Assessment of Low-Intensity Fluorescence Signals in Living Cardiac Cells Using Time-Resolved Laser Spectroscopy

D Chorvat Jr[1], F Elzwiei[2], V Bassien-Capsa[2], A Mateasik[1], A Chorvatova[2,3]

[1]International Laser Centre, Bratislava, Slovakia
[2]Research Centre of CHU Sainte-Justine, University of Montreal, Montreal, Canada
[3]Department of Pediatrics, University of Montreal, Montreal, Canada

Abstract

In the aim to study calcium distribution and dynamics in single living left-ventricular rat cardiomyocytes, we have applied a new method for discrimination of the calcium probe fluorescence signal from the intrinsically fluorescing cell constituents. In a case study investigating calcium-sensitive dye Fluo-3, we have characterized the spectral and the lifetime fingerprints of the Fluo-3 fluorescence and of the cell flavin autofluorescence by spectrally-resolved confocal microscopy and/or by multi-wavelength time-correlated single photon counting. We demonstrate that employing such spectral database, a low-intensity Fluo-3 signals comparable to the cell autofluorescence can be successfully resolved in cardiomyocytes using spectral linear unmixing algorithm.

1. Introduction

In the last two decades, a wide spectrum of fluorescent constructs and molecules have been developed, aimed as nanosensors for non-contact monitoring of the cell structures and biological functions. In this contribution, we focus on the measurement of spatial distribution and dynamics of intracellular calcium in isolated rat cardiac myocytes. This subject has been extensively studied using fluorescent calcium probes, traditionally exploiting the spatio-temporal changes of the probe intensity within the cell. Recently, the fluorescence lifetime detection started to gain interest in this field [1] due to its independence on the local probe concentration and potential for intrinsically calibrated measurement based on direct estimation of the ratio of molecules with bound and unbound calcium. However, in spite of great expectations from the fluorescence lifetime imaging introduced in mid-90s of the last century, only few reproducible applications to the living cell ion imaging have been reported so far.

To our knowledge, one of the most often overlooked issues in fluorescence experiments with labeled living cells is the presence of their endogenous autofluorescence (AF). As we will show later in this paper, the AF can constitute a large fraction of the signal detected in conditions such as cardiomyocyte resting state. Therefore, an efficient discrimination of the cell AF from the calcium-sensitive probe fluorescence is a prerequisite for an unbiased quantitative estimation of intracellular calcium concentration in cardiac cells in these conditions. Since the cell AF is inhomogenously spatially distributed within the cell, with its spectrum and intensity being highly sensitive to cellular metabolism [2], its contribution can not be easily substracted using a "constant background" model. Instead, we propose to use the fluorescence spectral/lifetime fingerprinting and sequential linear unmixing [3] as promising techniques capable to separate the signals of the externally applied fluorescence probes from the intrinsically fluorescing molecules. As a representative example, we show the application of the spectral unmixing on data from widely used fluorescence probe Fluo-3, recorded by two experimental techniques: spectrally-resolved confocal microscopy and multi-wavelength fluorescence lifetime spectroscopy.

2. Methods

2.1. Cardiomyocyte isolation

Left ventricular myocytes were isolated following retrograde perfusion of the heart with proteolytic enzymes from Sprague-Dawley rats (13-14 weeks old, Charles River, Canada) [3]. All procedures were performed in accordance with Institutional Committee accredited by the Canadian Council for the Protection of Animals (CCPA). Myocytes were maintained in a storage solution at 4 °C until used. Only cells that showed clearly defined striations were used in up to 10 hrs following isolation.

2.2. Solutions and drugs

The basic external solution contained (in mM): NaCl, 140; KCl, 5.4; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; HEPES, 10; adjusted to pH 7.35 with NaOH. Cells were loaded with Fluo-3-AM (3.3 μM) for 15 min at room temperature. All chemicals were from Sigma-Aldrich (Canada), whereas Fluo-3 (AM, as well free form) and Calcium Calibration Buffer Kit (with Magnesium #2) were from Molecular Probes (U.S.A.).

2.3. Confocal microscopy

Spectrally-resolved fluorescence imaging was done using the confocal laser scanning head LSM-510 Meta on Axiovert 200 inverted microscope (both Zeiss, Canada). Images were recorded with a PlanNeofluar 63x /1.3 oil objective, using the 488 nm Ar:ion laser for excitation, HFT KP 700/488 dichroic filter and 16 channels of the META detector in the 516 nm - 687 nm spectral range for emission detection. To avoid alteration of the autofluorescence spectral shape by photobleaching, spectral data were always recorded from the first scan of each cell.

