

Total Internal Reflection Fluorescence (TIRF) Microscopy Illuminator for Improved Imaging of Cell Surface Events

Daniel S. Johnson,¹ Jyoti K. Jaiswal,^{2,3} and Sanford Simon¹

¹The Rockefeller University, Laboratory of Cellular Biophysics, New York, New York

²Department of Integrative Systems Biology, The George Washington University Medical Center, Washington, D.C.

³Center for Genetic Medicine Research, Children's National Medical Center, Washington, D.C.

ABSTRACT

Total internal reflection fluorescence (TIRF) microscopy is a high-contrast imaging technique suitable for observing biological events that occur on or near the cell membrane. The improved contrast is accomplished by restricting the thickness of the excitation field to over an order of a magnitude narrower than the z -resolution of an epi-fluorescence microscope. This technique also increases signal-to-noise, making it a valuable tool for imaging cellular events such as vesicles undergoing exocytosis or endocytosis, viral particle formation, cell signaling, and dynamics of membrane proteins. This protocol describes the basic procedures for setting up a through-the-objective TIRF illuminator and a prism-based TIRF illuminator. In addition, an alternate protocol for incorporating an automated deflection system into through-the-objective TIRF is given. This system can be used to decrease aberrations in the illumination field, to quickly switch between epi- and TIRF illumination, and to adjust the penetration depth during multicolor TIRF applications. In the commentary, a description of the total internal reflection phenomenon is given, critical parameters of a TIRF microscope are discussed, and technical challenges and considerations are reviewed. *Curr. Protoc. Cytom.* 61:12.29.1-12.29.19. © 2012 by John Wiley & Sons, Inc.

Keywords: total internal reflection fluorescence microscopy • fluorescence • cell imaging

INTRODUCTION

TIRF microscopy improves image contrast by significantly reducing the thickness of the excitation plane compared to conventional widefield fluorescence microscopy. In TIRF microscopy no light propagates into the sample, but a thin evanescent field is generated by the total internal reflection (TIR) of excitation light at the glass-sample interface. Examples of samples include molecules in aqueous solution or glycerol, and cells in aqueous media. This evanescent field decays exponentially with distance from the interface, with a space constant that depends on the refractive index of the glass and the sample and the angle of incidence of the excitation light. The fluorophores at the glass-sample interface that are within the evanescent field are excited by this excitation light.

Excitation light for TIRF microscopy is commonly introduced at the glass-sample interface through either a prism or an objective. Each approach has advantages and disadvantages (see the Commentary). Briefly, through-the-objective TIRF utilizes the microscope's imaging objective to introduce an excitation field to the sample. This technique has the advantage that no other optical elements need to be attached directly to the sample coverslip, simplifying setup prior to imaging. Prism-based TIRF utilizes an optical element mounted on the sample glass to couple in the excitation light. Both approaches

are designed to bring incident light into the sample at an angle large enough for TIR to occur at the glass-sample interface. For many applications, a prism-based technique provides more flexibility and is less likely to suffer from aberrations introduced by the objective. Because of ease of use, the through-the-objective method is more commonly utilized for live cell imaging. In this protocol procedures are given for setting up both an objective and a prism-based illuminator. Commercial TIRF illuminators are available from all of the major microscope manufacturers. However, custom-built instruments offer more flexibility and fewer illumination artifacts, and are more affordable.

Basic Protocol 1 describes the configuration of an objective based system. A modification to this technique, which improves uniformity in the illumination field and allows for improved multicolor TIRF imaging, as well as sequential epi-fluorescence and TIRF imaging, is also provided (Alternate Protocol). Basic Protocol 2 describes how to set up a prism-based TIRF illumination system.

BASIC PROTOCOL 1

THROUGH-THE-OBJECTIVE TIRF PROTOCOL

Total internal reflection occurs when the excitation light impinges on the glass coverslip-solution interface at an angle higher than the critical angle (see Background Information). In order for the entire field of view to be in TIR, all of the incoming light needs to be above this critical angle. In addition, a collimated incident beam will produce the most uniform evanescent field. In a standard microscope, collimated light in the sample plane is achieved by focusing the laser light to a diffraction-limited spot in the back-focal-plane (BFP) of the objective. The propagation direction of this collimated light is determined by the radial position of the focused spot in the BFP. Focused light at the center of the BFP will propagate vertically along the axis of the microscope, while light away from the center will propagate at an angle (see Fig. 12.29.1A). The radially farther the focused light is from the center of the BFP, the steeper the angle of the light in the sample plane. To generate TIR in the sample plane, the focused beam must be near the periphery of the BFP of a high numerical aperture (NA) objective. NA characterizes the extent of the angles of light the objective collects or transmits. To produce light above the critical angle, the light has to be positioned in the BFP at an NA position that corresponds to a refractive index larger than the sample being imaged. Examples of samples include aqueous solution, glycerol, and cell cytosol in media.

Materials

- 40- to 100-nm fluorescent microspheres (e.g., Invitrogen FluoSpheres)
- Biological sample
- Upright or inverted infinity-corrected fluorescence microscope including appropriate emission filters and dichroics
- High numerical aperture (≥ 1.45) objective lens (see Critical Parameters)
- Laser(s) at desired excitation wavelength(s), with system of lenses and mirrors to combine lasers into similar beam diameter
- TIR focusing lens and TIR steering mirror; appropriate mounts with ability to translate optical components (if exciting with multiple wavelengths, select a lens with minimal chromatic aberrations)
- Periscope mirror systems, optional
- Beam expander
- Glass sample chamber [e.g. glass bottom dish (MakTek) or Sykes-Moore chamber (Bellco Glass)]

NOTE: For all of these studies, the excitation source will be a laser. However, it is also possible to use other light sources.

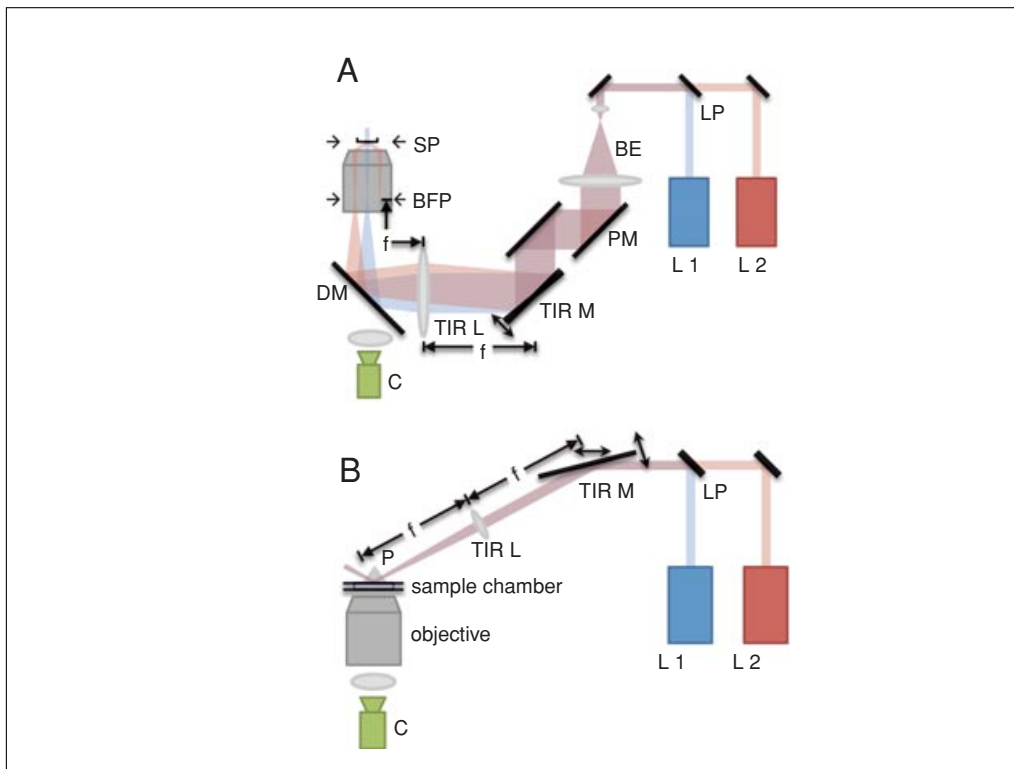


Figure 12.29.1 (A) Schematic of through-the-objective TIRF illuminator. Lasers are combined with longpass mirror(s) (LP) and then the beam diameter is increased with a telescope beam expander (BE). The periscope mirror system (PM) facilitates alignment by allowing translation of the beam on the TIR mirror (TIR M). Following the reflection of light from the TIR mirror, the collimated light is focused with a TIR lens (TIR L) onto the back-focal-plane (BFP) of the objective. Between the TIR lens and the objective is a dichroic mirror (DM), which allows emitted light from fluorophores to pass to an imaging camera (C). Light focused to the BFP becomes collimated in the sample-plane (SP), where the biological sample is on the surface of a glass dish. This figure illustrates two potential configuration scenarios of the TIR mirror. One laser is focused to the radial center of the BFP, which results in the excitation light propagating vertically through the sample. The other laser demonstrates a different tilt of the TIR mirror that results in the beam being focused radially away from the center of the BFP. In this scenario, the light incident on the glass sample chamber is above the critical angle, resulting in TIR. *NOTE:* The Alternate Protocol is realized by replacing the TIR mirror with an electro-mechanical mirror(s). (B) Schematic of through-the-prism TIRF illuminator. Separate beams are combined with longpass filters and then reflected off of a TIR mirror into a weakly focusing TIR lens. Light passes through a prism (P) where TIR occurs on a glass slide above the sample. Changing the tilt of the TIR mirror translates the position of the focused field, while translating the mirror position changes the angle of incidence.

