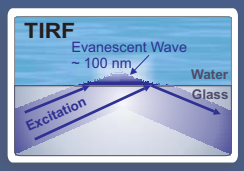


Surface selectivity ~0.1 um

TIRF Labs Technical Note 3

Compare TIRF Microscopy Geometries

Stray Light Errors in objective-, prism-, and lightguide-based TIRF

This Technical Note analyzes errors caused by the stray light in TIRF microscopy. It compares the advantages and limitations of three popular TIRF microscopy geometries: prism-lightguide, and objective-based TIRF, discusses the sources of stray light, and suggests ways for stray light mitigation. This Note will help you in choosing a TIRF geometry optimally suited for your studies.

Total Internal Reflection Fluorescence (TIRF) has established itself as an indispensable tool for single molecule detection, super resolution methods, cell membrane studies, and other areas. TIRF selectively excites only a ~100-nm layer of the specimen next to the surface, while the bulk of specimen is not illuminated and does not fluoresce. This superior spatial selectivity (in comparison, the confocal scheme excites ~1,000 nm) allows for the minimization of the background signal and providing the contrast sufficient for the detection of single molecules.

TIRF in theory:

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

$$d_p = \frac{\lambda_i}{2\pi n_1\sqrt{\sin^2\Theta_i-(n_2/n_1)^2}} \quad (2)$$

$$E_z = E_o\exp(-z/d_p) \quad (3)$$

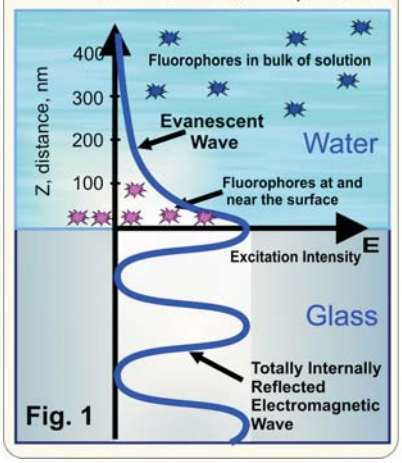


Fig. 1

objective-type TIRF in practice:

$$E_z = E_o\exp(-z/d_p) +$$

$$+ \text{Stray Light}(\Theta_i, x, y, z, \lambda)$$

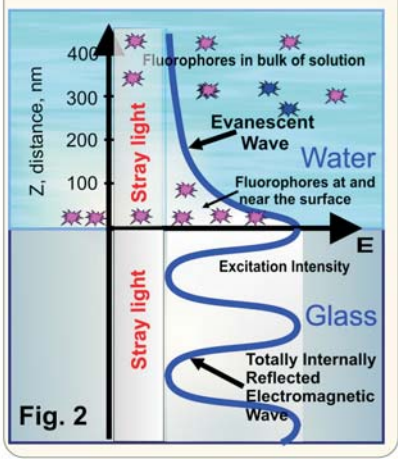


Fig. 2

The phenomenon of total internal reflection occurs at the interface between two optical media with different refractive indices, e.g. glass/water. If the angle of incidence is larger than critical, the incident light reflects back into the glass and generates a profile of exponentially decaying intensity at the interface, termed the Evanescent Wave (EW) (Fig. 1). The critical angle is given by eq. (1) (Fig.1). For the glass/water interface the angle is 62 degrees. EW intensity is maximal at the surface and decays exponentially with distance - eq. (2). The depth of penetration depends on the wavelength, the angle of incidence, and the ratio of refractive indices eq. (3). For a typical TIRF experiment with a glass/water interface, the depth of penetration is ~100 nm or less.

TIRF Theory and Practice. In theory, the intensity of EW exponentially decays with distance, as shown in Fig. 1. In practice, however, auto-fluorescnce of optical materials, scatter, reflections, and refractions produce undesirable rays of light, collectively termed “stray light.” Stray light contaminates the exponential decay of EW, excites the bulk of specimen, and deteriorates the TIRF effect, as shown in Fig. 2.

Sources of Stray Light. All optical materials, to a certain extent, autofluorese and scatter light. All surfaces and interfaces between optical parts, to a certain extent, reflect, refract, and scatter light. Due to the combination of these factors, the stray light is present in all practical systems. In certain cases, its intensity is too large to be neglected. With equal quality of the optical elements, the errors caused by the stray light critically depend on the optical scheme, the geometry.

