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Multi-Spectral Detection of Microfluidic Separation Products

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Multi-Spectral Detection of Microfluidic Separation Products

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Abstract

The objectives of this project were to develop a new scientific tool for studies of chemical processes at the single molecule level, and to provide enhanced capabilities for multiplexed, ultrasensitive separations and immunoassays. We have combined microfluidic separation techniques with our newly developed technology for spectrally and temporally resolved detection of single molecules. The detection of individual molecules can reveal fluctuations in molecular conformations, which are obscured in ensemble measurements, and allows detailed studies of reaction kinetics such as ligand or antibody binding. Detection near the single molecule level also enables the use of correlation techniques to extract information, such as diffusion rates, from the fluorescence signal. The micro-fluidic technology offers unprecedented control of the chemical environment and flow conditions, and affords the unique opportunity to study biomolecules without immobilization. For analytical separations, the fluorescence lifetime and spectral resolution of the detection makes it possible to use multiple parameters for identification of separation products to improve the certainty of identification. We have successfully developed a system that can measure fluorescence spectra, lifetimes and diffusion constants of the components of mixtures separated in a microfluidic electrophoresis chip.

Contents

Introduction and Background

This project had two; related goals, first, to develop a unique scientific tool for studies of chemical processes at the single molecule level, and second, to provide enhanced capabilities for multiplexed, ultrasensitive separations and immunoassays. Measurements on single molecules can reveal the heterogeneties in chemical processes, which are important for understanding detailed mechanisms of the chemistry of large molecules, particularly biological molecules. Micro-fluidic technology offers unprecedented control of the chemical environment and flow conditions, and affords the unique opportunity for single molecule studies of biomolecules without immobilization. In single molecule studies, molecular processes are revealed through their effects on the fluorescence properties of fluorophores. We have developed a detection system for a confocal microscope that simultaneously records fluorescence spectra and lifetimes. This detection system has been enhanced under this project to provide a continuous record of the emission times and properties of each detected fluorescence photon. We have combined microfluidic separation techniques with the lifetime, wavelength and fluorescence correlation measurements made possible with this detection system to provide multiple methods for differentiating microfluidic separation products. This approach provides a unique capability for microfluidic analysis.

The field of single molecule studies is rapidly expanding, largely due to its potential for detailed studies of the chemistry of biological processes. For example, the reactivity or function of a biomolecule can vary from place to place within a cell. These variations in properties between molecules and the dynamics of their reactivity changes are often obscured due to averaging in measurements on ensembles of molecules. The promise of single molecule studies is that they can reveal the detailed sequence of events involved in the reactions of complex molecules and measure the differences in properties between molecules. A critical problem in single molecule studies is the difficultly in making measurements without perturbing the molecule's activity, due to the need to immobilize the molecules. An important component of this research was to develop microfluidic methods for single molecule studies that do not require immobilization of the molecules of interest. With these techniques we can also study complexes of molecules from mixtures that we isolate by electrophoresis without the need for lengthy and likely destructive separation processes.

This research also has significance for development of analytical methods. Using the combination of micro-fluidic devices and our time-resolved multispectral detection system with single molecule sensitivity we have developed new methods for detection and identification of analytes in micro-fluidic devices. Most current microfluidic analysis schemes identify analytes based on a single measured property such as elution time in an electrophoretic separation. The capabilities developed in this work for simultaneous detection of multiple fluorescence properties can enhance the certainty in identification of analytes and single molecule detection takes sensitivity to a new level. In addition the time stamped detection method described below makes it possible to use fluorescence correlation techniques to measure quantities such as diffusion rates for the analytes. The methods we have developed should impact many application areas for micro-fluidic reaction and separation systems.

Instrumentation Development

This research relies on a multi-spectral fluorescence detection system we designed previously. The time-resolved multispectral microscope that we developed couples a confocal fluorescence microscope to a 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. 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. With the dispersion system we currently use the spectral resolution is approximately 5 nm per anode element. The detector thus acquires a spectral window of ~160 nm within the range from 400 to 750 nm. The instrument resolution for measuring the photon emission time relative to the excitation laser pulse is about 200 ps.

