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Advanced Microscopy: Time-Resolved Multi-Spectral Imaging of Single Biomolecules

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Advanced Microscopy: Time-Resolved Multi-Spectral Imaging of Single Biomolecules

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ABSTRACT

Over the past few years we have developed the ability to acquire images through a confocal microscope that contain, for each pixel, the simultaneous fluorescence lifetime and spectra of multiple fluorophores within that pixel. We have demonstrated that our system has the sensitivity to make these measurements on single molecules. The spectra and lifetimes of fluorophores bound to complex molecules contain a wealth of information on the conformational dynamics and local chemical environments of the molecules. However, the detailed record of spectral and temporal information our system provides from fluorophores in single molecules has not been previously available. Therefore, we have studied several fluorophores and simple fluorophore-molecule systems that are representative of the use of fluorophores in biological systems. Experiments include studies of a simple fluorescence resonance energy transfer (FRET) system, green fluorescent probe variants and quantum dots. This work is intended to provide a basis for understanding how fluorophores report on the chemistry of more complex biological molecules.

CONTENTS

Abstract	3
Contents	4
Introduction.....	5
Apparatus	6
Results.....	7
Single molecule FRET measurements on polyproline peptides	7
Single molecule studies of green fluorescent protein variants.....	12
Studies of fluorescence spectral and lifetime fluctuations in single quantum dots	14
Conclusions	17
References	19

FIGURES

Figure 1: Single molecule FRET measurement on a polyproline peptide labeled at the amino terminus with Alexa 555 (donor) and at the carboxyl terminus with Alexa 547 (acceptor)	9
Figure 2. Typical spFRET data for fluorophores on a 12-unit polyproline spacer	10
Figure 3. Single molecule FRET data for a 24-unit polyproline polypeptide showing fluctuations in the FRET efficiency	11
Figure 4. FRET efficiency histogram obtained from many 12-units polyproline molecules	12
Figure 5. Fluorescence data for a single GFP molecule excited at 488 nm.....	13
Figure 6. Histogram of the peak emission wavelength for many GFP molecules	14
Figure 7. Data from a single 655 nm quantum dot.....	16

INTRODUCTION

An important tool for the study of biological processes has long been the use of fluorescent probes that can be attached to specific constituents of biological systems. The location, distribution and transport of these chemical components can be studied by imaging the fluorescence of the attached probes. The development of probes such as green fluorescent protein (GFP), which cells can be genetically engineered to produce attached to specific sites on desired proteins, has greatly expanded the possibilities for the use of fluorescent probes in living organisms. Information about the chemical environment of fluorophore probes is contained in the detailed characteristics of their fluorescence. Chemical environments can be characterized by placing environmentally sensitive fluorophores within them and measuring the resulting fluorescence properties including spectrum, lifetime and polarization anisotropy.¹⁻⁵ Numerous probes have been developed that are sensitive to particular environmental characteristics such as pH, hydrophobicity or ion concentration. A particularly elegant example of the use of fluorescence to probe a chemical environment is fluorescence resonance energy transfer (FRET). In FRET a donor fluorophore is excited and can non-radiatively transfer energy to a nearby acceptor fluorophore. The efficiency of this energy transfer is dependent on the distance between the two fluorophores. Thus the fluorescence spectrum from this system can be used to monitor the spacing of the fluorophores and hence the distance between labeled molecules or sites on one molecule. More recently, the use of fluorescent probes has been extended to the level of single molecules where the fluorescence from one fluorophore must be detected. Studies on single molecules are valuable because they can reveal the sequence of events in biological processes.^{6,7} The microscopic sequence of events for a process is usually not evident in measurements on ensembles of molecules, because the processes in individual molecules are not synchronized with each other. Using appropriate fluorophores, changes in the local chemical environments and conformations of individual biomolecules can be monitored, in ways similar to ensemble measurements, to reveal the dynamics of complex processes.

