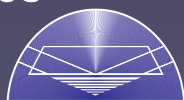
*i*TIRF Molecular Diagnostics
TIRF Spectroscopy
TIRF Microscopy

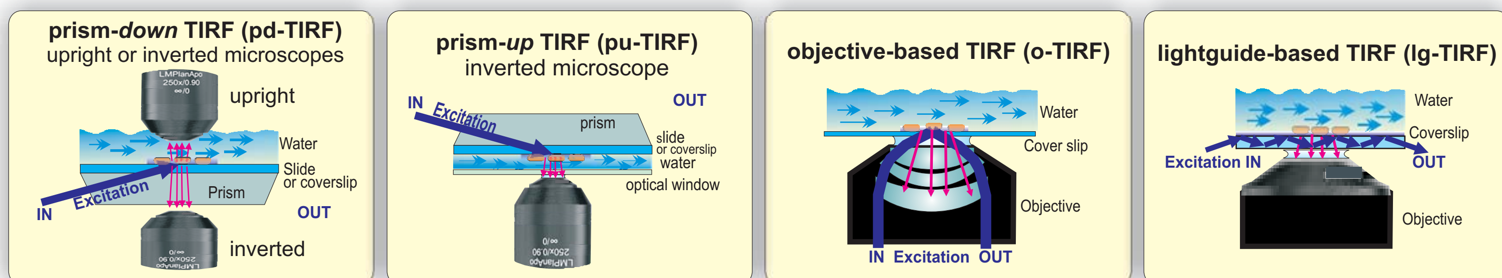


TIRF Labs

Total Internal Reflection Fluorescence

Compare TIRF Geometries

Total Internal Reflection Fluorescence Microscopy (TIRFM)

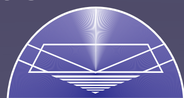


TIRF is a powerful analytical technique with applications in numerous areas of life sciences [1-3]. In particular, TIRF is "*...a method uniquely suited to image the plasma membrane with its associated organelles and macromolecules in living cells. The method shows even the smallest vesicles made by cells, and can image the dynamics of single protein molecules.*" [Steyer JA, Almers W. ***A real-time view of life within 100 nm of the plasma membrane***. Nat Rev Mol Cell Biol. 2001, 2(4), 268]. TIRF systems can be implemented in different ways. Prism-, objective-, and lightguide-based geometries produce the evanescent wave, which is well suited for analysis of biomolecular interactions, cell biology, and real-time microarray studies. Each geometry has its own set of advantages and limitations. Prism-based scheme provides the best signal-to-background ratio, but is difficult to implement with open perfusion chamber on an inverted microscope. Objective-based scheme is compatible with open perfusion chamber, but the background is larger, the intensity of evanescent wave is irreproducible, and one must use only specialized objectives with NA >1.43. Lightguide-based geometry yields exceptional flexibility - can be used with dry, water-, and oil-immersion objectives, is well-suited for multicolor TIRF, but requires larger optical power to obtain equal intensity of the evanescent wave. Table below compares three most popular TIRF geometries. Contact TIRF Labs for details to better determine which geometry is best suited for your applications.

Property \ Geometry	p-TIRFM*	lg-TIRFM	o-TIRFM
Depth of penetration of the evanescent wave	~100 nm	~100 nm	~100 nm
Signal-to-background ratio	best	good	fair
Excitation wavelengths	200-900 nm	200-900 nm	380-800 nm
Well-suited for multicolor TIRF studies	✔	✔	–
Reproducibility of the evanescent wave intensity	good	excellent	poor
Can be used with **dry objectives**	✔	✔	–
Can be used with **water-immersion objectives**	✔	✔	–
Can be used with **oil-immersion objectives NA<1.4**	✔	✔	–
Can be used with **oil-immersion objectives NA>1.4**	✔	✔	✔
Compatible with laser illuminators	✔	✔	✔
Compatible with LED, Hg- and Xe-arc lamp illuminators	✔	✔	–
Can be used for live cell studies with open perfusion chamber	** –	✔	✔
Can be used for single molecule detection studies	✔	✔	✔
Can be used for microarray studies (large area imaging)	✔	✔	–
Area of the evanescent wave	~5x5 mm	~10x20 mm	D ~0.1-0.3 mm
Volume of closed flow chamber	1-100 uL	1-100 uL	1-100 uL

[1.] Ambrose WP, Goodwin PM, Nolan JP. Single-molecule detection with TIRF: comparing signal-to-background and total signals in different geometries. Cytometry 1999, 36(3), 224.
[2.] Asanov A, Zepeda A, and Vaca L. A Platform for Combined DNA and Protein Microarrays Based on Total Internal Reflection Fluorescence. Sensors, 2012, 12, 1800.
[3.] See TIRF Labs' Application Notes for more details and additional literature.

