About the evaluation dependency of the of energy transfer efficiency on the emission wave length of the donor

The measurements of Resonance Energy Transfer (RET) and its efficiency (E) from the Donor molecule (D) to the acceptor molecule (A) are of obvious, tremendous importance. Briefly, the measurements of RET and E are applied in a great number of research areas, including structure and conformation of proteins, spatial distribution and assembly of proteins, receptor/ligand interactions, immunoassays, structure and conformation of nucleic acids, real-time PCR assays and SNP detection, nucleic acid hybridization distribution and transport of lipids, membrane fusion assays, membrane potential sensing, fluorogenic protease assays, indications for cyclic AMP and more.

The accepted methods of quantitative RET measurements utilize changes in the emission intensity of the donor D in the presence of the acceptor A, relative to the donor intensity in the absence of A, that is, without RET occurring. Despite the great need for RET measurements and E calculations, many errors occur in their estimation, particularly when these measurements are based on D emission in different states. The present results, as well as those of others, demonstrate that the measured values of E depend on the emission wavelength (in contrast to the theoretical prediction of Foerster), as well as on the measurement method, the measurement system characteristics and conditions of measurement. In other words, it appears that E is not an independent value, and therefore the application of this parameter is deficient and clearly questions the precision of studies published so far (as reviewed in chapter 2 of the present work).

Therefore, the present work conducts a comparative, theoretical and experimental examination of the measurement technique, and suggests new solutions to eliminate the measurement limitations.

Presently, the solutions attempting to rectify the problems of measuring donor emission intensity are all, without exception, deficient. The literature analysis demonstrated that many studies of RET are not aware of or ignore the problems posited by Bernard Valeur (Bernard Valeur, *Molecular Fluorescence: Principles and Applications*, 2001):

1. "The larger the spectral overlap, the larger the distortion. …for the usual right angle observation, a theoretical correction is difficult" (p. 163, ln. 2-4).
2. "All these …. effects are difficult to correct and it is advisory to work as much as possible with dilute solutions" Lines 19-20, p. 163

In cases when high concentrations of fluorophores are used, necessary for the majority of RET measurements, Valeur, as well as many others, suggest front-face measurements:

3. “When the use of high concentration is required, front-face illumination offers the advantage of being much less sensitive…..” (p. 163, ln. 23-24).

The present work proves both analytically and experimentally that neither of the above guidelines, representing the opinion of leading specialists in the field, provides a suitable practical solution, but rather an erroneous one, to the problems involved in quantitative measurements of RET and E in a solution containing high fluorophore concentrations.

Three principal achievements of this work are summarized below:

1. *The theoretical development and application of the correction function* $f^{-1}(\theta,\theta',d,d',C^A,\varepsilon^A(\lambda))$.

The function f depends on the spectroscopic properties of the fluorophores, on the concentration of A and on the characteristics of the measurement system. The development of the function is presented in Chapter 3 and its performance is tested in Chapter 6. The application of the correction function on raw data obtained from the emission intensity measurements of D in the presence of A restores the real values of D fluorescence in the state of radiative and non-radiative energy transfer. The corrected data allow the calculation of the real value of E. This value was found to be independent of the emission wavelength.

To the best of our knowledge, this correction equation is unique and it enables, for the first time, performing correct quantitative measurements of RET and E in ideal fluorescence measurement setups. An example for the successful application of this correction function on raw measurement data is presented in Figure 1.

