Total Internal Reflection Fluorescence for Single Molecule Studies.

PHY 552 Optical Spectroscopy

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Written by

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Abstract:

Since the development of optical instrumentation and data analysis we have opened the door for studying single molecules. One such technique is total internal reflection fluorescence microscopy (TIRFM). This technique has made an impact in single molecule experiments through improvements of high numerical aperture objectives and inverted microscopes. This paper will discuss the development of TIRFM since Daniel Axelrod to the present. Since the development researchers have formulated practical guides for biologists to utilize this technique in their research. Because the relative ease of set-up it is possible for one to learn the technique fairly quickly. However, it is the data analysis that will have your head spinning. Since we are dealing with single molecules, fluctuations in your sample will be prevalent and therefore care must be taken to insure consistent sample behavior. Overall single molecule detection is crucial in shining light on behaviors that were before overlooked due to averaging of a system model.

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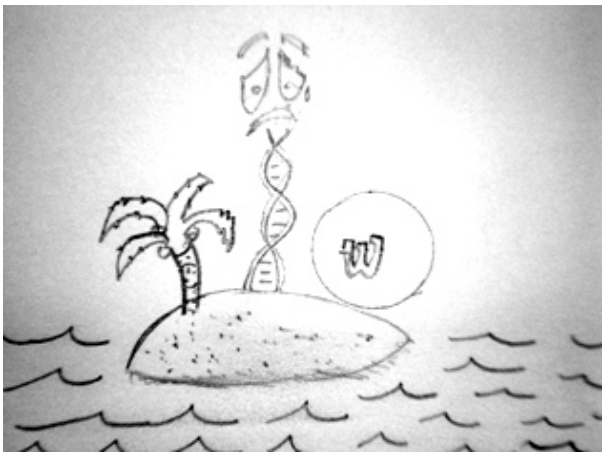
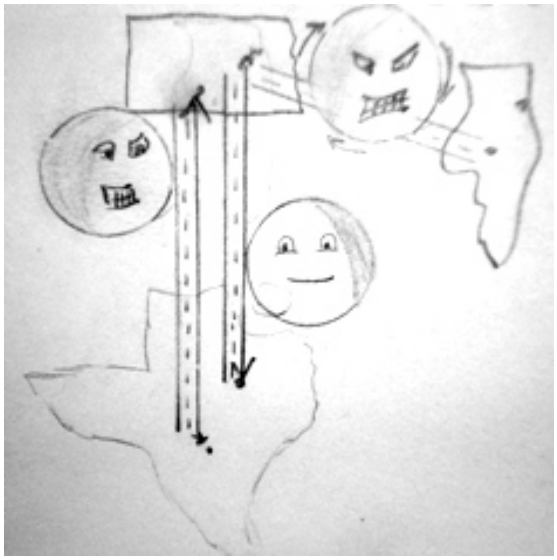
Introduction:

Since the development of optical instrumentation and data analysis we have opened the door for studying single molecules. These studies were at first done *in vitro* and because of the push for understanding biological systems as a whole we have turned to *in vivo* or better correlation between the two(Sako and Uyemura 357-365; Van Holde, Johnson and Ho 710, [27]). At the initial stages of single molecule isolation liquid He was used. Now, that we have revolutionized our designs and incorporated patch clamps, hydrodynamic flow, fluorophores and magnetism. These innovations have enables us to study single molecules at room temperature or physiological temperature (37^0C). With the development of detectors, fluorophores have taken the top drawer in our toolbox. Many fluorophores exist and many more are being discovered and synthesized. By exploiting the energy transitions of these systems with light we can target specific single molecules. The importance of studying single molecules is because nice smooth modeling is the average of a system as a whole(Miller ; Newman). Certain behaviors of a single molecule are shadowed by this averaging and therefore are overlooked(Miller ; Newman). To



1 "Single Molecule Measurement" It is lonely being isolated! At least there is "wilson" to keep him company, just be careful he gets easily excited at 280.