Set up the optics

1. Choose a location for the laser light to enter the microscope upstream from the objective.

If an epi-fluorescence lamp is not being used for imaging, then this microscope port can be used to bring in the light. The fluorescence lamp port also has the advantage that the dichroic/filter turret (slider) can be utilized to simplify switching of dichroics and emission filters during TIRF imaging. Alternatively (such as when the lamp is needed), a secondary illumination path between the fluorescence filter and objective can be utilized.

2. Install the TIR focusing lens (referred to in this procedure as the TIR lens so that it is not confused with the high NA microscope objective lens) on a mount that can be translated along the propagation direction of the light. This lens is used to focus collimated light to a spot in the BFP. The mount will allow for fine adjustment of the axial position of the focused spot. Locate the TIR lens at a distance from the BFP of

the objective that is equal to the focal length of the TIR lens (see Fig. 12.29.1A). The lens needs to be upstream of the microscope dichroic so that it is not in the imaging path of the optics.

Alternatively, instead of focusing the light onto the BFP of the objective, this lens can be used to focus the light onto an equivalent BFP in the optical system. For example, the TIR illumination beam could be combined with an epi-illumination lamp prior to entering the microscope.

See Critical Parameters for additional description on focusing the lens.

3. Place the TIR steering mirror upstream from the TIR lens at a distance equivalent to the focal length of this lens (see Fig. 12.29.1A). Utilize a mount that allows for easy adjustment of the deflection angle. In addition, to ease fine-tuning of the focus point of light on the BFP of the objective, place this mirror on the same translation stage as the stage used by the TIR lens.

With this optical configuration, a deflection of the beam by the TIR mirror results in translation of the focused spot on the BFP of the objective. In addition, this system can be adapted to a computer-controlled steerable mirror to facilitate automated adjustment of the TIR excitation field. This type of control makes it possible to rapidly switch the laser light from TIR to epi-illumination, enabling experiments that require imaging in both modalities, for instance, when there is a desire to monitor fluorophores prior to entering or after leaving the TIR field. In addition, an automated mirror system can be used to improve TIR field uniformity (see the Alternate Protocol).

The TIR mirror is in a conjugate plane to the sample plane, so a change in the deflection angle of the mirror will result in a change in the incident angle in the sample plane. In addition, a translation of the beam on this mirror will result in a translation of the illumination region in the sample.

4. If the excitation light will include multiple laser lines, then combine the lasers into a single path with long-pass dichroic mirrors, placing longer wavelength lasers farther from the microscope. To ease alignment of the lasers, utilize a periscope mirror system directly after each laser (two mirrors that provide the ability to translate and redirect the beam direction). Adjust the periscope mirrors at each laser so that the combined beams are aligned along their entire beam paths.

Use caution when working with the lasers. During alignment utilize low power whenever possible, use beam dumps where appropriate, and always remember to wear laser safety glasses.

It is best if the combined lasers have similar beam diameters. If there is a large deviation in beam diameters, it may be useful to first expand or contract one or more of the individual beams with a telescope lens system. In addition, beam shutters can be added to individual laser lines for selection of specific excitation wavelength(s) during experiments.

Depending on space constraints it may be desirable to couple the combined lasers into a fiber cable that brings the light to the optical system.

5. Install beam expander and periscope optical system in between the beam launch and the TIR deflection mirror. The beam expander is used to set the beam diameter to the desired size for the TIR deflection mirror and lens (see step 2 above). This periscope provides the ability to translate and redirect the combined beam direction.

These optical elements may not be necessary if using a fiber optic cable. After the beam launch, couple the beams into the fiber and align the output of the fiber with the TIR deflection mirror. Use appropriate optics at the output of the fiber to produce a collimated beam at the desired diameter.

Align light entering the microscope

6. Remove the microscope objective lens and align the laser along the entire beam line, starting at the beam launch. Adjust the mirrors and lenses so that the beam passes

through the center of each lens (with each lens normal to the beam propagation direction). The beam should deflect off of the center of the microscope dichroic and propagate vertically along the axis of the microscope optics (without the objective the beam will not be collimated in the sample plane). In the case of an inverted microscope, the light should travel vertically up to the ceiling when the brightfield illumination arm is not in place.

If the beam is not propagating vertically along the imaging axis of the microscope, then the illumination region in the sample plane will not be centered. This situation can be corrected by translating the beam on the TIR mirror. This can be accomplished by adjusting the periscope mirror system as described in step 5.

7. With the objective removed, translate the TIR lens/TIR deflection mirror (mounted on the same stage; see steps 2 and 3) along the beam propagation direction until the beam is focused at roughly the position of the objective thread mounts.
8. Reinstall the objective and fine tune the TIR lens/TIR deflection mirror until the light is collimated above the objective. Verify that the beam remains collimated when the deflection angle of the TIR steering mirror is modulated.

Projecting the light onto the ceiling or a distant wall (via the use of a mirror) may be useful for adjusting the collimation of the beam. With the beam shining on a distant surface, adjust the TIR lens until the beam diameter is minimized.

Fine adjustment of TIR

9. Prepare an imaging chamber/slide with fluorescent beads (microspheres) in water. Mount the chamber on the microscope and focus the microscope on the glass surface. Next, turn on the laser and verify that the beads are visible while using appropriate emission filters. With the TIR mirror, adjust the direction of the illumination beam away from vertical until the transmission of light through the chamber is no longer visible. At this point the light is totally internally reflected.

Dilute the beads in water so that individual microspheres can easily be distinguished with roughly 100 beads per field (with Invitrogen FluoSpheres diluted ~1:10,000).

When viewing the sample through the oculars with the field near the critical angle (but prior to entering TIR) the emission from the free beads may appear as an inclined beam, with more beads visible on the side of the sample toward which the light is deflected.

10. Verify that the entire field is in TIR. During TIR excitation, only beads stuck on the glass surface or close to the surface should be visible. The penetration depth of the evanescent field is dependent on the light's angle of incidence, or conversely on how far the focus beam is away from the center of the objective's BFP.

If no FluoSpheres beads are sticking to the glass, a little bit of salt can be added to the sample to facilitate some sticking (~1 mM NaCl).

With TIR illumination the entire imaging plane should be in TIR. If a portion of the field has transmitted light, then translate the TIR lens/TIR mirror along the propagation direction of the laser beam until the entire field is in TIR. This adjustment will move the position of the focused spot on the BFP of the objective. This spot can also be directly visualized by removing the eyepiece or by using a Bertrand lens.

If the illumination field is not centered in the eyepiece, then the propagation direction of the laser light entering the objective is not aligned with the normal direction of the objective. Adjust the beam optics to ensure the light enters the objective parallel to the objective's axis. Alternatively, the cone of light that makes up the spot at the BFP may not be symmetric. This could be caused by clipping within the beam path. Verify the absence of nonsymmetric clipping within the beam path. For this, remove the objective and place a piece of paper on the hole, and then ensure that the beam has an uninterrupted circular profile.

11. Replace the bead sample with a biological sample. If the refractive index of the biological sample is similar to the bead sample (step 10), then the necessary beam incident angle for TIR in step 10 should only excite fluorophores near the surface of the cell.

NOTE: As the cells' refractive index is typically higher than water, the incident angle of the illumination beam may need to be increased in order to achieve TIR at this new surface.