Probe fluorophores make it possible to investigate single molecule dynamics, however many factors determine the fluorescence properties that provide information on the microscopic chemical environment. In addition to the local environment around the fluorophore, the orientation of the fluorophore in the macromolecule and relative to the excitation laser polarization, the environmental sensitivity of electronic states of the fluorophore, and energy transfer from the excited fluorophore to a nearby quencher or acceptor fluorophore all influence the characteristics of the fluorescence. Accounting for the influence of all these factors on fluorescence-based measurements is important for the application of single molecule spectroscopy to probing fluctuations in the conformations and local chemical environments around biological molecules.⁸ In bulk systems the fluorescence properties are not time dependent so they can be measured sequentially to fully characterize the fluorescence. However, to fully describe the dynamics of single biological molecules using attached fluorophores requires simultaneous, correlated measurements of the relevant fluorescence properties with the sensitivity to detect single fluorophores. The simultaneous measurement of multiple fluorescence properties is particularly important for single molecule FRET measurements. Conformational changes in complex biological molecules, including proteins or nucleic acids, can produce correspondingly complex changes in fluorescence properties of fluorophores. For example, a conformational change that alters the spacing between two fluorophores that undergo spFRET may also change the polarity around one of the fluorophores. In many cases a polarity change will shift the spectrum and

change the fluorescence lifetime of the fluorophore involved. These changes in the fluorophore characteristics will affect the FRET efficiency as well.

Recently we have developed a unique time-resolved, multispectral, confocal microscope with single molecule sensitivity that will record the wavelength (λ) and emission time relative to excitation (τ) for each detected fluorescence photon along with its absolute detection time (Δ) so that correlations among all the fluorescence properties are maintained.⁹ Fully correlated photon records of λ , τ , Δ have not been previously available with single molecule sensitivity. Here we report preliminary results from studies of several interesting biomolecular systems using this new apparatus. Systems were chosen for study that represent examples of important fluorescence probe methods for biological systems. These include single molecule FRET measurements in a simple protein, single molecule fluorescence from two variants of green fluorescent protein, and fluorescence dynamics measurements on biologically functionalized quantum dots.

APPARATUS

The time-resolved multispectral microscope that we have developed couples a confocal fluorescence microscope to a new custom photon detection system. In the confocal microscope fluorescence is excited by a laser beam focused through a high magnification objective lens to form a ~ 250 nm spot on the sample plane. A high repetition rate mode-locked laser is used as the excitation source. The fluorescence emission is collected through the same objective, and scattered excitation light is eliminated with long-pass edge filters. The fluorescence from the laser spot on the sample is collected with confocal imaging through a pinhole, which eliminates light that does not come from the focal region. An image is created by raster scanning the sample through the excitation laser focal spot. These components comprise a fairly conventional confocal microscope. The novel aspect of our apparatus is the detection system. In our system the pinhole is imaged through a dispersing optical system onto a photon-counting time- and position-sensitive detector. The fluorescence signal is spectrally dispersed across the face of the detector so the position on the detector where the photon strikes is determined by the wavelength of the photon, while the measurement of arrival time of the photon relative to the excitation laser pulse enables us to determine the emission time.

The detection system is based on a 32-anode photomultiplier tube. Custom electronics allow us to determine the illuminated anode and hence the wavelength for each detected photon at high data rates. The photon arrival time relative to the exciting laser pulse is digitized by time-to-digital converters (TDCs). The TDCs are designed for high speed data transfer directly to the memory of a data acquisition computer. The information from each individual photon is also stamped with its absolute arrival time (time-stamped) to enable studies that make use of photon arrival time statistics. Thus, for each photon, the wavelength, the emission time relative to the laser pulse, and the absolute emission time are all recorded. With our design, measurements of photon characteristics can be made and transferred to the computer at photon count rates greater than 10 MHz. Collection of many photons and histogramming of the data yields the correlated fluorescence spectrum and lifetime. With the dispersion system we currently use the spectral resolution is approximately 4 nm per anode element at 600 nm. The detector thus acquires a spectral window of ~ 160 nm within the range from 540 to 700 nm. The instrument resolution for measuring the fluorescence decay is about 150 ps. The data acquisition approach we have developed is readily applicable to new types of time- and position-sensitive detectors that are currently in development with improved detection sensitivity or time resolution.

The data from this apparatus consist of a list of photons detected. For each photon, its wavelength, emission time relative to the excitation pulse and absolute emission time are all recorded. Thus, the data contain a time history of the fluorescence properties of each individual molecule studied. To display aspects of this data arbitrary time bins can be chosen. For each time bin the intensity can be calculated from the number of photons, the fluorescence spectrum and the fluorescence lifetime are extracted by histogramming the appropriate quantities. The data are displayed as time traces (trajectories) of the single molecule fluorescence intensity, lifetime or spectral characteristics. Averaged fluorescence spectra or lifetimes can also be obtained over the entire length of the time trace.

