What is Total Internal Reflection Fluorescence (TIRF)?

Introduction

The specific behavior of macromolecules at or near surfaces, interfaces, and membranes is of primary interest in the biological sciences. Important applications include: adsorption of blood proteins on biomaterials in thrombogenesis research; the binding to and triggering of living cells by hormones, neurotransmitters, and antigens; cell adhesion to various surfaces; the mechanism of electron transport in mitochondrial and photosynthetic membranes; and also reaction rate enhancement with membrane receptors by nonspecific adsorption and surface diffusion of ligands.

Most of the common analytical methods available for investigation of surfaces either lack the extent of surface selectivity required or demand relatively harsh sample handling that severely limits the biological relevance of any results obtained.

However, total internal reflection fluorescence **(TIRF)** spectroscopy has proven to be a very powerful and versatile technique for the study of surface and/or interfacial behavior of biological molecules and their aggregates [1,2]. TIRF has been successfully applied to numerous studies associated with solute adsorption, orientation, and rotational mobility associated with conformational changes. Surface selectivity is achieved in TIRF by detecting only the evanescent wave excited fluorescence signals that originate within approximately the first 100 nm from the waveguide surface.

This exceptionally short optical path length allows investigation of surface behavior even in the presence of highly concentrated solutions. In short, TIRF provides in situ, real-time, nondestructive, and highly sensitive detection suitable for studies on expensive biological materials available only in microliter quantities (~10 nl, minimum). The limit of detection is approximately 0.1% of a monolayer in most cases. Additionally, combination of TIRF with electrochemistry allows control of the physicochemical properties of the surface during a single TIRF experiment. This opportunity can provide new insight into mechanisms of interaction, as well as, facilitate modification of surface properties by an externally applied voltage.

Principles

The principles of TIRF are well documented in the literature [3-5]. In brief, when a beam of light propagating within a medium of refractive index (n_1) encounters an interface with a medium of lower refractive index (n_2), it can undergo total internal reflection for incidence angles (θ_i) greater than the critical angle (θ_C). Although the incident light totally reflects at the interface, a portion of the electromagnetic radiation penetrates the interface into the less dense medium. The intensity of this interfacial field, typically called the “evanescent wave”, decays exponentially with distance from the interface.

$$\theta = \sin^{-1} \frac{n_2}{n_1} \qquad [1]$$

The penetration depth (d_p) of the evanescent wave in the less dense medium is a function of incidence angle, refractive index ratio, and incident light wavelength, λ_i, (Eq. 2). The evanescent wave is primarily responsible for the electronic excitation of the fluorophore present in the lower refractive index medium. The penetration depth (path length) of the evanescent wave can be conveniently altered by changing the incidence angles. The extremely short path length of the evanescent wave (on the order of the wavelength of light) excites a very small sample volume and thereby minimizes primary absorption effects. Also depending on the optical geometry, the emitted fluorescence does not pass through the bulk of solution but rather through the waveguide, thus largely avoiding any secondary absorption effects.

Instrumentation

The design of the ISS TIRF Flow Cell accessory makes the normally difficult TIRF experiment a routine measurement. The ISS TIRF Flow Cell (Figure 1) was designed as an interchangeable sample compartment for ISS Spectrofluorometers, which enables fast switching of accessories. The flow cell comes with an easily assembled flow system to study kinetics of various surface interactions. This design provides high reproducibility of TIRF measurements by ensuring he exact positioning of optical elements against the excitation beam and emission axis. The ISS TIRF cell also differs from similar TIRF cuvettes by simple and fast assembly of the sandwich cell. The transparent gasket that forms the flow chamber (app. 20ml) and transparent back plate facilitate easy visualization of the surface and allows acquisition of microscopic pictures via a long-focus objective.

Figure 1. ISS TIRF Cell Accessory. The accessory fits all of the spectrofluorometers made by ISS. It is available for other selected instruments.

The standard cell comes equipped with a UV-quartz prism and optically coupled cover slide. The cover slide provides

an easily interchangeable working surface and minimizes wear on the TIRF prism. Cover slides with hydrophilic, hydrophobic, electroconductive thin films, or specific soluble protein docking films may be supplied as options. Additional options include three-electrode electrochemical control by application of an external voltage and temperature control by way of a thermostated block and water bath. The ISS TIRF cell is also available for use with most of the commercially available research-grade spectrofluorometers currently on the market.