2.4. Fluorescence lifetime spectroscopy

A custom-build time-correlated single photon counting (TCSPC) setup on inverted microscope (Axiovert 200M, Zeiss, Canada) was used to record fluorescence spectra and lifetimes, as described in details previously [3]. In brief, a beam of 475 nm picosecond diode laser (BDL-475, Becker&Hickl, Boston Electronics, U.S.A., running at 20 MHz) was reflected to the sample through the epi-fluorescence path of the microscope using standard filters (510 nm dichroic and 515 nm long-pass for emission). The emitted fluorescence was detected within the 390-670 nm range by 16-channel photomultiplier array (PML-16, Becker-Hickl, Boston Electronics, U.S.A), attached to the spectrograph (Solar 100, Proscan, Germany). Fluorescence decays were measured for 30 s with 25 ns time-base, sampled by 1024 points using the TCSPC interface board SPC-830 (Becker-Hickl, Boston Electronics, U.S.A). Cells were studied at room temperatures in 4-well chambers with coverslip-based slides (LabTech).

2.5. Data analysis

Data were analyzed using SPCImage software (Becker&Hickl, Boston Electronics, U.S.A), or Zeiss LSM Image Examiner (Zeiss, Canada), Origin 7.0 (OriginLab, USA) and custom-written procedures for data correction and analysis written in C++ with the help of NAG numerical libraries (Oxford, UK). Data are shown as mean ± standard error (SEM).

3. Results

3.1. Multispectral confocal microscopy of Fluo-3 and autofluorescence in cardiomyocytes

Spectrally-resolved spatial distribution of Fluo-3 signal was recorded in single cardiac myocytes using confocal microscopy. In the aim to determine underlying spectral components, reference spectra of Fluo-3 in calcium calibration solution and AF in cells were identified; both peaking with the spectral maximum at 532 nm channel (Fig. 1A).

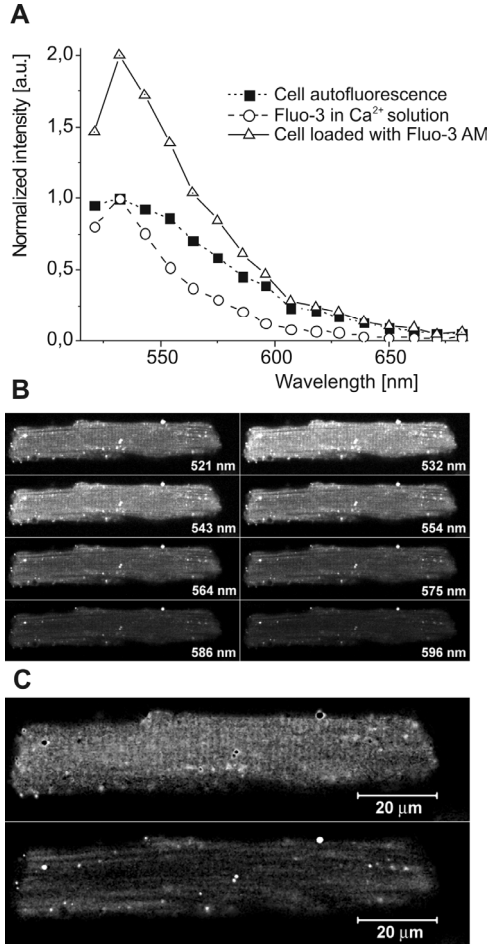

Figure 1. The signal from the cell loaded with Fluo-3 AM (3.3 μM) compared to reference spectra of the cell AF and Fluo-3 in Ca^{2+} solution **(A)**. Representative example of spectrally-resolved image of cardiac cell loaded with Fluo-3-AM **(B)**. Spectrally unmixed image of the same cell, showing the contributions of Fluo-3 (above) and of AF (below). For linear unmixing, a reference spectral database shown at A was used **(C)**.

The images of cardiac cells loaded with Fluo-3-AM (Fig. 1B) were then spectrally unmixed [2] on pixel-by pixel basis, using the equation

$$S(\lambda)_{sum} = \sum_{i=1}^{2} I_i \times S(\lambda)_i + S(\lambda)_b$$

where the recorded signal $S(\lambda)_{sum}$ was fitted using the reference spectra $S(\lambda)_i$, taken from Fig. 1A; I_i denotes the recovered intensity of each component and $S(\lambda)_b$ denotes the background signal measured outside the cell region. Unmixed images, corresponding to the pure Fluo-3 and AF signals in cardiac cells were of comparable intensities but featured distinctly different spatial distributions (Fig. 1C).

