BUILDING A SINGLE MOLECULE DEVICE AND MEASURING THE DIFFUSION CONSTANT OF ZSYELLOW

Chem 184, Biological Chemistry. Spring 2008

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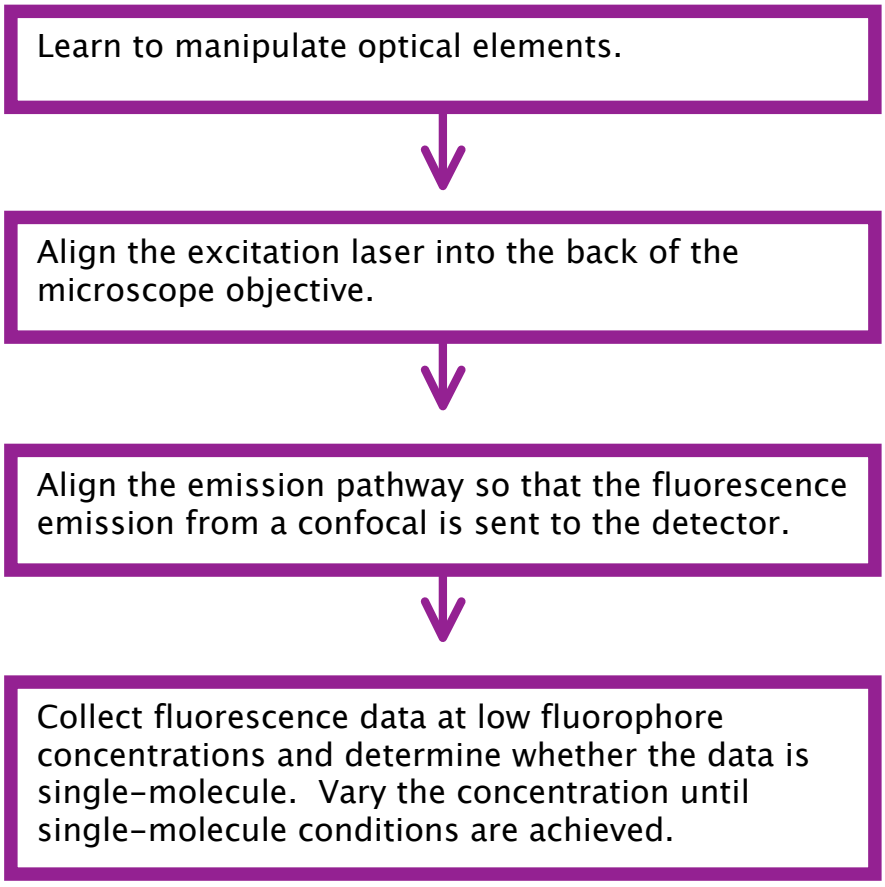
LAB OVERVIEW

The goal of this lab is to align and use an optical detection system that excites fluorescent molecules with laser light and detects the resulting emission with single fluorophore resolution. The system is based around a commercial inverted microscope, though the excitation laser and the detector are both external to the microscope. To achieve the extremely high signal-to-noise required for single fluorophore detection, the emission passes through an extremely small aperture. The aperture behaves as a spatial filter, only passing emitted light that arises from an extremely small volume (~1 fL = 10^{-12} L). This technique is called *confocal microscopy*.

Using the aligned system, you will excite a sample containing the Alexa-488 fluorescent dye and collect fluorescence emission time traces. By varying the concentration of the sample, you will find conditions at which the fluorescence from individual fluorophores can be detected. You will then statistically verify that fluorescent data arises from single molecules.



SCHEDULE

Day	Plan
1	Before lab • Read the Day 1 background and skim through the day's protocols • Read *Tools for manipulation of optical components.* During lab • Review *Laser lab rules* • *Introduction to manipulating light, Exercises 1-4*
2	Before lab • Read the Day 2 background and skim through the day's protocols During lab • *Coarse alignment of the excitation laser*
3	Before lab • Read the Day 3 background and skim through the day's protocols During lab • *Fine alignment of the excitation laser*
4	Before the lab and skim through the day's protocols • Read the Day 3 background and skim through the day's protocols Lab work • *Fine alignment of the emission laser*
5	Before the lab • Read the Day 5 background and skim through the day's protocols Lab work • *Optimizing data collection conditions*
6	Lab work • *Continue data collection*

LASER LAB RULES

1. Always know where your laser beam is! There is a single shutter to the laser that controls both systems. Before you open the shutter – ask the following questions:
   - Does the other group know that I am going to turn the laser on?
   - Do I know the *entire* path of the beam?
   - Have I made excessive use of beam stops?
   - Are there people next-door running DNA gels? Will the laser be directed toward the door?
   - Will the laser reflect/scatter off objects on the table?

The laser is not much stronger than a laser pointer. However, even laser pointers can be damaging to your eyes. Lasers also emit non-visible radiation. While exposure to your skin is not nearly the same hazard as laser exposure to your eyes, minimize this exposure as much as possible.

2. Make optical paths parallel to the table and as low as possible. Be aware of these optical planes, and do not pass your head through these planes – especially when aligning optics.
3. When switching optics in or out, either turn the laser off or block the laser path with a beam-dump. When moving an optical fiber from one mount to another, replace the end-cap.
4. When arranging optical elements on the table, ***always*** secure them to the table.
5. Use common sense.
6. At the end of the day - make sure the laser is off and store all fragile optical elements safely.
7. If you have ideas about different ways to arrange your optics, feel free to explore the *Thorlabs* website to explore their optical elements and mounts: **http://www.thorlabs.com.**

TOOLS FOR MANIPULATION OF OPTICAL COMPONENTS

1. *Screws and wrenches*

Most of the optical elements you will be using attach to the optics table and to each other by hexagonal socket screws. They come in two flavors, *set screws* and *cap screws*, which are pictured below. Note the protruding head of the cap screw and the inset socket of the set screw:

set screw

cap screw

You will use two types of wrenches. The first are *Allen keys* (or hex wrenches), which are L-shaped and have hexagonal ends.

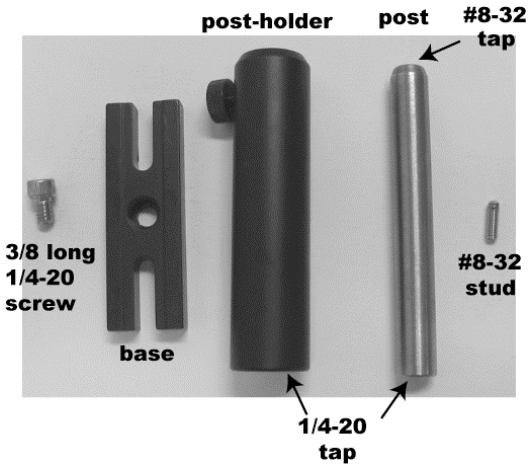
Ball end wrenches are similar to Allen keys except they have a hexagonal "ball" at their end, which makes them useful when you are coming to a socket at an angle.

The following table lists the most common screw sizes you will be using and what wrench to use with each:

	Wrench size (inches)
#8 set screw	*5/64*
¼ set screw	*1/8*
#8 cap screw	*9/64*
¼ cap screw	*3/16*

For a brief description of tap and screw nomenclature, see the following website: **http://www.wikihow.com/Read-a-Screw-Thread-Callout**. Understanding this nomenclature is *not* necessary for the lab.

2. *Post-assembly = post + post-holder + base*



The combination of these parts forms the most common support for optical elements.

A 3/8 inch long ¼-20 screw attaches the base to the bottom of the post-holder. The post rests in the post-holder and is held at a particular height and rotation by tightening the thumb-screw on the side of the post-holder. Most optical mounts can be attached to the tapped hole on top of the post via an #8-32 stud or screw. For example, in the following picture, an optical sensor is attached to the top of the metal post by a stud:

The post-assembly is attached to the optics table by ¼-20 screws passing through the arms of the base into the holes in the table.

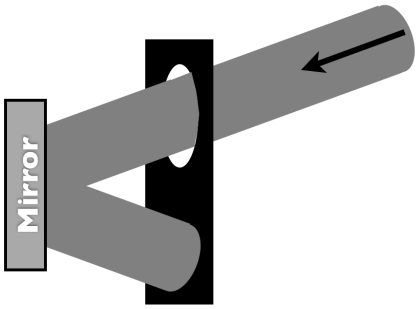
NOTE – It is tempting to attach post-holders directly to the table using a ¼-20 stud. However, using the base to attach the post assembly to the table gives you more freedom to move the optical element around the table without being limited by the positions of the tapped holes.

3. *Business cards wrapped in black electrical tape*

You will be provided with this low budget but very useful means of tracing the path of the laser. You can also use these to look at beam profiles and to see if the beam is falling entirely on the mirrors and lenses. These cards can be attached to post-assemblies using a filter clip (see the picture below on the left).

Particularly useful are black cards with holes, which we call *reflected-beam cards*. These are useful when looking at reflected light that is directed back toward the light source. For example, if you place a mirror in your optical path, this card allows you to look at the reflected beam without blocking the original light, as shown below on the right:

filter-clip attached to a metal post



using a reflected-beam card

When light passes through an optical element such as a lens or filter, there is always some reflected light. This reflected light is referred to as *back-scattered light* and can also be visualized using the *reflected-beam card*.

4. *Beam block*

Beam dumps are effective light absorbers that can be attached to the top of posts. Use these to block light that might be a hazard to others. These are especially useful during alignment when you are manipulating the laser light.

While a black card can also be used as a beam dump, the surface of the black card will reflect and scatter more light than the beam dump.

5. *Post-collar*

Post collars are clamped onto posts using a thumbscrew. These are useful when you want to rotate a post in a post-holder without changing its height. As a precautionary measure, it is a good idea to always attach a collar to an aligned post. If the optical element is bumped, the collar will prevent the height from changing.

DAY 1: INTRODUCTION TO MANIPULATING LIGHT

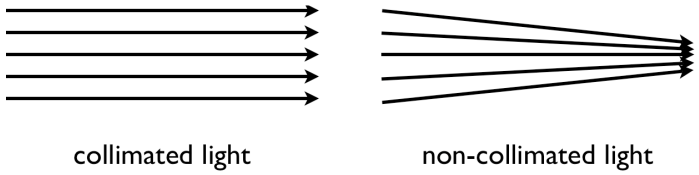
Today you will learn how to manipulate collimated laser light using optical elements. This includes:

- Reviewing laser lab safety.
- Learning how to turn on the laser.
- Using optical mounts to position the laser light on the table
- Using a lens to bend the light, and using the bent light to measure the focal length of the lens.
- Discussing how to leave the lab area at the end of the day.

Background: light, lenses, and mirrors

Collimated and non-collimated light

A beam of light is *collimated* if its rays are parallel. If unperturbed, rays of collimated light will never come together to a point. If non-collimated light converges to a point, the point at which the rays come together is called the *focal point*. Thus, collimated light is said to have its focal point at infinity.

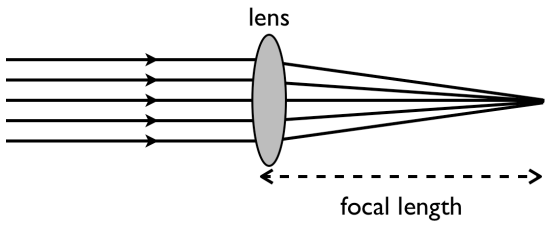


Lenses

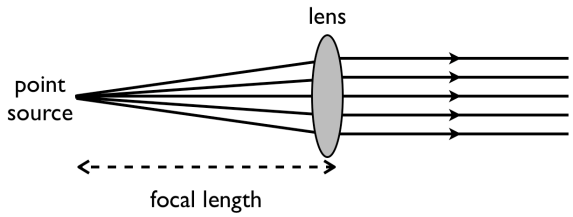
An optical *lens* is a device used to change the convergence of light rays. We will be working with lenses consisting of curved glass, though lenses can also exist in other forms.

The lenses used in our lab convert collimated light into light that converges – and are thus appropriately called *converging lenses*. There are also lenses that convert collimated light into light that diverges (*diverging lenses*), but these are not discussed here. We will use the term *lens* interchangeably with *converging lens*.

Lenses have the property that they bring rays of collimated light together at a single point. The distance from the lens to this point is called the lens' *focal length* (shown below):



Light rays that emanate from a point in space are said to arise from a *point source*. The focal length of a lens can also be defined as the distance that a lens must be placed from a point source to convert the diverging light into a collimated beam. This is depicted in the figure below, which is nearly a mirror image of the above figure.



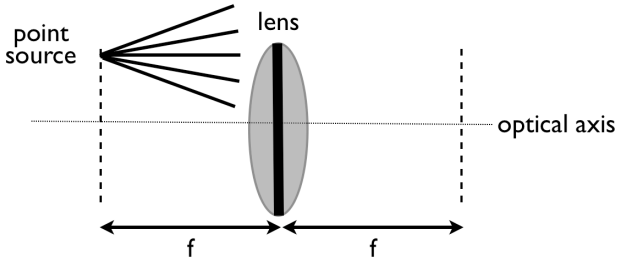
General properties of lenses

We have discussed the deflection by a lens of light that is either (a) collimated or (b) emanating from a light source one focal length from the lens. Using these two specific scenarios, we now seek a more general description of how a point source of light, located at any distance from a lens, is affected.

Up until now, we have only considered a light source located along the *optical axis* of the lens (the axis that is perpendicular to the lens and passing through its center). Let's now consider point sources off the optical axis.

RULE #1: *Light emanating from a point source one focal length (f) away from a lens, but not on the optical axis, is still collimated by the lens.*

PROOF:

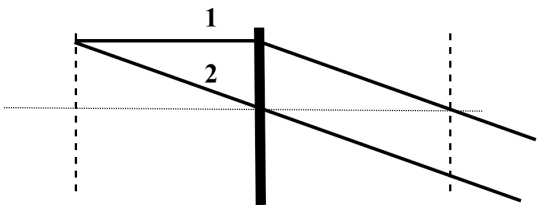


To describe how the lens affects the light, we make two assumptions.

(1) The path of the light is deflected at the exact center of the lens. Correspondingly, we replace the lens with a solid black line that represents the lens' center. In reality, light is deflected both when it enters and exits the lens, but this assumption is appropriate for thin lenses (and is thus called the *thin lens approximation*).

(2) Because the lens is un-curved only at the optical axis, we assume that light passing through the lens at the optical axis is not deflected. Again, this approximation is appropriate only for thin lenses.

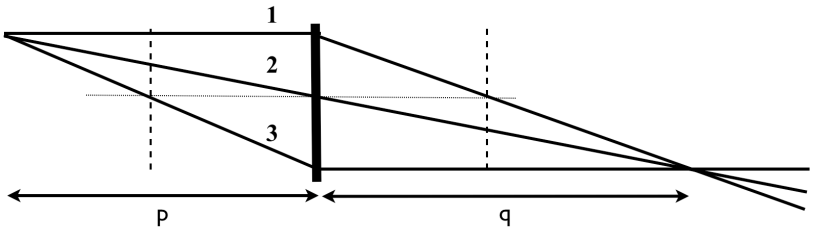
Using these two rules, we can predict the effect of the lens on light coming from the point source by drawing two rays: (1) a ray that travels parallel to the optical axis and (2) a ray that travels through the center of the lens. The former is deflected by the lens and crosses the optical axis at a distance *f* (the focal length of the lens) from the lens. The latter is not deflected by the lens. These rays are depicted below:



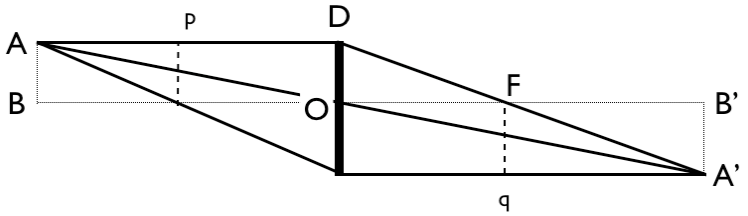
After passing through the lens, the two rays are now parallel. Thus, our two rules allow us to predict that light coming from a point source at a distance *f* from a lens is collimated after passing through the lens, even if the point source is not along the optical axis.

RULE #2: *Light emanating from a point source that is at a distance greater than f from a lens comes to focus at a distance q>f from the lens.*

PROOF: We again draw a ray that (1) is parallel to the optical axis and (2) passes through the center of the lens. We also draw a third ray (3) that passes through the optical axis at a distance *f* from the lens on the same side of the lens as the point source. This last ray is deflected such that it becomes parallel to the optical axis. The three rays are depicted below:



Light at a distance *p* from the lens is brought to focus at a distance *q* after the lens. Using the figure above, we can relate *p* and *q* through the focal length *f*. To do this, we consider the following triangles in our ray diagram:



The triangles with vertices ABO and A'B'O are similar, meaning the vertices have the same three angles and that the lengths of their sides only differ by a scaling factor. This allows us to relate the lengths of the sides AB and A'B' by the relation:

$$\overline{AB} \Big/ \overline{A'B'} = p \Big/ q$$

Similarly, the triangles with vertices DOF and A'B'F are similar, allowing us to relate the sides DO and A'B' by the relation:

$$\overline{DO} \Big/ \overline{A'B'} = f \Big/ q - f$$

Because the sides DO and AB are the same length, we can combine the above ratios to arrive at the relation:

$$p/q = f/q - f$$

This can then be simplified to the relation:

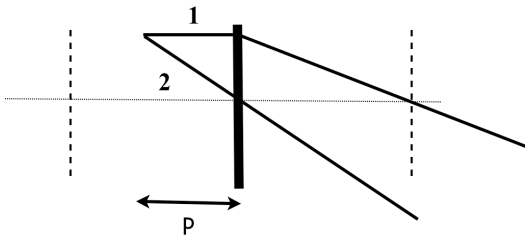
$$\frac{1}{p} + \frac{1}{q} = \frac{1}{f}$$

This is known as the *thin lens relation*.