RESULTS

Single molecule FRET measurements on polyproline peptides

Single-molecule or single-pair FRET (spFRET) is a powerful tool for studying conformational fluctuations in large molecules.¹⁰ By providing a measure of the distance between two fluorophores FRET measurements can address questions such as whether fluctuations involve transitions between quasi-stable states or continuously sample a continuum of available configurations. Time-resolved FRET measurements can determine the rate of conformation changes between states and establish the times scales for various types of conformational changes. There are many examples of the successful use of FRET to monitor single- molecule processes, but quantitative single-molecule FRET measurements are quite challenging due to the many factors that play a role in FRET efficiency.¹¹⁻¹⁶ FRET measurements determine the distance between fluorophores by measuring the energy transfer efficiency from the initially excited, donor, fluorophore to an acceptor fluorophore. The energy transfer efficiency is reflected in the relative fluorescence yields of the two fluorophores. Inherent in the usual interpretation of FRET efficiencies as distance measurements is the assumption that the spectral properties of the donor and acceptor fluorophores are not varying during the course of the measurement. However, in complex systems the local environment around fluorophores may fluctuate or change as the conformation changes and this leads to changes in the fluorescence characteristics of the individual fluorophores. Since the FRET efficiency depends on the spectral overlap between donor emission and acceptor absorption, changes in these individual spectra will also cause changes in FRET efficiency that are not related to distance changes. Changes in quenching, resulting in fluorescence lifetime changes, will also affect the measured FRET efficiency. Thus, there are several sources of fluctuations in measurements of FRET efficiency that do not correlate with distance changes between the fluorophores. The critical importance of understanding these variations in spectra, lifetime, and quantum yields for FRET is well recognized.^{8, 12, 17} These effects become increasingly important in practical applications in biological systems where fluorophores can encounter a wider range of local environments. Our new detection system provides a unique opportunity to quantify fluorophore spectral and lifetime fluctuations, and to determine their influence on model spFRET systems. The detection system can dynamically monitor the correlated spectra and lifetimes of the fluorophores in single FRET pairs. Thus, the photophysics of the individual fluorophores and the FRET efficiency are simultaneously probed. In this way, changes in the FRET efficiency resulting from macromolecule dynamics that change the fluorophore spacing can be separated from variations in the fluorescence due changes in the local environments of the fluorophores.

In collaboration with Prof. Haw Yang, at University of California, Berkeley we have studied spFRET using short polyproline peptides with 12 and 24 units as molecular spacers between donor and acceptor fluorophores. Polyproline has been assumed to be rigid, and was used as a spacer by Stryer and Haugland in their original work to check the dependence of the FRET efficiency on the donor-acceptor distance.¹⁸

The peptide was synthesized with a carboxyl-terminal cysteine residue which reacts with the maleimide derivative of the acceptor dye (Alexa 647, Invitrogen). The donor dye was attached to the N-terminus using the succinimidyl ester derivative of the donor dye (Alexa 555, Invitrogen). The labeling and the purification of the doubly labeled peptide were done according to the standard protocols. At the C-terminus, the synthesized peptide also contains a biotin molecule, which provides a strong attachment to a streptavidin-functionalized substrate. The streptavidin-functionalized substrate was prepared as follows. Plasma-etched coverslips were silanized and then activated with a polyethyleneglycol (PEG) mixture of 1:100 of PEG-biotin and PEG-SPA in 0.1 M NaHCO₃ for 3 hours. The coverslips were incubated for 10 minutes with a 0.2 mg/ml streptavidin (Invitrogen) solution in PBS buffer, and then incubated with 50 pM labeled polyproline in PBS for 10 minutes. Several washes with nanopure water were performed in between the different steps.

