TIRF Microscopy Overview

TIRF Microscopy uses the phenomenon of Total Internal Reflectance (TIR) to perform Fluorescence (F) illumination in a very thin layer barely into the sample side of a glass/sample interface. It has proved useful for studying surface regions of cells with minimal background from deeper regions.

Light incident at an interface between materials of different refractive index (RI) bends due to refraction according to Snell's Law. If the light begins in the higher-RI material and is incident at the surface with sufficiently large angle then Snell's law suggests the outgoing angle is greater than 90°. In fact the light is reflected. The threshold angle is called the critical angle; see the Wikipedia entry for Total Internal Reflection and Snell's Law. Total Internal Reflection (TIR) is the basis for fiber optic cables among other uses. However, the physics of TIR reveal that a small amount of the reflected light barely penetrates into the material with lower RI, which is called “evanescent” wave, with the intensity decaying exponentially deeper into the low-RI medium with a length scale of a few hundred nanometers, which is utilized in TIRF microscopy to enable selective illumination of a thin layer of the sample.

In TIRF microscopy, the illumination light is reflected off the interface between a glass substrate where the sample is mounted in water or similar medium. Even though the light is reflected off of the glass-water interface, the small evanescent wave of the illumination penetrates into the sample side for a few hundred nanometers depth and can excite fluorophores. Illumination can be done using the same objective lens used for imaging (objective-TIRFM) or with a separate glass prism (prism-TIRFM).

TIRF can be understood as a form of selective illumination where the thin layer at the glass/sample interface is illuminated. Any incident light below the critical angle will not be reflected and will go into the sample where it can excite florescence which will result in unwanted background signal. By changing the exact angle of the incident light within a narrow range, the penetration depth of the evanescent wave changes slightly and some degree of depth discrimination is possible.

Specially-designed objective lenses are available for TIRF. These are oil lenses that have been designed to have very high NA, nearing the RI of oil, so that the accessible optical angle is close to 90° and thus illumination can be introduced above the critical angle. The NA corresponding to the critical angle is the RI of the medium, nominally 1.33 for water but in practice usually around 1.4 for cells. Thus it is usually possible to barely achieve TIRF with an NA 1.42 objective lens, but objectives designed for TIRF usually have NA 1.49 or so. TIRF objectives also must have a flat field (plan correction).

More information can be found on Wikipedia's page on TIRFM.

Implementation Overview

To implement objective TIRF, an illumination beam is focused at the back aperture of the objective lens to create a collimated beam of light at the sample plane. Crucially, instead of shining this beam through the middle of the objective it is placed near the outside edge of the objective back aperture, so that the resulting beam of parallel light leaves the objective strongly tilted, specifically with angle sufficiently large to reflect off the interface of the coverslip and sample. It is important for the illumination light to be parallel (i.e. collimated) at the sample (i.e. focused at the back aperture) so that all of the illumination internally reflects; any light that doesn't undergo total internal reflection at

