General Procedure of Fluorometer operation

(QM-4 + TMII + Sphere, 2814 S/N with Felix32)

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A. Steady State (Xenon Flash Lamp)

1. **Preparation and warm up of the Instrument**

   Caution! The average life of 75 Watt Xenon Arc lamp is approximately 500 ~ 700 hours. If

   the voltage of the lamp is around 14, it is time to replace the lamp.

   To replace the lamp, read the manual first, and visit the PTI web tutoring movies

   (http://www.pti-nj.com/Technical-Movies.html)

   After lamp replacement, you should align the lamp carefully according to the manual.

   A. Turn on the lamp first!

      Click "power" on LPS-200B lamp power supply, the voltage should be below 14 V, the

      power will slowly increase to 72-73 (adjust "Current" to obtain a value of 72). Warm up

      the lamp for 15 minutes before taking any measurements.

   B. Open the Felix32 software on the desktop. Turn on the power for other parts: SC-500

      shutter control, two MD-5020 motor drivers (NOT necessary to turn on TCM-1000

      control module and Nitrogen Laser if you do not want to use laser).   The Brytebox is the

      last one to turn on.

   C. (Optional) Turn on the temperature control box and click "Run/Stop" button (DO

      NOT forget to plug in the Eheim Pump first), the initial setting temperature is 20.

      Then it is ready to go!

2. **Emission Scan (solution sample)**

   A. Choose "new acquisition" and click "emission scan".

   B. Choose "digital" HW configuration from the drop-down menu.

   C. Change the parameters as you want, such as excitation wavelength, emission range,

      integration time and averages (large average can smooth your spectra).

D. Click "ACQUIRE (PREP)" to initiate all of the parameter settings for the Instrument, you should hear the motor moving (clicking sound).   Then double-check the excitation wavelength and emission wavelength values displayed at the motors. If the numbers are incorrect (greater than ±0.5 nm) calibrate the instrument (see section 4).

E. Click "ABORT" instead of "START", then choose "More" to change the slit width for the Ex and Em windows (default is 5 nm), and also you can do "Real Time Correction" to get rid of system error, which comes from different intensities at different wavelength of the Xenon lamp. To do that, check the "Enabled" box, and uncheck the "XCOR" box, then click "Reference Source Gain" to pop out a new window. Open the Shutter, and "Goto" 468 nm wavelength, change the signal value to 1.00 V by adjusting the "Gain".

F. Once setup is complete, click "ACQUIRE (PREP)" again to change the parameters, then you can collect spectra by clicking "START".

G. If the signal saturated the detector (flat line), press "STOP" and change the slit widths and adjust the real time correction.

H. Once scan is complete, choose the best result and save data (and acquisition method). You can easily export your x and y data points by copying the files into Excel or Origin.

3. **Excitation Scan (solution sample)**

A. Choose "new acquisition" and click "excitation scan".

B. Choose "digital" HW configuration.

C. Change the parameters as you want, such as excitation range, emission wavelength, integration time and averages (large average can smooth your spectra).

D. Click "ACQUIRE (PREP)" to initiate all of the parameter settings for the Instrument, you should hear the motor moving (clicking sound).   Then double-check the excitation

wavelength and emission wavelength values displayed at the motors. If the numbers are incorrect (greater than ±0.5 nm) calibrate the instrument (see section 4).

E. Click "ABORT" instead of "START", then choose "More" to change the slit width for the Ex and Em windows (default is 5 nm), and also you can do "Real Time Correction" to get rid of system error, which comes from different intensities at different wavelength of the Xenon lamp. To do that, check the "Enabled" box, and uncheck the "XCOR" box, then click "Reference Source Gain" to pop out a new window. Open the Shutter, and "Goto" 468 nm wavelength, change the signal value to 1.00 V by adjusting the "Gain".

F. Once setup is complete, click "ACQUIRE (PREP)" again to change the parameters, then you can collect spectra by clicking "START".

G. Once scan is complete, choose the best result and save data (and acquisition method). You can easily export your x and y data points by copying the files into Excel or Origin.

4. **Calibration or Initiation**

A. Open the Felix32 software, go to "Configuration" and choose "Hardware Configuration", find and click "digital". That will open a diagram of the instrument setup. DO NOT change anything here.

