Total Internal Reflection Fluorescence Microscopy *for* Cancer Research

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Background

Total Internal Reflection Fluorescence Microscopy (**TIRFM**) is a unique tool in cancer research, offering high sensitivity and spatial resolution for studying molecular interactions near cell membranes. We present the design and application of a TIRFM system on a Nikon Ti Eclipse microscope, focusing on cancer biochemistry and biophysics. Careful setup optimization ensures exceptional performance for Fluorescence Recovery After Photobleaching (FRAP) and single molecule tracking experiments. To minimize background noise and achieve efficient evanescent wave penetration, special attention was paid to the exact control of excitation light, angle of incidence, and detecting optics. With this configuration, we ran tests to clarify important molecular processes for proteins that are known drivers of cancer. Our findings highlight the usefulness of TIRFM as a flexible instrument for researching cancer biology and provide hitherto unseen insights into the spatiotemporal dynamics of molecular events critical to the initiation and

Introduction

Total Internal Reflection Fluorescence Microscopy (TIRFM) is a cutting-edge imaging technique used to study molecular interactions near cell membranes with high sensitivity and spatial resolution. By employing total internal reflection (Snell's Law), fluorescence, evanescent field propagation, and various object detection algorithms, TIRFM enables precise tracking of individual molecules, such as liposomes and proteins, as they interact with cellular components. This capability is crucial for understanding dynamic processes like molecular diffusion, protein-protein interactions, and membrane dynamics, with significant implications for cancer research and biophysics.

Fluorescence & Stokes Shift

In Total Internal Reflection Fluorescence Microscopy (TIRFM), fluorescence is utilized to visualize molecular interactions near cell membranes. The Stokes shift, where fluorescent molecules emit light at longer wavelengths than they absorb, is crucial for enhancing signal specificity and reducing background noise. Dyes like Texas Red (TxRed) and Oregon Green are commonly used in TIRFM due to their favorable spectral properties, such as strong fluorescence and distinct emission wavelengths. These dyes allow for precise tracking and visualization of molecules near membranes, making TIRFM a valuable tool in cancer research and biophysics.

Instrumentation Research

Our research in instrumentation for biophysics and molecular biology aims to optimize the use of TIRFM by applying principles of applied physics and engineering. We studied applications of various principles in physics to determine the best parameters for optical configurations in TIRFM microscopes, focusing on maximizing efficiency and accuracy. Additionally, we explored methods to enhance the transmission of the excitation laser beam through optical fibers, including the use of index matching gel for power transmission optimization through adapters. Our work also involved CAD design of custom mount system for OBIS LS 488/561 nm lasers with cooling systems to improve overall performance and reliability. Furthermore, we delved into software and algorithm applications for single molecular movement tracking. The right combination of optical fibers, lenses, and channel filters ensures optimal performance, sensitivity, and reliability in tracking molecular interactions near cell membranes. This attention to detail in component choice and system design is key to obtaining meaningful and accurate results in TIRFM, particularly in the context of cancer research and biophysics.

Methods

- The **engineering stage** of our Total Internal Reflection Fluorescence Microscopy (TIRFM) setup involved meticulous optimization and integration of various components to achieve precise imaging capabilities for biophysics research. Initially, we focused on selecting the most suitable objective for TIRF applications on the Nikon TI Eclipse base, ensuring optimal penetration depth and resolution. Laser alignment at 488nm and 561nm wavelengths was meticulously calibrated to achieve accurate excitation of fluorophores. To capture the emitted fluorescence effectively, we identified the ideal filter cube configuration, ensuring that the desired fluorescent emission wavelengths were efficiently transmitted to the Hamamatsu sensitive sCMOS camera. Integration of excitation sources was achieved through fiber optics to minimize power scattering, allowing for consistent and high-quality imaging necessary for our cancer research investigations.
- Performing **FRAP** diffusion measurements on the Nikon TI Eclipse setup involved systematic experimentation with different color lasers and light intensities. Through precise alignment and calibration of the setup, we conducted FRAP experiments to investigate the diffusion dynamics of fluorescently labeled molecules within Support Lipid Bilayers (SLBs). Varied light intensities were applied to study the effects on bleaching efficiency and recovery rates, providing insights into molecular mobility and interactions. The integration of Total Internal Reflection Fluorescence Microscopy (TIRFM) capabilities allowed for enhanced spatial resolution and minimized background fluorescence, enabling accurate quantification of diffusion parameters crucial for our academic research endeavors.
- The analysis of collected data included measuring recovery kinetics and single molecule tracking. Utilizing custom scripts and software, we tracked fluorescence intensity changes over time within the bleached region. This thorough analysis enabled precise quantification of diffusion processes, crucial for our research in cellular membrane dynamics and interactions. Furthermore, our **TIRFM** system's capability to track fluorescence in both green and red channels adds versatility and depth to our studies, particularly in single molecule tracking and understanding molecular interactions.

