NEW FLUORESCENCE MOLECULAR TOOLS OF BIO-IMAGING TO MONITOR MEMBRANE POTENTIAL IN MULTIPHOTON MODE

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ABSTRACT

Fluorescent Resonant Energy Transfer (FRET) is a powerful technique for measuring intermolecular distances. Combined intensity (spectral) and Lifetime (FLIM) Imaging was used to discriminate the FRET signal of molecules on their different lifetimes whereas their emission spectra overlap. In this work, a two-component FRET sensor has been designed by using fluorescent phospholipids acceptor (DHPE-TRITC) bound on one side of the membrane and donor molecules (oxonol) which are sensitive to membrane potential. We used multiphoton excitation and FLIM to deliver contrast lifetimes to monitor membrane potential on cancerous cell in culture (HT-29 and fibroblasts).

Key-words: fluorescence lifetime, oxonol, FRET, microscopy.

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INTRODUCTION

In the recent past, non-invasive optical probes as fluorescent indicator dyes have been developed and applied to cell biology to monitor the excitable membrane properties in real time. Optical probes gain importance because they allow spatial resolutions and sampling frequencies that are not achieved using microelectrodes. Given the chromophore can directly interact with the transmembrane electric field, fluorescent optical probes are considered as a very effective tool to map variations of potential membrane directly in living and excitable cells[1]. Based on their response time to potential changes, this system is very sensitivity because the response time is faster than the duration of the action potential. An approach based on Fluorescence Resonance Energy Transfer (FRET) between a membrane bound donor molecule and a mobile Voltage-Sensitive Dye (VSD) acceptor has been introduced since 1997 as the FRET-VSD[2]. To monitor the membrane potential which can play a central role in carcinogenesis and tumour progression, we have caracterized a prototype molecular system based on a mobile dye (oxonol) which diffuses less into membranes of cells which are more negative, and thus are hyperpolarized. In a previous work[3], we have used this system (Figure 1) based on two-component dyes in which:

- the donor was a negatively charged mobile oxonol (DiBAC4[3]) and its partition across the plasmalemma is governed by a electrodiffusion process as a function of membrane potential.
- the acceptor (TRITC-DHPE) was a phospholipid (DHPE) located in the outer leaflet of the plasma membraneand bound to TRITC.

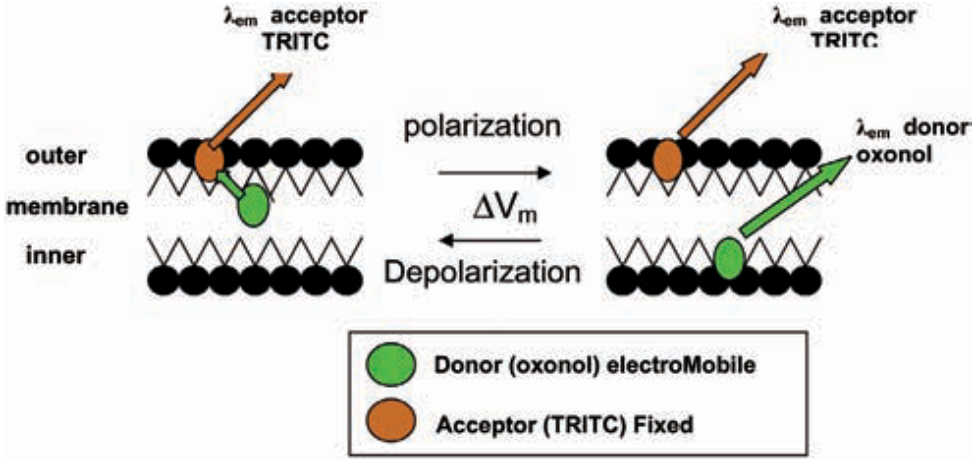

Fig. 1 – FRET system based on a electrodiffused donor (oxonol) and a membrane-bound acceptor molecule (DHPE-TRITC). Excitation of the donor results in FRET when the acceptor is in close proximity. After the action of valinomycin, upon depolarization, this changes in membrane potential can be detected by measuring donor and acceptor emission (spectra and/or lifetime fluorescence).

