RESEARCH ARTICLE

Total Internal Reflection Fluorescence Microscopy (TIRFM) – novel techniques and applications

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Abstract

Total Internal Reflection Fluorescence Microscopy (TIRFM) has been established almost 40 years ago for studies of plasma membranes or membrane proximal sites of living cells. The method is based on light incidence at an angle above the critical angle of total internal reflection and generation of an evanescent electromagnetic field penetrating about 100 nm into a sample and permitting selective excitation of membrane proximal fluorophores. Two techniques are presented here: prism-type TIRFM and objective-type TIRFM with high aperture microscope objective lenses. Furthermore, numerous applications are summarized, e.g. measurement of focal adhesions, cell-substrate topology, endocytosis or exocytosis of vesicles as well as single molecule detection within thin layers. Finally, highly innovative combinations of TIRFM with Förster Resonance Energy Transfer (FRET) measurements as well as with Structured Illumination Microscopy (SIM) and fluorescence reader technologies are presented.

Keywords: Optical microscopy, living cells, TIRFM, FRET, SIM, fluorescence reader

1. Introduction

Total Internal Reflection Fluorescence Microscopy (TIRFM) is a key technique providing selective illumination of fluorophores in close vicinity to a cell-substrate interface. TIRFM has been applied to living cells for almost 40 years to obtain high-contrast images of fluorophores within or close to the plasma membrane with very low background, reduced photodamage and short exposure times to analyse numerous reactions, biosynthesis or transport of various metabolites or organelles.

Originally TIRFM based on light incidence via a glass or quartz prism whose refractive index exceeds that of the specimen ("prism-type TIRFM" [1, 2]). Almost 20 years later "objective-type TIRFM" was introduced using a microscope objective lens of very high numerical aperture, so that upon illumination by a small annulus or a light spot close to the edge of the microscope aperture illumination occurs under total internal reflection with an evanescent wave penetrating about 100 nm into the sample [3].

2. Techniques

In a total internal reflection (TIR) microscope, an evanescent field is generated at the interface of transparent media with different refractive indices. When a light beam propagating through a medium of refractive index n_1 (e.g. glass) meets a second medium of refractive index $n_2 < n_1$ (e.g. cell membrane or cytoplasm), total internal reflection occurs at all angles of incidence Θ which 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 $d = (\lambda/4\pi) (n_1^2 \sin^2\Theta - n_2^2)^{-1/2}$ (with λ corresponding to the wavelength of light) penetration depths d range between about 70 nm and a few hundreds of nanometres, dependent on the angle of incidence Θ. Therefore, fluorophores located within or close to the plasma membrane can be detected selectively in living cells.

2.1 Prism-type TIRFM

Prism-type TIR microscopy where a laser beam hits the specimen under variable angles, e.g. in a special microscope condenser, is depicted in Figure 1. This technique requires an upright fluorescence microscope, e.g. equipped with a water immersion objective lens when measuring adherent cell cultures in aqueous solution. The main advantage of this approach is that the angle of incidence Θ can be varied over a wide range from EPI-illumination to extreme TIR illumination. Variable-angle TIRFM may thus be used to calculate cell-substrate distances for fluorescent membrane markers as well as for markers homogeneously distributed in the cytoplasm, for which an algorithm has been developed based on the angular dependence of penetration depth of the evanescent wave [4]. A further advantage of prism-type TIRFM is that there are no restrictions with regard to the aperture and magnification of the microscope objective lens.

