

Multi-Wavelength TIRF Enables Actin Filament Insights

Total internal reflection fluorescence (TIRF) microscopy provides a unique method of imaging isolated molecules and complexes *in vitro*. Additionally, the use of sensitive, low-noise cameras enables researchers to study this behavior in real time. A new plug-and-play method of combining several fiber-delivered, digitally modulated lasers into a single instrument, such as a TIRF microscope, now enables multiple labeled proteins to be imaged pseudo-simultaneously at high frame rates. In this article we see how multi-wavelength excitation is being combined with TIRF in the laboratory of Professor Dyche Mullins at University of California San Francisco (UCSF), and used to gain new insights into complex biochemical interactions that control the stability and function of actin filaments.

TIRF – Single Filament Studies

The Mullins laboratory is widely recognized as a leader in studying actin filaments. These protein filaments are fundamental to many processes in virtually every eukaryotic cell; they act as structural elements that enable movement of internal cargoes, amoeboid cell migration, cell division, etc. With so many different roles and formats, it is not surprising that the growth, branching, aggregation and movement of these filaments involve many subtle control options, mediated by a range of different proteins. Sam Lord, the Microscope Specialist in the Mullins lab, explains, “One area of our research is studying how various proteins bind to actin filaments to enable aggregation, branching and other actions, and more specifically how yet another set of proteins modulates these binding processes. Obviously we do bulk studies in a cuvette that reveal overall kinetic data about these binding processes, but we also want to *image* these processes in real time to study the structural biochemistry. We do this by observing single actin filaments using TIRF.”

In TIRF microscopy, excitation light is introduced into the sample region through a glass slide or coverslip. The microscope optics are configured so that the light hits the glass/sample interface beyond the critical

angle, meaning that all of it will undergo total internal reflection (TIR). However, even with TIR, some of the light electric field, called the evanescent wave, penetrates into the sample by an incredibly short distance (typically around 100 nanometers) beyond the interface. This means that TIRF can be used to selectively excite fluorescence in molecules and complexes that are somehow adhered to the interface. But, because the light does not penetrate into the bulk sample region, it will not excite fluorescence from the huge background of molecules floating free in this medium.

TIRF is thus a three-dimensionally resolved imaging technique. Its xy resolution is limited only by diffraction and/or the camera resolution, but the z axis sampling depth is much smaller than the diffraction limit. And, if there is sufficient signal for fast frame acquisition speeds, the important fourth dimension of time enables dynamic processes such as actin filament-protein binding to be followed on a single filament, or network of filaments, in real time.

In principle, both lasers and non-laser light sources may be used for fluorescence excitation in TIRF. However, for experiments with naturally low signal levels, such as single molecule monitoring, the extreme brightness of a laser beam is a critical advantage. In particular, the laser's unique spatial brightness means that it is relatively simple to collimate and then focus a beam into the sample with a narrow range of incidence angles, avoiding excitation of bulk (background) sample.

Through Objective TIRF

All TIRF microscope setups are based on one of two basic approaches: through-objective lens geometry or the prism-based method. In the former, light is directed in an off-axis geometry through an oil immersion microscope objective so that the angle of incidence at the coverslip/sample interface is greater than the critical angle, as shown schematically in figure 1.

In the prism based method, the orientation of the sample is reversed with respect to the imaging

objective. A light beam is introduced to the sample through a prism attached to the cover slip; the geometry of the prism ensures that the incidence angle at the sample is higher than the critical angle.

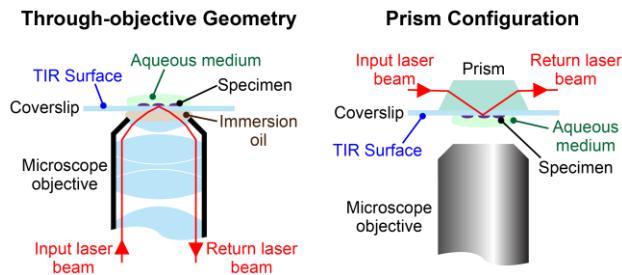


Figure 1 In TIRF microscopy, excitation light beyond the critical light is completely reflected. The evanescence of the light field at the refractive interface penetrates into the sample by about 100 nm causing selective excitation of molecules and complexes adhered to this interface. TIRF microscopes are available with a choice of either through-objective excitation or prism excitation.

There are advantages (and disadvantages) to both methods depending on the type of experiment. For example, the prism method limits physical access to the sample. The Mullins lab uses a Nikon microscope in the through-objective configuration with a very high numerical aperture ($NA = 1.49$) for several reasons, as explained by Lord. “For single molecule studies, fluorescence signal strength is always a major challenge, particularly since we are following processes that need fast frame rates. So we need a high NA objective with a small working distance to maximize light collection efficiency. These objectives require a coverglass of precise thickness and the sample near the top of the coverslip to minimize aberrations. Also we don’t want to introduce scattering and other losses due to viewing fluorescence through the bulk of the sample.”

