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Adjobo-Hermans, M.J.W.

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Combining TIRFM and FLIM to study annexin A4 translocation and protein-protein interaction at the plasma membrane

Merel J.W. Adjobo-Hermans¹, Alen Piljic², Erik B. van Munster¹, Carsten Schultz² and Theodorus W. J. Gadella Jr¹

¹Swammerdam Institute for Life Sciences, Section of Molecular Cytology, Centre for Advanced Microscopy, University of Amsterdam, Kruislaan 316, NL-1098 SM, Amsterdam, The Netherlands

²European Molecular Biology Laboratory Heidelberg, Meyerhofstrasse 1, 69117 Heidelberg, Germany
Summary

A myriad of cellular processes takes place at the plasma membrane. Conventional fluorescence microscopy enables visualization of these processes in living cells. However, in order to specifically observe events occurring at the plasma membrane with a high signal-to-noise ratio, the fluorescence contribution from the cytoplasm should be diminished. The technique that enables this is: total internal reflection fluorescence microscopy (TIRFM), which attains a typical resolution of about 100 nm in the z-direction. Combining TIRFM with Fluorescence Lifetime Imaging Microscopy (FLIM) permits the study of protein-protein interactions that occur at the plasma membrane of living cells with high specificity. Here, we report on the oligomerization of annexin A4 at the plasma membrane upon a long-lasting increase in intracellular calcium, as studied by means of the novel combination of TIRF and frequency-domain FLIM, dubbed TIR-FLIM.
Introduction

The annexin family in humans consists of 12 subfamilies. None of the family members is ubiquitously expressed (Markoff and Gerke, 2005). Annexins are present in the cytosol of cells at resting Ca\textsuperscript{2+} levels, but translocate to the plasma membrane when the intracellular calcium concentration is increased for extended periods of time. These proteins are built up out of a highly conserved annexin core and a variable N-terminal region that provides a protein interaction site and is a substrate for several kinases, e.g. protein kinase C (PKC). The annexin core domain that consists of 4 annexin repeats mediates binding to calcium ions, which subsequently increases the affinity of these proteins for negatively charged phospholipids in the plasma membrane (Gerke et al., 2005). At the membrane, annexins laterally assemble to form crystal-like structures by means of oligomerization. These structures decrease the diffusion of integral and other peripheral membrane proteins (Piljic and Schultz, 2006). The various family members vary in their affinities for calcium and will therefore be regulated differently depending on the amplitude of the calcium signal (for an overview, see (Raynal and Pollard, 1994)).

In general, annexins are implicated in Ca\textsuperscript{2+}-signalling, fibrinolytic action and display anti-inflammatory features by regulating membrane organization (e.g. in epithelial polarity), membrane transport, channel activity and transport (Markoff and Gerke, 2005). They are thought to exert their function as scaffolds or structural proteins (Rescher and Gerke, 2004). In the kidney of adult mice, annexin A4 is the most prevalent annexin member. Annexin A4 is also found in lung epithelium and many other epithelia (Markoff and Gerke, 2005). It is proposed to play a role in kidney development and to regulate calcium-activated epithelial chloride channels and membrane organization. Annexin A4 inhibits the interaction between calmodulin-dependent kinase II and the ion channel, thereby inhibiting the opening of the channel (Chan et al., 1994; Kaetzel et al., 1994). Inhibition is thought to be caused by annexin A4 translocation to the plasma membrane, leading to immobilization and steric hindrance of CaMKII, disabling the kinase to reach its substrate (Chan et al., 1994). Increasing our knowledge regarding this regulatory process is of utmost importance in finding drug targets to combat cystic fibrosis, a disease in which chloride channels do not function properly.

Both annexin A4 and A5 have been shown to translocate to the plasma membrane as well as the nuclear envelope upon high Ca\textsuperscript{2+}. Kaetzel et al. (2001) found annexin A4 to form trigonal crystals that assembled in ordered 2D-arrays on membrane surfaces. The arrays formed by annexin A4 are immobile and thereby cause immobilization of GPCRs, other integral membrane proteins (see above) and peripheral membrane proteins. Other array forming annexins may display similar properties. Array formation may play an important role in the protection of the cell’s integrity by reinforcing the plasma membrane and
inhibiting cell signaling upon the induction of mechanical stress or ischemia (Gerke and Moss, 2002; Piljic and Schultz, 2006).

Recently, Piljic and Schultz (2006) studied the formation of annexin A4 arrays at the plasma membrane by means of FRET ratio-imaging. A problem was encountered that is inherent to this technique, in which aspecific interaction in a 2D environment (the plasma membrane) between CFP- and YFP-tagged proteins influences the amount of FRET observed. In addition, the wide-field FRET setup was not able to differentiate between signals emanating from the plasma membrane or the cytoplasm when flat HeLa cells were used. These problems were circumvented by the use of a CFP-annexinA4-YFP fusion construct and high, pyramid-shaped N1E-115 cells.