As near-infrared light is far less damaging to living cells[4] and to reduce the photobleaching of oxonol and DHPE-TRITC, the cells were excited in two-photon mode at 835 nm. This multiphoton process (simultaneous absorption of 2 photons) is made possible by a very high intensity combined with time-related concentration of a very brief (femtosecond) and very high-frequency (about 80 MHz) laser flash[5,6]. For FRET technique, the oxonol molecule absorbs light and the close proximity of acceptor molecule results in a transfer of energy by a dipole-induced dipole interaction[7]. In agreement with the low absorbance of DHPE-TRITC at 835 as compared to that of oxonol, the contribution of the DHPE-TRITC upon excitation at 835 nm is only due to the FRET process. Classically, the changes in membrane potential were detected by measuring donor and acceptor emission wavelength (spectra). The transfer efficiency can be quantified as a decrease in oxonol fluorescence intensity or by an increase in the DHPE-TRITC fluorescence. The analysis of fluorescence intensity cannot solve the problems due to the probe photobleaching or a direct excitation of the acceptor (even reduced)[8]. To avoid these limitations in the case of multi-labelling FRET experiments, Fluorescence Lifetime Imaging Microscopy (FLIM) in dynamic-state provides a discrimination of molecules in their fluorescence lifetime, which allows to evaluate the underlying mechanism of energy transfer process. Importantly, the lifetime value revealed by FLIM is a property of individual fluorescent molecules and is independent of fluorophore concentration and photobleaching. FLIM provides a discrimination of molecules on their different lifetimes whereas their emission spectra overlap.

In this work, we investigated a VSD-FRET system and plasma membrane potential was measured by FLIM as a decrease in the lifetime of the oxonol. This prototype system has been validated with normal and cancerous cell in culture (HT-29 and fibroblast). These results show a significant response to depolarized cancerous cells from resting cells when compared to fibroblast normal cells.

MATERIALS AND METHODS

Experiments of depolarizing on culture cell lines

The depolarization of the plasma membrane has been realized by adding of valinomycin during the change in the medium of extracellular potassium (from 5 to 150 mM). Voltage-Sensitive Dyes have been calibrated by imposing a transmembrane potential using valinomycin (Sigma, V 0627) in conjunction with externally applied K+ solutions. Two cell lines have been used, a HT-29 type (human colorectal adenocarcinoma) and a CCD-1137Sk type (human skin fibroblasts, ATCC). These cells have been cultivated at 37°C in 5% CO2 on Labteck slides in RPMI 1640 (Gibco BRL, France) complemented with 10% decomplemented fœtal bovine serum (Dutscher, France), 4mM L-Glutamine (Gibco BRL, France), 2,5 µg/mL fungizone (Gibco, France), 1000

U/mL penicillin and 100 µg/ml streptomycin (Gibco, France). For the FRET-VSD experiment, the cells were stained with 0,5 µM bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC4[3], Molecular Probes) and 0,3 µM N-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3phosphoethanol-amine, triethylammonium salt (DHPE-TRITC) for 10 min and washed 3 times with the RPMI (without phenol red).

Two-photon microscopy and FLIM

For two-photon microscopy, we used a LEICA-SP2-MP-Laser Scanning Microscope (Leica Microsystems, Germany) and a mode-locked Ti:Sapphire laser (VERDI 8 W-MIRA 900F, Coherent) which delivers a pulse width from 120 fs with a repetition rate of 76 MHz. Images at a 0.195 µm (x,y) pixel size were obtained for each case in 512 x 512 matrices at x63 magnification (numerical aperture = 1.32). For lifetime imaging, SPC-730 TCSPC Imaging module (Becker & Hickl, Berlin) was interfaced (signals, Pixel Clock, Frame Sync) to the scan controller of the Leica TCS-AOBS Multiphoton laser scanning microscope equipped with an acousto-optical beamsplitter. The decay analysis measured by time-correlated single photon counting was performed, using the SPC Image software (Becker & Hickl GmbH) and the instrument response function was measured on erythrosine (full-width-half-maximum = 220 ± 20 ps) and used for deconvolution. For the fluorescence lifetime values, population t1 (two components τ_1^{ox} and τ_2^{ox}) for oxonol were measured in the absence of acceptor fluorescence by cutting with a special filter (480-540 nm).