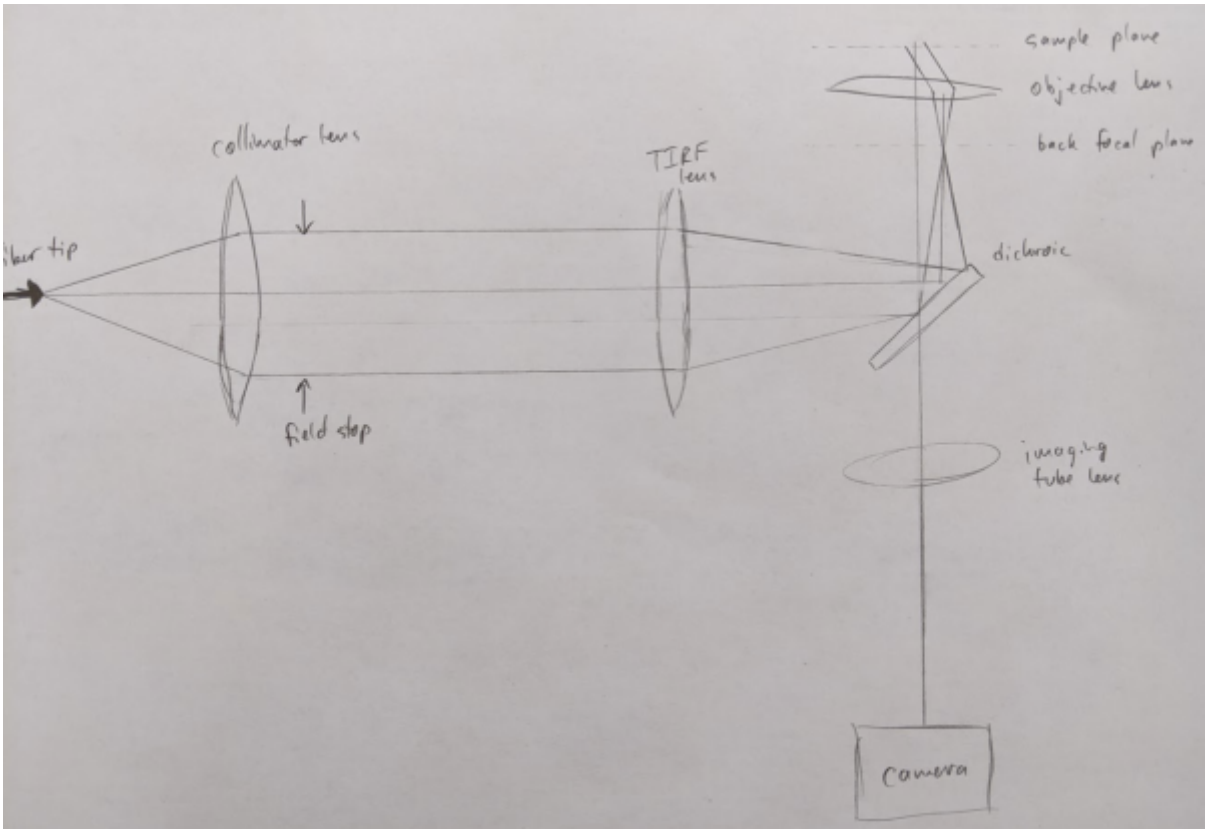
the coverslip/sample interface will go deep into the sample and result in undesired background fluorescence. For the same reason it is important to minimize any stray illumination light.

A single-mode fiber tip is imaged at the objective's back aperture or BFP to attain highly collimated illumination at the sample. A pair of lenses accomplishes the task; the fiber tip is placed at the focus of a collimating lens and then the collimated light enters a “TIRF lens” that is focused at the objective BFP. The lateral displacement of this assembly and/or the fiber tip sets the lateral position of the focused spot at the BFP and hence the angle of the light at the sample. The size of the collimated beam at the sample is given by “input beam” size exiting the collimation lens, demagnified by the ratio of the focal lengths of the “TIRF lens” and the objective lens.[1)] The “input beam” size is given by the fiber NA times the focal length of the collimating lens times 2.

An estimate of the allowable deviation from collimation at the sample reveals that as long as the “TIRF lens” is placed within 1 millimeter from the ideal axial position there are no important consequences.[2)]

The intensity distribution arising from a single-mode fiber is Gaussian, resulting in illumination that is brighter in the center than on the edges. It is possible to use an “apodizing” filter to reduce the intensity in the center of the field, or more commonly the resulting fluorescence images are normalized using flat-field correction in post-processing. Sometimes only the center of the Gaussian profile is used, making the illumination more uniform at the expense of illumination efficiency (in essence expanding the Gaussian so that only the center part is mapped to the imaged portion of the sample).

Next a dichroic is used to reflect the illumination into the objective lens and spectrally separate the emission light. Because the illumination light must be tightly focused at the objective and because the dichroics are usually long-pass (i.e. the illumination light is reflected), it is important that the dichroic mirror have low wavefront error, so ultra-flat dichroics on thicker substrates are usually used.



One variation is Ring-TIRF where instead of illuminating a single point at the back aperture instead a corresponding ring is illuminated (often by moving a point illumination in a circular pattern). Thus at the sample the illumination beam is coming from different directions and shadowing is avoided and uniformity is enhanced.

TIRFM with ASI components

ASI components can be used to easily implement objective TIRF.

A **C60-FIBER-LAUNCH** places the tip of the SM fiber at the focal point of the collimating lens, to which this part is attached, with a micrometer head allowing for precise lateral positioning of the fiber tip. The micrometer can be motorized if desired. There is also the capability of doing ring TIRF using an ASI scanner but that is outside of the scope of this document.

One of ASI's tube lenses is used as the collimating lens, most commonly the **C60-TUBE-100**. An iris – **C60-IRIS** – is often placed just after the collimating tube lens to act as a field stop.

ASI usually uses an achromat as the “TIRF lens” mounted in a short section of a cage system so that the lens can be axially moved along the cage rods during initial system alignment to place the focus of the beam at the back aperture of the objective. This is PN **C60-30CRM-30LM-xxx*** where xxx indicates the focal length in millimeters; standard possibilities include 100, 125, 150, 170, 200, 220, 250, 300, 350, 400 with values between 170 mm and 250 mm being the most common. It is usually advantageous for this lens to have as short of focal length as possible while still having the “reach” so that its focus is at the objective back aperture.

