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FRET and FLIM Techniques

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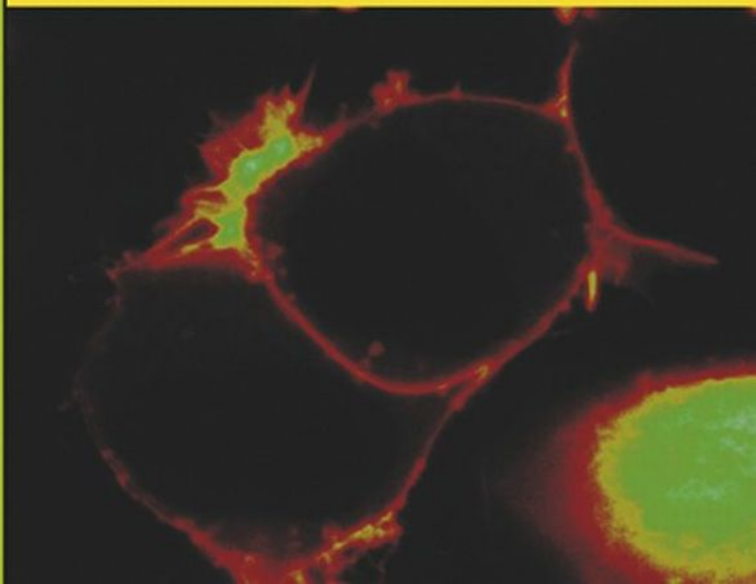

LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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FRET AND FLIM TECHNIQUES

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Preface

On September 13 2005 I received the invitation from professor P.C. van der Vliet (the editor of the Laboratory Techniques in Biochemistry and Molecular Biology Series) to become the editor of a new volume in the series on ''FRET and FLIM''. In the letter it was mentioned that ''in view of the rapid developments in single cell technology, we feel that a book on imaging techniques in living cells, such as FRET and FLIM, is appropriate and timely''.

Indeed FLIM and FRET (fluorescence lifetime imaging microscopy and Förster resonance energy transfer) have experienced a strong and (still exponentially) growing interest during the past years (for a quantitative assessment see Chapter 10, Fig. 10.1). The three major driving forces for this uplift are (i) the ease of in situ fluorescent labeling using the visible fluorescent proteins (since ~1996); (ii) the commercial availability of advanced fluorescence microscopes with FRET acquisition software and with special detectors capable of acquiring complete spectra or fluorescence lifetimes (since ~2000), and most importantly (iii) the unique information on in situ molecular conformation and-proximity that FLIM and FRET can extract from single living cells. In the more early days, factor (iii) was the only driving force available: for performing in situ FRET measurements it was required to first go through the burden of chemical (fluorescent) labeling of molecules and/or building an imaging microscope capable of acquiring digital (lifetime) images. My personal first FRET experiment dates back to ~1990 in the laboratory of Karel Wirtz in Utrecht, when I measured FRET between tryptophan residues of a membrane protein and (self synthesized) pyrene-labeled polyphosphoinositides (see the Verbist et al. reference in Chapter 6). At that time almost the only reference to FRET work in biochemistry was the famous spectroscopic ruler review of Lubert Stryer of 1978 (see Chapter 1

for reference). During my postdoctorate time in the Laboratory of Tom Jovin (1992–1994) I worked on (donor) photobleaching FRET (see Section 8.1 of Chapter 1) and I was introduced to FLIM by Bob Clegg (together being the α and ω of this book). To get it to work, software for analysis had to be written and a dedicated (non-commercial) FLIM setup was required, but in the end we could measure the oligomerization of EGF receptors in situ with both FRET techniques using fluorescein- and rhodamine-labeled EGF (see the reference by Gadella and Jovin in Chapter 5). To perform FLIM–FRET in the pre-GFP era, it was required that a laboratory (or scientist) combined the skills of biochemical labeling, microscope equipment construction and analysis software programming with relevant knowledge of (molecular) biology/biochemistry. Albeit this was a serious limitation for the technique to become more widespread, FRET-laboratories at that time typically could identify the sources of error, pitfalls and workarounds because they usually covered every aspect of the FRET experiment. In that respect I feel very privileged to have worked at that time with some of the pioneers of FRET–FLIM microscopy and fluorescence spectroscopy, most notably Tom Jovin, Bob Clegg and Ton Visser (incidentally, all of them worked in the lab of the ancestor of all fluorescence spectroscopy in biology: Professor Gregorio Weber).