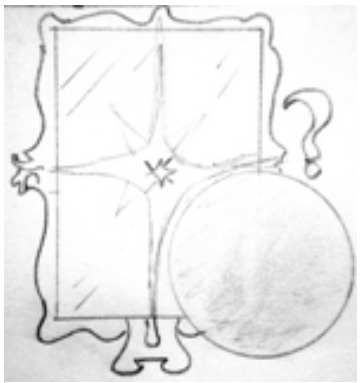


2 "Energy Transition" electrons jumping from "state" to "state". Watchout it looks one has used the "interstate" to "cross" over and now is a different spin "state"!

better understand single molecules total internal reflection microscopy has been further developed.

Total internal reflection (TIR) occurs when incident light passes from a medium of higher to lower refractive index. The refractive index is the property of the material to reduce the speed of light and is equal to the quotient of the speed light divided by the phase velocity. This ratio is used to calculate the angle at which incident light strikes a plane with respect to the normal. This critical angle is the minimum at which total reflection will occur with respect to the medium(s). Everyday uses of TIR include optical fiber and multi-touch screens. We see TIR by looking at the water/air interface when swimming under water. Remember though that this is only seen at certain angles greater than or equal to the critical angle. For water and air the indices of refraction are ~1 and 1.33 respectively. Thus our critical angle is 48.75^0 according to Snell's law.

$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \quad ; \quad \theta_c = \sin^{-1}(n_2 / n_1) \tag{1.1}$$



3 "Total Internal Reflection" – a photon trying to get ready in the morning. Does anyone really know what they look like?

TIR is used in many applications such as attenuated TIR, Fourier transform infrared TIR, and TIR fluorescence microscopy (TIRFM) Total internal reflection fluorescence microscopy (TIRFM) is a technique that utilizes the evanescent wave that is produce by total internal reflection. This technique was first developed from earlier concepts by Daniel Axelrod at the University of Michigan at Ann Arbor. What is interesting is that he actually presented his work at the Biophysical society meeting in 1981 and later published his work that year in the Biophysical Journal(Burghardt and Axelrod 455-467; Axelrod, Burghardt and Thompson 247-268; Thompson, Burghardt and Axelrod 435-454).