Applications

The principle applications of TIRF spectroscopy are:

- Protein absorption: kinetics and isotherms, effect of solvents, competitive adsorption, conformational changes, effects of detergents, surface mobility
- Immunoassay systems: antibody-antigen interactions, biosensor development
- Electron transport in mitochondrial and photosynthetic membranes
- Cell adhesion to surfaces

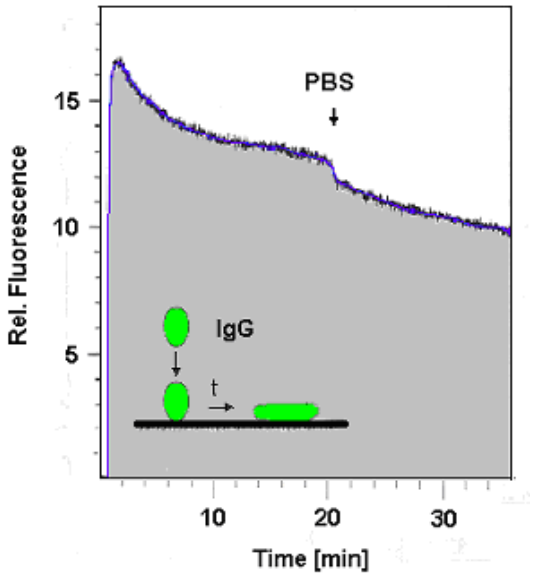

Figure 2. Fluorescence intensity changes caused by IgG-FITC conjugate adsorption onto hydrophilic silica from 0.3 mg/ml solution in pH 7.4 phosphate buffer. PBS arrow indicates switching to pure buffer.

The absorption kinetics of a fluorescently labeled immunoglobulin (IgG-FITC) on a hydrophilic silica surface are illustrated in Figure 2. About 70% of IgG is irreversibly adsorbed kinetically and does not desorb back into the flow of pure buffer solution. IgG molecules undergo structural changes after absorption that result in fluorescent lifetime changes of the covalently attached fluorescent label, pyrene sulfonyl chloride (not shown). The intensity increase observed within the first minutes of adsorption can be interpreted as the result of a flattening of the IgG molecules after attachment to the surface [6].

An example of multilayer protein absorption is illustrated in Figure 3. Bovine serum albumin (BSA) forms a monolayer when adsorbed from a 50 mM phosphate buffer (pH 6.2 - lower curve) onto a hydrophobic siliconized surface. Ammonium sulphate modifies the protein-protein interactions and enables adsorption of multiple layers.

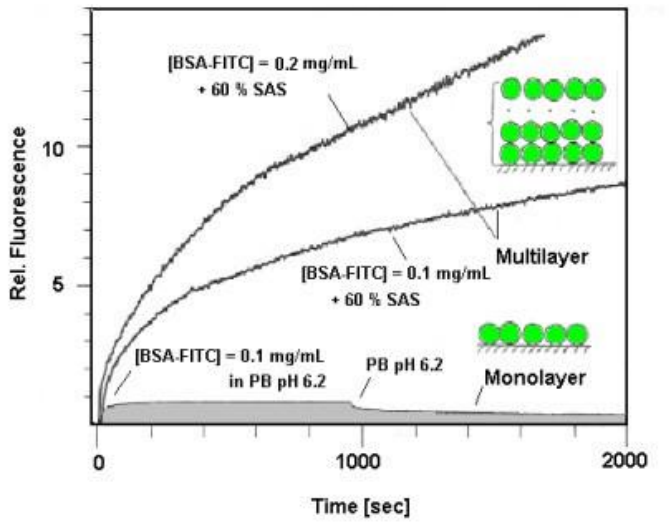

Figure 3. Plot of relative fluorescence intensity increases upon adsorption of a monolayer of bovine serum albumin-FITC (BSA-FITC) conjugate from 0.1 mg/mL solution in phosphate buffer pH 6.2 (lower curve) and multilayer BSA-FITC adsorption in presence of ammonium sulfate (60% v/v saturated solution).

Measurement of the excited state lifetime, rotational correlation time, fluorescence polarization, and quenching experiments can often provide important information about the molecular dynamics, and more specifically about protein conformation in the adsorbed state. The ISS K2 Spectrofluorometer equipped with the TIRF flow cell accessory is capable of selectively obtaining this important information from a surface under study.

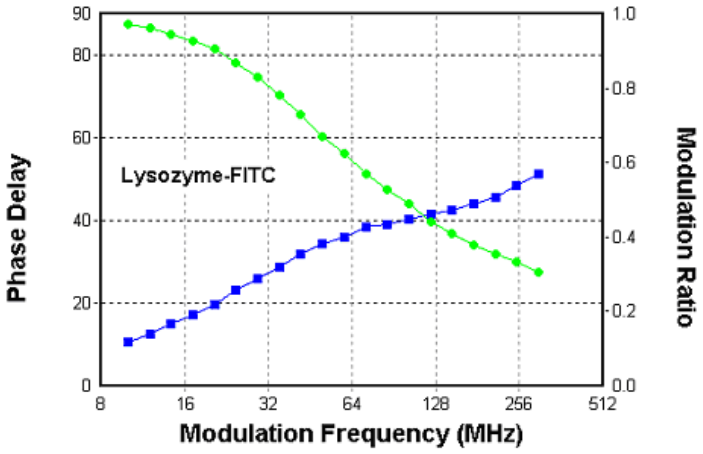

Figure 4. Fluorescence lifetime data obtained with ISS K2 and a TIRF cell. The fluorescence lifetime of FITC-labelled lysozyme adsorbed onto a quartz surface was measured. The single exponential decay of FITC changes into a bi-exponential decay. Two distinct lifetime were found for the adsorbed lysozyme: τ_1 =4.1 and τ_2 = 0.44 nsec, f_1 = 0.7.

References

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