The sample is excited at 532 nm with 7 ps pulses from a mode-locked laser. Correlated fluorescence emission spectra and decays for spFRET are detected. Figure 1a shows the fluorescence intensity time trace of a spFRET on a polyproline peptide containing 24 proline residues. The sudden decrease of the signal after ~250 ms to background level (step photobleaching) is indicative of a single molecule intensity trajectory. The correlated fluorescence emission and decay spectra for the spFRET are shown in Figures 1b-c, respectively. The fluorescence emission spectrum has two clearly separated peaks around 565 nm and 670 nm. Without the use of any bandpass filters, fluorescence properties can be analyzed after acquisition according to the actual emission characteristics of the donor and the acceptor. In this case, we chose 627 nm as the delimiting emission wavelength between the donor, to the blue, and the acceptor, to the red. Since the emission time of every photon is also recorded simultaneously with the emission wavelength, the decay traces directly correlating to the two different emission spectrum features can easily be determined. The decay traces for the donor and for the acceptor are represented by blue and red curves in Figure 6c, respectively. The fluorescence lifetimes obtained by mono-exponential fitting the decay curves are markedly different, namely 270 ps for the donor and 1.29 ns for the acceptor. The 60% FRET efficiency for this spFRET system, which was computed based on the above spectrum split, is in good agreement with the theoretical value computed based on molecular parameters of the polyproline and the Förster radius of the donor-acceptor pair. To our knowledge, this is the first measurement of the correlated spectrum and decay associated with a FRET process at the single-molecule level. From the data one can construct, post acquisition, any combination of time-gated spectra or wavelength-gated decays and extend the powerful techniques of photon statistics to gain additional insights into the system.

Figure 1: Single molecule FRET measurement on a polyproline peptide labeled at the amino terminus with Alexa 555 (donor) and at the carboxyl terminus with Alexa 547 (acceptor). a) The total intensity trajectory using donor excitation at 532 nm. b) The emission spectrum corresponding to the first 250 ms of the trajectory. The donor contribution is shown in blue, the acceptor in red. c) The fluorescence decays and their mono-exponential fits (black curves) for the two spectral features: in blue, donor = 0.27 ± 0.03 ns, and in red, acceptor = 1.29 ± 0.18 ns.

The fluorescence intensity trace (trajectory) for most single molecules is more complex. Typically the acceptor fluorophore bleaches first so there is a time interval where FRET is observed followed by a period where only donor fluorescence is observed. An example is shown in Figure 2 for a polypeptide with 12 proline units. The overall intensity trace has an initial time period where FRET occurs with an efficiency of $\sim 80\%$, up to a time of ~ 0.5 s. The FRET efficiency is determined from the fluorescence spectrum (not shown) as discussed for Figure 1. After 0.5 s the acceptor photobleaches and only donor emission is observed. The donor fluorophore shows several intervals of blinking when its emission goes to zero. The fluorescence lifetime of the donor also shows fluctuations reflecting changes in its environment. It is important to monitor the fluorophore lifetimes because they affect the calculation of FRET efficiency and hence the determination of the spacing between fluorophores.

Figure 2. Typical spFRET data for fluorophores on a 12-unit polyproline spacer. The overall fluorescence intensity time trace is shown in the upper left plot. In the plot at the lower left the FRET efficiency derived from the spectrum is plotted as a function of time along with the fluorescence intensities of the donor and acceptor individually. Note the acceptor bleaches (intensity drops to background) at ~ 0.5 s accompanied by an increase in the donor fluorescence. Note the variations in the fluorescence lifetimes for the color-coded intervals in the intensity trace that correspond to different FRET and donor emission characteristics.

Short polyproline polypeptides are expected to be relatively rigid spacers between the fluorophores, however we observe changes in FRET efficiency with time for one molecule and differences in FRET efficiency from molecule to molecule. While this observation could indicate conformational changes such as isomerization of the polyproline, the conformational fluctuations of this small protein are expected to be rapid and thus would not be expected to yield different FRET efficiencies for different molecules. Our experimental approach allows us to explore other possibilities for changes in the calculated FRET efficiencies. Figure 3 shows an example of a single molecule FRET trajectory that has rapid changes in the FRET efficiency. The plot in the upper left shows the time traces of the fluorescence intensities of the donor and acceptor separately. The donor and acceptor emission intensities are separated using the fluorescence spectrum as discussed for Fig 1. The time traces show anticorrelated changes in the emission intensities of the fluorophores which are reflected in the FRET efficiency plot at the lower left of Figure 3. On the right of Fig. 3 is a histogram of the FRET efficiency over 10 ms time intervals. This plot shows a bimodal distribution of efficiencies suggesting that there are two configurations of the FRET system. One explanation for the changes in FRET efficiency is that the length of the spacer changes, perhaps due to isomerization. However, it is important to consider whether other effects may be causing the changes in FRET efficiency.