IMPROVED UNIFORMITY IN THE EXCITATION FIELD PROTOCOL

The ability of lasers to produce a narrow beam of monochromatic light makes them an ideal illumination source for fluorescence imaging techniques such as TIRF microscopy. However, the coherent nature of laser light is also capable of introducing undesired interference patterns into the illumination field, which can make it difficult to accurately quantify TIRF images. These aberrations are produced by undesired scattering within the optical elements (see Technical Challenges). Several techniques have been utilized to overcome these aberrations, and in this protocol a technique in which the azimuthal angular direction of the illumination field is continuously scanned 360° is described (Mattheyses et al., 2006; Fiolka et al., 2008; van't Hoff et al., 2008). Fast scanning of the TIRF excitation light (faster than the rate of image acquisition by the camera) averages out the interference patterns produced due to coherent laser illumination, thus generating a uniform illumination field.

The incorporation of an automated deflection mirror into the illumination system also enables the capability of rapidly switching between TIR and epi-illumination. Switching between illumination methods is useful when there is a desire to differentiate molecules near and away from the glass interface (Merrifield et al., 2002; Mattheyses et al., 2011).

Additional Materials (also see Basic Protocol 1)

Steerable mirror, such as a 2-axis Galvo scan head (Nutfield Technology, Cambridge Technology), a fast steering mirror (Newport, Optics in Motion, Thorlabs), or a tip-tilt piezomirror (PhysikInstrumente, MadCity Labs, Piezosystem Jena)

Two-channel function generator or a function-generating computer card (such as a National Instruments multifunction DAQ card with analog output channels); if using a computer card, control software will also be necessary

Set up the steerable mirror

1. Follow Basic Protocol 1 (step 3), but instead of using a fixed TIR mirror utilize an electronic steerable mirror. Place the mirror upstream from the TIR lens at a distance equivalent to its focal length (see Fig. 12.29.1A).
2. Align the system as described in Basic Protocol 1 (steps 6 and 7). With the objective removed and a piece of thin paper placed at the position of the BFP, verify that the focused beam is centered on the BFP (with the steering mirror at its central position). In addition, verify the light is propagating vertically along the axis of the microscope.
3. Test the deflection system. Change the electrical control signal sent to one axis of the steerable mirror to verify the spot translates in the BFP. Next drive one axis of the mirror with a sine wave while fixing the other direction to confirm the spot near the BFP traces out a straight line. The amplitude of the control signal should determine the amplitude of the deflection. Repeat on the other axis to verify the spot moves along a line perpendicular to the first. Confirm the spot travels along a 45° line relative to the other axes when both directions are driven with the same sine wave. Finally, verify the spot traces out a circle when one axis is driven by a sine

wave, and the other is driven by a sine wave that is 90° out of phase (i.e., a cosine wave).

4. Install the microscope objective and verify the beam is collimated when it propagates vertically from the objective. If the beam is not collimated, correct the beam focus in the BFP by translating the steering mirror (and consequentially the TIR lens if they are mounted together) along the propagation direction of the light. Confirm the beam remains collimated when it is deflected by the steering mirror.
5. Prepare a bead solution in a sample chamber (see Basic Protocol 1, step 9). Mount the chamber on the microscope and focus the objective on the glass surface. Verify the beads are visible when the beam is vertical. Adjust one axis of the TIR steerable mirror until TIR occurs. If the illumination field is not centered or if a portion of the sample plane is not in TIR, then adjust TIR optics following Basic Protocol 1 (see step 10).

Calibrate the beam rotation

6. Drive the two axes of the TIR steerable mirror with sine waves that are 90° out of phase. This signal will direct the inclined collimated beam to rotate azimuthally around the axis of the objective. By increasing the amplitudes of the sine waves, the zenith angle of the beam should increase until TIR is achieved.

As the beam rotates near the critical angle, some azimuthal angles may result in TIR while others may not. While viewing beads through the oculars or watching the beam from above the sample chamber, adjust the amplitude and offset of each sine wave so that the rotating beam of light is near the critical angle throughout its rotation around the azimuthal axis. These adjustments will in effect change the center point and ellipticity of the circle being traced by the focused laser in the BFP.

In practice, it may be easiest to first adjust the bias offset of each signal in order to get the rotation of the beam centered. As the beam is rotating, if the light is close to the critical angle (but not at the critical angle in any direction), the excitation of the beads will appear in the oculars like the rotating spot light of a lighthouse when viewed from above. There will be an outward pointing beam of light that is rotating circularly. Near the critical angle, if the rotation is not centered, then the excitation of the beads will appear and disappear with respect to the azimuthal angular position of the light. This effect is because the beam is going in and out of TIR. If, for example, the appearance of the beam is more apparent in the up direction (defined here as North in directional coordinates), then an offset bias should be added to the control signal that drives the mirror along the North/South direction. If needed, an adjustment should also be made for the East/West direction. Once the beam is centered in the North/South and East/West directions, it may also be necessary to make small changes to the relative amplitude of the two directions. For example, if during azimuthal rotation of the beam, the East/West direction is in TIR while the North/South direction is not, then the amplitude of the sine wave controlling the North/South direction should be increased relative to the East/West direction to make the appearance of TIR similar. Finally, if the illumination is elliptical along a 45° axis relative to the North/South and East/West directions, it may be necessary to make small changes in the phase between the two control signals (with the nominal difference being 90°).

With the system aligned correctly and the incident beam rotating around the azimuthal angle, the emission from beads or fluorescence dye on a glass coverslip should appear better than when the system is not rotating.

7. Replace the bead solution with a biological sample. When the beam is above the critical angle and rotating azimuthally, only fluorophores near the glass surface should be visible.

THROUGH-THE-PRISM TIRF PROTOCOL

In a prism-based TIRF system, the excitation light is introduced into the sample using a glass block instead of the objective. Most configurations of prism-based TIRF microscopy consist of the prism being mounted on the condenser side of the sample. In this configuration, the sample needs to be attached to a glass surface that is coupled to the prism with oil that is preferably matched (or greater than) the refractive index of the glass. Either an upright or an inverted microscope can be utilized, with the prism being below the sample in an upright microscope or above the sample in an inverted microscope (see Critical Parameters). If an upright microscope is used, the imaged sample will be on the top surface of the glass coverslip situated just above the prism. In this configuration a wide variety of objective lenses can be used, including air, oil immersion, water immersion, or water dipping. If using either an oil immersion or water immersion lens, a second glass coverslip above the aqueous solution will also be necessary. Many options for separating the two pieces of glass are possible, including cellophane tape, vacuum grease, or rubber gaskets. Alternatively, commercially available flow chambers are an option. If using an inverted microscope configuration, with the prism mounted above the sample, a similar two-glass-surface sample chamber will be necessary. In this approach either an air, oil, or water immersion objective can be used. As an alternative to having the prism on the condenser side of the sample, the prism can be mounted next to the objective on the objective side of the sample chamber. Due to the size of the objective, it is typically difficult to get the prism close to the imaging field, though the excitation light may be brought to the imaging field by utilizing multiple internal reflections of the light off of both surfaces of the glass (Weis et al., 1982).

Materials

- Immersion oil
- Biological sample
- Upright or inverted microscope with appropriate emission filters
- Triangular- or hemispherical-shaped glass prism (such as right angle BK7 or fused silica prism) and mount to hold the prism on the appropriate side of the sample (to ease adjustment, the mount should be able to translate easily away from the sample)
- Appropriate microscope objective for imaging (in order to minimize spherical aberrations over long working distances, it is advisable to use a water-dipping or water-immersion objective)
- Focusing lens (~100- to 200-mm focal length) in a mount that can be easily translated; select a weakly focusing lens so that laser light is focused to a diffraction limited spot roughly the size of the viewable imaging plane
- Beam steering mirror in a mount so that it can be easily translated and rotated around small angles
- Laser (and beam combining optics if using multiple lasers)

Set up the optics

1. Set up a standard upright or inverted fluorescence microscope with the appropriate emission filters for the lasers being used and the fluorophores being observed.
2. Set up mounts to hold the prism, the focusing lens, and the beam steering mirror (see Fig. 12.29.1B). The prism mount can be attached directly onto the microscope. For instance, it can be fastened to the condenser assembly. The focusing lens should be positioned at a distance from the sample that is roughly equivalent to the focal length of the lens, and should be aligned so that its optical axis is along the direction of the beam path. Install the beam steering mirror upstream from the focusing lens at a distance roughly equal to the focal length of the lens and along the same beam path.

