TIRF/TIRFM

C. Michael Stanley, PhD, Chroma Technology Corp

Total Internal Reflection Fluorescence (Microscopy) is a technique that was developed to restrict the background fluorescence and increase the signal-to-noise ratio (s/n) in the resultant images. This is accomplished in TIRF by using the ability of light to create an evanescent wave (or field) at a very limited range within the sample beyond an interface of two substrates differing in refractive index. In practice, this involves imaging a specimen that is in direct contact with a glass slide or tissue chamber. If the angle of the light is greater than the critical angle, this refractive index mismatch will create a field/wave with properties that are identical in frequency to the light. In other words, a fluorescent molecule that would normally absorb light at 488nm can be 'excited' by the electromagnetic field created by a 488nm laser (or other monochromatic source) that is reflected off of the lower refractive index material. Since this field will decay in intensity exponentially with distance, the resultant fluorescence signal will occur in less than 100 nanometers of the surface. This effectively restricts the excitation to and therefore reduces the z-axis signal but that significantly increases the s/n ratio of the sample Of course, this does mean that the molecules of interest must be within that limited range of the evanescent field.

TIRF has been used for some time and in a variety of fields. The initial work done with microscopy was with prisms and beam steering devices to bring the laser/light to the sample from above using an inverted microscope design. The recent developments of ”through-the-lens” microscope systems (via high numerical aperture lenses) have made the application much more available to researchers who do not want to align their systems nor worry about lasers shooting around the room.

The “through-the-lens” approach to TIRF places an added burden on the optical filters since the reflected beam is collected by the objective lens and follows the beampath backwards. This means that the emission/blocking optics not only have to deal with huge amounts of excitation energy, but also high angle scatter and reflections from this light. A typical laser emission filter must block the excitation wavelengths to OD 6, but in TIRF this blocking must exceed OD 8 in most applications (note that many modern spectrometers will not even measure to OD 8 across the full visible spectrum). Our solution is to use proprietary blocking mechanisms and optics to achieve OD 8, even at the higher angles of these systems.

In addition to the added blocking requirements, the dichroic mirrors in these applications must be incredibly stable in reflected wavefront characteristics. Where a standard laser scanning confocal might require only a reflected wavefront distortion (RWD) of less than 2 waves per inch, TIRF applications require less than ½ wave distortion from the reflected surface. This reflected wavefront demand is after substrate coating as the coating process always exerts stresses on the fused silica substrates. This makes producing dichroics that meet these flatness requirements nearly impossible for some coating techniques and for some physical requirements of the substrates (i.e. very thin materials that are still fairly large in overall size).

We have found that using a modified magnetron sputtering coater, coupled with proprietary methods for stress relief and starting with 2-3mm thick substrates at 1/10 wave flatness, we can control the RWD to 1/2 wave. However, we have also had to design and produce our own cubes/mounts/holders for these commercial TIRF microscopes (patent pending), since the standard microscope cubes will not hold these thicker substrates and do not possess the ability to adjustment the dichroic angles that make the TIRF application more efficacious. These new cubes are also designed so that we can adjust the mirror position using a laser jig arrangement for exact positioning. The cubes are also built with an emission port that will accept 2 different blocking/attenuation in series to increase the total blocking efficiencies (see figures 1 and 2, below).

Figure 1: Chroma custom TIRF cube for Nikon Te/Ti series (Chroma part # 91032)

Figure 2: Chroma custom TIRF cube for Olympus BX2 Series (Chroma part # 91041)

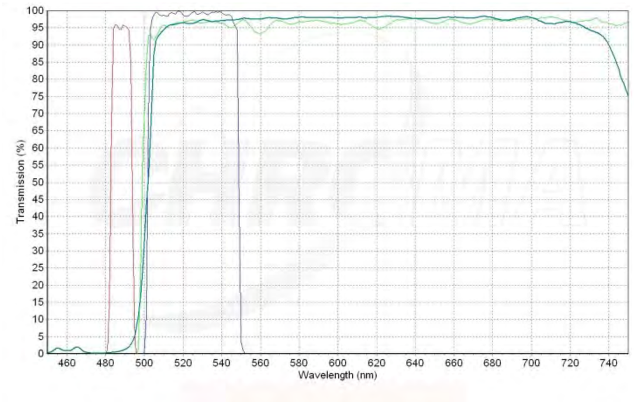

Figure 3: Typical single laser line set. ZET488/10x, ZT488/491rdc-xr, HHQ500lp, ET525/50m.

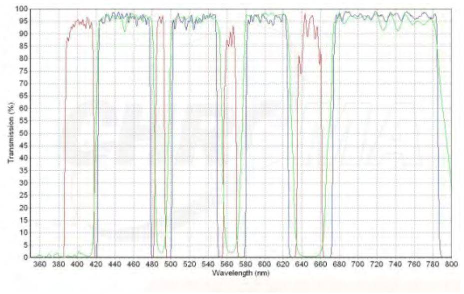

Figure 4: Typical quad set. ZET405/488/561/640-7x, with ZET405/488/561/640-7rdc and ZET405/488/561/640-7m (all installed in cube)

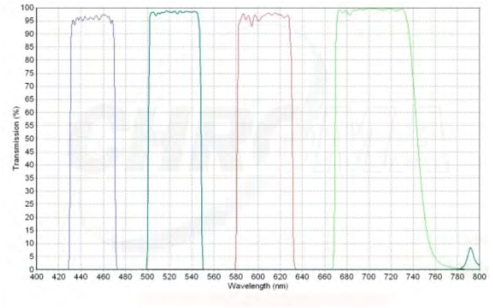

Figure 5: separate single emission filters for quad set above (for emission filter wheel) ET450/40m, ET525/50m, ET605/52m, ET705/72m.