INNER FILTER EFFECT CORRECTIONS FOR PROTEIN FLUORESCENCE MEASUREMENTS IN MICROPLATES USING VARIABLE *z*-FOCUS POSITION

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Inner filter effect (IFE)

- Absorption of the excitation (primary IFE) and/or emission (secondary IFE) radiation.
- A function of: optical density, solution composition and geometric parameters of the sample illumination.
- Significant IFE will cause deviation from the linear response of the measured relative fluorescence (*F*) and analyte concentration.
- Should be taken into account even for low emission or excitation wavelength absorbance ($A \approx 0.05$).

Fluorescence measurements in microtiter plates

- Outstanding tool for the protein binding studies (low sample volume, multisample approach, replicated measurements).
- Different optical systems of the microplate readers and traditional fluorimeters (measurements performed in a cuvette).
- Most modern plate readers allow precise adjustments of the optical element's position in the *z*-axis (perpendicular to the sample well).

IFE minimisation strategies

- Simplest approach is to avoid significant IFE by using the diluted solutions (if possible) for the fluorescence measurements.
- Correcting IFE *via* absorbances at the excitation and emission wavelengths is a widely used method proposed by Lakowicz [1]:

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

Initial (uncorrected) fluorescence F_1 is corrected (F_A) by multiplication with the exponential term which is a function of both the emission and excitation wavelength absorbance.

- IFE-corrected fluorescence by means of Lakowicz's method seems to have a systematic trend (obvious from the residual plot inspection, Figures 4 and 5). This method also requires the usage of rather expensive transparent-bottom microtiter plates in order to measure both the fluorescence and the absorbance of the solutions.

- For measurements in cuvettes, IFE can be corrected using the cell-shift method [2]. This method requires fluorescence to be measured at two different geometric positions (F_1 and F_2) with different respective optical pathlengths l_1 and l_2. Correction (F_0) is performed as following:

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

Main downside of the cell-shift method is a rather complicated experimental setup in order to shift the cuvette to different positions.

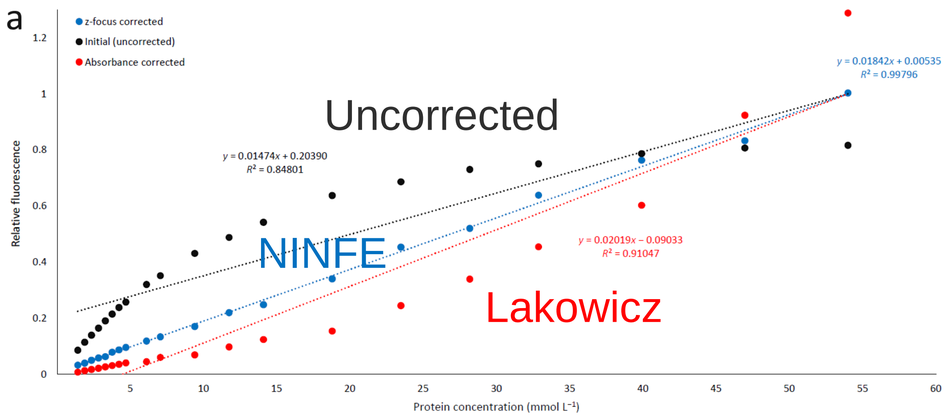


Figure 4. Comparison of the linear interpolations for the: uncorrected, z-focus corrected and absorbance corrected relative fluorescence in transparent microplate (a). *z*-focus corrected relative fluorescence in transparent and non-transparent microplate (b).