When the prism is located above the sample chamber (in the case of an inverted microscope), it does not have to be mounted to a holder, and instead can be placed directly on the glass coverslip. However, a mount makes it easier to laterally translate the stage during imaging and also makes it easier to return the prism to the same position during sample changes, thus minimizing the need to make adjustments to the incoming beam between samples.

The laser light can be brought in from the back, the side, or even the front of the microscope. The choice of location will affect where the focusing lens and steering mirror are placed. Consider the geometry of the microscope, as well as peripherals that may be used during imaging when determining the ideal position of the entering laser light. If necessary, additional mirrors can be used to bring the light to the sample.

The optics should be set up so that the beam will have an incident angle with the sample that is greater than the angle necessary for total internal reflection. This angle depends on the refractive index of the glass, as well as the refractive index of the sample (e.g., aqueous solution, glycerol, cell cytoplasm—see Background Information). For a glass-water interface, the critical angle is $\sim 62^\circ$. However, the incoming beam angle may be different if there is refraction occurring at the air-prism surface.

If the light's angle of incident on the prism is close to normal, dispersion artifacts will be minimized. If the polarization of the light is perpendicular to the plane of incidence (s-polarized), the maximum transmission of light into the prism will occur at normal incidence. However, if the polarization of the light is parallel to the plane of incidence (p-polarized), then the maximum transmission of light (100%) into the prism will occur at a Brewster's angle of incidence.

3. Set up the laser so that the light reflects off of the beam steering mirror, through the focusing lens, through the prism, and then reaches the position of the glass interface that is close to the imaging plane (at the objective's optical axis).

To ease the initial alignment, it may be helpful to first remove the focusing lens and adjust the steering mirror until the light intersects the sample at the position of the objective axis. Then reinsert the lens and make fine adjustments to the position of the lens until the light is focused on the surface.

Adjust the illumination field

4. Prepare a sample chamber with fluorescent beads (see Basic Protocol 1, step 9) and mount it on the microscope. Focus the objective on the glass-liquid solution surface. Add immersion oil to the prism and translate the vertical position of the prism so that it interfaces with the sample chamber glass.
5. Adjust the steering mirror so the laser light intersects the glass-solution interface along the objective axis. The focus can be adjusted by translating the focusing lens along the propagation direction of the beam. Verify that only beads close to or stuck to the glass surface are visible when the incident angle is above the TIR critical angle.

With this optical configuration, fine adjustments to the position of the excitation light can be made by tilting the mirror. During this movement, the incident angle of the light should remain roughly the same. Fine adjustments to the incident angle of the beam are made by translating the position of the mirror. Throughout this adjustment, the position of the focus should remain roughly the same.

6. Replace the bead solution sample chamber with a biological sample and verify TIRF is working as expected.

Having the prism on a vertical translation stage makes it possible to temporally displace and then return the prism to its original position when the sample chamber is being changed.

COMMENTARY

Background Information

Total internal reflection of light

TIRF microscopy improves image contrast in fluorescence microscopy by significantly restricting the depth of illumination relative to the axial (\hat{z}) resolution of the microscope. This thin excitation depth is produced by TIR of light at the glass-liquid (typically aqueous) interface of the sample chamber. During TIR of light there is a thin evanescent field in the aqueous solution that can excite fluorophores efficiently near the interface. Moving away from this interface the excitation intensity drops exponentially such that beyond the evanescent field there is no detectable excitation. Since fluorophores far away from the interface are not excited, the background signal is decreased. The phenomenon of TIR is based on the bending of the light at the interface of two media of different refractive indices. To elucidate this principle it is helpful to first review the basic interactions of light that occur in dense materials such as glass or water.

Light's interaction with atoms can be thought of in terms of two phenomena, absorption and scattering. In absorption, the capture of photon energy by a molecule results in electron orbitals, typically in the valence band, transitioning into a different quantum state. The molecule then returns to its original ground state by dissipating the energy through thermal motion (heat) or through the reemission of a photon. A blue-colored glass is an example of absorption where light in the red/yellow spectrum is absorbed and then thermally dissipated, while light in the blue spectrum is not absorbed. Another example of absorption is the excitation of fluorophores, such as those used in TIRF microscopy. In this process a fluorophore absorbs a photon, thermally dissipates some energy (producing the Stokes Shift), and then reemits a photon at a lower energy (longer wavelength). Alternatively, scattering is a process in which a photon is absorbed and reemitted by a molecule that remains in its ground state. The captured energy results in the molecule vibrating like an oscillator, thus producing an oscillating dipole that emits a new photon. This process is typically elastic, in that the new photon has the same energy as the initial photon, and has the property that the new photon can be emitted in any direction. As an example, the process of scattering results in the sky appearing blue as opposed to black. Molecules in the upper

atmosphere (where the density is low) preferentially scatter the sun's blue light, which deflects in all directions, including towards the earth. In dense materials (such as glass, liquid, or even the earth's lower atmosphere), photons are also scattered in all directions, but as a consequence of the high density of scatterers, the light destructively interferes in all directions except along the primary propagation direction. This interference phenomenon is typically studied in terms of the wave properties of light (for a more thorough discussion, see Hecht, 2002). Scattering in dense materials is what allows a beam of light to propagate in a single direction over long distances.

In materials with changing atomic properties, such as when moving from glass to water, the propagation direction of any light that is not parallel to normal will change following the transition between mediums. This deflection of light is called refraction, and is a manifestation of differences in scattering in each material that result in the phase velocity of the light not being the same in each material. This velocity difference occurs because scattering absorption and reemission take time. This delay, in combination with the continuous emission and reemission of photons, creates a phase shift in the light that effectively establishes a change in the wavelength (λ) of the light. The new wavelength can be expressed in terms of the vacuum wavelength (λ_0) and the refractive index (n) of the material as: $\lambda = \lambda_0/n$. The refractive index is material-specific and frequency dependent (dispersion effects). It is a quantity related to how atoms in a material oscillate (producing the dipole emitters described above) when light interacts with it. For example, at 589 nm the refractive index in air is 1.0003, in water, 1.33, and in optical glass (such as BK7), 1.52. The frequency of light during scattering remains constant. Therefore, the wavelength change effectively results in the phase velocity of the light changing since $v = \lambda f$ (v is the velocity, and f is the frequency). Alternatively, the velocity can be expressed in terms of the vacuum speed of light c as $v = c/n$.

At the boundary of two media of different refractive indices, light wavefronts are joined because the electromagnetic field must remain continuous across the boundary. However, as discussed above, the wavelength on each side of the interface is different. In order to maintain these boundary conditions the direction of the wavefronts must change (see Fig. 12.29.2A).

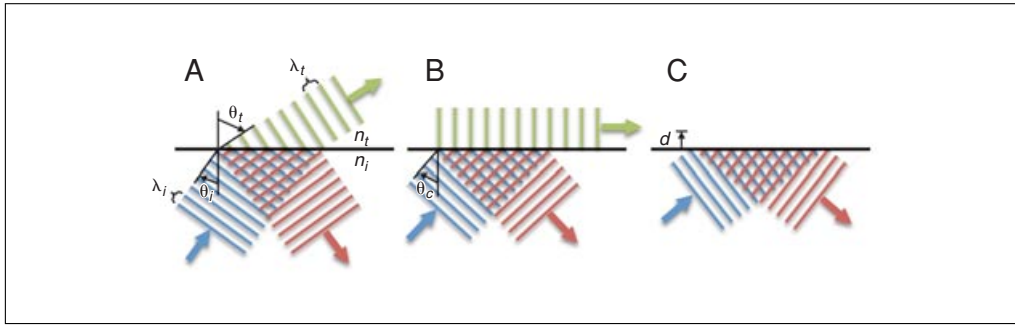


Figure 12.29.2 Illustration of refraction of light between media of different refractive index. Three different incident angles are shown: **(A)** below the critical angle, **(B)** at the critical angle, and **(C)** above the critical angle. The incident medium has a refractive index, n_i , which is higher than the refractive index of the transmitted medium, n_t . Incident light is illustrated as an infinite plane wave, with the distance between the wavefronts (solid lines) being the wavelength (λ). The speed of light is faster in the transmitted medium; therefore, as illustrated by the larger distance between wavefronts, the wavelength (λ_t) is longer in the transmitted medium. At the boundary, wavefronts must remain continuous. In order for (1) the wavefronts to remain continuous and (2) the wavelength to increase, the light must bend further from normal at the interface. (B) At the critical angle (θ_c), the transmitted light propagates along the surface of the interface. (C) Beyond the critical angle, light does not propagate into the transmitted medium. TIR results in an exponentially decaying evanescent field with a depth constant of d .