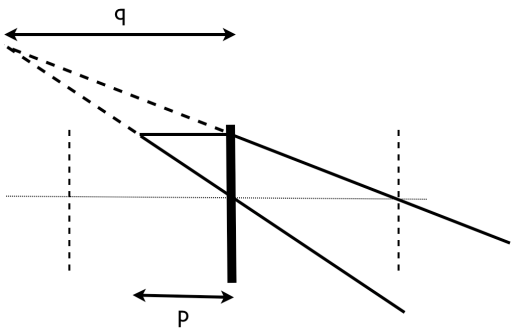
NOTE – when p is equal to f, the relation reduces to $1/q = 0$. In other words, q goes to infinity as p approaches f. Thus, we predict that a point source that is one focal length in front of a lens is focused at infinity – or it is collimated. Our thin lens relation thus also predicts **RULE #1**.

RULE #3: *A point source that is at a distance less than f from a lens is not focused by the lens.*

PROOF*:* Again, we draw a ray emanating from the point source that (1) is parallel to the optical axis and (2) passes through the center of the lens.



The light rays diverge following the lens, never coming together at a focus. Tracing these rays backward, we see that the light behaves as though it had radiated from a different point source on the same side of the lens as the actual point source:



This point from which the light appears to radiate is referred to as a *virtual source*. Using a similar geometrical approach as we used in the previous section, the relation between p and q can again be derived. Though we don't derive it here, you can show that p and q are still related by the thin lens relation:

$$\frac{1}{p}+\frac{1}{q}=\frac{1}{f}$$

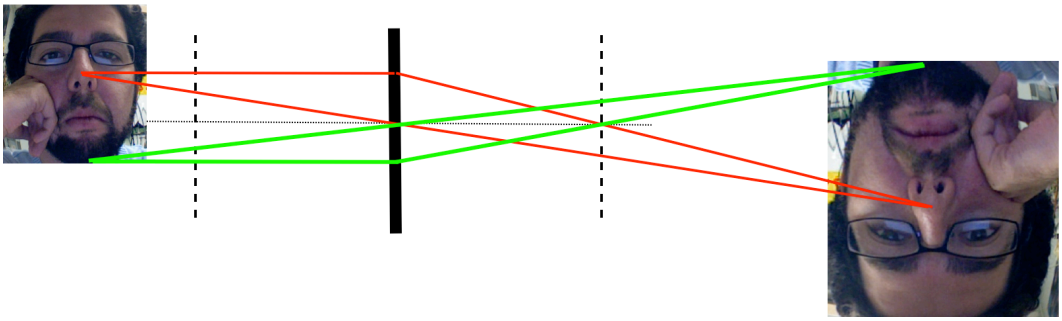
However, because $p < f$, q is negative – meaning that it is on the same side of the lens as the point source.

Imaging objects

Up until now, we have considered how a lens affects rays of light coming from a point source. But, how does this relate to the imaging of an entire object by a lens?

A simple way to consider this problem is to envision that each point in the object is emitting light (or reflecting light from the sun or a lamp). If each point behaves as a point source, we can apply our ray diagram approach to see how the lens affects each point separately.

This approach allows us to determine the effect of a lens on an entire image. For example, in the figure below, we see that an image that is further than one focal length from the lens is magnified and inverted.



Mirrors

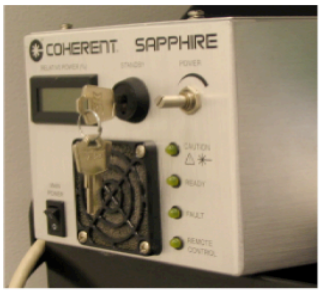
When light strikes a mirror, the angle between the light beam and mirror surface is identical to the angle between the reflected beam and mirror surface:

Turning on the laser

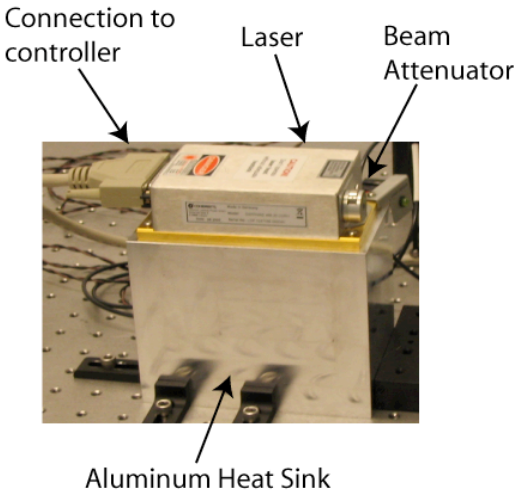
The laser is sent into a laser-to-fiber coupler with 50/50 beam splitter. This device splits the laser into two beams and couples each beam into an optical fiber. Each group will work with one of these two fibers.

The laser has already been aligned into the laser-to-fiber coupler – please be careful; do not knock this out of alignment!

The laser system consists of three parts: the laser unit, heat sink, and controller. The aluminum block on which the laser sits acts as a heat sink. The laser controller is kept off the table. Pictures of these components are shown below:



Laser controller



1. There is a beam attenuator on the front of the laser. Rotate the attenuator to the CLOSED position.
2. Turn the power knob on the controller to zero (counterclockwise). NOTE – the knob turns more than 360°, so continue turning until it stops.
3. Make sure the key on the controller is set to STANDBY.
4. Turn the black switch to the ON setting.
5. Turn the key to ON. On the controller front panel, the light next to CAUTION will turn on, and on the front of the laser, the *laser emission* light will turn on. These lights remain lit while you are using the laser.
6. Allow the temperature to stabilize – this takes about 90 seconds. When the system is stable, the light next to READY on the controller will turn on.
7. Turn the power knob clockwise to increase the power.
    - If the FAULT light turns on, the temperature of the laser was not stable. Turn off the laser, and start again at step 1.
    - The numbers corresponding to laser power on the controller are in units of percentage of maximum power (20 W). For example, a value of 50 corresponds to 10 W. The maximum percentage is 111% (I don't know why!)
8. When you are ready to use the laser, open the beam attenuator.

Because the laser is shared between the two groups – communicate with each other! Make sure everyone knows when the laser is turned on and if the beam attenuator is being opened. Always announce what you are doing with the laser.

Using your optical tools

Exercise #1 – Manipulating a point source of light.

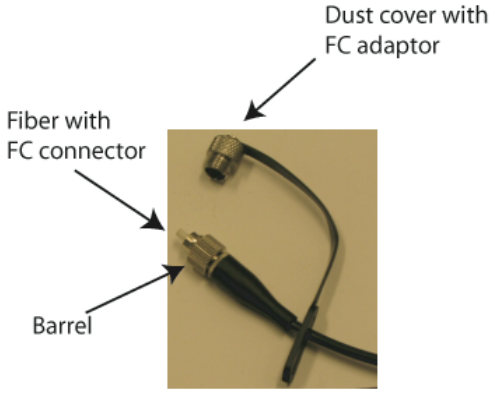
1. Put together a post-assembly with a 2-inch post and 3-inch post-holder.
2. Attach the optical fiber mount with an FC adaptor to the top of the post.

   An FC connector and adaptor are shown below. Note the key on the connector, which fits into the notch on the adaptor. The FC connectors on your optical fiber also contain a threaded barrel that screws into the adaptor to hold the connection in place.

FC connector

FC adaptor

3. At the end of your optical fiber, a dust cover is attached. To remove the cover, unscrew the threaded barrel that connects the FC connector and adaptor.



4. Attach the end of your fiber to the optical fiber mount on the post.

   NOTE – before screwing the barrel into the adaptor, ensure that the FC connector key has slid into the FC adaptor notch. Typically, you can do this by rotating the FC connector until you feel the key slip into the notch. You will hear a click when this occurs.

5. Aim the mounted optical fiber toward a piece of black cloth on the wall. Black surfaces absorb most of the laser light. Avoid looking directly at laser light coming off a white or light surface, which will reflect more of the light
6. Using a black card, follow the path of the laser from the mount to the wall.

QUESTIONS:

(1) Does the end of a fiber optic act as a point source? Why?

(2) What determines the rate at which the light diverges as it moves from the end of the fiber?

Exercise #2 – Manipulating collimated light.

1. Attach the fiber to the FC adaptor on the beam collimator. The collimator is already mounted on a post-assembly for you.

   Whenever you move the fiber, remember to attach the dust cover if the laser is on and the beam attenuator open. Direct the laser light toward the black surface.
2. Using a card wrapped in black electrical tape, follow the path of the laser from the mount to the wall.

QUESTION:

How effective is the beam collimator at collimating light? To answer this, measure the rate at which beam diameter increases as the laser travels away from the collimator. Assume that the beam diameter increases linearly with distance from the collimator. We are only looking for an approximate value here.

Exercise #3 – Measure the focal length of a lens.

You have been provided with two identical magnifying glasses. Meet with the other group working on the lab and discuss ways in which you can measure its focal length using your fiber optic and the tools from steps *Exercises #1 and #2*.

Come up with two methods to make this measurement, one based on the effect of a lens on collimated light and one based on its effect on a point source of light. Have each group perform one of these methods, and compare your results. Do they match? If not, why?

Exercise #4 – Verify the thin lens relation.

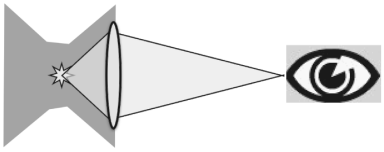
Attach the fiber optic to the mount used in *Exercise #1*, so that the light behaves as a point source. Place the fiber at a distance from the magnifying lens that is arbitrary but greater than the focal length of the lens. Measure the distance from the fiber optic to the magnifying lens and from the magnifying lens to the point where the lens focuses the light. Do your measurements confirm the thin lens relation?

Place the magnifying lens at a distance from the fiber optic that is less than the focal length of the magnifying lens. Make the appropriate measurements that will allow you to verify the thin lens relation.

DAY 2: ALIGNMENT OF THE EXCITATION PATHWAY - PART 1

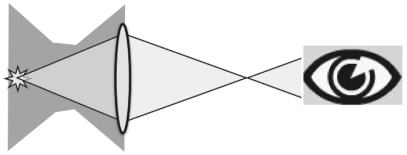
Background: Confocal microscopy

Marvin Minsky patented the principle of confocal microscopy in 1957. On his website (*http://web.media.mit.edu/~minsky/papers/ConfocalMemoir.html*), he provides an article, published in *Scanning*, in which he describes the process of inventing the scanning confocal microscope. A brief description of how confocal microscopy works is provided below:



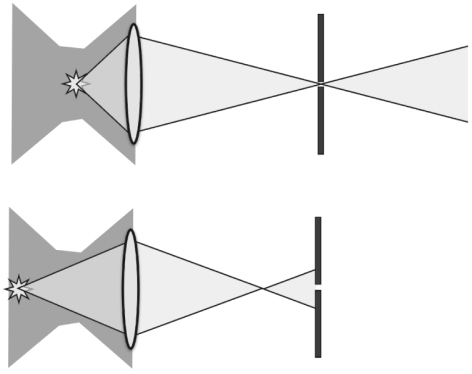
In the figure above, a laser (represented as an hourglass) has been tightly focused by an objective lens into a sample containing fluorescent molecules. The laser light excites a single fluorophore (the star), and the fluorophore emits light upon returning to its ground state. The emitted light is collected by the emission optics (the oval lens) and is directed to the detector (the eye).

The detector has been positioned so that, if the excited fluorophore is at the laser's focus, the emission optics will focus its emitted light at the detector, as depicted above. Light emitted from a fluorophore that is *not* at the focus of the excitation will *not* be focused at the detector:



The out of focus light that reaches the detector results in background signal and thus reduces the signal-to-noise of your fluorescence measurement.

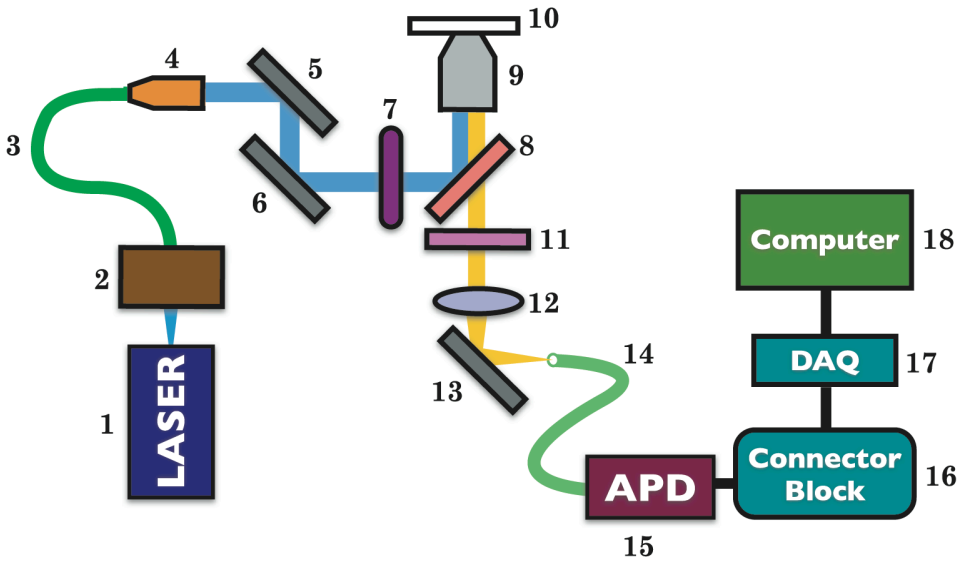
Using a pinhole, we can remove much of this background signal. In the figures below, a pinhole following the emission optics selects for emitted light from the laser's focus. In the upper figure, the light from a fluorophore at the focus passes through the pinhole. In the lower figure, most of the emission from a fluorophore not at the focus is blocked:



By collecting only the light that passes the pinhole, the emitted light from an extremely small region in the sample (denoted a *confocal volume*) is measured. The dimensions of this volume are determined by the width of the focused laser's waist and the diameter of the pinhole. The limit of the former is set by half the wavelength of the laser light (~240 nm for a blue laser).

Your confocal system

The following diagram details the main components of the optical system you will be aligning. This system is capable of imaging fluorescence from a confocal volume through the mechanism described above. The excitation laser light is show in blue and the fluorescence emission is shown in orange.



Components 1-7 comprise the optical path prior to the microscope:

1. Blue laser, Sapphire 448-20 CRDH System (Coherent)

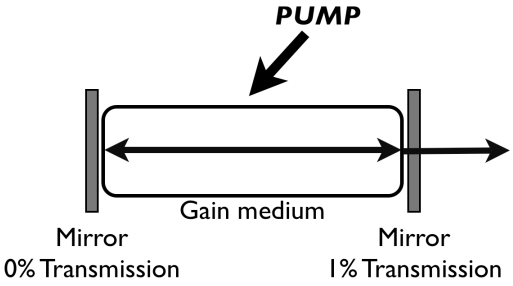
Laser is an acronym for *Light Amplification by Stimulated Emission of Radiation*. A laser primarily consists of (a) an active laser medium (*gain medium*) and (b) a *resonant cavity*. The gain medium is energized (*pumped*) by an external energy source. This energy is absorbed by the gain medium, transitioning some of its particles from a lower energy quantum state into a higher energy, "excited" state.

For a laser to function, more particles must be in the high-energy state than in the low energy state, a condition known as *population inversion*. When this condition is met, *stimulated emission* occurs. The interaction of a photon with an excited particle in the gain medium results in stimulated emission, leading to amplification of the number of photons.

An important characteristic of stimulated emission is that the emitted photon has a similar frequency, phase, and polarization as the stimulating photon. The resulting light, in which frequency, phase, and polarization are identical for all photons, is denoted as *coherent***.**

The gain medium is contained within an *optical resonant cavity*, a cavity that contains mirrors on both sides. One of these mirrors reflects 100% of the light while the other is designed to reflect only 99% of the light. The 1% of light transmitted through this mirror is the output of the laser.

An optical cavity allows photons to pass through the gain medium numerous times before being emitted, thus increasing the photon amplification.



For the laser you will use, the gain medium is a semiconductor that is optically pumped by a diode laser. This results in a continuous wave output of up to 20 mW at 488 nm.

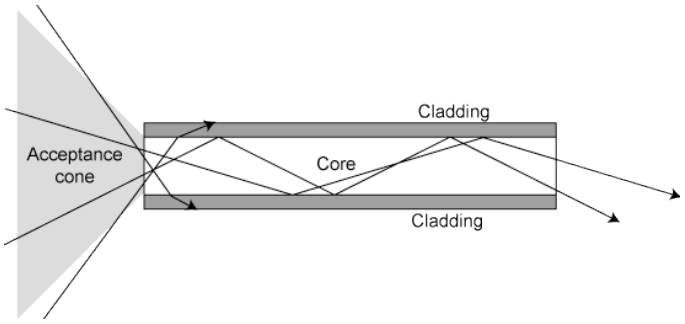
2. Laser-to-fiber coupler with a 50/50 beam splitter (Oz Optics)

This optical element divides the laser input into two outputs of approximately equal power. The two outputs are coupled to optical fibers (only one output is shown in the schematic above). This element allows us to use a single excitation laser for the two optical setups.

3. Multimode optical fiber (Oz Optics)

An optical fiber is a glass or plastic fiber that guides light. For multimode fibers, the refractive index of the outside of the fiber (*the cladding*) is less than the refractive index of the inside (*the core*). *Refractive index* is the measure of how much the speed of light is reduced by the medium.

Rays that meet the boundary between cladding and core at a sufficiently large angle relative to a line perpendicular to the boundary are completely reflected; this phenomenon is called *total internal reflection*. These rays are propagated through the fiber through repeated reflections (see below).



Because rays of light are propagated only if they strike the core-cladding boundary at a steep angle, only those rays within a cone of light at the end of the fiber optic (the *acceptance cone* in the diagram above) are transmitted by the fiber. Notice the two rays in the figure that are not in the acceptance cone, and thus are not totally internally reflected.

Light exiting the end of the fiber is also contained within a cone of light (consider the figure above reversed, from right to left). Because this cone of light radiates from an extremely small opening at the end of the fiber, the end of the fiber is similar to a point source of light.

All the fibers used in the lab are 2 meters long with a 3 mm outer diameter. They are high-powered multimode fibers and terminate with FC/PC connectors at both ends. FC (or *fixed connection*) connectors offer precise positioning of the fiber optic cable with respect to the optical

source emitter and the optical detector. This connection is discussed in more detail in the section: *Introduction to Manipulating Light*. PC refers to the polishing of the fiber, which is denoted as *physical contact* or *polished convex*.

