Absorption Measurements on PC1™

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Introduction

The ISS absorption accessory enables measuring of the wavelength-dependent optical density (OD) of a sample on PC1 or K2™ spectrofluorimeters. The transmitted intensity follows the Lambert-Beer law for non-scattering, optically homogeneous non-aggregated samples [1]. Highly scattering samples, aggregates or fluorescent samples require additional correction. This accessory is installed in place of the sample compartment in K2 or PC1 spectrofluorimeters [2].

Figure 1. Absorption Accessory - Side View.

Instrumentation

The absorption spectra shown below were measured on PC1™, the photon-counting spectrofluorimeter from ISS. PC1™ features parallel beam geometry for reliable polarization measurements. Polarization measurements can be performed in the L or T-format. Vinci – Multidimensional Fluorescence Spectroscopy, a comprehensive and flexible fluorescence analysis software package, also enables instrument control and data acquisition directly from the PC.
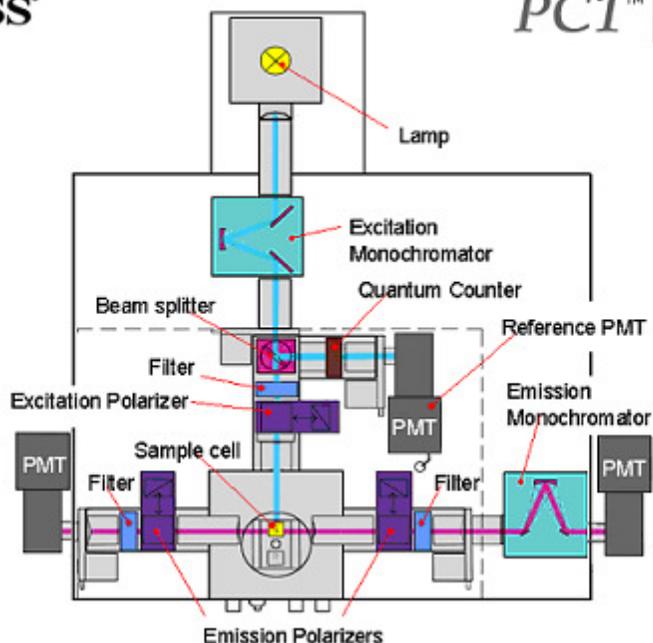
Figure 2. Schematic drawing of PC1, the photon-counting spectrofluorimeter from ISS.

Installing the Absorption Accessory

Although the standard use of the accessory will be for the left emission channel of the instrument (filter holder channel), the accessory can be used also with acquisition through the right emission channel (monochromator channel). In the following, we will assume that the accessory will be utilized with the left emission channel.

Please keep the left emission shutter closed. Turn the left emission PMT voltage temporarily off. This protects the detector from receiving an excessive amount of room light.

Remove the standard sample holder from the instrument. Remove the four 4/40 flat head screws holding the front of the sample compartment to the instrument. Also remove the two 8/32 socket head screws located next to the cooling can, under the emission lenses. Slide the turret halfway out and carefully disconnect all cable connectors. Remove the excitation channel lens holder and the two collecting lenses. Insert the absorption measurements accessory into the instrument.

Insert in the excitation channel the lens holder supplied with the accessory (focal length 200 mm). Move the excitation monochromator to 500 nm wavelength. Open the excitation shutter: the light beam is reflected via the first mirror onto the second and, from there, it will cross the cuvette holder. If not, adjust the mirrors in order to make the beam pass through the optical center of the cuvette holder (Figure 1 red).

Absorption Measurements

For this type of measurements it is necessary to use slits with a 1 mm bandwidth (8 nm) or less in the excitation monochromator and to insert neutral density filter with an OD=3 in the filter holder of the excitation channel. Finally the iris located in the excitation channel behind the sample compartment of the instrument needs to be closed to its full extent. All these operations are meant to reduce the light intensity reaching the sample and eventually the light detector. The level of PMT counts should be around 100K. It is important to keep the PMT gain knob position at its maximum (10).

The absorption measurement is performed in two steps:

1. Measurement of the blank (Cuvette containing pure solvent) is collected setting the excitation monochromator to the selected wavelength;
2. Measurement on the sample

Calculation of the OD:

$$OD(\lambda) = \varepsilon x = \log \frac{I_0}{I} = 0.434295 \ln \frac{I_0}{I}$$

The symbols are defined as follows:

- ε = Molar extinction coefficient $[M^{-1}cm^{-1}]$
- c = concentration [M/liter]
- x = optical path [cm]
- I_0 = incident intensity (blank light intensity)
- I = light intensity after passing through the sample

The absorption coefficient, K=εc, can be derived once the optical path is known. Standard cuvettes have an optical path of 1 cm. Other cuvettes can have a lower (or higher optical path), which can be entered in the equation to calculate the extinction coefficient:

$$K = \frac{OD}{x}$$

Absorption spectroscopy utilizes quantities, which can be related to the optical density and the absorption coefficient. The Absorbance A is defined as:

$$A = \log \frac{I_0}{I} = 0.434295 \ln \frac{I_0}{I}$$

Analysis

After the measurement of the sample and the blank, the absorption spectrum is obtained using Vinci Analysis software. For the calculation of the absorption values in Vinci the following steps are required:

Open the *sample transmission* file in Vinci Analysis and choose *Spectral* and then Determine Absorption Spectrum in the menu which brings up the *Compute Absorption Spectrum* dialog box, which asks to select the 2nd *blank* transmission spectrum. Select the spectrum by double-clicking on it and then click OK. This will produce the background corrected absorption spectrum.