For this research we have enhanced the data acquisition system by adding the capability for recording the absolute detection time for each photon, known as time stamping. Thus, for each photon, the wavelength, the emission time relative to the laser pulse, and the absolute emission time are all recorded.[1] Collection of the data in this manner is very powerful because it maintains correlations between the measured photon properties including spectral and lifetime correlations as well as photon-to-photon temporal correlations. The data can be histogrammed in many ways to display correlated fluorescence spectrum and lifetime, or time histories of the intensity spectrum or lifetimes. In addition, fluorescence correlation spectroscopy[2] (FCS) analysis can be performed on the same data to determine diffusion rates and detect binding. The data collection approach is shown schematically in Figure 1. For this project software for photon-by-photon calculation of auto-and cross-correlation calculations was developed that provides for very rapid calculation of the correlation functions.

The time stamping of the data for this project was implemented using Xilinx complex programmable logic devices (CPLDs). The absolute time is determined from a 32-bit counter clocked at 20 MHz, synthesized into a CPLD. The arrival of a photon latches the current state of the counter. This value is interleaved with the TDC values for the photon that determine the wavelength and emission time relative to the laser excitation. These data are continuously being stored into the data acquisition computer via direct memory access (DMA) through a commercial PCI data acquisition card. The data acquisition approach we have developed is readily applicable

to other detector types. We frequently used with multiple single channel detectors such as avalanche photodiodes (APDs).

