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Spectrally and spatially resolved fluorescence lifetime imaging in living cells: TRPV4–microfilament interactions

Radhan Ramadass *, Daniel Becker, Marina Jendrach, Jürgen Bereiter-Hahn

Kinematic Cell Research Group, Institute for Cell Biology and Neuroscience, JW Goethe University, Max-von-Laue-Strasse 9, D-60438 Frankfurt/Main, Germany

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Abstract

Time- and space-correlated single photon counting method has been used to demonstrate the interactions of cation channel "transient receptor potential vanilloid 4" (TRPV4) and microfilaments. Living cells co-expressing TRPV4-CFP and actin-YFP, when excited for the donor molecules (CFP) exhibited an emission peak at 527 nm and decrease of the lifetime in the wavelength band 460–490 nm; corresponding to resonance energy transfer to YFP. CFP fluorescence decay was fitted best by a dual mode decay model. Considering the average lifetime of the donor, both in the presence and absence of acceptor yielded an apparent FRET efficiency of ~20%. This is rather high placing the minimum distance of chromophores in the two fluorescent proteins in the range of 4 nm. Thus, this study shows for the first time that TRPV4 and actin intimately associate within living cells. The significance of this finding for cell volume regulation is highlighted.

Keywords: TRPV4; Actin; Regulatory volume decrease; Osmoregulation; FLIM; Time-resolved FRET; Single photon counting; CHO cells; CFP; YFP; Fluorescent proteins; Decay associated spectra; Spectrally resolved fluorescence decays

Investigation of protein–protein interactions in living cells is essential to understand the underlying signal cascade mechanisms in their natural environment. Many interactions in living cells are made probable by the creation of specific environment like complex organisation and compartmentalisation for the molecules to interact. Using fluorescence microscopy, the sub-cellular localisation and the distribution of proteins could be easily determined. To envisage and study the extent of interaction of proteins in a complex, dynamic and highly dense environment like in a living cell; a non-invasive approach with high spatial sensitivity in the range of few nanometers is necessary.

Förster resonance energy transfer (FRET)[1] is a non-radiative phenomenon between two fluorochromes requiring the emission spectra of the donor molecule overlapping with the absorption spectrum of the acceptor molecule. This energy transfer due to long-range dipole–dipole interactions [1] occurs only when the interacting molecules are in the distance of 10–100 Å. Hence, FRET can be used as a spectroscopic ruler to study the interactions of proteins in living cells [2]. Proteins fused with green fluorescent protein and its variants make the study of protein–protein interactions in living cells possible [3–6].

However, resonance energy transfer measurements based on steady-state fluorescence signal change and acceptor photobleaching are not sufficient proof to confirm the interactions, especially, when the photophysics of the fluorochrome is not fully understood [7]. Fluorescence lifetime imaging based on time- and space-correlated single photon counting is a non-destructive, non-invasive and reliable method to investigate interactions of proteins in living cells [8,9]. Fluorescence lifetime imaging microscopy (FLIM; [10–13]) based experiments are independent of fluorochrome concentration and

* Corresponding author. Fax: +49 69 79829607.
E-mail address: ramadass@bio.uni-frankfurt.de (R. Ramadass).

[1] *Abbreviations used:* TRPV4, transient receptor potential vanilloid 4; FRET, Förster resonance energy transfer; FLIM, fluorescence lifetime imaging microscopy; RVD, regulatory volume decrease; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; DAS, decay associated spectra.

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excitation intensity in contrast to intensity based FRET measurements. Single photon counting methods [14] have the striking benefits of using low level excitation light and picosecond time resolution to directly investigate the photophysical process involved.

Resonance energy transfers are revealed by a wavelength related shift in the emission spectrum and fluorescence lifetime changes in the donor molecules. Spectrally resolved fluorescence decays offer an insight into the different ground state species of the donor and acceptor molecules and the nature of their interaction. Spatially resolved fluorescence decays over a two-dimensional image of a living cell can be used to elucidate the key mechanisms involved in the regulation of protein interactions in differing environments in the different regions of single living cells. Hence, the method of choice is time- and space-correlated fluorescence lifetime imaging including spectral resolution to prove interactions on a molecular basis by Förster resonance energy transfer determinations.

In this study, we investigate the proximity of the membrane protein TRPV4 involved in volume regulation with the actin cytoskeleton, by applying spectrally and spatially resolved fluorescence decay measurements, for the detection of FRET. TRPV4 is a Ca^{2+} permeable non-selective cation channel and is a member of the vanilloid subfamily of transient receptor potential (TRP) channels, which preferentially form homo- oligomeric complexes [15,16]. TRPV4 has six predicted membrane-spanning subunits with a pore loop [17]. The amino-terminal domains with three ankyrin repeats as well as the C-terminus are located on the cytoplasmic side [16]. Its wide distribution in mammals including heart, brain, lung, kidney and liver indicates its prominent physiological function [18]. The ability for cells to adapt to changed osmotic conditions is essential for cellular homeostasis and disequilibrium could lead to dramatic events like apoptosis and necrosis [19]. Therefore, many cell types are able to counteract to anisotonic environment by processes called regulatory volume decrease (RVD) and regulatory volume increase (RVI). Recently, a direct participation of TRPV4 in RVD at cellular level and osmosensing in whole organism has been shown [20–23].