Multiple “Simultaneous” Laser Wavelengths

The mechanisms controlling the binding of regulatory proteins to actin filaments are quite complex as already noted. To better understand these processes, the Mullins lab is consequently using increasingly sophisticated multi-wavelength TIRF experiments. Lord explains that, “To image multiple fluorophores, we can use either multiple sequenced lasers or a scope

equipped with multiple cameras – we have setups for both arrangements. Multiple excitation wavelengths that sequence at high rates enable us to selectively image multiple differently labelled targets using a microscope equipped with a single high sensitivity camera, and ensures near perfect image registration.”

“When using multiple lasers, the two technical challenges are to perfectly co-align the lasers into the microscope objective, and then to be able to switch between the different wavelengths. In order to follow fast binding processes in real time, we typically must switch wavelengths between alternate camera frames, so that we build up pseudo-simultaneous (interleaved) videos at two or even three laser wavelengths. And we need to do this switching with no undesirable dead time or shifts in the beam path, i.e., without using mechanical shutters or a complex and costly approach such as an AOTF. As recently as five years ago, we simply didn’t have low-cost options to conduct single molecule studies using multiple laser wavelengths pseudo-simultaneously at the requisite frame rates (30 frames/second) in order to follow critical binding processes. For example, using a filter wheel and dichroic turret would involve hundreds of milliseconds for each wavelength change. Digitally controllable diode or solid-state lasers, hardware sequencing electronics and quad-band optical filters make it possible to achieve nearly simultaneous multicolor imaging with a single camera.”



Figure 2 The OBIS Galaxy (shown with the top cover removed) allows plug & play combining of up to eight separate fiber coupled lasers into a single output fiber. Image courtesy Sam Lord.

In 2014, the Mullins lab acquired several digitally modulatable smart lasers to enable multi-wavelength TIRF. These were the fiber-pigtailed Coherent OBIS FP modules at 488 nm, 561 nm and 640 nm. Just as important, they also acquired the OBIS Galaxy, which enables simple plug-and-play combining of up to eight fiber-coupled lasers into one, single-mode output fiber. This passive module enables lasers to be added or subtracted (hot-swapped) to any fiber coupled instrument or setup in a few minutes or less, via standard fiber connectors (FC/UFC and FC/APC).

Because these smart lasers support direct digital modulation, the timing hardware is very simple in the Mullins lab setup. In each experiment, the frame rate is set by the microscope's high-sensitivity camera (Andor model DU897).

The camera's TTL output trigger pulses are processed in a programmable board (Arduino) or ESIO controller (www.esimagingolutions.com) which then directs TTL pulses to fire one of the three lasers with no hardware or software delay. Alternating wavelengths are typically used in most experiments, but any sequence of wavelength frames can be easily programmed using the Arduino and Micro-Manager software [Edelstein, A. D., Tsuchida, M. a, Amodaj, N., Pinkard, H., Vale, R. D., & Stuurman, N. (2014). Advanced methods of microscope control using μ Manager software. *Journal of Biological Methods*, 1(2), 10].

Lord also explains that the flexibility of this arrangement supports future, even more complex, experimental setups. "We may well add a 405 nm laser option in the near future. If/when this arrives, we can simply plug it in and we are ready to go."

Investigating Modulation of Actin Binding Process

In their work on the binding of actin filaments, this flexible TIRF setup enables the Mullins lab to conduct experiments with several different approaches. For example, in typical two-wavelength experiments, the actin filament is labeled with one fluorophore, and the protein of interest is labeled with another fluorophore. The protein fluorophore only appears in the TIRF images if/when it binds to the actin sitting on the cover slip. One use of the third wavelength is to image a second protein labeled with a different fluorophore.

The image sequences then reveal, for example, if the proteins are interspersed at different sites on the filament, or whether the second protein promotes filament growth or branching from a new site, or whether the second protein competitively displaces the first.

In a study just published in Current Biology [Hsiao, J. Y., Goins, L. M., Petek, N. A., & Mullins, R. D. (2015). Arp2/3 Complex and Cofilin Modulate Binding of Tropomyosin to Branched Actin Networks. *Current Biology*, 1–10.], researchers in the Mullins lab used their multi-laser TIRF setup to investigate the details of control mechanisms associated with the binding of tropomyosins to actin filaments. Tropomyosins are coiled-coil proteins whose known function is to bind actin filaments and thereby regulate multiple cytoskeletal functions, including actin network dynamics near the leading edge of motile cells.

Professor Mullins explains, "The binding of tropomyosins to actin filaments is known to be fundamentally important in actin dynamics. But we do not yet fully understand how this binding is regulated, especially near the leading edge of migrating cells. Why, for example, are filaments in the lamellum coated with tropomyosin while filaments in the adjacent lamellipod are not? (The lamellum and lamellipod are distinct actin-based substructures involved in cell migration.)

