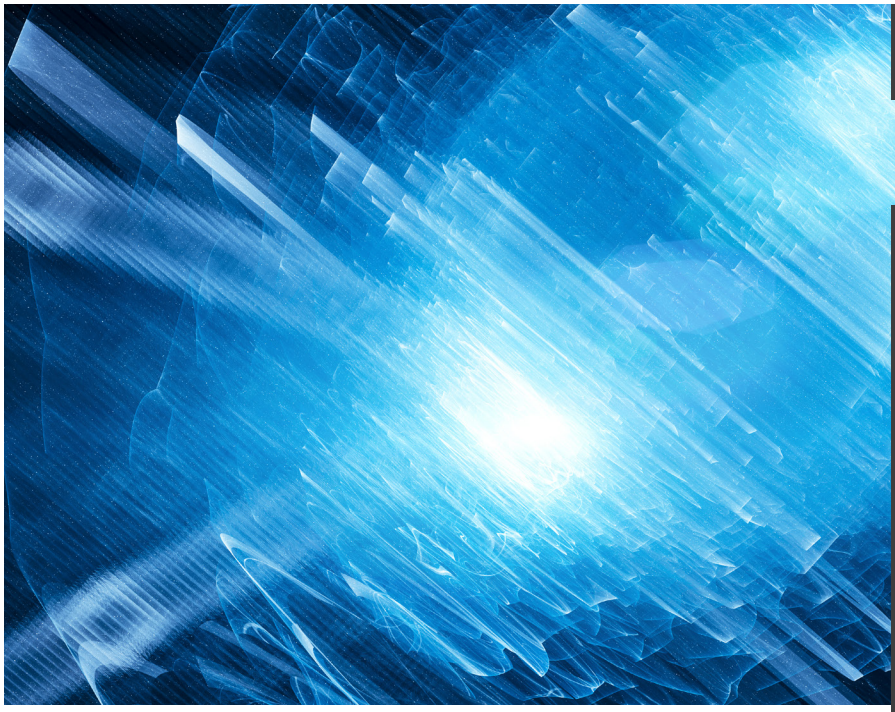

APPLICATION NOTE

Fluorescence Spectroscopy

Authors:

Kathryn Lawson-Wood

Steve Upstone

Kieran Evans

PerkinElmer, Inc.
Seer Green, UK

Determination of Relative Fluorescence Quantum Yields using the FL6500 Fluorescence Spectrometer

Introduction

Fluorescence quantum yield (Φ_F) is a characteristic property of a fluorescent species and is denoted as the ratio of the number of photons emitted through fluorescence, to the number of photons absorbed by the fluorophore (Equation 1). Φ_F ultimately relates to the efficiency of the pathways leading to emission of fluorescence (Figure 1), providing the probability of the excited state being deactivated by fluorescence rather than by other competing relaxation processes. The magnitude of Φ_F is directly related to the intensity of the observed fluorescence.[1]

$$\Phi_F = \frac{\textit{Number of photons emitted through fluorescence}}{\textit{Number of photons absorbed}} \qquad (1)$$

Fluorescence quantum yield can be measured using two methods: the absolute method and the relative method. Relative Φ_F measurements are achieved using the comparative method. Here, the Φ_F of a sample is calculated by comparing its fluorescence intensity to another sample of known Φ_F (the reference). Unlike absolute quantum yield measurements, which require an integrating sphere, the relative method uses conventional fluorescence spectrometers with a standard single cell holder.[2] The relative method does, however, require knowledge of the absorbance of both the reference and the sample.

PerkinElmer®
For the Better

The importance of Φ_F has been shown in several industries, including the research, development and evaluation of audio visual equipment, electroluminescent materials (OLED/LED), dyes/pigment and fluorescent probes for biological assays.[3] This application note demonstrates the use of the PerkinElmer FL6500 fluorescence spectrometer and Spectrum FL software for the determination of relative Φ_F of rhodamine B using the comparative method. The FL8500 may also be used. Spectrum FL provides an easy-to-use inbuilt method for measuring the Φ_F of samples with the option of choosing either the relative or absolute measurement. Rhodamine 6G, which has a known Φ_F of 0.95, was used as the reference material in this study.[4]