With the advancement of microscopes, TIRF has been incorporated into inverted

Early Model of TIRFM

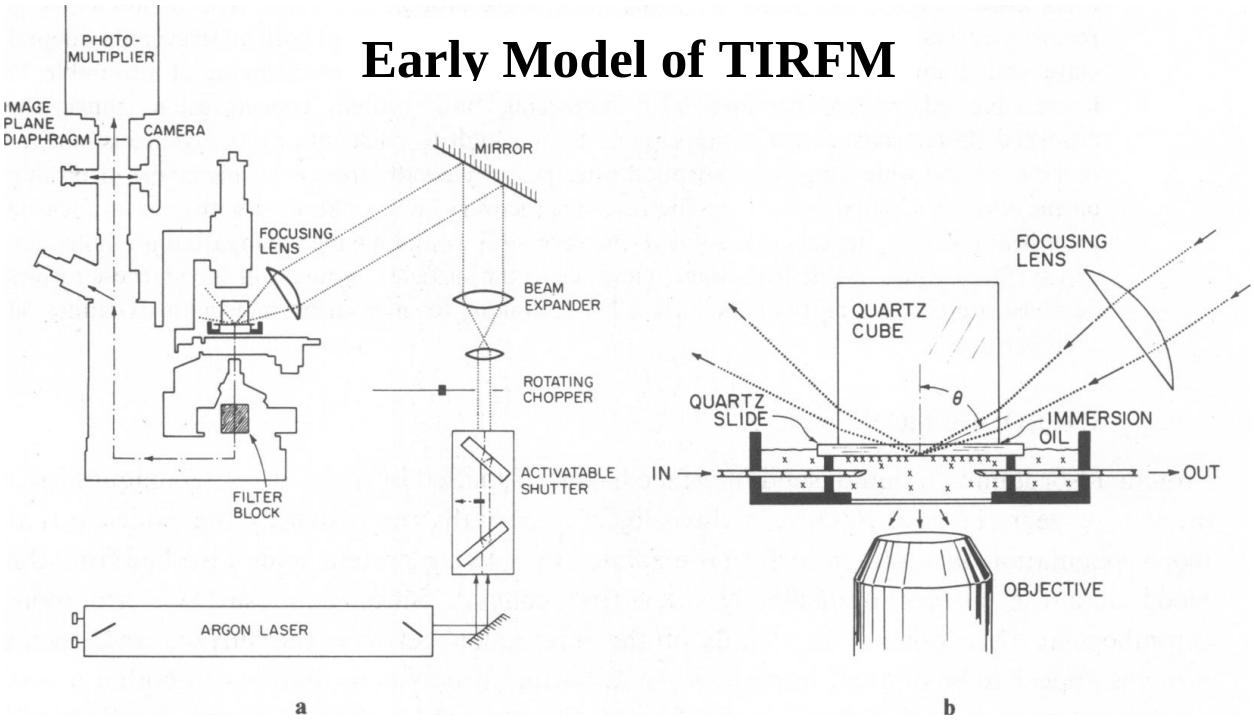


Figure 4(a) Optical apparatus for TIR/FPR. The activatable shutter is based on the design of Koppel (16). The slowly rotating chopper wheel, which transmits the laser beam with a 20% duty cycle, is used during times long after the photobleaching flash to avoid photobleaching during extended observation. The filter block, composed of a dichroic mirror and barrier filter, is identical to one used for fluorescence microscopy by epi-illumination. (b) Detail of the plastic dish in which R-BSA is adsorbed to a quartz slide. R-BSA molecules are schematically indicated by x. The median incidence angle 0 = 750 for these experiments; the critical angle is 0, = 65.40 for this system. The fused Suprasil quartz cube (Precision Cells) is 1.5 cm on a side. The hole in the bottom of the dish is covered with a glass cover slip and sealed in **with encapsulating resin. The objective has a x 10 magnification and 0.25 numerical aperture**(Burghardt and Axelrod 455-467)**.**

microscopes systems. The Olympus IX71 is a system that I will focus on in the scope of this paper. This technique is based on the use of high numerical aperture (NA) objectives. In single molecule detection, attached fluorophores are excited by the evanescent wave that is produce by total internal reflection. A method to distinguish your sample from the background and free fluorophores is flourescence recovery after photobleaching (FRP/FRAP)(Axelrod, Burghardt and Thompson 247-268; Thompson, Burghardt and Axelrod 435-454; Axelrod et al. ; Axelrod 1-33). This analysis separates the single step (bound) and exponential (free) recovery of the fluorophores. For applications towards single molecule detection I will discuss DNA genotyping and Förster resonance energy transfer (FRET), both using TIRFM.

Methods:

For a TIRFM setup either prism (PTIR) or objective (OTIR) based techniques, a couple of things are required. I will briefly go over PTIR and discuss in detail (OTIR) using an Olympus IX71. In the early model of PTIR (Figure 1) a fused quartz cube was used to reflect the light at the sample slide. In order to prevent differences in refraction a fused quartz sample slide was used as well (n = 1.4585). Thus our first requirement is meet assuming that the medium which suspends the sample is similar to water (n=1.33). Therefore the incident beam passes through the cube and slide as if one material and then comes to slide/water interface where it is reflected. The result is an evanescent wave that decays exponential as the distance from the slide increases. The intensity of this wave is given by

$$I(z) = I_0 e^{-z/d} \tag{1.2}$$

Where d is the penetration depth given by

$$d = \frac{\lambda_0}{4\pi}[n_1^2 \sin^2\theta - n_2^2]^{-1/2} \tag{1.3}$$

Previously this wave had a limit of excitation to ~100nm. An evanescent wave penetration depth is independent of the incident light polarization. With advances and affordability of laser systems and inverted fluorescence microscopes this optimal penetration depth currently ranges from 30-300nm(Axelrod et al.). Therefore this technique is beneficial for detecting single molecules or binding to coated slides.