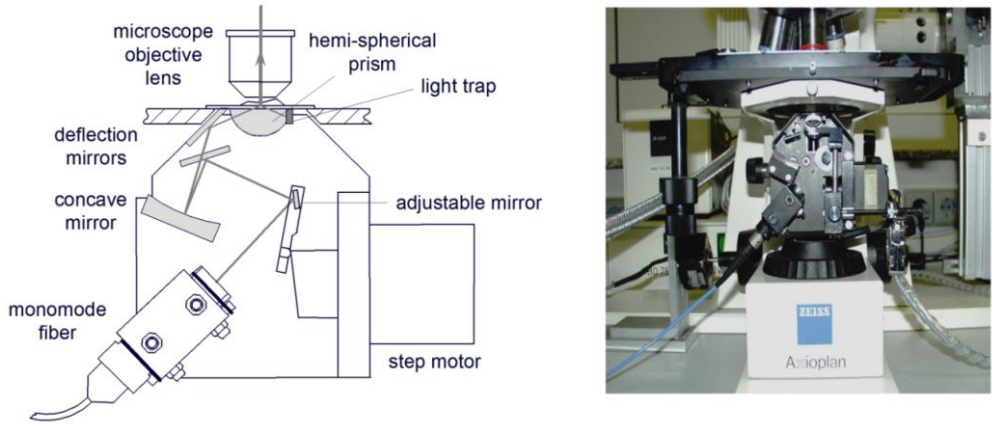

Figure 1. Condenser for prism-based TIR illumination under variable angle; schematic (left) and laboratory equipment with an additional light path for transillumination and phase contrast microscopy (right) (reproduced from Ref. 5 with modifications).

2.2 Objective-type TIRFM

Objective-type TIR Microscopy with numerical apertures $A_N \geq 1.45$ is now a well-established technique [3], which meanwhile has been commercialized by several industrial companies (Carl Zeiss, Olympus, Nikon). Here a laser beam is focused close to the edge of the aperture, so that the angle of incidence is larger than Θ_c. Disadvantages of this technique, however, are a limited range of angles of incidence and a small number of available objective lenses with high numerical aperture and magnification. Objective-type TIRFM has been combined with various methods of super-resolution microscopy, e.g. standing wave microscopy [6, 7] or Structured Illumination Microscopy (SIM) with up to 6 interfering laser beams hitting the sample under TIRF conditions [8–11], as further described in Section 3.3. Using these techniques a lateral resolution around 100 nm has been attained.

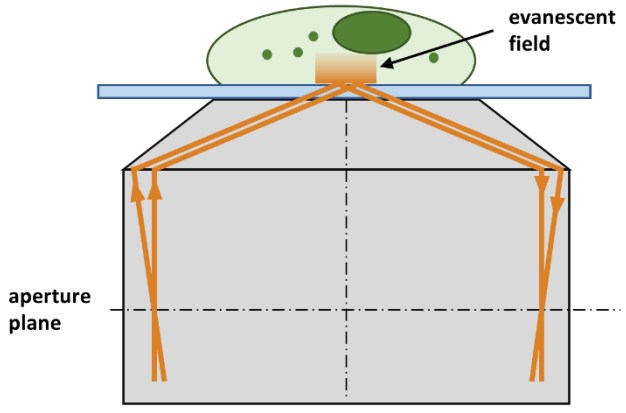

Figure 2. Objective-type TIR illumination (schematic).

3. Applications

3.1 Overview

TIRF microscopy has been applied for measurements of focal adhesions, cell-substrate contacts [1], protein dynamics [12], as well as endocytosis or exocytosis [13, 14]. Figure gives an example for HeLa cervical carcinoma cells, where membrane associated paxilline has been fused with Enhanced Yellow Fluorescent Protein (EYFP). While conventional epi-illumination excites EYFP all over the cells, TIR illumination excites selectively EYFP bound to paxilline in focal adhesions of the cells towards the substrate.

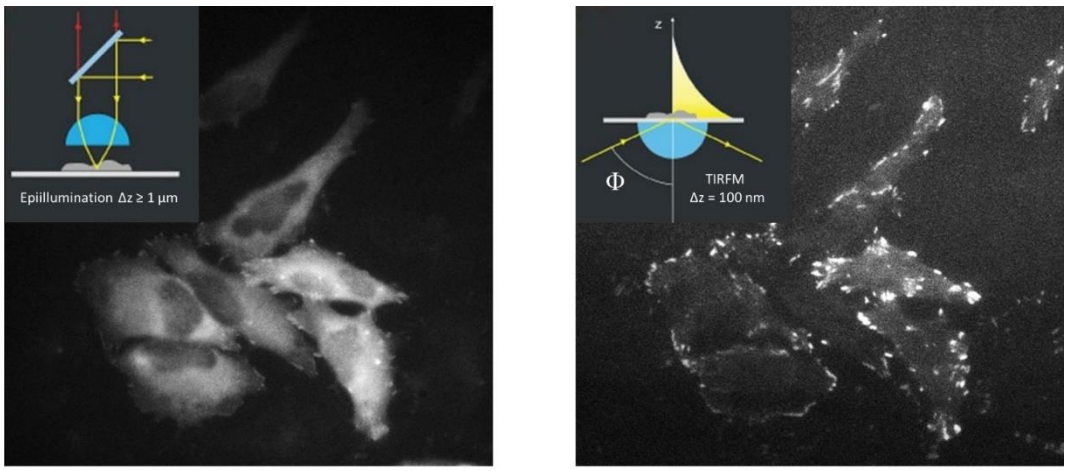

Figure 3. Comparison of epi-illumination (left) and TIR illumination (right) for excitation of membrane associated Paxilline – EYFP in HeLa cervical carcinoma cells. Insets: Beam alignment for epi-illumination and TIR illumination (reproduced from Ref. 5 with modifications).