B. Click "Configuration" from the tool bar and choose "Initialize", the instrument will perform a self-check. After both excitation and emission motors stop running, read the numbers and put the real values into the corresponding windows.

C. Next, go to new acquisition and set up a scan to compare the numbers from the software to the values from motors. It is done!

* Before calibration, you should see "ACQUIRE (PREP)" instead of "START" on the parameter window. If you can see "START", "Initialize" menu will not be activated.

5.   Thin Film and Powder Sample (solid) Measurement

Thin film and powder samples measurements need different sample holder compared with solution measurements. Before doing solid sample measurement, you have to change the sample holder manually (thin film and powder samples share the same base, which can tune the angle, but they use different holder heads which are easily switched).

A. First unscrew the black board C on the bottom right corner, and detach the Emission lens. Then carefully unscrew the solution sample holder, which is connected with several cables (undetachable) and put the solution sample holder away (be careful of the wires). Install the thin film and powder sample holder on the same position of solution sample and put the Emission lens back (see Figure 1.)
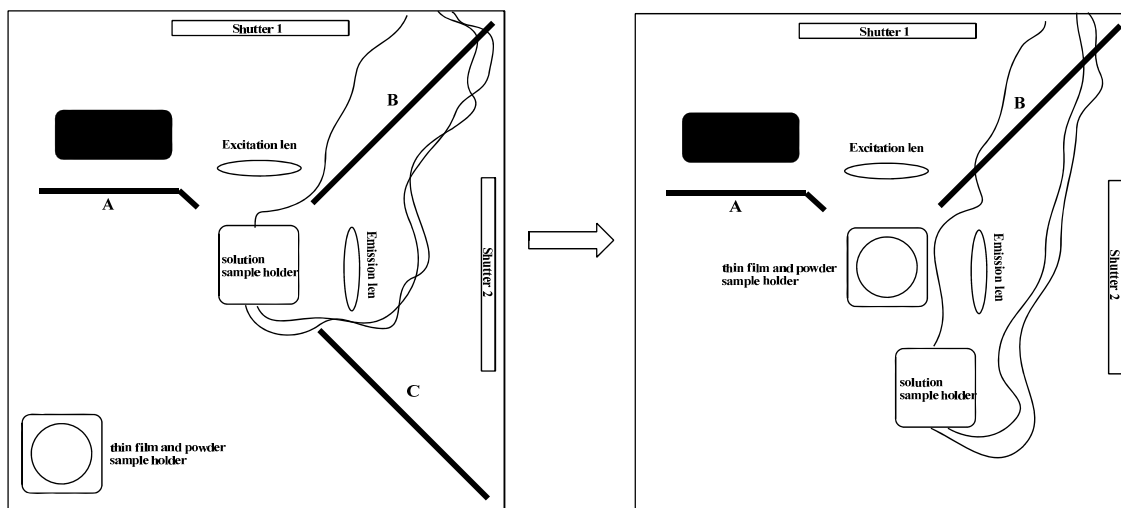

Figure 1. Fluorometer sample chamber (top view)

B. After inserting your thin film glass slide (or powder sample), adjust the angle to approximately -12°, which gives the best fluorescence intensity with minimum reflection from excitation light.

C. The following steps are same as Emission Scan (solution sample). But for film or powder sample, you need to collect background scan.

   * For powder sample measurements, you have to clean the sample holder carefully by unscrewing the cover and rinsing with acetone or dichloromethane, then methanol. Allow sample holder to air dry.

6. **Absolute Quantum Yield Measurement**

* Intensity at excited wavelength should be below 2million count which limits slit width below ~2nm.

*If there is overlap between absorption and emission band, getting accuracy becomes difficult.

*Sample holder position should be consistent. (Mark the sample holder and sphere)

*Corrected spectrum should be used. (Do NOT use uncorrected one.)

A. Before installation, adjust detector dial the position from "chamber" to "sphere" and "Shutter 2" should be closed manually.