4. Beam collimator (Oz Optics)

Multimode fiber collimator with a 33mm outer diameter and an FC receptacle; used for 400-700 nm light. This optical element attaches directly to an optical fiber with an FC connector and outputs a collimated beam.

5. 1-inch diameter aluminum mirror (Thorlabs).

6. 1-inch diameter aluminum mirror (Thorlabs).

The above two mirrors direct the laser light to the dichroic beam-splitter in the microscope.

7. Neutral density filter (Thorlabs)

This is a glass filter that reduces transmission of all wavelengths equally.

Components 8-12 comprise the optical path within the microscope:

8. Dichroic beamsplitter (Chroma)

This reflects the excitation light into the objective and filters back-scattered excitation light from entering the emission path. The dichroic is part of the *Fluorescence Filter Cube*.

9. 60x oil immersion objective, 1.2 numerical aperture (NA) (Nikon)

The objective serves two important purposes: 1) It focuses the collimated laser beam to a small spot in the experimental sample. The plane where the laser is focused is the referred to as its *focal plane*. 2) It collects fluorescence emission from excited fluorophores in the sample. If the fluorescence arises from the focal plane, the light will be collimated by the objective.

10. Experimental Sample

11. Emission filter (Chroma)

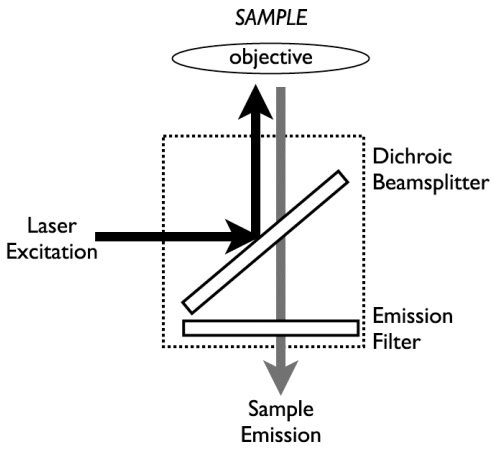
This optical element greatly reduces the amount of excitation light that passes through to the emission optical pathway. This is part of the *Fluorescence Filter Cube.*

12. Tube lens (Nikon)

This focuses the emission light from the sample.

Fluorescence Filter Cube

The fluorescence filter cube consists of the *dichroic beamsplitter* (component #8) and *emission filter* (component #11) in the following configuration:



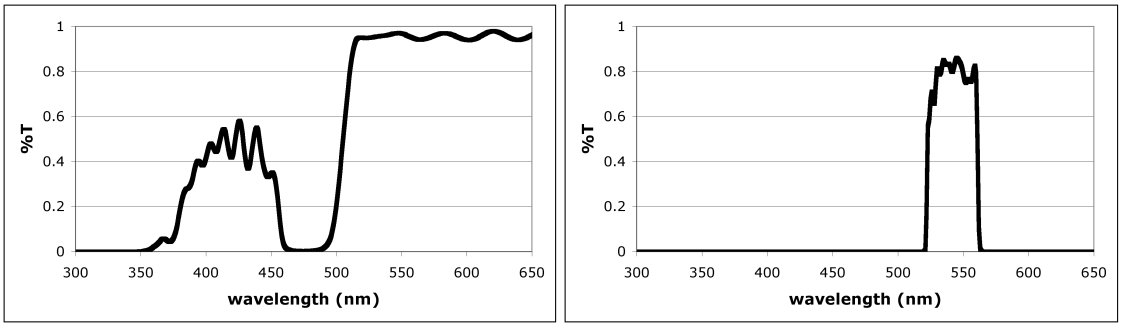
The excitation laser light is shown as a black line and the emission is grey.

The *dichroic beamsplitter* is a thin piece of coated glass set at a 45-degree angle to the optical path of the microscope. The coating reflects or transmits light, depending on its wavelength. For a typical fluorescence microscope, the excitation light is reflected and the fluorescence emission is transmitted. Current dichroic beamsplitters allow for 90% reflectivity of the excitation and 90% transmission of the emission. A plot of the transmittance of your dichroic beam splitter (HQ540/40) as a function of wavelength is provided below.

The *emission filter* transmits fluorescence emission and blocks any excitation light that has passed to the emission path as a result of scattering or reflection from the sample. Either band-pass or long-pass filters can be used as emission filters. A plot of the transmittance of your emission filter (z488RDC) as a function of wavelength is provided below. This is a band-pass filter.

Often, filter cubes also contain an *excitation filter* that transmits only light of a particular wavelength to the dichroic beamsplitter. However, because we are exciting with laser light, which is comprised of a narrow range of wavelengths, this is not necessary for our setup.

Transmittances of the z488RDC dichroic beamsplitter (left) and HQ540/40 emission filter (right)



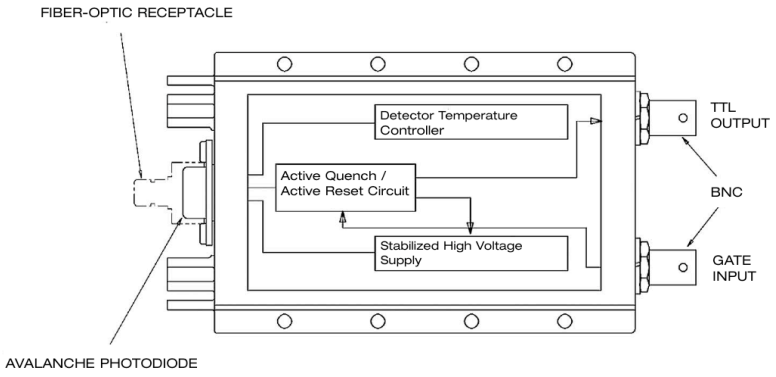
Components 13-15 comprise the optical path following the microscope:

13. *1-inch diameter aluminum mirror (Thorlabs)*
14. *Multimode optical fiber (Oz Optics)*

15. Single-photon counting module, SPCM-AQR avalanche photodiode (PerkinElmer)

An avalanche photodiode (APD) is a photodetector – it converts a photon signal to a measurable electrical signal.

Our detector is a single-photon counting module containing an APD. The APD is located at the head of the detector, directly following the optical fiber input. The bulk of the detector, however, consists of a temperature control module and an electric circuit that converts a single photon signal into a digital output voltage pulse (~2.5 V) that is sent to the detection computer. A block diagram of the detection device is shown below. The 2.5 V pulse exits the detector from the BNC connector labeled as *TTL output*.



Components 16-17 are responsible for *data acquisition*:

National Instruments describes data acquisition as "the process of gathering or generating information in an automated fashion from analog and digital measurement sources such as sensors and devices under test. Data acquisition uses a combination of PC-based measurement hardware and software to provide a flexible, user-defined measurement system."

16. BNC-2121 Connector Block (National Instruments)

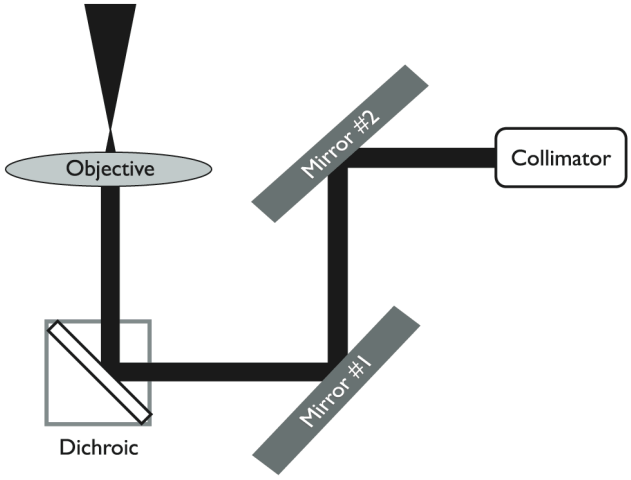
The connector block is connected to the single-photon counting module. It transfers the detection output of the counting module to the data acquisition board.

17. PCI-6040E Real Time Multifunction Data Acquisition (DAQ) Board (National Instruments)

18. Desktop computer for data collection and analysis (Dell)

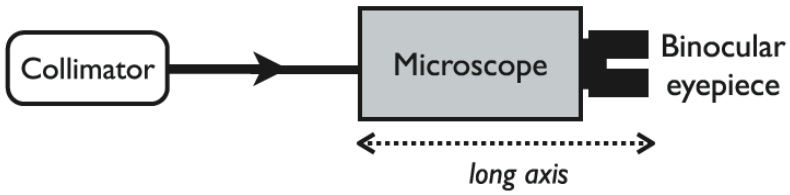
Coarse alignment of the excitation laser

GOAL – Use two mirrors to guide the collimated excitation laser light into the microscope and through the center of the objective. The focused beam should pass perpendicular to and through the center of the objective lens, as depicted below. The excitation laser, which emanates from the collimator, is shown in black.



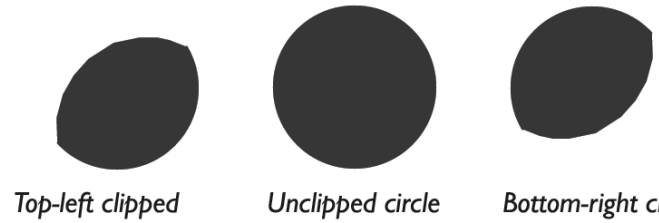
Step 1: Adjust the height of the collimated laser source.

1. Prepare the microscope and laser for the alignment:
   a. Rotate the microscope nosepiece to the 10x objective.
   b. Make sure the filter cube is in position to direct the laser light into the objective.
   c. Make sure the metal stage with with the smaller aperture is in place. Adjust the x-y position of the stage so the aperture is directly over the objective.
   d. Turn the laser on, but keep the shutter closed.
2. The collimator is held in a mount attached to a post-assembly. Place the assembly on the table as far behind the microscope as possible. Direct the beam toward the filter cube. Roughly, the light beam should be parallel to the long axis of the microscope and parallel to the surface of the table.



QUESTION – Why do we want the collimator as far as possible from the objective during the alignment?

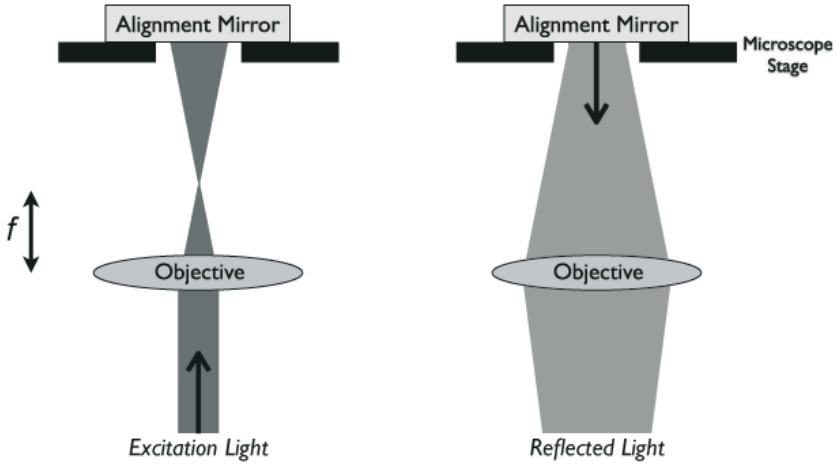
3. Adjust the position of the post-assembly on the table and the height and rotation of the post until you see the collimated beam passing through the objective. The laser will be visible on the ceiling above the objective as a blue, speckled circle. Unless you are very lucky, the blue circle will be partially clipped. For example, you may see one of the following:



Clipping indicates that not all of the beam is falling on the dichroic. Continue your adjustments to remove as much clipping as possible.

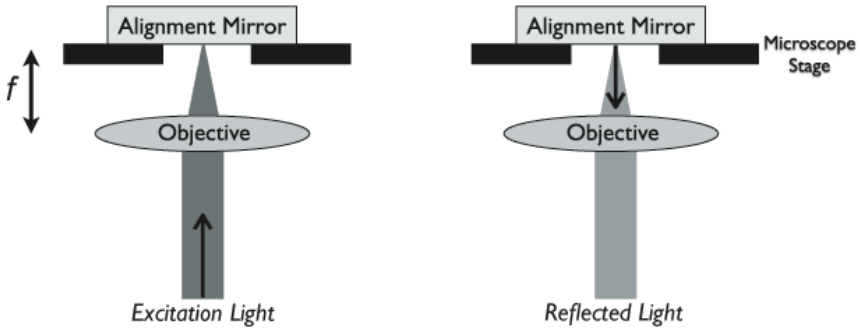
4. Using the two thumb-screws on the collimator mount, remove any remaining clipping of the blue circle.
5. Place an alignment mirror upside down on the stage to reflect the light back toward the dichroic. To view the reflected beam, place the *reflected-beam card* in front of the collimator, making sure the collimated light passes completely through the hole in the card.

The distance between the alignment mirror and objective will affect what you see at the *reflected-beam card*. In the picture below, the excition beam is shown on the left, and the corresponding reflected beam is shown on the right (arrows indicate the direction of propagation).



The reflected beam is *not* re-collimated by the objective because the distance between the mirror and objective is greater than the focal length of the objective (*f*).

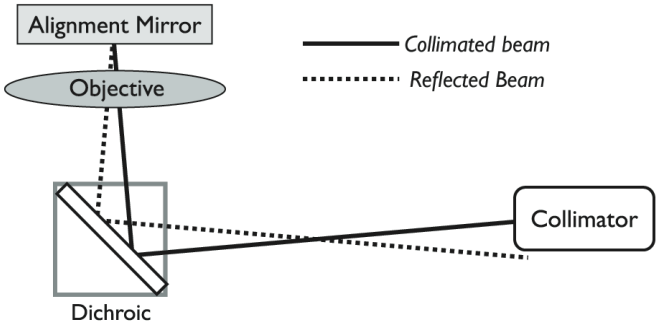
However, if the mirror is placed one focal length away from the objective, the objective will re-collimate the reflected beam:



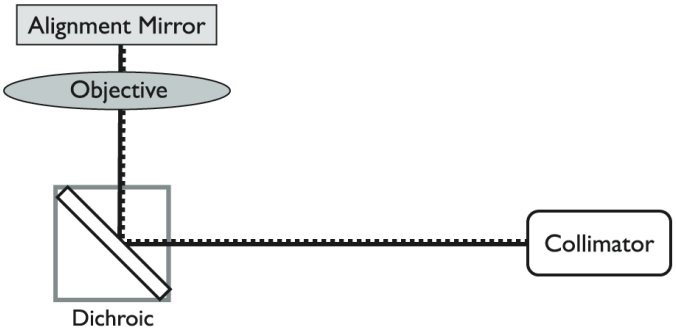
As a result, the beam profiles of the excitation and reflected beams will be the same size.

6. Adjust the distance between the alignment mirror and objective using the microscope's focus knob. Move the mirror one focal length from the objective, so that the profiles of the excitation and reflected beams are similar in size.
7. Adjust the two screws on the collimator-holder until the incoming beam and the reflected beam overlap completely. This ensures that the beam is passing through the center of and perpendicular to the objective:

UNALIGNED



ALIGNED



NOTE – as you adjust the thumbscrews, you may have to adjust the *reflected-beam card* to ensure that the laser still passes completely through the hole.

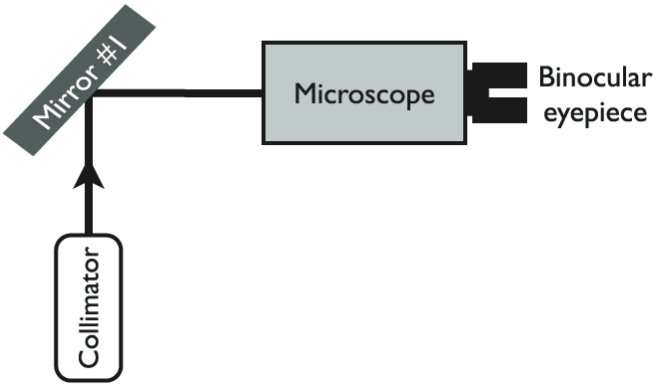
The excitation beam is now horizontal to the surface of the table, and the collimator is at roughly at the same height as the dichroic. Use a C-clamp to fix the height of the collimator.

For the remainder of the protocol, do not alter the height or tilt of the collimator holder.

8. Place a *reflected-beam card* directly behind the microscope so that the laser beam passes entirely through its hole. You will use this card as an aid for the rest of the alignment - be careful not to disturb it.

Step 2: Coarse Alignment of Mirror #1.

1. Close the shutter on the front of the laser.
2. Mirror #1 is in a mount attached to a post-assembly. Place the mirror and collimator behind the microscope in the following arrangement:



   Place the mirror 2-3 feet behind the microscope, and put as much distance between the collimator and mirror as possible.

3. Roughly, adjust the positions of Mirror #1 and the collimator on the table so that:
   - The path of the beam toward the dichroic is parallel to the long axis of the microscope.
   - The mirror is at an angle of ~45° relative to the collimated beam.
4. Open the shutter on the front of the laser, and make sure the collimated beam strikes the mirror's surface.
5. Adjust the height of the mirror so that the laser light strikes the mirror at its center height (don't worry about side-to-side alignment yet). Use a C-clamp to fix the mirror's height.
6. Adjust the thumbscrews on the mirror's mount until the light reflected off of it is at the same height as the hole in the *reflected-beam card* behind the microscope (don't worry about side-to-side alignment yet).

The height and tilt of the mirror are now coarsely aligned.