Compare TIRF Geometries



Stray Light Causes Errors

of TIRF Penetration Depth and Variable Angle Measurements

Total Internal Reflection Fluorescence (TIRF) has established itself as a powerful tool for several areas of life sciences, including analysis of biomolecular interactions, single molecule detection, imaging of the dynamics and organization of cell membranes. TIRF efficiently rejects the background and achieves sub-diffraction axial sectioning by confining fluorescence excitation to a thin submicron layer close to TIRF substrate. Typically, it is stated that the depth of penetration of the Evanescent Wave (EW), or optical sectioning in TIRF is from 100 to 200 nm. The theory predicts exponential decay of EW intensity $I(z) = I(o)e^{-z/d}$ as shown in Fig 1. Depth of penetration, d_p, depends on the wavelength of the excitation light λ, refractive indices of the substrate n_1 and aqueous solution n_2, and the angle of incidence Θ: $d_p = \lambda_0 / \left(2\pi \cdot n_1 \sqrt{\sin^2\theta_1 - (n_2/n_1)^2}\right)$

In theory, there is no stray light as it is shown in Fig. 1. In practical TIRF systems, however, EW is contaminated with stray light as shown in Figure 2. Stray light excites the bulk of specimen, which diminishes surface selectivity of TIRF and deteriorates its effect of optical sectioning. The effect of stray light is minimal in the case of prism-based scheme (pTIRF), and maximal in the case of objective-based geometry (oTIRF). See for details Technical Note ***Compare TIRF Geometries***. In oTIRF geometry, where the excitation light and emission channel share numerous optical elements of TIRF objective and other optical parts inside the microscope, the intensity of stray light is not negligibly small; it can be comparable with that of EW. Large deviations from the expected exponential decay have been reported for oTIRF geometry [1-4].

The intensity of stray light increases with the amount of imperfections located on the path of the excitation light. Certain types of imperfections are distributed randomly, other types exhibit more systematic pattern of their occurrence. If the angle of incidence increases, the depth of penetration and the intensity of EW excitation decreases, while the intensity of stray light typically remains the same, or increases with the angle of incidence. The decreased ratio of intensities EW/(stray light) results in large errors in determination of the penetration depth. If you are performing variable angle TIRF experiments using oTIRF geometry, the effect of stray light should be carefully taken into account. The intensity of stray light should be measured and compared with that of the evanescence wave.

Recent study [2] performed with oTIRF geometry identified two major sources of stray light: (i) originating from TIRF objective itself, and (ii) from the rest of the microscope optics. The authors detected only minor contributions due to evanescent-wave scattering at the TIRF coverslip/water interface and at refractive-index boundaries within the live cells. Local variations of cell adhesion and refractive index within live cells also complicate the determination of the exact penetration depth. Depth of penetration is often unknown and the interpretation of biological TIRF images remains difficult [3-5]. It is good idea to measure the intensity of stray light in independent experiments, determine and report the experimental error. Reporting only calculated values of the penetration depth without reporting the experimental error may lead to misconceptions. Additional problem of oTIRF geometry are the interference fringes caused by coherent laser illumination [6,7].

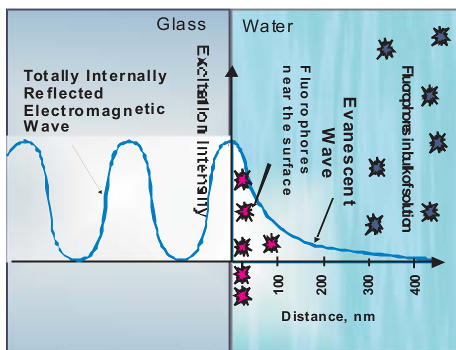


Figure 1. Theory predicts exponential decay of the intensity of Evanescent Wave (EW) with distance from the interface. EW excites fluorophores located at the surface and in close proximity to the surface. Fluorophores in the bulk of solution are not excited.

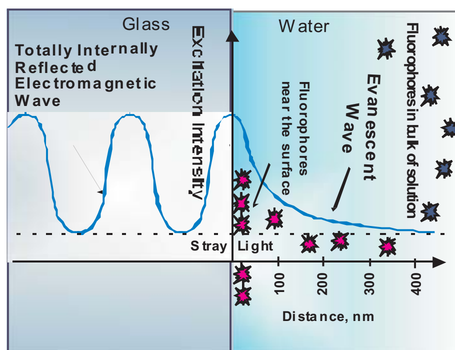


Figure 2. Stray light, which intensity is comparable with that of the EW in objective-based TIRF geometry, contaminates the evanescent wave and excites fluorophores located in the bulk of solution.

Literature:

1. Ambrose W, Goodwin P, Nolan J. *Single-molecule detection with TIRF: comparing signal-to-background and total signals in different geometries.* Cytometry 1999, 36(3), 224.
2. Brunstein M, Teremetz M, Tourain C, and Oheim M. *Combined evanescent-wave excitation and supercritical-angle fluorescence detection improves optical sectioning.* http://arxiv.org/ftp/arxiv/papers/1302/1302.1615.pdf; Simon S. *Partial internal reflections on total internal reflection fluorescent microscopy.* Trends Cell Biol, 2009, 19: 661; Brunstein M, Tourain C, Oheim M. *Glare and other sources of non-evanescent excitation light in objective-type TIRF microscopy.* In: 12th European Light Microscopy Initiative Meeting, 2012, RMS, Leuven.
3. Teremetz M. *Advantages of spinning TIRF illumination for subcellular fluorescence imaging.* In: Master in Cell Biology and Pathology 2011, Université Paris Diderot.
4. Toomre D, Bewersdorf J. *A new wave of cellular imaging.* Annual Rev Cell Dev Biol, 2010, 26: 285-314.
5. Schwarz J, König I, Anderson K. *Characterizing system performance in total internal reflection fluorescence microscopy*. Meth Mol Biol, 2011, 769: 373-86.
6. Conibear P, Bagshaw C. *A comparison of optical geometries for combined flash photolysis and TIRF microscopy*. J Microsc, 2000, 200(3): 218-29.
7. Mattheyses A, Axelrod D. *Direct measurement of the evanescent field profile produced by objective-based TIRF*. J Biomed Opt, 2006, 11: 014006A.