This phenomenon is a result of destructive interference of scattering waves, resulting in only a single propagation direction (at a different angle) being maintained in the transmitted material.

When light propagates from a material of higher to lower refractive index, the angle of incidence increases (Fig. 12.29.2A). Mathematically this change in angle is described by Snell's law, which states the incident light angle (θ_i) and the transmitted light angle (θ_t) are related as follows:

$$n_i \sin(\theta_i) = n_t \sin(\theta_t)$$

where n_i and n_t are the refractive index of the respective medium. As the incident angle is increased, there is an angle at which the transmitted light is completely parallel to the boundary (Fig. 12.29.2B). At this critical angle, light no longer propagates in the second medium. The critical angle is defined as follows (where θ_t is set to 90°):

$$\theta_{i \text{ critical}} = \arcsin(n_t / n_i)$$

Whenever $\theta_i > \theta_{i \text{ critical}}$ no light propagates into the second medium, and instead the light is reflected in what is termed total internal reflection (TIR; Fig. 12.29.2C). Beyond the critical angle 100% of light is reflected, whereas below the critical angle the amount of reflected

light is angle and polarization dependent. The above equation reveals the critical angle depends on the refractive index of each material. At a glass-air interface the critical angle is $\sim 41^\circ$, whereas at a glass-water interface it is $\sim 62^\circ$.

As in the scenario in which the incident angle is below the critical angle, above the critical angle the electromagnetic field is also continuous across the boundary. However, the field does not propagate into the second, lower refractive index, medium. Instead there is only a near-field effect, termed the "evanescent field," with an intensity that falls off exponentially with distance. The intensity of the field depends on distance from the interface and is related to the intensity at the boundary (I_o) based on the following equation:

$$I(z) = I_o e^{-\frac{z}{d}}$$

where

$$d = \frac{\lambda_o}{4\pi \sqrt{n_i^2 \sin^2(\theta_i) - n_t^2}}$$

The field intensity at the boundary I_o is dependent on the incident angle, as well as the polarization of the light. For light in which the electric field is in the incident plane (\hat{p} polarized) $I_o^{\hat{p}}$ is

$$I_o^{\hat{p}} = E^{\hat{p}} \cdot \frac{4 \cos^2(\theta_i) [2 \sin^2(\theta_i) - (n_i/n_o)^2]}{(n_i/n_o)^4 \cos^2(\theta_i) + \sin^2(\theta_i) - (n_i/n_o)^2}$$

where $E^{\hat{p}}$ is the \hat{p} polarized component of the electric field at the interface. For light with an electric field perpendicular to the incident plane (\hat{s} polarized) $I_o^{\hat{s}}$ is

$$I_o^{\hat{s}} = E^{\hat{s}} \cdot \frac{4 \cos^2(\theta_i)}{1 - (n_i/n_o)^2}$$

where $E^{\hat{s}}$ is \hat{s} the polarized component of the electric field at the interface.

Using TIR for fluorescence microscopy

In order to utilize the evanescent field for fluorescence microscopy, light must be incident on the glass-sample interface (water, glycerol, cell cytoplasm, etc.) above the critical angle. In principle, introducing light above the critical angle is straightforward; however, in practice, the refraction of the light off of other surfaces besides the glass-liquid interface must also be considered. For example, with a flat microscope slide, when laser light enters the glass at the air interface its angle of incidence will decrease at the air-glass interface because the refractive index of air (~ 1) is lower than the refractive index of glass (~ 1.5). In addition, since air has a lower refractive index than water (~ 1.33), even light incident close to parallel ($\sim 90^\circ$ from normal) on the air-glass surface will not have a high enough incidence angle on the glass-liquid interface for TIR. To overcome this situation, an additional optical element is needed, such as a prism or high-numerical aperture oil immersion objective. A prism has the property that it has a similar refractive index to the glass used in a slide, but also has a shape such that the incoming and exiting light surfaces are not parallel to one another. Thus, the incident angle of the light on the air-prism surface can be closer to normal, while still preserving an incident angle above the critical angle at the glass-liquid interface. In order to couple the prism to the glass coverslip, high refractive index oil is needed to prevent TIR of light within the prism. Another option is to use an objective with a numerical aperture (NA) that is larger than the refractive index of the sample. With an objective lens, light that enters the center of the back-focal-plane (BFP) produces light at the front-focal-plane that propagates along the axial direction of the objective, creating

normal incident light on the glass-liquid interface. Light that is off axis in the BFP will produce light with a nonnormal incident angle. The angle of incidence is determined by how far away from the center of the BFP the light enters the objective. Thus, light at the periphery of the BFP will produce the highest angle light. Therefore, in practice, TIR is achieved by focusing light to the BFP off axis from the center of the objective. The maximum angle of incident of the light is set by the following equation:

$$\theta_i = \arcsin(NA/n_{oil})$$

where n_{oil} is the refractive index of the immersion oil between the objective and the glass coverslip. In the case of a 1.49 NA oil objective, the maximum incident angle is $\sim 80^\circ$. However, because of diffraction, it is not possible to focus to a single point. Instead, for TIR to occur, the focused beam needs to remain between the radial edge of the BFP and a position where the NA of the objective is 1.33 (refractive index of the liquid) or even farther for samples with higher refractive index (cell cytoplasm is ~ 1.38).

As a technique, TIRF microscopy has been used to study a number of biological processes including those occurring at or near the cell membrane. Examples include endocytosis, exocytosis, membrane-bound protein dynamics, and viral particle formation. In addition, TIRF has commonly been used for in vitro single-molecule studies of biological molecules such as DNA and associated proteins, cytoskeleton molecules, and molecular motor proteins. Applications of TIRF microscopy are summarized in Axelrod (2008), Mattheyses et al. (2010), Trache and Meininger (2008), and UNIT 12.18.

Critical Parameters

Prism-based versus objective-based TIRF microscopy

Through-the-objective and through-the-prism TIRF microscopy each have advantages and disadvantages. Through-the-objective TIRF microscopy is typically more convenient for imaging because only one optical element needs to be in contact with the sample. This facilitates quicker loading and unloading of the sample, lessens the need to make adjustments to the optics between sample changes, minimizes photo bleaching by ensuring the illumination is only in the imaging

region, and makes it possible to use open-top sample dishes. These advantages make through-the-objective imaging ideal for conventional live cell imaging. However, through-the-objective TIRF is typically more difficult to set up, requires a high NA oil objective, and often costs more to build. Through-the-prism TIRF microscopy has an advantage that there are far fewer optical elements in the illumination path than in through-the-objective TIRF microscopy (including the multiple lenses in the objective); therefore, it is possible to create an evanescent field with fewer aberrations in intensity and polarization. In addition, background noise is usually lower since the excitation light does not pass through the imaging optics. With prism-based TIRF microscopy it is also possible to generate a thinner illumination field because the excitation light can be introduced at a higher incidence angle. The low background noise and minimum illumination aberrations make through-the-prism TIRF ideal for single-molecule biophysical/biochemical studies and quantitative cellular biology studies.

Microscope

A TIRF illuminator can be used on either an upright or an inverted microscope. The choice will likely depend on the imaging application, as well as other equipment that is needed during imaging. For imaging cultured cells, it is common to use an inverted microscope because the cells can easily be cultured on the top surface of a glass coverslip. If TIR is produced through an objective or with a prism below the glass, then the top of the sample chamber can be left open to allow easy access for tasks such as adding reagents, microinjection, micromanipulation or CO₂ control. An inverted microscope is commonly used in prism-based TIRF systems because there is usually more space for the optics above the sample, making it easier to build and adjust the system. However, upright systems are useful when there is a desire to specifically use a prism-based TIRF system to image cells plated in a glass-bottom dish. Because it is more straightforward to bring in light with a prism on the condenser side of the sample, the prism is placed below the sample and an objective (such as a water dipping objective) images from above. To bring the light to the sample it may be necessary to use the bright field port in combination with small mirrors. Alternatively, reflections with a trapezoidal prism can be used to bring the light to the sample at the proper angle for TIR (Axelrod et al., 1984).

If building a prism-based TIRF system, consider using a microscope in which the focus is controlled by moving the nosepiece as opposed to the stage. This will allow the prism to remain fixed relative to the TIR beam optics when the focus is adjusted.

