Protocol for Setting up Anisotropy Measurements in FluorEssence

Calibrating the polarizers
These are instructions given to me by the applications specialist Lin Chandler at Horiba Jobin Yvon. They are a little different from what's written in the manual.
First calibrate the excitation and emission monochromators with the polarizers out of the light path, just as you normally would. Then calibrate the polarizers as follows:

> Make up a ludox solution. Lin said that you usually get more accurate values with more dilute samples. Try 1 drop of ludox in 25mL MilliQ water. Fill the standard 1cm x 1cm cuvette with the ludox solution. In the FluorEssence software menu, go to anisotropy single point measurement and set:
> Excitation wavelength = Emission wavelength = 370nm
> Slits = 3nm - 5nm

Take a measurement with both polarizers in the vertical (0°) position: S1_VV
You want S1_VV to be ~1 - 1.5 $x10^6$ cps. If it is too high (> 2 $x10^6$ cps), you will be out of the linear range of the detector and you must dilute your ludox sample or narrow your slits. Be sure to keep the slits within the above range—you don't want them to be too wide or too narrow. Once you've found sample & slit conditions that give you S1_VV ≈ 1 $x10^6$, measure the anisotropy. It should be close to 1. If anisotropy < 0.97, you need to realign the polarizers. Follow the instructions in the manual (page 10-8), save the new values and then remeasure the anisotropy as above. It should be closer to 1. Values of > 0.98 are typical.

Things to remember when setting up anisotropy measurements

- Be sure to filter all of your solutions. Scattered light is 100% polarized and will systematically distort anisotropy measurements.
- Use temperature control (even for ambient temperatures of 25°C). Fluorescence measurements are highly sensitive to temperature.
- The fluorescence of many fluorophores is pH dependent. For example, fluorescein has a pKa of ~6.5 and its fluorescence is dependent on the charge state, pH ≥ 7.5. To ensure that small changes in pH do not affect the fluorescence, make sure your samples are buffered sufficiently.

The FluorEssence manual (page 10-21) says that you should adjust your sample concentration and slits so that S1_VV is ~1 x 10^6 cps. Values less than that give excessive contributions from dark noise and if signal is >2 x 10^6 cps, you are in the non-linear range of the detector.
Jim Mattheis (Horiba apps. specialist) said you should NOT set your slits > 10nm as you run the risk of increasing contributions of scattered light. The full width half maximum (FWHM) of each monochromator is 10 nm.

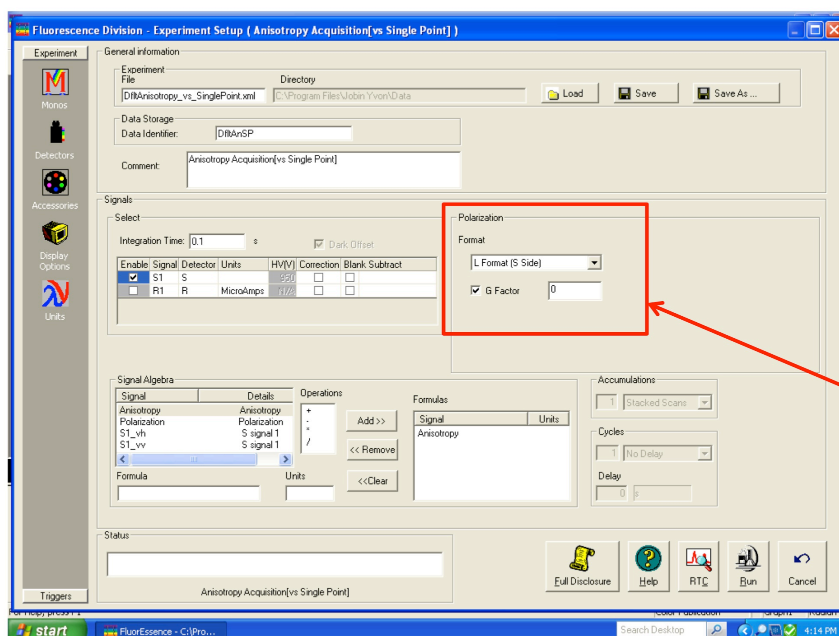
Cuvettes You cannot use the round microcuvette for anisotropy measurements. The CSB has a 500uL square microcuvette + adaptor that will accommodate a stir bar (ask Laura for details). The viewed volume of the cuvette is 250uL, but people have used less (220-240uL) and reported no problems with anisotropy measurements.

G-factor

The G-factor is an instrument-dependent correction factor that requires empirical determination for each fluorophore to be used. It is temperature and wavelength dependent. According to the FluorEssence manual (page 10-2):

G-factor (λem) = S1_HV/S1_HH

This definition may be the inverse of some that are in the literature. A pre-calculated G-factor can be applied to experiments in which instrumental factors (emission wavelength and bandpass) are kept constant. In this case, you would measure the G-factor for your sample, check the G-factor box under the polarization parameter menu (see figure below), and enter in the experimentally-determined value in the box next to it. Alternatively, you can have the instrument calculate the G-factor for you. To do this, you must <u>either</u> leave the G-factor box unchecked OR check the G-factor box and set G-factor=0 (see figure below). If you check the G-factor box and leave the value set at the default=1, it will give you a false anisotropy reading. If you leave the G-factor box unchecked, it doesn't matter what the value is in the box next to it, but you can't leave the box blank.



The G factor will automatically be calculated if the box is left unchecked OR if the box is checked and G factor =0.

Jim Mattheis said that if you have a low signal, you may want to calculate the G-factor yourself and enter it in the G-factor box to increase the sensitivity of the measurement. Entering a manual G-factor automatically fixes S1_HH and S1_HV, leaving more photons for the detection of S1_VV and S1_VH.

Also, in order to increase your signal to noise (S/N), you can increase your integration time. Remember that in order to increase your S/N by a factor of two, you must increase your integration time by a factor of 4.