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In vivo detection of membrane protein expression using surface plasmon enhanced fluorescence spectroscopy (SPFS)

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Abstract

Surface plasmon enhanced fluorescence spectroscopy (SPFS) was applied for the detection of expression and functional incorporation of integral membrane proteins into plasma membranes of living cells in real time.

A vesicular stomatitis virus (VSV) tagged mutant of photoreceptor bovine rhodopsin was generated for high level expression with the semliki forest virus (SFV) system. Adherent baby hamster kidney (BHK-21) cells were cultivated on fibronectin-coated gold surfaces and infected with genetically engineered virus driving the expression of rhodopsin. Using premixed fluorescently (Alexa Fluor 647) labeled anti-mouse secondary antibody and monoclonal anti-VSV primary antibody, expression of rhodopsin in BHK-21 cells was monitored by SPFS. Fluorescence enhancement by surface plasmons occurs exclusively in the close vicinity of the gold surface. Thus, only the Alexa Fluor 647 labeled antibodies binding to the VSV-tag at rhodopsin molecules exposed on the cell surface experienced fluorescence enhancement, whereas, unbound antibody molecules in the bulk solution were negligibly excited. With this novel technique, we successfully recorded an increase of fluorescence with proceeding rhodopsin expression. Thus, we were able to observe the incorporation of heterologously expressed rhodopsin in the plasma membrane of living cells in real time using a relatively simple and rapid method. We confirmed our results by comparison with conventional wide field fluorescence microscopy.

Keywords: Surface plasmon enhanced fluorescence spectroscopy (SPFS); Immunofluorescence; Living cells; Membrane protein expression; Time course; Fluorescence interference contrast microscopy (FLIC)

1. Introduction

Membrane proteins play a central role in the physiology of living cells and fulfill a number of important functions, for example, energy metabolism, cell–cell interaction, or uptake of nutrients and ions (von Heijne, 1999). Many interesting targets in drug design are membrane-bound proteins, an important subclass of which are G-protein coupled receptors (GPCRs) (Pierce et al., 2002).

Despite their importance, detailed functional studies of membrane proteins are scarce because of their sensitivity to degeneration as soon as they are removed from the native lipid bilayer by solubilization with detergents (Hurley, 2003). Exploring the biological function of these proteins has proven to be an intricate task, mainly due to their hydrophobic nature and low abundance. Furthermore, high yield purification of membrane proteins in the purity needed for investigation is difficult and time-consuming to achieve by classical methods of purification and cell fractionation.

Therefore, one of the benefits of analyzing membrane proteins in their natural environment is the avoidance of strenuous and intricate purification procedures. Moreover, significant differences in affinity and interactions of membrane proteins were recorded in the plasma membrane compared to measurements of

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reconstituted proteins in artificial membrane systems (Claasen et al., 2005; Fierens et al., 2002). Therefore, investigation of membrane proteins in their natural environment might provide more comprehensive results considering the highly dynamic and complex structured plasma membrane where intricate molecular reactions take place.

In this regard, wide field fluorescence microscopy and the more sensitive confocal laser scanning microscopy (CLSM) as well as variations thereof like near-infrared (NIR) multiphoton microscopy have been applied on vital cell investigations (Konig, 2000). The dynamic behavior of membrane proteins has been characterized by fluorescence CLSM and fluorescence correlation spectroscopy (Kahya et al., 2005). However, difficulties like photooxidation processes arise when living cells are illuminated. Even when focused on the plasma membrane, CLSM look into cells to a depth of nearly half a micron. Hence, confocal and in particular wide field fluorescence microscopy show strong background fluorescence from the cytosol that obscures the weaker fluorescence from small structures or molecular assemblies near the plasma membrane.