TIRF Geometries. There are three popular geometries suited for TIRF microscopy: through-objective, prism-, and lightguide-based optical schemes (o-TIRF, p-TIRF, and lg-TIRF, respectively). For a number of reasons Nikon, Olympus, Zeiss, and Leica have aggressively marketed only o-TIRF geometry. Objective-TIRF employs expensive specialized high NA objectives and other sophisticated optics. A typical o-TIRF microscopy system costs ~$80,000 or more. Paradoxically, it has been reported in the literature that o-TIRF is the worst from the standpoint signal-to-background ratio. Additionally, it is too rigid - can be used only with specialized TIRF objectives. On the other hand, TIRF geometries that demonstrate superior signal-to-background ratios, namely p-TIRF and lg-TIRF, are cost-efficient (~$10k). However, they were not offered commercially, until recently - 2010, when TIRF Labs started to market them. In the past, each research group built p-TIRF and lg-TIRF systems on their own. Since 2010, our customers generated unique TIRF data and demonstrated superior advantages of p- and lg-TIRF geometries on a number of applications, including single molecule detection [1, 2]. The sections below compare the geometries, analyze sources of stray light, and outline ways to mitigate the stray light interferences.

Stray Light Causes Large Errors in Objective-type TIRF Microscopy

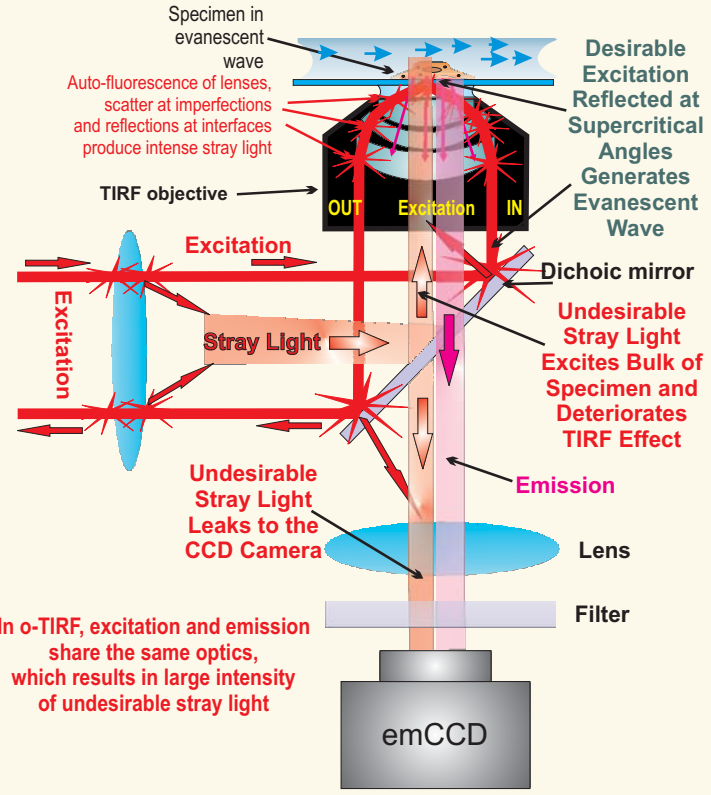
Compare TIRF Microscopy Geometries

Stray light errors in objective-, prism-, and lightguide-based TIRF

Objective-type TIRF Geometry. Fig. 3 shows the schematics of o-TIRF system. The main feature, which might appear elegant at first glance, is the use of the emission path for delivering the excitation light to the glass/water interface. This feature has been acquired from epi-fluorescence. The o-TIRF scheme uses large angles of incidence, greater than the critical angle for glass/water interface, > 63 degrees. Microscope objectives with a Numerical Aperture (NA) smaller than 1.38 do not support such angles. Therefore, the o-TIRF scheme depends on specialized high-NA objectives.

