Spectral Unmixing of Flavin Autofluorescence Components in Cardiac Myocytes

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ABSTRACT We applied linear unmixing approach to reveal individual components of intrinsic flavin fluorescence signal recorded in living cardiac cells by spectrally resolved confocal microscopy. Responses of whole-cell autofluorescence to modulators of cell metabolism and respiration were used as a tool of separation of its components; their spectral profiles, estimated by principal component analysis, correspond to free FAD and FAD bound to different enzymes of electron transport chain.

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Numerous cardiac diseases, including hypertension and diabetes, are associated with mitochondrial dysfunction. It is therefore highly relevant to develop appropriate tools for investigation of metabolic changes directly in living cells, such as redox fluorimetry and imaging based on the intrinsic cellular fluorophores. The main sources of cell autofluorescence, when excited by visible light, are oxidized mitochondrial flavins and flavoproteins (1–4). However, several naturally occurring fluorophores based on the FAD cofactor have emission spectra that are superimposed in a broad band in the wavelength region of 490–560 nm, making their separation particularly difficult. In our previous contribution we demonstrated the sensitivity of cardiomyocyte autofluorescence spectra to changes in the redox state (5). In this study, we aim to separate the individual spectral components of the flavin autofluorescence. The enzymes fluorescing in the cells can be hardly isolated in their native state; we therefore attempted to resolve individual components of flavin autofluorescence directly in cardiac cells using their responsiveness to modulators of cell metabolism and respiration and apply spectral decomposition techniques for their separation.

MATERIAL AND METHODS

See Supplementary Material for Material and Methods.

Modulation of autofluorescence

Despite large number of enzymes containing flavin cofactors, previous research has established that α-lipoamide dehydrogenase (LipDH) and electron transferring flavoprotein (ETF) of the mitochondrial respiratory chain are the most significant contributors to bound flavin cellular autofluorescence. The fact that the LipDH is in redox equilibrium with NADH and the ETF is redoxly associated with ubiquinone (UQ) allows one to selectively monitor their redox state (6) and to use the inhibition of mitochondrial respiratory chain for their selective reductions. In the first instance, we therefore attempted to isolate the LipDH and the ETF by sequential application of rotenone and cyanide in cells placed at low redox potential in 2 mM glucose (Fig. 1, *ii*) (3,6). The control emission spectra (Fig. 1, *i*) of cardiac cells were obtained from the multispectral images (Fig. 1, *inset*) by plotting the mean intensity values within the whole cell area as a function of emission wavelength.

To modulate the fluorescence of LipDH, we applied rotenone, an irreversible inhibitor of NADH dehydrogenase. This treatment raised mitochondrial NADH by blocking the electron transport chain at complex I (3) and prevented the NADH-linked flavin of LipDH from being oxidized (4). The change of the redox state of the LipDH pool toward its reduced form was observed as a small drop in the fluorescence emission in blue-green region (Fig. 1, *iii*).

Second, we applied sodium cyanide, well-known blocker of electron transport at the level of the complex IV of the respiratory chain (3,6). The addition of the cyanide to cells pretreated with rotenone allowed us to discriminate between flavoproteins tight to the respiratory chain below the complex I from the flavoproteins that are reduced via ubiquinone above the complex I. As expected, this treatment resulted in a sharp drop in the autofluorescence emission intensity, predominantly in the shorter-wavelengths range (Fig. 1, *iv*). This is typical for the blue-shifted autofluorescence of FAD bound in electron transfer protein (1) of the mitochondrial β-oxidation pathway for which redox changes are ubiquinone dependent (6). Although the reduction of ubiquinone pool should lead to reduction of various flavoproteins (besides ETF), such as electron transferring protein ubiquinone reductase ETF:UQ and succinate dehydrogenase, their contribution to overall flavoprotein fluorescence can be neglected, as demonstrated previously (7).

After the treatment with both rotenone and cyanide, a significant amount of cell fluorescence remained in yellow spectral region with the emission maximum at 550–560 nm (Fig. 1, *iv*). This remaining component was suggested to be linked to enzymes of fatty acid β-oxidation (7). Indeed, the octanoate (5 mM), when applied to cells in the presence of the rotenone, significantly enhanced the fluorescence in this spectral region (Fig. 3, *v*). We hypothesize that dehydrogenation of octanoylCoA derivates, catalyzed by acylCoA dehydrogenase reaching its maximum activity in the presence of octanoate (8), could be responsible for the origin of this signal.

We finally attempted to increase the fluorescence intensity of free oxidized FAD/FMN in cardiomyocytes by addition of 2,4-dinitrophenol (DNP), the uncoupler of mitochondrial oxidation, which is known to increase oxidized state of mitochondria, leading to generation of oxidized flavins (4). We observed significant elevation of overall fluorescence 5–10 min after

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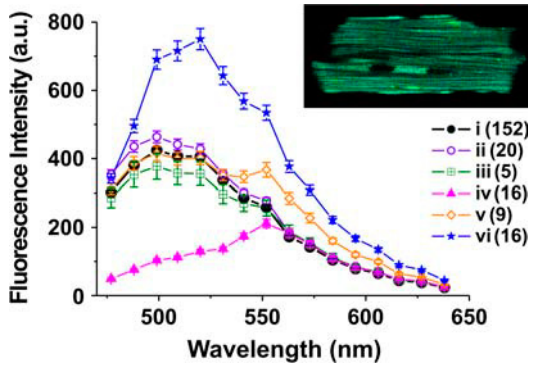