Before our latest studies, previous work had demonstrated that tropomyosins inhibit actin nucleation by the Arp2/3 protein complex and prevent filament severing by the protein cofilin [Blanchoin L, Pollard TD, Hitchcock-DeGregori SE. (2001) Inhibition of the Arp2/3 complex-nucleated actin polymerization and branch formation by tropomyosin. *Current Biology* 11(16):1300-1304; Iwasa JH, Mullins RD. (2007). Spatial and temporal relationships between actin-filament nucleation, capping, and disassembly. *Current Biology*, 17, 395–406]. So we have recently used TIRF and other methods to investigate if and how the Arp2/3 complex and cofilin in turn modulate the binding of tropomyosins to actin filaments. We have studied these interactions in the specific case of non-muscle *Drosophila* tropomyosin Tm1A. We also compared some of these interactions in Tm1A to the same interactions in rabbit skeletal muscle tropomyosin,

since other researchers had previously found that mammalian skeletal muscle tropomyosin is the least effective Arp2/3 inhibitor [Hsiao, J. Y., Goins, L. M., Petek, N. A., & Mullins, R. D. (2015). Arp2/3 Complex and Cofilin Modulate Binding of Tropomyosin to Branched Actin Networks. *Current Biology*, 1–10.]

Some dual-wavelength excitation TIRF single filament data is seen in figure 3, which shows that Tm1A preferentially binds near the pointed-end of actin filaments. By comparing similar data under different experimental conditions, the researchers showed that pointed-end binding is dependent on the nucleotide state of the actin and the Tm1A concentration.

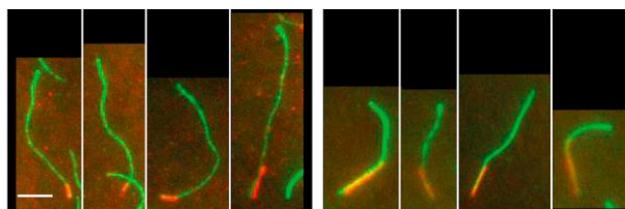


Figure 3 TIRF images showing Tm1A binding preferentially to the pointed end of single actin filaments. The red signal is from Cy5 labeled Tm1A fluorescence excited at 640 nm, and the green signal is due to Alexa488 labeled actin excited at 488 nm. Reproduced with permission from Hsiao, J. Y., Goins, L. M., Petek, N. A., & Mullins, R. D. (2015). Arp2/3 Complex and Cofilin Modulate Binding of Tropomyosin to Branched Actin Networks. *Current Biology*, 1–10.

A full discussion of all the results, conclusions and wider implications from their latest research is beyond the scope of this article. But Mullins summarizes some of the key points, “Binding of cytoskeletal tropomyosin to actin filaments turns out to be more complicated than previously appreciated. Both nucleation and spreading of tropomyosin are strongly influenced by the conformation of the actin filament and the presence of other regulatory proteins. Based on our TIRF images and other data, we have been able to propose a model where the cooperation of the severing activity of cofilin and tropomyosin binding help establish the border between the lamellipod and lamellum.” The role of cofilin in this model is shown in Figure 4.

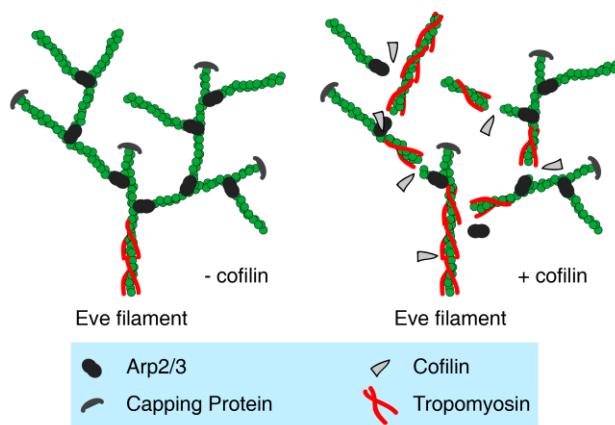


Figure 4 These images summarize the role of cofilin in the model proposed by Hsiao et al [ref]. The branched actin network on the left shows the situation in the absence of cofilin where tropomyosin binding is blocked by Arp2/3 branches in the absence of cofilin. The branched actin network on the right illustrates that in the presence of cofilin, new pointed ends are created, which allows tropomyosin to bind. Once tropomyosin is bound, it protects the actin filaments from further cofilin severing, possibly resulting in the transition from the lamellipod to the lamellum. Tropomyosins. Reproduced with permission from Hsiao, J. Y., Goins, L. M., Petek, N. A., & Mullins, R. D. (2015). Arp2/3 Complex and Cofilin Modulate Binding of Tropomyosin to Branched Actin Networks. *Current Biology*, 1–10..

Conclusion

TIRF is well established as a technique that can image single molecular structures and protein complexes, and that also enables their dynamics to be followed in real time. By providing a method to rapidly switch between two or more excitation wavelengths, the latest lasers and laser-combining technologies are now enabling researchers to perform TIRF experiments with a larger number of separate labels. This capability is already delivering unique insights into important multi-faceted processes in cell biology.

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