Standard Operating Procedure for Absorption, Emission and Excitation Spectra

- Initialise CD Spectrometer.
- Open Pro-Data Chirascan and Pro Data Viewer software.
- In Pro Data Viewer create a file for experiment series – including date and solvent in title are recommended.
- Press "Files Name" and fill in details and experiment numbers as required.

UV Spectroscopy

Baseline

- Place cuvette filled with solvent (it is recommended to fill the cuvette at least at its half volume) in CD holder ensuring all surfaces of the cuvette are clean.
- Ensure the software is in Circular Dichroism mode: this is the default setting.
- Adjust the spectral width of the spectrometer by changing "Low", "High" and "Step" values. The "Low" value should be set above the solvent UV cut-off value. The High value depends upon the absorption spectra of the sample you are studying.
- If "Enable Adaptive Sampling" is ticked, the instrument will measure each wavelength with different times depending on the energy required at each region. In this case, the time-per-point should be kept as the default (0.5 sec).
- Press "Baseline".
- N.B. WHEN SPECTRUM IS RUNNING, MONITOR THE **HT** – IF VALUE EXCEEDS 700 V, IMMEDIATELY STOP THE RUN.
- A "Baseline" is required for acquiring Absorption spectra.

Absorption Spectra

- Fill a cuvette (it is recommended to fill the cuvette at least at its half volume) of the same path length with your sample dissolved in your baseline solvent.
- Check the box marked "Auto Subtraction".
- Ensure all the spectral width parameters are as before
- Press "Acquire".
- N.B. WHEN SPECTRUM IS RUNNING, MONITOR THE **HT** – IF VALUE EXCEEDS 700 V, IMMEDIATELY STOP THE RUN

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Fluorescence Spectroscopy

- A 10 x 10 mm cuvette with the 4 sides transparent MUST be used for ALL fluorescence measurements because florescence spectroscopy is highly sensitive, thus the bigger the pathlength, the higher the intensities.
- Select "Fluorescence" from the drop-down list on the "Signal" panel
- Tick the "Emission Mono" box as shown in Figure 1
- Double-click on "PMU" box and adjust the HV according to how strong your fluorophore is (750 or 1000 V are the recommended values). Click "Apply".
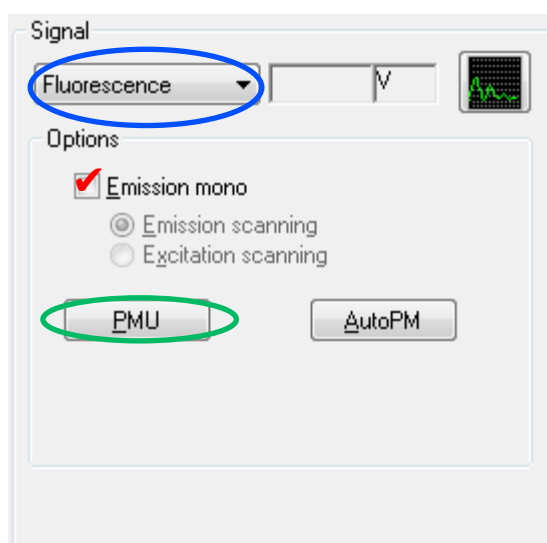- N.B. REOPEN "PMU" BOX AND ENSURE THE HV VALUE HAS BEEN SAVED.

Figure 1. The "Signal" panel for fluorescence mode.

Measuring Fluorescence Emission Spectra:

For **emission** spectra, all the **excitation** wavelengths should be taken as the λ_{max} from the absorption spectra. It is indicated to perform an experiment for each main peak observed in the absorption spectra. The emission spectra will display similar patterns at different excitation wavelengths, but will have different intensities: the higher the irradiation wavelength, the higher the intensity. For accurate fluorescence measurements, the intensity of absorbance spectra should be around 1 a.u. (this is strongly related to the chromophore present in the molecule), otherwise other phenomena such as re-absorption and self-absorption are seen in the fluorescence spectra, leading to misinterpretation.

The time-per-point used for measuring absorption spectra should be maintained the same during fluorescence measurements for consistency. It is indicated to record fluorescence spectra straight after recording the absorption.

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- Select the "Emission Scanning" using the radio button, a Monochromator panel will show up (Figure 2).

- In the box marked "Excitation mono (Mono 1)", set the desired excitation wavelength from the absorption spectra previously acquired. Press "Set" twice.

- Set the bandwidth depending on the fluorophore present (12 nm is recommended in most of the cases). Press "Set" twice. If the detector is maximised (i.e., the intensity in the fluorescence spectrum is 10 a.u.), the bandwidth should be lowered. Please bear in mind that the higher the bandwidth the better signal-to-noise ratio, but at the same time the resolution is lost (broadness of the peaks).

- Set the spectral width (wavelength range) for the emission spectra. The "Low" value can match the wavelength in the corresponding absorption spectrum, but the "High" value should be much higher than the excitation one (in the case of near IR absorbing molecules, set the "High" wavelength to 800 nm). Press the "Set" box next to the "Low" one twice.

- The "Step" is the same as in CD/Absorbance mode (the default is 1.0 nm). Also, a longer time-per-point will reduce the noise of the spectrum.

- If the spectrum is too noisy at PMU 1000 V and bandwidth 12 nm, and no appropriate response is observed (unless in the case of fluorescence quenching), the light intensity can be further increased by changing the slit width knobs on the CS/SEM detector (the default is 1 mm = 4.65 nm bandpass). Both knobs should be set at the same value. Please do not change it by yourself and ask a member of GDP group if this operation is required.