Note: Both the sample and blank data files require smoothing with Vinci Analysis before the actual absorption spectrum is calculated: Go to **Math** in Vinci Analysis, then choose **Smooth** and then **Savitzky-Golay** (or any other) for smoothing of the raw spectra.

Measurement Examples

In the following measurements we compared the ODs of Cy5-solutions that were obtained on PerkinElmer's Lamda25 to those measured on PC1 using the absorption accessory. The plots below show the spectra of these solutions measured on these two instruments as well as the calculated difference of their absorption maxima [in %].

Experimental conditions: excitation slits: 0.5 mm, lamp current: 18A, PMT gain: 10, OD=3 neutral density filter in excitation channel. The original data were smoothed by averaging with Savitzky-Golay by a factor of 6.
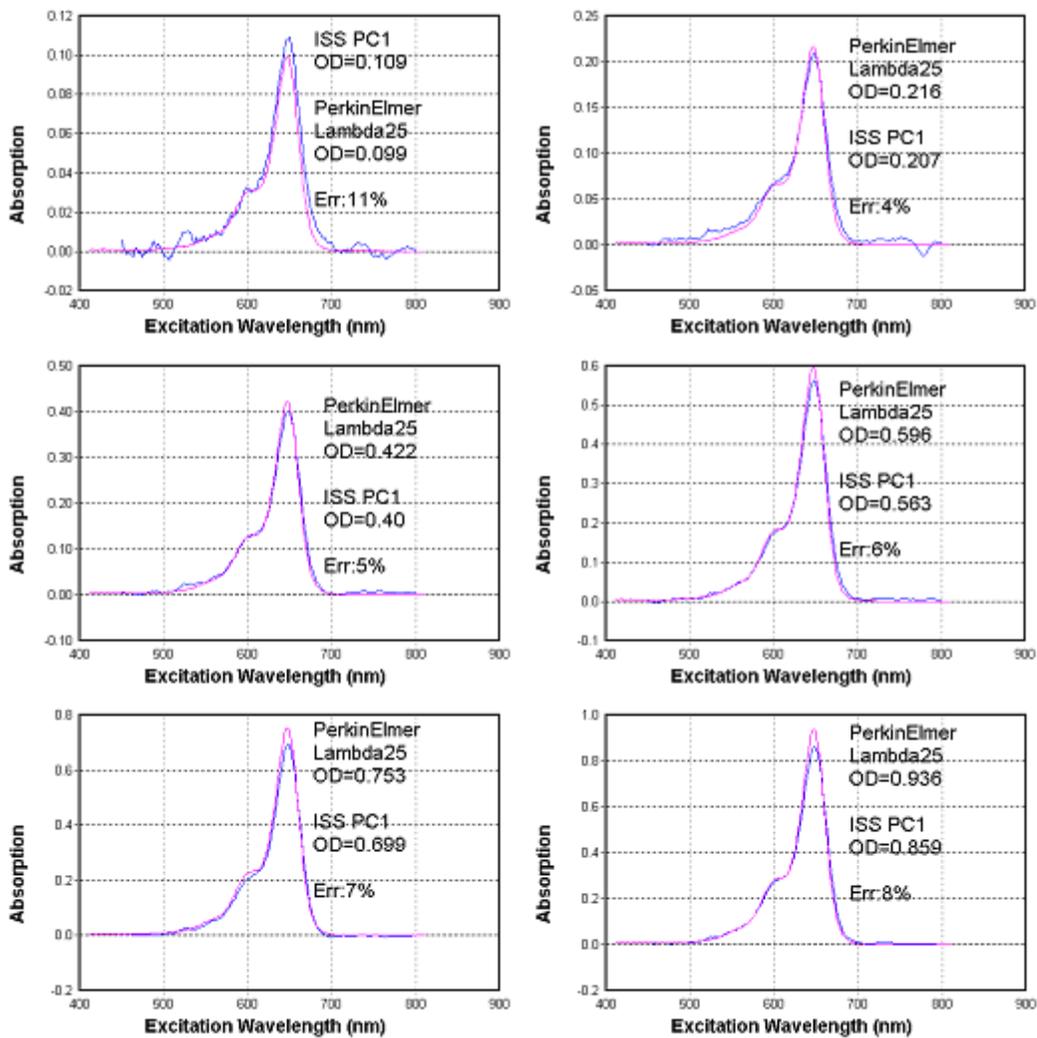
Figure 3.Comparison of absorption spectra of Cy5 measured on PC1 using the absorption accessory and on PerkinElmer's Lambda 25 Spectrophotometer.

References

1. H. Langhals, G. Abbt-Braun, F. H. Frimmel. Association of Humic Substances: Verification of Lambert-Beer Law. Acta hydrochimica et hydrobiologica 28 (6), 329 – 332 (2001).

2. H. E. Rose. Breakdown of the Lambert-Beer Law. Nature 169, 287 - 288 (1952).