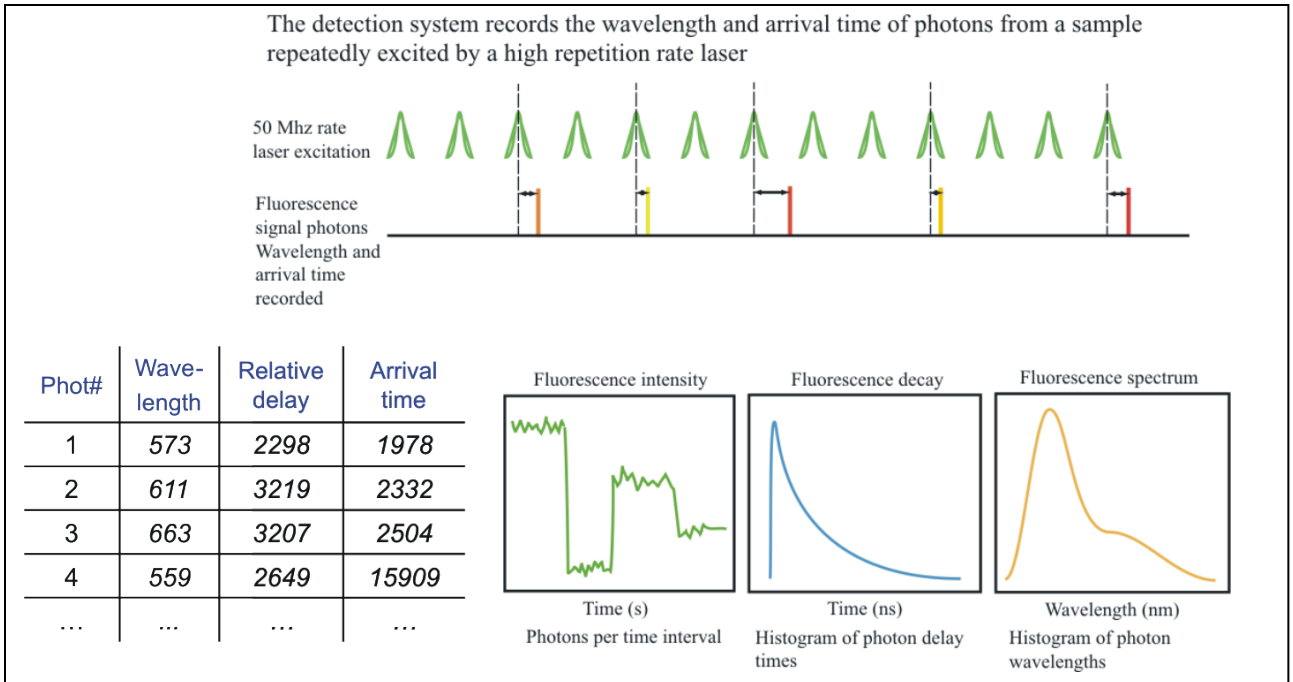

Figure 1. Data collection. The sample is excited by a high repetition rate pulsed laser. Eacg time a fluorescence photon is detected its wavelength, delay relative to the excitation pulse and its absolute arrival time is recorded. Thus, the data consist of a list of photons tagged with their associated information. This data can be histogrammed in many ways after acquisition, and also used to correlation analysis.

Both single molecule detection and FCS methods require high fluorescence collection efficiency. In our microscope, collection efficiency is optimized by using high numerical aperture oil immersion microscope objectives for excitation and light collection. These objectives are designed to image through 170 micron thick microscope coverslips. Thus to use these objectives with microfluidic chips we designed and had fabricated custom chips with thin bases to image through.

The versions of the microfluidic chips designed for electrophoretic separations make use of intersecting channels, one for sample introduction and the second for separation. In operation, using the Sandia designed μChemLab computer programmable power supply, a voltage is first applied between reservoirs connected by the sample introduction channel. Once this channel is filled with the mixture to be analyzed a voltage is applied to the intersecting separation channel in which the electrophoresis analysis is performed.

Results

Streptavidin interaction with tetramethylrhodamine (TMR) biocytin

The effect of interactions with proteins on the fluorescence characteristics of fluorophores is fundamentally important to understand for many applications of single molecule spectroscopy. This is a particularly appropriate problem to address with our new capability to simultaneously measure multiple fluorescence properties from single fluorophores. We have chosen to investigate the interaction between the streptavidin protein and the TMR fluorophore. Streptavidin is a 53 kDa molecular weight protein that has an extremely strong binding affinity for its ligand, biotin (244 Da). Biotin can bind in any of four equivalent deep pockets in the streptavidin molecule. The strong binding of biotin by streptavidin has numerous applications in biotechnology and is the basis of many affinity assay techniques. The biotin binds in a deep hydrophobic pocket so the polarity of the environment around a fluorophore linked to biotin should be markedly different depending on its location relative to the binding pocket. There are also tryptophan residues in the binding pocket that can cause fluorophore quenching.[3]

The TMR fluorophore is widely used as a donor in FRET systems, and its high quantum yield makes it important for single molecule studies. It is chosen for these studies because its fluorescence is substantially quenched when it is bound to streptavidin using biotin with a short linker between the TMR and biotin. Quenching is a commonly observed phenomenon when fluorophores are conjugated close to some amino acids or nucleic acids. Our experiments using TMR/biotin attached to streptavidin are probing the mechanism and range of the quenching interaction by measuring whether the degree of quenching is essentially static or undergoes sudden changes as the protein environment around the TMR fluctuates. This type of information is only available through single molecule studies.