FIGURE 1 Responses of autofluorescence to mitochondrial modulators. Emission spectra of cardiomyocyte autofluorescence in control conditions (*i*), in low glucose (*ii*) in the presence of 10 μM rotenone (10 min) (*iii*) and after addition of sodium cyanide (4 mM for 5 min) (*iv*), or octanoate (5 mM for 10 min) (*v*), and/or in the presence of DNP (50 μM) in the external solution (*vi*). Data are represented as mean ± SE, with the number of cells in brackets. (*Inset*) Example of the cardiomyocyte image.

perfusion of cells with DNP in control external solution, especially in the green spectral region with maximum at 530 nm (Fig. 1, *vi*), which closely resembles the emission reported for free oxidized FAD.

Separation of spectral components

To identify major spectral components in autofluorescence images of isolated cardiomyocytes, we have applied principal component analysis (9). First step was to define the number of significant, independent sources of the component variation, which could regenerate matrix of experimental data. By single value decomposition technique (9) we have estimated that three significant components were present in our data. The spectra of these components were determined using the target transformation method described by Beauchemin (10). As an initial estimation of the potential target spectra we have used the difference spectra obtained after the specific modulation of autofluorescence, constructed from profiles described at Fig. 1. The final spectral profiles (Fig. 2) were computed using constrained least-square optimization.

In the next step, we applied the linear unmixing approach to separate the individual components in the recorded images. We fitted the measured emission signal at each image pixel with the function $S(\lambda)_{sum}$, computed according to the equation

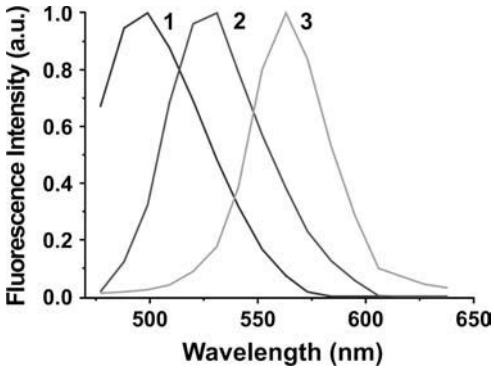

FIGURE 2 Reference spectra of individual flavin autofluorescence components, computed by principal component analysis.

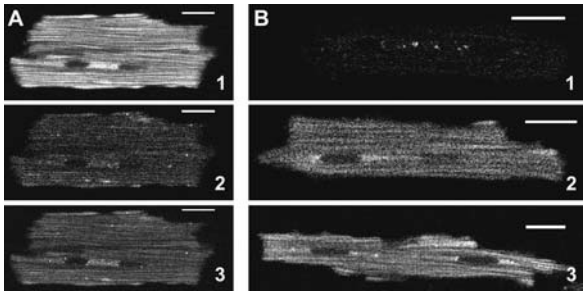

FIGURE 3 (*A*) Image of cardiomyocyte autofluorescence in control conditions (from Fig.1, *inset*) after linear unmixing of its spectral components. Channel numbers correspond to the components with the reference spectra given by Fig. 2. (*B*) Example of changes in the autofluorescence intensity at the respective channels after modulation by (*1*) 10 μM rotenone + 4 mM cyanide; (*2*) 50 μM DNP; (*3*) 10 μM rotenone + 10 mM octanoate. Only affected components are shown in panel *B*. White line corresponds to 20 μm.

$$S(\lambda)_{sum} = \sum_{i=1}^{3} I_i \times S(\lambda)_i + S(\lambda)_b, \quad (1)$$

where the spectral profiles $S(\lambda)_i$ are taken from Fig. 2, I_i denotes the recovered intensity of each component, and $S(\lambda)_b$ denotes the background signal.

Unmixed images (Fig. 3 *A*) revealed the predominance of the blue-fluorescent first component with the spectral maximum around 500 nm in control conditions. This component was almost completely abolished using cyanide in the presence of rotenone (Fig. 3 *B*, *1*), suggesting that it mainly images the flavin bound to the ETF. Contribution of the LipDH could not be resolved in this case due to its overlap with the ETF component; other approaches such as the use of multiple excitation wavelengths (3) may be needed in addition to spectral imaging for further separation of these components. On the other hand, the DNP clearly enhanced the second component peaking at 530 nm (Fig. 3 *B*, *2*), in agreement with its free FAD origin. Finally, the third component with the maximum around 560 nm was significantly enhanced using octanoate (Fig. 3 *B*, *3*), supporting its acylCoA dehydrogenase origin. The residual signal with maximum at 615 nm (not shown) was small and mostly compartmentalized; porphyrin-filled lipofuscin deposits are most likely to be responsible for its origin.

Our results indicate that the different components of cardiomyocyte flavin autofluorescence can be identified and separated using spectrally resolved confocal microscopy and enzyme-selective modulation of the mitochondrial metabolic state, followed by linear unmixing. This approach can greatly improve the analysis of the flavin autofluorescence and its use for diagnostics of cardiomyocyte mitochondrial dysfunction.

SUPPLEMENTARY MATERIAL

An online supplement to this letter can be found by visiting BJ Online at http:\\www.biophysj.org.

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