How to deal with inner filter effect in fluorescence experiments

J. L. Chavez[1], L. Ceresa[1], J. Kimball[1], Sunil Shah[2], I. Gryczynski[2], and Z. Gryczynski[1,2]

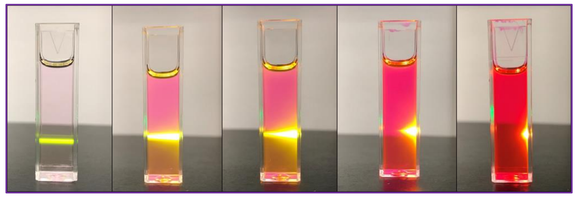
[1] Department of Physics and Astronomy, Texas Christian University, 2800 S University Drive, Fort Worth, TX, 76129, USA

[2] Department of Molecular Immunology and Genetics, University of North Texas Health Science Center, Fort Worth, TX, 76107, USA

Background

Fluorescence is a very useful tool and a very popular technique which has been used in many fields of research including biology and medicine. Despite being relatively simple from the theoretical point of view, it is not easy to use. For example, a fundamental obstacle in its use is the nonlinearity of the dependence of fluorescence intensity on fluorophore concentration. This is referred to as inner-filter effect. In the literature, such effect is divided into a "primary inner-filter effect" and a "secondary inner-filter effect". The former is caused by the absorption of the excitation light, which results in a lowering of the intensity of the light reaching deeper regions of the solution. The latter is represented by the reabsorption of the emitted fluorescence. If the primary inner filter effect is a direct consequence of the high concentration of the solution, to observe the secondary inner filter effect it is necessary to have a chromophore which absorbs part of the light that is emitted by the main fluorophore.

$$A = \varepsilon l C$$

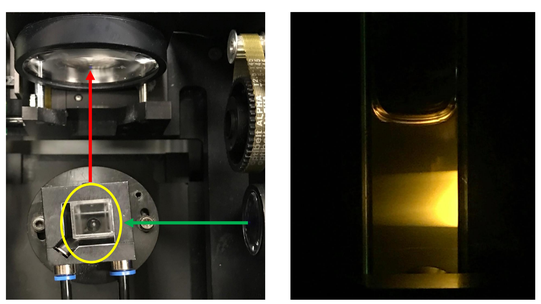


Rhodamine B increasing concentration shows a decreasing light penetration through the sample

An increase in sample absorbance may lead to a significant distortion of the emission spot that consequently will strongly affect (decrease) the detected signal. This is due to two main factors:

- The detection center of any spectrofluorometer is focused/limited to a very narrow cone in the center of the detection (cuvette).
- The excitation light intensity decreases as it penetrates the sample.

In other words, the portion of sample closest to the cuvette's wall facing the excitation light source will produce a higher intensity emission light than the center and opposite side of the cuvette.

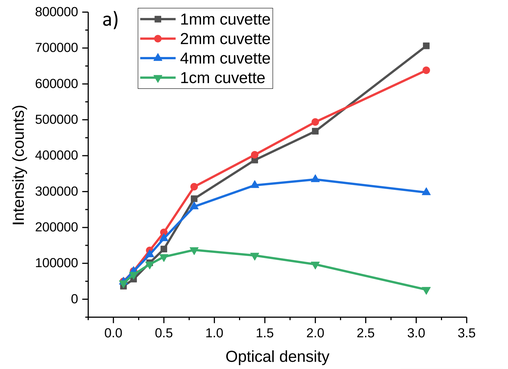


Square geometry setup of spectrofluorometer FT 300 and cone of excitation light showing higher brightness at the edge of the cuvette facing the excitation source.

Results

The emission spectra of Coumarine 152 have been obtained for samples with increasing concentrations (optical density). Moreover, different cuvette dimensions have been tested in order to explore the effect of an increasing path length from 1 mm to 1 cm.

The linearity of the dependence between optical density and emission is shown exclusively when the used path lengths are 1mm or 2mm. On the contrary, if the path length is increased to 4 mm and 1 cm the inner filter effect is clearly visible. In fact, for samples characterized by an optical density larger than 0.5 the linearity is lost. Furthermore, if higher optical densities are investigated, the fluorescence signal is decreased.

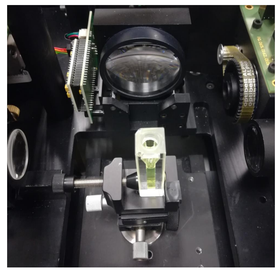


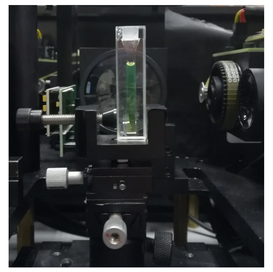
Dependence of intensity on optical density for different dimension cuvettes (a) and dependence of intensity on cuvette dimension for increasing optical densities (b)

Although working with low concentrations is generally recognized as a good practice to avoid artifacts related to inner filter effects, it can be observed that the primary inner filter effect occurs even at low absorbances (< 0.05). Furthermore, it is possible that using solutions with high absorbance is strictly necessary in studying the photophysical properties of fluorescent dyes and the interactions of biological macromolecules. Therefore, a good correction method for inner filter effects is necessary.

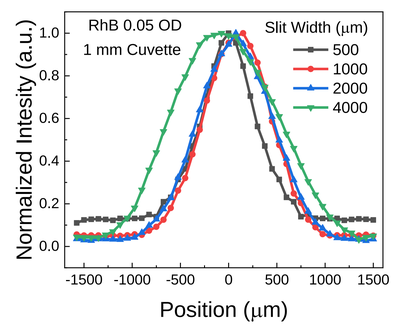
Solution

Since it has been reported that the existing methods for correcting the fluorescence intensity are hard to implement in practice, we propose a strategy based on the previous calculation of the so called "sensitivity factor" of a spectrofluorometer. By mounting a cuvette on a movable holder in a square geometry setup, we can modify the position of the cuvette during a regular emission/excitation experiment. This allows us to determine the sensitivity factor, or rather the resolution of the detector. This result can be effectively used to correct the emission/excitation spectra to restore the linearity between absorbance and fluorescence intensity in samples characterized by high concentrations.





The excitation intensity of Rhodamine B was measured in order to demonstrate how the intensity is affected by the sample's position. The optimal position corresponding to the maximum intensity of emission has been labelled as "0" on the X axis. A decrease in emission was observed away from the center. More specifically, the experiment has been conducted with a single dimension cuvette (1mm) and a varying slit's dimension to allow an increasing amount of light to reach the sample.



Normalized excitation intensity vs. position obtained from a single cuvette geometry with different emission slit's dimensions

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