B. Remove the plastic caps on the beam pathway to install the Integration Sphere.

C. Carefully install the sphere in the front of chamber and then adjust the height of the sphere by adjusting the leg.

D. Connect optical fiber to the left side of sphere.

E. Prepare the sample holder and cuvette, then fill with your sample (~1mL)

F. Fix the cuvette to the holder gently.

   Caution! In case of solid sample, the sample should be powder-like solid. After put the powder sample into the holder, very gently screw down the plug to make it film-like sample. Otherwise, solid sample can easily break the quartz window of solid sample holder.

G. After select excitation wavelength, set up the scan range which should include excitation wavelength.

Ex) Excitation wavelength: 300nm, Scan range: 290 ~ 580nm

H. Adjust slit width very small such as 1.0, 1.5, or 2.0 in order to prevent of saturation at excitation wavelength.

I. Obtain the emission of solvent, and sample separately, then subtract sample emission to solvent emission. For powder sample, the blank reference is $BaSO_4$.

J. Calculate the quantum yield which is the ratio the emission total area to absorption total area. (You can use PTI software or Excel)

K. Repeat at different slit width which should show the consistent quantum yield value.

7. **Standard Method for Quantum Yield Measurement**

The most reliable method for recording Φ_F is the comparative method in which involves the use of well characterized standard samples with known Φ_F values. Essentially, solutions of the standard and test samples with identical absorbance at the same excitation wavelength can be assumed to be absorbing the same number of photons. Hence, a simple ratio of the integrated fluorescence intensities of the two solutions (**recorded under identical conditions**) will yield the ratio of the quantum yield values.

Standard samples. The standard samples should be chosen to ensure they absorb at the excitation wavelength of choice for the test sample, and, if possible, emit in a similar region to the test sample. The standard samples must be well characterized and suitable for such use. A number of lists of good fluorescent standard samples are available, including: http://omlc.ogi.edu/spectra/PhotochemCAD/index.html

Concentration range. In order to minimize re-absorption effects absorbance in the 10 mm fluorescence cuvette should never exceed 0.1 at *and above* the excitation wavelength. Above this level, non-linear effects may be observed due to inner filter effects, and the resulting

quantum yield values may be perturbed. Remember that this maximum allowable value of the recorded absorbance must be adjusted depending upon the path length of the absorption cuvette being used (for example, 10 mm = 0.1 maximum, 20 mm = 0.2 maximum etc).

Sample preparation. It is vital that all glassware is kept scrupulously clean, and solvents must be of spectroscopic grade and checked for background fluorescence.

Procedure:

A. Record the UV-vis absorbance spectrum of the solvent background for the chosen sample. Note down the absorbance at the excitation wavelength to be used.

B. Record the fluorescence spectrum of the same solution.

Calculate and note down the integrated fluorescence intensity (that is, the area of the fluorescence spectrum) from the fully corrected fluorescence spectrum.

C. Repeat steps 1. and 2. for five solutions with increasing concentrations of the chosen sample.

(There will be six solutions in all, corresponding to absorbance at the excitation wavelength of 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10.)

D. Plot a graph of integrated fluorescence intensity *vs* absorbance. The result should be a straight line with gradient m, and intercept = 0.

E. Repeat steps 1. to 4. for the remaining samples.

Calculation of Fluorescence Quantum Yields from Acquired Data:

The slope of the graphs obtained above is proportional to the quantum yield of the different samples. Absolute values are calculated using the standard samples which have a fixed and known fluorescence quantum yield value, according to the following equation:

$$\Phi_X = \Phi_{ST}\left(\frac{Grad_X}{Grad_{ST}}\right)\left(\frac{\eta_X^2}{\eta_{ST}^2}\right)$$

