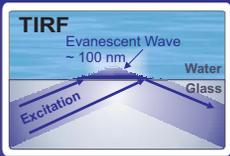


Selecting the Optimum TIRF Microscopy Geometry for Your Application



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To ensure that the TIRF technique serves efficiently to your specific application, it is important that the correct geometry of TIRF microscopy is chosen. This white paper will help you to select the optimum TIRF geometry. It compares the advantages and limitations of prism-(pTIRF), lightguide- (lgTIRF), and objective-based (oTIRF) geometries, analyzes potential sources that cause deviations from theoretically predicted behavior, discusses trade-offs between performance, flexibility, and cost. The table at the end summarizes the comparison.

In theory, Total Internal Reflection Fluorescence (TIRF) produces an evanescent wave, which exponentially decays with the distance, as shown in Fig. 1. Typical depth of penetration is ~100 nm. In practice, however, there are scatter, reflections, refractions, and auto-fluorescence that produce undesirable rays of light collectively termed as “stray light”. The stray light penetrates far beyond ~100 nm, excites the bulk of the specimen, as shown in Fig. 2, and deteriorates the TIRF effect, in certain cases enormously.

Different TIRF microscopy geometries treat the problem of stray light differently. pTIRF and lgTIRF geometries allow for minimizing the interference of stray light, by separating the excitation lightpath and the emission channel. In contrast, in oTIRF geometry, the excitation light is delivered via the emission channel. The latter scheme “maximizes” the problem: large intensity of stray light is generated at multiple optical parts of the common optical path, and the emission channel efficiently transmits this contaminating light to the TIRF surface. Not surprisingly, significant deviations from anticipated exponential decay have been reported in the literature for the case of oTIRF geometry [1-5]. These deviations deteriorate signal-to-background ratio and make the interpretation of experimental data difficult. Therefore, selecting the optimum TIRF microscopy geometry is critical and will permit you to achieve the desirable signal-to-background ratio, facilitate interpretation of experimental data, and reach the flexibility suitable for your specific application.

Fig. 3 illustrates how stray light is generated in an optical lens. In theory, there is no scatter and no auto-fluorescence. Light propagating in the lens should not be visible, as it is not visible in vacuum or clean air. However, in practice, the zigzag path of a 405 nm laser pointer beam is easily visualized due to the scatter at surfaces and in the bulk of lens, reflections at surfaces, and auto-fluorescence of the lens material.

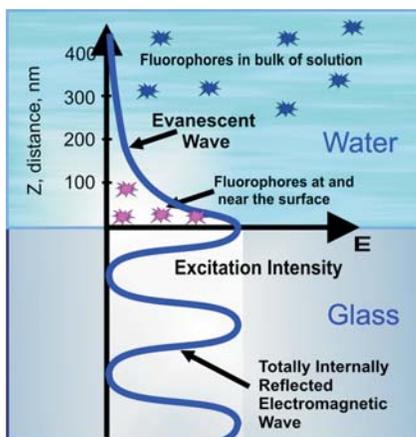


Fig.1. Theory predicts exponential decay of the evanescent wave with the depth of penetration ~100 nm.

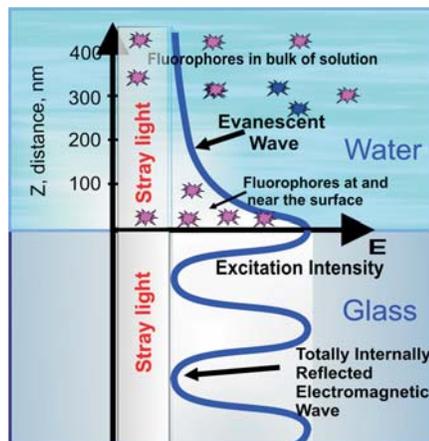
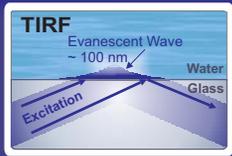


Fig.2. In practice, stray light contaminates the evanescent wave and excites fluorophores that are located far beyond the penetration depth.



Fig.3. Origination of stray light in optical lens. Light of 405 nm laser pointer is invisible in clean air, but visualized at the surface and in the bulk of the lens due to scatter, reflections, and auto-fluorescence.



