

## Long-Wavelength Polarization Standards

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### Introduction

Fluorescence polarization (FP) is a parameter that has found widespread use in high throughput screening and in clinical diagnostics. Due to the ratiometric nature of FP measurements they are more robust than intensity measurements and to some extent they are insensitive to fluctuations of the sample concentration and the light source as well as the sample thickness and inner filter effects. Nevertheless, reliable measurement of FP requires frequent calibration of the instrument with polarization standards. For a more detailed theory on FP, the authors would like to refer the reader to the ISS Technical Note: Fluorescence Polarization.

### Long-Wavelength Quantum Counter

For the measurement of excitation polarization spectra of long-wavelength dyes it is necessary to change the quantum counter from rhodamine B to a compound that does not have the wavelength limitation of this compound. The origin of the 600-nm limit on corrected fluorescence excitation spectra is due to the use of rhodamine B as the standard quantum counter in most of all commercial spectrofluorimeters. We used 1,1',3,3',3'-hexamethylindotricarbocyanine (HITC) as a quantum counter for measurement of the excitation polarization spectra of the standards that are presented in this note [1].

### What are Suitable Criteria for a Polarization Standard?

For a more detailed discussion about this topic we refer the reader to the ISS Technical note: Polarization Standards [2].

In order to extend the list of polarization standards into the red and NIR region we measured the excitation polarization spectra of several dyes that have lifetimes in the low picosecond range: Rose Bengal (Sigma-Aldrich 330000) was used as a reference to compare the data obtained with two different quantum counters; Indocyanine Green was purchased from Sigma-Aldrich (I2633); Alexa 750 (Invitrogen) and K8-1351 [3]. Rose Bengal has a fluorescent lifetime of 76 ps [2], the average lifetime of K8-1351 is 290 ps, Indocyanine Green's lifetime is 520 ps and that of Alexa 750 around 660 ps in water.

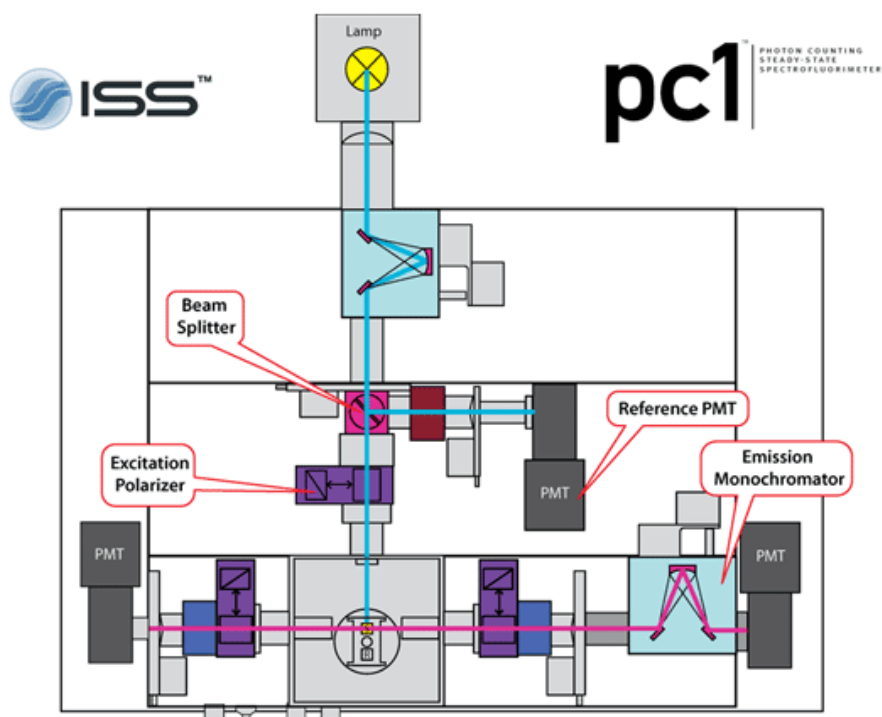
We measured the excitation, emission and excitation polarization spectra of these dyes in water at room temperature using PC1<sup>™</sup>. The excitation polarization values of these dyes at RT are adequate ( $P = 0.28$  to  $0.38$ ) to be measured with satisfactory precision. Another useful characteristic for these dyes is that the excitation polarization is constant for a wide range of excitation wavelengths (50 – 100 nm).

### Instrumentation

Excitation, emission and excitation polarization spectra were measured on PC1<sup>™</sup>, 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, enables instrument control and data acquisition directly from the PC.