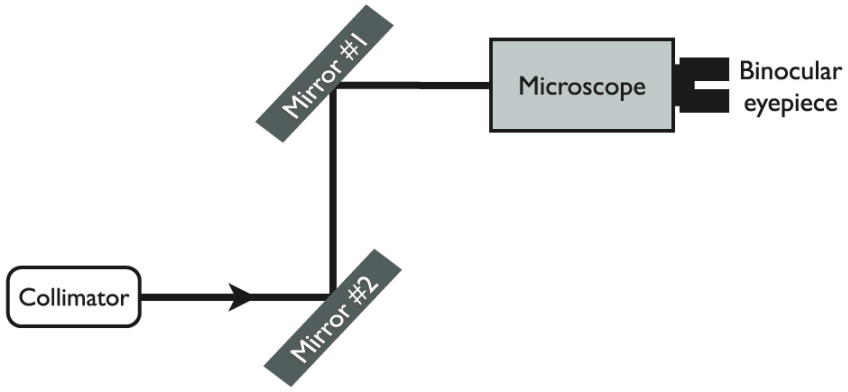
7. Adjust the positions and rotations of Mirror #1 and the collimator so that the light reflected off the mirror passes completely through the *reflected-beam card* and forms a blue circle on the ceiling. Remove as much clipping of this blue circle as possible.
8. Adjust the screws on the mirror mount to remove any remaining clipping.

9. Place a mirror upside down on the stage to reflect the laser light back toward the dichroic. Using a second *reflected-beam card* and the focus-adjust knob, move the mirror one focal length away from the objective. Follow the same procedure as you used in *Step 1*.
10. Adjust the screws on the mirror mount so that the excitation and reflected beams overlap completely. This ensures that the excitation is perpendicular to the objective and passes through its center (see the figure at the end of *Step 3*).

Mirror #1 is now coarsely aligned. Make sure the beam profile is not clipped anywhere alng the light path. If it is, adjust the position of the mirror mount appropriately and repeat the alignment.

Step 3: Coarse Alignment of Mirror #2.

1. Close the shutter on the laser.
2. Mirror #2 is in a mount attached to a post-assembly. Place Mirror #2 and collimator behind the microscope as shown below. (Don't move Mirror #1, or it will no longer be aligned):



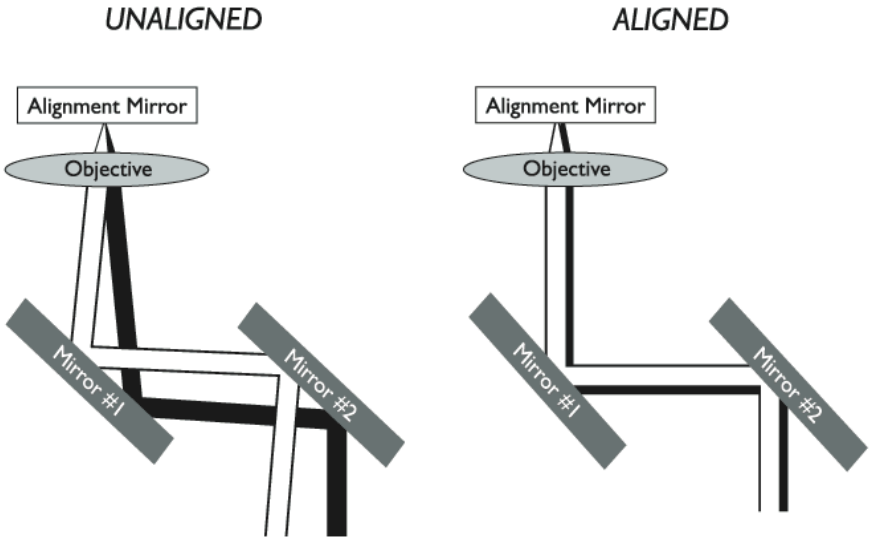
The distance between the two mirrors should be about 2 feet, and the distance between the collimator and Mirror #2 should be as long as possible.

3. Roughly, adjust the positions of Mirror #2 and the collimator on the table so that Mirror #2 is at an angle of ~45° relative to the path of the laser.

Repeat steps 4-10 of *Step 2: Coarse Alignment of Mirror #1*, adjusting Mirror #2 instead of Mirror #1.

Mirror #2 is now coarsely aligned. Make sure that the beam profile is not clipped anywhere alng the excitation path. If it is, adjust the position of the mirror mount appropriately and repeat the alignment.

Using a mirror to aid in the coarse alignment of the excitation laser:
The collimated excitation beam (black) is directed to the objective lens by two mirrors. The objective focuses the beam, which is then reflected back by a mirror. The reflected beam (white with black border) is re-collimated by the objective and is directed away from the objective by the two mirrors. For clarity, the filter cube and dichroic have been omitted from the diagram.
On the left, the incoming excitation beam is misaligned; it is not perpendicular to the objective lens. As a result, the reflected beam does not return along the same path as the excitation beam.
On the right, the incoming excitation beam is aligned. As a result, both beams traverse identical paths.



Step 4: Add a neutral density filter wheel to the optical path.

To allow both groups to independently adjust the power of their excitation laser, a neutral density filter wheel will be placed in the excitation pathway. The fraction of light transmitted by a neutral density filter is described by its optical density *d* according to the relation:

$$Fractional\ \ T = 10^{-d}$$

Your wheel (see the picture below) contains 6 ports. One part is kept empty, allowing for 100% transmission (d=0). The other ports contain filters with optical densities of d = 1, 2, 3, 4, and 5.

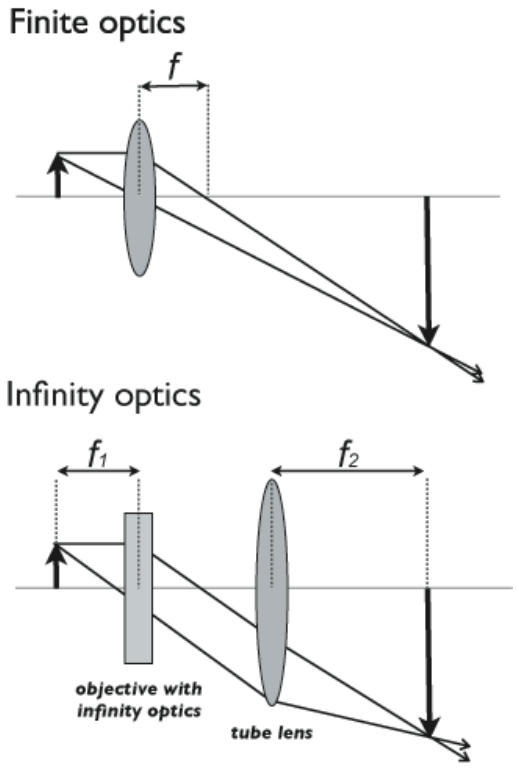
Place the neutral density filter wheel, which is already attached to a post assembly, in the excitation pathway. It is not important where the filter wheel is placed since it will not perturb the collimated beam. Adjust the height of the post so that the light passes through one of the ports. The filters will result in back-scattered laser light – be aware of where this reflected light is going.

DAY 3: ALIGNMENT OF THE EXCITATION PATHWAY – PART 2

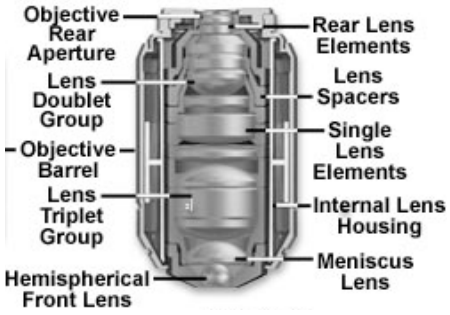
Background: The optical train of the microscope

The infinity optics of the objective

The objectives you will use (and most objectives used today) contain *infinity corrected optics*. This means that light collected by the objective from the sample plane is collimated (i.e. focused at infinity). A sexcond lens, called the *tube lens*, then focuses the collimated light at the tube lens' focal length. As a result, the magnification of the image is determined by both the focal length of the objective and tube lens. The figure below shows how an image is formed by *finite optics*, consisting of a single lens with focal length *f*, and *infinity optics*, consisting of an objective with focal length f_1 and a tube lens with focal length f_2:



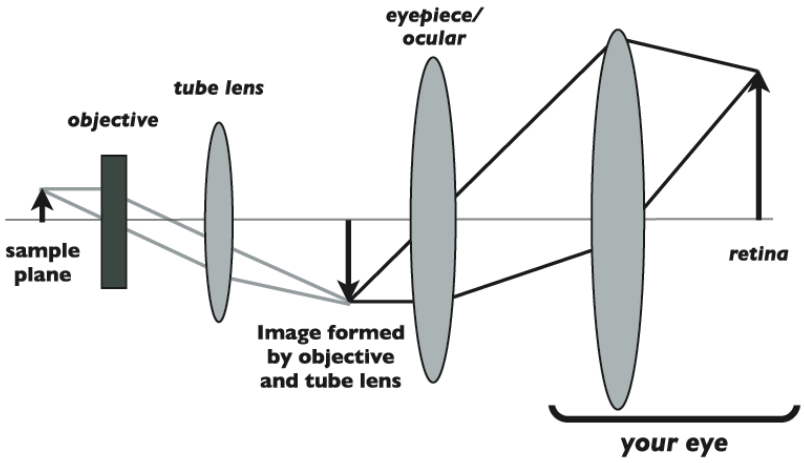
Because an infinity corrected objective consists of numerous elements, it is simply depicted above as a rectangle. The figure below provides an example of a particularly complicated objective from *Olympus*, which contains a ridiculous number of optical components:



The question remains: what's so special about infinity corrected objectives? The answer lies in the space between the objective and tube lens, referred to as the *infinity space*. When optical components are placed in the path of non-collimated light, aberrations are introduced. When they are placed in the path of collimated light, such as the light in the infinity space, aberrations are reduced. Quoting the *Nikon* website: "Infinity optical systems allow introduction of auxiliary components, such as differential interference contrast (DIC) prisms, polarizers, and epi-fluorescence illuminators, into the parallel optical path between the objective and the tube lens with only a minimal effect on focus and aberration corrections."

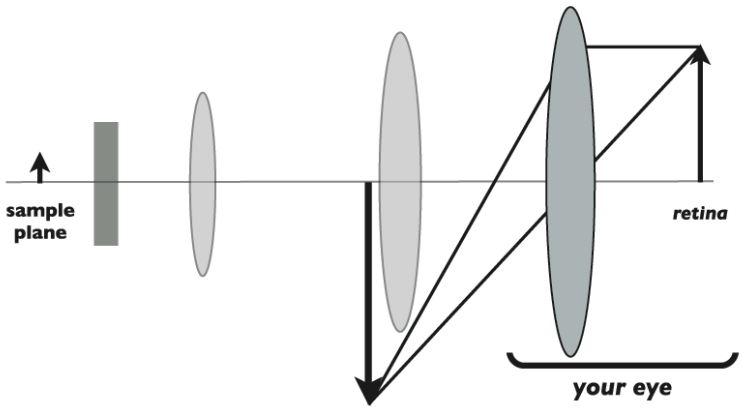
Images in the eyepiece

To allow you to view the sample image through the *eyepiece* (or the *ocular*), a lens in the eyepiece collects the light from the tube lens and sends it to the lens of your eye. Together, the eyepiece lens and your eye focus the sample image on your retina:



Since your eye plays an important role in this optical train, it is not surprising that a sample that appears focused for you may appear slightly out of focus for your partner.

One last note about the eyepiece: the image you see through the eyepiece appears ~10 inches behind the eyepiece, giving you the sense that the image comes from within the microscope. This is because the image on your retina appears to come from a virtual image that is approximately 10 inches from the lens of your eye. This virtual image is depicted below:



Fine alignment of the excitation laser

You are now ready to finely align the two mirrors. During this alignment, pay close attention to the collimated beam and its position on the two mirrors. If the beam falls off the edge of either mirror, resulting in clipping of the beam profile, adjust the appropriate mount and, if necessary, return to the coarse alignment protocols.

As discussed previously, at the end of this alignment, the excitation light should be perpendicular to the objective and pass through its center.

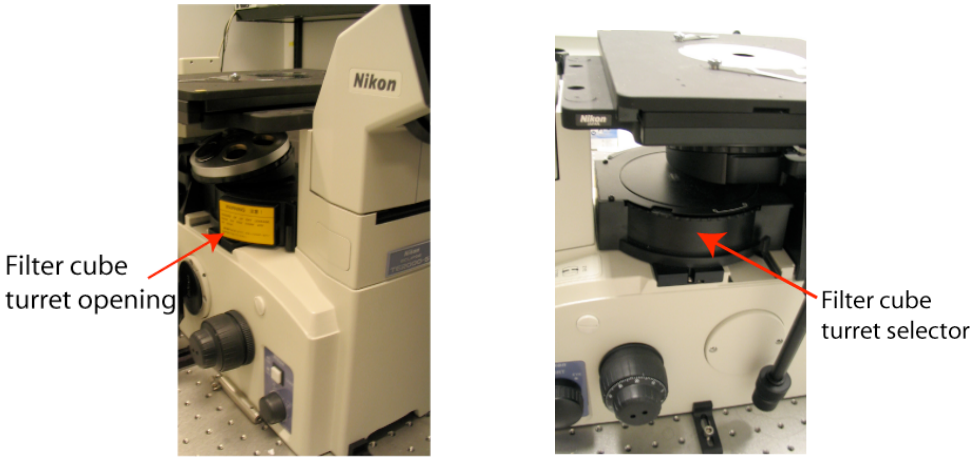
You will finely align the laser by reflecting it off a glass surface and looking at the reflected light through the eyepiece.

1. Close the shutter on the laser.
2. Remove the emission filter from the filter cube. To avoid damaging the emission filter, you must wear gloves during the next steps.

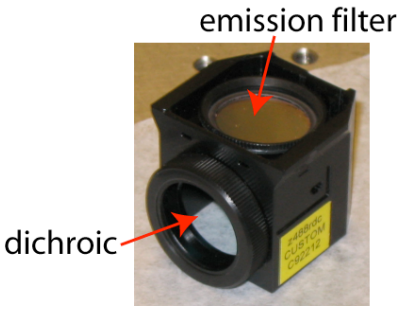
   a. Put on gloves.

   b. Pull the filter cube from the microscope's *filter cube turret*.

      To do this, pull the yellow cover off the turret opening (see the pictures below). Then, rotate the turret selector until the filter cube is visible in the turret opening. Gently pull the cube from the turret opening, being careful not to touch the filter.



c. Hold the filter cube with the emission filter facing you (note the picture below). Use your gloved fingers to gently pry off the plastic ring that holds the filter in the cube.



d. Invert the cube so that the emission filter and ring fall gently into your hand.

e. Place the ring and emission filter onto a piece of filter paper, using the ring to keep the emission filter raised off the surface of the paper. Place the emission filter somewhere very safe and memorable.

3. Using the focus-adjust knob, lower the objective mount as much as possible. Turn the objective mount to an empty port and remove the circular metal stage.

4. Screw the 60x objective into the empty port. Replace the circular metal stage with the small aperture, making sure that the lens of the 60x objective is situated below the aperture.

   Because the 60x objective is very large, it is possible to bang it into the stage using the focus-adjust knob. Be careful to never do this!

5. CAREFULLY, place a single drop of objective oil (~25 µL) on the objective lens. Be careful not to introduce any air bubbles. Never touch the objective with the tip of the pipette.

   Excess oil can drip down the objective and onto the filter cube, where it will ruin the dichroic and emission filter. Only use as much oil as you need.

6. Place a glass coverslip on the microscope stage, and slowly raise the objective using the focus-adjust knob. Stop when you see the oil just make contact with the coverslip.

The interface between the coverslip and air reflects a small amount of laser light. Because there is no emission filter and because a small amount of light passes through the dichroic, some of the scattered light will pass to the emission pathway.

7. Turn the laser on. You will see a blue spot on the coverslip.
8. Set the neutral density filter wheel to the least transparent setting. Turn the knob on the right side of the microscope to EYE. This sends the light collected by the objective to the eyepiece.

   Look through the eyepiece – do you see blue scattered laser light? If not, turn the neutral density wheel to the next least transmitting filter. Repeat this until you find a filter that allows you to see scattered light but still blocks most of the scattered light.
9. You will see blue light from the scattering of the laser off the coverslip-air interface. Adjust the stage height (and thus the coverslip height) using the focus-knob until you see a bright blue spot. Focus the blue light to a tight spot that is as small and bright as possible.

CAUTION – it is extremely easy to smash the objective against the glass coverslip, potentially cracking the coverslip and damaging the objective. Pay attention to where the objective is and which way you are moving it with the focus-adjust knobs. Use the fine focus-adjust knob as much as possible.

10. Use the two adjustment-screws on one of the mirror mounts (it doesn't matter which one) to move the spot to the center of field of view. One of the eyepieces contains a crosshair to help you locate the center.
11. While looking at the blue light through the eyepiece, use the fine focus-adjust knob to slowly move the objective up and down.

QUESTION: What do you see as you move away from the bright focused spot. Describe what happens to the blue spot. Draw pictures if it will help in your explanation.

You are going to use the reflected light to precisely align the two mirrors. The appearance of the beam profile in the eyepiece field of view depends on the distance between the coverslip and objective. This is illustrated in the figure on the next page, which depicts the beam profile observed when the beam is aligned (perpendicular and through the center of the objective) and when it is misaligned.