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As mentioned above, pTIRF and lgTIRF geometries separate the excitation lightpath from the emission channel (Figs. 4, 5). Unlike, in the case of oTIRF geometry, the delivery of the excitation light to the TIRF interface employs the emission channel (Fig. 6). This difference results in clean TIRF effect in the case of p- and lg-TIRF geometries, and large deviations from theoretical predictions in the case of oTIRF geometry. The difference has been documented in the literature [see, for example, Refs.1-5]. Typical TIRF images taken with p-, lg-, and o-TIRF geometries are shown in Figs. 7-9.

Prism-based TIRF

If your application permits to enclose the sample, e.g. immobilized molecules, into a closed flow cell, as shown in Fig. 4, the best choice for your studies will be the prism-based TIRF geometry. The cleanest TIRF effect and the best signal-to-background ratio are the main advantages of pTIRF [1]. pTIRF provides the intensity of the evanescent wave, which ~10,000-fold or more exceeds the intensity of stray light, provided that the optical materials are selected correctly and the elements manufactured accurately. pTIRF geometry yields the highest contrast of imaging in the evanescent wave. Since the TIRF effect is clean, the interpretation of experimental data is a straightforward task. *In vitro* single molecule FRET for protein-protein and protein-DNA interactions represents an application, for which prism-based geometry is the best choice. See the webpage <http://tirf-labs.com/applications.html> for more examples of pTIRF applications.

Stray light and its mitigation. The scatter and reflections at the interfaces, including that at the glass/water interface, scatter and autofluorescence in the bulk of TIRF prism and TIRF slide are the main sources of stray light in pTIRF. Fortunately, most of these sources are located away from the TIRF interface and the emission channel. Only small portion of the stray light reaches the bulk of the specimen or enters the emission channel. Mitigation for the minimal stray light is seldom necessary for pTIRF and involves use of autofluorescence-free and scatter-free materials, such as fused silica, and manufacturing of optical parts with the highest quality of surfaces to ensure minimal scatter.

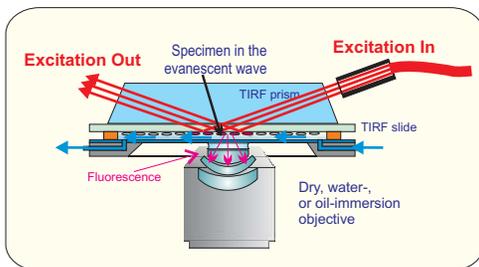


Fig.4. Prism-based TIRF (pTIRF). Excitation lightpath and emission channel are naturally independent. Interference of stray light is minimal.

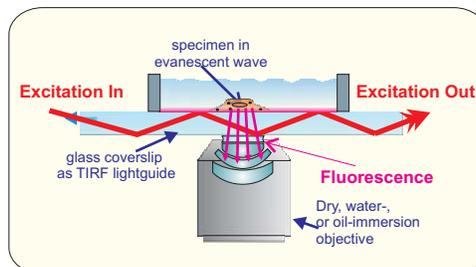


Fig.5. Lightguide-based TIRF (lgTIRF). Excitation lightpath and emission channel are independent. Interference of stray light is minimal.

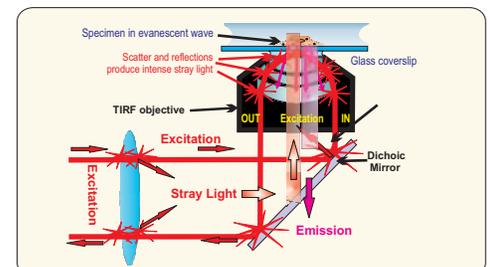


Fig. 6. Objective-based TIRF (oTIRF). Excitation light is delivered via the emission channel. Large intensity of stray light is generated, which deteriorates TIRF effect.

Comparison of TIRF images obtained with prism-, lightguide-, and objective-based TIRF microscopy geometries



Fig.7. Prism-based TIRF,100X, NA 1.25 oil-imm. objective. 1-micron fluorescent beads.

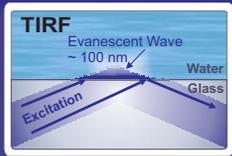


Fig.8. Lightguide-based TIRF,100X, NA 1.0 water-imm.objective. 1-um fluorescent beads.



Fig. 9. Objective-based TIRF,100X, NA 1.46 oil-imm. objective. 1-um fluorescent beads.