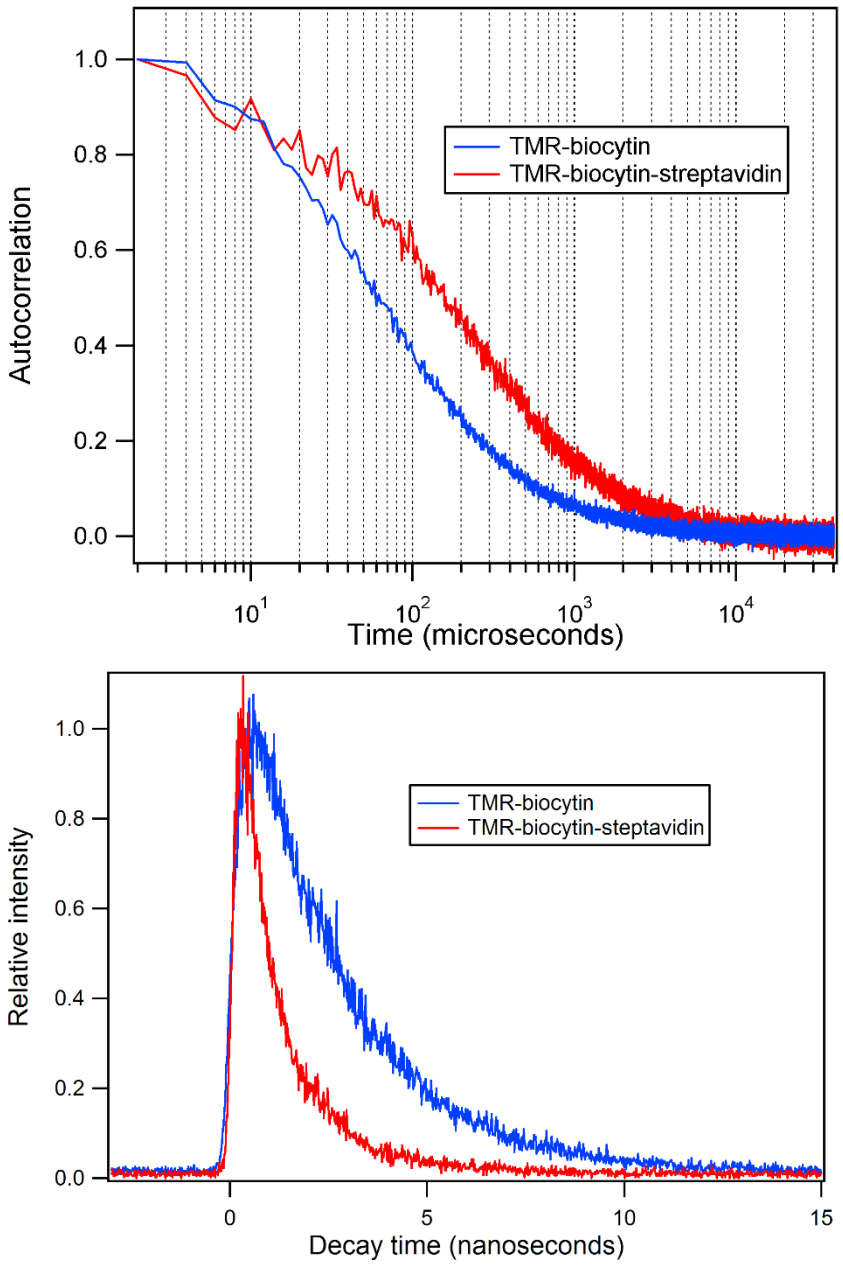

Figure 2. Streptavidin-TMR biocytin binding. The top panel shows FCS autocorrelation curves that illustrate the increase in the diffusion time of the TMR fluorophore when TMR biocytin binds to streptavidin. The bottom panel shows the decrease in the TMR fluorescence lifetime when TMR biocytin is bound by

For our experiments we used TMR biocytin as the labeled biotin ligand for streptavidin. Initial experiments used fluorescence correlation spectroscopy to verify the binding of the labeled ligand to the protein. The intensity autocorrelation of the small ligand shows its rapid diffusion. With the addition of the streptavidin protein the ligand binds quantitatively and the diffusion time of the labeled species increases accordingly. The fluorescence decay curves can be extracted from the same data records and show a marked decrease in the fluorescence lifetime of the TMR due to quenching by the protein. These results are shown in Figure 2. To understand this quenching mechanism on a molecular level it is important to ask whether the quenching is a static process that is the same for all molecules at all times or whether the fluorophore quenching varies as the dye molecule explores different environments on the protein. We examined the fluorophore quenching at the single molecule level initially in a very dilute ~50 pM solution containing the streptavidin TMR biocytin complex. At this low concentration individual molecules are observed as they diffuse through the focal spot of the excitation laser by the burst of fluorescence photons they emit. For each burst of photons

corresponding to a single molecule we determined the fluorescence lifetime. A very broad distribution of lifetimes was determined from these measurements. This suggests that on the millisecond diffusion time scale different protein-fluorophore complexes exhibit different lifetimes and hence quenching efficiencies. However, since the number of photons obtained from each molecule is small, the uncertainty in the lifetime determination is great. Therefore we also examined the quenching behavior by immobilizing the complexes on a polyethylene glycol (PEG) surface. In these experiments individual molecules are located by raster scanning the surface and then they are probed by illuminating them and recording the fluorescence over a several second time scale. A sample result is shown in Figure 3. These results show a complex quenching behavior effecting both the TMR fluorescence spectrum and lifetime. Further studies of this process are ongoing under outside funding.