Where the subscripts ST and X denote standard and test respectively, Φ is the fluorescence quantum yield, *Grad* the slope from the plot of integrated fluorescence intensity *vs* absorbance, and η the refractive index of the solvent.
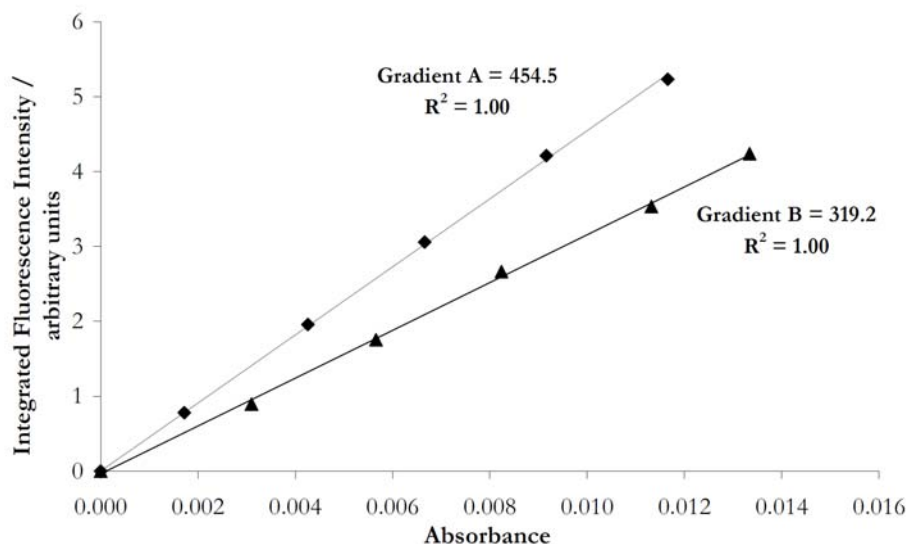
Figure. Example of linear plots for two standard samples. The gradient for each sample is proportional to that sample's fluorescence quantum yield. Conversion into an absolute quantum yield is achieved through the equation given in the text.

You can cross-calibrate with two standard compounds by using this equation. This is achieved by calculating the quantum yield of each standard sample relative to the other. For example, if the two standard samples are labeled A and B, initially A is treated as the standard (ST) and B as the test sample (X), and the known Φ_F for A is used. Following this, the process is reversed, such that B is now treated as the standard (ST) and A becomes the

test sample (X). In this manner, the quantum yields of A and B are calculated relative to B and A respectively.

The values of $\Phi_F(A)$ and $\Phi_F(B)$ obtained by experiment in this way should match the literature values. The match is generally considered valid and acceptable if the data obtained is of good quality (i.e., good linearity with a zero intercept) and the experimental quantum yields match their literature counterparts within ±10%. If the values show a larger error than this, fresh standard solutions should be prepared, and the process repeated. Possible reasons for a poor match include contamination or degradation of the samples or inaccurate spectral correction of the spectrofluorimeter.

Once the standard samples have been cross-calibrated and an acceptable match obtained, the quantum yield values for the test samples can then be calculated, using the same equation above. For each test sample, *two* Φ_F values will be obtained, one relative to standard A, the other to standard B. The simple average of these two values represents the quantum yield of the test sample. The error in the reported value must be considered based upon the observed errors in the gradients; a value of ±10% is normal.

8. **Fluorescence Anisotropy**

    Rhodamine B in glycerol is a good standard to check the performance of fluorescent anisotropy. See a reference, *Analytical Biochemistry* **1974**, *58*, 361-367

    A. Install the anisotropy tools (polarizer) in both emission and excitation position inside the chamber.
    B. Click "New Acquisition" and then choose "Timebased Polarization" and set up the parameters such as excitation and emission wavelength.

C. As a scatter blank, cream in water should be checked first; The cream is dispersed in water but you need tiny amount of cream for scattering. If the cream solution looks cloudy, then make more diluted solution.

(Use the same emission wavelength to excitation wavelength because you are watching scattering not emission)

D. After checking the blank solution, then you are ready to measure anisotropy.

(During the measurement, G-factor is automatically calculated)

9. **Kinetic Measurement**

– Ref. "*An Introduction to Chemical Kinetics*" by Margaret R. Wright

The reference book is available as e-book version in chemistry library web site.

(http://www.libraries.iub.edu/index.php?pageId=5188)

A. Install the cuvette holder cap and syringe holder.

B. Click "New Acquisition" and then choose "Timebased"

C. Set up the parameters such as duration time – The excitation and emission wavelength should remain fixed through the experiment.

D. Prepare the cuvette; after filling the sample into the cuvette, cover the mouth of cuvette with aluminum foil and then wrap with parafilm to fix.

