Chirascan
FP.3 Fluorescence Polarisation Accessory
User Manual

USE OF THIS DOCUMENT

This document is intended to inform the operator of Applied Photophysics' FP.3 fluorescence polarisation accessory on its design, installation and operation. The FP.3 is used with the Chirascan circular dichroism spectrometer, and this document should be used in conjunction with the User Manuals applicable to that instrument. It is assumed that the user of this document is familiar with the operation of the Chirascan, and with Applied Photophysics Pro-Data software. In particular it is assumed that the user is familiar with the hazards associated with the operation of the spectrometer, and has read the hazard and other safety information contained in its User Manuals.

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FP.3 INSTALLATION AND OPERATIONAL REQUIREMENTS

Safety information

There are no hazards associated with the operation of the FP.3, fluorescence polarisation accessory beyond those described in the main Chirascan User Manual. Please ensure that you are familiar with these before installing or operating the fluorescence accessories.

Environmental and electrical requirements

The FP.3 has no environmental requirements additional to those of the Chirascan spectrometer. Operation of the FP.3 is controlled by the Chirascan electronics, and there are no additional electrical requirements.

Bench space

No additional bench space is required for the FP.3.

Nitrogen purge gas

The ORD.3 has no purge gas requirements additional to those of the Chirascan spectrometer

Servicing

Servicing of the TF.3 and SEM.3 should only be undertaken by qualified personnel. If you are in any doubt at all please contact the Applied Photophysics Technical Support Department.

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CONTENTS

FIGURES

1 INTRODUCTION

The FP.3 fluorescence polarisation accessory is designed to be used with the Chirascan circular dichroism spectrometer.

Fluorescence occurs when electromagnetic radiation absorbed by a sample is re-emitted at a longer wavelength. The difference between fluorescence and the related effect of phosphorescence is sometimes said to be that fluorescence stops when the irradiation source is removed, whereas phosphorescence continues, but it is really a matter of timescales: re-emission in the case of fluorescence is very rapid, of the order of nanoseconds or less, but it cannot be instantaneous.

The fluorescence polarization technique is based on the two effects just mentioned: the change in wavelength and the timescale of re-emission. If the sample is illuminated by a beam of plane polarised light, this will be only be adsorbed by fluorophores (fluorescent species) oriented in a specific way relative to the plane of polarisation, and the re-emitted light will also be polarized. However, in solution the fluorophore is not stationary, it is undergoing continuous rotational and translational diffusion. Since there are numerous fluorophore species present in any sample, the rotational orientation of the absorbing fluorophores will become randomized over time, and hence the plane of polarization of the emitted light will also become randomised. The result will be a decrease in the measured polarisation of the emitted light.

The magnitude of the decrease will depend on the relative timescales of the fluorophore rotational diffusion, and the absorption – re-emission process. If the rotational diffusion is relatively rapid, the decrease will be large; if it is relatively slow, the decrease will be small. The polarisation, P, is quantified by:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

where I is the intensity of the emitted light, and the subscripts indicate the directions parallel to and orthogonal to the plane of polarization. Thus for completely polarised light $P = 1$, whereas for unpolarised light $P = 0$.

It is sometimes more convenient to use the anisotropy, r, rather than the polarization. This is related to P by $r = 2P/(3 − P)$; either function can be selected in the Pro-Data software. If $P = 1$ or 0, r will take the same value, but P and r are not equal at intermediate values.

Because the wavelength of the excitation radiation differs from that of the emitted radiation, there are several types of fluorescence polarization experiment that can be performed. The simplest is total fluorescence at a single excitation wavelength, when all the emitted radiation is detected simultaneously. For example, the sample may be irradiated at a wavelength of 280 nm. A cut-off filter of 305 nm is placed between the sample and the detector to exclude scattered excitation radiation, and all the radiation emitted by the sample at wavelengths longer than 305 nm is detected.

A development of this technique is to scan the excitation wavelength, perhaps between 200 nm and 300 nm using a 320 nm filter. The spectrum thus collected by the detector is an *excitation* spectrum: it is still the total emitted radiation that is being detected, but this varies as a function of the wavelength of the excitation radiation.

For more information on fluorescence polarisation spectroscopy see J.R. Lakowicz "Principles of Fluorescence Spectroscopy", 3rd edition, Plenum, New York, 2006.

The FP.3 fluorescence polarisation accessory consists of a detector comprising a housing fitted with a photomultiplier tube capable of measurement in the range 300-650 nm; a 550 nm cut off filter, 25 mm diameter, with storage box; a filter holder and filter clip, and a fluorescence aperture blanking plate. Alternative PMTs are available for extended wavelength range operation, and alternative filters are also available on request.

