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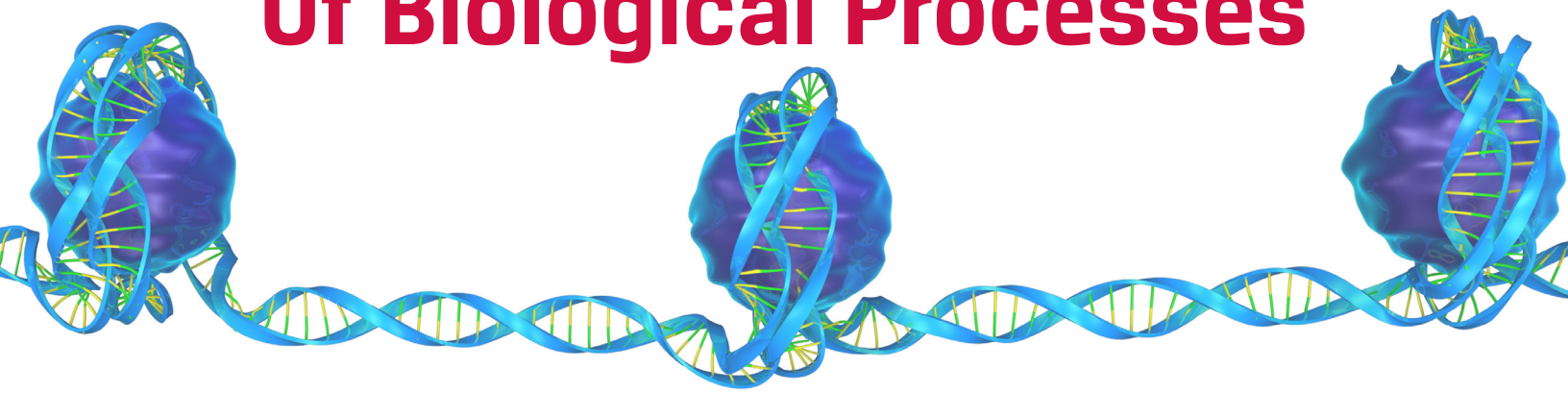
Fluorescence Microscopy Technique Provides New Views Of Biological Processes



Micromirror TIRF Microscopy: Technique and Application

Fluorescence microscopy has long been a powerful tool in biological research. A form known as total internal reflection fluorescence (TIRF) microscopy has more recently been used to watch biological processes unfold in real time. By taking advantage of the ability to label individual molecules with different colors of fluorescent tags, TIRF microscopy now affords scientists a view into the complex molecular assemblies that govern cellular processes. But TIRF microscopy can be limited by its signal-to-noise ratio. As scientists seek to understand ever-more complex processes, they have to label more components with more colors, and this signal-to-noise problem worsens. Fortunately, a newer version of TIRF microscopy that relies on micromirrors allows researchers to view their samples with unprecedented clarity, opening up new vistas in biological research.

Viewing Molecular Assembly

Almost all biological processes — DNA replication, RNA splicing, the opening and closing of ion channels that conduct neuronal signaling — are governed by so-called molecular machines. These machines are transient assemblies of molecular complexes often made up of many components, which come together, perform a task, and then break apart. How are these associations and disassociations ordered and regulated? What happens when these processes go awry? Since these questions are central to an understanding of both normal and pathological cellular processes, scientists are hard at work seeking answers to them.

One way they do this is through a method known as colocalization single-molecule spectroscopy (COSMOS). Imagine a particular molecular machine is made up of four molecules — A, B, C, and D — and each can be labeled with a uniquely colored fluorescent tag. Researchers can anchor molecule A to a microscope slide and wait for the other molecules to join up with it — to colocalize, in other words. There are many ways the molecular machine could assemble. For instance, perhaps C and D have to bind together before they can bind to A, and only after all three are together does B join in. By watching the order in which the various components come together and seeing