E. Check the base line; record the initial intensity at 0 minute, which is zero intensity.

F. Put the syringe into the syringe holder.

(Make sure the needle tip position should be in between solution and parafilm)

G. At the exact time such as 10 second, push the syringe plunger (delivery).

(After deliver the guest solution, usually do not take the syringe out from the syringe holder to prevent evaporation of solvent)

H. Check the shape of intensity change during the initial 20sec.

(Until deliver the guest solution, there should be no change of the intensity. If there is a change, which means that the guest solution was delivered a little before the delivery time or there is a leaking in the syringe)

B. Time Master (Nitrogen Laser or Dye Laser)

Caution! Before using Nitrogen Laser, check the pressure of nitrogen gas cylinder then close the valve of gas regulator again.

1. Turn on the Laser

Before performing any measurements, turn on the laser! Turn on the TCM-1000 control module (switch on the back) and turn on nitrogen cylinder (~ 80 psi) as well as the nitrogen laser power (using the key, like starting a car). The regulator G1 should be 45 psi and G2 should be 80 psi. Then hold the "GAS PURGE" button (you can hear the gas flow) until the "LASER ON" light is on.

2. Aligning the Laser

Once the laser is on, you should align the laser to obtain the maximum intensity. Open the "Dye Laser" and "Frequency Doubler" boxes to make sure the laser is operating correctly. The original wavelength from the nitrogen laser is 337.1 nm. By using dye molecule solutions in the "Dye Laser" box and different filters in the "Frequency Doubler" box, we can obtain a desired excitation wavelength. On the left side of the "Frequency Doubler" box, the laser beam will shoot into optical fiber, which can transfer the laser signal to the sample chamber.

Turn off lab light, and switch "INT MODE" (on the right side of "Nitrogen Laser" box) the laser will begin to pulse (you will hear it!). Then tune the FP-1A holder to obtain the maximum laser signal by checking the intensity from the other end of optical fiber (usually takes longer time than expected). Then it is good to go!

* DO NOT forget to switch to the laser detector (the switch is beneath the detector).

3. Rewire the Optical Fiber

Due to the weak intensity of nitrogen laser, rewire the optical fiber position to get better signals and results. As shown below (Figure 2.), carefully pull out the optical fiber and move to the front of the sample chamber. Install another lens (same as Excitation lens) between fiber and solution sample holder. Adjust the position of the lens to obtain the best intensity from the optical fiber. After that, **DO NOT forget to switch to the laser detector (the switch is beneath the detector).**
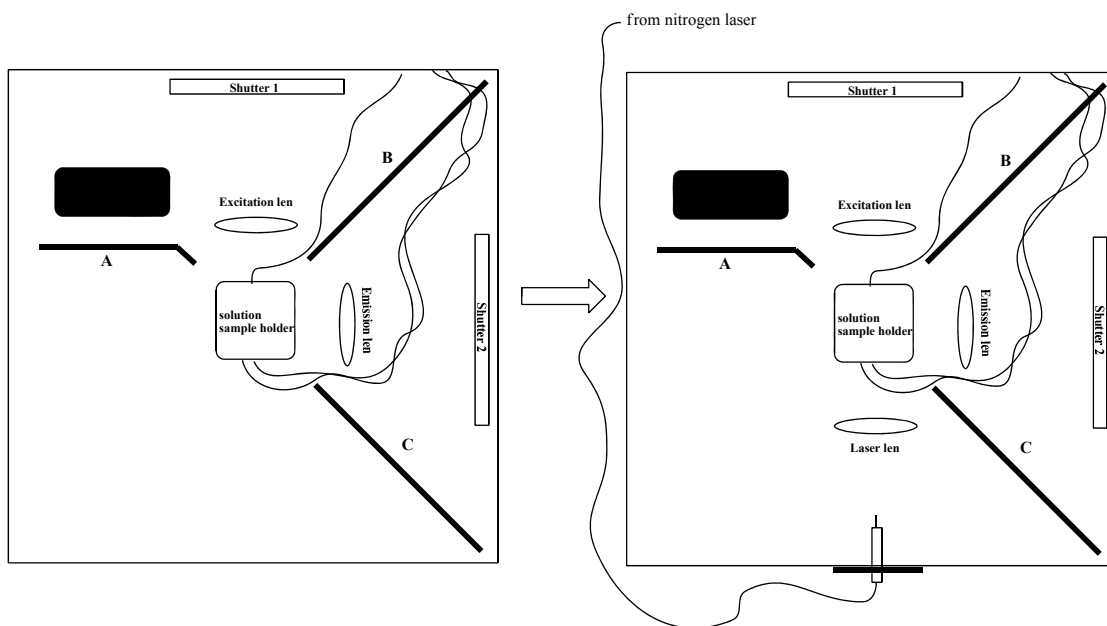