2 OPERATING PRINCIPLES

The FP.3 accessory enables the measurement of anisotropy and fluorescence polarisation on the Chirascan. The technique makes use of the existing polarisation optics in the instrument, including the polarising monochromator and the photo-elastic modulator (PEM) to modulate the polarisation of the excitation light. Fluorescence is detected using a single dedicated detector, which can isolate the DC and 100 kHz AC components in the fluorescence signal. Noise is de-coupled from the AC signal using a phase sensitive lock-in circuit in a manner identical to that used for CD measurement.

Mathematical transform equations are then used to convert the AC and DC signal levels into polarization/anisotropy and total fluorescence measurements. Calibration only needs to be performed once at installation time unless the sample cell is changed, and since no additional optics or mechanics are required, the setup and measurement process is extremely quick and straightforward.

3 INSTALLATION

3.1 Hardware installation

Figure 3.1: filter mounted within the filter holder

Before mounting the detector onto the Chirascan, insert the required filter into the rear of the filter holder, and hold in place using the filter clip (Figure 3.1). Fit the filter holder onto the neck of the detector and attach the flexible tip to the holder (Figure 3.2).

Figure 3.2: filter holder mounted on the detector

The detector mounts in the ESHU side port, i.e. at right angles to the light beam (the CD detector can either be left in place in the port in line with the beam, or replaced with the blanking plate). Insert the detector into the port with the flexible tip in contact with the sample holder (Figure 2.3). Tighten the knurled ring by rotating clockwise to hold the detector firmly in place.
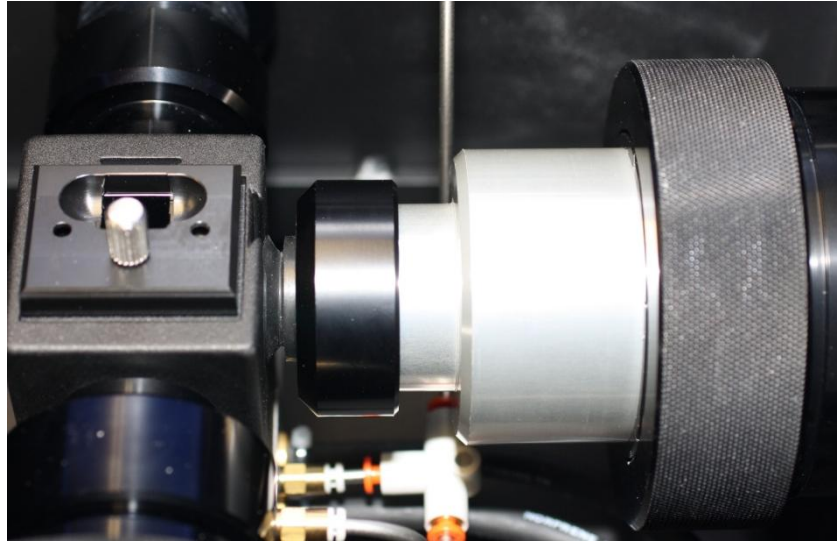
Figure 3.3: detector mounted in the Chirascan ESHU

3.2 Electrical connection

⚠️ **CAUTION: Do not connect or disconnect any electrical leads to any of the electronic units without first ensuring that the units are turned off.**

The fluorescence polarization detector connects to the PMT port on the PEM module at the rear of the Chirascan electronics box, replacing the CD detector. The PMT cable need not be removed from the module when changing between the fluorescence and CD detectors, the same cable can be used for both detectors

4 OPERATION

4.1 The signal panel

The software settings for fluorescence polarisation are set on the **Signal** panel of the Pro-Data Chirascan control software. Select **Fluorescence Polarisation** from the drop-down list. The Signal panel fluorescence polarisation option will appear (Figure 4.1).
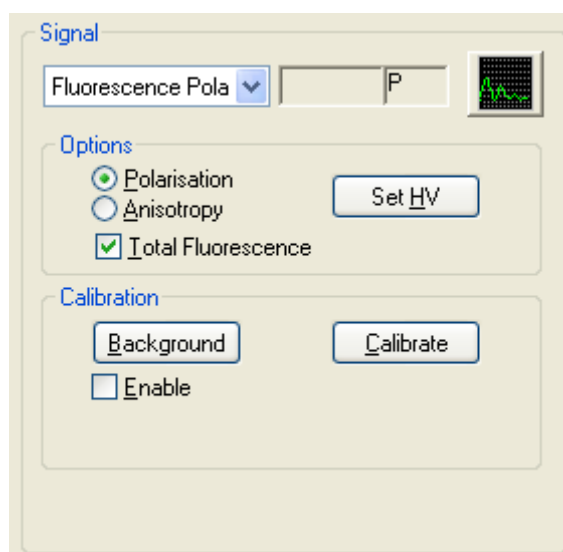

Figure 4.1: the Signal panel fluorescence polarization option

Select the required measured variable (Polarisation or Anisotropy), and tick the **Total Fluorescence** box if that measurement is also required.

