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Total internal reflection fluorescence microscopy: technical innovations and novel applications

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Recent years have seen the introduction of novel techniques and applications of total internal reflection fluorescence microscopy (TIRFM). Key technical achievements include miniaturization, enhanced depth resolution, reduction of detection volumes and the combination of TIRFM with other microscopic techniques. Novel applications have concentrated on single-molecule detection (e.g. of cellular receptors), imaging of exocytosis or endocytosis, measurements of adhesion foci of microtubules, and studies of the localization, activity and structural arrangement of specific ion channels. In addition to conventional fluorescent dyes, genetically engineered fluorescent proteins are increasingly being used to measure molecular conformations or intermolecular distances by fluorescence resonance energy transfer.

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Abbreviations

CFP	cyan fluorescent protein
FRET	Förster (fluorescence) resonance energy transfer
GFP	green fluorescent protein
TIR	total internal reflection
TIRFM	total internal reflection fluorescence microscopy
YFP	yellow fluorescent protein

Introduction

For more than 20 years the total internal reflection (TIR) of light has been used to study cell-substrate surfaces and to acquire detailed information about cell membranes. When a light beam propagating through a medium of refractive index n_1 (e.g. glass) meets an interface with a second medium of refractive index $n_2 < n_1$ (e.g. the cytoplasm), TIR occurs at all angles of incidence Θ that are greater than a critical angle $\Theta_c = \arcsin(n_2/n_1)$. Despite being totally reflected, the incident beam establishes an evanescent electromagnetic field that penetrates into the second medium and decays exponentially with the distance z from the interface. According to the relation $d = (\lambda/4\pi)\,(n_1^2 \sin^2\Theta - n_2^2)^{-1/2}$ (where λ corresponds to the wavelength of light) penetration depths (d) can be adjusted between about 70 nm and 300 nm. Therefore, fluorophores located within or close to the plasma membrane are detected selectively in living cells. In addition, by varying the angle of incidence Θ cell-substrate topography can be determined. So far, total internal reflection fluorescence microscopy (TIRFM) has been used for measuring the topography of cell-substrate contacts [1,2] and in studies of protein dynamics [3], membrane-proximal ion fluxes [4], endocytosis or exocytosis [5,6], as well as membrane-associated photosensitizers [7].

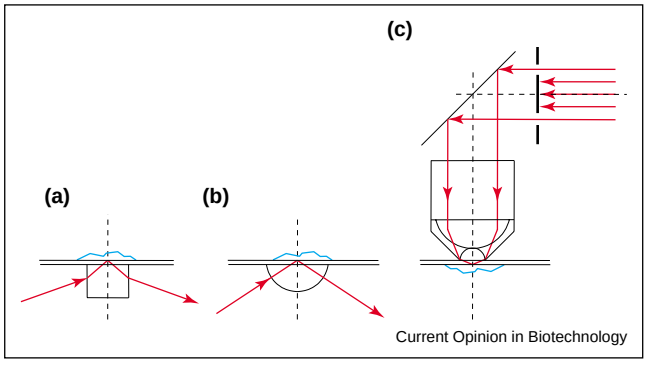
Two different technical solutions for TIR illumination have been established, as depicted in Figure 1. In the first case, the cell substrate is optically coupled to a glass or quartz prism of cubic, hemicylindrical or hemispherical shape (Figures 1a,b). By using a hemicylindrical or hemispherical prism or a multiple laser-scanning system [8], the position of the illuminating light spot on the sample is maintained upon variation of the angle of incidence. The second technical solution (Figure 1c) is based on extreme dark-field illumination by an objective lens of high numerical aperture A_N (up to 1.45 for glass lenses and up to 1.65 for sapphire lenses). When using $A_N = 1.45$ an aperture angle $\Theta_A = 72^\circ$ is attained, which exceeds the critical angle for TIR Θ_C by about 7°. Only a small annulus in the aperture plane or a small spot close to the edge of the aperture is illuminated in this case [9]. Therefore, within the small angular range between Θ_C and Θ_A, variation of the penetration depth (needed for studies of surface topography) is difficult to perform; however, thin layers of biological samples can be selected (e.g. for single-molecule detection [10]).

Here we present an overview of the novel techniques and applications of TIRFM developed over the past few years. We consider recent technical innovations, which have focused on combining TIRFM with confocal or two-photon microscopy, atomic force microscopy and fluorescence lifetime imaging. Key findings of TIRFM are also summarised and include the imaging of single molecules, single ion channels and granules during exocytosis.

