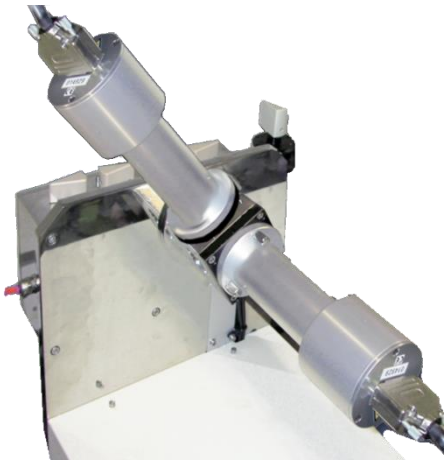
Stopped-Flow Fluorescence (Förster) Resonance Energy Transfer (FRET)

- Study complex biomolecular interactions such as that between TCRs and their target pMHC ligands
- Understand competition between phospholipid-containing and non-phospholipid vesicles, yielding important implications in understanding the evolution of the cell membrane.
- Representative understanding of the kinetics of the structural dynamics of nucleosomes contained in an extended chromatin structure.

Stopped-flow FRET is a useful technique for studying many types of biomolecular interactions. The method provides kinetic information regarding distance changes occurring on the millisecond timescale. This application note gives an introduction to the technique along with a review of some example experiments from the scientific literature. Applied Photophysics Ltd. can provide the accessories and application support required to perform such experiments on the SX series of stopped-flow spectrometers.



KEYWORDS

- SX20
- Stopped-Flow
- FRET
- Reaction Kinetics
- Biomolecular Interactions
- Rapid Mixing
- Dual Fluorescence
- Time-Dependent Spectroscopy

Introduction

FRET is a phenomenon that can be observed between two fluorophores in which the emission wavelength of a donor species (D) overlaps with the absorption wavelength of an acceptor species (A). Depending on several factors, a transfer of energy may occur from the excited state of D (D*) to the ground state of A. This may be observed experimentally as an increase in fluorescence intensity at A's emission wavelength and/or a decrease in intensity at D's emission wavelength. Applied Photophysics Ltd. offers dual fluorescence stopped-flow capabilities, enabling the measurement of both D* and A* emissions. FRET is a useful tool as it is distance dependant; its measurement can give information regarding the dynamics of molecular interactions. Stopped-flow FRET experiments can provide unique information on many types of biomolecular interactions on a millisecond to second timescale. The following presents examples from the literature to illustrate some of the applications stopped-flow FRET can be used for.

Case Study 1: Kinetics of Protein-Ligand Interactions

This example describes how stopped-flow FRET can yield useful kinetic and mechanistic information regarding complex biomolecular interactions. Stopped-flow FRET was used to study the interaction between T-cell receptors (TCRs) and their target peptide ligands which are known as pMHCs (major histocompatibility complexes bound to a particular peptide). The study, described in reference [3], determined the kinetics of the binding interaction between a TCR specific for Cytomegalovirus Peptide (TCRCMV) and its ligand pp65-HLA-A*201. The TCR was labeled with tetramethyl-rhodamine (acceptor) and the pMHC was labeled with fluorescein (donor).

It was found that the interaction was bi-phasic characterized by a fast step followed by a much slower step. This is demonstrated in the kinetic traces observed on different timescales. **Figure 1** (top panel) shows a representation of the published data of the decrease in donor emission as the binding interaction proceeds over a long timescale. The decrease in emission intensity of the donor can be attributed to a binding interaction as the donor/acceptor pair are brought together, decreasing the distance between them. **Figure 1** (middle panel) shows the trace obtained with a