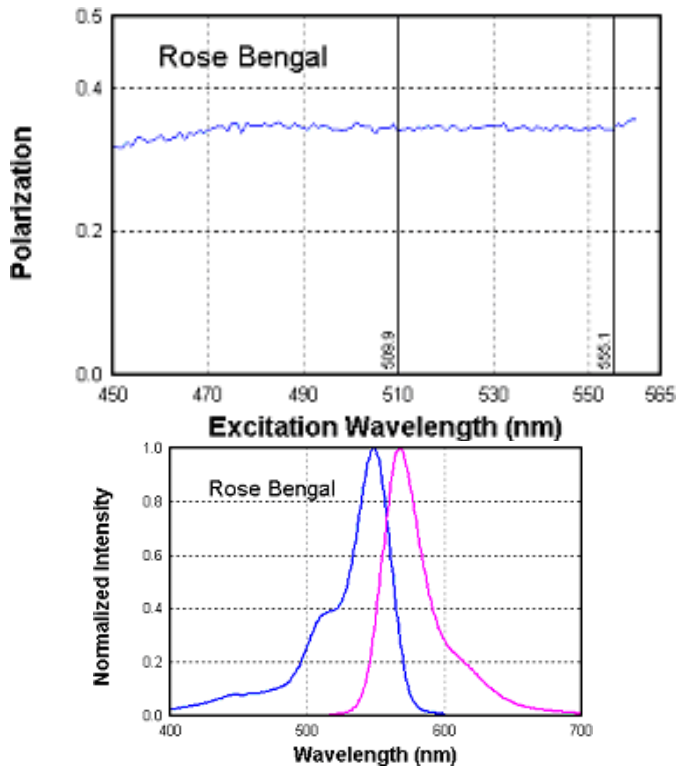
PC1™ can be fully upgraded to the K2™- Multifrequency Phase Fluorometer for fluorescence and phosphorescence lifetime measurements. A variety of light sources and accessories are available for a wide range of applications.



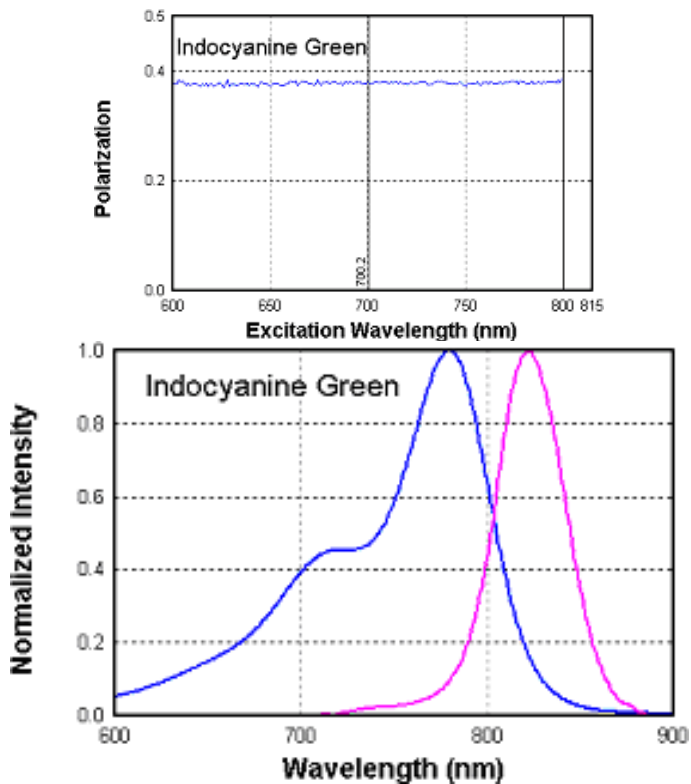
**Figure 1.** Schematic drawing of PC1, the photon-counting spectrofluorimeter from ISS.

## Spectra

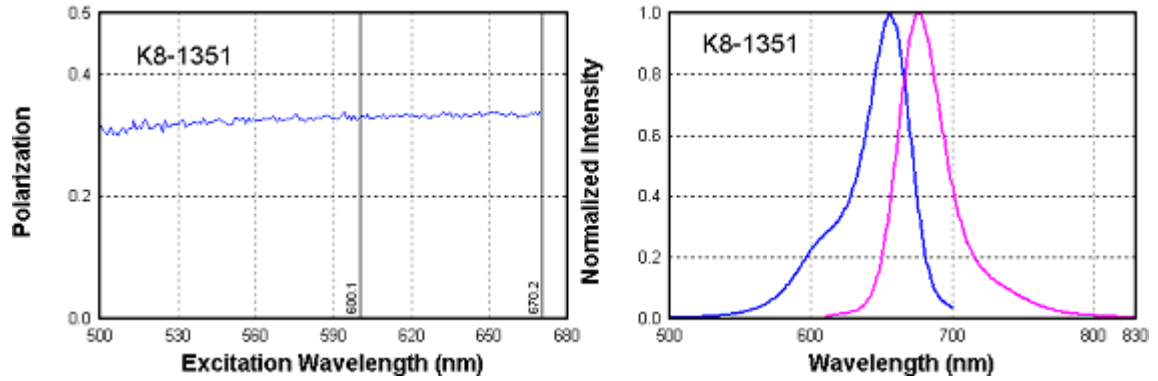
For the measurement of the excitation polarization spectra shown below the excitation and emission slits were 2mm, the solvent was water and the T was 25oC. All excitation measurements were performed using 1,1',3,3,3',3'-hexamethylindotricarbo-cyanine (HITC) (triangular cuvette with a saturated solution of HITC in acetonitrile). Prior to measurement of the long-wavelength standards, we obtained the excitation polarization spectrum of Rose Bengal with this set up (Figure 1.) and compared the data to that obtained with Rhodamine B as a quantum counter (ISS technical note: Polarization Standards). The polarization spectra of Rose Bengal obtained with different quantum counters are in good agreement. Figures 2 to 4 show the excitation polarization spectra of Indocyanine Green, K8-1351 and Alexa750.