Adjusting the illumination angle can in theory be done by adjusting the tilt of the dichroic, but this is avoided because it changes the illumination position as well (the dichroic is between the “TIRF lens” and objective where its tilt affects both the angle and position of the beam). Rather the lateral position of the fiber tip is adjusted via the **C60-FIBER-LAUNCH**.

It is desirable to mechanically offset the TIRF lens and other upstream components so that the illumination is already offset at the objective's BFP so that only a slight lateral adjustment of the fiber position is required to achieve the desired beam angle at the sample. ASI's **C60-EXT-10-2** provides a 2 mm lateral offset in 10mm axial space; any offset between 0 and 4 mm can be achieved by combining two at different rotation angles. To estimate the target mechanical offset, multiply the objective focal length by 1.4.[3)]. If the aforementioned mechanical offset is not implemented for some reason, is helpful to have roughly 4f spacing between the collimating lens[4)] but implementing the mechanical offset of the illumination path minimizes the amount of fiber tip adjustment and renders the 4f spacing unnecessary.

The SM fiber will create a Gaussian intensity profile at the sample plane. By changing the focal lengths of the collimating lens and the “TIRF lens”, the size of the illumination pattern at the sample plane will be adjusted. Here is a spreadsheet which does some of these calculations:

gaussian_profile_for_tirf.xlsx

. Shorter focal length “TIRF lens” leads to larger illumination patterns, but there is a mechanical limit on reducing the focal length because the lens focal plane has to mechanically reach the objective back aperture.

Ring TIRF

ASI offers scanners originally designed for light sheet that are suitable to implement Ring-TIRF, and ASI offers special firmware with the FAST_CIRCLES module for moving the beam in a circle at high speed and synchronizing this to the camera.

The maximum illuminated FOV at the sample can be calculated by considering the size of the image of the MEMS mirror at the sample. To boost illuminated FOV it is advantageous to:

- have as short of focal length for the “TIRF lens” as possible (but needs to be sufficiently long to “reach” the objective back aperture
- have as long of focal length for the “collimating lens” as possible (but will increase optical path length)
- have as short of focal length for the scan lens as possible (75mm for the traditional light sheet scanner, 39mm for the “Scan-SL”)
- have as large of MEMS mirror as possible (1.2mm and 2.4mm are the standard sizes; the 2.4mm is significantly slower but in our hands fast enough for e.g. 50 frames per second (20ms circle times))

However there is an upper limit to how far the illuminated FOV can be boosted because the MEMS mirror deflection must be able to trace a large enough circle at the BFP so that the beam undergoes total internal reflection.

The ASI Ring TIRF Control plugin can help you operate an ASI based Ring TIRF microscope and it comes with the latest nightly build of Micro-Manager 2.0.

tirf

1)
the fact that the illumination beam is tilted at the sample doesn't matter in this calculation

2)
The divergence in radians is given by the the FOV radius divided by the axial distance to the focal point the not-quite-collimated light; the axial distance to focus is infinity when the beam is exactly focused at the objective BFP. Using the thin lens equation, the distance to focal point resulting from a deviation δ from the BFP is given by EFL_{obj}^2/δ assuming that $\delta<<EFL_{obj}$. Thus the divergence of the light is given by $FOV_{radius} * \delta / EFL_{obj}^2$. The residual divergence must be small enough so that the entire illumination undergoes total internal reflection when the beam is tilted coming into the glass/sample interface, meaning it must be in the TIR range of approximately 65-80°. It can be readily seen that the largest possibility for too-high divergence occurs with high-magnification objectives when imaging large fields of view. In the extreme case of a Nikon 100x objective and 20mm diameter sensor (0.1mm FOV radius), then a 1mm deviation δ in “TIRF lens” placement leads to 0.025 radian divergence or 1.4°. In a more typical case of a Nikon 60x with 10mm diameter sensor, 1mm axial delta leads to 0.0075 radian divergence or 0.4°. Thus a 1mm axial deviation is expected to have negligible impact.

3)
Multiplying by the objective NA gives the outer edge of the back aperture corresponding to the maximum acceptance angle, multiplying by 1.33 is the critical angle for water, and normally the TIRF angle is set between these values. The objective focal length is e.g. 3 mm for an Olympus 60x and 2 mm for a Nikon 100x.

4)
this way adjusting the lateral position of the fiber tip mainly changes the angle of the illumination at the sample and not its position; in an extreme situation the illumination might be displaced off the

central “imaging sweet spot” of the objective lens.