Variable-angle TIRF microscopy has further been applied to studies of cell-substrate topology, e.g. after depletion of cholesterol or application of photosensitizing agents [15] as well as after cell transfection with tumor suppressor genes [16]. In these cases cell-substrate distances were calculated from about 10 TIRFM images recorded under various angles of illumination. While glioblastoma cells with a tumour suppressor gene showed a "folded" surface topology with cell-substrate distances varying between about 100 and 300 nanometres, the wild type tumour cells were characterized by almost constant distances of about 100 nm from the substrate (Figure 4). This might offer a possibility to distinguish cells of different malignancy.

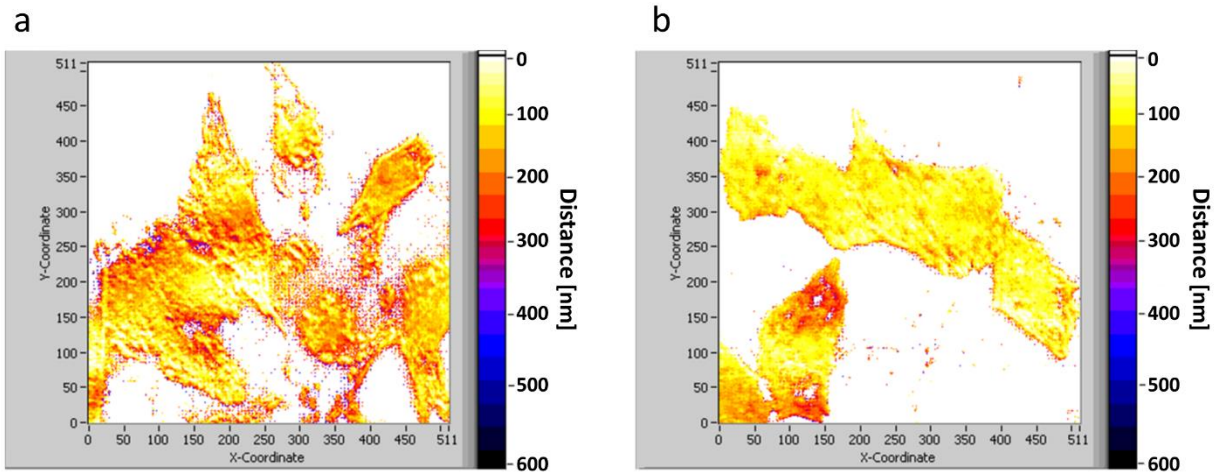

Figure 4. Cell-substrate distances in the range of 0–600 nm calculated from variable-angle TIRFM for U251-MG glioblastoma cells with activated TP53 suppressor gene (a) and for U251-MG wild type glioblastoma cells (b) after incubation with the fluorescence marker laurdan (8 µM; 60 min.)**.** Excitation wavelength: 391 nm, detection range: $\lambda \geq 420$ nm; image size: 210 µm × 210 µm. Reproduced from Ref. 16 with modifications.

3.2 TIR-FRET

Measurements of Förster Resonance Energy Transfer (FRET) [17] between a donor and an acceptor molecule (intermolecular FRET) or between different chromophoric groups of a larger molecule, e.g. a protein (intramolecular FRET), have been reported for about 30 years [18, 19], but only in very few cases their plasma membranes were assessed selectively, e.g. upon total internal reflection (TIR) [20–22]. The method is based on optical excitation of a so-called donor molecule and interaction of optical transition dipoles with an acceptor molecule, which is able to fluoresce. This method has become a valuable tool for probing either molecular interactions or conformational changes of a molecule in the nanometer range. Selective examination of the plasma membrane and adjacent cellular sites by TIRFM excludes superimposing signals from the cytoplasm or the supernatant and permits measurements at the interface of a cell with its environment including interactions of molecules with ion channels [23] as well as protein-protein interactions, which may play a role in the pathogenesis of various diseases [24, 25].