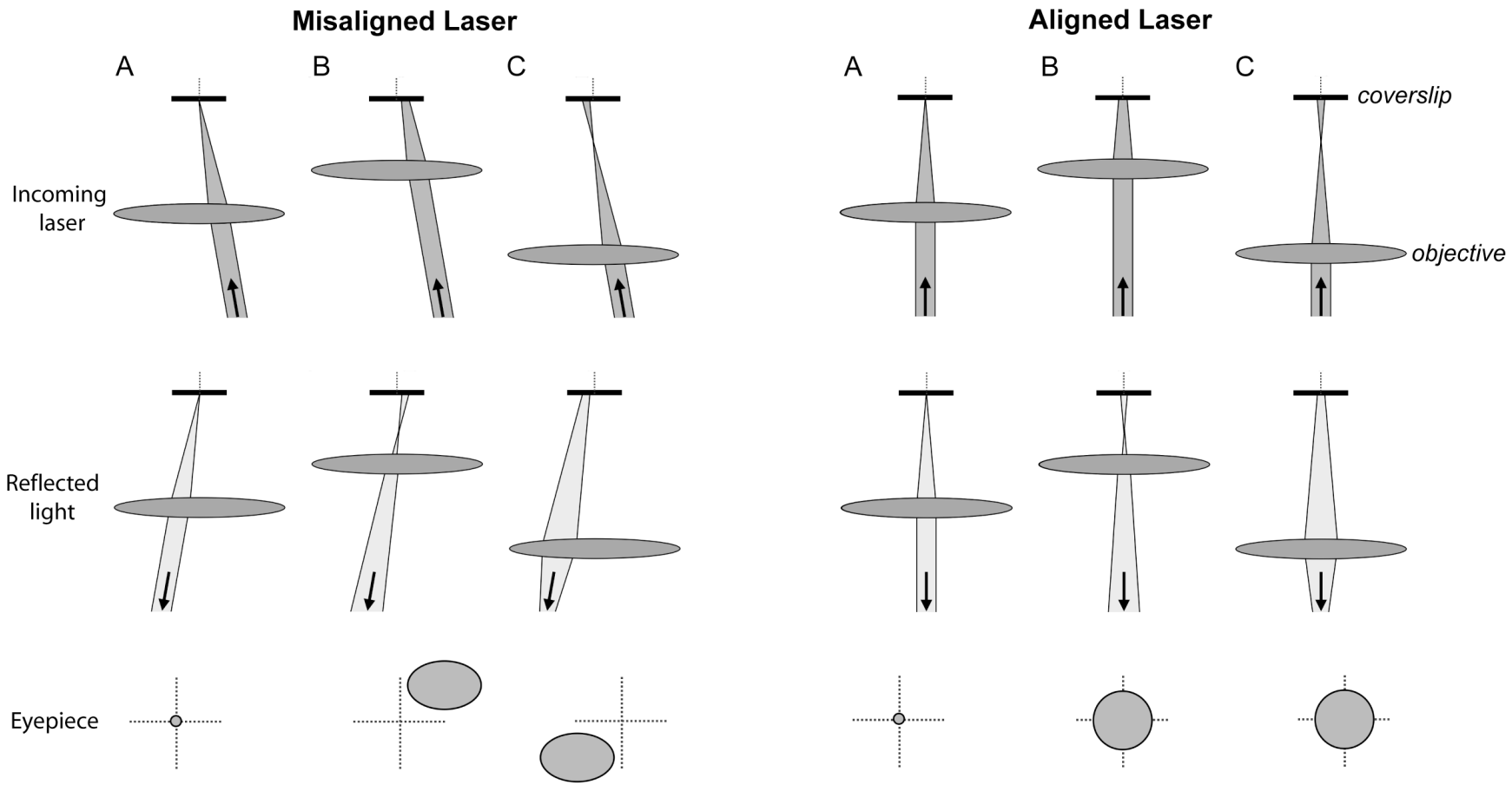
When the coverslip is one focal length away from the objective, it is situated at the laser's focus. As a result, the reflected light will be collimated when it exits the objective, as shown in **panel A**. This also means that the light will be a focused spot in the eyepiece field of view. It is hard to differentiate between the aligned and misaligned laser using this bright spot.

In **panels B and C**, the objective has been moved either closer to or further from the coverslip, respectively. The reflected light is no longer collimated by the objective, and thus the light will not be a focused spot in the field of view. Thus, for both the aligned and misaligned laser, the beam profile will expand, increasing in diameter as you move further from the focal length. For the aligned laser, this expansion is symmetric, resulting in a circular profile. For the misaligned laser, on the other hand, the expansion is asymmetrical, resulting in a more oval profile.

From **panels B and C**, we also see that, for the misaligned beam, the position of the beam shifts from side-to-side as you move the objective up and down. For the aligned beam, on the other hand, the center of the beam profile does not change.

You will take advantage of these two observations to align the mirrors.

The goal of the fine alignment is to make the reflected blue spot expand and contract symmetrically and about a fixed point when the distance between the objective and coverslip is changed.


Misaligned Laser
Aligned Laser
A
B
C
A
B
C
coverslip
objective
Incoming laser
Reflected light
Eyepiece

The following steps require you and your partner(s) to coordinate with each other – come up with a game plan before you start.

12. Designate one of the adjustable mirrors as M1 and the other as M2. Designate each of the adjustable screws on a mirror as S1 and S2. Which is which doesn't matter, just be consistent.

13. Iteratively adjust the mirrors as follows:

(A) Rotate the focus-adjust knob on the microscope until the reflected spot is tightly focused.

(B) If it isn't already, use both screws on M1 to center the bright spot on the eyepiece crosshair.

(C) Move the objective up and down using the focus-adjust. How does the spot expand? Is it symmetric? Is it stretched up-down or side-to-side?

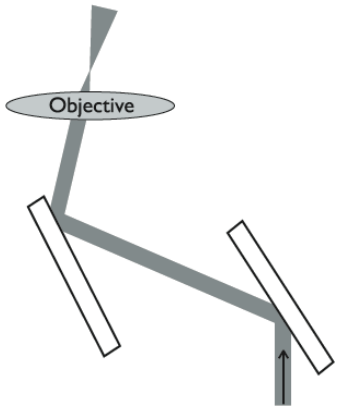
(D) Using S1 on M1, move the bright spot in one direction along the axis of S1. Do not move it out of the field of view.

Remember both the direction in which you turned the screw and the direction the spot moved in the field of view.

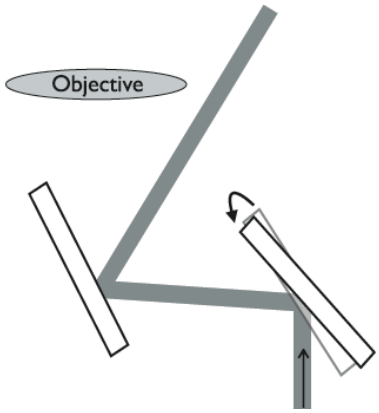
(E) Using both screws on M2, move the spot back to the center of the field of view.

(F) Move the objective up and down using the focus-adjust. How does the spot expand? Does it look better or worse than in step (C)?

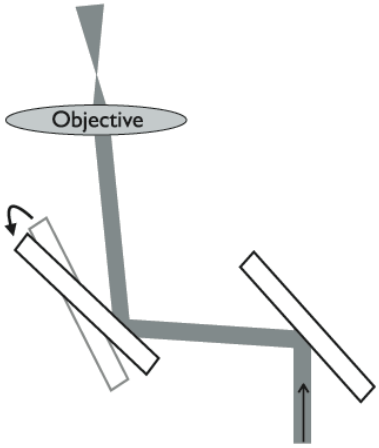
After completing steps **A-F**, the laser is still passing through the center of the objective. However, you have changed the angle at which the laser enters the objective. To make this clear, let's draw this process out. Consider the following misaligned laser, which is being guided toward the objective by two mirrors (for clarity, we have not included the dichroic):



The laser is passing through the center of the objective but is not perpendicular to the lens. To fix this, we adjust the mirror furthest from the objective:



This moves the laser away from the center of the objective. In this case – the laser doesn't hit the objective anymore. Using the second mirror, we bring the laser back to the center of the objective:



The laser now passes through the center of the objective, but the angle at which it strikes the objective is changed.

In the above scenario, the laser is better aligned following the movement of the two mirrors (i.e. it is closer to being perpendicular to the objective). This was just luck, however, and we could have just as easily rotated the mirrors in the opposite directions. This would have then resulted in a worse alignment.

The trick is to identify whether the alignment is better or worse following the adjustment. If it is better, then continue the alignment by repeating the same motions of the mirrors. If it is worse, adjust the mirrors in the opposite direction. To identify is the alignment is better or worse, you will look at the reflected light through the eyepiece. As you move the objective up and down using the focus-adjust, you will compare what you saw before the alignment with what you now see in the field of view, and answer the questions:

1) *Is the expansion and contraction of the blue spot more symmetrical?*

2) Is there reduced movement of the expanding and contracting spot across the field of view?

If the answer to these questions is yes, then the alignment has improved.

Of course, your alignment will be a little more complicated since your system has more degrees of freedom then my 2-dimensional example, but the basic idea is the same. Continuing with the alignment:

(G) If the expansion of the bright spot in (F) was an improvement – return to step (D) and continue to move the bright spot in the same direction using S1. If the expansion looks worse, return to step (D) and move the bright spot in the ***opposite*** direction using S1.

(H) Repeat steps (D)-(G) until you can no longer improve the alignment of the laser.

(I) Repeat step (D)-(H), but instead of moving the beam with S1 on M1, use S2 on M1.

The laser should now be aligned so that it passes perpendicular to and through the center of the objective.

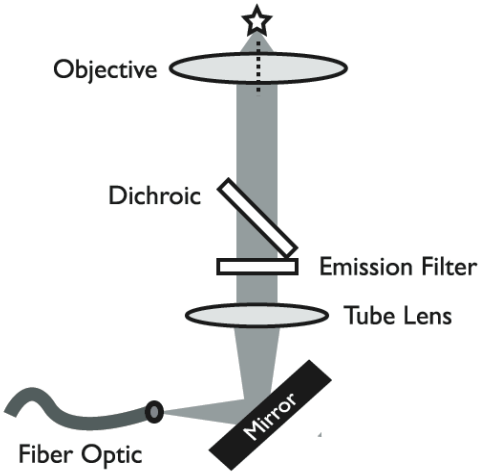
DAY 4: ALIGNMENT OF THE EMISSION PATHWAY

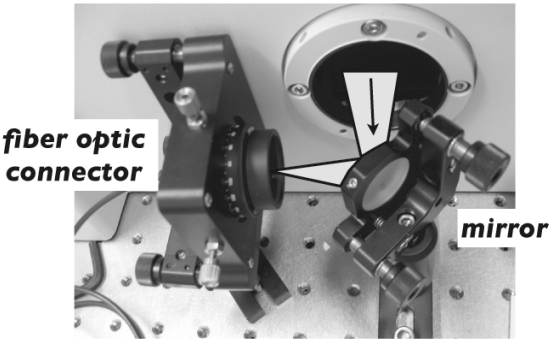
The emission pathway has already been coarsely aligned. However, variations in the excitation alignment may lead to small differences in the alignment of the emission path. Thus, you will perform a fine alignment of the emission pathway.

Background: The emission pathway

The collimated laser that you aligned in the previous section is focused to a point just above the objective, one focal length away. When a fluorophore sample is placed on the microscope stage, this light will preferentially excite fluorophores near this focused point.

The resulting fluorescence emission is collected by the objective. The path traversed by the emitted light is detailed in the picture below. The objective collimates the light emitted by fluorophores one focal length away from the objective. The light then propagates through the dichroic and emission filter of the filter cube, and a tube lens focuses the emitted light to a point. Before reaching a focused point, the light exits from the side port of the microscope. A mirror directs the light to the entrance of a fiber optic, which acts as a confocal pinhole. The light entering the fiber optic is then sent to the single-photon counting module. A picture of the mirror and fiber optic mount are shown below on the left, with the path of the fluorescence emission depicted in white:





Because the emission path has been previously aligned, we will assume that the placement of the mounts on the table do not need to be adjusted and that the mirror is oriented correctly. You will only adjust the mount that holds the end of the fiber optic. The mount contains four adjustment screws, two that translate the end of the fiber optic from side-to-side and three that adjust its tilt (the latter are similar to the thumb-screws on the mirror mounts).

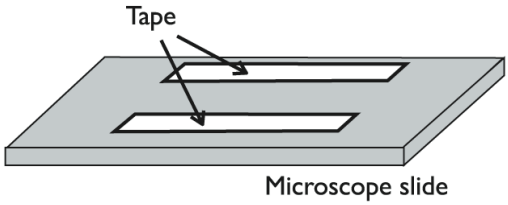
We will align the emission path using a concentrated fluorophore sample. The sample will be placed in a *sample flow chamber*. The following protocol describes how to make a sample chamber, place sample in the chamber, and place the chamber on the microscope. Practice making a chamber and filling it with buffer before beginning the alignment.

Making and using a sample flow chamber

You will repeat the following protocol whenever you collect data. A chamber holds ~15 μL of sample

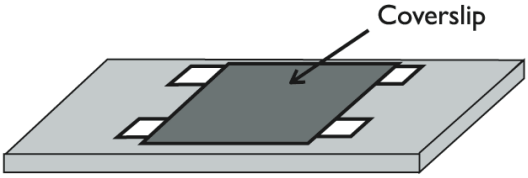
1. Take a 1-2 inch piece of double-sided tape (it must be longer than a coverslip, which is a 22x22 mm square). Split it lengthwise down the center to make two strips of tape of equal length. Either use a razor or split it carefully by hand.

   Put the strips of tape on a microscope slide as shown:



   The tape should be centered along the length of the slide. Using the end of another slide, push the tape against the slide until it forms a tight seal with the glass. If there are any trapped bubbled, wrinkles, or bumps, start over. Otherwise, you will have leaks in your chamber.

2. Place a clean glass coverslip on the tape.

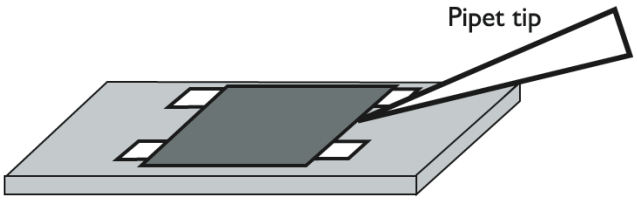


   Using the end of another slide, gently push against the portions of the coverslip that are in contact with the tape to form a tight seal. If you see any leaks or if the coverslip cracks, start over.

   The experimental chamber is the volume between the slide and coverslip.

3. Flow sample through the chamber using capillary action.

   Using a pipetman, pick up 20 μL of sample. At one of the open ends of the chamber (where the coverslip is not in contact with tape), push the pipet-tip against the coverslip edge. Slowly push liquid out of the tip. If properly positioned, the liquid will be "sucked" into the chamber through capillary flow. Slowly empty the tip until the chamber is filled. Throw away the extra sample in the tip.



4. Seal the sample chamber to prevent evaporation.

   Liquid will rapidly evaporate from the edges of the sample cell. This is annoying and detrimental to your experiment because the evaporation creates flows in the chamber. To

avoid this problem, place a small streak of vacuum grease at each of the open edges of the sample chamber. To ensure the grease has formed a complete seal, use a pipette tip to spread the grease along the edge of the chamber. Be as neat as possible – DO NOT get any grease on the objective.

5. Prepare the microscope for use with the 60x objective.
    a. Remove the metal disk on the stage and lower the position of the microscope nosepiece as much as possible.
    b. Screw the 60x microscope objective into an empty port. Keep track of which of the two objectives you are using, and use this same objective for the remainder of the lab.
    c. Rotate the nosepiece to the 60x objective position.
    d. Place the metal disk with the small aperture on the stage, adjusting the xy-position of the stage so that the aperture is directly above the objective.
6. Place a *very* small drop of immersion oil (~20 μL) on the top of the objective. DO NOT touch the objective lens with the oil dropper! Let gravity do the work. Excess oil will drip down the edges of the objective and ruin the spring-loaded mechanism that provides crash-protection. Excess oil can also drip down to the filter cube and ruin the filters. The oil drop should be so small that it rests on the top of the objective lens without running towards the edge.
7. Invert the sample chamber so that the tape faces the floor. Place the slide on the microscope stage so that the coverslip is directly above the objective.
8. SLOWLY raise the objective until the oil makes contact with the sample.
9. Adjust the xy-position of the stage so that the objective lens is below the sample and not the tape.

Fine alignment of the emission pathway

1. A fiber optic cable is attached to the fiber optic mount. Attach the other end of this fiber optic to the single-photon counting module.

AN IMPORTANT ASIDE ON THE PHOTON COUNTING MODULE

As discussed in the DAY 2 section entitled *Your confocal system*, the single-photon counting module contains an avalanche photodiode (APD). These are extremely sensitive detectors that can be destroyed by being exposed to too much light. The APD is accessed through its FC adaptor. This adaptor should remain covered when the room light is on.

Whenever the detection device is turned on (i.e. plugged in), the room lights must be off and the detector must be covered with a black box. You can use the small desktop lamp provided for you – direct the light toward the ceiling.

When fluorescence emission is collected by the fiber optic attached to the detector, be careful not to send too much light. The emission filter must be in the filter cube. Always start your measurements by blocking most of the excitation light with the most opaque neutral density filter. If the signal is too low, go to the next filter.

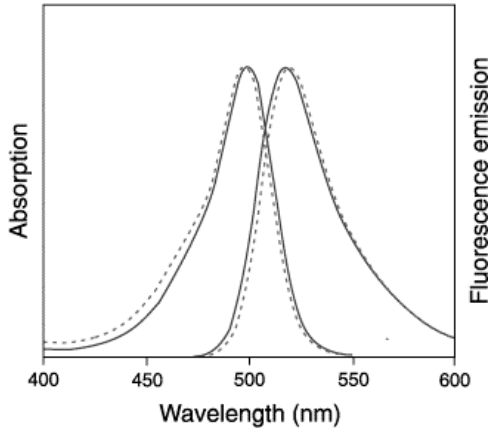
Make a sample of the Alexa Fluor 488 Antibody (*Invitrogen*) stock diluted 50-fold in PBS. You only need 15 μL of this sample, so please conserve the stock. The stock must be kept on ice at all times. The following recipe can be used to make the dilution:

	50 μL of 50x fluorescent sample
Alexa Fluor 488 antibody stock	1 μL
PBS	49 μL

Alexa Fluor dyes are stable, bright, and small fluorophores developed by *Molecular Probes*. The excitation and emission spectra of these dyes cover the visible spectrum and some of the IR spectrum.

Because our dye is attached to an antibody, the antibody is useful for applications such as immunostaining. In your experiments, however, the antibody simply makes the fluorophore larger, resulting in slowed diffusion

The absorption and emission spectra of Alexa Fluor 488 are shown as blue-dashed lines in the plot below, which comes from the *Invitrogen* website):



2. Make a sample chamber, place your fluorophore sample in the chamber, and place the chamber on the 60x objective of the microscope (use the protocol **Making and using a flow chamber** provided above).
3. Position the coverslip of the sample chamber one focal length from the objective:
   a. Remove the emission filter from the filter cube (*always use gloves!*). If you have forgotten how to remove the emission filter, go back to **Day 2, Fine alignment of the excitation laser**; removing the emission filter is the second step.
   b. Set the microscope PORT to EYE.
   c. Open the attenuator on the laser, and change the filter wheel to a neutral density filter that blocks most of the light.
   d. Adjust the height of the objective using the fine focus-adjust knob. Start with the objective at a low height, where it is clearly too far from the sample to image it. While looking through the eyepiece, move the objective upward so that it is approaching the

coverslip. Do this slowly and carefully. If you are not cautious, you can jam the objective and crack the coverslip.