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Flexibility. pTIRF can be used with dry, water-, and oil-immersion objectives. For the latter, however, additional aberrations may be anticipated, because there is a layer of aqueous solution, which is not assumed to be in the oil-immersion scheme. pTIRF geometry is well-suited for multicolor TIRF, if used in conjunction with a multicolor illuminator. Typical Single Molecule Detection (SMD) experiment requires the intensity of excitation light $\sim 1 \text{ kWatt/cm}^2$. If used with illuminator coupled into a 100-micron fiber, an excitation spot ~ 100 micron wide with the SMD intensity $\sim 1 \text{ kW/cm}^2$ is obtained at the TIRF surface at the optical power of the illuminator $\sim 100 \text{ mW}$. pTIRF geometry is well-suited for producing uniform and reproducible intensity of the evanescent wave with larger or smaller TIRF areas in the flow cell. If optical parts of the system are made from silica, UV excitation can be used for TIRFing novel synthetic nucleic acid and amino acid fluorophores. These fluorophores absorb UV light, but due to large Stoke's shift emit fluorescence at wavelengths longer than 380 nm - within the transmittance range of fluorescence microscopes.

Trade-offs. Different configurations of pTIRF with fixed and variable angles of incidence, compatible with inverted or upright microscopes are available for different life science applications. The trade-offs between the best signal-to-background ratio and the flexibility are associated with the fact that it is difficult to use pTIRF geometry with an open perfusion chamber on an inverted microscope. There is an exception though: for water-immersion or dry objectives with working distance 2 mm or longer a pTIRF geometry system is available for TIRFing with Petri dishes on inverted microscopes. See for details the web page <http://tirf-labs.com/prismtirf.html>.

Lightguide-based TIRF

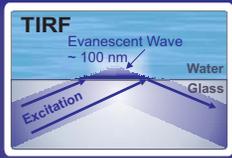
If your application involves live cells and requires an open perfusion chamber, IgTIRF geometry is a sensible alternative to pTIRF (Fig. 5). Similar to prism-, IgTIRF geometry uses the excitation lightpath independent from the emission channel. If optical elements of IgTIRF system are manufactured from low scatter, low autofluorescence materials, the intensity of the evanescent wave exceeds that of stray light $\sim 1,000$ -fold or more. IgTIRF system (as well as pTIRF) can be rapidly installed/uninstalled as add-on accessory on a motorized or manual XY translation stages of inverted or upright microscopes. In IgTIRF geometry the excitation light undergoes multiple reflections from the top and bottom surfaces of the lightguide, as schematically shown in Fig. 5. There are several methods to couple the excitation light into the lightguide: from the top, from the bottom, and through the side end of the lightguide (see for details webpage <http://tirf-labs.com/lightguidetirf.html>). Disposable and reusable open perfusion chambers installed on the surface of glass or silica coverslips, as well as closed flow cells surrounding the TIRF area are compatible with IgTIRF geometry.

Stray light and its mitigation. Similar to pTIRF, the main sources of stray light in IgTIRF geometry are: scattering at the interfaces, including the glass/water interface, scatter and autofluorescence in the bulk of the TIRF lightguide. Fortunately, significant portion of these sources are located away from the working TIRF surface and the emission channel. Only small fraction of the stray light reaches the bulk of the specimen and enters the emission channel. Mitigation for the stray light in IgTIRF involves the use of autofluorescence-free and scatter-free materials, such as fused silica, and using TIRF lightguides with high surfaces quality to ensure minimal scatter at the interfaces. There are specialized optical traps that allow for further decreasing the intensity of stray light in IgTIRF geometry. Additionally, these traps can be used for reducing the depth of penetration. See for details the webpage <http://tirf-labs.com/lightguidetirf.html>

Flexibility. IgTIRF is an exceptionally flexible geometry. It can be used with dry, water-, and oil-immersion objectives. There is plenty of room on the top of IgTIRF stage for combining TIRF with AFM, optogenetic probes, patch clamp electrophysiology, optical tweezers, electric field and dielectrophoresis control. The most popular version of IgTIRF microscopy system is mounted onto a 110 mm x 160 mm K-frame, common for all motorized stages. The excitation light is delivered to IgTIRF system via a 100-micron silica fiber, which being coupled to a multicolor illuminator, enables multicolor TIRF capabilities. The intensity of excitation light necessary for SMD studies ($\sim 1 \text{ kWatt/cm}^2$) is attained at optical power of the illuminator $\sim 500 \text{ mW}$. Multicolor and single color illuminators with such power are available from TIRF Labs. IgTIRF geometry is convenient for real-time microarray studies that require larger area of the evanescent field. If used with lightguides made from silica, UV excitation can be employed for TIRFing fluorophores that are excited by UV light, but emit fluorescence with wavelengths longer than 380 nm.