Nowadays, with some background in molecular biology, almost any scientist can perform a FRET experiment using commercial microscopes. Some microscopes even are equipped with a ''FRET-button'' so that all image acquisition and data processing is automated but ''hidden'' for the experimenter. The ease of performing automated FRET experiments by non-FRET experts also encompasses a danger: the underlying principles and pitfalls are often not well understood, leading to all sorts of misinterpretations, errors and frustration. Whether or not the correct filters, probes, laser sources, acquisition strategies, or image processing routines were used is often not completely (to completely not) known by the much larger community of FRET-scientists nowadays. Some of

these frustrations culminate into statements that FRET technology is "unreliable" or produces "false negatives".

Hence, for modern FRET and FLIM techniques in Molecular Biology and Biochemistry it is important to keep the enthusiasm for the in situ technique, yielding unprecedented rich information on molecular states in live cells, and to keep the advantages of easy labeling techniques, modern microscopes and automated data processing. However, we need to "educate" the new generations of FRET scientists in the theoretical background of the technique, how it should be done correctly, and what the sources of errors are. Only then it will be clear that FRET–(FLIM) is a very direct, robust, extremely sensitive, and reliable technique.

This thought convinced me that I should accept Professor van Vliet's invitation to become editor of a FRET–FLIM volume. What I intended with this FLIM–FRET volume is to make a compilation of chapters that would be useful for the new generation of FRET scientists, but also interesting enough for the experts. So while nowadays, a variety of "exotic" FRET applications, theory, and instrumentation is around, I aimed to highlight the most straightforward and mainstream FRET work in this volume with sometimes also giving a peek into more advanced and future directions. Hence, this volume will not cover every aspect of FLIM–FRET. For instance, it does not cover single molecule, low temperature, detailed spectroscopic FRET work or very detailed hardware issues. In addition, triplet state conversion, or saturation effects (ground state depletion) giving rise to nonlinear excitation power-fluorescence intensity relationships are generally ignored (the reader is referred to Chapter 12, the FRET calculator for further information). Finally treatment of very complex FRET situations with (i) movement between donor and acceptor within the donor lifetime, (ii) multiple (n) acceptors for one donor (multiplying Eq. (1) of Chapter 1 with n), (iii) situations of a coexistence of many energy transfer states due to different conformations, (iv) effects of geometry leading for instance to a fourth power distance dependency for energy transfer to a planar surface of acceptors,

and (v) changes in local refractive index in the cell leading to different local values of R_0 are not considered. It is of note however that in reality, in cells, (a multitude of) these situations will be applicable. So the basic FRET concepts and measurement strategies are illustrated for a situation with uniform FRET efficiency E in the cell in the absence of the above "problems" but with (local) variation in fractions of molecules (f_D and f_A) experiencing FRET, since these parameters are most interesting for biologists. In the more complex example cases (i)–(v) listed above, the equations listed in this volume will still be correct for defining an "average" or "apparent" energy transfer value in a microscopy image; this value will still represent an interaction or conformational state(s); but it will not be possible to make a statement on the fraction of molecules involved in such interactions and/or states with these formulas: simulation or more exotic equations are required.

