Electronic Supplementary Material (ESI) for Analyst.
This journal is © The Royal Society of Chemistry 2019

Fluorescence Lifetime Imaging of Intracellular Magnesium Content in Live Cells

Azzurra Sargenti [1§], Alessia Candeo [2§], Giovanna Farruggia [1,3], Cosimo D'Andrea[2], Concettina Cappadone [1], Emil Malucelli [1], Gianluca Valentini[2], Paola Taroni[2], Stefano Iotti [1,3]

[1] Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy;

[2] Department of Physics, Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milano, Italy

[3] National Institute of Biostructures and Biosystems (NIBB), Rome, Italy;

Supplementary Information

1 Experimental Procedures

1.1 General spectroscopic methods

All reagents were from Sigma Aldrich (Italy), if not differently stated, and were of ultrapure grade. DCHQ5 was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg/mL (1.4 mM). Aliquots were kept at 4 °C in the dark. Dulbecco's Phosphate-Buffer Saline (DPBS) was purchased without Ca^{2+} and Mg^{2+} and the acronym DPBS in the text will indicate this formulation.

1.2 Cell Culture

Human Osteosarcoma (SaOS-2) and human colon carcinoma (LoVo) cells (purchased from American Type Culture Collection ATCC, Manassas, VA) were grown at 37 °C and 5 % CO_2 in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 10% FBS, 100 units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin.

To reduce intracellular magnesium of SaOS-2, the cells were cultured for 24 h in Mg-free MEM medium (Invitrogen) with 0.5 % of dialysed FBS. After 24 h of Mg and serum deprivation, the cells were switched to a 5 % FBS Mg free medium for 24 h and then analyzed (the sample was named SaOS-2 –Mg).

FBS without Mg^{2+} was prepared by membrane dialysis of FBS: CelluSep T1 membranes with nominal MWCO 3500 (Membrane Filtration Products Inc., Seguin, TX, USA) were used and the dialysis process was performed in Puck Buffer Solution (800 g L_1 NaCl, 4 g L_1 KCl, 3.5 g L_1 $NaHCO_3$, 5 g L_1 anhydrous Dglucose, pH 7.4) for 5 days at 4 °C (fresh solution was changed every day and 1 mM EDTA was added in the first 2 days). At the end of the dialysis, 1.8 mM CaCl2 hydrate was added to FBS to restore its normal Ca^{2+} content.

For FLIM measurements, LoVo cells and control SaOS-2 cells (Ctrl) were seeded (10^5 cells/cm^2) on glass dish and grown for 24h before experiment were performed. Mg-deprived SaOS-2 (-Mg) were seeded (10^5 cells/cm^2) on glass dish and treated as described above to reduce intracellular magnesium. Before the analyses, LoVo cells and control and Mg-deprived SaOS-2 cells were washed twice with DPBS and directly incubate in the dish with DCHQ5 5 µM in the dark for 15 minutes.

1.3 Confocal microscopy

For confocal microscopy measurements SaOS-2 cells were seeded (10^5 cells/cm^2) on well slide (8 wells type) (Nunc Lab-Tek Chamber Slide System, ThermoFisher, Waltham, MA, USA) and grown for 24 h before experiments were performed. After two brief washes in DPBS cells were loaded with DCHQ5 5µM in the same buffer and incubated at room temperature in the dark for 15 minutes. At the end of the incubation, cells were immediately placed on the microscope stage for imaging. Imaging was performed using a laser scanning confocal miscoscope (olympus FV-10i-LIV, Olimpus, Shinjuku, Tokyo, Japan). Fluorescence of DCHQ5-loaded cells was excited by excitation at 488 nm and the emission signal was collected at 510 nm.

1.4 Fluorescence spectroscopy

For the fluorescence spectroscopy measurement, uncorrected emission and corrected excitation spectra were obtained with a PTI Quanta Master C60/2000 spectrofluorimeter (Photon Technology International, Inc., NJ, USA).