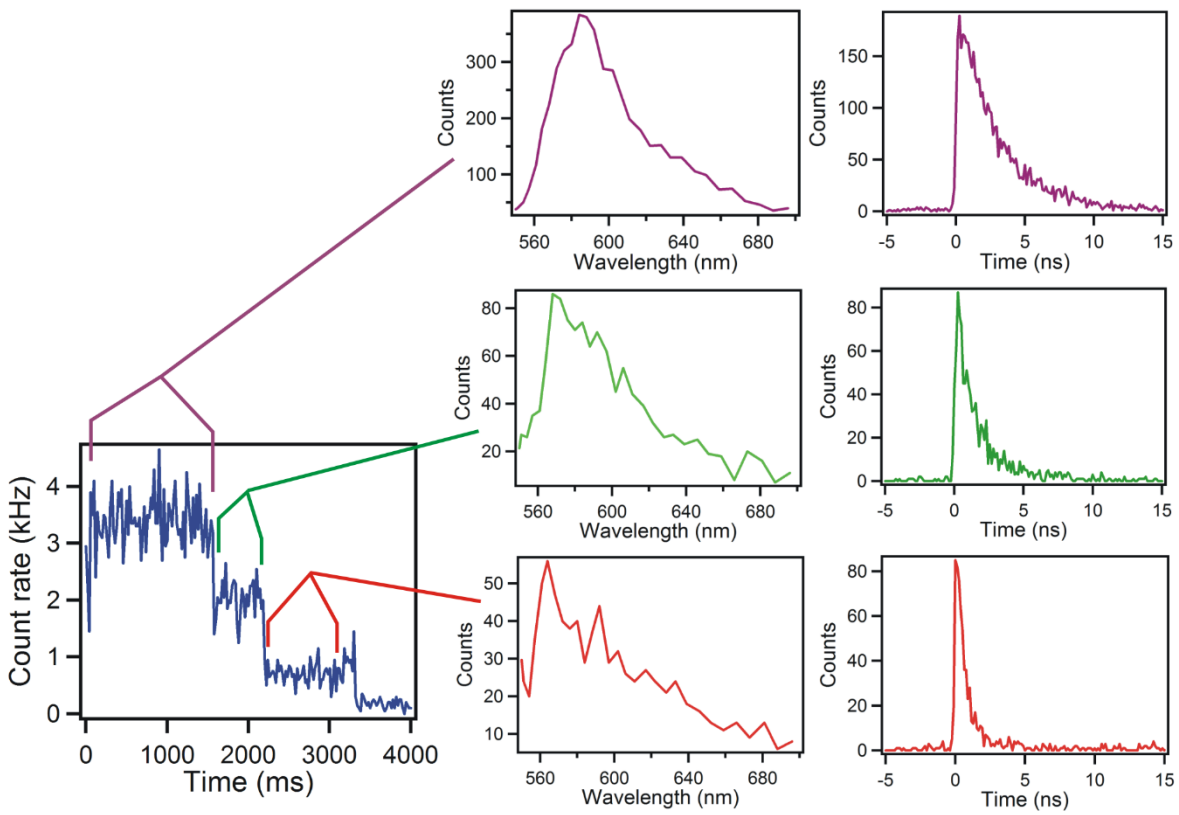

Figure 3. Results on TMR biocytin bound to streptavidin immobilized on a PEG surface. On the left is an intensity time trace of the TMR fluorescence. The fluorescence intensity shows discrete steps. On the right are fluorescence spectra and lifetimes for each intensity step. The decreasing intensity correlates with a shift of the fluorescence spectrum to the blue and a decreasing fluorescence lifetime (increased quenching).