3.2. Multi-wavelength fluorescence lifetime detection and sequential unmixing of Fluo-3 and autofluorescence

Spectrally-resolved TCSPC recordings of fluorescence decays of Fluo-3 in calcium calibration solution showed the best fit by bi-exponential analysis. At the maximum emission wavelength of 522 nm, we have identified following fluorescence lifetimes and their relative amplitudes for Fluo-3: $\tau_1 = 0.57 \pm 0.01$ ns (79 ± 1%) and $\tau_2 = 2.09 \pm 0.05$ ns (21 ± 1%), yielding the mean lifetime of the decay $\tau_{mean} = (\tau_1 a_1 + \tau_2 a_2)/ (a_1+a_2) = 0.89 \pm 0.03$ ns (n = 5 samples, range of 0.1-1.2 μM Ca^{2+}). While the τ_{mean} values obtained for all studied calcium concentrations show no concentration dependence (Fig. 2A), the fluorescence intensity estimated as total photon counts was, as expected, linearly dependent on calcium concentration (Fig. 2B).

In cells loaded with Fluo-3-AM (see fluorescence decays at Fig. 3A), direct subtraction of the mean AF background lead to two-exponential decay of Fluo-3 at 522 nm with lifetimes and relative amplitudes of $\tau_1 = 0.49 \pm 0.17$ ns (68 ± 9%) and $\tau_2 = 2.20 \pm 0.29$ ns (32 ± 9%), yilding the $\tau_{mean} = 1.04 \pm 0.28$ ns (n = 4 cells). These values were in a reasonable agreement with the Fluo-3 measured in standard calcium-calibrating solutions. However, following background subtraction, the reduced χ^2 values of the double exponential fit significantly increased. Moreover, high dependence of cardiomyocyte AF on their metabolic state [2] is making the assumption of its steadiness questionable. Another approach was therefore tested: a spectral unmixing of the Fluo-3 and AF signals.

To spectrally unmix our TCSCP data, we have applied the same procedure as in the case of spectrally-resolved microscopy. Since we presumed that the shape of recorded spectra can vary at different time delays after laser pulse excitation, we included the time coordinate into the spectra database construction set. The time-resol-

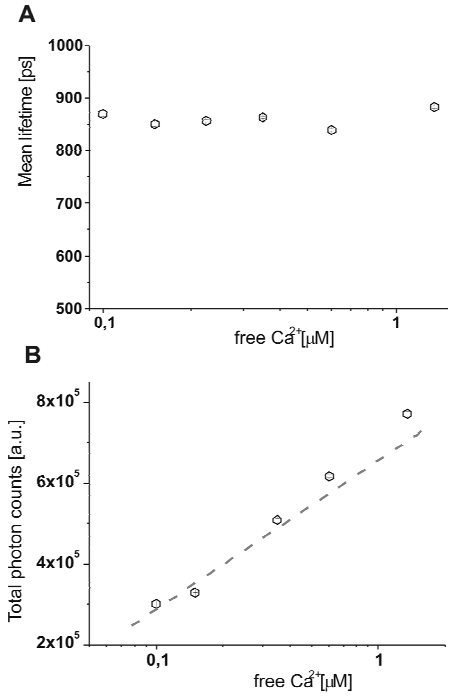

Figure 2. Mean fluorescence lifetime (**A**) and total photon counts at 522 nm (**B**) of Fluo-3 in calcium calibration solution (n=5).

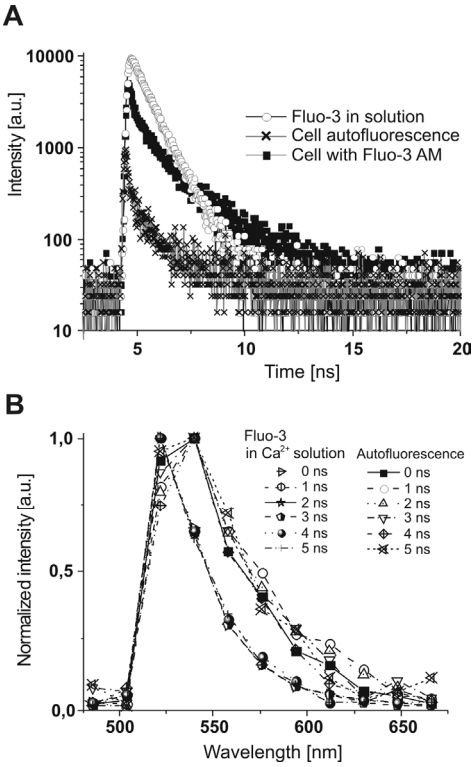

Figure 3. Example of the original recording of fluorescence decays of Fluo-3 in 1.2 μM Ca^{2+}, of cell loaded with Fluo-3-AM (3.3 μM) and of cell AF at 522 nm (**A**). Normalized time-resolved emission spectra of AF and of Fluo-3 in cardiac cells (**B**).

