FLUORESCENCE ACCESSORY

TIRF Flow Cell

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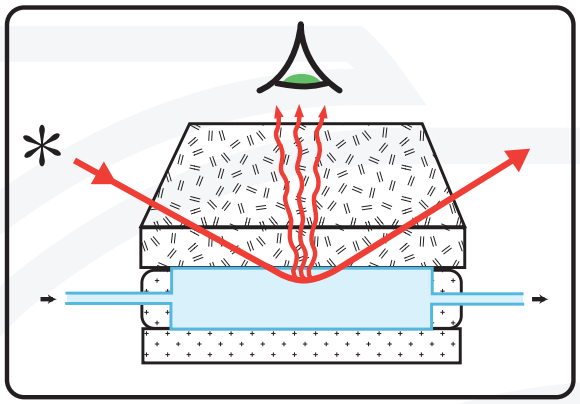

Fig. 2 - Optical scheme of TIRF Flow Cell

Introduction

The specific behavior of macromolecules at or near surfaces, interfaces, and membranes is currently of primary interest in nanotechnology and 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 which originate within approximately the first 100 nm from the waveguide surface. This exceptionally short optical pathlength 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, in this case, the optional combination of TIRF with electrochemistry allows control of the physicochemical properties of the surface during a single TIRF experiment. This opportunity can provide new insights into mechanisms of interaction, as well as, facilitate modification of surface properties by an externally applied voltage.

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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.

$$\theta_c = \sin^{-1}(n_2/n_1)$$

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 (pathlength) of the evanescent wave can be conveniently altered by changing incidence angles. The extremely short pathlength 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 solution but rather through the waveguide, thus largely avoiding any secondary absorption effects.

$$d_p = \lambda_i / (2\pi n_1[\sin^2\theta_i - (n_2/n_1)^2]^{1/2})$$

Instrumentation

The design of the ISS TIRF flow cell makes the normally difficult TIRF experiment become routine. Figure 1 is a photograph of the TIRF accessory and Figure 2 illustrates the basic optical design. The ISS TIRF flow cell comes prealigned for the ISS spectrofluorometers. along with an easily assembled flow system to study kinetics of various surface interactions. High reproducibility of TIRF measurements is ensured by the exact positioning of optical elements against the excitation beam and emission axis. The ISS TIRF cell differs from similar TIRF cuvettes by simple and fast assembly of the sandwich cell. The transparent gasket which forms the flow chamber (app. 20µl) and transparent back plate facilitate easy visualization of the surface and allows acquisition of microscopic pictures by a long-focus objective.

The standard cell comes equipped with a UV-quartz prism and optically coupled cover slide, as shown in Figure 2. 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 can 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 adsorption: kinetics and isotherms, effect of solvents, competitive adsorption, conformational changes, effect of detergents, surface mobility
- immunoassay systems: antibody-antigen interactions, biosensor & nanotechnology development
- electron transport in mitochondrial and photosynthetic membranes
- cell adhesion to surfaces.

Figure 3 illustrates the adsorption kinetics of immunoglobulin (IgG) at a hydrophilic silica surface. 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 adsorption that result in fluorescent lifetime changes of the fluorescent label, pyrene sulfonyl chloride (not shown). The maximum observed within the first minutes of adsorption can be interpreted as a result of a flattening of the IgG molecules after attachment to the surface [6].