Fluorescence resonance energy transfer (FRET) in single quantum dot-dye hybrids.

Fluorescence based probes can be used to sense many properties such as pH, ion concentration and binding in complex chemical and biological systems. FRET between donor and acceptor fluorophores is a sensitive detection mechanism for these probes if the separation

between the fluorophores can be designed to depend on the quantity of interest. As donor fluorophores, semiconductor quantum dots (QD) offer significant advantages over fluorescent dyes including, superior photostability, broad absorption, and narrow, size-tunable emission. For assays in microfluidic systems QD labels offer particular advantages because the overlapping absorption of QDs with distinct, narrow emission spectra allow for multiplexed detection of

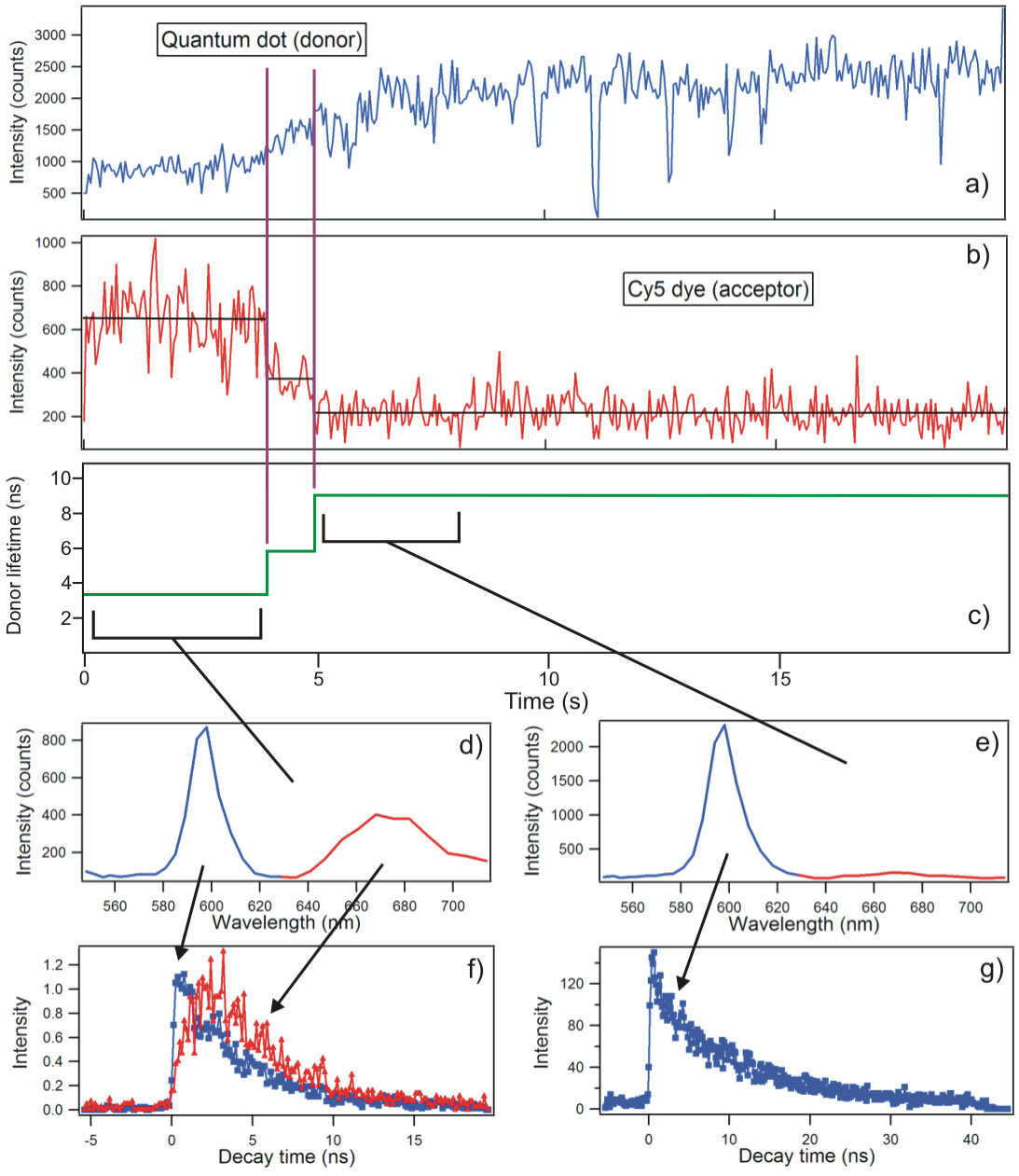

Figure 4. Data from a single QD-dye hybrid. Panels (a) and (b) show intensity traces with time for the QD and dye channels respectively. At short times the energy transfer is efficient so the QD intensity in a) is low and the dye intensity b) is high. Trace c) is the donor lifetime. FRET spectra are shown in d) and e) with fluorescence transients before and after acceptor photobleaching in f) and g). The transients in f) show the time-resolved energy transfer from initially excited donor (blue) to acceptor (red).

multiple labeled species. The bright emission with narrow spectra also facilitates the identification of the label even in rapid flows with short observation times. QD-organic dye hybrids are becoming especially popular for FRET-based sensing.[4-7] We have studied some fundamental characteristics of QD-organic dye hybrids in the process of evaluating their suitability for assays in microfluidic devices.

For most purposes where the use of QD based FRET sensors is anticipated multiple acceptors are bound to a single QD hub that serves as the sole energy donor. A major obstacle to single-particle measurements with these QD-multiple-acceptor systems is that the FRET signal must be distinguished from acceptor dye photo-bleaching events, flickering emission caused by the QD donor blinking, and direct excitation of the acceptors. To study the important photodynamics we have worked with relatively simple assemblies immobilized for long time measurements. By measuring changes of multiple spectral parameters synchronized with emission intensity jumps, we are developing methods to identify events with high confidence even in these noisy situations.