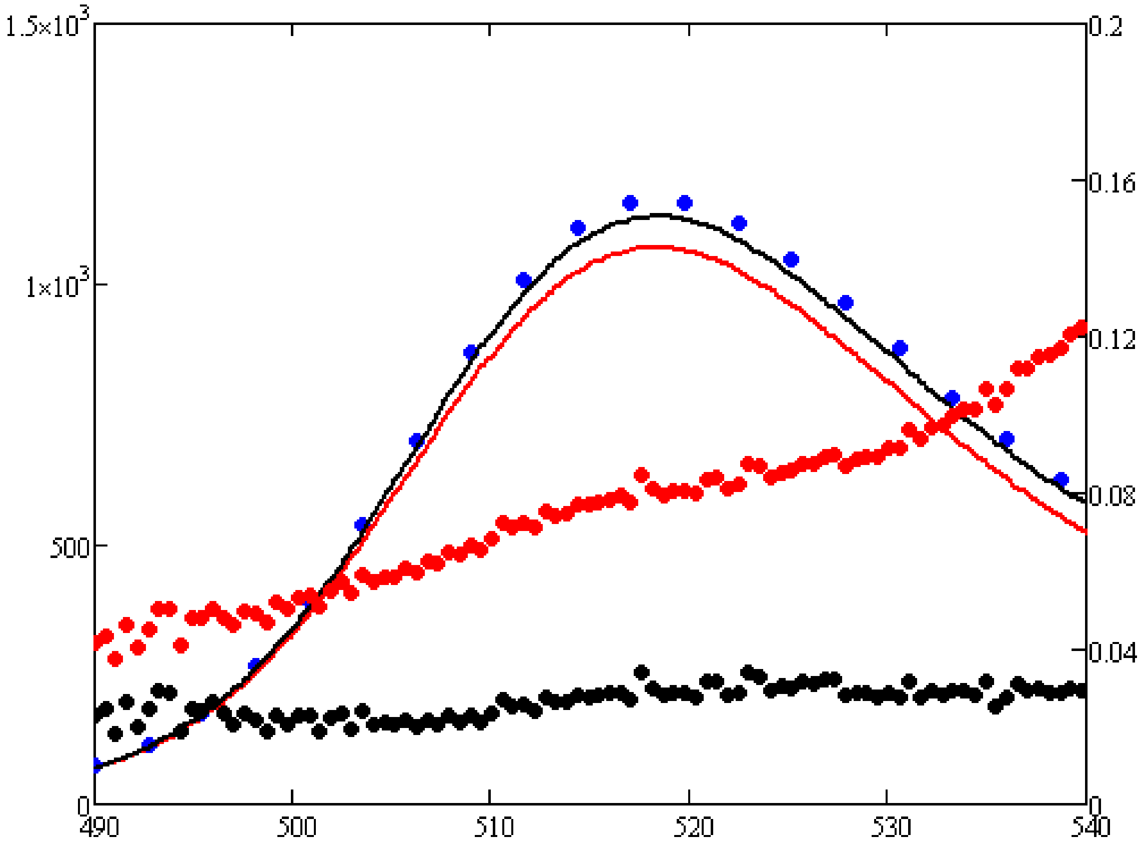

Figure 1. Recovery of correct D^A emission values. Axes: the measured and corrected values of emission intensity (left ordinate) and the efficiency of energy transfer (E, right ordinate) as a function of the emission wavelength (abscissa). The concentration of D (fluorescein) is 10 μM, the concentration of A (TMRM) is 4 μM. Blue dots: emission of D alone. Continuous red curve: emission intensity of D measured in the presence of A. Continuous black curve: the corrected value of D

emission intensity in the presence of A. Red dotted line: Measured E. Black dotted line: corrected E.

The results shown if Figure 1 indicate that the application of the correction function improves the results at least in two ways. First, the “corrected E”, in contrast to the “measured E” does not depend on the emission wavelength, as it is required according to Foerster’s theory. Secondly, the “corrected E” value is much lower than the “measured E” value, in agreement with the theoretical prediction.

This and other similar results obtained in the course of this work support the application of the correction function as an effective solution to the problems of E measurement. In addition, the application of the correction function makes front-face measurements unnecessary, as the latter provide only a partial and problematic solution for the evaluation of energy transfer efficiency.

2. *The analytical examination and application of E evaluation via donor fluorescence polarization measurements*.