Trade-offs. The trade-off between superior performance of IgTIRF and its flexibility is associated with the necessity to move the excitation spot to follow the XY translations of the specimen. However, this disadvantage can be circumvented by using a holder, which fixes the position of the illuminator with respect to the objective.





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Additional trade-off involves the necessity of using higher optical power for producing equal intensity of the evanescent wave. In the case of IgTIRF, the optical power necessary for SMD experiments is greater (~500 mW) than that in the case of pTIRF (~100 mW).

Objective-based TIRF

Nikon, Olympus, Zeiss, and Leica are marketing only oTIRF geometry. One of the reasons for this choice is the expertise in making and selling objective lenses. The microscope companies comfort zone is in solving problems with specialty objectives that are sold in small quantities, mandating high price. A number of advanced high numerical aperture lenses have been recently developed. These lenses collect superior portion of emitted light and are well-suited for a number of applications. However, in the case of TIRF method these objectives, unfortunately, produce evanescent wave contaminated with large intensity of stray light. Compromised quality of TIRF effect in the case of oTIRF geometry has been documented in the literature [1-5]. Only in theory there is no stray light. In practice, all optical parts generate certain amount of stray light (refer to Fig. 3). oTIRF geometry maximizes the problem, because (i) there are numerous sources for stray light, including that inside the microscope (Fig. 6); (ii) unlike in p- and Ig-TIRF, these sources are in the emission channel, which efficiently transmits stray light to the TIRF surface. Mattheyses and Axelrod estimated that the intensity of stray light in oTIRF geometry is 10-15% relative to the intensity of the evanescent wave (EW) at the surface [4]. Since EW exponentially decays with the distance, the relative intensity of stray light exponentially increases. It is too difficult to interpret the data, taking into account that stray light is a complex function of X, Y, Z coordinates and the irradiance parameters of the excitation light that are typically unknown. In 2014, Oheim et al. partially reviewed and summarized publications on the interferences of stray light with TIRF effect [2, 3]. The literature also documented irreproducible and inhomogeneous intensity of the evanescent wave in oTIRF.

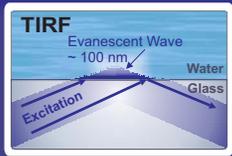
Stray light and its mitigation. Fig.6 illustrates several sources of stray light in TIRF objective itself and inside the microscope, including the dichroic mirror. See for details the webpage <http://tirf-labs.com/objectivetirf.html>. Unlike p- and Ig-TIRF, these sources are located inside the emission channel, which efficiently transmits the stray light to the TIRF interface. The amount of stray light in oTIRF critically depends on the design, the quality of materials and manufacturing of the TIRF objective and other elements inside the microscope. Mitigation for the stray light in oTIRF involves careful selecting of the objective with minimum level of stray light, as well as optimizing the other optics, including the dichroic mirror. See for details the webpage <http://tirf-labs.com/objectivetirf.html>.

Flexibility. oTIRF is a rigid geometry, which can be used only with specialized TIRF objectives. It is not compatible with dry or water-immersion objectives. On the other hand, advantageous features include spacious room on the XY translation stage well-suited for combining with other methods. Additionally, the intensity of excitation light necessary for SMD ~ 1 kWatt/cm² can be attained in oTIRF at optical power of the illuminator ~10-100 mW, which provides more flexibility at the illuminator end. However, the necessity of using single mode optical fiber and respective lasers diminishes the value of this advantage. Transmittance of the microscope optics limits the range of excitation wavelengths to >380 nm; UV excitation is not available.

Trade-offs. In the case of oTIRF geometry, there is a paradox of trade-offs between the compromised performance and the cost. oTIRF geometry belongs to infrequent exceptions, when the cost of a system with compromised performance (\$40k-\$80k) is significantly greater than that for superior quality TIRF systems, p- and Ig-TIRF (\$6-12k). In the past, each research group built pTIRF and IgTIRF systems on their own, until 2010, when TIRF Labs started to market pTIRF and IgTIRF microscopy products. As mentioned above, Nikon, Olympus, Zeiss, and Leica are marketing only oTIRF geometry.