Note: disabling the measurement of the total fluorescence will prevent the data being converted from Polarisation units to Anisotropy units and vice versa

4.2 Calibration

4.2.1 The FP detector

The fluorescence polarisation detector collects the fluorescence over a relatively large cone angle. This improves the detection efficiency, but means that the mathematical transforms used must take this into account to compute accurate values for r and P.

In practice, a single calibration value can be used for each any given experimental arrangement. Moreover, this value can be determined

empirically using a sample with a known anisotropy. The value is stored in the instrument software and applied automatically at the point of acquisition. The calibration value will depend on the sample cell used, but the software cannot store more than a single value. However, the calibration only needs to be performed once for each cell, its value can be recorded separately and re-entered into the software when that cell is in use.

The calibration value is relatively insensitive to excitation wavelength, but for best accuracy it is good practice to determine a value for each cell for each wavelength used.

4.2.2 Recommended calibration samples

For accurate calibration, it is recommended that a sample with as large a value of r as possible is used. The ideal sample for calibration is one that scatters with high efficiency and has no fluorescent component, giving a theoretical value of $r = 1$. A glycogen suspension in water is a good example, and APL recommend its use at a concentration is 2 mg/ml.

4.2.3 Performing a calibration

To perform a calibration, use the following procedure:

1. Load the cell with a sample having a known steady state anisotropy (r). Make sure you have selected the appropriate excitation wavelength and cut-off filter for your sample (a filter should not be used with a scattering sample).
2. Set the sampling time on the **Sampling** panel of the ProData control page. The longer the sampling time, the more accurate the calibration.
3. Click the **Calibrate** button in the **Calibration** panel. The **FP Calibration** dialog box will appear (Figure 4.2).
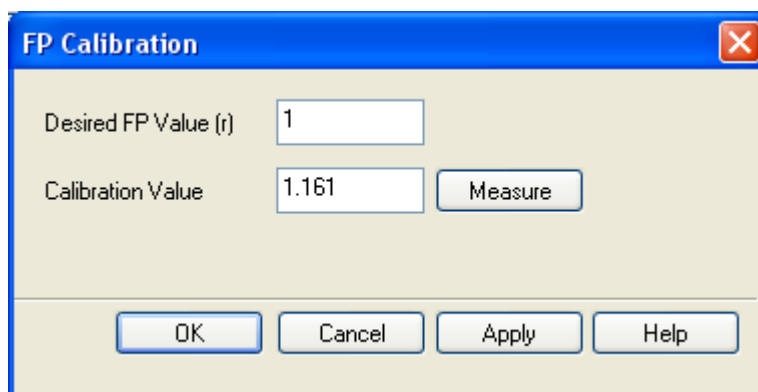
Figure 4.2: the FP Calibration dialog box

4. Enter the known value of anisotropy (r) for your sample in the **Desired FP Value** box. The default value is 1 (one) for a scattering sample.
5. Click **Measure**. Measurements will be made and a newly calculated value will be displayed in the **Calibration Value** box. This will generally show a value slightly larger than 1.
6. To accept the new calibration value, click **Apply**. This will overwrite the existing value. To continue with the existing value click **Cancel**. A pre-recorded value can be entered manually in **Calibration value** box.
7. To close the dialog box click **OK**.

4.3 Measuring a fluorescence polarisation spectrum

4.3.1 Setting the HV

Ensure that you have the required filter in place, and that the correct calibration value is set.

Load the cell containing the sample into the sample holder.

In the **Monochromator** panel of the control software, set the range, step size and bandwidth to those needed for the experiment; in the **Sampling** panel, set an appropriate time per point (see the main Chirascan User Manual for further information).