The present work demonstrates that the estimation of E using fluorescence polarization of D in the presence of A is not influenced by an emission exhaustion of D by A. An example for a successful application of this method is presented in Figure 2.
Figure 2 shows the dependence of the fluorescence polarization of D in the presence of A. Values up to ~560 nm represent the polarization of D in its emission spectrum area, and further on are the values of the polarization of A in its emission spectrum area. It can be seen that, in the emission area of D, the measured polarization value is constant and therefore it is not influenced by the fact that the emission of D is exhausted by A, starting from 490 nm onward. Consequently, the E value, estimated from these polarization values, is also found to be independent of the emission wavelength of D (as shown in Figure 3). For an illustration of this phenomenon, compare the determination of E shown in Figure 3 with that measured and presented in Figure 1.

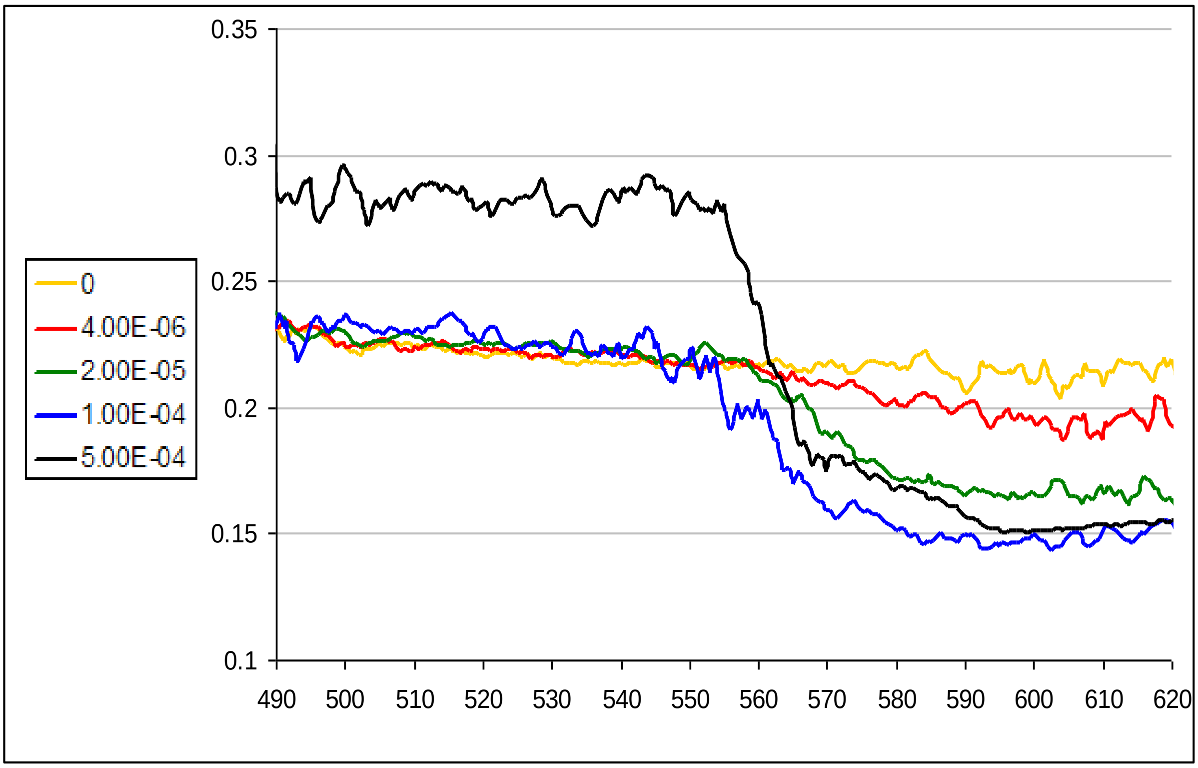

Figure 2. Polarization spectra of D at different concentrations of A (the curves are denoted in the insert). The ordinate shows the degree of fluorescence polarization, and the abscissa represents the emission wavelength (nm).