Sources of stray light in o-TIRF. Fig. 3 illustrates numerous potential sources of the stray light in the o-TIRF. Not surprisingly, significant interferences of stray light have been reported for o-TIRF geometry [3-8].The intensity of stray light at the surface amounts 10-15% of that of the evanescent wave; the ratio increase exponentially with the distance. Fluorophores in the bulk of the specimen are excited and the TIRF effect is compromised. In many instances, the intensity of stray light changes unpredictably. Two major sources of stray light were identified as (i) originating from the TIRF objective, and (ii) originating from the rest of the microscope optics [4, 5].Only minor contributions were detected due to the scatter at the TIRF glass/water interface and at refractive-index boundaries within the specimen, including live cells [4, 5].

Fig. 3 **Objective-based TIRF (o-TIRF)**
Stray Light Deteriorates TIRF Effect



The first group of sources (i) is related to undesirable autofluorescence, scatter and reflections inside the objective. The intense excitation light travels through multiple lenses and interfaces on its way to and from the TIRF surface (Fig. 3). The quality of the optical glass, which should minimally fluorescent and scattering, and the surface quality are critically important for minimizing the intensity of stray light. To our knowledge, the systematic comparison of TIRF objectives from the standpoint of the intensity of stray light has not been performed yet. Our own tests on small number of TIRF objectives, the analysis of the literature, and reports of our customers and colleagues indicate that all TIRF objectives demonstrate significant intensity of stray light due to autofluorescence and scatter. Autofluorescence and scatter of the front lens, which is in direct contact with the specimen, appears to be the most contributing factor.

The second group of stray light (ii) originates from the optics inside the microscope. A significant amount of stray light is generated at the dichroic mirror. Even a high-quality dichroic mirror scatters and transmits certain portion of light, which, in a perfect world, would be ideally reflected and blocked. The leaking of the excitation light through the dichroic mirror, as well as through the emission filter, results in increased background at the photodetector.

In o-TIRF, the intensity of stray light changes unpredictably with the angle of incidence and XYZ coordinates. It increases with the amount of imperfections located on the path of the excitation light. Certain types of imperfections are distributed randomly, while other types exhibit more systematic patterns 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 remains the same or increases. If you are performing variable angle TIRF experiments using o-TIRF geometry, the effect of stray light should be carefully taken into account. In certain cases, the intensity of stray light is comparable with that of EW. In such cases, the depth of penetration calculated using eq. (3) (Fig.1) does not describe the intensity profile anymore and can mislead the interpretation of biological TIRF images [3-8].

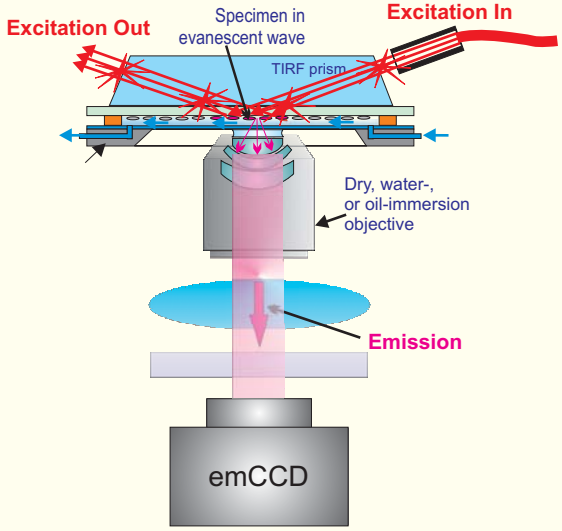
Dichroic mirror and emission filter. The dichroic mirror is the central element of o-TIRF geometry; its quality is critically important for the objective-type TIRF. The beam of excitation light reflected from the dichroic beamsplitter must be focused at the back focal plane of the objective. A significant focal shift or a change in the focal spot size caused by a bend of the dichroic mirror can make it difficult to achieve TIRF, especially if the microscope has a limited ability to adjust the collimation of the excitation beam. Chroma and Semrock recently made progress in improving technical performance of dichroic and bandpass filters. The companies increased the thickness of dichroic mirrors to keep their flatness, which is necessary for precision focusing in o-TIRF, minimized surface roughness, as well as the density of pinholes [9, 10].

o-TIRF stray light mitigation. First, it appears to be rational to explore the opportunity of using alternative TIRF geometries for your study. If your study dictates the use of o-TIRF scheme, select a TIRF objective with minimal amount of stray light. 405 nm laser pointer allows for rapid visualization of autofliuorescece and scatter in TIRF objectives and coverslips. Select the best quality objectives, coverslips, dichroic mirror and the emission filter. Use an additional excitation filter to block undesirable lines in the excitation light. Make sure that the dichroic mirror, emission filter and other accessible optical parts are free from dust particles and other contaminations.