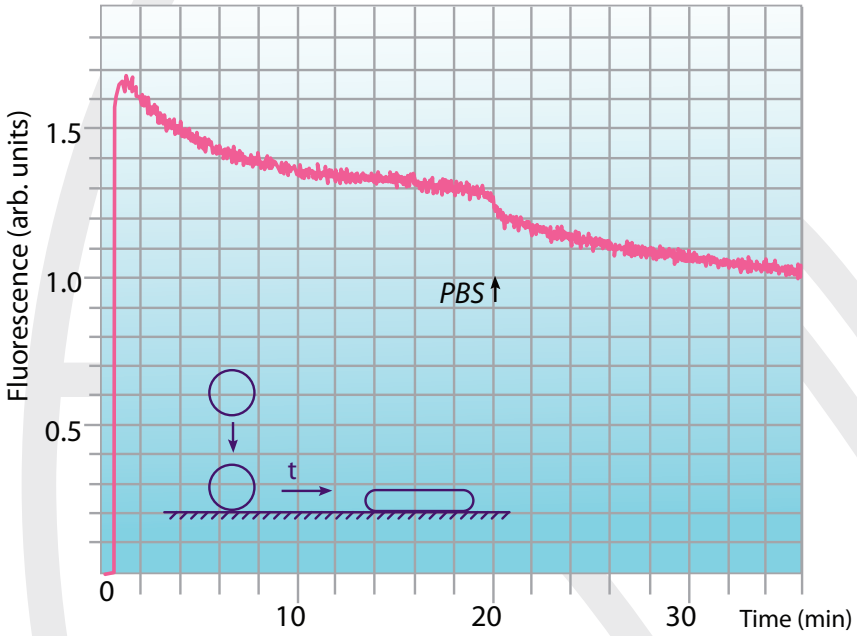

Figure 3.
Immunoglobin (IgG-FITC conjugate) adsorption onto hydrophilic silica from 0.3 mg/ml solution in pH 7.4 phosphate buffer. PBS arrow indicates switching to pure buffer.

An example of multilayer protein absorption is illustrated in Figure 4. 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 allows multilayer adsorption to occur.

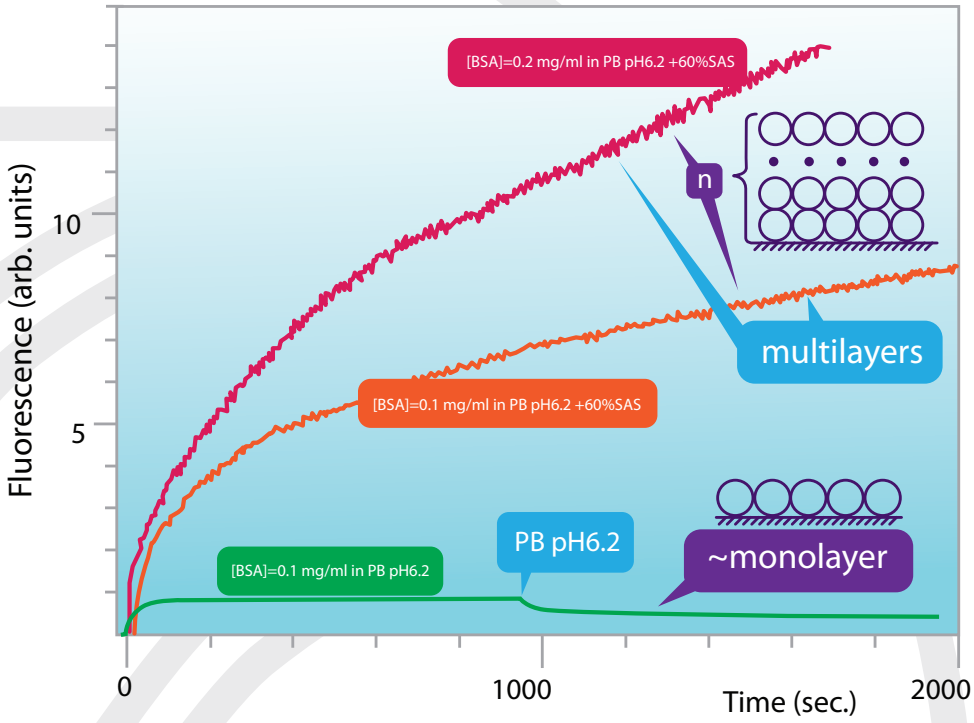

Figure 4. Monolayer bovine serum albumin (BSA-FITC conjugate) adsorption from solution in phosphate buffer pH 6.2 (lower curve) and multilayer adsorption in the presence of ammonium sulfate (60% v/v saturated solution).

Figure 5 illustrates the effect of electrochemical control of human serum albumin (HSA) adsorption at a tin dioxide transparent electrode. Cathodic polarization increases surface affinity for HSA, probably, because of negative polarization which enhances hydrophobicity of the surface. Application of an anodic polarization stimulates desorption of HSA from the tin dioxide surface [7].