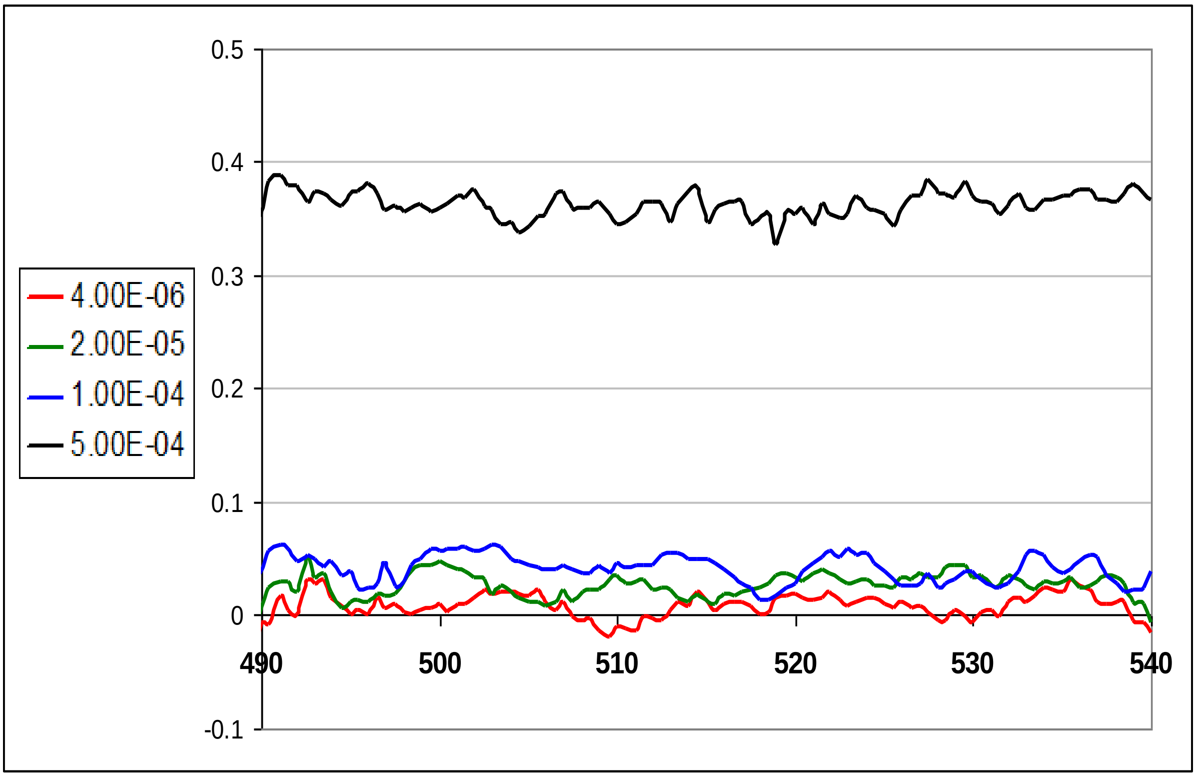

Figure 3. Energy transfer efficiency – E (ordinate) is calculated from the emission polarization of D, and found to be independent of the emission wavelength (nm, abscissa).

3. *Design and construction of a unique cuvette for measurements from the internal front surface (IFS)*

The “improved cuvette” is made of PDMS. It was designed to allow for fluorescence measurements in general and RET measurements in particular, providing a quality higher than currently available. The measurement compartments provide an original and appropriate answer to the problem of reproducibility and miniaturization of the optical pathways, in cases when measuring fluorescence in solutions containing high fluorophore concentrations. The IFS is represented in Figure 4.