In OTIR set ups the key components is the high NA objective and the immersion oil used. The numerical aperture of an objective is defined as the product of the index of refraction

the lens is surrounded by and the sine of the angle formed by the ray between the focal point that lies on the normal to lens and the edge of the lens.

$$NA = n \sin \theta \tag{1.4}$$

Therefore in this objective a series of lenses are needed to shorten the focal length without having to change the characteristic size of the lens. This allows the diameter of the objective to be small and the focal length smaller. The immersion oil used is to keep the index refraction the same with the glass slide. Also, advances in developing this oil have made it able to filter out auto-fluorescence thus enhancing the single to noise ratio. As similar to the early model the incident excitation beam reflects back through the objective and is filtered out and only emission is reflected back to the eye piece or CCD camera. To target a specific excitation from a mercury-xenon arc lamp a filter tray is used and can be rotated based on experiment specifications. In improvements over the Olympus IX70 have been to separate the power controls from the base of the unit to a separate apparatus(Olympus). This has given a reduction in noise during measurements.

In preparing a single molecule experiment one beneficial component is a micro fluidic device. These devices allow the user to flow cells or single molecules at a controlled rate. In order to make this device one can use a method outline by MicroChem Corporation. A silicon wafer is used to make a mold for polydimethylsiloxane (PDMS). PDMS is a chemical compound found in silly putty. In order to create this device a mask with your custom micro-fluidic channel design must be prepared to protect the area against negative photoresist. A chemical polymer is then spun at certain rpm to gain a certain thickness. After this it is baked to harden the cross linkers in the polymer. It is then put under a UV lamp with the mask protecting the area for etching later. After this light bath, it is submerged in a chemical compound that eats

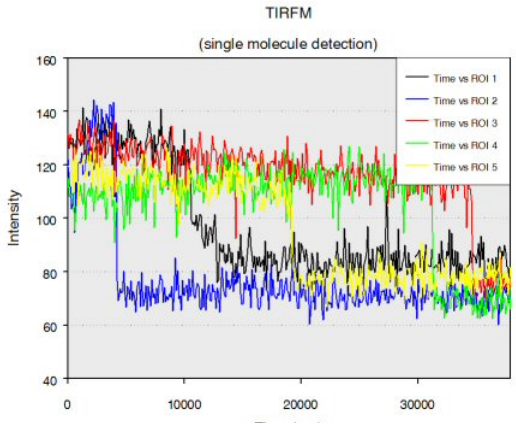
away the area that was not hardened by the UV light. It is then dried off using nitrogen gas and then baked again. This is now your mold for the PDMS. This method is more important for studying systems that have specific requirements to function normally. One specific example is *M. acetivorans* which have all three metabolic pathways for methanogenesis. This is the production of methane. For this particular Archaea it is the production of methane form carbon dioxide and monoxide.

Analysis:

Analysis for these experiments is determined by bulk and surface diffusion coefficients of solute molecules, area of observed region, surface association and dissociation rate constants(Thompson, Burghardt and Axelrod 435-454). Ultimately the excited samples are those attached to the surface of the microscope slide. Simply the reaction is represented by

$$A(\mathbf{r},z,t)+B(\mathbf{r},t)\underset{k_2}{\overset{k_1}{\rightleftharpoons}}C(\mathbf{r},t) \qquad (1.5)$$

where A is the concentration of solute molecules freely diffusing and B is the surface binding site concentration. They form the complex C. After mathematical intuition it can be found the differential equations which govern this system only rely on the concentration of A at z=0.