We assemble QD-dye hybrids from biotin conjugated, 605nm QDs, bound to streptavidin labeled with Cy5 dye. Our time-resolved, multi-spectral microscope measures the wavelength, emission delay relative to excitation (excited-state lifetime), and chronological time (intensity) for each fluorescence photon detected from individual hybrid particles. The initially excited QD donor emission at ~605nm is readily distinguished from the acceptor dye emission around 675nm in the spectrally resolved data.

Typical data from a single hybrid are shown in Figure 4. In this example dye photobleaching is the event to be detected. The acceptor intensity traces are analyzed using a changepoint method.[8] This model-free statistical analysis quantitatively determines the emission intensity changes identifying stepwise decreases (vertical lines) in the acceptor emission due to individual dye photobleaching. This assignment is confirmed by synchronized increases in the donor lifetime, Fig. 4c, due to reduced acceptor quenching. The emission spectrum, Fig. 4d shows initial strong FRET, but after ~5 seconds the FRET and hence red emission disappears due to photobleaching of the acceptor dyes (Fig. 4e). The time evolution of the QD and dye spectral components, Fig.4f,g directly illustrates the energy transfer process. Before dye photo-bleaching, the donor lifetime is quenched due to energy transfer to the acceptor. The acceptor emission exhibits a slower rise because it is excited by energy transfer from the donor. In addition, the decay of the acceptor emission is prolonged from its ~1.4 ns intrinsic lifetime to match the donor lifetime, because energy transfer can occur throughout the longer donor lifetime (Fig. 4f). The form of the acceptor emission transient demonstrates that the acceptor emission is from FRET and not direct acceptor excitation. Once the dyes photobleach the QD lifetime increases as it is no longer quenched (Fig.4g). The results of this work provide a robust approach for using QD-dye hybrids in single particle applications.[9,10]

Multiparameter fluorescence measurements of analytes in microfluidic separations.