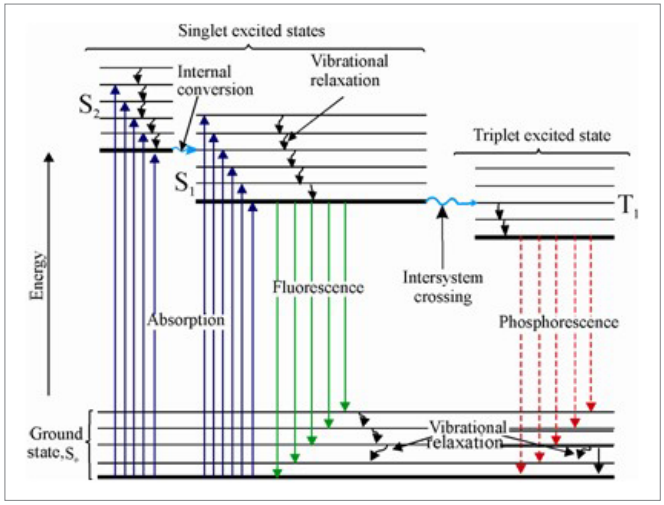

Figure 1. Jablonski diagram illustrating the main primary deactivation processes following excitation from the ground electronic singlet state (S_0) to excited electronic singlet states (S_1 and S_2).[5]

Relative vs. Absolute Fluorescence Quantum Yields

The absolute method is applicable to both solid and solution phase measurements and the Φ_F can be determined in a single measurement without the need for a reference standard or absorbance data. It is especially useful for samples which absorb and emit in wavelength regions for which there are no reliable quantum yield standards available. This method, however, requires an integrating sphere which allows the instrument to collect all of the photons emitted from the sample. By simply comparing the total number of emitted photons with the total number of absorbed photons, the absolute Φ_F of the sample can be calculated.[6,7]

The relative comparative method, on the other hand, is the most common way of determining the Φ_F of samples. It relies on the use of well-characterised reference standards, with known Φ_F values and optical properties closely matching the sample of interest. It uses a conventional fluorescence spectrophotometer which detects only a fraction of the light emitted due to a wide range of factors including the refractive index of the solvent, scattering of light by the sample, the emission wavelength and the 90° arrangement of the excitation and emission optics.[2] To calculate the Φ_F value of the unknown, the relative method compares the integrated fluorescence intensity of a sample of known Φ_F, generally referred to as the reference, against the sample of unknown Φ_F. This approach is only applicable to samples which can go into solution because the measurement requires knowledge of the refractive index of the solvent, and the absorbance of both reference and sample. The relative method provides good accuracy for solution phase samples (as it uses the gradients to calculate the Φ_F), with the added benefit of not requiring an integrating sphere.[8]

The fundamental principle of the comparative method is that two solutions (i.e. the reference and the test sample) with identical absorbance at the excitation wavelength can be assumed to be absorbing the same number of photons. Therefore, providing the experimental conditions remain the same, the ratio of the integrated fluorescence intensities of the two solutions can be related to the ratio of the Φ_F values.[9]

It is possible to estimate the Φ_F by simply using the absorbance and integrated fluorescence intensities of a single reference and test sample, generally referred to as the single-point method. Although this single-point method is quick, it can result in large inaccuracies in the quantum yield measurements due to inaccurate measurement of solution absorbance. It is, therefore, advisable to measure the fluorescence intensity of the reference and test samples over a range of absorbances at the excitation wavelength. Two calibration curves of the integrated fluorescence intensity (emission peak area) as a function of solution absorbance may then be plotted, and the quantum yield obtained using the gradients.[10]

The Φ_F of the test sample using the comparative method is determined using Equation 2 when a rhodamine quantum-corrected reference system is used. Equation 2 can be simplified to Equation 3 by combining the E_R and A_R terms (and also the E_S and A_S terms) as their ratio is equal to the gradient of the calibration line produced when plotting the integrated fluorescence intensity against solution absorbance. If the same solvent is used for both the reference and unknown samples then the value of $(n_s/n_r)^2$ is equal to 1. Therefore, the fluorescence quantum yield of the unknown is obtained from the product of the quantum yield of the reference and the quotient of the two gradients.[9,11,12]