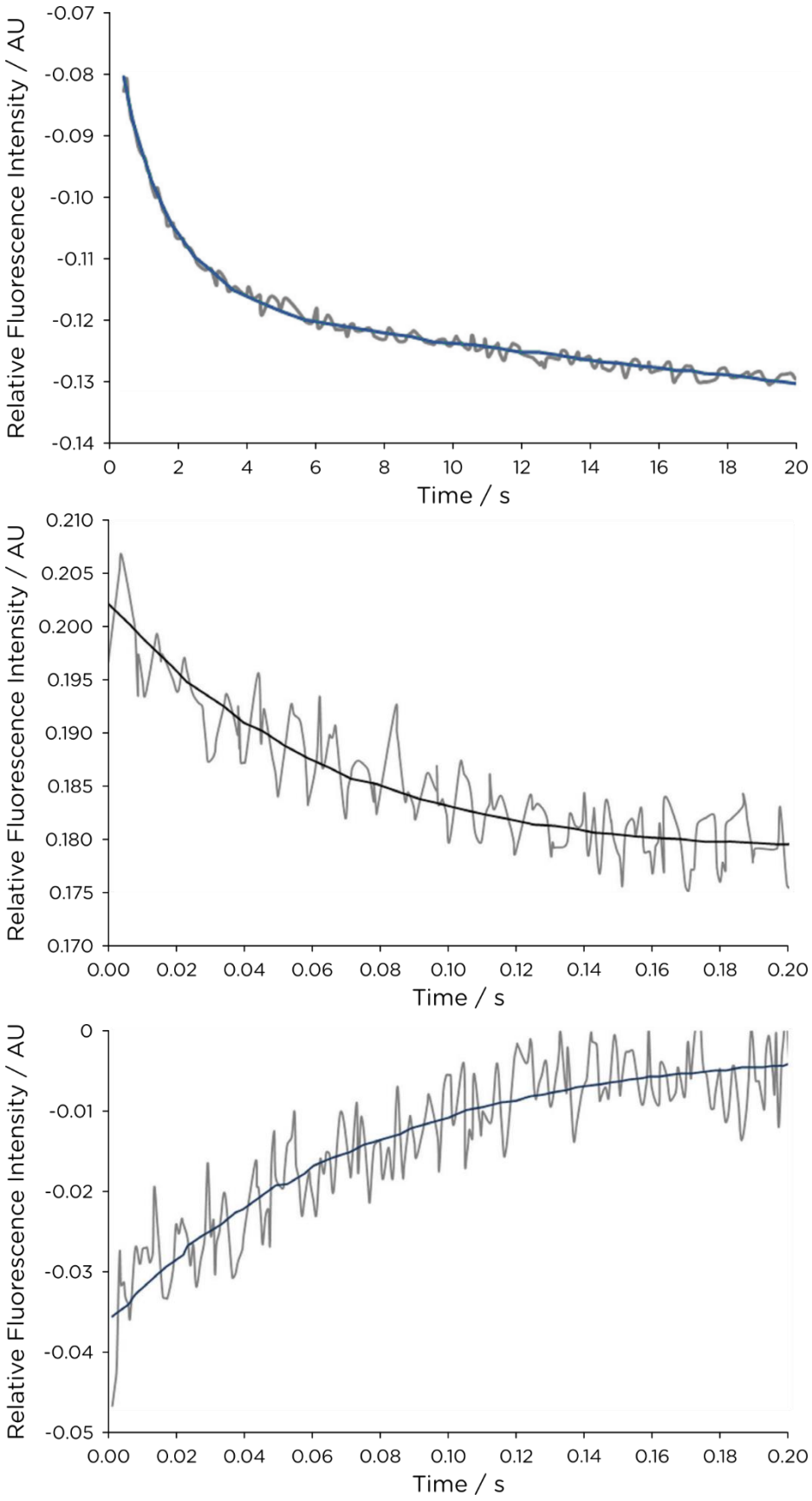


Figure 1: Stopped-flow FRET for the interaction between a T-cell receptor and its peptide ligand as monitored by donor emission on two different timescales (top and middle) and acceptor emission (bottom).

much higher temporal resolution. **Figure 1** (bottom panel) shows an equivalent trace obtained from measuring the increase in fluorescence intensity of the acceptor. The observed traces can be treated as a direct observation of the binding interaction and those of donor emission can be fit to obtain observed first order rate constants k_2^{obs} and k_1^{obs} for the fast and slow phases of the reaction, respectively.

Fitting to longer timescale traces needs the addition of an extra rate constant (k_3^{obs}) to account for photobleaching of fluorescein. Likewise, the rate constants can be extracted from the acceptor emission traces as seen in **Figure 1** (bottom panel). It was determined that k_2^{obs} showed a linear dependence on the concentration of labeled TCRCMV under pseudo first order conditions ([TCRCMV] >> [pMHC]) whereas k_1^{obs} showed no such dependence.

Applying these results to those from previous crystallographic studies, the following reaction scheme is implied:

$$TCR + pMHC$$
$$k_{12} \quad \Downarrow\Uparrow \quad k_{21}$$
$$(TCR \cdots pMHC)^{tr}$$
$$k_{23} \quad \Downarrow\Uparrow \quad k_{32}$$
$$(TCR \cdots pMHC)^{st}$$

where $(TCR\cdots pMHC)^{tr}$ and $(TCR\cdots pMHC)^{st}$ represent a transient and stable complex, respectively. The fact that the first step in the above mechanism is independent of [TCR] (when

[TCR] >> [pMHC]) implies that the rapidly formed $(TCR\cdots pMHC)^{tr}$ rapidly dissociates back to TCR and pMHC (k_{21} approximately 18-30 s^{-1}). The second step of the reaction is much slower (k_{23} approximately 2-4 s^{-1}) which represents the transition of the transient complex to a stable TR···pMHC complex and is rate determining. This final step is believed to be brought about by a conformational change in the $(TCR\cdots pMHC)^{tr}$ complex via an "induced fit" mechanism.