DCHQ5 was dissolved to a 15 uM final concentration in a mixture which contains 10% of sonicated samples in a 1 : 1 solution of MeOH : MOPS (methanol:H_2O 1:1 buffered at pH 7.4 with 3-morpholinopropane1-sulfonic acid (2 mM) at room

temperature). Fluorescence emission spectra of sonicated samples of control (Ctrl) and Mg-deprived (-Mg) SaOS-2 were recorded, upon excitation at 360nm, in the range from 400 to 650 nm.

1.5 Quantification of total cell Mg by spectrofluorimetric assay

Total Mg content was assessed on sonicated cell samples of SaOS-2 cells by the fluorescent dye DCHQ5 as reported as previously described (Sargenti et al 2017, Sargenti et al 2014).

1.6 Fluorescence Lifetime Imaging Microscopy set-up

Fluorescence Lifetime Imaging Microscopy (FLIM) was performed using a wide field microscope (Leica DM R) in epifluorescence configuration, coupled to a fast gated ICCD camera.

The excitation light was provided by a supercontinuum laser source based on a photonic crystal fiber (SuperK EXTREME, NKT photonics, Denmark). The laser is made of two Ytterbium (Yb) doped optical fibers, in the master oscillator/power amplifier configuration, which emits 10 ps pulses at 1080 nm, with a repetition rate selectable from 10 MHz to 40 MHz. The laser light is then input to a photonic crystal fiber, which, through non-linear effects, transforms the narrowband Yb radiation into white light pulses extending from 400 nm to 2400 nm. For the FLIM experiments the repetition rate was set to 20 MHz, and an acousto-optic modulator was used to select a 10-nm wide portion of the white spectrum centered around 485 nm. The laser light was then coupled to a large core optical fiber (400 μm), whose distal end was imaged through a home-made optical system into the object plane of the microscope, in such a way that the same region of the field of view (about 2/3 of the field diameter) was uniformly illuminated using any objective. The discrimination between fluorescence emission and laser light was achieved through a dichroic mirror at 505 nm. A high pass emission filter, with a cut-on wavelength of 500 nm, was employed to remove the residual excitation light from the detection path.

The detection system was based on a fast light intensifier that can be gated with window width of 200 ps to 1 ns, synchronously with the laser pulses. The intensifier (Picostar, LaVision GmbH, Göttingen, Germany) is coupled to a low-noise, cooled CCD camera by means of a high-aperture lens. The light intensifier is synchronized with the laser pulses by a trigger unit made of a fast photodiode coupled to a constant-fraction discriminator and by a jitter free delay generator capable of delays of 0 to 22 ns in 25-ps steps. The jitter of the overall system was estimated to be <50 ps. The whole apparatus is computer controlled by means of dedicated software that automatically sets the measurement parameters (delay, gain of the light intensifier, etc.) and controls the acquisition of the set sequence of the gated images.

1.6.1 Acquisition parameters

For the selection of the field of view a 10X objective was used, while all the fluorescence measurements were collected with a 63X, 0.9 NA water immersion objective (HCX APO, Leica Microsystems). Overall, the spatial resolution is of about 0.5 μm. The optical power on the image plane was 300 μW, corresponding to a power density of about 15 mW/cm2 with the 63X objective. For each field of view, a sequence of 22 images was acquired with gate width of 1 ns and delays of 0, 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 20, … , 70 ns with respect to the excitation pulses.

1.6.2 Data analysis

The data analysis was performed using a custom written software based on the Matlab engine (MathWorks®, Natick, USA). In each image set, an Area Of Interest (AOI) was selected by visual inspection in order to identify a discrete number of cells (typically 1 to 3 per field) for data analysis. Then, the dataset made by the AOIs of all the gated images was processed using a parallel fitting algorithm working, pixel by pixel, with a modified bi-exponential model function. More specifically, the model function for fitting is:

$$F(d) = A_1 exp\left(-d/\tau_1\right)\left[1 - exp\left(-G/\tau_1\right)\right] + A_2 exp\left(-d/\tau_2\right)\left[1 - exp\left(-G/\tau_2\right)\right] + C$$

where F is the fluorescence, i.e. the integral of the fluorescence intensity within the gate time, d is the acquisition delay for each image of the sequence, A1, A2, τ1 and τ2 are the amplitudes and lifetimes of the two fluorescence components, respectively. The terms [1 - exp(-G/τ)] are due to the finite width of the sampling window, and an offset C was introduced to account for any background noise.