The first chapter written by Bob Clegg introduces the FRET theory and basic equations. Also the original work and historic background of the FRET theory is presented in this chapter. Because sometimes it is difficult to picture a situation from an equation, Bob Clegg describes an analogy between FRET and monkeys escaping through doors of a dark room. Another highlight of this chapter is a description of a measurement of FRET without measuring fluorescence (arguing strongly in favor of FRET being "Förster resonance energy transfer" instead of "fluorescence resonance energy transfer").

The second chapter by Peter Verveer and Quentin Hanley describes frequency domain FLIM and global analysis. While the frequency domain technique for fluorescence lifetime measurement is sometimes counterintuitive, "the majority of the 10 most cited papers using FLIM have taken advantage of the frequency domain method" as stated by these authors. The global analysis of lifetime data in the frequency domain, resolving both E and f_D has contributed significantly to this advantage.

The third chapter by Alessandro Esposito et al., describes the time domain counterpart of FLIM. When photon economy and

fast decaying components are considered, the time-domain implementation of FLIM is the method of choice. Most commercial (multiphoton) confocal FLIM systems implement this technology.

The fourth chapter by James McGuinty et al. describes the more advanced forms of time-domain FLIM. While not immediately available on commercial instruments this chapter should give the reader an idea what the current state-of-the-art is in terms of FLIM instrumentation, and perhaps what to expect on future commercial instruments. Real-time FLIM, combined FLIM-spectral imaging, hyperspectral FLIM-imaging, combined lifetime-anisotropy imaging and some of their applications are covered here.

Besides FRET theory and instrumentation, also probes are a key issue for performing a valid FRET experiment in cells. Chapter 5 by Gert-Jan Kremers and Joachim Goedhart highlights the various visible fluorescent proteins (VFPs) for the use of FRET. These authors argue that "with the large number of spectral classes and several variants within each spectral class, choosing the right VFP FRET pair for FRET can be a daunting task". To assist in this choice, a unique table with all Förster radii (R_0) between the major monomeric VFPs (Chapter 5, Table 5.1) and spectra highlighting five different VFP combinations with theoretical FRET spectra, are included.

While VFPs have boosted the applications of FRET–FLIM, chemical FRET probes should not be dismissed. The advantage of chemical probes is that they are much smaller in size and that they often have much better spectral readout than VFP probes. In Chapter 6, Amanda Cobos Correa and Carsten Schultz highlight the various small molecule-based FRET probes and their use in bioimaging.

For many scientists dedicated FLIM instruments are too expensive and/or too complicated to work with. Therefore, Chapter 7 by Jacco van Rheenen and Kees Jalink is included dealing with "low budget" but "high quality" Filter FRET. Filter FRET has the advantage that it is fast, sensitive, direct and inexpensive. However, if you want to do it quantitatively and without errors, you need to

go through a lot of formulas and correction factors. In this chapter, the reader is guided through these issues and a full comprehensive description is given to perform correct calibration of a filterFRET microscope (both wide-field and confocal).

Chapter 8 written by Steve Vogel et al. also deals with sensitized emission based FRET methodology, but now using a spectral imaging detector device. Because a spectral detector and spectral unmixing software nowadays are standard options on the major commercial confocal microscopes, here a complete description is given how to quantify FRET from unmixed spectral components.

The smallest Chapter 9 (written by undersigned) deals with total internal reflection FRET–(FLIM) imaging. This technique enables the measurement of FRET with high contrast in a layer of only 80 nm above the cover glass, which is very useful for cellular membrane-related events. It is explained how an existing FLIM-system can be ''upgraded'' to incorporate the TIRF contrast (with thanks to Carsten Schultz for proofreading/editing).

In Chapter 10, Riyaz Bhat highlights FLIM–FRET applications in plant systems. Particularly plant sciences suffering from notoriously difficult biochemistry can profit most from the detailed in situ molecular imaging and contrast provided by FRET–(FLIM) imaging. With the help of genetically encoded probes and the ease of plant transformation and (back)crossing, plant scientists increasingly see the benefit of FRET–FLIM. For non-plant scientists it may be interesting to read how shooting gold bullets into plant material can be used for performing FRET microscopy.