Case Study 2: Monitoring Vesicle Growth

Stopped-flow FRET was used to demonstrate that fatty acid vesicles which had been doped with phospholipids competitively acquired fatty acids from equivalent non-phospholipid containing vesicles. The phospholipid-containing vesicles therefore grow at the expense of their non-phospholipid containing counterparts. The results of this study may have important implications in understanding the evolution of the cell membrane.

Either fatty acid vesicles or phospholipid-containing vesicles were fluorescently labeled with low concentrations of the fluorescent phospholipids NBD-PE (donor) and Rhodamine-DHPE (acceptor) in a 1:1 ratio. The fluorophores are assumed to remain on the surface of the vesicle throughout the experiment. In this study, the authors converted the data from fluorescence intensity measurements into relative surface area. This is a valid

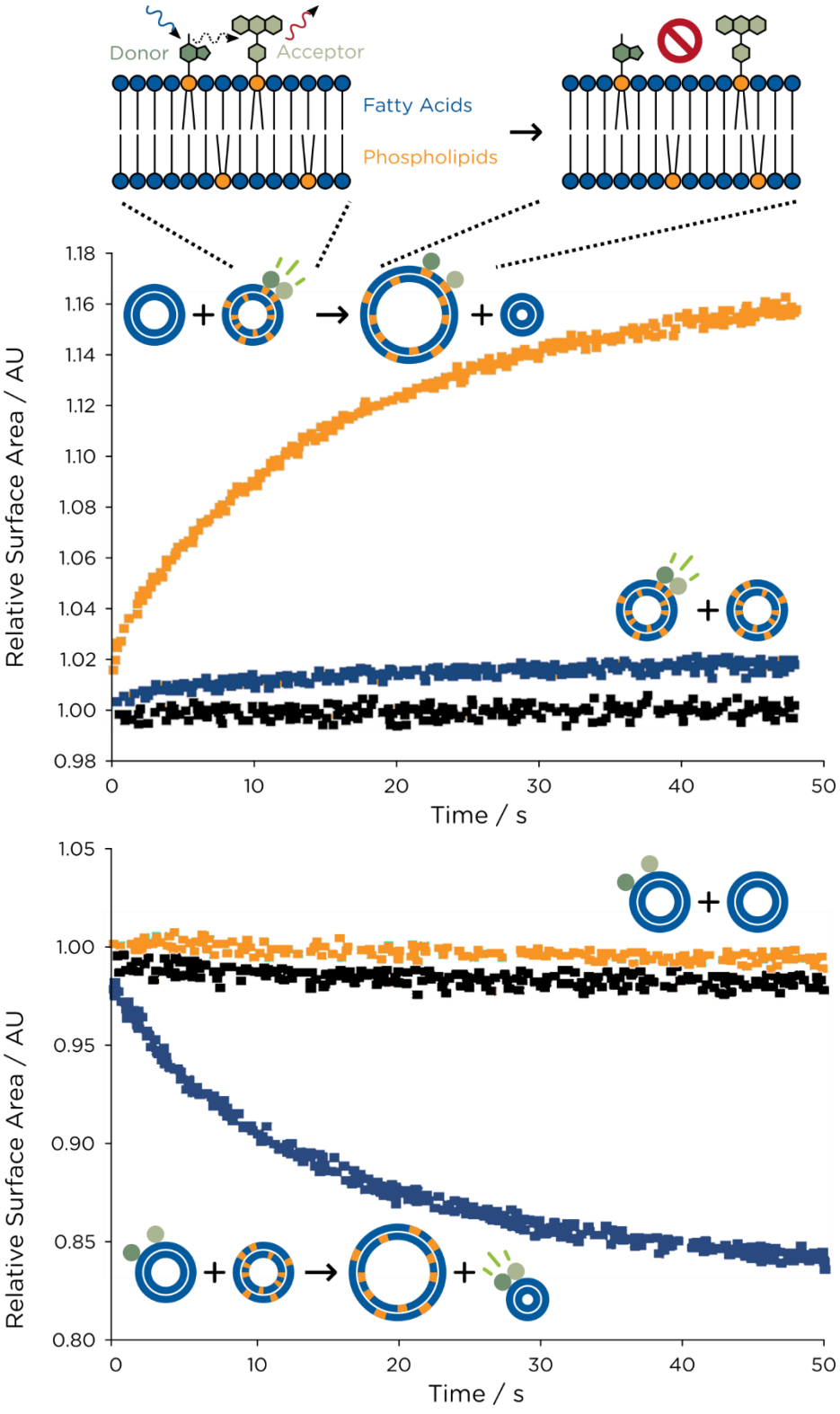


Figure 2: Change in relative surface area of labeled vesicles. Top: phospholipid-fatty acid vesicles rapidly mixed with equimolar unlabeled fatty acid vesicles (orange), buffer (black) and equimolar unlabeled equivalent phospholipid-fatty acid vesicles (blue). Bottom: fatty acid vesicles rapidly mixed with equimolar unlabeled phospholipid-fatty acid vesicles (blue), buffer (black) and equimolar unlabeled equivalent fatty acid vesicles (orange). Data adapted from [4].