Figure 2. Rewire the optical fiber to the front of the sample chamber

4. Preparation of Scatterer Solution (for IRF)

Before going further, first understand the stroboscopic technique (strobe technique) with consulting PTI manuals.

In order to obtain the fluorescence lifetime, the profile of ***instrument response function*** (***IRF***, excitation pulse) has to be measured in addition to the fluorescence decay. This is because the lamp (laser) pulse has a finite temporal width, which distorts the intrinsic fluorescence response from the sample. This effect is called ***convolution***.

 In a typical experiment, two curves are measured: the ***instrument response function*** (***IRF***) using a scatterer solution and the ***decay curve***. Analysis is then performed by convoluting the IRF with a ***model function*** (e.g. a single exponential decay or a double exponential decay or some other function) and then comparing the result with the experimental decay. This is done by an iterative numerical procedure until the best agreement with the experimental decay curve is achieved.
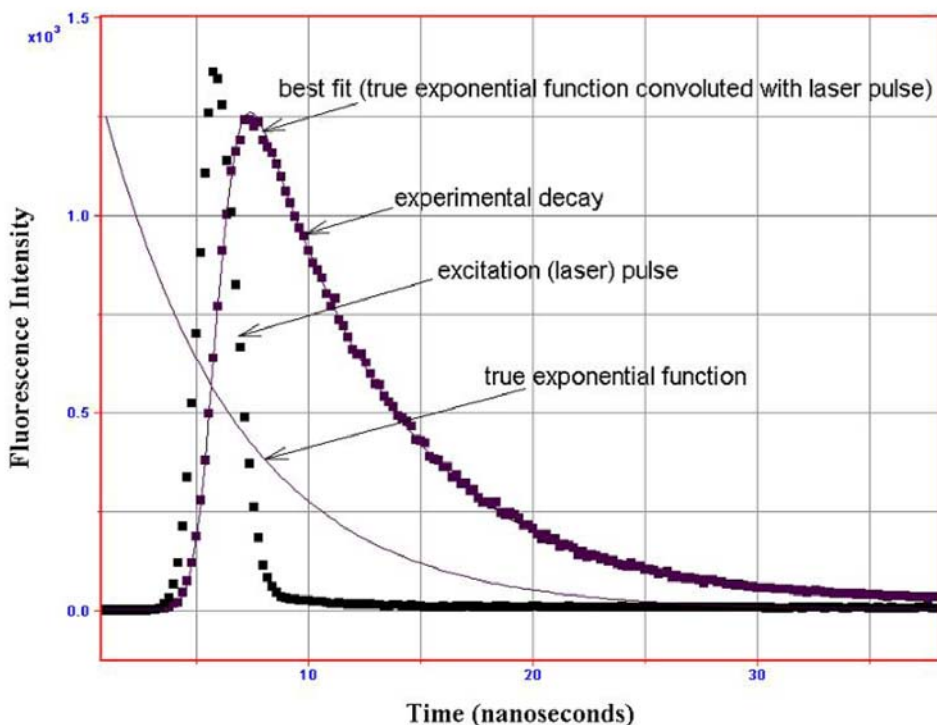
Figure 3. Experimental laser profile and decay (dotted functions) and the best numerical fit in a typical time-domain experiment. The true exponential function represents the model decay, i.e. a hypothetical decay if the laser pulse was infinitely narrow.

The scattering solution can be made from powdered non-dairy creamer. In all cases the concentration of the scatterer should be adjusted to be *as high as conveniently possible while still not causing the scattering signal to saturate*. The reason that the scatterer should be relatively intense is that data analysis by convolution methods requires an accurate measurement of the scatterer intensity. If the scatterer is too weak, noise in the IRF will make the analysis of the sample decay difficult or unreliable.