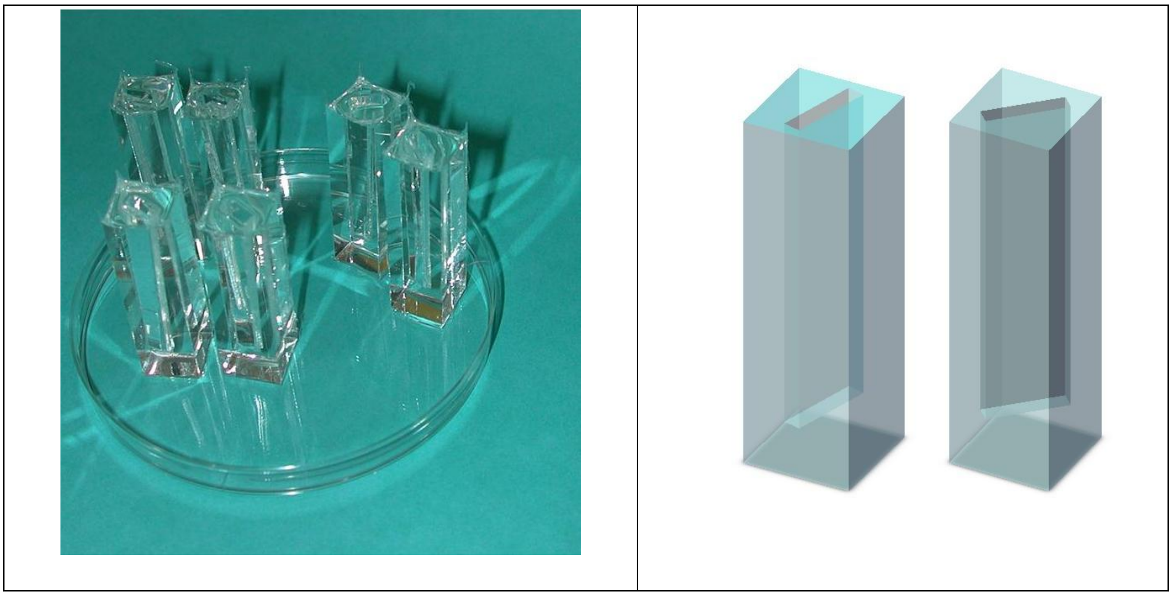

Figure 4. The cuvette (made of PDMS) for Internal Front Surface (IFS) measurements. Molding design (right) for the production of the improved cuvette (left)

This work includes 2 parts. The first part includes 3 chapters, and the second 7 chapters.

Part 1. *Background, analytical and theoretical survey of energy transfer measurement arrangements.*

Chapter 1. Background for the present research, including brief explanations, definitions and equations necessary for the performance of this work.

Chapter 2. Presentation and definition of the problems inherent in RET and E measurements. The chapter includes a critical, calculative survey of a number of characteristic works by others. The data presented in these works are thoroughly analyzed, demonstrating that the problems defined at the beginning of the chapter are indeed present in the works of leading specialists in the field.

Chapter 3. This theoretical chapter provides an analytical investigation of the influence of various factors on the standardization and accuracy of radiative and non-radiative energy transfer measurements and its efficiency E. In addition, the chapter presents an analytical critique of the solutions suggested by special literature. Furthermore, the chapter proposes analytical solutions, experiments and applications for correcting raw measured data. These solutions are successfully applied in the forthcoming experimental chapters of this work.

Part 2. *Experimental*

Chapter 4. Presentation of the materials, methods, calculation procedure and measurement apparatus. The latter is described in this chapter in brief and elaborated on in the Appendix.

Chapter 5. Discussion of RET measurements in a regular cuvette. The chapter presents the problems associated with its use and their origin.

Chapter 6. Experimental confirmation of the theoretical prediction discussed in Chapter 3.9. The correction function $f^{-1}\left(\theta,\theta',\lambda,d,d',C^{A},\varepsilon^{A}_{(\lambda)}\right)$ that was developed for the rectification of erroneous raw data measurements of D^A is applied. The results indicate the correctness of the proposed approach, the validity of the correction function and the effectiveness of its use.

Chapter 7. Experimental confirmation of the theoretical prediction presented in Chapter 3.9 regarding the correct evaluation of E using the fluorescence polarization parameter.

Chapter 8. Presentation and testing of front-surface and micro-cuvette measurements, which are the two major techniques recommended by special literature.

Chapter 9. Presentation of the research and development of Internal Front Surface (IFS) measurement containers at selected illumination angles.

Chapter 10. Presentation of the results of measurement with the new IFS measurement setup. Initially, the theoretical predictions of Chapter 3.3. were confirmed using model 1 of the cuvette and the results of measurements from the regular front surface cuvette that were obtained in Chapter 8.1. Additional examples are presented demonstrating the measurements of polarization intensity and RET that were performed in the IFS cuvette in models 2 and 3.