assumption because a decrease in FRET corresponds to an increase in donor emission and a decrease in acceptor emission, meaning there has been an increase in the distance between the fluorophores i.e. the vesicle has acquired fatty acids resulting in growth and thus, a larger surface area.

Relative surface area is estimated from the ratio of the donor fluorescence to the acceptor fluorescence (F_{don}/F_{acc}) by using a standard curve [5].

Figure 2 (top panel) shows that, when the phospholipid-containing vesicles are mixed with the equivalent fatty acid vesicles, the phospholipid containing vesicle grows, i.e. competitively obtains fatty acids from the fatty acid vesicles. The data in **Figure 2** (bottom panel) further support the competitive growth of the phospholipid containing fatty acid vesicles.

If the earliest cell membranes were composed of fatty acid vesicles (as currently suspected), the presence of small proportions of phospholipids may have provided a selective advantage for these cells to grow. This may have driven the conversion from primitive to modern cell membranes [4].

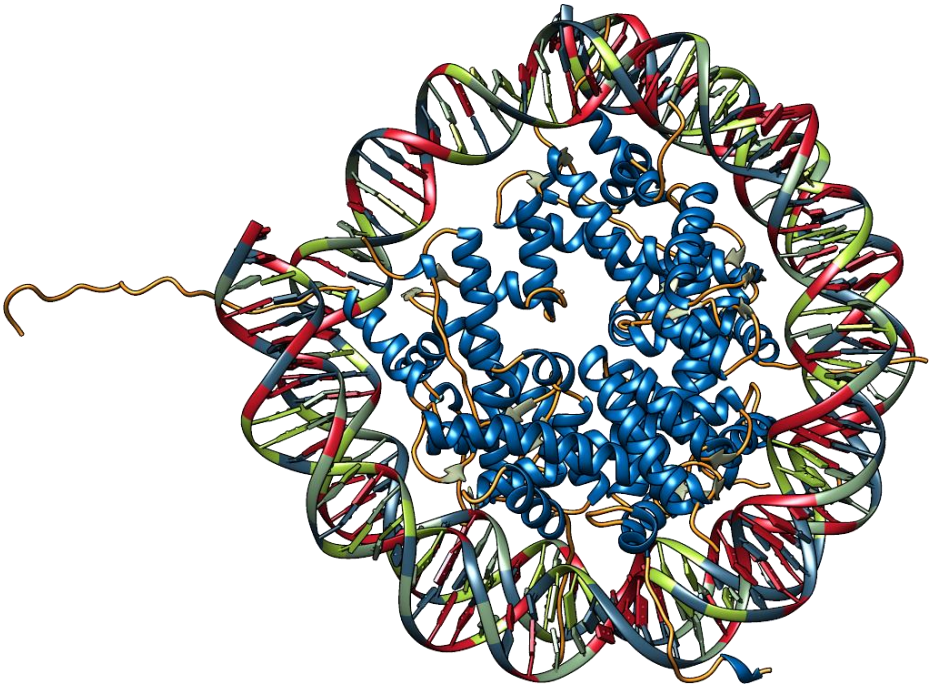


Figure 3: X-ray crystal structure of a nucleosome. PDB ID: 1AOI.