If the microscope will be used to image over long periods of time or if solutions will be added to the sample during imaging, such as by using a flow chamber, potential focus drift should be considered. In TIRF microscopy, if the glass-aqueous solution interface moves even a little in the axial direction relative to the focus, then the sample will appear out of focus. To overcome this problem a focus compensation system can be used. These systems work either by monitoring the position of the glass or by maximizing the image contrast. In practice, monitoring the glass surface is usually more ideal because the position can be monitored without exposing the sample to light, and, additionally, multiple image slices are not necessary. However, this style of focus adjustment is usually more complicated to implement and costs significantly more.

Excitation light path in the microscope

If an alternative path is desired but not available for the laser light to enter the microscope, it may be possible to use a “stage up kit” or home-built brackets to create a new beam path between the objective (the objective nosepiece) and the fluorescent filter turret. This can be accomplished by creating a mounting bracket for the objective nosepiece, which extends the distance between the filter turret and the objective. This extension of the distance is possible because of the infinity-corrected optics of the microscope. Between the objective nosepiece and turret, install a 45° dichroic mirror mount, which will be used to reflect the laser light into the objective. Extending the position of the nosepiece may also require brackets to be used to adjust the height of the stage, as well as the position of the bright field illumination source.

Objectives

The numerical aperture (NA) of the objective for through-the-objective TIRF microscopy needs to be larger than the refractive index of the sample. Often the sample is in a water-based solution with a refractive index of ~1.33. However, careful consideration should be taken when imaging cells, which have a cytoplasmic refractive index of roughly 1.35 to 1.4 (Bereiter-Hahn et al., 1979; Curl et al., 2005; Liang et al., 2007). Because of these

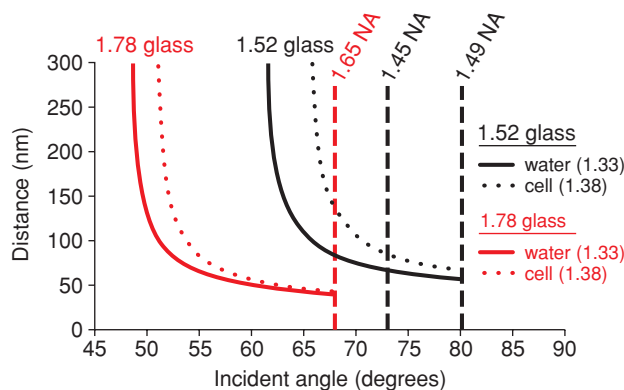


Figure 12.29.3 Illustration of evanescent field penetration depth, d , versus incident angle. Depth is given for two different types of microscope glass coverslips; conventional ($n_t = 1.52$) and high refractive index ($n_t = 1.78$). For each incident glass, two transmitted media are given: water ($n_i = 1.33$) and cell cytoplasm ($n_i = 1.38$). The maximum potential incident angle is given for three objectives: a 1.45 NA objective (using 1.52 glass), a 1.49 NA objective (using 1.52 glass), and a 1.65 NA objective (using 1.78 glass).

conditions and because of diffraction in the excitation beam, it is useful to have an objective with an NA significantly above 1.33. In addition, the depth of the evanescent field is nonlinear with respect to incident angle, so it is possible to significantly decrease the thickness of the evanescent field by moving a little beyond the critical angle (see Fig. 12.29.3A). All of the major microscope manufacturers make oil immersion objectives specifically designed for TIRF microscopy in the 1.45 to 1.49 NA range. These objectives use immersion oil, which matches the refractive index of a conventional glass coverslip (1.52). It is possible to produce incident angle light up to $\sim 73^\circ$ with a 1.45 NA objective, and up to $\sim 80^\circ$ with a 1.49 NA objective. The critical angle between a conventional glass coverslip and an aqueous solution is $\sim 62^\circ$, and between glass and cell cytoplasm is $\sim 65^\circ$. A 1.45 NA provides sufficient range to produce TIR light. However, a 1.49 NA objective provides significantly more room to work and allows for thinner penetration depth (Fig. 12.29.3). There are also objectives that use special high refractive index oil such as the Olympus 100×1.65 NA Apochromator and the Zeiss 100×1.57 NA Plan-Apochromat. The Olympus 1.65 NA Apochromat uses special coverslips with a refractive index of 1.78. Since the ratio of the objective NA to the glass coverslip refractive index is lower, the maximum incident angle for this objective is lower ($\sim 65^\circ$). However, since the refractive index of the glass is higher, the TIR critical angle is significantly lower. With a 1.78 coverslip, the critical angle with water is $\sim 48^\circ$, as opposed

to $\sim 62^\circ$ with conventional glass. Therefore, it is possible to easily produce a thin illumination field with the 1.65 NA objective. Unfortunately, the use of special oil and glass coverslip comes with a drawback of significantly increased imaging expense because of the high cost of the uncommon glass slides. Moreover, the chromatic properties of this glass and this objective make them poor for multicolor imaging.

Focusing lens

The size of the illumination area in the sample plane is dependent on the focal length of the TIR lens, the effective focal length of the objective, and the beam diameter that enters the lens. Therefore, these parameters need to be considered when selecting a suitable focal length for the TIR lens. The TIR lens and objective act as a beam contractor, taking a wide beam and making it narrower based on the following relationship:

$$\text{diameter at sample} = \text{diameter at TIR lens} \cdot \left(\frac{\text{objective focal length}}{\text{TIR lens focal length}} \right)$$

For example, a 15-mm entering beam diameter, 3-mm effective objective focal length, and 150-mm TIR lens focal length will illuminate a 300- μm region in the sample plane. The effective focal length of the objective can be calculated based on the magnification of the objective and the focal length of the microscope's tube lens (manufacturer specific) based on the following relationship:

$$\text{objective focal length} = \frac{\text{tube lens focal length}}{\text{objective magnification}}$$

As an example, a 60 \times objective on an Olympus microscope with a tube lens focal length of 180 mm has an objective focal length of 3 mm.

Prisms

A variety of prism shapes and glass types can be used in TIRF microscopy. Commonly used shapes include right angle triangle, equilateral triangle, and trapezoidal dove, Pellin-Broca. With \hat{s} -polarized light (polarization perpendicular to the plane of incidence) a prism geometry in which the laser beam is incident close to normal on the air-glass surface will maximize transmission of light to the sample by minimizing back reflections. One option to ensure normal incidence is the use of a hemispherical prism centered directly above the imaging field. With \hat{p} -polarized light (polarization in the plane of incidence) the maximum transmission of light (100%) occurs when the light is incident at the Brewster's angle. With any of these geometries having the laser beam incident close to normal will help to minimize spatial splitting of different wavelengths of light (dispersion). In addition, to minimize dispersion a glass that has a refractive index that does not depend strongly on wavelength can be utilized. In general, when selecting glass type the prism needs to have a refractive index above the refractive index of the sample being imaged; otherwise, it will not be possible to introduce light above the critical angle. Fused-silica and BK7 are both commonly used in prism-based TIRF microscopy.

Light sources

Common laser light sources for TIRF microscopy include gas, diode, and diode-pumped solid state (DPSS). A major factor when selecting lasers is the output power. Required power will depend on the quantity and brightness of fluorophores being imaged, as well as the camera exposure time. For most applications, ≥ 20 mW is sufficient, but it is often useful to have ≥ 100 mW per line when imaging fast or dim systems. Traditionally, air-cooled gas lasers have been used for fluorescence imaging, but DPSS and diode have been becoming more prevalent because they are smaller, quieter, more energy efficient, have a more stable output intensity, and are easier to maintain. Diode lasers are particularly popular because they are the more affordable and

allow direct, fast modulation of output power via an electrical control signal. Diode lasers are also less susceptible to temperature and power changes than DPSS lasers, which can result in undesirable mode hopping. DPSS lasers generally have better beam properties, including a narrower frequency band and a smaller beam diameter. As of the time of this writing there were very few diode lasers in the 500- to 600-nm range. However, new diodes are constantly being developed and presumably more options will be available in the future.

The bright, collimated light produced by lasers makes them useful for creating the TIRF field. However, a conventional fluorescence lamp, such as a xenon or mercury arc lamp, can also be used to create the TIR field. A lamp light source has a wide spectrum of available excitation wavelengths, making it possible to easily and relatively cheaply modify the system for specific fluorophores by just changing excitation filters (as opposed to buying new lasers). In addition, since lamp light is not coherent, the illumination field is less likely to have interference patterns like those experienced with laser light. TIR with a lamp is achieved by illuminating an annular-shaped ring in the BFP of the objective. For TIR, the light needs to be in the objective's high NA region, the portion of the BFP corresponding to light above the critical angle. This situation can be achieved by using an opaque disk in the objective BFP (or an equivalent plane). The use of an opaque disk means a significant amount of the potential excitation light is lost, a major disadvantage for lamp-based TIR. In addition, they are not as flexible for controlling the angle of excitation as the laser-based systems.