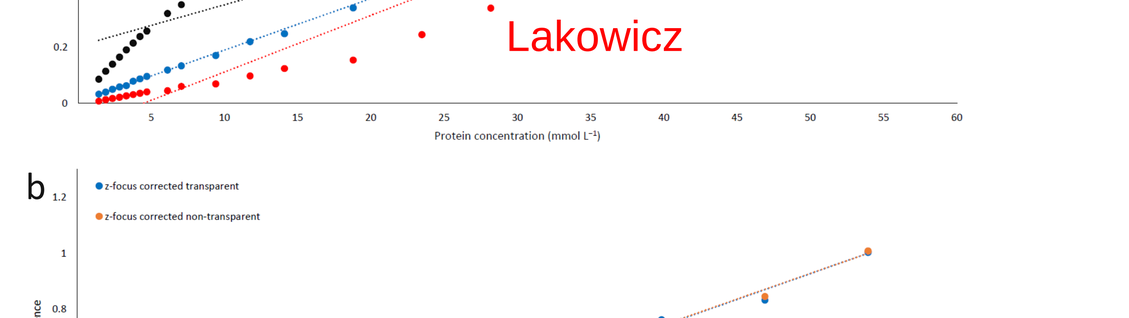
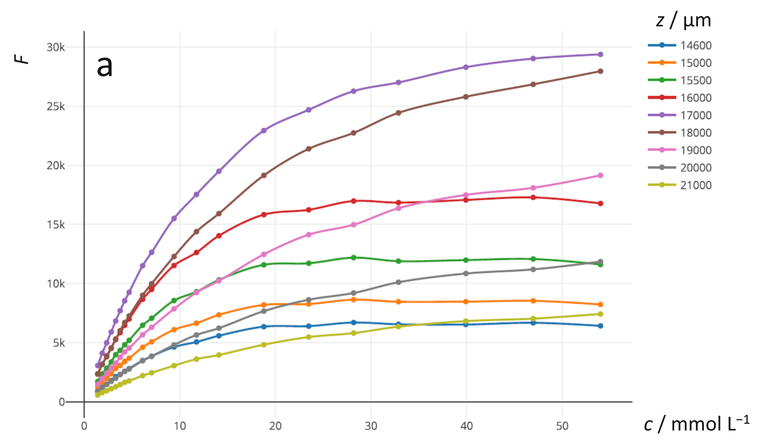


Figure 5. Scaled residual ($(F_{obs}-F_{calc})/F_{obs}$) values for the *z*-focus (blue) and absorbance corrected (red) values (transparent microplate). Distribution is not random in case of the absorbance correction.



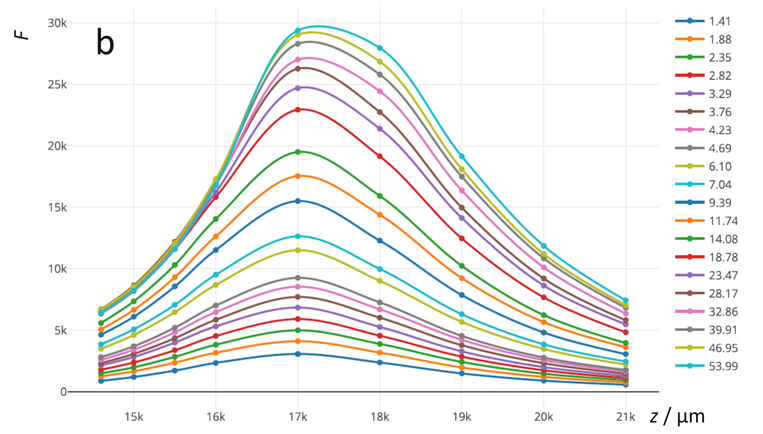


Figure 1. Uncorrected relative fluorescence (*F*) as a function of the concentration and *z*-position. Concentration plotted on the *x*-axis (a), *z*-position on the *x*-axis (b).