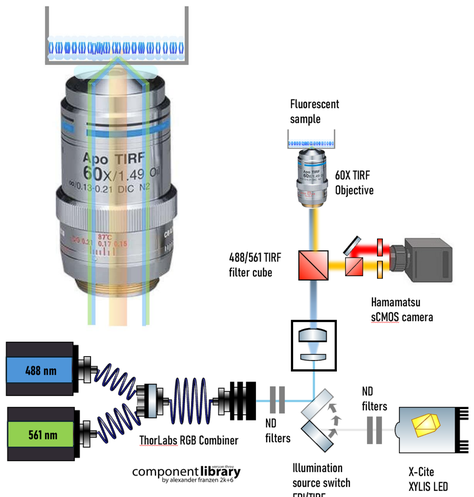


Figure 1. a)TIRFM setup principle diagram
b)TIR effect in 60X TIRF objective

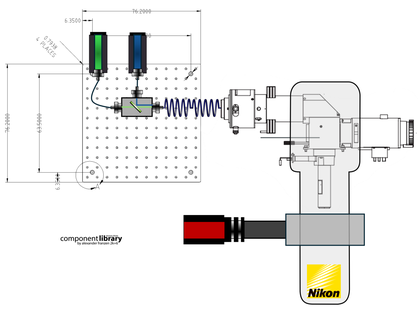


Figure 2. The Optical Setup Design for TIRF Microscope Upgrade (488nm & 561nm excitation system)

Figure 3. Nikon Eclipse Ti-E TIRF Microscope

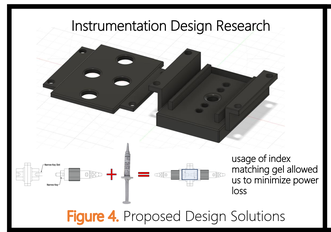
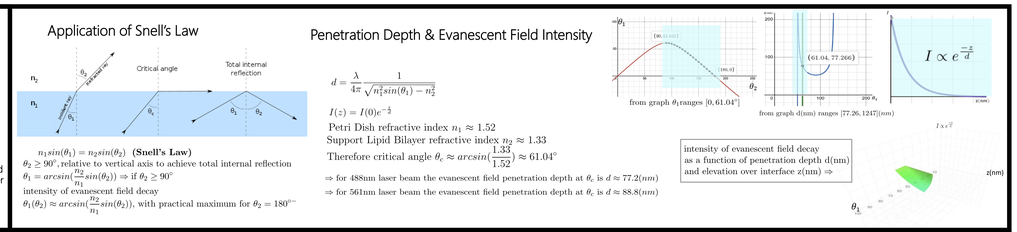