- The "Enable Adaptive Sampling" does the same thing as in the CD/Absorbance mode.
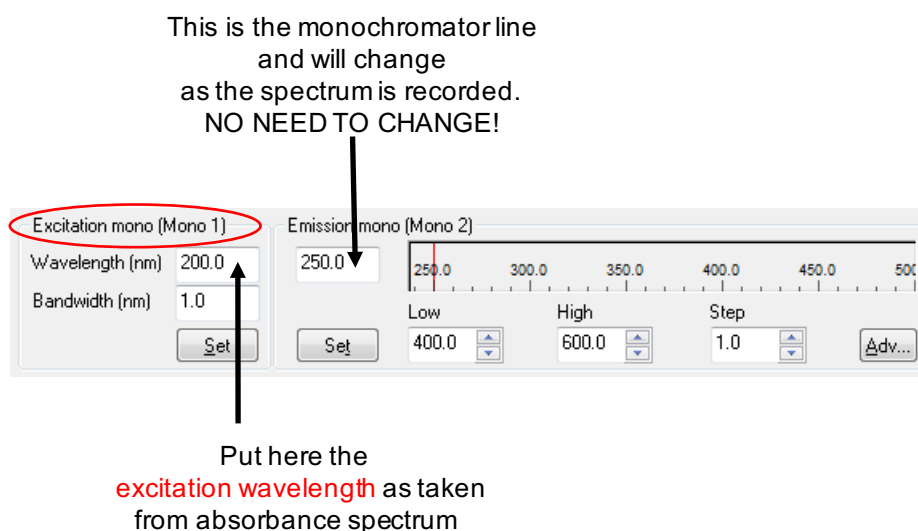
- Press "Acquire".

This is the monochromator line
and will change
as the spectrum is recorded.
NO NEED TO CHANGE!

Put here the
excitation wavelength as taken
from absorbance spectrum

Figure 2. The Excitation mono ("Mono 1") panel.

Measuring Fluorescence Excitation Spectra:

Always acquire the emission spectrum first, and then the excitation one!

For **excitation** spectra, all the **emission** wavelengths should be taken as λ_{max} from the emission spectrum previously acquired. It is indicated to perform an experiment for each main peak observed in the emission spectra. The excitation spectra should display similar pattern to absorption spectra (same λ_{max} but can have different intensities). For accurate fluorescence measurements, the intensity of absorbance spectra should be around 0.1 a.u. (this is strongly related to the chromophore present in the molecule), otherwise other phenomena such as re-absorption and self-absorption will distort the fluorescence spectra.

- Select the "Excitation Scanning" using the radio button, a Monochromator panel will show up (Figure 3).
- In the box marked "Emission mono (Mono 2)", set the desired emission wavelength (λ_{max}) from the emission spectra previously acquired. Press "Set" twice.
- Set the spectral width (wavelength range) for the excitation spectrum. It should cover the range used in the absorption spectrum. Press the "Set" box next to the "Low" one twice.
- The "Step" is the same as in CD/Absorbance mode (the default is 1.0 nm). Also, a longer time-per-point will reduce the noise of the spectrum.
- The "Enable Adaptive Sampling" does the same thing as in the CD/Absorbance mode.
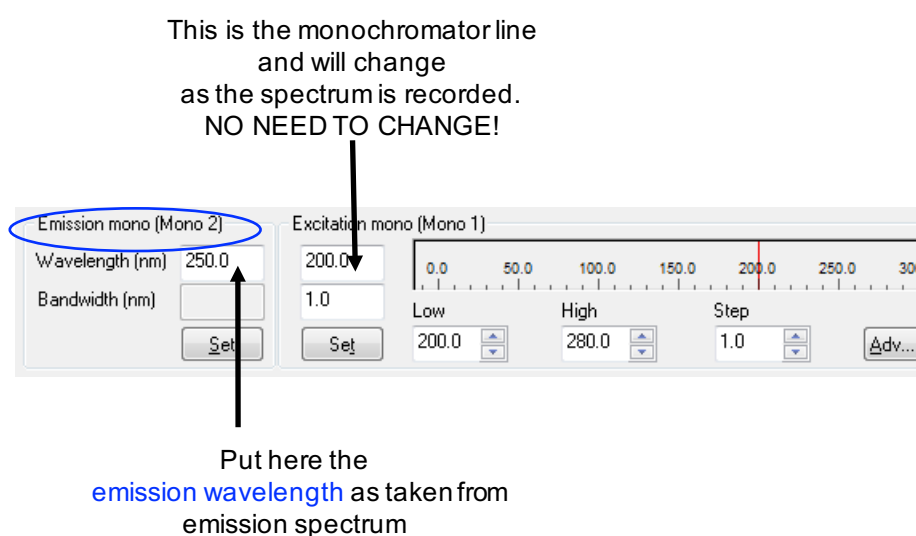- Press "Acquire".

This is the monochromator line
and will change
as the spectrum is recorded.
NO NEED TO CHANGE!

Put here the
emission wavelength as taken from
emission spectrum

Figure 3. The Emission mono ("Mono 2") panel.

Quantum Yield Determination:

A standard compound ("reference") with a known quantum yield is required for determining the quantum yield of a new molecule. The "reference" should absorb as close as possible to the region where the new molecule absorbs.

The λ_{max} from the emission spectrum (corresponding to the same fluorophore as in the reference) is used for quantum yield calculation. The intensity of the absorption spectrum should be under 0.1 a.u. for accurate determination, and both emission and absorption spectra must be zero-corrected.

Please note the quantum yield is temperature dependant, thus, it is important to set the temperature controller at 20 °C (or the temperature at which the quantum yield of the "reference" has been determined).

A comprehensive guide for determining the quantum yield and a list of common "references" can be found at the following link:

http://www.horiba.com/fileadmin/uploads/Scientific/Documents/Fluorescence/quantumyieldstrad.pdf

LME 10/15; TMG 1/16; DMR 8/17

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