Prism-based TIRF. p-TIRF geometry has been shown to provide the best signal-to-background ratio [1-3]. Indeed, p-TIRF is a scheme, where excitation and emission channels are naturally independent, as is illustrated in Fig. 4. The excitation light travels through the prism, reflects at the glass/water interface, and escapes through the opposite facet of the prism. Potential sources of the stray light are a few reflections at the prism facets, prism/slide interface, autofluorescence and scatter in the prism and slide/coverslip. These few sources of stray light are located away from the EW region and the detection channel. In p-TIRF, stray light minimally interferes with the EW region. Only a small portion of stray light enters the emission channel. Crisp, high-contrast TIRF images have been reported for p-TIRF [1-3, 8].

p-TIRF stray light mitigation. If your application permits to enclose your specimen into a closed flow cell, p-TIRF geometry is the best choice for your studies. In p-TIRF scheme, the intensity of stray light is negligibly small, provided that high-quality optical materials were selected to minimize the scatter and autofluorescence inside the prism and the TIRF slide. For demanding experiments, the surface quality of the TIRF prism and TIRF slide should be high to ensure minimal scatter. At the emission channel, the selection of an efficient emission filter, which blocks the stray light, will reduce the background.

Fig. 4. **Prism-based TIRF (pTIRF)**
excitation and emission channels are independent
stray light is minimal and does not enter emission channel



pTIRF - the best signal-to-background ratio
the cleanest TIRF effect, well-suited for single molecule detection
UV-visible excitation. Use with dry, water-, or oil-immersion lenses

A limitation of p-TIRF is related to the fact that experiments with live cells in open dishes is difficult to perform with p-TIRF on an inverted microscope. An upright microscope with a prism-down geometry and water-immersion objective is a geometry well-suited for live cell studies. Visit www.tirf-labs.com for additional information.

Lightguide-based TIRF geometry. Lg-TIRF is a sensible alternative to o-TIRF. It is a flexible geometry available with open perfusion chambers on inverted microscopes, and closed flow cells [11]. Lg-TIRF can be used with dry, water- and oil-immersion objectives, with UV and visible excitation. Novel method of Shallow Angle Fluorescence Microscopy, in which objects a few microns away from the surface are illuminated, can be implemented at the same platform [12]. Lg-TIRF principles are close to that of p-TIRF: the excitation light is naturally independent from the emission channel. In lg-TIRF, the excitation light undergoes multiple reflections from the top and the bottom surface of glass or silica coverslip, which serves as the TIRF lightguide. The excitation light escapes from the opposite end of the coverslip. Unlike in o-TIRF, it does not enter the emission channel. The optical scheme of lg-TIRF allows for using Petri dish chambers for experiments with live cells. Potential source of scatter and autofluorescence is the coverslip material. Additionally, the top, bottom, and end surfaces of the coverslip play role in lg-TIRF. For demanding TIRF experiments these surfaces should be of the higest quality.