Method

$$Q_s = Q_r \left(\frac{A_r}{A_s}\right)\left(\frac{E_s}{E_r}\right)\left(\frac{n_s}{n_r}\right)^2 \quad (2)$$

$$Q_s = Q_r \left(\frac{m_s}{m_r}\right)\left(\frac{n_s}{n_r}\right)^2 \quad (3)$$

Where:

- Q = Fluorescence quantum yield
- m = Gradient of the plot of integrated fluorescence intensity against absorbance
- n = Refractive index of the solvent
- A = Absorbance of the solution
- E = Integrated fluorescence intensity of the emitted light
- Subscripts 'r' and 's' refer to the reference and unknown fluorophore respectively

Quantum yield measurements require the absorbance (optical density) of the solution at the absorption maxima to be < 0.1, thus appropriate sample concentrations must be prepared. Limiting the absorbance to below 0.1 minimizes non-linear effects arising from inner filter (re-absorption) effects, which may otherwise skew the resulting quantum yield.[13]

Fluorescence grade rhodamine 6G, fluorescence grade rhodamine B, and 200-proof anhydrous ethanol (≥ 99.5 %) were obtained from Merck (previously Sigma-Aldrich). Rhodamine 6G (used as the reference sample) and rhodamine B (used as the unknown sample) were chosen as they have similar excitation and emission profile.

Five standard solutions in ethanol were prepared each for rhodamine B and rhodamine 6G, with absorbance values between 0.01 – 0.1 at the excitation wavelength of 537 nm. The absorbance of each solution was determined using a PerkinElmer LAMBDA 265 UV/Vis Spectrophotometer using PerkinElmer UV Lab™ software. In the absence of a separate UV-Vis instrument, the PerkinElmer FL6500 or FL8500 Fluorescence Spectrometer's absorbance module accessory (P/N: N4201018) can be used.

All samples were measured using the PerkinElmer FL6500 Fluorescence Spectrometer (Figure 2) with a standard single cell holder accessory (Figure 3, P/N: N4201010). The PerkinElmer FL8500 Fluorescence Spectrometer may also be used. Quartz fluorescence cuvettes (10 x 10 mm) were used with a white PTFE stopper to prevent solution evaporation. Emission spectra were background corrected using a solvent blank, in order to correct for any emission arising from the solvent. The 'Quantum Yield Method Setup' in Spectrum® FL software was used in relative mode with the instrument settings specified in Table 1.

Results

Fluorescence spectra of the varying concentrations of rhodamine 6G and rhodamine B are shown in Figure 4, with the absorbance values and integrated fluorescence intensities of these samples shown in Table 2.

Spectrum FL automatically calculates the integrated fluorescence intensity (peak area) of each of the reference and unknown samples of varying concentration. Peak calculation points (peak start, peak end and peak bases) can be manually refined, as demonstrated in Figure 6. The software simultaneously creates two calibration curves by plotting the magnitude of the integrated fluorescence intensity against the absorbance of the solution for both sample types, and calculates the quantum yield of the unknown sample using Equation 3. This automation allows quick analysis and high sample throughput. The calibration curves for rhodamine 6G (reference) and rhodamine B (unknown) are shown in Figure 5, with the calculated gradients and quantum yield shown in Table 3.

In this study, ethanol, which has a refractive index of 1.36, was used for both reference and unknown samples, thus the value of $(n_s/n_r)^2$ was equal to 1. The fluorescence quantum yield of rhodamine B using the relative method was determined to be 0.69, which was close to the published literature value of 0.70.[4]

Figure 2. PerkinElmer FL6500 Fluorescence Spectrometer.

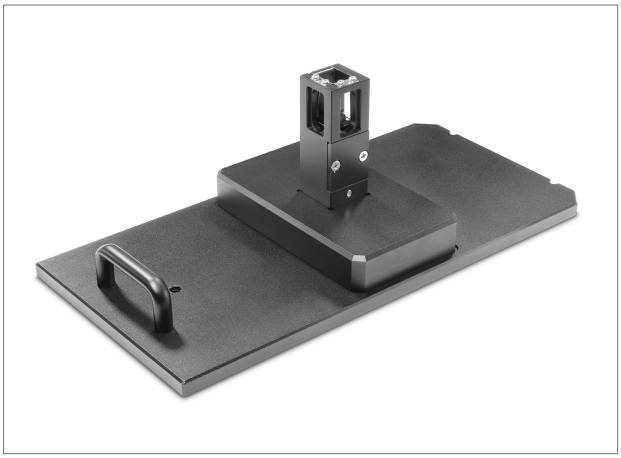

Figure 3. Standard single cell holder accessory (P/N: N4201010) in the FL6500 and FL8500 Fluorescence Spectrometers.