Because there is no emission filter, you will see blue laser light through the eyepiece. As you move the objective higher, the blue light will converge to a bright spot in the center of the eyepiece. This is similar to the spot you observed through the eyepiece on Day 2, when you looked at light reflected from the coverslip-air interface. This time, however, you are reflecting light off of the interface between the coverslip and sample. Adjust the objective height until the blue spot is as small as possible.

NOTE: If you were to continue moving the objective even higher, you would see the light diverge and eventually converge once more to a bright spot. This is the result of reflection off the interface between the sample and the slide.

The coverslip is now at the focus of the laser, and is thus one focal length from the objective.

4. Move the objective lens "slightly" closer to the coverslip.

   When you move the objective toward the coverslip, you are moving the focus of the excitation laser from the coverslip surface to a point just above the coverslip. In other words, you are moving the focused laser into your sample chamber. However, as you move the laser further into the sample, aberrations from the water-glass interface will compromise the tight focus of the laser. This is why we only want to move the objective only *slightly* closer to the coverslip, so that the laser is focused within the sample chamber just a short distance from the coverslip.

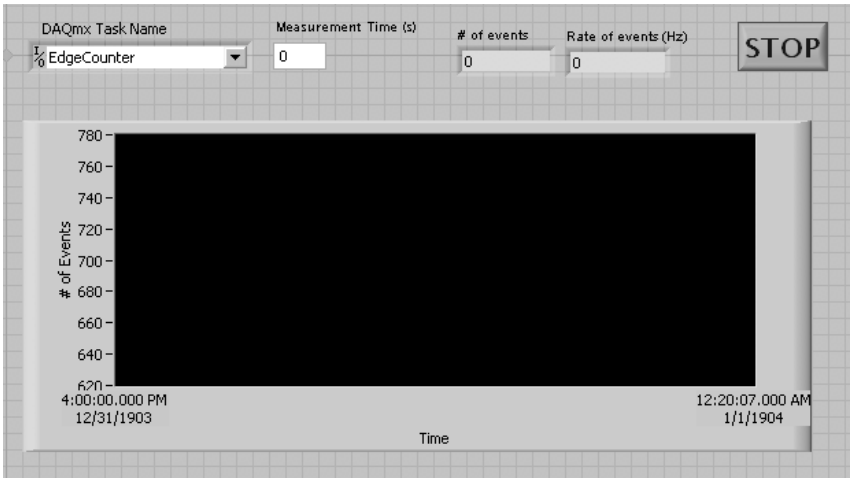
   This adjustment should be approximately a ¼-rotation of the fine-focus-adjustment knob. The bright spot in the eyepiece will expand slightly. Be sure you are moving the objective closer to the coverslip, and not further away!

5. Close the attenuator on the laser.
6. Replace the emission filter.
7. Set the neutral density filter wheel to the highest setting.
8. Turn off the lights in the room and turn on the small desktop lamp. Close the black curtain as completely as possible.
9. Cover the microscope stage and detector with their cardboard black boxes.
10. The coaxial cable coming from the single-photon counting module should be in the BNC adaptor on the BNC-2121 labeled with orange tape.
11. Open the LabView program *Data Reader* by double-clicking the icon on the Desktop.

Data Reader.vi

This program counts the events received from the single-photon counting module, and integrates these counts over a particular interval of time.

When you double-click the *Data Reader* icon, the following panel appears:



COMPONENTS ON THE FRONT PANEL:

DAQmx Task Name – Use this menu to choose the data acquisition *task*. The *task* tells the data acquisition system what to do with detected events (e.g. the task used by *Data Reader* tells the system to simply count events)

Measurement Time (s) – *Data Reader* counts the number of events detected during the time interval indicated here. The program repeats this measurement until the program is stopped.

# of events – This output corresponds to the number of events counted during the most recent measurement interval.

Rate of events (Hz) – This output corresponds to the calculation: *# of events / s*.

STOP – This button stops the program.

The bulk of the front panel consists of a plot of *# of events* as a function of time.

Using the LabView program

This description assumes no knowledge of LabView. When you open *Data Reader*, you will see both the program's front panel and the *tools palette*:

If you do not see this, pull down the *View* menu on top of the front panel and click *Tools Palette*.

This palette contains the tools to control and alter the program. You will only use two of these icons. Selecting the *hand icon* (top left) allows you to interact with elements on the front panel (i.e. click buttons, move switches, adjust scrollbars). Selecting the *letter icon* (top right) allows you to type text and numbers. For example, you will need to click the letter icon in order to type in a value for *Measurement Time*.

NOTE - In LabView, white boxes are inputs and grey boxes are outputs.

12. Make sure *MyCounterTaks2* or *EdgeCounter* is selected from the *DAQmx Task Name* menu. This should be the default selection when you open *Data Reader*.
13. Set the *Measurement Time* to 0.2 seconds.
14. Open the attenuator on the laser.
15. Start the program by pushing the play icon at the top left of the front panel. Because the APD is not plugged in, the number of detected events will be zero.
16. Plug in the single-photon counting module.

    Because the microscope is set to EYE, the signal you are seeing corresponds only to (1) background light and (2) dark signal. The former results from ambient light and can reduced by making the boxes light-tight and removing sources of stray light. The dark signal, however, is noise inherent to the device and is independent of signal. The price of an APD detector is inversely proportional to the magnitude of this dark signal.

    To get a feel for the sensitivity of the detector and the degree to which noise affects your measurements – use *Data Reader* to measure detection rate for the following conditions:

    - Keep the small desk lamp on.
    - Turn the small desk lamp off.
    - Keep the desk lamp off and remove the box from the microscope stage.
    - Move both computer screens so that neither faces the detector.
    - How low you can get the count rate to go? Try reducing the light as much as you possibly can.
17. Turn the microscope port to SIDE. What count rate do you see? If your signal is not noticeably larger than background, switch to a neutral density filter with higher transmittance.
18. Align the emission path fiber optic using *Data Reader*. Though the fiber optic is close to being aligned, you will adjust the mount slightly to maximize the observed signal. Use *Data Reader*'s plot to determine whether an adjustment increases or decreases the rate of detection. To refresh the plot while the programming is running, left-click on the plot and select *Clear Chart*:

    a. Slowly adjust the x-translation on the mount holding the fiber optic to maximize event count rate.
    b. Adjust the y-translation on the mount to maximize the count rate.
    c. Adjust Knob #1 on the mount to maximize the count rate.
    d. Adjust Knob #2 on the mount to maximize the count rate.
    e. Adjust Knob #3 on the mount to maximize the count rate.
    f. Repeat the above steps until you can no longer improve the signal.

Congratulations – you are finished aligning the device. You are an alignment guru!

DAY 5: DATA COLLECTION AND ANALYSIS

You are now ready to use your system to collect fluorescence emission from single fluorophores. Collecting single molecule data requires you to understand the following three concepts:

(1) Maximizing the signal-to-noise of your fluorescent measurements.

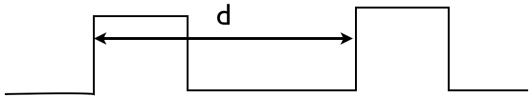
(2) Identifying sample concentrations where data is single-molecule.

(3) Confirming that a series of measurements results from single fluorophores.

Today, you will focus on the first two.

Background: Your fluorescence measurements

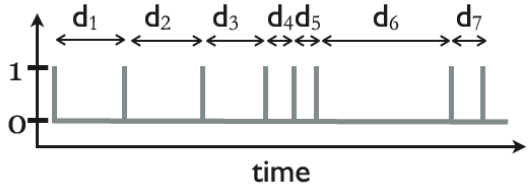
You have aligned a system that uses a focused laser to excite fluorescent molecules and collects the fluorescence emission from a confocal volume centered at the laser's focus. Today, you will use your system to collect data from fluorophore samples. The data collection program you will be using, which is from National Instruments, measures the duration of time between two signals received by the data acquisition card. For example, if the single-photon counting module sends out two pulses corresponding to two detection events, the program will save the following duration, *d*.



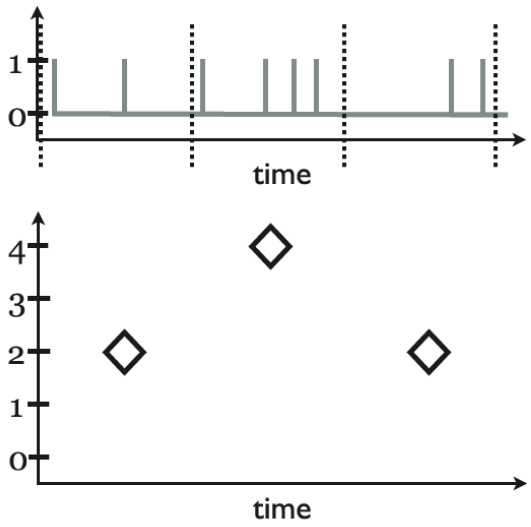
where *d* is the time between the leading edge of the events.

Your data will thus be comprised of a series of *N* durations $\{d_i\}$. To help us interpret these durations, we can envision the time trace of detection events described by this data. This time trace is zero everywhere except at *N+1* points where it goes to 1. These points correspond to *N+1* detection events, and the times between consecutive detection events are given by the *N* duration measurements.

For example, a series of 7 durations can be interpreted as describing the following time trace, consisting of 8 detections events:



Plotting the data in this way, however, it is not easy to discern by eye regions with a large number of detection events (for example, when a fluorophore enters the confocal volume) from regions with only a few detection events (for example, when the signal arises solely from dark counts from the detector). To make these differences more clear, you can bin the time trace into windows of fixed width, Δt. For example, for the above time trace, binning the data using a time window that is ~1/3 the length of the time trace results in the following plot:



The value for a particular bin describes the number of detected events within an interval of time Δt. We refer to this value as the detection count n_i at time $i*\Delta t$, and the rate of event detection is $n_i/\Delta t$. For the time trace above, our binning has allows us to identify a region in the middle of the data with a higher detection rate.

You will be provided with software that converts a series of duration measurements $\{d_i\}$ into a time trace, and bins this time trace using a window of fixed width Δt. In this way, the data is converted into a series of detection counts $\{n_i\}$. For the remainder of our discussion, we will focus on this form of the data.

Background: Optimizing data collection

Maximizing the signal-to-noise of your fluorescent measurements

A single detection count n will contain two contributions: (a) n_f, counts arising from fluorescence emission (if there is a fluorophore in the confocal volume), and (b) n_s, counts resulting from scattering (Rayleigh and Raman) of the excitation light:

$$n = n_f + n_s \tag{1}$$

The former contribution is the signal and the latter is noise.

For a series of counts $\{n_i\}$, the mean value μ is the sum of the mean count from fluorescence emission and from scattering:

$$\mu = \mu_f + \mu_s \tag{2}$$

Successful single-molecule detection requires the fluorescent signal to be distinguished from the noise. Thus, we seek to maximize the ratio of the mean fluorescent count to the mean fluctuation in the scattering:

$$\mu_f / \sigma_s \tag{3}$$

The term in the denominator is the standard deviation of the scattering counts. Because scattering events occur at a fixed rate and are independent of one another, the probability of measuring a particular value for n_s is described by a Poisson distribution. (*If you are not familiar with the*

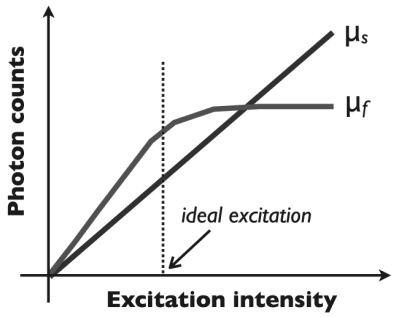
Poisson distribution, it will be discussed in detail on Day 6.) The standard deviation of a Poisson distribution with mean μ is given by $\mu^{1/2}$, and so the ratio in equation (3) can be rewritten as:

$$\mu_f / \mu_s^{1/2} \tag{5}$$

As the power of the excitation laser is increased, both the mean fluorescent and mean scattering signals increase linearly with the excitation laser intensity *I*. The signal-to-noise can thus be improved by increasing *I*, since equation (5) dictates that the mean fluorescent count will increase more rapidly than the variance in the scattering.

As *I* continues to increase, however, the fluorescent signal eventually stops its linear increase and approaches a maximal value. This is referred to as saturation of the fluorescence emission. One reason for saturation is that, at sufficiently high excitation photon flux, fluorophores cannot decay back to their ground state fast enough, and the ability of fluorophores to absorb incoming photons is compromised. This saturation is exacerbated by intersystem crossing of excited fluorophores to their triplet state. This process renders the fluorophore incapable of fluorescence for the relatively long lifetime of the triplet state, which can be on the order of 100 ns. The result is saturation of the emission at lower excitation intensities.

Because of fluorescence emission saturation, it is important to find the ideal laser intensity where signal-to-noise is maximized. This optimal intensity is qualitatively depicted in the following figure:



Unfortunately, it is not easy to determine how much of your photon count measurement is the result of fluorescence emission and how much arises from scattering, so we will need an experimental strategy to find this optimal laser excitation. Our trick will be to calculate the autocorrelation of measured time traces.

Autocorrelation

For a continuous time-dependent function *f(t)* with mean value μ, we define the autocorrelation function *G(τ)* as the time-average of the product of values of *f(t)* - μ separated by duration τ:

$$G(\tau) = E\left[\left(f(t) - \mu\right)\left(f(t+\tau) - \mu\right)\right] \tag{6}$$

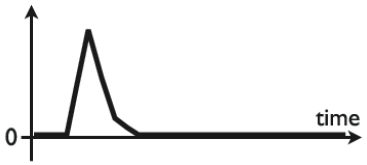
E[...] represents the expected value of what is in the brackets. (The autocorrelation function is often defined as the above relation divided by the standard deviation of *f(t)*, but we will ignore this additional factor.)

For discrete data consisting of *n* measurements $\{f_1, f_2, \ldots f_n\}$ separated by duration Δt, *G(τ)* is written as:

$$G(k\Delta t) = G(\tau) = \frac{1}{(n-k)} \sum_{i=1}^{n-k} [f_i - \mu][f_{i+k} - \mu] \qquad (7)$$

where $\tau = k*\Delta t$.

To help us understand what the autocorrelation function represents, consider the following function *f(t)* that is zero everywhere except where a positive peak occurs:

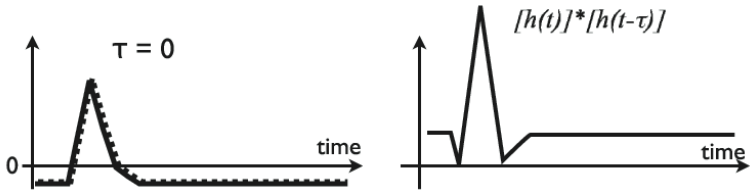


To calculate the autocorrelation of *f(t)*, we first subtract the mean value μ from the data:

We denote this new function as $h(t) \equiv f(t) - \mu$. An important property of *h(t)* is that its time-integral is zero.

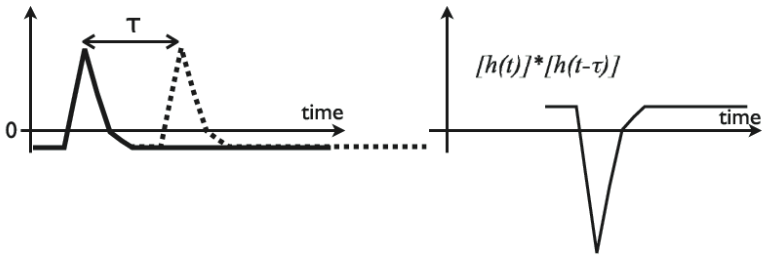
Next, we multiply *h(t)* by a copy of itself that is shifted along the time-axis by τ (i.e. $h(t-\tau)$). According to equation (7), $G(\tau)$ is proportional to the time-integral of the resulting function. In the following figures, we represent *h(t)* with a solid line and the time-shifted function with a dashed line. The product of these functions is shown on the right.

For $\tau = 0$, we simply multiply *h(t)* by itself.



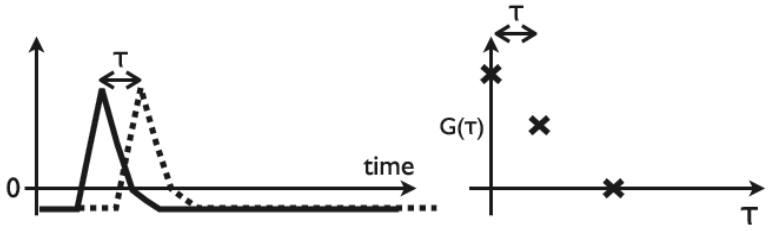
$G(\tau)$ is clearly positive for this scenario since the peak in *h(t)* completely overlaps with the peak in $h(t-\tau)$.

For sufficiently large values of τ, there is no overlap between the peak in *h(t)* and the peak in the time-shifted function.

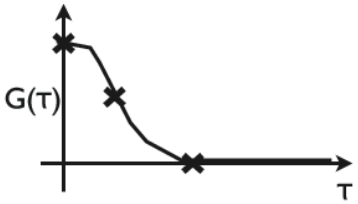


Because the function on the right is simply *h(t)* multiplied by a constant, and because the time integral of *h(t)* is zero, the time-integral of the resulting function is zero. (This is actually only true for a time trace of infinite length. For a finite time trace, our result is approximate.)