Figure 3. Single molecule FRET data for a 24-unit polyproline polypeptide showing fluctuations in the FRET efficiency. At upper left is a plot of the intensity time traces for the donor and acceptor fluorophores. The lower left plot shows the FRET efficiency time trace. Note changes in FRET efficiency that correspond to anticorrelated changes in the emission intensities of the donor and acceptor fluorescence intensities. On the right is a histogram of the FRET efficiency over 10 ms intervals.

Figure 4 shows a similar FRET efficiency histogram only in this case derived from many 12-unit polyproline molecules. This histogram also shows contributions from two different FRET efficiency distributions. Further analysis of the contributions to the lower FRET efficiency distribution found that the intrinsic donor lifetime was shorter for this distribution. Thus an environmental effect on the donor was responsible for the lower calculated FRET efficiency rather than a difference in the length of the spacer polypeptide. One possible explanation of this shorter lifetime is interaction of the donor fluorophore with the immobilizing surface.

This work shows the importance of our multiparameter measurement technique for accurate spFRET measurements. With our technique the fluorophore fluorescence characteristics, including lifetime and spectra, that determine FRET efficiency are continuously recorded. Thus, environmental effects on the fluorophores can be taken into account so that fluorophore separation, which is the quantity usually of interest, can be accurately determined. Work on fully characterizing the polyproline spFRET system is ongoing because it has become an important prototype system for quantitative spFRET measurements.

Figure 4. FRET efficiency histogram obtained from many 12-units polyproline molecules.

Single molecule studies of green fluorescent protein variants

Fluorescent proteins are often used in studies of cellular processes because they can be introduced into cell proteins through genetic mutation. We have studied two variants, green fluorescent protein (GFP) and yellow fluorescent protein (YFP). These proteins have multiple ground state configurations with different fluorescence characteristics. Understanding the variations in fluorescence characteristics is important because they can provide information on the chemical environment around the fluorescent protein, but they can also interfere with FRET measurements that use fluorescent proteins.¹⁹ We have measured the time variations in the fluorescence spectra and lifetimes of many individual GFP and YFP molecules. This data will provide better understanding of the mechanisms and time scales for transitions between the ground state configurations of GFP and YFP and determine the fluorescence properties of specific configurations.

For our studies of GFP and YFP highly dilute solutions of the fluorophores were spin coated onto coverslips in a pH buffered polyvinyl alcohol (PVA) solution. This immobilizes the proteins in an environment that is similar to aqueous. The samples are excited with light pulses at 488 nm from a doubled, mode-locked Ti:Sapphire laser.

An example of the data obtained from a single molecule of GFP is shown in Figure 5. We have taken data of this type for many different single GFP molecules. Our studies so far do not show a great deal of temporal variation in the fluorescence spectrum of GFP. This suggests that it does not readily change between its ground state configurations on the time scale of our experiments. However, there is a substantial variation in the fluorescence spectrum from molecule to molecule. This spectral variation can result from different GFP molecules being in different ground state configurations that do not interconvert rapidly or it can result from differences in the local environment for GFP molecules located in inequivalent sites in the PVA matrix. The data for many GFP molecules are shown in Figure 6. There is a very wide range of spectral peaks. The maximum in the histogram of Figure 6 corresponds to the "normal" emission of GFP around 510 nm.

There are hints of secondary maxima at ~ 530 nm and 550 nm. This is consistent with the presence of different GFP ground states, but further experiments are needed to confirm this. The time independence of the spectral properties shows that light induced photophysics are not responsible for the wide variation in peak emission wavelengths, but rather there is a heterogeneous distribution of GFP molecules either in different initial states or local environments. Experiments in different environments, such as liposomes will investigate this question.

Figure 5. Fluorescence data for a single GFP molecule excited at 488 nm. At the upper left is a plot of the intensity time trace. Note the repeated blinking of the molecule with different periods. The lower left plot is the time trace of the maximum of the emission spectrum. On the upper right is a histogram of the emission spectrum maximum over 100 ms increments. The plot on the lower right shows the average spectrum from the time the molecule is first illuminated until it photobleaches.