Figure 4. Proposed Design Solutions



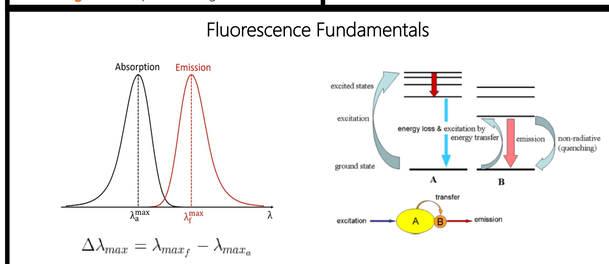


Figure 5. Stokes Shift & Fluorescence Principle

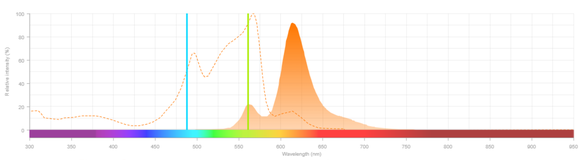


Figure 6. Absorption/Emission Spectrum for Texas Red Fluorescent Dye

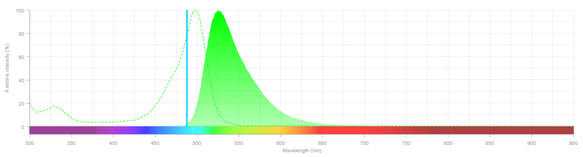


Figure 7. Absorption/Emission Spectrum for Oregon Green Fluorescent Dye

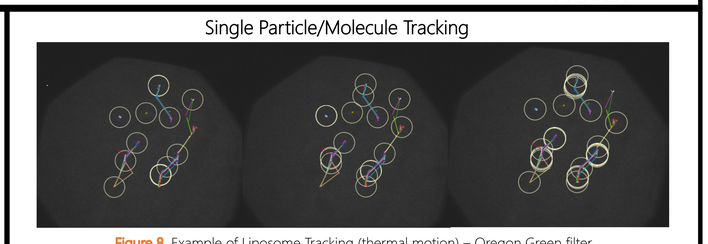


Figure 8. Example of Liposome Tracking (thermal motion) – Oregon Green filter

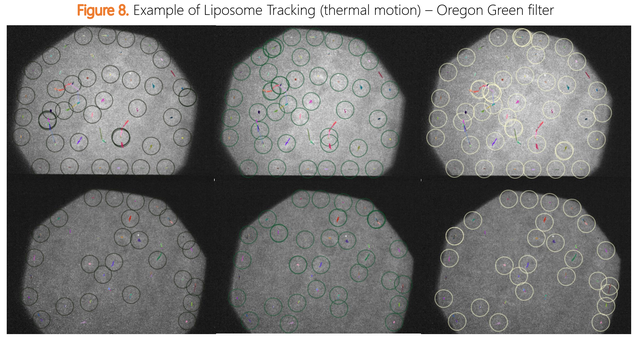
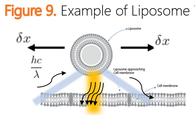


Figure 9. Example of Liposome Tracking (thermal motion) – Oregon Green & TxREed (both channels)



$$\Delta x \Delta p \geq \frac{\hbar}{2} \Rightarrow \Delta x \cdot m\Delta v \geq \frac{\hbar}{2} \Rightarrow \Delta x \cdot \Delta v \geq \frac{\hbar}{2m}$$

No Violation of Heisenberg Principle = Fundamental Limit in Precision

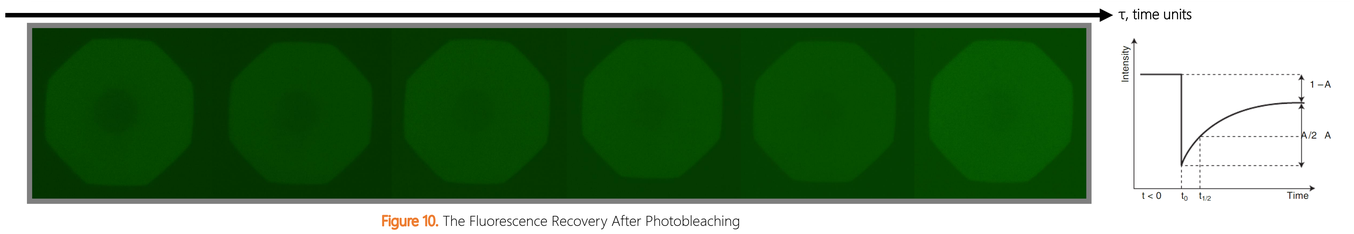


Figure 10. The Fluorescence Recovery After Photobleaching

Conclusion

Our TIRFM system has demonstrated remarkable capability in tracking liposomes, proteins, and other molecules near cell membranes with high accuracy and reliability. Through rigorous experimentation and optimization, we have achieved our goal of establishing a setup capable of performing tracking and Fluorescence Recovery After Photobleaching (FRAP) experiments effectively. Our system also provides room for future upgrades and enhancements to further improve performance. Moreover, we have identified and implemented optimal parameters tailored to our specific TIRFM system, ensuring optimal functionality and usability for ongoing research in biophysics, molecular & cancer biology.

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