3.3 TIR-SIM

Structured Illumination Microscopy (SIM) with a periodically modulated illumination pattern created by two interfering beams leads to a resolution enhancement around a factor 2 compared to the value given by the Abbe criterion [26, 27]. For SIM, interference patterns resulting from the first diffraction orders of a spatial light modulator (SLM) are recorded for three phases (0, $2\pi/3$, $4\pi/3$) and three rotation angles (0°, 60° and 120°) in order to obtain an optical transfer function (OTF) of rotational symmetry up to high frequencies. Our experimental technique is based on a setup for SIM reported previously [28] with the optical components adapted to an objective lens for TIRF-imaging (Plan Apochromat 63×/1.46 Oil, Carl Zeiss Jena, Germany). By adjusting the angle of

incidence above the critical angle Θ_c for TIR, the structured illumination (SIM) is limited to a very thin layer, and super-resolution can be used to study the plasma membrane selectively. Previously, we applied SIM-TIRFM for measurement of the stimulus-dependent translocation of the glucose transporter GLUT4 (fused with green Fluorescent Protein, GFP) from intracellular storage compartments to the plasma membrane [11]. For this purpose, insulin or the insulin-mimetic phytochemical compounds tannic acid as well as *Bellis perennis* extract were used as stimulating agents [29, 30]. As depicted in Figure 5 (left) we observed fluorescence arising from small vesicles and – preferentially after simulation – from larger areas within the plasma membrane by SIM. When we measured the plasma membrane almost exclusively by SIM-TIRFM (Figure 5, right), we observed a pronounced increase of fluorescence over the whole cell surface after stimulation.

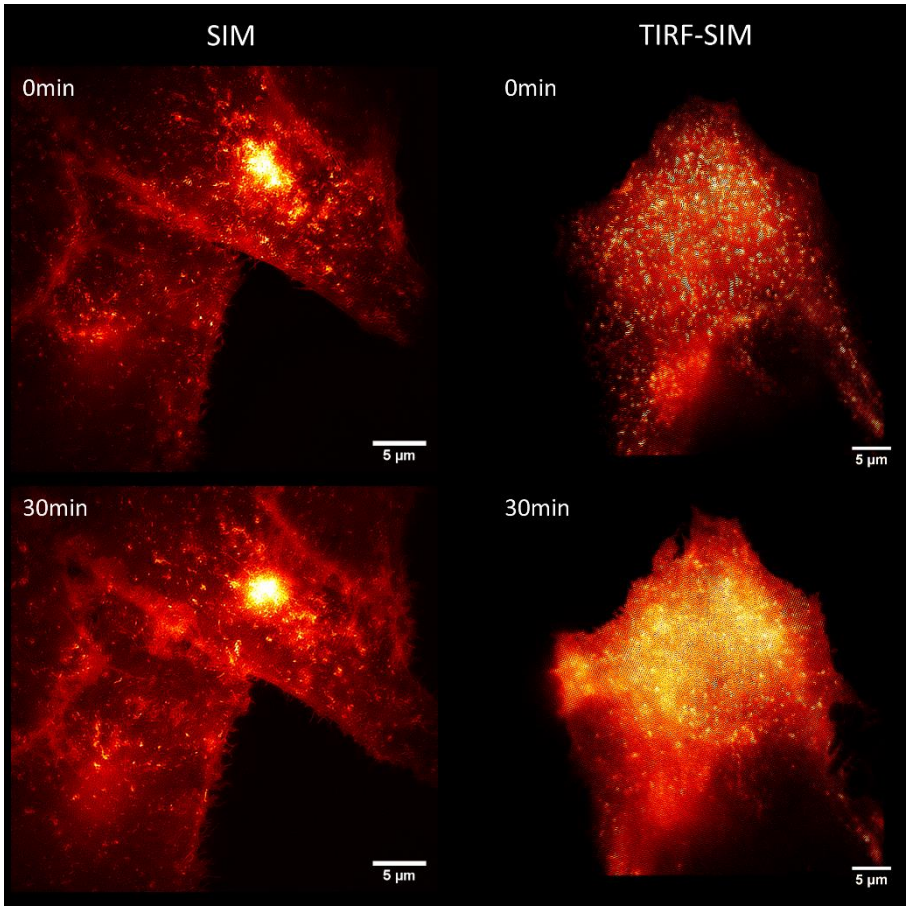

Figure 5. Comparison of SIM (left) and SIM-TIRFM (right): CHO-K1-hIR-myc-GLUT4-GFP cells prior to (0 min) and subsequent to stimulation (30 min) with *Bellis perennis* extract (common daisy; 10 mg/l); excitation wavelength: λ_0 = 488 nm; detection range: $\lambda_D \geq 515$ nm; Plan Neofluar 40×/1.30 oil immersion (left) or Plan Apochromat 63×/1.46 oil immersion objective lens (right). Reproduced from Ref. 11 with modifications.