This paper reports on the oligomerization of annexin A4 at the plasma membrane upon a prolonged high concentration of calcium in the cytosol, as measured by means of combinatorial microscopy. We opted for a combination of TIRF and FLIM in order to diminish the distorting effects on ratiometric FRET measurements that occur as a consequence of increased effective protein concentrations upon translocation of proteins to the plasma membrane (a quasi-2D surface) and the aspecific interactions that are a result of this. Using these techniques, we were able to ascertain that annexin A4 translocation to the plasma membrane is a prerequisite for oligomerization to occur.

**Microscope set-up**

In order to ascertain whether oligomerization of annexin A4 occurs at the plasma membrane or prior to translocation in the cytosol, we combined TIRF microscopy with FLIM (TIR-FLIM). Total internal reflection can only occur when the incident beam of light reaches the interface between a medium of higher refractive index (e.g. a glass cover slip) and one of lower refractive index (e.g. cell culture aqueous medium) at an angle larger than the critical angle (with $\theta_2 = 90^\circ$). This critical angle can be calculated using Snell’s law of refraction (Eq.1).

$$n_1 \cdot \sin \theta_1 = n_2 \cdot \sin \theta_2$$  \hspace{1cm} \text{Eq.1}

$n_1$ = the refractive index of medium 1;  $\theta_1$ = refracted beam angle

Upon the occurrence of TIRF, a standing evanescent wave (i.e. an electromagnetic field) is formed. The intensity of this wave decays exponentially in the z-direction (Figure 1, Eq.2).

$$I(z) = I(0) \cdot e^{-\frac{z^2}{d}}$$  \hspace{1cm} \text{Eq.2}

$I(z)$ = the intensity at distance $z$ from the interface;  $I(0)$ = the intensity at the interface
The penetration depth ($d$) that can be attained is dependent upon the angle of incidence and the wavelength of the applied light (Eq. 3).

$$d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta_1 - n_2^2}}$$

Eq. 3

Typically, a depth of ~100nm is attained, which means that only in this region fluorophores are excited. Obviously, this phenomenon can only be used to study processes that specifically occur at the basal plasma membrane of cells with a high signal to noise ratio, since background fluorescence from out-of-focus planes is effectively eliminated. Importantly, the depth of the evanescent wave provides a $z$-resolution that is about 10 times higher than the resolution that can be reached by confocal laser scanning microscopy. Two TIRF setups are possible on a microscope; the prism method and the objective lens method. The last option is most useful for live cell imaging and is used here, since easy manipulation of the cells is possible, in contrast to the prism method (Axelrod, 2001).

When TIRF is combined with FRET imaging, ‘super-resolution’ can be achieved. In general, the combination of a XY spatial resolution of ~200 nm, a $z$-resolution of ~100 nm and a 3-10 nm molecular FRET contrast are feasible in TIR-FLIM. We selected FLIM to measure FRET, because this technique obtains intensity-independent and generally bleach-insensitive FRET data, as opposed to the more commonly used FRET ratio-imaging approach. In addition, the use of FLIM for the detection of FRET enables the use of global analysis, which can be used to quantify populations of molecules in a particular physical state (Verveer et al., 2000). To upgrade a wide-field frequency-domain FLIM setup (Van Munster and Gadella, 2004) to TIR-FLIM, the microscope was equipped with a 100x NA 1.45 oil objective. Such a high numerical aperture (NA) objective is required in order to achieve total internal reflection at the interface with living cells ($n=1.38$), as exemplified in the following equation (Eq. 4). Only at high enough aperture can the appropriate illumination incidence angles ($\sin(q)$) be attained.

$$NA = n \times \sin(q)$$

Eq. 4

In order to obtain an angle greater than the critical angle, the excitation light (a laser) enters the back focal plane of the objective not centered as normally but off-axis. The high NA of the objective causes the excitation light to leave the front lens at a very shallow angle, thereby causing total internal reflection at the interface between the cover slip and the aqueous medium in which the cells reside (Figure 1).
This off-axis entrance into the objective can be modulated by means of a micromanipulator that moves the output of the laser fiber with respect to the lens in the side port of the microscope. This lens is positioned in parallel to the front lens in the objective and therefore enables direct regulation of the incident angle to facilitate the formation of the most adequate evanescent field.