In Chapter 11, by Phill Jones et al., biomedical FRET–FLIM applications are reviewed and illustrated. The molecular background of a variety of diseases (e.g., Alzheimer's disease) can be uncovered by using FRET–FLIM. In this major funding area, the ''killer''-applications of the technology are and will be found, leading to a further boost of the implementation and commercial availability of high-end microscopes with automated acquisition and standardized analysis features.

Chapter 12, by Eli Erijman and Tom Jovin concludes the volume. This special chapter introduces a new and quantitative definition of FRET-measurements without requiring knowledge of the donor quantum yield Q_D or the energy transfer efficiency E. Furthermore, it highlights the recent explosion in labeling strategies, ranging from genetic encoded FlAsH labeling, through AGT, NTA technology, photochromic labels to quantum dots and further. Hence, the potential of getting the most sensitive probe attached to a biomolecule of interest nowadays is phenomenal (and still increasing). They conclude their chapter with "Quo vadis"...

So approximately 3 years after receiving the invitation letter from professor van Vliet, the FLIM and FRET volume is ready and I believe the combined chapters make an excellent statement for FRET and FLIM technology. Hereby, I want to thank all chapter authors for their efforts and the fine work they have delivered. It was a pleasure from my side to work on the diverse chapters and for me it was also a good learning experience especially to go through the equation rich Chapters 1, 7, 8, and 12. For that I am especially grateful for the list of common symbols that we could agree on (see Table). The equations in the volume may scare off scientists with training mainly in (molecular) biology. Although they may appear as difficult, the vast majority of the equations represent "simple mathematics" being not more difficult than subtracting, adding, multiplying or dividing (elementary school stuff). Let the reward of understanding the equation (quantitative information on molecular interactions and conformation in situ) be a motivation to go through the math.

Having said this, I hope the above thoughts and chapters highlights make you curious and eager for reading the volume, contributing to more good, reliable and enthusiastic future FRET–FLIM work in Biochemistry and Molecular Biology.

Dorus Gadella, 5 August 2008

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FRET and FLIM Techniques
T. W. J. Gadella (Editor)

CHAPTER 1

Förster resonance energy transfer—FRET what is it, why do it, and how it's done

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The applications of Förster resonance energy transfer (FRET) have expanded tremendously in the last 25 years, and the technique has become a staple technique in many biological and biophysical fields. Many publications appear weekly using FRET and most of the applications use FRET as a spectroscopic research tool. In this chapter, we have examined some general salient features of resonance energy transfer by stressing the kinetic competition of the FRET pathway with all other pathways of de-excitation. This approach emphasizes many of the biotechnological and biophysical uses of FRET, as well as emphasizing the important competing processes and biological functions of FRET in photosynthesis.

1.1. Introduction

There are numerous excellent reviews and original literature about Förster resonance energy transfer (FRET) where one can read detailed descriptions and get lists of earlier references [1–11]. This chapter is neither a review of the literature, nor a detailed account

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of experimental techniques and methods of analysis, nor a how-to-do manual, nor an appraisal of theoretical descriptions of energy transfer for the specialist. The chapter focuses on a few critical essentials concerning the fundamentals of energy transfer and the methods of measurement. These basic aspects of FRET are helpful for understanding the important features and interpretations of energy transfer measurements. It is also useful to see historically how these ideas were developed. If one understands these simple fundamentals, it is usually straightforward to appreciate specific theoretical and experimental details pertaining to methods of acquisition and analysis.