Visit for more information the webpage: <http://tirf-labs.com/tirfmicroscopy.html>.





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Conclusions

If your application, for example *in vitro* single molecule FRET, permits to enclose your sample (e.g. immobilized protein or DNA molecules) into a closed flow cell surrounding the TIRF area, prism-based geometry (pTIRF), is the best option for your application. In the case of live cell studies and other applications that require open perfusion chambers and/or open access to TIRF area, the lightguide-based geometry (lgTIRF) is the option, which will provide excellent signal-to-background ratio and superior flexibility. Objective-based TIRF geometry (oTIRF) exhibits TIRF effect of compromised quality: the evanescent wave is contaminated with large intensity of stray light, minimum 10-15%. The stray light excites fluorophores in the bulk of specimen and deteriorates TIRF effect.

Table 1. Comparison of TIRF microscopy geometries.

Property \ Geometry	pTIRFM	lgTIRFM	oTIRFM
Depth of penetration of the evanescent wave	~100 nm	~100 nm	~100 nm
Evanescent wave/Stray light intensity ratio (at TIRF surface)	>10,000	>1,000	<10
Signal-to-background ratio	The Best	Excellent	Compromised
Excitation wavelengths	190-900 nm	190-900 nm	380-800 nm
Can be used for multicolor TIRF studies	yes	yes	yes
Reproducibility of the evanescent wave intensity	good	excellent	poor
Can be used with dry objectives	yes	yes	no
Can be used with water-immersion objectives	yes	yes	no
Can be used with oil-immersion objectives NA<1.4	yes	yes	no
Can be used with oil-immersion objectives NA>1.4	yes	yes	yes
Compatible with laser illuminators	yes	yes	yes
Compatible with LED, Hg- and Xe-arc lamp illuminators	yes	yes	no
Can be used for live cell studies with open perfusion chamber	no	yes	yes
Can be used for single molecule detection (EW>1kW/cm ²)	yes	yes	yes
Can be used for microarray studies (large area imaging)	yes	yes	no
Area of the evanescent wave	0.1-10 mm	0.1-20 mm	~0.1-0.3 mm
Volume of closed flow chamber	1-100 uL	1-100 uL	1-100 uL
Can be combined with AFM and other techniques	no*	yes	yes
Cost	\$6-8k	\$8-12k	\$40-80k

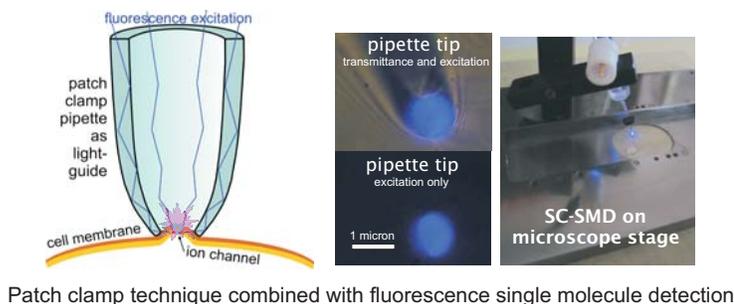
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Modular TIRFM systems include:

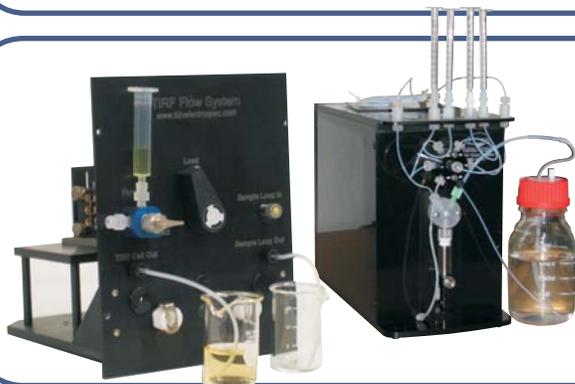
- Fluorescence microscope
- Ig-, p-, or/and o-TIRF microscopy flow systems
- Low light EM CCD camera
- Multi-color computer-controlled illuminator
- Digital fluidics SmartFlow
- Optional temperature and electric field control
- Software for instrument control and data analysis

Prism- and Lightguide-based TIRF Microscopy Accessories

- Single molecule detection, cell membrane studies
- Superior signal-to-background ratio
- 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 stage
- Optional temperature and electric field control



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