Inner filter effect correction for fluorescence measurements in non-transparent microplates

Tomislav Friganović, Davor Šakić, and Tin Weitner*

University of Zagreb, Faculty of Pharmacy and Biochemistry, Zagreb, Croatia

*Corresponding author: tin.weitner@pharma.unizg.hr

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Inner filter effect (IFE) – important consideration in fluorescence

- apparent fluorescence intensity and spectral distribution is dependent on:
  - sample optical density
  - geometry of sample illumination
  - primary and secondary IFE
- PROBLEM: nonlinear dependence of the fluorescence intensity on the concentration

IFE minimization – common strategies

AVOIDING IFE

- use of DILUTE SOLUTIONS is considered best practice

CAUTION: IFE correction should be performed even for low fluorophore concentrations

USING ABSORBANCE-CORRECTED FLUORESCENCE

- simple and approximate method for IFE correction of observed fluorescence:[1]

$$F_A = F_1 \times 10^{[(A_{ex}+A_{em})/2]}$$

DISADVANTAGES:

- requires 3 separate measurements in 2 separate instruments: fluorescence and absorbance at excitation and emission wavelengths
- measurements in microplates require expensive UV-transparent microplates for the excitation in the UV range (e.g. unlabeled proteins)

USING CELL SHIFT METHOD

- sample fluorescence intensities (F_1 and F_2)): are measured at 2 different positions in the cuvette with different effective light path lengths (l_1 and l_2):[2]

$$F_0 = F_1 \times \left(\frac{F_1}{F_2}\right)^{\left(\frac{l_1}{l_2 - l_1}\right)}$$

- does NOT require separate absorbance measurements

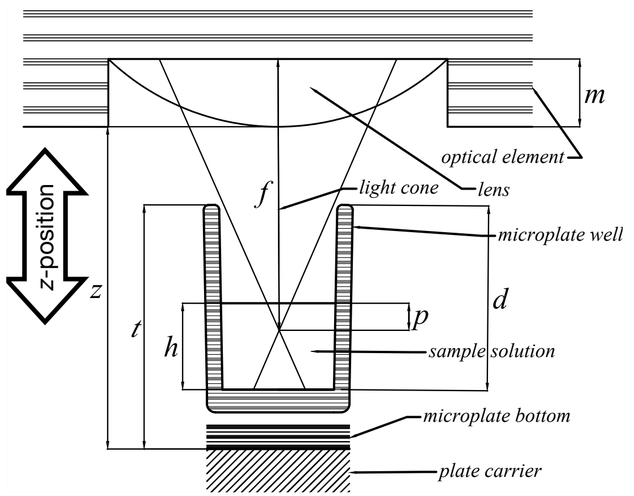
DISADVANTAGE:
COMPLICATED experimental setup with custom hardware for measurements in cuvettes

ADVANTAGES:

- measurements are performed on the same sample in the same well using a single instrument without additional manipulation
- black non-transparent microplates can be used for the entire spectral range
- applicable to optical densities of at least A~2

Fluorescence measurements in microplate samples

- outstanding tool for investigating the structure and dynamics of matter or living systems
- multi-sample approach for binding studies, quenching, and cell-based assays
- optics used in microplate readers are different than in an instrument designed for use with a cuvette
- modern instruments allow precise z-positioning above the well
- STILL - IFE PROBLEMS



NEW: IFE correction in non-transparent microplates

- adapting cell-shift method to microplate readers
- movement of the optical element used for excitation and emission in the z-axis (perpendicular to the sample well) allows simple modification of sample geometry
- a single overall geometric parameter k for a particular sample volume, microplate and microplate reader type:

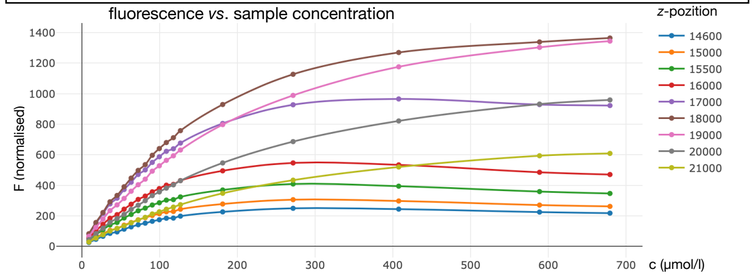
$$k = (h + t - d) + (f - m)$$

- the resulting z-position IFE correction:

$$F_0 = F_1 \times \left(\frac{F_1}{F_2}\right)^{\left(\frac{k - z_2}{z_2 - z_1}\right)}$$

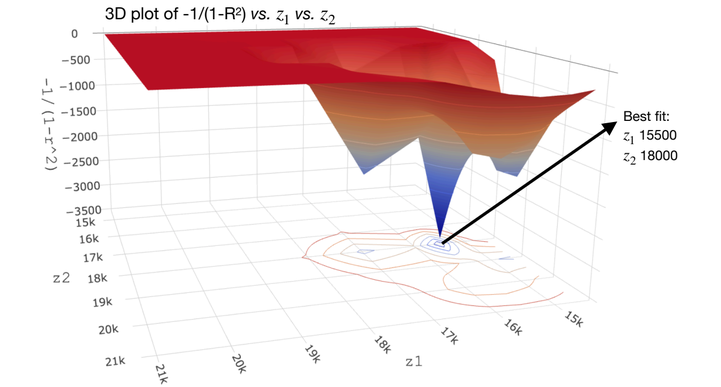
-requires 2 fluorescence measurements (F_1 and F_2) at 2 different z-positions (z_1 and z_2)

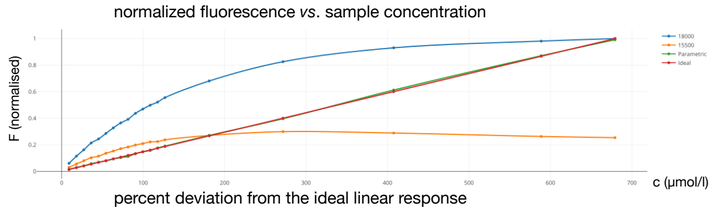
Example measurements – obvious nonlinearity of fluorescence due to IFE

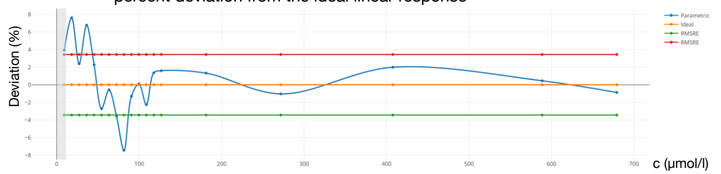


Results – significantly improved linearity of the fluorescence signal

-linear response **over >98% of the analyte concentration range** with approximately **1% overall deviation** of the calibration slope from the ideal signal







References:

1. J. R. Lakowicz, Ed., in Principles of Fluorescence Spectroscopy, Springer US, Boston, MA, **2006**, 27–61.
2. H.-P. Lutz and P. L. Luisi, Helvetica Chimica Acta, **1983**, *66*, 1929–1935.

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