The fitting method is based on a standard least mean square algorithm, with initial conditions randomly varied within proper intervals to avoid the convergence of the algorithm toward local minima.

Finally, the maps of the amplitudes A1 and A2, and of the lifetimes τ1 and τ2 are plotted in pseudo colors with a linear colorbar. Amplitude and lifetime maps achieve a spatial resolution of about 1 μm.

2 Figure and table

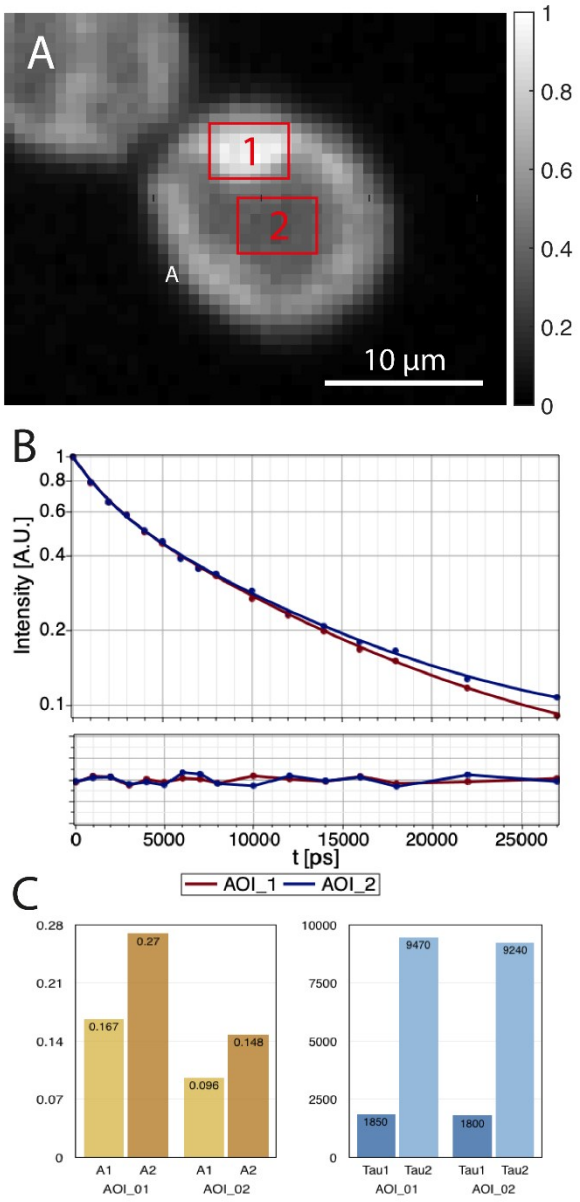

Figura 1S. Steady state fluorescence image of a typical LoVo cell; B) Examples of bi-exponential fit with residuals on the fluorescence emission decay integrated over the pixels of AOI 1, (high intensity) and AOI 2 (low intensity); C) Histograms of the amplitudes (a.u.) and lifetimes (ns) in AOIs 1 and 2.

LOVO

Cel	Intensity	A1/A2	Tau1/Tau2
Cel_01	H	0.57	0.18
Cel_01	M	0.83	0.29
Cel_01	L	0.67	0.19
Cel_02	H	0.67	0.18
Cel_02	L	0.57	0.11
Cel_03	H	0.63	0.17
Cel_03	L	0.70	0.16
Cel_04	H	0.88	0.21
Cel_04	L	0.74	0.18
Cel_05	H	0.67	0.18
Cel_05	L	0.99	0.18
Cel_06	H	0.81	0.32
Cel_06	L	0.63	0.19
Cel_07	H	0.89	0.24
Cel_07	L	1.06	0.18
Cel_08	H	1.05	0.18
Cel_08	L	0.64	0.18
Average		**0.77**	**0.20**
Std		**0.16**	**0.05**

Table 1S. Fluorescence amplitude ratio (A1/A2) and lifetime ratio (Tau1/Tau2) obtained in 8 LoVo cells from areas having different intensity (L= low; M=medium; H=high) as detected in steady state fluorescence image.