Table 1. PerkinElmer FL6500 scanning parameters using relative mode.

Scan Settings		
Source	Excitation Correction	On
	Initial Dark	On
	Power (kW)	80
	Frequency (Hz)	100
Excitation	Excitation Wavelength (nm)	537
	Slit Width (nm)	5
Emission	Wavelength Range (nm)	450 – 750
	Slit Width (nm)	5
	Scan Speed (nm/min)	240
Acquisition	Voltage (V)	300
	Response Width (nm)	0.5
	Emission Correction	On
	Gain	Auto

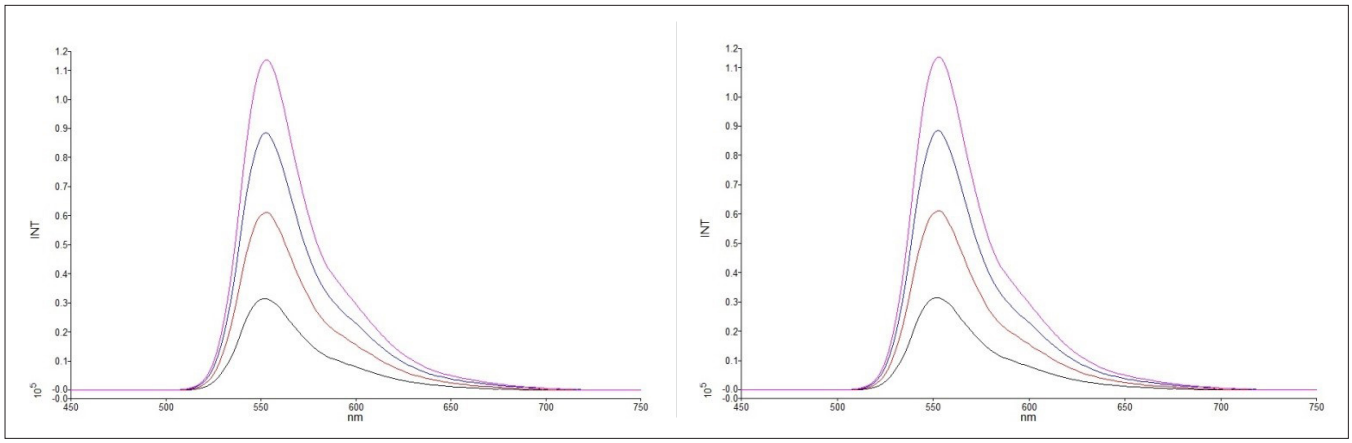

Figure 4. Fluorescence emission spectra of rhodamine 6G (left) and rhodamine B (right) samples with varying absorbance using the PerkinElmer FL6500.

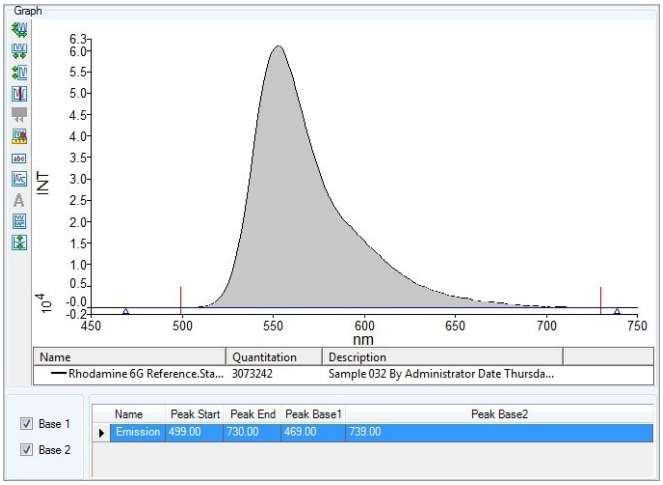

Figure 5. Automatic peak integration using the relative quantum yield module.