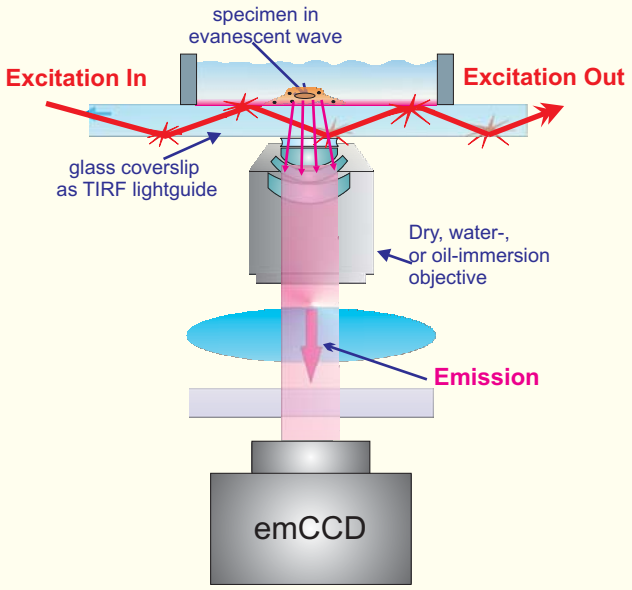
Compare TIRF Microscopy Geometries

Stray light errors in objective-, prism-, and lightguide-based TIRF

lg-TIRF stray light mitigation. To minimize the intensity of stray light and to maximize the signal-to-background ratio in lg-TIRF, it is recommended to use coverslips made from high-quality synthetic silica, autofluorescence of which is low. The use of coverslips polished to the highest surface quality reduces scatter from the surfaces. However, regular flame-polished coverslips work well for many lg-TIRF applications. Higher quality coverslips as well as superior emission filters are necessary only for demanding TIRF applications.

In summary, it appears that using of the emission channel for delivering TIRF excitation results in too large intensity of stray light in the case of o-TIRF. Indeed, p-TIRF and lg-TIRF geometries provide superior signal-to-background ratios, because the excitation light is naturally separated from the emission channel. lg-TIRF performs all the functions that o-TIRF does. Additionally, it can be used with UV excitation. If your applications involve TIRFing of living cells in open chambers, lg-TIRF is the optimal system for this purpose. If you are TIRFing single molecules with bright fluorophores, an inexpensive objective 1.20 NA will be sufficient. Use of expensive >1.4 NA lenses will increase the amount of light collected by the objective, and the lg-TIRF geometry give you the freedom to select an objective, which is better fitted for your specific task.

Fig. 5 **Lightguide-based TIRF (lgTIRF)**
excitation and emission channels are independent
stray light is minimal and does not enter emission channel



lgTIRF - superior signal-to-background ratio
well-suited TIRF geometry for cell biology and SMD studies
UV-vis excitation. Use with dry, water-, or oil-immersion lenses

CCD camera. The TIRF signal is inherently low. Therefore, a low light camera is a necessary module for TIRFing. An electron-multiplying CCD camera is a rational choice. Scientific sCMOS cameras, and low light CMOS cameras are sufficiently sensitive for many TIRF applications, including single molecule detection [13].

UV-absorbing-blue-light-emitting fluorophores. p-TIRF and lg-TIRF systems can be used with UV excitation, a feature not available in o-TIRF. There is a new class of fluorescent probes that absorb UV light and emit in the range 360-450 nm. These fluorophores, including modified nucleic and amino acids, are useful for many lifescience applications. p-TIRF and lg-TIRF systems are well suited for studies with UV fluorophores.

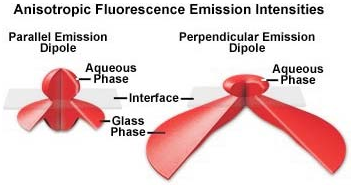
Selecting microscope objective. Unlike o-TIRF, which can be used only with specialized TIRF objectives, p-TIRF and lg-TIRF systems can be used with high and low NA, dry, water- and oil-immersion lenses. Many TIRF applications, e.g. real-time microarrays, deal with relatively large amount of fluorescence emitted from relatively large area; dry objectives with low magnification are suitable for such studies. For low light applications, such as single molecule detection and super resolution methods, objectives with high NA are necessary; they collect more light (equation (4)). When you are choosing the objective, take into account that the efficiency of collecting light, G, is proportional to the square of numerical aperture of the objective, NA, and reverse proportional to the square of magnification, X:

$$G = k\ (NA)^2\ (X)^{-2} \quad (4)$$

where k- is a coefficient, constant for the given microscope. The table below enumerates the relative efficiency of objectives with different NA and X values. For example, a 60X/1.40 objective, which is available as a cost-efficient oil-immersion lens, provides 87% efficiency, relatively to the costly 60X/1.49 lens. The light-collection efficiency of the 60X/1.40 lens 2.4 times exceeds that of 100X/1.49 objective.