Technical Challenges

Interference fringes

The coherent nature of the laser light in a TIRF illuminator can produce undesired interference fringes in the evanescent excitation field. These non-uniformities are attributed to scattering of light from mirrors and lenses in the beam path. This process affects the phase of combined light at the sample field, creating undesirable constructive and destructive interference fringes (see Fig. 12.29.4A). Uneven illumination makes it difficult to discern intensity differences in different spatial parts of a sample, such as distinct regions of a cell, and can make it very difficult to perform quantitative measurements. Selection of optics designed for TIRF and minimizing dust

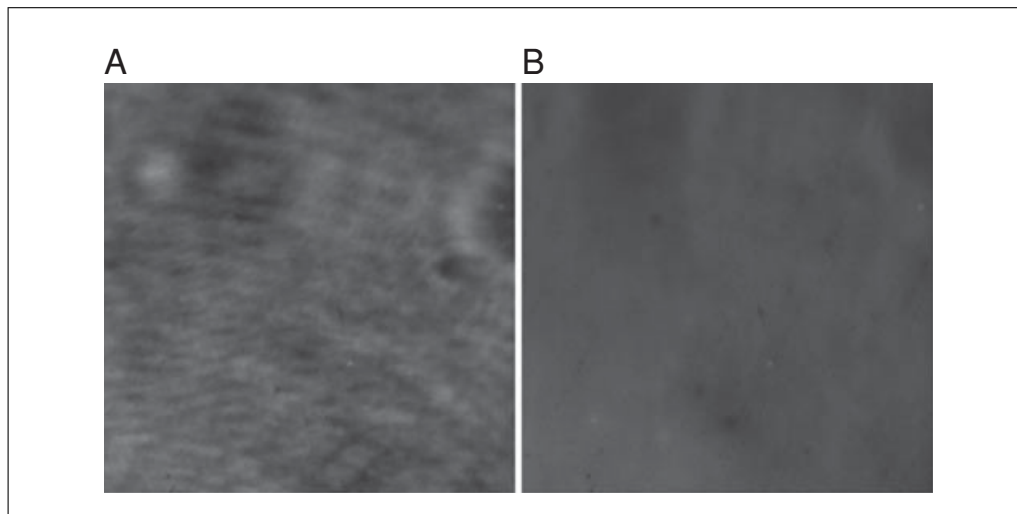


Figure 12.29.4 TIRF microscopy image of acridine orange stuck to glass coverslip (**A**) with the azimuthal incident angle of the light fixed during imaging or (**B**) with the azimuthal incident angle rotating during imaging. Presence of patterned interference fringes decrease significantly when the excitation field is rotating.

on the optics can help to decrease interference patterns, though it is difficult to completely overcome non-uniformities. A more uniform field can be produced by modulating either the phase or the angular orientation of the light during the time course of capturing an image. A method as described by Kuhn and Pollard (2005) consists of a rapidly spinning transparent petri dish in the laser beam of a prism-based TIRF microscope. The spinning of the dish continuously and spatially modulates the phase of the incoming light, in effect changing the position of the interference fringes in the sample plane. Assuming the phase is modulated at a rate much faster than the imaging rate, a uniform field will be visible in the captured image. In an alternative method the azimuthal angle of incident light is rapidly modulated, again changing the position of the fringes rapidly during the imaging period (Fig. 12.29.4B). This method has been demonstrated using multiple methods including a wedge-shaped glass element (Mattheyses et al., 2006), with the use of acousto-optical deflectors (AODs; van't Hoff et al., 2008), or by using a tip-tilt scanning mirror (Fiolka et al., 2008). These techniques are similar to the scanning mirror method described in the Alternate Protocol.

Undesired coupling of TIR light into sample

Although cells are often imaged in aqueous media with a refractive index of 1.33, they typically have a refractive index between 1.35 to 1.4 (Bereiter-Hahn et al., 1979; Curl et al., 2005; Liang et al., 2007). If the angle

of incidence is set just beyond the critical angle for the glass-aqueous solution interface, then propagating light may enter the solution. Therefore, an incident angle beyond the maximum sample refractive index is recommended. A higher incident angle will also help to decrease the illumination depth. In addition, objects (such as cells or beads) that have a refractive index different from the surrounding medium can scatter propagating light into the solution. This light may excite fluorophores away from the glass surface, thus decreasing the signal-to-noise, and so creating the false impression that these fluorophores are near the glass surface. This problem can be minimized by using a surrounding medium that closely matches the refractive index of the sample, and by minimizing the number of objects in the excitation field that could potentially scatter light. Another potential source of light propagation into the solution is via scattering within the objective lens (Mattheyses and Axelrod, 2006).

Multi-color excitation

The penetration depth of the evanescent field is wavelength dependent (see Background Information). Therefore, if multiple excitation wavelengths are used during imaging with the same incident angle, the characteristic penetration depth (d) will vary for each wavelength. Shorter wavelengths will have a thinner penetration depth than longer wavelengths. This situation is typically not a problem for qualitative multi-color imaging applications where the relative axial position of

fluorophores is not critical or for quantitative studies where the excitation wavelengths of the fluorophores are relatively close to each other (e.g., CFP, 445 nm and GFP, 488 nm). However, in situations where axial depth is critical and in particular when there is a large difference in excitation wavelengths (e.g., CFP and RFP, 561 nm), it will be necessary to set up an optical system in which the deflection angle of each laser line can be adjusted independently. This can be achieved using one of two methods. The most straightforward method is to independently image each fluorophore sequentially in time. While imaging each fluorophore the respective laser can be activated (through direct modulation or by opening a shutter) while using a wavelength specific deflection angle. Specific deflection angles for each wavelength can be achieved by using a steerable mirror system like the one described in the Alternate Protocol. If simultaneous imaging of multiple colors (with penetration depth correction) is needed, then independent control of the deflection angle of each laser should be added prior to combining the lasers in the beam launch. This can be achieved by mapping the imaging plane to a deflection mirror behind each beam combining longpass mirror. Commercially available systems are also capable of achieving multiple independent laser line illumination (e.g., Olympus cell[^]TIRF and OMAC-TIRF).

When utilizing multiple-color excitation, chromatic aberrations in the optical system should be considered. Each lens in the beam path (including the TIR focusing lens and the objective) may introduce variations in focus based on wavelength-dependent refraction within the optical elements. Therefore, lenses that minimize chromatic aberrations should be selected. Further focusing control, such as an additional lens or translatable fiber, can be incorporated into each laser line prior to combining the beams so that focus can be independently controlled for each excitation color.

Polarization

Laser light is typically polarized, and the resulting evanescent field from a polarized laser will also have specific directional components. The polarization of light incident on a surface (such as the glass-aqueous solution interface) is usually expressed in terms of the electric field in the plane of incidence (\hat{p} -polarized) and the electric field perpendicular to the plane of incidence (\hat{s} -polarized). If the normal direction of the interface is defined as the \hat{z} direction, and the propagation direction of the light

is in the \hat{y} - \hat{z} plane, then the \hat{s} -polarized light is directed along the \hat{x} axis and the \hat{p} -polarized light is in the \hat{y} - \hat{z} plane. Thus, incident light that is \hat{s} -polarized only has components of the electric field that are along the surface plane of the interface (\hat{x}) while \hat{p} -polarized incident light has components perpendicular to the surface (\hat{z}), as well as along the surface (\hat{y}). However, since a steep incident angle is necessary for TIR to occur, the field will primarily be directed in the perpendicular \hat{z} direction. The polarization direction is an important consideration if the excitation dipoles of the fluorophores being imaged are situated in a preferential direction. For example, when DiI is in a lipid bilayer, its dipole is preferentially oriented along the \hat{x} - \hat{y} plane of the bilayer (Axelrod, 1979; Bradley et al., 1973). Therefore, if DiI is in a membrane that is attached to the glass surface, an \hat{s} -polarized field will preferentially excite DiI. This preferential excitation can be used as a technique to study the orientation of biological molecules, such as cell membrane orientation (Sund et al., 1999), and exocytosis (Anantharam et al., 2010). For most cell biology applications of TIRF microscopy, polarization is not a concern because fluorophores are usually freely oriented. However, if there is a concern about polarization and both \hat{s} and \hat{p} excitation directions are simultaneously desired, linearly polarized laser light can be circularly polarized by adding a quarter-wave plate to the beam path. Alternatively, if the light is scanned azimuthally in a circle at a rate faster than the camera exposure time, as described in the Alternate Protocol, then both \hat{s} and \hat{p} excitation polarizations will be present in each image frame. TIRF microscopy with a lamp (as opposed to a laser) will also produce mixed \hat{s} and \hat{p} polarized excitation field.

