “bound” and “free” flavins respectively. Another possibility is to use a monochromator in front of the detector with the advantage of choosing the exact wavelength detected.

- 4.7. Always verify the state of the recorded cells before and after recording. Discard all recordings during which the cells presented change in their shape and/or state. In case of cardiac cells, do not use cells presenting hypercontractions. Consequently, although shorter recordings are more suitable, the length of the recording has to be appropriate to reach a sufficient number of photons for multiexponential decay data analyse (for more details, refer to [2] or the Becker & Hickl TCSPC handbook <https://www.becker-hickl.com/literature/handbooks/>).
- 4.8. The recording time can be adjusted depending on the rapidity of the studied processes. For fast processes, synchronized recording can be considered, as described in [26].
- 4.9. Other fluorescence probes for viability testing can also be used, depending on the cell type and/or setup characteristics.
- 4.10. Effect of metabolic modulators can vary depending on the cell type and/or pathway that it employs. To test the oxidative phosphorylation, we have employed rotenone (the inhibitor of the Complex I) and DNP to inhibit and/or uncouple the ATP production by the respiratory chain, respectively. At the same time, rotenone can be replaced by CCCP and/or cyanides (NaCN or KCN, the inhibitors of the Complex IV), while 9,10-Dinitrophenol can be replaced by FCCP [4].
- 4.11. In cells preferentially using glycolysis instead of oxidative phosphorylation [4, 27, 28], modulators mentioned in the **Note 4.10** would induce only limited effect and other pathways, such as modulation of glucose production etc. need to be tested.
- 4.12. Comparably, sensitivity of cells to oxidative stress can be tested by applying compounds such as hydrogen peroxide [4, 7, 24].
- 4.13. For precise evaluation of the fluorescence lifetimes, measure the IRF [20] and apply it for the fluorescence lifetime data analysis. The Becker-Hickl SPCImage analysis software does not require the measured IRF, fast lifetime verification can be done with the pre-defined IRF. However, IRF measurement should always be performed on the new sample and verified regularly to achieve a precise lifetime evaluation, especially in case of very short lifetimes. IRF measurement could also be needed when custom analysis software is employed.
- 4.14. Goodness of the fit can be enhanced (the χ^2 lowered) by optimizing the offset and scatter parameters in the analysis, but also by improving the recorded image, for example, by insuring that less background light is entering the setup – darkening of

the setup can significantly improve the goodness of the fit(for more details, refer to [2] or the Becker & Hickl TCSPC handbook <https://www.becker-hickl.com/literature/handbooks/>).

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Figure legends.

Fig. 1. Intensity (left) and FLIM (right) image of flavin fluorescence recorded in cardiomyocytes in control conditions after excitation with 473 nm picosecond laser diode and emission with LP 500 nm. Fluorescence decay image scale is between 0.5 and 2.0 ns.

Fig. 2. Intensity (left) and FLIM (right) image of Rhodamine 123-stained cardiomyocytes after excitation with 473 nm picosecond laser diode and emission with LP 500 nm. Fluorescence decay image scale is between 0.5 and 2.0 ns.

Fig. 3. Intensity (left) and FLIM (right) image of flavin fluorescence recorded in cardiomyocytes in a reduced state, in the presence of rotenone ($1 \mu\text{M}$) after excitation with 473 nm picosecond laser diode and emission with LP 500 nm. Fluorescence decay image scale between is 0.5 and 2.0 ns. Note low intensity in the presence of the rotenone.

Fig. 4. Intensity (left) and FLIM (right) image of flavin fluorescence recorded in cardiomyocytes in an oxidized state in the presence of DNP (50 μ M) after excitation with 473 nm picosecond laser diode and emission with LP 500 nm. Fluorescence decay image scale between is 0.5 and 2.0 ns.