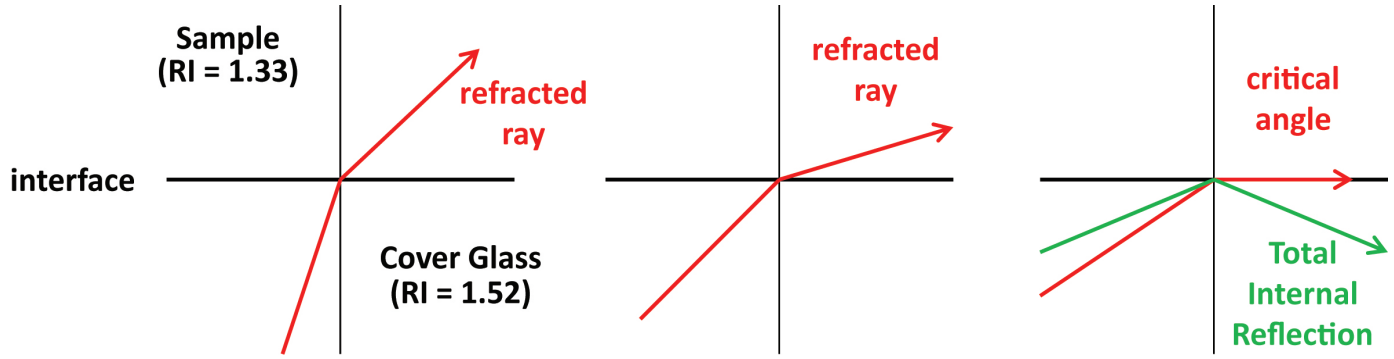


Figure 1. As a light ray passes from the sample medium to a cover glass, it is refracted based on the different indexes of the two materials. Changing the angle of incidence changes the angle of refraction, until the ray reaches a critical angle and undergoes total internal reflection.

how long each step takes, the COSMOS methods allows researchers to gain mechanistic insights into the overall process. The key point here is that TIRF lets them see colocalization in action. If researchers were to shine the various excitation lasers on the whole sample, all the fluorescent tags, even those not associated with the anchored molecule A, would shine at once and the output would just be a colorful haze. Using TIRF to confine the excitation light to a small volume of the sample allows researchers to see the interactions of molecules as they take place. It is critical to understand how this excitation confinement works to understand the unique power of the COSMOS method.

TIRF relies on an aspect of Snell's law, which describes how light refracts, or bends when it passes from one medium to another with a different refractive index. For materials with different refractive indexes, such as glass and water, there is a certain angle at which the light beam no longer passes through the interface but instead reflects back into the material, a phenomenon known as total internal reflection. That angle is determined by the difference between the refractive indexes.

Even though the light is reflected, however, a small amount does penetrate a short distance, perhaps 100 or 200 nanometers, into the second medium, creating what's called an evanescent wave. This is key, as that wave can act as an excitation source, causing tagged molecules within that small volume to fluoresce, while those elsewhere in the sample are untouched, and thus dark. By anchoring molecule A to the glass slide (medium 1), TIRF ensures that molecules B, C, and D will only fluoresce when very close to the slide-water (medium 2) interface, and the COSMOS methods can then be used to monitor the ordered assembly of the ABCD complex.

Loss in Dichroic Mirrors

While TIRF confines the excitation and thus can eliminate the background haze that would otherwise come from molecules outside the evanescent wave, labeling single molecules with fluorescent tags typically give only weak signals. Seeing each uniquely labeled molecule as it assembles into a complex thus poses additional challenges.

The traditional version of TIRF microscopy relies on dichroic mirrors, which reflect some wavelengths of light and let others pass through. In a typical inverted microscope setup, light from a laser beam comes in parallel to the focal plane of the objective lens and strikes a dichroic mirror that is at a 45-degree angle, which reflects the beam up through the lens. To get the correct angle for total internal reflection, the beam must enter the lens at its periphery.

The beam comes out of the objective lens and passes through the sample slide, which is immersed in oil with

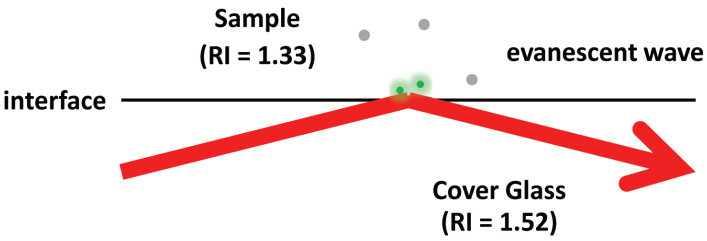


Figure 2. A light ray hitting the interface at the critical angle is reflected back into the cover glass, but produces an evanescent wave in the sample, exciting the molecules that encounter it.

the same refractive index of the slide, then hits the interface between the slide and the sample medium. The beam reflects back into the slide, leaving only the shallow evanescent wave in the sample medium, which excites the fluorescent tags. The wavelengths emitted by the fluorescence come back down through the lens, and the dichroic mirror lets those photons pass through to a detector because they're a different wavelength than the input beam.