1.1.1. Fluorescence and FRET are popular methods

Fluorescence has exquisite sensitivity for detecting very low concentrations of molecules over broad spatial and temporal dimensions. By choosing luminophores (fluorophores and phosphors) with lifetimes from subnanoseconds to milliseconds, molecular dynamics can be observed over a large time scale; nevertheless, the FRET measurement can be carried out on macrosystems. FRET is frequently applied to determine molecular distances or to show whether or not molecular complexes are present. Lifetime-resolved FRET has been carried out on fluorescence images [12–20]. FRET is increasingly occupying a center stage in biological studies and in biotechnology (especially dealing with DNA chips and other massively parallel assay systems). It has been applied in single molecule studies to provide information on conformational changes [21, 22] and the pharmaceutical industry has developed major microscopic fluorescence assay detection systems with very low sample volumes, even on the single molecule level, using fluorescence correlation spectroscopy [23–29].

Information about molecular interactions, spatial juxtapositions, and distributions of molecular and supramolecular components constituting biological structures are of crucial importance

for understanding functions on a molecular scale in biology. This information is especially vital when we consider that a major part of biology takes place at the interface between interacting molecules and supramolecular organizations. Many of these macromolecular systems are ideally suited for FRET applications. For this reason, FRET has received so much interest in biotechnology and medicine as well as in biophysics [30–38]. Applications for FRET extend from more traditional cuvette spectroscopic measurements on larger volumes (from 100 μm to 100 ml) to FRET imaging experiments in the fluorescence microscope [16, 17, 39–44] and single molecule experiments [21, 22]. The recent applications of FRET in the optical microscope have become very popular because of its interpretive power on the molecular scale with regard to statically and dynamically associating molecular systems in cellular biology [45–48]. Using geometrical and stereochemical information that can be attained from FRET measurements, we can more confidently propose models how biological structures carry out their functions, for instance in ribosomes [49, 50]. Knowing the spatial distribution of the parts of a structure makes it possible to ask more specific questions concerning the dynamics of intermolecular interactions.

1.1.2. FRET—A molecular detective, transmitting molecular dimensional information to the experimenter

Fluorescence molecules are analogous to roaming molecular spies with radio transmitters, radiating information to the experimenter about the state of affairs on the molecular scale, and informing us where the spies are located and how many there are. A feature unique to FRET is the capability to inform us whenever two or more molecules (usually biological macromolecules) are close to one another on a molecular scale ($\leq$80 Å), and whether they are moving relative to each other (Section 1.3.1.1). It is even sometimes possible to detect how the D and A transition moments are oriented

relative to each other, because the efficacy of transfer depends on the relative angular dispositions of the two dipoles (Section 1.3.1.2). As with all other fluorescence methods, we can couple FRET with other physical and biological methods, and this greatly extends the usefulness. Such broad application is characteristic to fluorescence. Very importantly, FRET (and fluorescence in general) can be carried out in most laboratories, whether the "samples" are large (such as in cuvettes, or even on whole mammalian bodies) or small (such as in the fluorescence microscope, and on the single molecule level). No matter what scale of the sample, the information on the molecular scale derivable from FRET remains accessible. In this regard, FRET is like a spectroscopic microscope, providing us with distance and orientation information on the molecular scale regardless of the size of the sample (Stryer [9] dubbed FRET a molecular ruler). In addition, by observing FRET over time (such as in stopped-flow), we can follow the dynamics of changes in molecular dimensions and proximities.

As should be apparent from the above discussion, and a perusal of the contents of the recent literature, the range of applications of FRET is extremely broad.

In Section 1.2, we first introduce some historical facts concerning the development of FRET and indicate how FRET is interrelated with several scientific disciplines. If one is not interested in this historical account, then you can skip to Section 1.3 without losing the thread.