Table 2. Absorbance and integrated fluorescence intensities (areas) for rhodamine 6G standards and rhodamine B samples.

Sample	Absorbance	Integrated Fluorescence Intensity
Rhodamine 6G (Reference)	0.0216	1580446.95
	0.0433	3073237.32
	0.0631	4466636.16
	0.0828	5724564.54
Rhodamine B (Unknown)	0.0240	1154648.76
	0.0441	2098566.73
	0.0645	3052074.71
	0.0801	3956476.48

Table 3. Rhodamine B and rhodamine 6G calibration curve gradients and subsequent rhodamine B fluorescence quantum yield calculated using Spectrum FL software.

Rhodamine 6G Gradient (m_r)	Rhodamine B Gradient (m_s)	Rhodamine B Calculated Φ_F (%)
67990219.91	49453828.63	69.0998

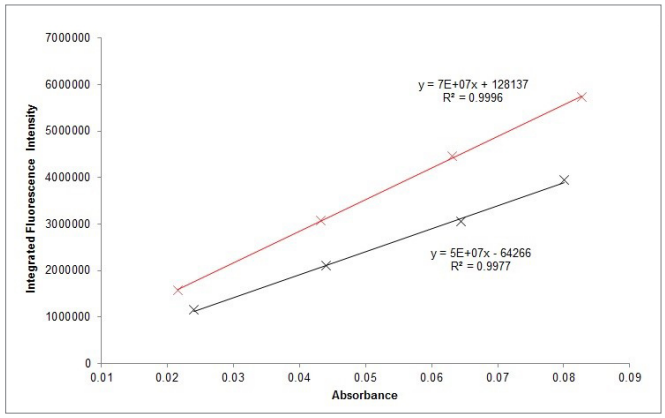

Figure 6. Calibration curves of integrated fluorescence intensity (area) against absorbance for rhodamine 6G (red) and rhodamine B (black).

The results may also be obtained manually by taking the measurements using the Spectra Scan method in Spectrum FL. The integrated fluorescence intensity of all spectra can then be calculated using the 'peak area' function within the data analysis tab in the software.

Conclusion

This application demonstrates the use of the PerkinElmer FL6500 (the FL8500 may also be used) with the standard single cell holder accessory for the determination of relative fluorescence quantum yield, using rhodamine 6G as the reference and rhodamine B as the unknown. The fluorescence quantum yield of rhodamine B was determined to be 0.69, close to the literature value of 0.70. The ease of use of the FL6500 and Spectrum FL software makes it highly suitable for the determination of relative quantum yield of liquid samples in academia and research laboratories. Additionally, the relative method requires no integrating sphere, allowing the use of a simple conventional fluorescence spectrometer. Spectrum FL software provides a step-by-step approach with automated calculation to allow easy analysis and fast sample throughput.