5.   Fluorescence Decay

Fluorescence Decay is used to determine the fluorescence lifetime of a sample (usually in nanoseconds). As standard samples, Rhodamine B and Erythrosin B in water are available, which are on the reference and web site below.

Anal. Chem. **2007**, *79*, 2137-2149, and http://www.iss.com/resources/lifetime.html

Since the laser strobe technique is intensity based, it is always advantageous to have the sample *as intense as possible* without saturating the detector.

   A. Choose "new acquisition" and click "Fl. Decay".

   B. Choose "Istrobe" HW configuration (only choice).

   C. Change the parameters:

   For example, excitation wavelength (337 nm for nitrogen laser), emission wavelength (337 nm to obtain IRF using aq. creamer solution), then change to the wavelength of your sample to get decay.

   D. Notice that every new decay scan the "Background: Acq" box will be checked. Then Click "ACQUIRE (PREP)" to initiate all of the parameter settings for the instrument, you should hear the motor moving (clicking sound).

E. Click "START", then you should hear the laser pulse, but it will finish soon without any data, that is for background.

F. Then click "ACQUIRE (PREP)" again and you can collect spectra by click "START".

G. Depending on the signal intensity, adjust your slit width, channels, average and shots number. Large channels number (suggest 200 or above) will give more data points, which is good for fitting data.

H. Usually the pulse will come out at 160 ns, due to delay from traveling through fiber optic cable, and delay from communication between detector and computer. To save time, "Start Delay" can be set at 100 ns.

I. After you finish scan, choose "math"→"analysis"→"1 to 4 Exp. Lifetime" to start fitting fluorescence decay data.

   i.   Choose the IRF spectra (which you collected with creamer aq. solution) and click "IRF".

   ii. Choose the sample decay and click "Decay".

   iii. Select the fitting range (suggest start 100 ns, end 500)

   iv. By click "Start Params" to change numbers of lifetime (1, 2 …) and estimate the lifetime value first. If you have reliable data, no matter what lifetime value you start at, the results should be same.

   v.   Then "start fit" to generate a fitting spectra and text file. Save them separately. Typically in the text file, several parameters should be reasonable, such as: chi2 (Good results produce $0.9 < \chi^2 < 1.2$), lifetime (value should have small error i.e. less than **10%**). The fitting spectra should superimpose with original spectra. It takes a while to get an appropriate data fit, so be patient.

* **Start/End Delay**: The excitation pulse is typically situated 50 to 100 ns for fluorescence and at approximately 100 μs for phosphorescence, after the beginning (0) of the delay allowing data to be collected before the excitation pulse to establish a baseline. The exact position of the pulse must be found from a scattering experiment. The start delay is normally chosen a little before the excitation and the end delay is some 5 to 10 lifetimes after the excitation.

* **Channels**: The number of data points to be collected for each scan. Although the limit imposed by the software is very large, the time taken to collect and analyze decays may become excessive for large numbers of points. A maximum of 1000 points is reasonable.

* **Int Time**: This is the time window in microseconds within which the signal is integrated for each laser pulse. The window should be long enough so that the emission signal is fully contained within it. Set this parameter to 50 μs.

* **Shots**: The number of laser shots to be collected and averaged at each delay for each scan. Extra shots will improve the signal to noise ratio at the expense of additional acquisition time. For statistical reasons, it is generally preferable to average over several scans than over more shots on a single scan. Thus averaging three scans with five shots each scan is better than one scan with fifteen shots.

* **Frequency**: This determines the frequency of laser firing and may be set at up to 20 Hz. Higher frequencies shorten the time required to acquire decay data. However, the consumption of nitrogen gas increases substantially at higher frequencies and the energy per pulse drops. Ten pulses per second is a reasonable choice for most experiments.

6. **Fl. Time Resolved Spectra**

In time resolved spectra, selecting different decay times along the decay curve can be useful for resolving individual fluorophores within a sample mixture.