FRET efficiency by FLIM

Fluorescence resonance energy transfer, as described by Förster 's theory, explains FRET as a dipole-dipole interaction between neighboring molecules and derives the dependence of the energy transfer efficiency E on their actual proximity R. The direct non-linear dependence between E and R is described as : $E = 1/ [1+(R/R0)^6]$ with R_0 the distance between the donor (oxonol) and acceptor (TRITC-DHPE) for E = 0.5. The properties of oxonol/ TRITC pair including acceptor quantum yield, spectral overlap between oxonol-emission and TRITC-excitation and the relative spatial oxonol-TRITC orientation are reflected by R_0. Emission within the band of 480-540 nm and above 580 nm was collected for oxonol and TRITC fluorescence respectively. To quantify the FRET signal, we calculated the effective FRET efficiency as:

$$FRETeff= (\tau_D-\tau_{D=>A}) / \tau_D$$

where $\tau_{D=>A}$ was the fluorescence lifeteime of the donor in the presence of the acceptor and ôD the fluorescence intensity of the donor alone.

RESULTS: FRET-VSD BY FLIM

The fluorescence lifetime distribution of oxonol in the presence of DHPE-TRITC has been used to cha-

racterize a FRET-VSD system, through the heterogeneity of the two lifetime population of the donor. The reduced lifetime values of oxonol indicated a molecular interaction with TRITC incorporated in the plasma membrane. For control fibroblasts, the donor exhibited the expected lifetime with one component around of 1420 ± 320 ps (Figure 2, curve t1). A very efficient FRET-VSD process has been precised in the presence of valinomycin (Figure 2, curve t1-val), as suggested by two fluorescence lifetime components τ_1^{ox} (735± 250 ps for 71 %) and τ_2^{ox} (998 ± 110 ps for 29%) with a high amplitude of the shorter τ_1^{ox} component. Interestingly, for HT29 cells in the abscence of valinomycine, a reduced lifetime component has been observed similarly to an effective FRET (Figure 3, curve t1), respectively around of 439 ± 120 ps for 28% and 1478 ± 310 ps for 72%. This reduced value of lifetime distribution showed the effect of depolarized cells membrane under resting condition. After depolarization by valinomycin (Figure 3, curve t1-val), the proximity oxonol-TRITC requirement for the occurrence of FRET was met for HT29 cell line as shown by two fluorescence lifetime components τ_1^{ox} (451± 380 ps for 75 %) and τ_2^{ox} (1380 ± 185 ps for 25%) with a high amplitude of the shorter τ_1 component. The effective FRET was calculated based on mean lifetime values. FRET efficiency showed a significant FRET for fibrobasts (45%) whereas HT29 showed a higher rate of FRET (69 %) for species which have the longest fluorescence lifetime values.