The proximity to the surface results in anisotropy of the fluorescence emitted by a fluorophore [14, 15]. The emission patterns of fluorophores located at ~50 nm are shifted toward the coverslip (Fig.6), which is an additional rational for using high NA objectives.

Fig. 6



Stray Light Causes Large Errors in Objective-type TIRF Microscopy

Table. Relative light-collecting efficiency of microscope objectives with different values of numerical aperture, NA, and magnification, X.

Numerical Aperture, NA / Magnification, X	1.49 X60	1.40 60X	1.20 X63	1.00 X60	1.49 X100	1.20 X100	0.80 X40
Relative efficiency of collecting light	100%	87%	58%	45%	36%	23%	65%

In summary, the problems of stay light, poor signal-to-background ratio, and compromised quality of the evanescent wave are inherent only to o-TIRF geometry. Significant interferences of stray light with TIRF effect - undesirable excitation of the bulk of the specimen have been reported only for the case of o-TIRF geometry [1-6]. These interferences are caused by the fact that the excitation and emission channels share the same optics in o-TIRF microscopy. On the other hand, p-TIRF and lg-TIRF geometries demonstrate excellent quality of TIRF effect, minimum amount of stray light, crisp and high-contrast TIRF images. Both p-TIRF and lg-TIRF geometries employ optical schemes in that the excitation light is independent from the emission channel. Published articles show that p-TIRF and lg-TIRF geometries are well-siuted for single molecule detection, living cell membrane studies, and other areas of lifesciences. For supersensitive TIRF experiments it is rational to use p-TIRF and lg-TIRF with microscope objectives of high or moderate NA numbers (>1.0 and more) to ensure that the objective collects sufficient portion of the emitted fluorescence. For additional information please visit www.tirf-labs.com or contact TIRF Labs: *info@tirf-labs.com.*

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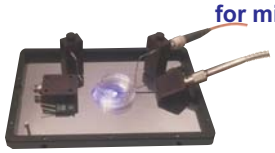
TIRF Labs

Total Internal Reflection Fluorescence

Prism- and Lightguide-based TIRF Microscopy Accessories

- Single molecule detection
- Super-resolution microscopy: STED, PALM, STORM, ...
- Minimal stray light, crisp, high-contrast TIRF images
- Work with dry, water-, and oil-immersion objectives
- Use UV or visible excitation light 190-900 nm
- Use Petri-dish, open perfusion, or closed flow chamber
- Nested design - fits inside 96-well plate, K-frame, 4-inch round, or manual XY stages
- Optional temperature, dielectrophoresis, electric field control

p-TIRF and lg-TIRF accessories for microscopy



Turnkey Single Molecule Detection TIRF Microscopy Station

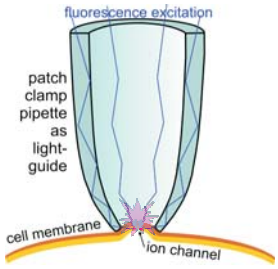
Modular TIRFM stations include:

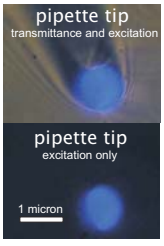
- Fluorescence microscope
- lg-, p-, or/and o-TIRF microscopy flow systems
- Low light EM CCD or sCMOS camera
- Multi-color computer-controlled illuminator
- Digital fluidics SmartFlow
- Optional temperature and electric field control
- Software for instrument control and data analysis

TIRF Accessories for Fluorometers

- **TIRF Accessory** TA-1004 transforms a spectrofluorometer into a super-sensitive TIRF biosensor instrument
- Optional electrochemical, DEP and temperature control
- **SmartFlow** Fluidic System allows to run unattended TIRF experiments, measure sensograms to derive k_{on} and k_{off}
- Microfluidic system allows for handling nanoliter volumes

Single ion Channel Single Molecule Detection





Patch clamp technique combined with fluorescence single molecule detection

*i*Diagnostics

cellphone based molecular diagnostics

Real-time TIRF microarrays: Parallel supersensitive detection of protein, nucleic acid, and metabolite biomarkers

We extended TIRF into 3rd dimension and invented iDiagnostics
Now you can hold a hospital laboratory in the palm of your hand