    A. Choose "new acquisition" and click "Fl. Time Resolved Spectra".

    B. Choose "Istrobe" HW configuration (only choice).

C. Change the parameters:

Int. Time = 50 µs

For example, excitation wavelength (337 nm for nitrogen laser), emission range is same as standard solution emission scan range; Delay 1 is the delay time at which detection window will be opened, should be larger than 160 ns.

Other parameters are similar to "Fl. Decay" scan.

D. Notice that, every new decay scan, the "Background: Acq" box will be checked. Click "ACQUIRE (PREP)" to initiate all of the parameter setting for the instrument, you should hear the motor moving (clicking sound).

E. Click "START", you should hear the laser pulse, but it will finish soon without any data, that is for background checking.

F. Then click "ACQUIRE (PREP)" again and you can collect spectra by click "START".

G. Change "Delay 1" time to collect spectra again. If there are multiple chromophores in your sample with different lifetime, the spectra at different delay time should be different, not the intensities, but the λ_{max}. It is similar to deconvolution.
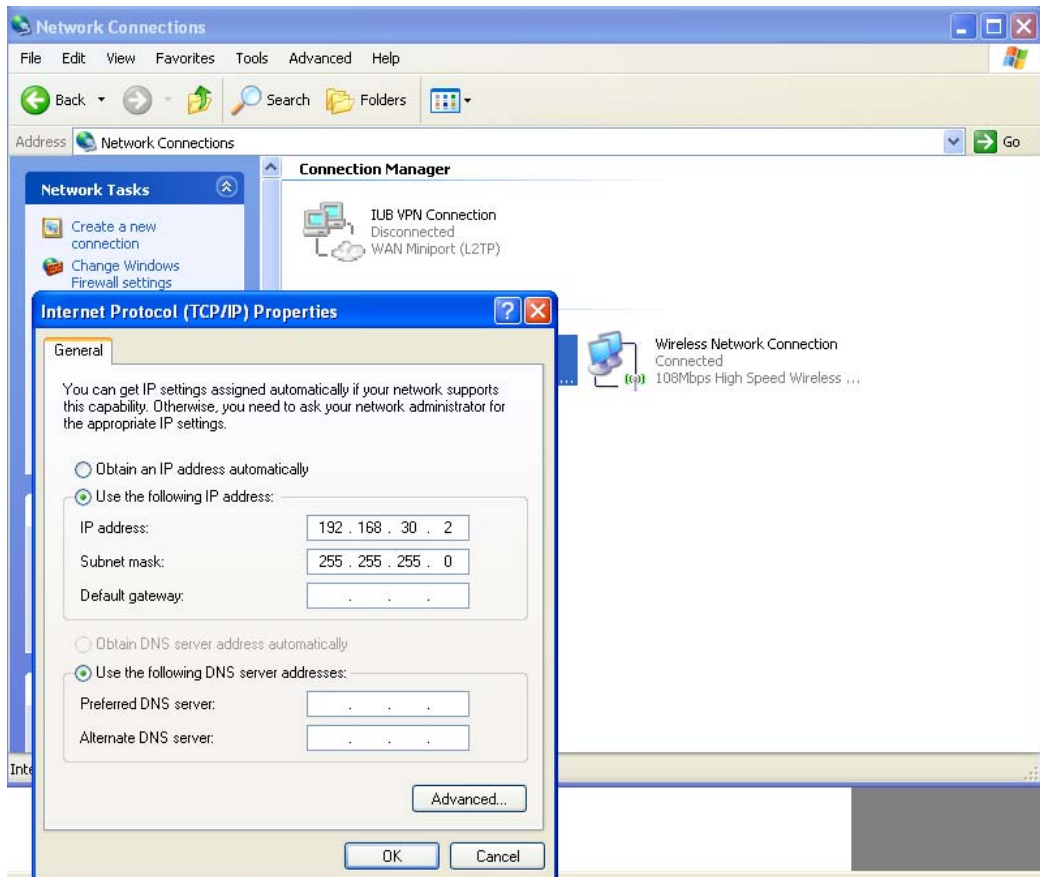
C. Appendix

1. Network Connection between computer and BryteBox

Set IP Address and Subnet mask (see below figure)
IP address: 192.168.30.2
Subnet mask: 255.255.255.0

Set as an **Exception** for Windows Firewall blocking (see below figures)