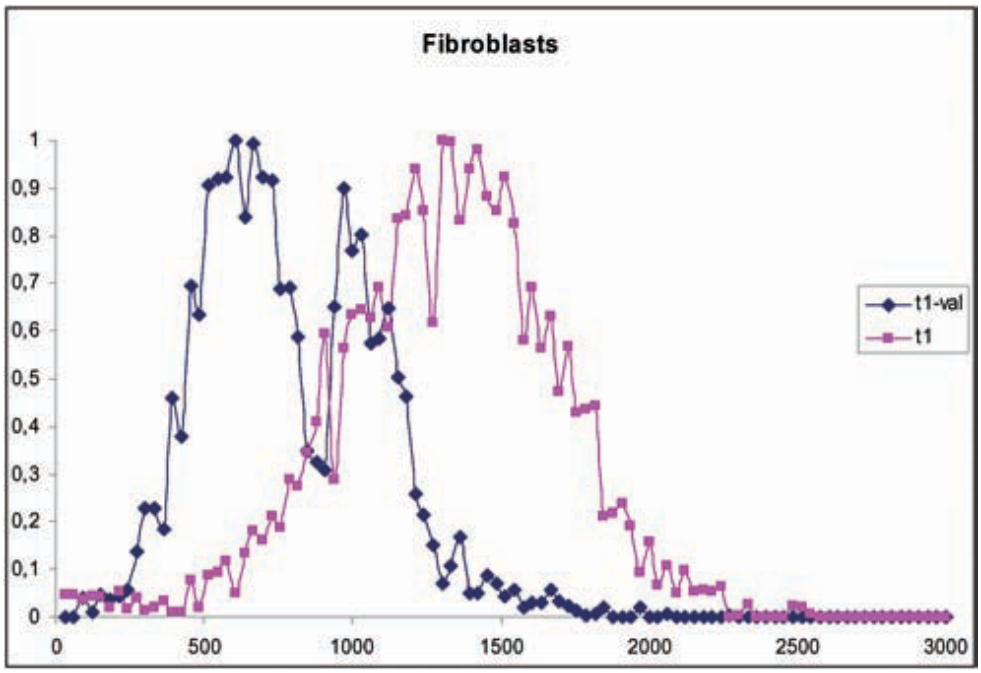

Fig. 2 – Temporal historigram showing the distribution of fluorescence lifetime for the oxonol under resting condition for fibroblaste cells (data t1). Upon membrane depolarization by valinomycin, FRET-VSD is effective as shown by the reduced fluorescence lifetime of oxonol (data t1-val) with a lower component.

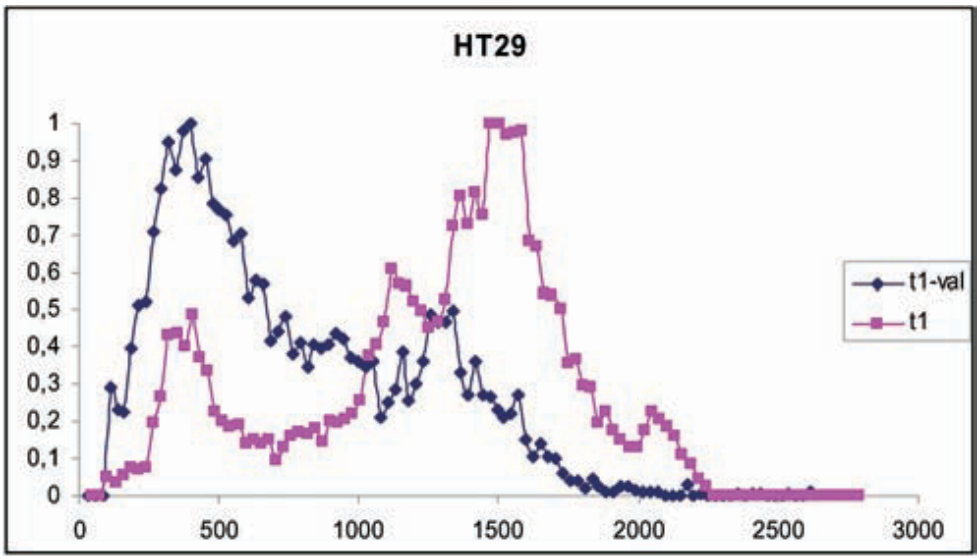

Fig. 3 – Temporal historigram showing the distribution of fluorescence lifetime for the oxonol under resting condition for HT29 cells (data t1). Upon membrane depolarization by valinomycin (data t1-val), FRET-VSD is very effective as shown by lowest fluorescence lifetime value.

DISCUSSION – CONCLUSION

As it is governed by electrodiffusion of mobile Voltage-Sensitive Dye (DiBAC4[3] oxonol), this two-component FRET (oxonol-TRITC) system improves on other systems of potentiometric fluorescence probes. The sensitivity of this system is increased because the response time of electrodiffusion is faster than the duration of the action potential. Under depolarization, the donor electrophoresis across the membrane and a change in fluorescence due to FRET is observed. Fluorescence Lifetime Imaging Microscopy (FLIM) combines the advantages of lifetime spectroscopy with fluorescence microscopy by revealing the spatial distribution of a fluorescent molecule together with information about its microenvironment. By FLIM, the FRET efficiency can be quantified as a decrease in oxonol fluorescence lifetime. At 835 nm, the reduced lifetime values of oxonol indicated a molecular interaction with TRITC incorporated in the plasma membrane. Upon resting conditions, the FRET from oxonol to TRITC was weakly found for HT29 cells line but not at all for fibroblast. Upon depolarization by valinomycine, the FRET was clearly found for both cells line tested, 45% for fibroblast and 69% for HT29. As revealed by these results, comparison of the FRET-VSD obtained from two line cells upon depolarization showed that the membrane depolarization could be a reliable indicator to characterize a cancerous cell line. Given the sensitivity and the fast time response, this molecular tool based on FRET system may be particularly useful for applications involving compression of tissues by mechanical forces developed in the field of tissue engineering.

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