In this work, we present surface plasmon field-enhanced fluorescence spectroscopy (SPFS) (Attridge et al., 1991; Liebermann and Knoll, 2000; Neumann et al., 2002), an extension of surface plasmon resonance spectroscopy (SPS) (Bernard and Lengeler, 1978; Liedberg et al., 1983; Flanagan and Pantell, 1984), that provides enhanced sensitivity, as a novel method for investigating membrane proteins in the plasma membrane of living cells with the advantages of low background and little damage by photooxidation. SPFS exploits the strong evanescent field generated by optical resonance of surface plasmons to excite fluorophores located near the metal–dielectric interface. The field intensity is enhanced 16× on gold by laser light at $\lambda = 633$ nm (Liebermann and Knoll, 2000; Neumann et al., 2002). This enhancement excites the fluorophores within the evanescent field. The evanescent field decays exponentially in the adjacent dielectric medium with a penetrating depth of approximately 150 nm (Vareiro et al., 2005). Due to this surface sensitivity, the application of SPFS on living cells provides specific signals from a fluorescently labeled membrane protein of interest with a considerably low background from cell autofluorescence and debris. In order to prove applicability of SPFS for detection of membrane proteins in living cells, we monitored the expression of the fluorescently labeled model GPCR bovine rhodopsin in real time. We used light intensities of less than 1 mW that were minimal invasive for the used cell lines during the SPFS measurements. Simultaneous monitoring of the reflected light and the fluorescence light emitted from the same surface enables appreciation of the highly sensitive nature of SPFS in a biosensor format.

2. Materials and methods

2.1. Chemicals

All used reagents were obtained in analytical grade quality from Sigma–Aldrich (Taufkirchen, Germany) or Roche Diagnostics GmbH (Penzberg, Germany). The fluorescently labeled antibody, goat anti-mouse-Alexa Fluor 647, was purchased from Molecular Probes (Leiden, The Netherlands).

2.2. Plasmids

The semliki forest virus construct pSFV3-lacZ encoding *Escherichia coli* β-galactosidase (lacZ) as negative control has been described elsewhere (Liljestrom and Garoff, 1991). pSFV1cap was used for the construction of pSFV1-rhodopsin (Sjoberg et al., 1994). The construct pSFV1-rhodopsin for expression of bovine rhodopsin contains the vesicular stomatitis virus (VSV)-tag and a so-called cap sequence, described as coding sequence for a translation enhancing protein (Sjoberg et al., 1994). As the cap protein is cleaved off autocatalytically (Nicola et al., 1999), an N-terminally VSV-tagged rhodopsin is produced. The VSV-tag was attached to rhodopsin by PCR using the following primers: forward primer, 5′-GCATGTACACCGACATCGAGATGAACAGACTGGGCAAGAACGGGACCGAGGG CCCAAAC-3′; reverse primer, 5′-GCGGATCCTTAGGCAGGCGCCACTTGGCTGG-3′.

The resulting PCR-product as well as pSFV1cap were restricted by *Sma*I to yield blunt ends, then ligation occurred. pSFV1-rhodopsin was verified by DNA-sequencing.

pSFV3-lacZ and pSFV1cap were kindly provided by Kenneth Lundström (BioXtal, Epalinges, Switzerland).

2.3. Cell culture

Baby hamster kidney (BHK-21) cells (catalogue no. CCL10 from American Type Culture Collection, ATCC, Rockville, MD, USA) were cultured in a 1:1 mixture of Dulbecco's modified F12 medium and Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (BHK medium) at 37 °C in humidified 5% CO_2 atmosphere. All media and supplements were obtained from Gibco (Karlsruhe, Germany).

2.4. Heterologous expression of bovine rhodopsin and β-galactosidase

For heterologous expression of bovine rhodopsin in BHK-21 cells, we used the semliki forest virus (SFV) expression system highly versatile for the expression of membrane proteins (Liljestrom and Garoff, 1991; Berglund et al., 1993; Lundstrom et al., 1994, 1995, 2001). Preparation of replication-deficient infectious SFV particles was carried out using the pSFV-Helper2 vector as described (Lundstrom et al., 1994). Briefly, RNA from in vitro-transcribed pSFV3-lacZ encoding *Escherichia coli* β-galactosidase (rSFV3-lacZ) and accordingly pSFV1-rhodopsin encoding VSV-tagged rhodopsin (rSFV1-rhodopsin) was co-electroporated with RNA from pSFV-Helper2 (Berglund et al., 1993) into BHK-21 cells. Recombinant rSFV1-rhodopsin and rSFV3-lacZ particles were harvested after 24 h. Prior to infection, recombinant SFV particles were activated with 500 μg/ml α-chymotrypsin for 30 min at 20 °C and the reaction was terminated with 250 μg/ml aprotinin for 15 min at 20 °C. Test infections with serial dilutions of viral particles containing rSFV1-rhodopsin and rSFV3-lacZ were carried out for titer