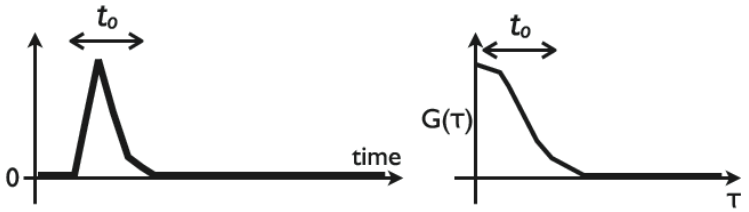
For other values of τ, the value of $G(\tau)$ will be determined by the extent of overlap between the two peaks and will lie somewhere between zero and the maximum value:



$G(\tau)$ is thus maximal at $\tau = 0$ and decreases toward zero as τ increases:



We can describe the shape of the autocorrelation function in terms of the width of the peak in *f(t)*, which we denote as t_o. When $\tau < t_o$, $G(\tau)$ is greater than zero because there is overlap between the peaks in *f(t)* and $f(t-\tau)$. When $\tau > t_o$, $G(\tau)$ is zero because there is no overlap. Thus, $G(\tau)$ drops to zero when $\tau \sim t_o$:



This is a feature of the autocorrelation function that we will take advantage of. During the peak in *f(t)*, time points are said to be *correlated*. This means that, if a particular time point is positive, nearby time points are also likely to be positive, and this correlation extends for the duration of the peak t_o.

We can generalize this observation with the following statement:

> *The autocorrelation function of a time trace drops to zero at a value of τ that is comparable to the duration of correlated features in the time trace.*

Autocorrelation of detection count time traces

Let's connect this picture of the autocorrelation function with experimental measurements. For a detection count time trace, you can calculate the autocorrelation using equation (6):

$$G(\tau) = E\left[\left(n(t) - \mu\right)\left(n(t+\tau) - \mu\right)\right] \tag{8}$$

We incorporate equations (1) and (2) to account for the separate fluorescence emission and scattering contributions to the measurements:

$$G(\tau) = E\left[\left(n_f(t) - \mu_f + n_s(t) - \mu_s\right)\left(n_f(t+\tau) - \mu_f + n_s(t+\tau) - \mu_s\right)\right] \tag{9}$$

Rearranging (9) gives us:

$$G(\tau) = G_f(\tau) + G_s(\tau) + G_{fs}(\tau) \tag{10}$$

where the three terms on the right side of the equation are:

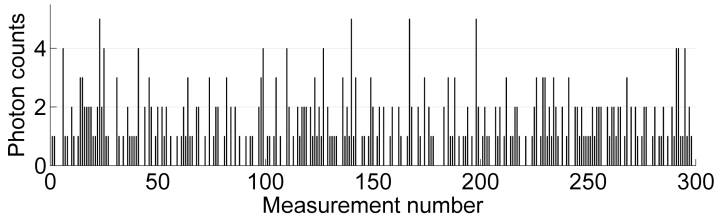
$$G_f(\tau) = E\left[\left(n_f(t) - \mu_f\right)\left(n_f(t+\tau) - \mu_f\right)\right] \tag{11}$$

$$G_s(\tau) = E\left[\left(n_s(t) - \mu_s\right)\left(n_s(t+\tau) - \mu_s\right)\right] \tag{12}$$

$$G_{fs}(\tau) = E\left[\left(n_f(t) - \mu_f\right)\left(n_s(t+\tau) - \mu_s\right)\right] + E\left[\left(n_s(t) - \mu_s\right)\left(n_f(t+\tau) - \mu_f\right)\right] \tag{13}$$

Let's consider each term separately:

$G_s(\tau)$ – This term describes the autocorrelation of the scattering signal. Scattering is a time-independent process since the probability of a particular value of n_s is independent of the previous value. A modeled series of measurements of n_s is shown below:

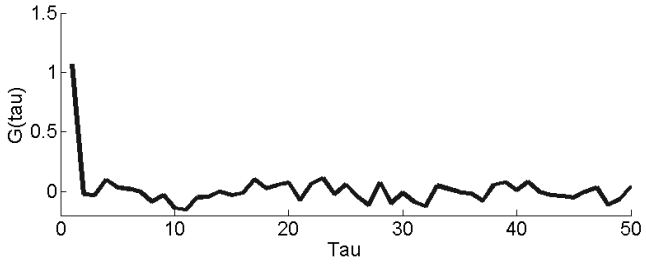


In this example, n_s has been "measured" 300 times (a measurement of zero photon counts appears as a blank space). The probability of measuring a particular value of n_s has been modeled according to a Poisson distribution with mean value of 3 photon counts.

For $\tau > 0$, $n_s(t)$ and $n_s(t+\tau)$ are completely independent of each other. This allows us to simplify (12):

$$G_s(\tau) = E\left[\left(n_s(t) - \mu_s\right)\right]E\left[\left(n_s(t+\tau) - \mu_s\right)\right] = 0 \tag{14}$$

Thus, $G_s(\tau)$ is zero for $\tau > 0$. This is apparent when we calculate the autocorrelation of modeled data using equation (7):



As expected, the autocorrelation is positive when $\tau = 0$ but immediately goes to zero for $\tau > 0$.

$G_{fs}(\tau)$ – The two terms in the brackets are independent of each other since the fluorescence and scattering processes are independent of each other. As with equation (12), this causes both terms in (13) to go to zero when $\tau > 0$.

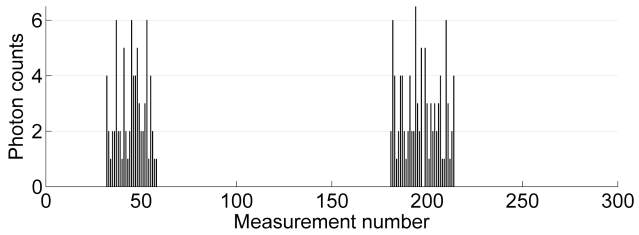
Equation (10) thus reduces to the simple form for $\tau > 0$:

$$G(\tau) = G_f(\tau) \tag{15}$$

$G_f(\tau)$ – This term describes the autocorrelation of the fluorescence signal. Because you have aligned your system so that the excitation light is focused within the confocal observation volume, we will assume that, once a fluorophore enters the confocal volume, it is immediately excited. We also assume that the excited fluorophore returns to its ground state via fluorescence emission, is then immediately excited again, and repeats the process again until it exits the confocal volume. This ignores photobleaching and triplet state dynamics.

Fluorescence emission of an excited fluorophore is a time-independent process. Like scattering, for sufficiently small Δt, the probability of observing a particular value of n_f is described by a Poisson distribution. However, your time trace will contain regions where the confocal volume contains 0 fluorophores, 1 fluorophore, 2 fluorophores, etc. This variability in the number of fluorophores will result in a time-dependent signal.

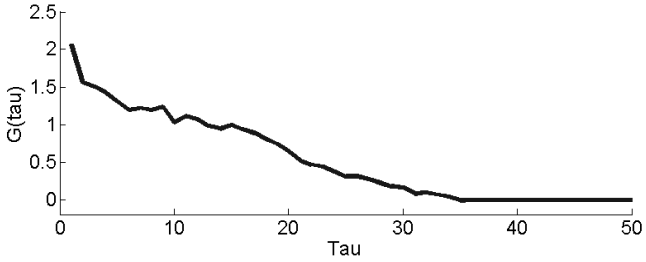
For example, below is a modeled time trace in which the confocal volume is almost always empty, but twice during the data collection, a single fluorophore enters the confocal volume:



This data consists of 300 measurements. When a fluorophore enters the confocal volume, the probability of measuring a particular photon count is modeled as a Poisson distribution with mean value of 8 photon counts. When the confocal volume is empty, n_f is zero. Most of the measurements are zero because the confocal volume is almost always empty. Near measurements 30 and 180, a fluorophore enters the confocal volume and stays in the volume for ~30 measurements.

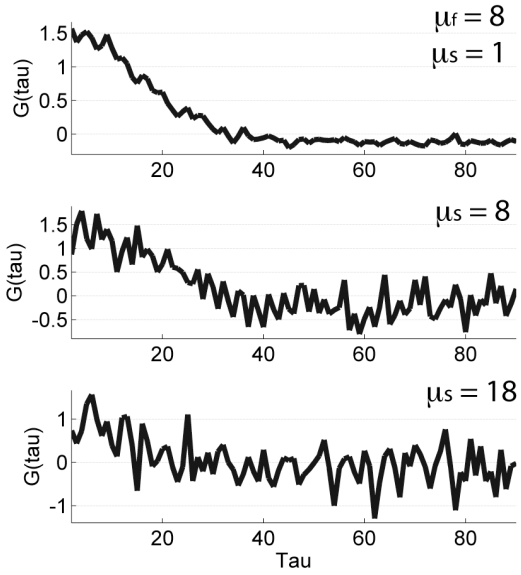
Unlike n_s, it is clear that the time trace of n_f is not time-independent. Positive values of n_f are correlated over a duration of about 30 measurements. Thus, the resulting autocorrelation will

decrease to zero at $\tau \sim 30*\Delta t$. This is confirmed when we calculate the autocorrelation of the modeled data using equation (7):



Optimizing signal to noise

Although fluorescence emission is the only component of the data count measurements that results in a non-zero contribution to the autocorrelation function for $\tau > 0$, the scattering signal introduces noise that makes it difficult to discern this contribution. For example, the three plots below show the autocorrelation of modeled data in which the mean fluorescence signal is 8 photon counts per measurement and the mean scattering contribution is 1, 8, and 18 photon counts per measurement.



Clearly, as the contribution from scattering becomes more predominant, the change in the autocorrelation resulting from fluorescence becomes more obscured. You can thus use the appearance of the autocorrelation function as a means of gauging the signal-to-noise of your signal.

In order to maximize the signal-to-noise of your measurements, you will vary the laser excitation intensity until the contribution to the autocorrelation from fluorescence is as clear and distinct as possible. When the fluorescence signal dominates the scattering signal, the drop in autocorrelation due to fluorescence is most clearly discernable. In order to look for this drop in the autocorrelation in your signal, however, you first need to estimate the time-scale over which

this drop will occur. In the next section, you will estimate how long a fluorophore that has entered the confocal detection volume remains there.

Motion of a fluorophore in solution.

The reason that fluorophores move in and out of the confocal volume is because they are undergoing a jittery, random motion due to their interactions with surrounding water molecules. Thus, if we are going to understand the contribution of fluorescence to the autocorrelation function of your data time traces, we must first understand this motion.

In aqueous solution, a fluorophore constantly experiences randomly directed forces arising from collisions with surrounding water molecules. These are called *thermal forces* because they are the result of the rapid motions of water molecules that dictate the water's temperature. The resulting jittery, random motion of the protein is called Brownian motion

Over time, the fluorophore undergoes a random walk (*rw*) as result of its Brownian motion. Because the direction of each step is random, the mean distance traversed is zero:

$$< x_{rw} >= 0$$

However, over time, the fluorophore is more likely to be further from its starting point. This effect is captured by the mean squared displacement, which increases linearly with time:

$$< x_{rw}^{\ 2} > \propto t$$

In one of his seminal papers from 1905, Albert Einstein linked the microscopic process of Brownian motion to the macroscopic phenomenon of diffusion, the flow of particles from regions of high to low concentration. The diffusion constant *D* describes how a concentration gradient evolves over time. Einstein derived a relation for *D* (known as the *Einstein relation*) that involved the viscosity of the liquid in which diffusion occurs (η = 0.001 kg*m^{-1}*s^{-1} or Pa-s for water) and the radius of the diffusing particle (*r)*. For a diffusing spherical particle, the Einstein relation is:

$$D = \frac{k_B T}{6\pi\eta r}$$

where k_B is the Boltzmann constant (1.38 * 10^{-23} J/K) and *T* is the temperature of the system (K). The left side contains a constant that describes macroscopic diffusion while the right side contains the microscopic radius of the diffusing particle, and the link between the macroscopic and microscopic is thermal energy (k_BT)

Einstein went on to demonstrate that the increase in the mean squared displacement of a particle undergoing Brownian motion over time is proportional to *D*. In three dimensions, this relation is:

$$< r_{rw}^{\ 2} >= 6Dt.$$

According to this relation, an object with a larger diffusion constant will more rapidly explore regions far from its starting point (though on average, it will go nowhere.) The diffusion constant thus dictates how long a fluorophore will spend in the confocal volume before Brownian motion causes it to leave.

QUESTIONS:

For your experiments, you will be collecting fluorescence emission time traces for fluorophore labeled antibodies. The radius of an antibody is ~6 nm, and the size of the Alexa dye is much smaller.

(1) What is the approximate diffusion constant of the antibody? Assume it is a sphere with radius 6 nm.
(2) Roughly, how long will a fluorophore that has entered the confocal volume stay in the confocal volume. To estimate this, calculate how long until the root-mean-squared distance $\sqrt{< r^2 >}$ of an antibody's Brownian motion is equal to the radius of the confocal volume. Your answer here will be similar to the value of τ for which the autocorrelation of your detection count measurements will decrease to zero. Compare your answer to the other group's calculated value.

Identifying sample concentrations where measurements are single-molecule.

In the previous section, we discussed how to utilize the ACF of a detection count time trace to identify the laser intensity at which the signal-to-noise of your measurements is maximized. You will also use the ACF to aid you in finding fluorophore concentrations for which your data is single-molecule.

To reiterate the previous section, correlation in the fluorescence emission signal results from the changing number of fluorophores in the confocal volume. This results in fluctuations in the signal whose time-scale is determined by the Brownian motion of the fluorophores, which carries them into and out of the confocal detection volume.

These fluctuations due to Brownian motion are obscured by stochastic fluctuations in the fluorescence emission signal. The magnitude of the former is dictated by the fluorescence emission of a single fluorophore, and is relatively unaffected by the fluorophore concentration. The standard deviation of the fluorescence emission, on the other hand, decreases with fluorophore concentration. Thus, as you reduce the fluorophore concentration, fluctuations resulting from changing numbers of excited fluorophores become predominant in the fluorescence emission signal, and the decay in the ACF resulting from these fluctuations becomes more pronounced.

Summarizing the important conclusions from our background sections:

- The fluorescence signal is correlated over a time-scale set by the Brownian motion of a fluorophore traversing the confocal volume. This correlation results in an ACF that decays from a positive value to zero over this same time-scale.
- Increasing the signal-to-noise of your measurements improves your ability to discern the drop in the ACF.
- Decreasing the fluorophore concentration improves your ability to discern the drop in the ACF.

Collecting data

When working with the fluorophore-labeled antibody stock – always keep the protein stock and any protein dilutions on ice.

1. Calculate a fluorophore concentration (molar) that will yield approximately one fluorophore within the confocal volume at any given time. Assume the confocal volume is 1 fL and that the fluorophores are homogeneously distributed.
2. Calculate the appropriate dilution of the fluorophore stock that will achieve the concentration you calculated in (1). Remember, your fluorophore stock consists of 2 mg/mL of IgG antibody with a small dye molecule attached. IgG antibodies have molecular masses of ~150 kDa.
3. Turn on the laser and close the attenuator.
4. Make a flow chamber that contains your fluorophore sample diluted *10-fold less* than the dilution you calculated in (2). Use the protocol **Making and using a flow chamber** from Day 4 to make the flow chamber and place it on the 60x objective.
   - You will start with a concentration that is likely too high, and then work your way down toward single-molecule concentrations.
   - Use serial dilutions rather than a single large dilution. For example, if you want a final dilution of 1/10,000, make two serial dilutions of 1/100. Typically, I avoid dilutions greater than ~1/100 or 1/1000.
   - You only require 10 μL of your final dilution for a flow cell – please conserve the fluorophore stock!
5. Position the coverslip of the sample chamber one focal length from the objective. To do this, use laser light that is reflected off a coverslip:
   a. Remove the emission filter from the filter cube (*always use gloves!*). If you have forgotten how to remove the emission filter, go back to **Day 2, Fine alignment of the excitation laser**; removing the emission filter is the second step.
   b. Set the microscope PORT to EYE.
   c. Open the attenuator on the laser.
   d. Adjust the height of the objective using the fine focus-adjust knob. Start with the objective at a low height, where it is clearly too far from the sample to image it. While looking through the eyepiece, move the objective upward so that it is approaching the coverslip. Do this slowly and carefully – if you are not cautious, you can jam the objective and crack the coverslip.

      Because there is no emission filter, you will see blue laser light through the eyepiece. Change the filter wheel to a neutral density filter that blocks most of this light. As you move the objective higher, the blue light will converge to a bright spot in the center of the eyepiece. This is similar to the spot you observed through the eyepiece on Day 2, when you looked at light reflected from the coverslip-air interface. This time, however, you are reflecting light off of the interface between the coverslip and sample. Adjust the objective height until the blue spot is as small as possible.

      NOTE: If you were to continue moving the objective even higher, you would see the light diverge and then eventually converge once more to a bright spot. This is the result of reflection off the interface between the sample and the slide.

The coverslip is now at the focus of the laser, and is thus one focal length from the objective.

6. Move the objective lens "slightly" closer to the coverslip:

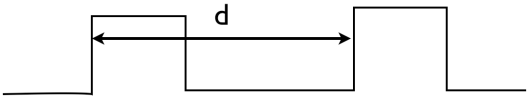
   When you move the objective toward the coverslip, you are moving the focus of the excitation laser from the coverslip surface to a point just above the coverslip. In other words, you are moving the focused laser into your sample chamber. However, as you move the laser further into the sample, aberrations from the water-glass interface will compromise the tight focus of the laser. This is why we only want to move the objective only *slightly* closer to the coverslip, so that the laser is focused within the sample chamber just a short distance from the coverslip.

   This adjustment should be approximately a ¼-rotation of the fine-focus-adjustment knob. The bright spot in the eyepiece will expand slightly. Be sure you are moving the objective closer to the coverslip, and not further away!