The measurement of a fluorescence polarisation spectrum requires the setting of a suitable high voltage on the detector. To set a high voltage, click on the **SET HV** button to open the **FP Set HV** dialog box (Figure 4.3).
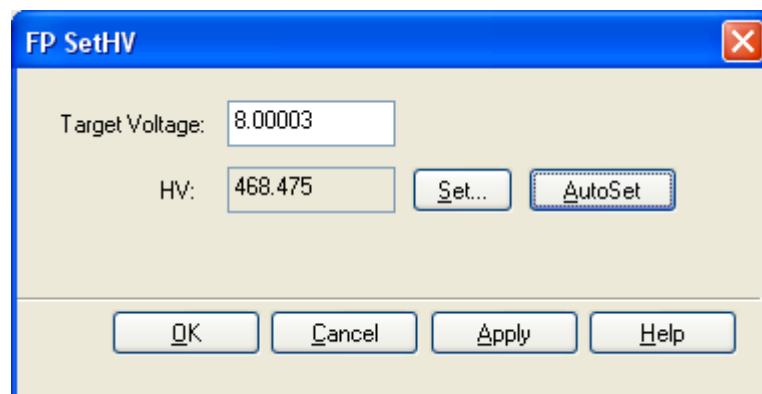
Figure 4.3: the Set HV dialog box

There are two writable fields on the dialog box. The **Target Voltage** is the DC signal voltage that the instrument software will try to achieve by adjusting the HV when the **AutoSet** button is clicked. The default value is 8 volts. The **HV** box displays the voltage that will be applied to the PMT when the **Apply** button is clicked (on first opening the dialog box, this box displays the voltage that is currently set on the detector).

You will not normally need to change the values in these fields manually, but can use the AutoSet function as follows:

1. Open the **SetHV** dialog box
2. Click **AutoSet**. The proposed new HV value will be displayed.
3. Click **Apply**.

If the signal is very weak, it may not be possible to achieve the specified target voltage. A warning message will appear and the HV field will show 1000 V. Although this will not affect the measurement accuracy, you may wish to consider using a wider bandwidth setting on the monochromator in order to improve the noise level in the measurements. If you change the bandwidth, remember to repeat the Set HV procedure.

4.3.2 Taking a measurement

If the above procedure has been followed, the current value of anisotropy (r) or polarisation (P) will be displayed in the field at the top of the **Signal** panel of the ProData control software. The live display panel can be opened by clicking on the icon to view the r or P signal in real time. This gives a useful indication of the amount of noise present in the signal.

It is not possible to view the total fluorescence in real time, although it will be recorded with the fluorescence polarisation acquisition if the **Fluorescence Polarisation** box is ticked.

You may toggle between the anisotropy and polarisation modes by selecting the appropriate radio button in the signal panel. Whichever mode is selected, the interconversion can be simply made after the data has been acquired.

Begin the acquisition in the usual way be clicking the **Acquire** button.

4.3.3 Background correction

Background correction allows for the removal of residual scatter contributions to the detector signal, which can lead to measurement inaccuracies. It may need to be used for samples containing particulate matter, or which have a low fluorescence yield, and for which the scattered light cannot completely be removed using a cutoff filter. Scattering tends to artificially increase the measured values of P or r. If r values are obtained that are greater than 0.4 (or P values greater than 0.5), there almost certainly a scattering contribution.

Background correction requires a determination of the residual AC and DC signals, which are present in the absence of any fluorescence from the sample. When background correction is enabled, these values are subtracted from the incoming AC and DC data streams before the mathematical transforms are applied, yielding corrected values for r, P and the total fluorescence.

To perform a background determination, use the following the procedure:

1. Load the sample and perform the Set HV procedure as described in Section 4.3.1
2. Replace the sample with solvent or a blank equivalent, using the same sample cell. For example, if the experiment involves a fluorescent probe binding to a (non-fluorescent) protein, the blank should contain only the protein. Make sure that the concentration of the protein in the blank is the same as that of the sample.
3. Click the **Background** button on the **Calibration** panel. The background AC and DC values will be measured for each wavelength defined in the current scanning setup. After the background has been measured, the **Enable** box will be ticked.
4. Re-load the sample. Subsequent measurements will be background corrected.

Background correction can be enabled or disabled using the **Enable** tick box. The background values will be stored until the next time background determination is performed. These can be viewed by clicking **Show**.

If the SetHV function is used to change the detector HV, the background will become invalid, and the above procedure will need to be repeated.

4.3.4 Using the detector for standard fluorescence

The fluorescence polarisation detector can be used as a direct replacement for the standard fluorescence detector for measurements that do not require collection of P or r, for example for simultaneous fluorescence and CD determination, or with the scanning emission monochromator. For operation in this mode see document 4207Q113, the TF.3 User Manual.

APPENDIX 1: Technical Specifications

Measurement Accuracy (P or r):   ± 5%
Sensitivity: 1 nM fluorescine (steady state).
Excitation range: 180 nm to > 700 nm (instrument limited)
Detection range: 300 nm to 600 nm. (Detector limited)

If you require detection at other wavelengths, please contact applied Photophysics Ltd. about alternative detector options.