**Results and Discussion**

HeLa cells were transfected with both RFP-annexin A4 and YFP-annexin A4 and TIR-FLIM images were collected of several cells. The localization of annexin A4 and the interaction between YFP- and RFP-annexin A4 was imaged upon addition of 5μM of the calcium ionophore ionomycin. Phase stacks were recorded in TIRF mode with intervals of 15-30 seconds. After ~1 minute the fluorescence intensity increased, indicating that the annexin A4 protein started moving to the plasma membrane. At 2-3 minutes the translocation to the plasma membrane was complete, showing clear fluorescent filopodia and ruffle-like structures. Often, translocation to the nuclear envelope was also observed, displaying a speckle-like pattern, and lagging behind with respect to the translocation to the plasma membrane. Importantly, plasma membrane anchoring coincided with a decrease in fluorescence phase lifetime (2.84 ± 0.04 ns → 2.12 ± 0.22 ns) and modulation lifetime (3.03 ± 0.04 ns → 2.42 ± 0.26 ns) (n=5), which stabilized upon completion of the translocation (Figure 2). Such a large decrease is indicative of significant FRET after translocation.

![Figure 1.](image1.png) In wide-field illumination, the excitation light can enter the living cell and excite fluorophores everywhere in the cell. In TIRF illumination, the excitation light is coupled in off-axis and is totally reflected at the glass/medium interface, leading to an evanescent wave that decays exponentially. Therefore, only fluorophores close to the coverslip (i.e. close to/at the plasma membrane) will be excited.
We hypothesize that annexin A4 has to be in a specific orientation to interact, which may be coordinated by the coincidence detection of calcium ions and phospholipids at the plasma/nuclear membrane.
intensity image of the cell after addition of ionomycin (t=120 seconds). E) YFP phase lifetime image of the cell shown in (D). F) YFP phase lifetime histogram of the cell shown in (D). G) YFP intensity image of the cell after addition of ionomycin (t=240 seconds). H) YFP phase lifetime image of the cell shown in (G). I) YFP phase lifetime histogram of the cell shown in (G).

Control experiments were done in HeLa cells exclusively expressing the donor, YFP-annexin A4. In this case, addition of ionomycin did not induce a change in fluorescence lifetime, whereas the fluorescence intensity did increase, indicating that YFP-annexin A4 translocated to the plasma membrane (Figure 3). The fact that the lifetime remains constant indicates that the change in location (from cytosol to plasma membrane) and the increase in concentration at the plasma membrane do not change the fluorescence lifetime of YFP-annexin A4.

An important question still remains as to the physiological relevance of the annexin translocation phenomenon upon addition of ionomycin. High enough Ca\(^{2+}\) increases may arise in polarized epithelial cells at the apical membrane where annexins reside (Mayran et al., 1996). Unfortunately, TIRF does not enable the study of these cells, since only the basal membrane attaches to the glass surface. Future research will be directed towards the study of the impact agonist-mediated GPCR activation has on annexin self-association. Certainly, oligomerization has not been observed in wide-field applications upon stimulation of conventional heterotrimeric G protein signaling (Piljic and Schultz, 2006), however, formation of small arrays at discrete plasma membrane sites might be detectable by virtue of the high signal to noise ratio obtained in TIRF microscopy. In conclusion, the combination of TIRF and frequency-domain FLIM enables the study of translocation and protein-protein interaction at the plasma membrane in living cells with nanoscale resolution.

**Materials and Methods**

**Constructs**

EYFP(YFP)-annexin A4 and mCherry(RFP)-annexin A4 DNA constructs were described formerly (Piljic and Schultz, 2006).

**Cell culturing & transfection**

HeLa cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in DMEM-Glutamax (#61965-059), supplemented with 10% FBS, Penicillin (100U/ml) and Streptomycin (100µg/ml) (Invitrogen, Breda, The Netherlands) at 37°C, 5% CO\(_2\).

Cells were transfected on a glass cover slip (24 mm Ø #1) with 1.5 µl lipofectamine and 0.5 µg plasmid DNA in 50 µl OptiMEM (Invitrogen, Breda, The Netherlands). Samples were imaged after 24h and mounted in an Attofluor cell chamber (Invitrogen, Breda, The Netherlands), submerged in medium (140mM NaCl; 5mM KCl; 1mM MgCl\(_2\); 1mM CaCl\(_2\); 10mM glucose; 20mM HEPES, pH 7.4).

**Microscopy**

Cells were imaged at room-temperature on the TIR-FLIM setup described above. The TIRF objective used is a Zeiss α-Plan Fluar 100x NA 1.45 oil objective (Carl-Zeiss GmbH, Germany). EYFP-annexin A4 was excited with a
514 nm argon-ion laser line through a 525 nm dichroic mirror and YFP emission was selected using a 545-30 nm bandpass filter, thereby eliminating interference from mCherry fluorescence. Ionomycin (5 μM; Sigma, Zwijndrecht, The Netherlands) was added to induce translocation of annexin A4 and every 15 or 30 seconds a phase stack (12 phase images each, exposure time 150 ms) was recorded.

References