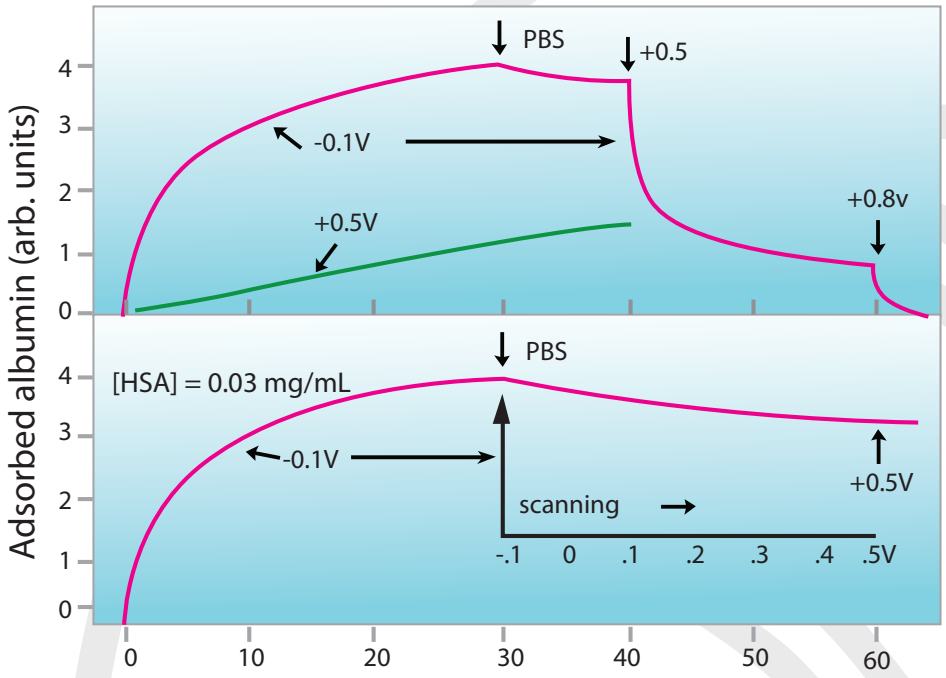

Figure 5. Effect of electrochemical polarization of a tin dioxide surface on the adsorption of human serum albumin (HSA-FITC conjugate) from a 0.03 mg/ml solution in phosphate buffer pH 7.4

Excited state lifetime, rotational correlation time, fluorescence polarization, and quenching experiments often provide important information concerning molecular dynamics, in general, and protein conformation related to adsorption, more specifically. The ISS spectrofluorometers equipped with the TIRF flow cell accessory are quite capable of selectively obtaining this type of information from the surface under study.

Figure 6 illustrates fluorescence lifetime data obtained directly using the K2™ and TIRF cell. Lifetime data for FITC-labelled lysozyme adsorbed onto a quartz surface were obtained. These data provide evidence of at least two types of adsorbed lysozyme characterized by lifetimes of 4.1 and 0.44 nsec, respectively.

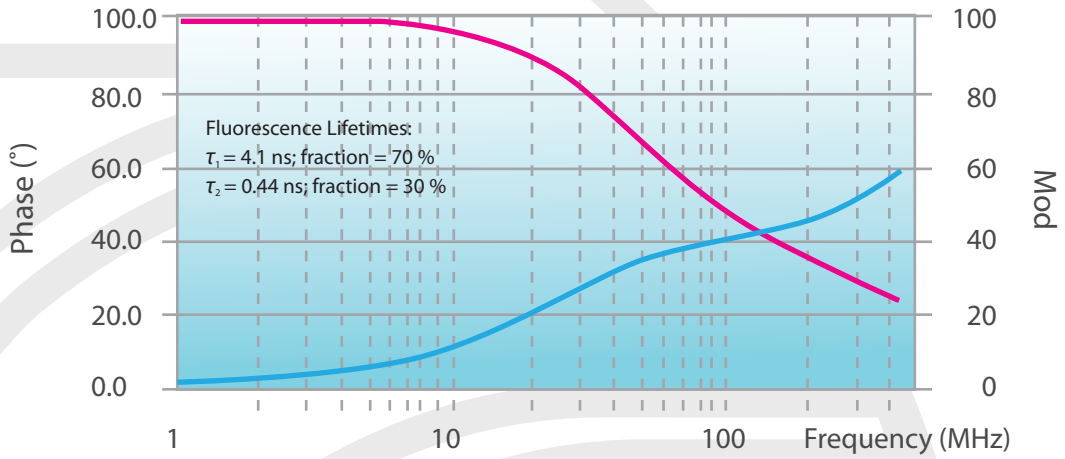

Figure 6. Multifrequency lifetime data obtained with the ISS TIRF cell for lysozyme-FITC conjugate adsorbed onto a quartz surface from a 0.3 mg/ml solution in 50 mM acetate buffer, pH 4.5

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