5 Olympus site
Data courtesy of Jesper Donsmark, University Leiden, NL

Therefore for understanding FRAP we are targeting emission intensity jumps of one step. Instead of free fluorophores which decay exponential as shown by Figure 5. The black data line shows the exponential decay of free fluorophores as opposed to those bound to a molecule. The main obstacle is resolving images obtained from your CCD camera. Image resolution plays a key role in truly calculating the

distance between components. However, it has been shown that one nanometer accuracy is capable with FIONA(Yildiz and Selvin 574-582). With this, resolution to 10nm is possible and also capable in bright field as well.

Examples:

The first example is using TIRFM for genotyping(Ruttinger, Lamarre and Knight 1021-1026). In this article researchers have developed a technique for genotyping single nucleotide polymorphisms (SNP). One in every thousand bases in a human genome experiences a SNP. This alteration could contribute to disease. That is why it is important to exhaust all possible SNP locations to help predict or confront the onset of disease. The experimental design has four principles that are (1) each allele must have a different fluorescent label, (2) the DNA are immobilized on a microscope slide and the density must coincide with the image resolution, (3) using TIRFM with two laser and two filters simultaneously and (4) the frequency of the allele can be calculate by

$$f_A = n_A / (n_A + n_B) \tag{1.6}$$

where f_A is the frequency of allele A and n_A is the number of allele A detected and n_B is the same for allele B and that only these two alleles of SNP are present.

Data acquisition was performed through a five step process, (1) images were averaged, (2) background calculated from mean image, (3) average subtract background, (4) the image was put into a threshold to differentiate between objects and the background, (5) large or too small objects were removed especially those that overlapped with the fluorescent probes and (6) finally the number of objects were counted. This research used a PTIR based instrument to conduct the experiments with two lasers. This count is said to follow a Poisson distribution and that the standard deviation is the $\sqrt{n}$. Therefore the more your sample population the better sound your

value is. In the end they discuss the adaptation of their work into a high through put screen by forming a microarray format.

The second example is Dr. Taekjip Ha's compilation of a practical guide for using single molecule FRET(Roy, Hohng and Ha 507-516). FRET is the ability of a donor molecule to become excited and transfer energy (nonradiative) to the acceptor molecule within ~10nm. This article compares fluorophores and suggests that bright with extinction coefficient > 50,000 M^{-1} cm^{-1} is ideal for single molecule detection. They should also have large separation of emissions and similar quantum yield in order to make a good FRET pair. He reflects on the use of quantum dots (QD) and how methods are used to inhibit their blinking. However, their large size limits their use. Dr. Ha supports the use of Cy3, 5.5, and 7 with some substitutes that offer better photostability. He also advocates the use an oxygen scavenger to enhance and prolong photostability. Some Fret schemes mentioned in the article are two-color, three-color cascade, three-color with independent acceptors, three-colors, and two FRET pairs. I am most intrigued by the three-color which has one donor that can transfer with the second or third acceptor and the second can also transfer with the third acceptor. This would be useful for determining the order of contact within an experiment.

Some limitations to single molecule FRET are that two external dyes are required because intrinsic ones such as tryptophan are not bright enough. Also weakly interacting fluorescent probes are difficult to study. FRET is also insensitive to changes outside the 2-8nm inter-dye range of Förster radius 5nm. To optimize signal to noise ratio at least ~100 photons must be counted. Last by not least the time resolution is limited by the CCD camera. Advances in this technique have to lead utilization of a three fluorophore system to study the DNA four-

way junction also known as a Holliday junction. Also with this method and TIRFM, protein motion on single stranded DNA was observed.

Overall I am excited to learn about TIRFM and hope to utilize it in my future graduate studies. Hopefully, I will further advance the technique through my scientific contributions.

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