Technical innovations

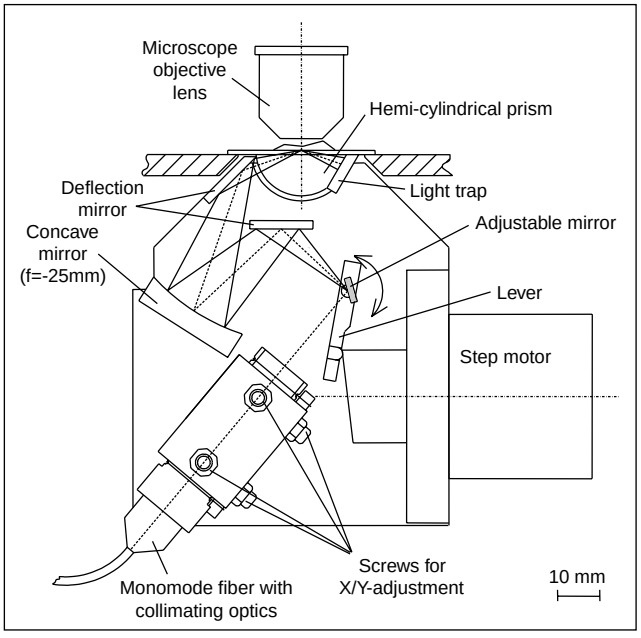
During the period 2003–2004 novel techniques have been introduced in both prism-type and objective-type TIRFM. The well-known problem that different

Figure 1



Technical solutions for TIR illumination. TIR illumination can be performed using **(a)** a cube shaped prism, **(b)** a hemispheric prism or **(c)** an objective lens of high numeric aperture. In all cases the angle of incidence of light propagating in a glass substrate and meeting the surface of a cell (blue line) is larger than the critical angle of total internal reflection. Therefore, an evanescent electromagnetic wave arises on the cell–substrate interface and penetrates a small distance into the cell (the dashed line represents the optical axis).

Figure 2



Compact illumination device for variable-angle TIRFM replacing a conventional microscope condenser. Laser light is delivered via a glass fibre and collimating optics, deflected on an adjustable mirror, and focused on the sample using a concave mirror, a deflection mirror and a hemicylindrical prism. By rotation of the adjustable mirror the angle of light incidence on the sample and therefore the penetration depth of the evanescent wave can be varied. (Figure reproduced from [12] with permission of Blackwell Publishing.)

polarizations of incident light can excite different patterns of fluorophores has been overcome by using a prism combination that permits excitation by two orthogonal beams [11]. The complex equipment previously used for variable-angle TIRFM was recently replaced with a miniaturized illumination device, which can be used instead of a conventional microscope condensor [12•]. As depicted in Figure 2, a light spot on an adjustable mirror is imaged in the plane of the sample under variable angles in steps of 0.15°. Light can be delivered from different laser sources via monomode [12•] or multimode [13] fibres, which can be easily exchanged. This setup is well suited for measuring cell-substrate topology and might also be combined with transillumination or phase-contrast microscopy. Recently, objective-type TIRFM has been combined with two-photon microscopy using either wide-field detection [14] or sample scanning [15]. Thus, the excited sample volume could be further localized and background emission was reduced. An inherent disadvantage in this case was that the incident laser beam could not be focused simultaneously in the aperture and in the object plane. To overcome this problem, Ruckstuhl and Seeger [16] replaced the microscope objective lens by a parabolic mirror, which focused a parallel beam of circular shape to submicrometer dimensions. The detection volume in this case could thus be reduced to less than 5×10^{-18} litres. Even smaller volumes have been assessed by immobilizing a few protein molecules on the tip of an atomic force microscope before TIRFM measurement [17•]. TIRFM has also been combined with fluorescence lifetime imaging microscopy to compare molecular interactions within the plasma membrane with those of intracellular membranes or organelles [18,19]. As depicted in Figure 3, the fluorescence lifetime of the mitochondrial marker rhodamine 123 (R123) applied in rather high concentration (50 μM) to endothelial cells is reduced within the mitochondria (indicating some fluorescence quenching), whereas an additional accumulation of R123 occurs in the plasma membrane. Therefore, for measurements of mitochondrial metabolism the concentration of R123 should be below 50 μM.

Single-molecule detection using TIRFM

Single-molecule imaging of living cells by TIRFM dates back to 2000 [10]. In particular, the epidermal growth factor (EGF) and its receptor (EGFR) located on the cell surface were of some interest in single-molecule detection. Sako *et al.* [20] now discuss the dimerization of EGFR upon binding of EGF, activation of EGFR through tyrosine phosphorylation, the recognition of activated EGFR by a cytoplasmic adaptor protein, and intracellular calcium responses induced by EGF. TIRFM was the major technique to excite sufficiently thin layers and to reduce background luminescence from outside. So far, various fluorophores emitting in the green–red part or the spectrum (e.g. Alexa dyes or tetramethylrhodamine) have