ved emission spectra (TRES) of Fluo-3 in calcium calibration solution (Fig. 3B) were used as one spectral set in the reference database. Based on our recent study of the time-resolved flavin AF in cardiac cells [4], we have employed a two-component model to better describe AF variability in the reference spectral database. This model was derived for flavin adenine dinucleotide (FAD) bound to enzyme(s) of mitochondrial metabolic chain, and to free FAD. To increase the signal-to-noise ratio, we have carried out the unmixing procedure on the averaged data sub-sets in the subsequent time delays after the excitation pulse (0.25 ns step). After the linear unmixing over all temporal delays with the three components (one for Fluo-3 and two for AF), a biexponential decay of Fluo-3 fluorescence has been resolved, with $\tau_1 = 0.29 \pm 0.02$ ns (66 ± 1%) and $\tau_2 = 1.67 \pm 0.04$ ns (34 ± 1%), and with $\tau_{mean} = 0.75 \pm 0.04$ ns (n = 20 cells).

4. Discussion

In this study, we measured and characterized fluorescence spectra and fluorescence decay parameters of cytoplasmic calcium-sensitive dye Fluo-3 in isolated cardiac cells by spectrally-resolved microscopy and fluorescence lifetime spectroscopy. As the signal levels of the Fluo-3 probe are small in myocytes with unchanged contractility, its fluorescence can be substantially distorted by the endogenous background AF of each cell. Indeed, we found that in cell resting state, the AF constitutes approximately half of the total intensity observable in the confocal image. Having characterized the reference spectra of Fluo-3 and AF, we were able to spatially separate their signals using spectral linear unmixing. As expected, the intracellular localizations of the Fluo-3 was confined within the cytoplasm and T-tubular regions of the cell, while the AF was co-localized with the mitochondria along the characteristic longitudal spatial pattern.

Highly variable autofluorescence signal in cardiac cells, demonstrated by our previous studies [2-4], points to the necessity to describe its nanosecond decay by at least two exponential components. Adding the bi-exponential decay of Fluo-3 makes the kinetics of the detected fluorescence in the cell even more complex. Owing to the low signal levels attainable in our experiments, instead of analyzing the spectrally-resolved TCSPC data as the sum of 4 exponentials we have rather tested whether the use of fluorescence unmixing can be a more suitable aproach to discriminate between the AF and Fluo-3 signals. Both the subtraction of the AF as a simple background, as well as the spectral unmixing resulted in a reconstructed Fluo-3 fluorescence kinetics in a form of a two-exponential decay. While the direct AF substraction lead to increased value of estimated mean Fluo-3 AM lifetime, spectral unmixing of the Fluo-3 and AF reduced values of both estimated lifetimes. Obtained results, with the exception of the value of its shorter fluorescence lifetime, were in qualitative agreement with the decay of Fluo 3 measured in calcium calibration solution, as well as with previously reported lifetime values for the Fluo-3 dye [1]. Computer-simulated data (not illustrated) showed that the spectral unmixing algorithm is sensitive to both the spectral resolution of the detection system, as well as the signal-to-noise ratio, limiting its applicability in the case of low signals and narrow spectral differences. While possible interaction of the probe with complex intracellular environment can not be ruled out, in regard to almost complete spectral overlap of the Fluo-3 and flavin AF signals we attribute the lower lifetime values of the Fluo-3 after unmixing to the inability of the unmixing algorithm to completely discriminate the short-living AF component from the observed Fluo-3 decay.

We demonstrated that spectrally-resolved TCSPC provide a perspective tool for discrimination of intrinsic fluorescence from signals of calcium probes directly in living cardiac cell and thus for study of their characteristics in physiological conditions.

Acknowledgements

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Address for correspondence

Dr. Chorvatova Alzbeta
Research Center of CHU Sainte Justine, University of Montreal
3175 Cote Sainte Catherine, H3T 1C5 Montreal, Canada
Email. alzbeta.chorvatova@recherche-ste-justine.qc.ca.