The exposure of cells to a hypotonic medium results in activation of TRPV4, as indicated by Ca^{2+} influx [24]. During RVD many cell types show a transient intracellular Ca^{2+} increase, which may be mediated through TRPV4 [20,21,25–29]. An intact cytoskeleton, mainly microfilaments have been shown to be required for the mechanosensitive or swelling-induced Ca^{2+}-entry in several cell types, suggesting possible interaction of TRPV4 and F-actin [30].

The sub-cellular localisation of exogenously expressed green fluorescent protein (GFP) fused to TRPV4 and counterstained with TRITC-Phalloidin in different cell lines revealed co-localization of TRPV4 in the cell membrane with the underlying cortical actin cytoskeleton. Whether this co-localization represents molecular interactions in living cells can be assessed by FRET measurements. For this purpose, TRPV4-CFP and actin-YFP plasmids were transfected in vertebrate cells; to evaluate the possible interactions by FLIM. Both, spectrally and spatially resolved fluorescence decays indicated an interaction of these proteins. Apart from the fluorescence lifetime changes of the donor in the presence of acceptor, an additional emission peak appeared at 527 nm. This is a stringent indication of the energy transfer taking place. In addition, spatially resolved fluorescence decays were used to check for the spatial heterogeneity of environment and interaction.

Materials and methods

Cell culture

HaCaT keratinocyte cells (passage 47–52) were cultivated in keratinocyte SFM medium (Invitrogen, Karlsruhe, Germany), containing 10% fetal calf serum (FCS), 10 mM Hepes (Invitrogen) at 37 °C in 5% CO2 atmosphere. CHO-K1 cells (passage 28–50) were cultivated in F-12 HAMs cell medium (Invitrogen), containing 10% FCS and 10 mM Hepes (Invitrogen) buffer at 37 °C in 5% CO_2 atmosphere.

HaCaT and CHO cells were either transfected with Effectene transfection reagent (Quiagen, Hildern, Germany) according to manufacturer's instructions or by electroporation (BTX electro cell manipulator 600, Holliston, USA). The transfected cells were seeded onto coverslips after 24 h.

For F-actin staining, cells (72 h post-transfection) were washed twice with PBS and fixed with 10% formaldehyde in PBS for 20 min at room temperature. Fixed cells were washed thrice and stained for 20 min with 2 μg/ml TRITC-Phalloidin (Sigma–Aldrich, Munich, Germany) in PBS at RT. Cells were washed 3 times and embedded in Mowiol.

Constructs

For construction of TRPV4-CFP, TRPV4-GFP [21] was digested with BamH1 and Not1 and the GFP tag was exchanged for the CFP tag of plasmid CFP-C1 (Clontech, CA, USA). For construction of actin-YFP, actin-GFP (Clontech) was digested using BamH1 and BglII and cloned into the EcoR1 and BglII sites of the vector YFP-C1 (Clontech, CA, USA).

Steady state fluorescence images

Co-localization studies were performed using a Leica TCS 4 D confocal laser scanning microscope fitted with the appropriate filters and PL Fluotar objective (100×, 1.3 NA) that was controlled by the SCAN Ware 5.10 software (Leica, Bensheim, Germany). Images were processed using Imaris v. 4.1.3 and auto deblur, edition gold v. 9.3.

Steady state fluorescence imaging was done using an IX70 (Olympus, Hamburg, Germany) inverted fluorescence microscope. Mercury arc lamp (50 W) was used for excitation, in combination with filters 450–490 nm band pass filter, 480/30 nm (Omega opticals, VT, USA) filter and BP530–550 nm (Omega opticals, VT, USA) emission filters. Imaging was done using a 100× oil objective and 12 bit sensi-cam (Imago).

Time- and Space-resolved Fluorescence Decay Microscopy

A Millennia Xs ($Nd:YVO_4$ gain medium; Spectra – Physics, Darmstadt, Germany) pumped, mode-locked Ti:sapphire laser (Titanium-doped sapphire; Tsunami, Spectra – Physics, Darmstadt, Germany) with a repetition rate of 82 MHz and about 50 fs pulse width was tuned to 880 nm. For the stable operation at the wavelength tuned, it had to be purged in nitrogen. The Ti:saphire laser was coupled to a pulse stretcher, pulse selector and frequency doubler (Model 3980;