Figure 6. Histogram of the peak emission wavelength for many GFP molecules.

Similar experiments have been performed for YFP. As with GFP, the YFP molecules do not show much variation in their spectra with time over the interval of observation before photobleaching. The histograms of the emission spectra maxima for many molecules are strongly peaked, but the actual wavelength of the peak varied from sample to sample. Further work on the GFP and YFP samples is ongoing to explore the questions raised by these initial experiments.

Studies of fluorescence spectral and lifetime fluctuations in single quantum dots

Quantum dots are becoming increasingly important as fluorophores for biological studies because of their tremendous resistance to photobleaching. However, quantum dots exhibit some nonideal behaviors, the best known of which is blinking, or having their fluorescence turn on and off. Quantum dots can also show large fluctuations in their fluorescence intensity as well as fluctuations in their fluorescence spectrum. We are measuring the correlation between changes in fluorescence intensity, lifetime and spectrum of individual quantum dots. The purpose of these studies is to determine what characteristics of the quantum dots are responsible for their non-ideal optical behavior.

In collaboration with Prof. Haw Yang, University of California, Berkeley we have begun studying correlated fluorescence spectral and lifetime fluctuations in commercially available, biologically functionalized quantum dots. We have chosen 655 nm emitting quantum dots (Quantum Dot Corporation) for primary study because they are the largest core CdSe quantum dots available and are thus most likely to show nonideal behavior. The quantum dots are immobilized on a PEG-biotin coverslip similar to that used for polyproline FRET studies discussed above. Plasma-etched coverslips were silanized and then activated with a PEG mixture of 1:100 of PEG-biotin and PEG-SPA (Nektar Therapeutics) in 0.1 M NaHCO₃ for 3 hours. The 655 nm quantum dots have a streptavidin coated surface so they adhere to the biotin functionalized PEG. The experiments are performed with the immobilized quantum dots in an aqueous buffer solution. The sample is excited with 7 ps laser pulses at 532 nm. The excitation intensity is kept very low (~200 nW) to minimize the effect of the laser excitation on the sample.

Typical data from a 655 nm quantum dot are shown in Figure 7. All three time traces are derived from a single record of photons from which we have extracted the fluorescence intensity, the fluorescence lifetime and the spectral mean. The results show the unique capability of our apparatus to make simultaneous correlated measurements of fluorescence spectra and lifetimes from single particles. The fluorescence intensity trace shows large fluctuations with occasional blinking where the fluorescence temporarily ceases. All the 655 nm quantum dots observed exhibit large intensity fluctuations although the pattern of the fluctuations varies widely. Comparing the top two plots in Fig. 7 it is clear that the fluorescence intensity and lifetime are almost perfectly correlated because the two traces are very similar. This result shows that the quenching mechanism that controls the fluorescence lifetime is responsible for the intensity fluctuations. On the other hand the spectral mean fluctuates over a fairly wide range but is not so obviously correlated to the intensity or the lifetime. This result is somewhat of a surprise because it was expected that different emitting states that result in such drastic changes in the fluorescence intensity and lifetime would also have different spectra, but the spectral fluctuations appear to be influenced by other factors. Determining the relation between fluctuations in the different fluorescence properties of the quantum dots will require detailed analysis of the data we have taken for a large number of quantum dots. This work is ongoing.

Figure 7. Data from a single 655 nm quantum dot. The top plot is an intensity time trace the middle plot is a time trace of the fluorescence lifetime and the bottom plot is a time trace of the spectral mean.

CONCLUSIONS

This report describes initial results from a simultaneous time- and wavelength-resolved, single-molecule, confocal microscope system on a number of molecular systems of biological interest. In these experiments the simultaneous measurement of the fluorescence wavelength and emission time for individual detected photons reveals correlations between the fluorescence intensity, wavelength, and lifetime. This method is well suited for studying dynamics of processes that change the local environment of fluorophores and hence their fluorescence characteristics. It will enable new studies at the single molecule level that take advantage of the detailed information on the fluorescence characteristics of probe fluorophores to monitor the progress of biological processes. Perhaps the most obvious application of this method is in studies of spFRET where the fluorescence spectra and fluorophore lifetimes are inherently correlated by the dynamics of the energy transfer process. We have shown here results from spFRET experiments. We have also shown initial results of experiments to characterize other fluorophores important for probing biological systems.

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