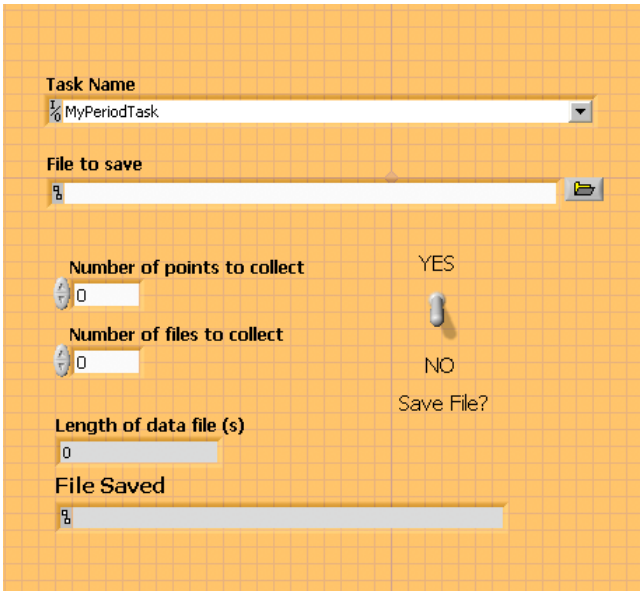
7. Close the attenuator on the laser.
8. Replace the emission filter.
9. Set the neutral density filter wheel to the highest setting.
10. Turn off the lights in the room and turn on the small desktop lamp. Close the black curtain as completely as possible and cover the microscope stage and detector with their cardboard black boxes.
11. Place coaxial cable coming from the single-photon counting module should be in the BNC adaptor on the BNC-2121 labeled with red tape.
12. Open the LabView program **Data Collect.vi** by double-clicking the icon on the Computer *Desktop.*

Data Collect.vi

This program measures the duration *d* between two rising edges in the voltage signal. For example, when the single-photon counting module emits two consecutive pulses corresponding to two separate detection events, the following measurement is made:



When you double-click the **Data Collect** icon, the following panel appears:



Components:

DAQmx Task Name – pull-down menu that lets you choose the data acquisition task you want to use for data collection.

File to save – the file-path and base name for your saved file. The data files will consist of this name followed by the file number. For example – if you type in *davidisgreat*, the saved files will be *davidisgreat0*, *davidisgreat1*,…

Number of points to collect – the number of durations you want saved to a single file.

Number of files to collect – the number of files you want saved.

Save File? – toggle switch that allows you to choose whether or nor to save the file.

Length of data file – the total time elapsed from the beginning to end of the most recent file.

File saved – the file path and name of the most recently saved file.

13. Create a folder on the *Desktop* where you would like to save your data.
14. Click on the folder icon next to the path indicator labeled **File to save**. Navigate to the *Desktop*, and select any file on the *Desktop*. In the indicator bar, delete the file's name and type in the name of your folder followed by the backslash key, and then the name you would like for your saved files.
15. In the **Number of points to collect** box, type in the number of durations you would like collected and saved in a single file. 100-300 works well.
16. In the **Number of files to collect** box, type in the number of files you would like to collect.
17. Toggle the **Save File** switch to YES.

You are ready to collect data!

18. Plug in the single-photon counting module.

19. Change the microscope side PORT to SIDE. The fluorescence emission is now directed toward the emission pathway.
20. Open the attenuator on the laser.
21. Push the play button at the top of the panel. The button will switch from a white arrow to a black arrow, and as each file is saved, you will see the total length of the saved file in the **Length of data file** box. When all the files haves been saved, the arrow will switch back to white.

HINT – it is sometimes useful to first figure out how long it will take to collect a single file before you collect your data. For example, if it takes 1 second to collect a file, then you may want to collect 60 files since it will only take a minute. But, if it takes 10 seconds, then you may only want to collect 6 files, which will also take a minute.

To estimate how long a single file takes, type in "1" in the **Number of files to collect** box, and toggle the **Save File** switch to NO. When you push the play button, the length of the single file will appear in the **Length of data file** box.

TROUBLESHOOTING Data Collection –

A. My program seems to be taking forever to finish!

The most likely problem is either (a) the coaxial cable with your data signal is not connected to the correct connected or (b) you chose to collect too many points (i.e. if you chose to collect 1000 points, but you only collect 1 event every 10 seconds, they you are going to wait for a long time.) Push the Stop-Sign button on the front panel to interrupt the program, and fix the problem.

B. I am getting an error message about over-writing the file before it can be read.

The data acquisition card can only read data so quickly before it gets overwhelmed. Because detection of a photon by the APD is a Poissonian process, there is always a probability that the duration between two consecutive events will be too rapid for the card. However, you can reduce the probability of this occurring by reducing the frequency of detection events. You will find that at some fluorophore concentrations and some laser intensities, it is not possible to collect a significant amount of data without the program crashing.

If this error occurs – the program will ask you if you want to stop the program or continue. Stop the program and delete the last saved file, which now contains bad data. All other saved files can still be used.

---

22. Close the attenuator on the laser. If you are going to turn the lights on, unplug the single-photon counting module.
23. Switch to the next most transparent neutral density filter.

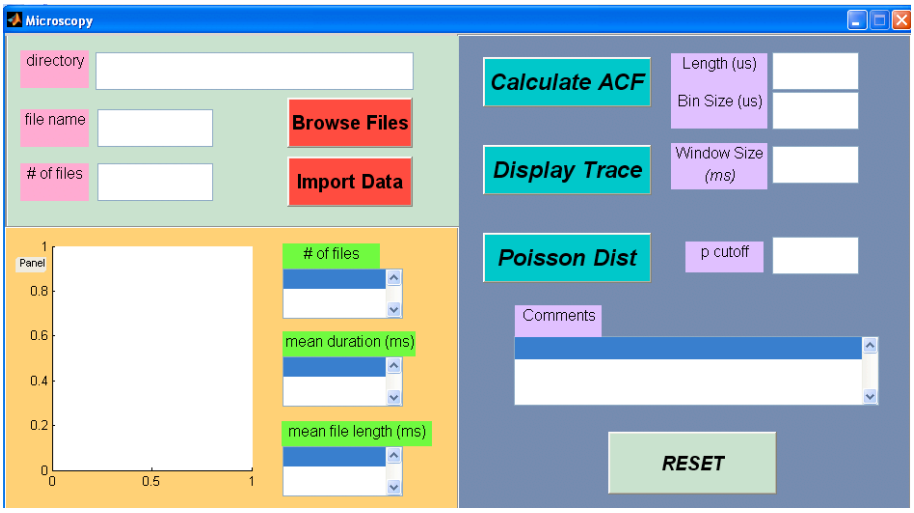
Because we are using one laser for both groups, you will not be able to search for the optimal laser intensity for data collection by adjusting the output of the laser itself. Instead, you are going to collect data using the various neutral density filters. While this does not give you complete freedom to adjust the laser power, it should be sufficient for our purposes.

24. Repeat the data collection process for this higher laser intensity (Steps (14)-(21)).

25. Repeat (23) and (24), collecting data at different laser intensities. At some point, the rate of detection events will be so rapid that you will find that you are constantly overwhelming the data acquisition card (see **TROUBLESHOOTING Data Collection, B**). Stop collecting data at this (or any higher) laser intensity.

Analyzing your data

You will be doing all of your analysis in Matlab using the GUI *Microscopy.fig*. Open Matlab by double-clicking the Matlab icon on the desktop. At the command-line, type in *Microscopy*. The following window will appear:



This program will allow you to import your data, display the averaged time trace, calculate its autocorrelation function, and help identify single molecule events in your data. Let's go through these functions one at a time:

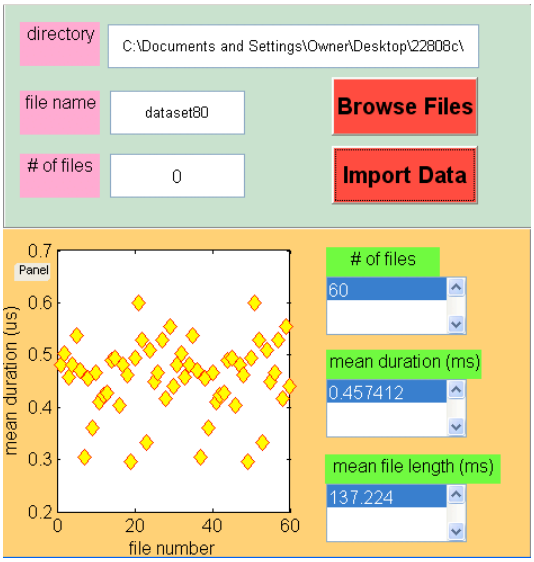
Import data

To import data, you will use the top left corner of the GUI, where the background is tea green.

1. Click the **Browse Files** button. This opens a window that allows you to search for your data files on the computer.
2. Select the folder where your data is located, and select the first file from your data set. The directory and file name will be displayed in the *directory* and *file name* boxes.
3. In the *# of files* box, type in the number of files you would like to import. The program always starts at the first file. Typing in "0" tells the program to import all the data files whose names are the same as the file you selected (except for the trailing number).
4. Click the **Import Data** button.

The program opens the files and creates a list of all the measurements. It also calculates the mean duration and total file length for each. Information about the data files is displayed in the bottom left corner, where the background is orange. The total number of files imported is displayed in the *# of files* box. The mean duration and mean file length for all the imported files is displayed in the *mean duration* and *mean file length* boxes. On the axes, the mean duration is plotted as a function of file number.

For example – when I opened the files from dataset8, the program opened 60 files and displayed the following:



5. Repeat steps 1-4 to import more data files. The data will be appended to the end of the durations that have already been imported. All the information in the lower left orange panel will describe *all* imported files.
6. To refresh the program, clearing all data that has been imported, click the RESET button in the bottom right corner.

All actions that you will perform on imported data involve buttons in the blue panel on the right.

Display the data as a time trace of detection counts

1. In the *window size* box, type in the window size Δt you would like to use for binning your data. NOTE – the units are in μs.
2. Push the **Display Trace** button. Your calculated time trace of detection counts will appear in a new window. Each point is the number of events detected within an interval of time Δt.

Calculate the ACF

To calculate the ACF of your imported data, you must provide the program with two inputs. The first is the total length of the ACF you would like to calculate. It becomes more computationally intensive to calculate the ACF out to larger values of τ. There is also a point at which calculating the ACF for larger values of τ does not provide you with any more information. For example, if you are looking for a feature in the ACF at τ=1 ms, you may only want to calculate the ACF out to 5-10 ms.

You must also input a bin size over which the ACF will be averaged. The program will be unable to resolve any structure in the ACF that is smaller than the smallest measured durations. Similarly, you will be unable to resolve any structures in the ACF that are smaller than the bin size.

You should play around with both of these values, and try to find the optimal values for your data. Remember, you are looking for a drop in the ACF around the value of τ that you calculated at the end of the background, for Question #2.

1. Type in the length of the ACF file you would like to calculate and the size of the averaging window in the *Length* and *bin size* windows. NOTE – the units are in μs.
2. Push the **Calculate ACF** button. Your calculated autocorrelation function will appear in a new window.

QUESTION:

The program is taking individual time traces from different data files and attaching them end-to-end to form one large time trace. In other words, it assumes that all the durations, which have been imported from *different* data files, came from the *same* time trace. What errors could be introduced by assuming all the durations came from one continuous time trace? Do you think that it is reasonable to make this assumption?

Approaching single molecule conditions

You now have the tools to collect single molecule data. The following protocol will allow you to search out the optimal data collection conditions.

1. Collect data at a particular fluorophore concentration and at various laser intensities.
2. Using the **Calculate ACF** function, calculate and examine the ACF for your various laser intensities. Can you see the decay in the ACF due to correlation in the fluorescence signal? Play around with the *Length* and *bin size* – in case you are not looking at the right time-scale.

   If you do not see the drop in the ACF in any of your data files, possible problems are:

   a. The scattering signal is too large. Are you using clean coverslips? Did the coverslips or sample get dirty?

   b. The fluorophore concentration could be so low that you are not getting any fluorescent signal,

   c. The fluorophore concentration could be so high that the ACF is dominated by stochastic fluctuations in the fluorescence.

   d. You are looking for a change in the ACF at the wrong time-scale. Did you miscalculate the time for the antibody to diffuse across the confocal volume? Is your prediction similar to the other group's prediction?

   Determine which you think is most likely, make a new sample that addresses this problem, and start again at 1.

If you see a drop in the ACF:

3. Before moving on, make sure that the drop in ACF is at a time-scale that makes sense.
4. Determine which laser intensity yielded the most pronounced decay in the ACF. The signal-to-noise should be most optimal for this data set.

5. Using the **Display Trace** feature, display the data as a time trace of detection counts . Use a variety of window sizes. Do you think this is single molecule data? Why or why not?

If you suspect your data may contain single-molecule events, then it is time to be more rigorous. Up until now, we have been extremely qualitative, with our primary criterion being how well we can see a drop in the ACF. Move on to Day 6, where you will determine how likely it is that your data is single molecule.

If you do not believe that your data is single molecule, what changes do you need to make? Make a new sample, and start again at *Step 1*.

DAY 6: VERIFYING YOUR DATA IS SINGLE MOLECULE

It is important to quantitatively test whether your measurements contain fluorescence from single fluorophores. Unfortunately, we cannot trust our eyes alone. An important rule in single molecule studies is: *if you are looking for it, you will probably see it whether it is there or not.*

While it is not possible to say with 100% certainty that a particular set of measurements is the result of fluorescence from individual molecules, it is possible to test the likelihood that this is true. More precisely, by convincing yourself that features in your measurements are unlikely to be the result of noise or fluorescence from numerous fluorophores, you can increase your confidence that they arise from fluorescence of a single molecule.

Before continuing, you first need to understand the statistical tests that will allow you to quantify your confidence.

Background: Statistical analysis of your data

For a process that occurs randomly at a fixed rate, with each event occurring independently of the time since the previous event, we can ask: What is the probability that N events will occur in a fixed period of time Δt. The Poisson distribution is the discrete distribution describing this probability:

$$P(N;\lambda) = \frac{\lambda^N e^{-\lambda}}{N!} \tag{1}$$

λ is the mean number of events that occur within the interval Δt, and so the mean rate of events is $\lambda/\Delta t$. Though people often associate the Poisson distribution with rarely occurring events, this equation is also appropriate for large values of λ, for which it approaches the more familiar normal distribution.

An important property of the Poisson distribution (which we will not prove here) is that its variance is equal to λ. Thus, a series of measurements of a Poisson process will be centered at λ, and the standard deviation about the mean will be $\lambda^{1/2}$.

How does the Poisson distribution relate to your experiments?

When you use the **Display Trace** function, you are calculating the detection counts $\{n_i\}$ observed in a series of intervals of duration Δt (whose value is the input in the *window size* box). Since we are looking at a superposition of both the scattering and fluorescence signals, we expect that the distribution of values $\{n_i\}$ will be the sum of two independent Poisson distributions.

We denote the mean scattering count for the time interval Δt as μ_s. At high fluorophore concentrations, there will always be a fluorophore in the confocal volume. Thus, we expect that the mean of the distribution $\{n_i\}$ will be much larger than μ_s due to the contribution from fluorescence emission.

As the fluorophore concentration is reduced, however, it will become more rare for the detection volume to contain a fluorophore. Eventually, when you approach concentrations appropriate for single molecule fluorescence detection, the confocal volume will almost always be empty. Only on relatively rare occasions will a fluorophore enter the confocal volume.

At these low concentrations, the mean of $\{n_i\}$ will approach μ_s. Similarly, the distribution of $\{n_i\}$ will approach the Poisson distribution of the scattering signal alone. Only occasionally will a fluorophore enter the confocal volume, during which time the photon count measurements will be much higher than this Poisson distribution would predict. We can take advantage of this behavior at low fluorophore concentrations to identify detection counts that are likely the result of fluorescence emission.

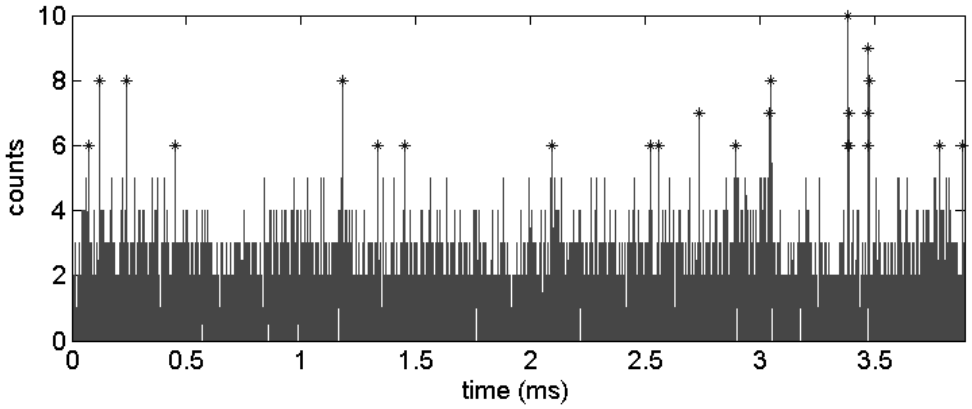
Identifying measurements that contain fluorescence emission

When you push the **Poisson Dist** button, the Matlab program analyzes the distribution of detection counts $\{n_i\}$ that were calculated when you binned your data using the **Display Trace** function. The program then calculates the mean value μ of the detection counts. As discussed above, for very low fluorophore concentrations, nearly all the values $\{n_i\}$ are described by a Poisson distribution with mean value μ_s. When a fluorophore enters the confocal volume, the number of detection events will no longer be described by this distribution, but this occurs so infrequently that the calculated value of μ does not deviate significantly from μ_s.

The program scans the time trace and calculates $P(n_i; \mu)$, the probability that a particular n_i arose from a Poisson distribution with mean value μ, using equation (1). In the $p <$ box next to the **Poisson Dist** button, you must input a probability cutoff. If $P(n_i; \mu)$ is below this cutoff value, the value of n_i in the plotted time trace is marked with a blue asterisk.

What can we say about these marked values? These values of n_i have a probability equal to or lower than the cutoff probability that they came from the distribution of scattering measurements. Assuming that you chose a small cutoff probability, this means it is unlikely that they arose from the scattering distribution. Thus, we have an increased confidence that these values arose from fluorescence emission, and are not the result of random variations in the scattering signal.

For example, in the following trace, a dataset consisting of 30 files, each containing 300 durations, is displayed as a detection count time trace:



The *window size* used to bin the data is 0.5 ms, and the $p <$ cutoff is 0.001. Thus, for all the counts marked with asterisks, the probability that these measurements are part of a Poisson distribution with mean value equal to the mean μ of the time trace is less than or equal to 0.1%.

It is important to ask the question: how many of these asterisked points would we have expected if all the data in the time trace were described by a Poisson distribution? In the *Comments* window, the program tells you how many bins are in the time trace. For the trace above, the program tells us there are 7787 windows.