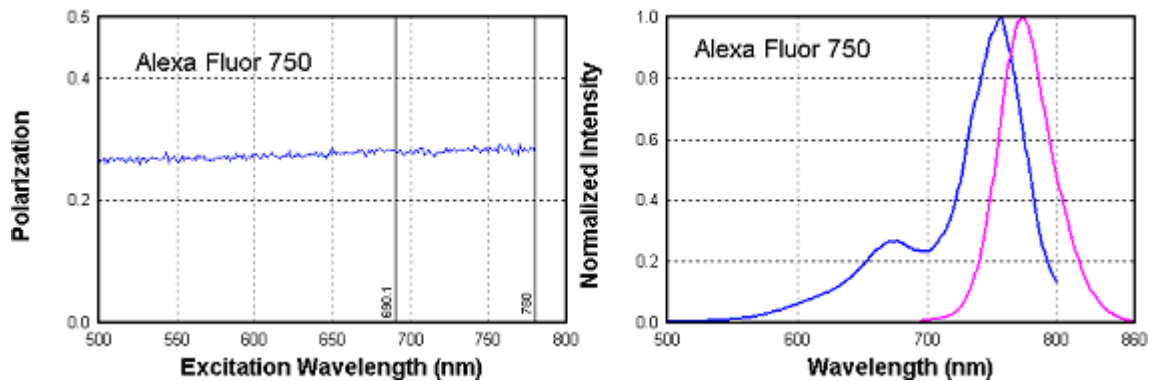
**Figure 2.** Plot a shows the excitation polarization spectrum of Rose Bengal with a polarization value of  $P = 0.344$  for the recommended excitation range for polarization measurements. Plot b shows the excitation and emission spectra ( $\lambda_{\text{max}}(\text{ex})=548 \text{ nm}$ ,  $\lambda_{\text{max}}(\text{em})=567 \text{ nm}$ ) of Rose Bengal in water. Data was acquired on PC1 using a 300W Xenon lamp.



**Figure 3.** Plot a shows the excitation polarization spectrum of Indocyanine Green in water with a polarization value of  $P = 0.379$  for the recommended excitation range for polarization measurements. Plot b shows the absorption and emission spectra ( $\lambda_{\text{max}}(\text{abs})=780 \text{ nm}$ ,  $\lambda_{\text{max}}(\text{em})=822 \text{ nm}$ ) of Indocyanine Green in water. Data was acquired on PC1 using a 300W Xenon lamp.



**Figure 4.** Plot **a** shows the excitation polarization spectrum of K8-1351 in water with a polarization value of  $P = 0.333$  for the recommended excitation range for polarization measurements. Plot **b** shows the excitation and emission spectra ( $\lambda_{\max}(\text{ex})=656 \text{ nm}$ ,  $\lambda_{\max}(\text{em})=676 \text{ nm}$ ) of K8-1351 in water. Data was acquired on PC1 using a 300W Xenon lamp.



**Figure 5.** Plot **a** shows the excitation polarization spectrum of Alexa Fluor 750 in water with a polarization value of  $P = 0.282$  for the recommended excitation range for polarization measurements. Plot **b** shows the excitation and emission spectra ( $\lambda_{\max}(\text{ex})=756 \text{ nm}$ ,  $\lambda_{\max}(\text{em})=774 \text{ nm}$ ) of Alexa Fluor 750 in water. Data was acquired on PC1 using a 300W Xenon lamp.

## References

1. E. A. Nothnagel. *Quantum Counter for Correcting Fluorescence Excitation Spectra at 320 –800 nm Wavelengths*. *Anal. Biochem.* 163, 224-237 (1987).
2. R. B. Thompson, I. Gryczynski, and J. Malicka. *Fluorescence polarization standards for high- throughput screening and imaging*. *BioTechniques*, 32, 34 – 42 (2002).
3. L. Tatarski et al. *Synthesis of water-soluble, ring-substituted squaraine dyes and their evaluation as fluorescent probes and labels*. *Anal. Chim. Acta* 570, 214–223 (2006).

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