Troubleshooting

Correct TIR illumination field

If TIR is not achieved in the imaging field, then the excitation light is not incident on the sample above the critical angle. In through-the-objective TIRF microscopy, the angle is increased by shifting the radial position of the focused beam on the BFP. In through-the-prism TIRF microscopy, the angle can be increased by translating the position of the TIR mirror. Additional discussion on these corrections is given in the protocols. Propagating light can also enter the illumination field by scattering within the beam optics or sample. Dust on optical surfaces (including in the immersion

oil) can couple light into the sample chamber. In addition, samples (such as cells) in a solution of different refractive index may scatter light (see Technical Challenges). Decreasing the density of scatters and using media with the same refractive index can decrease unwanted coupling of light into the solution.

When near the critical angle in objective-based TIRF microscopy, if a portion of the field is in TIR while another portion is not the focus position of the beam in the BFP should be adjusted. In prism-based TIRF microscopy this problem can be corrected by adjusting the focus position of the light on the sample. In addition, the convergence angle can be decreased by using a weaker focusing lens and/or decreasing the diameter of the beam entering the focusing lens.

In through-the-prism TIRF, if the illumination field is not centered in the imaging field then the position of the focused beam can be adjusted by changing the deflection angle of the light with the TIR mirror. If this problem is occurring in through-the-objective TIRF, then the light may not be entering the objective parallel to the optical axis. Translate the position of the laser beam on the TIR mirror by adjusting periscope mirrors upstream of the TIR mirror. Alternatively, there may be clipping of the light in the beam optics. Verify clipping is not occurring. These corrections are also discussed in more detail in the protocols

Alternative method to verify TIR field

If it is difficult to determine if the excitation field is in TIR, it may be helpful to use two different fluorescent dyes (excited by the same wavelength), with one dye stuck to the surface and the other free in solution. If the excitation field is in TIR, the stuck dye will primarily be visible. For example, with a 488-nm excitation source, fluorescein (green) free in solution can be used in combination with DiI (red) stuck to the surface. First allow DiI (0.5 mg/ml in ethanol) to stick to the surface for 10 min. Then wash away any remaining free DiI with water and add carboxyfluorescein (0.1 mg/ml). When imaging with 488 nm, the green fluorescein will primarily be visible when the incident angle of the excitation light is below the critical angle. Above the critical angle the red DiI will primarily be visible.

Anticipated Results

Compared to conventional fluorescence microscopy the illumination thickness in TIRF microscopy is significantly thinner. The thinner excitation field decreases background fluo-

rescence, which results in an improved signal-to-noise. In addition to the improved contrast, users of this technique should anticipate being able to distinguish events near the cell surface from events occurring deeper within a cell. This selectivity occurs because only fluorophores near the glass interface are excited in TIRF microscopy. Therefore, only tagged molecules near this interface (and thus the cell membrane) will be visible. For instance, an investigator can use TIRF microscopy to distinguish actin bundles near the cell surface involved in cell migration and adhesion from actin deeper within the cell.

Time Considerations

The time to build and align a TIRF microscope illuminator will depend somewhat on the complexity of the system being constructed, the availability of components (parts that do not need to be custom machined), the precision requirements of the system, and the user's experience working with optics. Typically, a through-the-objective system is more time consuming to assemble because multiple optical elements are needed to correctly focus light in the BFP of the objective. In addition, it might be necessary to reconfigure the microscope to handle the TIRF illumination beam path. The investigator can expect to spend one to multiple months configuring this type of system. A very basic through-the-prism TIRF system on an inverted microscope, such as one consisting of a single mirror, lens and prism, can be configured relatively quickly, potentially in the time frame of a week. A more sophisticated system with beam combining optics, multiple mirrors, and machined mounts might take multiple months to assemble.

In an azimuthal illumination system as described in the Alternate Protocol, periodic (weekly) calibration of mirror offsets and amplitudes may be necessary (~30 min). Once a TIRF microscope is properly aligned, the set up time for imaging biological samples is similar to imaging with a conventional fluorescence microscope. Fine adjustments to ensure the field is in TIR typically only take a few minutes.

Literature Cited

Anantharam, A., Onoa, B., Edwards, R.H., Holz, R.W., and Axelrod, D. 2010. Localized topological changes of the plasma membrane upon exocytosis visualized by polarized TIRFM. *J. Cell Biol.* 188:415-428.

- Axelrod, D. 1979. Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization. *Biophys. J.* 26:557-574.
- Axelrod, D. 2008. Total internal reflection fluorescence microscopy. *Methods Cell Biol.* 89:169-221.
- Axelrod, D., Burghardt, T.P., and Thompson, N.L., 1984. Total internal reflection fluorescence. *Ann. Rev. Biophys. Bioeng.* 13:247-268.
- Bereiter-Hahn, J., Fox, C.H., and Thorell, B. 1979. Quantitative reflection contrast microscopy of living cells. *J. Cell Biol.* 82:767-769.
- Bradley, R.A., Martin, W.G., and Schneider, H. 1973. Dynamic behavior of fluorescent probes in lipid bilayer model membranes. *Biochemistry* 12:268-275.
- Curl, C.L., Bellair, C.J., Harris, T., Allman, B.E., Harris, P.J., Stewart, A.G., Roberts, A., Nugent, K.A., and Delbridge, L.M. 2005. Refractive index measurement in viable cells using quantitative phase-amplitude microscopy and confocal microscopy. *Cytometry A* 65:88-92.
- Fiolka, R., Belyaev, Y., Ewers, H., and Stemmer, A. 2008. Even illumination in total internal reflection fluorescence microscopy using laser light. *Microsc. Res. Tech.* 71:45-50.
- Hecht, E. 2002. Optics, 4th Ed. Addison Wesley, San Francisco, Calif.
- Kuhn, J.R. and Pollard, T.D. 2005. Real-time measurements of actin filament polymerization by total internal reflection fluorescence microscopy. *Biophys. J.* 88:1387-1402.
- Liang, X.J., Liu, A.Q., Lim, C.S., Ayi, T.C., and Yap, P. H. 2007. Determining refractive index of single living cell using an integrated microchip. *Sensor. Actuat. A-Phys.* 133:349-354.
- Mattheyses, A.L. and Axelrod, D. 2006. Direct measurement of the evanescent field profile produced by objective-based total internal reflection fluorescence. *J. Biomed. Opt.* 11:014006.
- Mattheyses, A.L., Shaw, K., and Axelrod, D. 2006. Effective elimination of laser interference fringing in fluorescence microscopy by spinning azimuthal incidence angle. *Microsc. Res. Tech.* 69:642-647.
- Mattheyses, A.L., Simon, S.M., and Rappoport, J.Z. 2010. Imaging with total internal reflection fluorescence microscopy for the cell biologist. *J. Cell Sci.* 123:3621-3628.
- Mattheyses, A.L., Atkinson, C.E., and Simon, S.S. 2011. Imaging single endocytic events reveals diversity in clathrin, dynamin and vesicle dynamics. *Traffic* 12:1394-1406.
- Merrifield, C.J., Feldman, M.E., Wan, L., and Almers, W. 2002. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. *Nat. Cell Biol.* 4:691-698.
- Sund, S.E., Swanson, J.A., and Axelrod, D. 1999. Cell membrane orientation visualized by polarized total internal reflection fluorescence. *Biophys. J.* 77:2266-2283.
- Trache, A. and Meininger, G.A. 2008. Total internal reflection fluorescence (TIRF) microscopy. *Curr. Protoc. Microbiol.* 10:2A.2.1-2A.2.22.
- van't Hoff, M., de Sars, V., and Oheim, M. 2008. A programmable light engine for quantitative single molecule TIRF and HILO imaging. *Opt. Express.* 16:18495-18504.
- Weis, R.M., Balakrishnan, K., Smith, B.A., and McConnell, H. M. 1982. Stimulation of fluorescence in a small contact region between rat basophil leukemia cells and planar lipid membrane targets by coherent evanescent radiation. *J. Biol. Chem.* 257:6440-6445.