3.4 TIR-Reader

High throughput or high content screening using fluorescence techniques requires simultaneous illumination of multiple samples, e.g., for drug discovery [31] or for examination of diseases accompanied by altered membrane properties, e.g., Morbus Alzheimer [32], Morbus Crohn [33] or Niemann-Pick disease [34]. For these purposes, we developed a microtiter plate reader system based on multiple TIR excitation and fluorescence detection [35]. The method is based on splitting of a laser beam and multiple total internal reflections within the bottom of a microtiter plate (cell substrate), such that up to 96 individual samples are illuminated simultaneously by an evanescent electromagnetic field. Main prerequisites are an appropriate thickness and a high transmission of the glass bottom, which is attached to the 96-well cell culture plate by a noncytotoxic adhesive. Glass rods of rectangular cross sections are optically coupled to this bottom for TIR illumination. Fluorescence arising from the plasma membrane of living cells is detected simultaneously from all samples using an integrating charge-coupled device (CCD) camera. In addition to fluorescence intensities we measured fluorescence lifetimes in the nanosecond range by using a pulsed laser system and replacing the CCD camera by a highly sensitive time-resolving camera (e.g. Picostar HR12 image intensifier coupled to a cooled ICCD camera, LaVision, Göttingen, Germany) [22], as depicted in Figure 6. This permits to detect membrane-associated fluorophores as well as their interactions with adjacent molecules simultaneously for up to 96 individual samples.

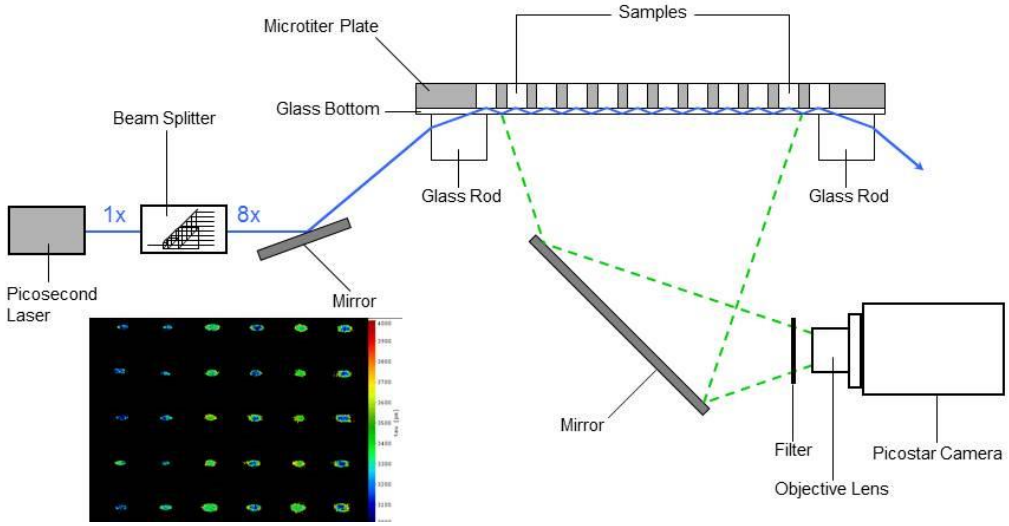

Figure 6. TIR fluorescence intensity and lifetime reader for a 96-well microtiter plate. Inset: example of a fluorescence lifetime measurement.

4. Conclusion

Meanwhile TIRFM is a well-established technique, which can be combined with further methods of modern microscopy, spectroscopy or biomedical screening. In particular, it is appropriate for studies of cell surfaces, plasma membranes or, more general, thin layers. For this reason it has been used for about 20 years for imaging of single molecules [36, 37], e.g. in super-resolution microscopy [38–40].

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