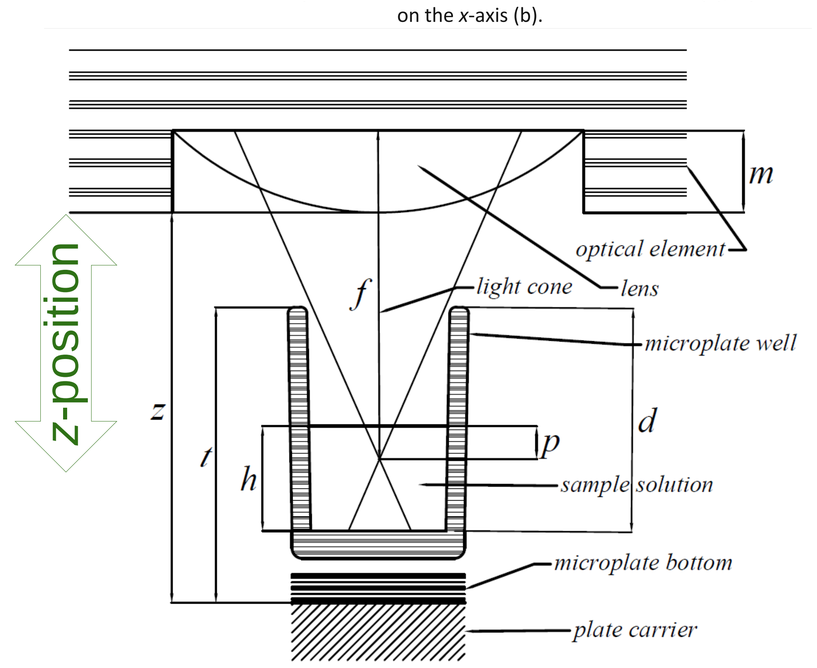


Figure 3. Geometric parameters used for the *z*-position (*z*-focus) IFE correction in microplate readers.

Numerical INner Filter Effect Corrector – NINFE

- Adaptation of the cell-shift method for the microplate readers (shift only in *z*-axis).
- Optical pathlength depends on the volume of the solution and is determined from the calibration function by correlating the absorbance measurements with the added volume.
- For *n* different *z*-positions there are $n\cdot(n-1)$ possible z_1, z_2 combinations that can be used.
- Modified correction formula is:

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

Geometric parameter *k* (Figure 3.) is defined as:

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

Parameters z_1 and z_2 are the *z*-position lengths adjustable on the microplate reader.

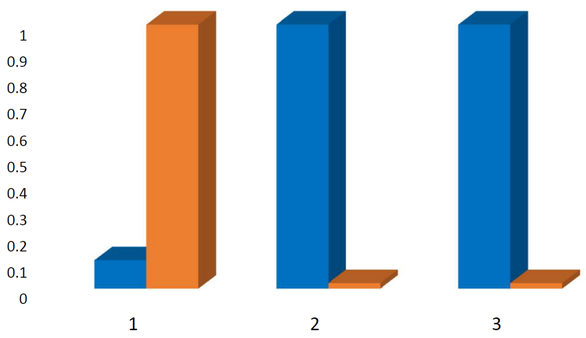


Figure 6. Scaled ratios of the lack of fit sum of squares (orange) and pure error sum of squares (blue) for: absorbance correction (1), *z*-focus correction in transparent plate (2), *z*-focus correction in non-transparent plate (3). Fluorescence corrected by *z*-focus is fitted well by the linear model, unlike the absorbance corrected data.

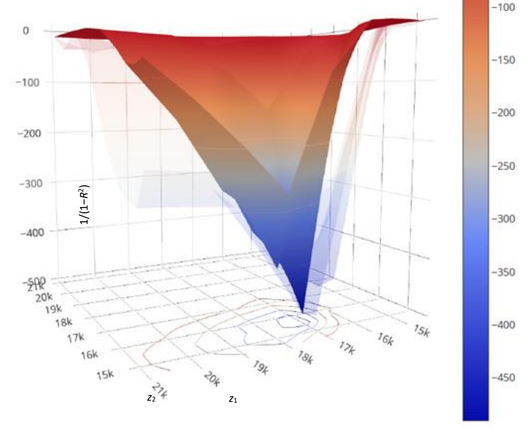


Figure 7. $1/(1-R^2)$ calculated from various linear interpolations as a function of z_1 and z_2 values used for the related IFE correction interpolations.

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[1] J. R. Lakowicz, Ed., in *Principles of Fluorescence Spectroscopy*, Springer US, Boston, MA, **2006**, pp. 27–61.

[2] H.-P. Lutz and P. L. Luisi, *Helvetica Chimica Acta*, 1983, **66**, 1929–1935.

[3] J. Kimball, J. Chavez, L. Ceresa, E. Kitchner, Z. Nurekeyev, H. Doan, M. Szabelski, J. Borejdo, I. Gryczynski and Z. Gryczynski, Methods Appl. Fluoresc., **2020**, 8, 033002.

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