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Total internal reflection fluorescence microscopy as a powerful tool to follow dynamic events at the cell membrane

E.S. Di Cairano, S. Moretti, F. Daniele, V.F. Sacchi, C. Perego

Department of Pharmacological and Biomolecular Sciences, University of Milan, Italy

Cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signaling. Understanding how these processes are dynamically regulated is fundamental. Total internal reflection fluorescence microscopy (TIRFM) is ideally suited to study these events. It is based on the use of fluorescent labeled proteins associated to a microscope set-up that allows selective excitation of molecules at the plasma membrane. Indeed, fluorescent molecules alone provide information about the expression and localization of proteins and other molecules, but the temporal and spatial resolution is confounded by signal from outside the area of interest. TIRFM overcomes this limitation by using an evanescent wave generated by the reflection of a laser beam; this wave is relatively low powered and it is able to illuminate just a narrow (<100 nm) strip at the surface of a cell, thereby excluding the signals arising from structures in the cell interior (Figure 1).[1]

As a result, spatial and temporal resolutions are increased, thus making it possible to measure dynamic events occurring at or immediately below the plasma membrane such as exocytosis and endocytosis, single molecule interactions, and ionic changes. This technique allows not only qualitative analysis, but also a quantitative measure of these events, by evaluating variation in fluorescence intensity during time-lapse recording. Analysis of these processes may open novel perspectives in the study of cell signaling, membrane trafficking and cytoskeleton remodeling.

In our laboratory, we have taken advantage of TIRFM for evaluating the glutamatergic signaling in the nervous system and in peripheral organs, in particular we have investigated the following.

First, dynamic modulation of glutamate transporter density at the plasma membrane. The excitatory amino acid carrier 1 (EAAC1) is a plasma membrane high affinity glutamate transporter expressed in the nervous system and in absorptive epithelia. EAAC1 activity can be rapidly regulated by its redistribution between intracellular compartments and the plasma membrane, a process controlled by protein-protein interactions and extracellular signals. We analyzed the molecular mechanisms of this modulation in two different physiological contexts: in epithelial cells, where EAAC1 is important for absorption of dicarboxylic amino acids, and in Schwann cells, where it may participate in cell myelination and proliferation by regulating the level of extracellular glutamate or by providing Schwann cells with glutamate.

In epithelial cells, we investigated the molecular mechanisms that control the surface density of EAAC1. We detected in its cytoplasmic C-tail a consensus sequence for interaction with class I PDZ proteins and a tyrosine-based internalization signal ($^{-503}$YVNG^{506}). To understand their role in transporter trafficking, we generated green fluorescent protein (GFP)-tagged transporters-lacking the PDZ target motif (ΔTSQF) or carrying the Y503A substitution. We expressed them in the Madin Darby Canine Kidney epithelial cell line, and we monitored their residence on the plasma membrane by time-lapse TIRF imaging. In these experiments, if the GFP transporter is internalized, the fluorescence signal recorded by TIRFM should progressively decrease. Quantification of the fluorescence changes in the different mutants indicated that the PDZ target sequence controls the transporter resident time at the plasma membrane and that Y503 is involved in the constitutive endocytosis of EAAC1 (Figure 2).[2]

In Schwann cells, we investigated the mechanisms of action of allopregnanolone (ALLO), a steroid with neuroprotective effects, synthesized by Schwann cells. We found that incubation with ALLO rapidly increases the activity of the glutamate transporter EAAC1, with a mechanism that involves protein trafficking to the plasma membrane. We investigated this phenomenon by time lapse TIRFM imaging in primary cultures of Schwann cells transfected with EGFP-EAAC1. By alternatively blocking the exo- or endocytic pathways, we found that ALLO promotes the surface delivery of EAAC1 and increases its plasma membrane residence time by tethering it to the submembrane cytoskeleton. This recruitment is important to control Schwann cell proliferation.[3]

Second, vesicle dynamics. Vesicle exocytosis is a common mechanism to control neurotransmitter and hormone release in different bio-

Correspondence: Eliana Di Cairano, Department of Pharmacological and Biomolecular Sciences, University of Milan, via Trentacoste 2, 20134 Milan, Italy.
E-mail: eliana.dicairano@unimi.it

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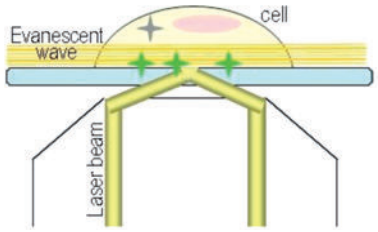

Figure 1. Schematic representation of TIRF microscopy. When the laser beam is reflected by the glass slide, it generates an evanescent wave that diffuses in the specimen with the same wavelength but decades in a short distance, thereby illuminating only fluorescent molecules at or immediately below the plasma membrane.

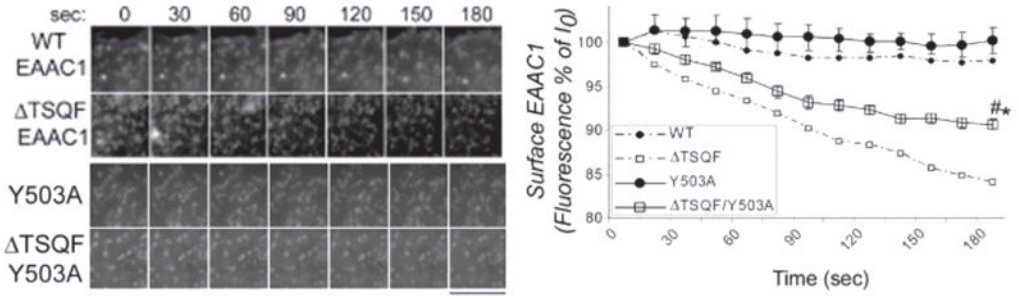

Figure 2. Representative TIRFM image sequences showing the membrane resident time of the indicated transporters (left), together with the averaged fluorescence intensity curves (right).

logical systems. To investigate the molecular mechanisms of vesicle exocytosis modulation, we labeled mice cortical synaptosomal membranes with the fluorescent organic dye FM1-43. Under TIRFM, we monitored the effect of corticosterone treatment on dynamics of vesicles docking and fusion and we found that the glucocorticoid promotes the docking of vesicles to the synaptic plasma membrane.[4]

In addition to conventional fluorescent dyes, genetically engineered fluorescent proteins such as vesicles-resident proteins, cargo molecules (neurotransmitter and hormones) are increasingly being used to measure membrane trafficking and to monitor cell signaling.[5] We are currently setting up the experiments to measure the dynamics of hormone release in endocrine cells of the pancreas.

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