Dichroic mirrors consist of layers of thin films of various thicknesses, made from materials with different refractive indexes to select where individual wavelengths are directed. That naturally leads to some photons being absorbed, which decreases the signal-to-noise ratio in the system. More complex molecular machines require a larger variety of fluorescent tags, which in turn means the mirrors must have more layers of thin film to handle more colors. With more material for the photons to pass through, absorption increases and the signal-to-noise ratio worsens. Filters put in place to keep the excitation light from getting into the imaging pathway also cut down on the number of photons that get through to the detector.

A New Approach

The problem of photon loss can be solved by replacing the dichroic mirror with micromirrors, strategically placed near the back aperture of the objective lens, at its periphery to achieve the proper angle of illumination to achieve TIRF. These broadband mirrors are not wavelength-dependent, and because they're tiny they obscure very little of the objective lens. The micromirrors spatially, rather than spectrally, separate the excitation pathway and the emission pathway within the objective lens, and in doing so significantly improve the signal-to-noise ratio. The more colors an experiment uses, the bigger advantage the micromirrors have in terms of increasing signal strength.

In the setup, the input beam passes through the objective lens, the oil, and the cover slide to the interface with the sample medium, creating the evanescent wave. The reflected beam then returns to another mirror, which directs it out of the system so it does not interfere with the signal. An iris placed just downstream of the micromirrors in the emission pathway can also reduce the amount of scattered light and auto-fluorescence from the objective lens entering the imaging pathway.

The end result of micromirror based TIRF microscopy is a much stronger output signal than a system with dichroic mirrors, and it also has the ability to add many more colors of fluorescent tags without degrading the signal.

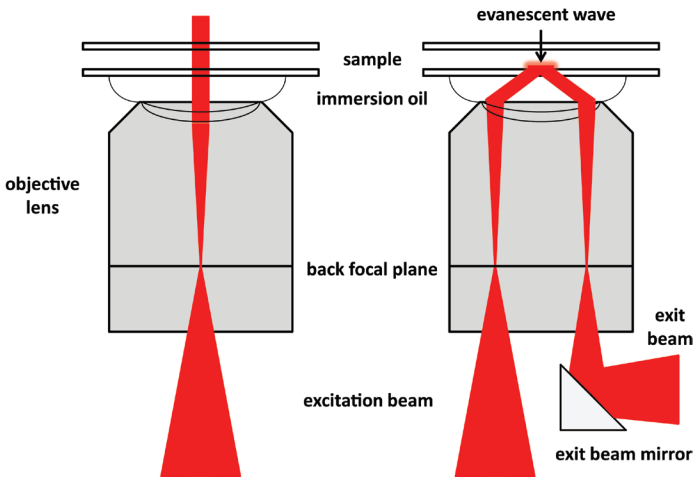


Figure 3. A beam going straight through the focal point of the lens simply passes through the sample (left), but offset to the edge it produces an evanescent wave, while the reflected light is directed out of the system by a mirror.

New Views Of Biology

Micromirror TIRF microscopy is opening up many key biological processes to scrutiny. DNA replication and transcription, RNA splicing, and signal transduction are all performed by complexes of macromolecules that assemble themselves, start a particular process, and then break apart once they've done their job. Often these complexes assemble and disassemble through a number of steps, each of which can lead down different possible pathways. If scientists can observe the construction and deconstruction one step at a time, they can identify which of these pathways is important for correct function and which might lead to damage or disease.

For instance, researchers use micromirror TIRF to study the spliceosome, a multi-subunit molecular machine that edits RNA after it is transcribed from its DNA template. The spliceosome assembles into an ordered structure on a substrate RNA transcript, where it catalyzes the removal of introns — intervening, non-coding RNA — and the splicing together of exons — coding RNA — to form a messenger RNA, which then can be translated into a protein by the ribosome. Micromirror TIRF shows the assembly process in action, allowing scientists to learn more about how it works.

Another area generating much interest is epigenetics, which alters how genes are expressed even though the genes themselves don't change. One key factor in epigenetics involves chromatin remodeling, a processes which involves how DNA wraps around proteins called histones. Being able to view changes in how the chromatin is structured, how it unwraps and remodels itself, can open new insights into epigenetics.

Biologists are learning more all the time about the cellular processes that take place in living organisms. They are combining studies of gene sequencing, protein structure, and epigenetics to form a greater understanding of these processes and move toward an era of personalized medicine. Being able to get a fine-grained picture of how core processes such as replication, transcription, RNA splicing, and chromatin remodeling work in various circumstances can advance their understanding, but it requires tools capable of capturing molecular activity as it takes place.

By substantially increasing the signal-to-noise ratio for multi-spectral TIRF applications, micromirror TIRF microscopy is providing scientists with new insight into the fundamental processes of life. Scientists can learn more about the microscopy by contacting Mad City Labs, the exclusive supplier of the technology.

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