1.2. Historical background; setting the groundwork

In a series of remarkable papers, Theodor Förster revealed the correct theoretical explanation for nonradiative energy transfer [7, 51–55]. He was partly motivated by his familiarity with the extreme efficiency of photosynthetic systems in funneling the energy of absorbed photons to a relatively small number of reaction sites [51]. The average number of photons striking the total area of a leaf is much larger than that expected, considering the small area of the leaf containing the reaction centers, where the photosynthetic

electron transfer reactions take place. In order for the absorbed energy to be channeled efficiently into the reaction centers, he, and others, reasoned that the excitation energy is rapidly and efficiently transferred throughout an area that is large compared with the reaction center. Eventually, this energy is captured by a reaction center. This process increases the effective capture area of the reaction center. This process was pictured as a random diffusive spreading of the absorbed photon energy that is captured by a sink (the reaction center). Although such a mechanism was suspected at the time, the physical mechanism responsible for this energy transport in photosynthesis was not understood.

1.2.1. Pre-Förster: Dipole–dipole interaction; the Perrins

Early dipole–dipole models of energy transfer were developed by the Perrins (father (J.) and son (F.)) [56, 57]. Dipole interactions had already been used in descriptions of molecular interactions in bulk matter, including dipole-induced–dipole-induced van der Waals forces, dipole–dipole-induced forces, and dipole–dipole interactions [58]. Classically, the electric field emanating from an oscillating dipole, $\vec{E}\tilde{\mu}$, (real dipoles or transition dipoles) decreases as distance between the dipoles, R, is increased. The functional form of the oscillating dipole can be divided into three zones: the near-field zone ($\vec{E}\tilde{\mu}^{nf} \propto R^{-3}$), the far-field zone ($\vec{E}\tilde{\mu}^{ff} \propto R^{-1}$), and in the intermediate transition zone between the near- and far-field. We are concerned here only with the interaction energy between two dipoles in near field, which is very large compared with the other two zones. The near-field dipole–dipole transfer mechanism had been first proposed to explain energy transfer between atoms [59]. A nonradiative dipole–dipole model successfully accounted for energy transfer in gas mixtures arising from near collision processes between the atoms [58, 60–63]. It was found that energy could be transferred over distances beyond the hard core collision distances between molecules, in the near-field zone of dipole–dipole interactions.

The Perrins were the first to attempt a quantitative description of nonradiative (no emission of a photon) energy transfer *in solution* between an excited molecule (originally called the sensitizer; in this chapter called the *donor*) and a neighboring molecule in the ground state (originally called the activator; in this chapter called the *acceptor*). The Perrins' reasoned that the depolarization that occurs in a solution of a fluorophore at higher concentrations resulted from the transfer of excitation energy between molecules with different orientations, before a photon was emitted. The Perrins' model involved a near-field energy of interaction, E_{int}, between the oscillating dipoles of two molecules, D and A ($E_{\text{int}} \propto \overrightarrow{E}^{nf}_{\vec{\mu}_D} \cdot \vec{\mu}_A$); $\vec{\mu}_A$ is the dipole of the acceptor. This is simply the general form of the interaction energy of a dipole in an electric field. This interaction is identical to the perturbation employed in a quantum mechanical representation of FRET. An interesting account of the early history of energy transfer is given in a recent review of the Perrins' accomplishments [64], and a recent historical review of FRET [58]. They assumed identical molecules [56, 57, 65]. Initially, they considered a classical model involving oscillating point dipoles; later they presented a quantum mechanical model. In modern quantum mechanical descriptions, the dipoles are the transition dipoles [1, 6, 7]. They were correct that the energy transfer involved dipole perturbations, as had also been realized by earlier researches studying molecular interactions. However, their model did not account quantitatively for the energy transfer between identical molecules in solution. It was known that fluorescence becomes depolarized at concentrations of fluorophores where they are separated by ~2–5 nm; and that depolarization can be detected below concentrations required for quenching effects. However, their models for explaining this depolarization predicted energy transfer (and therefore depolarization) between two molecules separated by a much larger distance, on the order of a fractional wavelength of light (~100–500 nm). Because of this large discrepancy, F. Perrin's theory of energy transfer lay dormant for about 20 years.