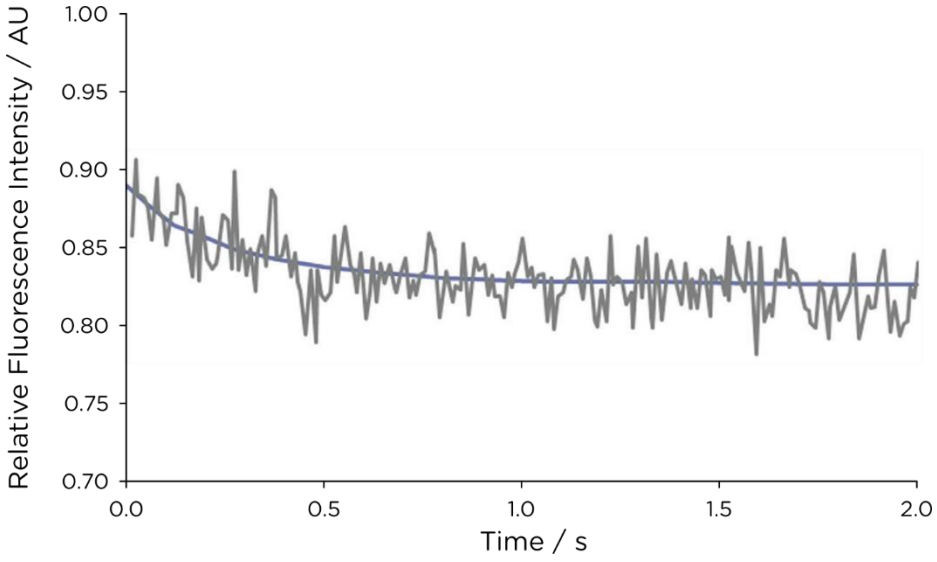


Figure 4: Histone unwrapping as followed by stopped-flow FRET. Data adapted from [6].

Case Study 3: Kinetics of Nucleosome Structural Dynamics

In the nucleus of eukaryotic cells, the genomic DNA is organized into chromatin that consists of repeating units, the nucleosomes. An individual nucleosome consists of an octameric protein which the DNA wraps around, the histone (**Figure 3**). For many essential biological processes including transcription and regulation thereof, the DNA surface needs to be accessible so that other species can interact with it.

Therefore, the nucleosome must unwrap so that these events can occur. The rate at which unwrapping/rewrapping occurs has a direct effect on processes that govern an organism's metabolism. Stopped-flow FRET was used to determine the

kinetics of DNA unwrapping from the nucleosome, giving important insights into the biological processes mentioned above.

A 147-base pair DNA sequence containing a binding site for the transcription factor LexA was used to prepare nucleosomes. In addition, this DNA strand had a Cys3 (donor) group attached to its 5' end. The histone protein was labeled with Cys5 (acceptor) so that changes in distance between the DNA and histone could be observed by a change in FRET signal. In the wrapped nucleosome the DNA binding site is buried within the structure. However, when the nucleosome becomes transiently unwrapped, the DNA binding site is exposed. In the presence of LexA, the unwrapped nucleosome becomes 'trapped' in its unwrapped state because transcription factors generally bind target DNA sequences extremely rapidly and tightly. The unwrapped nucleosome exhibits a decreased FRET efficiency, allowing this process to be observed using stopped-flow spectroscopy by rapidly mixing the nucleosome with LexA (**Figure 4**). When there is a large excess of LexA, it can be assumed that the observed first order rate constant corresponds to the rate of unwrapping.

A value of approximately 4 s^{-1} was obtained for the first order rate constant. The equilibrium constant between wrapped and unwrapped states has previously been determined using steady-state FRET experiments (K_{eq} ~4.5*10^{-2}). This allows an estimation of the reverse rewrapping process via the relationship:

$$K_{eq} = k_1/k_{-1}$$

This gives a rewrapping rate constant of ~90 s^{-1}. These rate constants correspond to lifetimes of approximately 250 ms and 10 ms for the wrapped and unwrapped forms of the nucleosome, respectively. There is good reason to believe that the data acquired in this study are representative of nucleosomes contained in an extended chromatin structure.

References

[1] P. R. Selvin, 2000, *Nat. Struct. Biol.*, 7, 730-734.

[2] H. S. Tims and J. Widom, 2007, *Methods*, 41, 296-303.

[3] D. M. Gakamsky, et al., 2007, *Proc. Natl. Acad. Sci. USA*, 104, 16639-16644.

[4] I. Budin and J. W. Szostak, 2011, *Proc. Natl. Acad. Sci. USA*, 108, 5249-5254.

[5] M. M. Hanczyc, S. M. Fujikawa and J. W. Szostak, 2003, *Science*, 302, 618-622.

[6] G. Li, M. Levitus, C. Bustamante and J. Widom, 2005, *Nat. Struct. Mol. Biol.*, 12, 46-53.