References

1. P. Atkins, J. De Paula and J. Keeler, *Atkins' Physical Chemistry.* Oxford University Press, Oxford, 2018.
2. C. Würth, M. Grabolle, J. Pauli, M. Spieles and U. Resch-Genger, *Relative and Absolute Determination of Fluorescence Quantum Yields of Transparent Samples,* Nature Protocol, 2013, 8, p 1535 – 1550.
3. L.A. Moreno, *Absolute quantum yield measurement of powder samples,* Journal of Visualized Experiments, 2012, 12.
4. A. Brouwer, *Standards for Photoluminescence Quantum Yield Measurements in Solution (IUPAC Technical Report),* Pure Applied Chemistry, 2011, 83, p 2213 – 2228.
5. University of Richmond, Molecular Fluorescence, Retrieved from https://facultystaff.richmond.edu/~rdominey/301/local/Molecular%20Fluorescence.pdf. Date accessed: May 2018.
6. D.O. Faulkner, J.J. McDowell, A.J. Price, D.D. Perovic, N.P. Kherani and G.A Ozin, *Measurement of absolute photoluminescence quantum yields using integrating spheres – Which way to go?,* Laser & Photonics Reviews, 2012, 6, p 802 – 806.
7. L. Porres, A. Holland, A. Monkman, C. Kemp, A. Beeby and P. Lars-Olof, *Absolute Measurements of Photoluminescence Quantum Yields of Solutions Using an Integrating Sphere,* Journal of Fluorescence, 2006, 16, p 267 - 272.
8. K. Rurack, *Fluorescence Quantum Yields: Methods of Determination and Standards,* Standardization and Quality Assurance in Fluorescence Measurements, 2008, 5, p 101-145.
9. J. Li and N. Wu, *Biosensors Based on Nanomaterials and Nanodevices,* CRC Press, 2017.
10. D. Nagaraja, R. M. Melavanki, N. R. Patil, H. S. Geethanjali and R. A. Kusanur, *Solvent effect on the relative quantum yield and fluorescence quenching of a newly synthesized coumarin derivative,* Luminescence: The Journal of Biological and Chemical Luminescence, 2015, 30, p 495 – 502.
11. A. T. R. Williams, S. A. Winfield and J. N. Miller, *Relative fluorescence quantum yields using a computer controlled luminescence spectrometer,* Analyst, 1983, 108, p 1067-1071.
12. B. Brandner. *Implementation of a Comparative Method for Measuring Photoluminescence Quantum Yields of Novel Compounds in Solution,* Oregon State University, 2016.
13. S. Dhami, A. J. de Mello, G. Rumbles, S. M. Bishop, D. Phillips and A. Beeby, *Phthalocyanine fluorescence at high concentration: dimers or reabsorption effect?* Photochemistry and Photobiology, 1995, 61, p 341-346.

Appendix – Example Screen Captures of Relative Quantum Yield Method using Spectrum FL

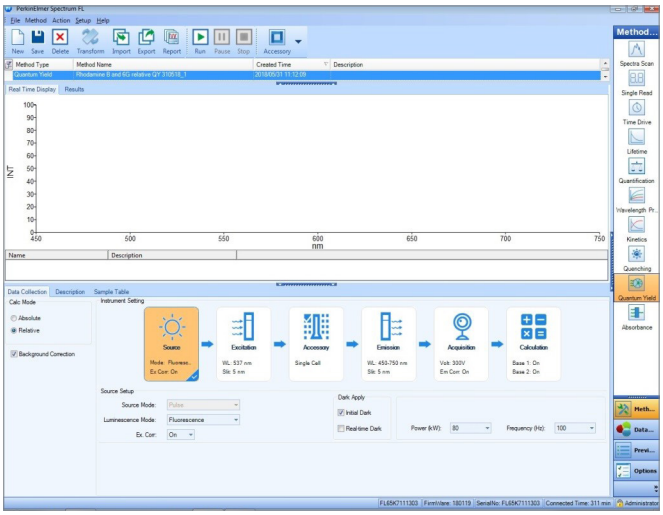

Figure 7. Spectrum FL quantum yield method setup module.

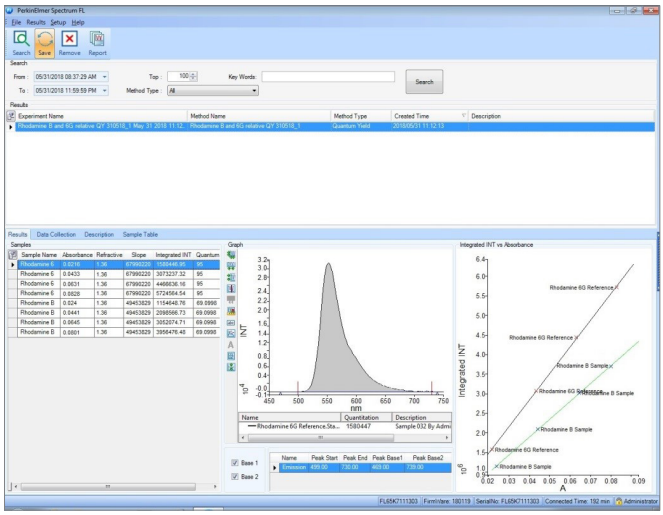

Figure 8. Spectrum FL software demonstrating the automated relative quantum yield determination of rhodamine B.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

014216_01 PKI