A primary purpose of this project was to develop new detection capabilities for microfluidic separations that include fluorescence spectral and lifetime measurement with improved sensitivity. We have been developing our detection scheme using labeled tetanus toxin C (TTC) fragment and its antibody. Figure 5 shows some initial results that demonstrate

fluorescence spectral and lifetime measurements in a microfluidic separation at ~1nM injection concentration. These results were obtained with a 40X, 0.7 NA objective and demonstrated the feasibility of our approach.

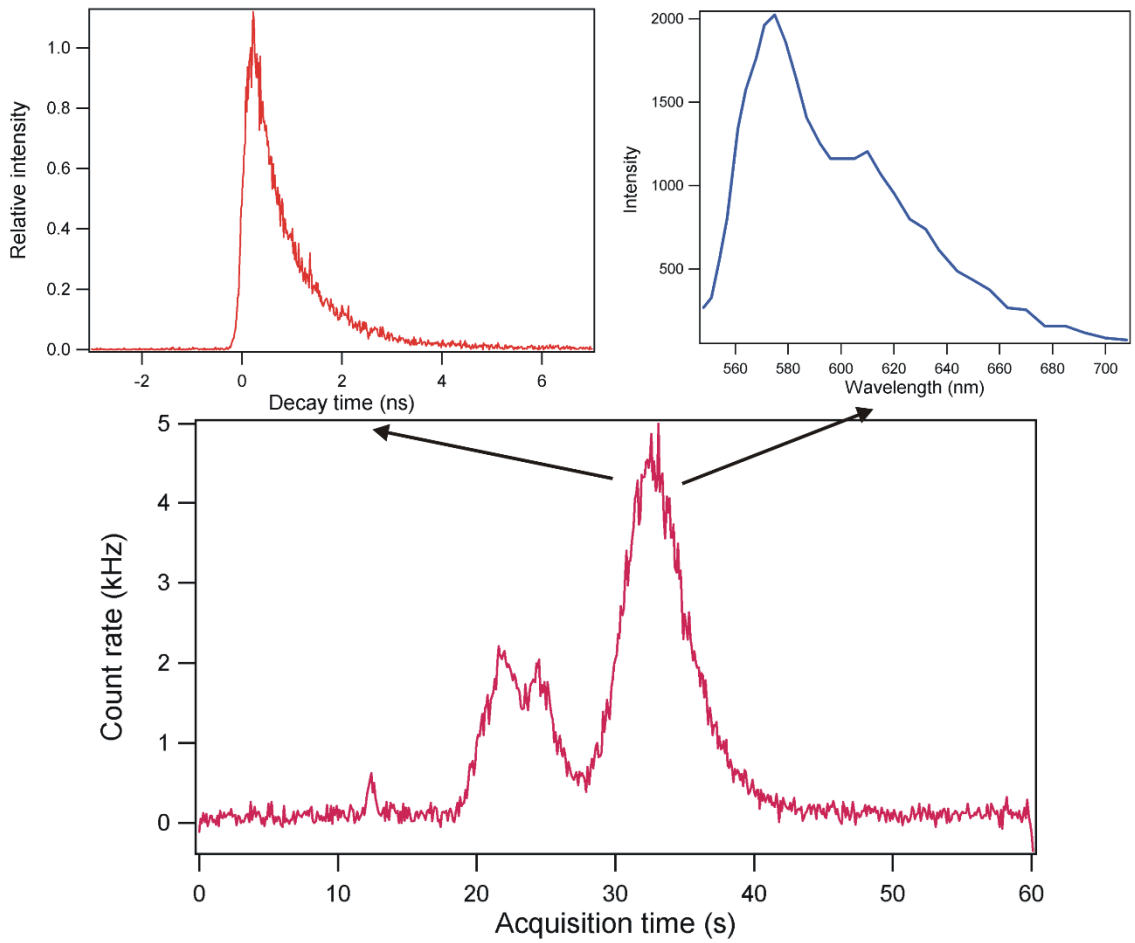

Figure 5. Separation of labeled TTC from its antibody complex on a microfluidic chip by electrophoresis. The large peak at shorter acquisition time is the TTC fragment split due to different numbers of labels. The large peak at longer times is the TTC-antibody complex. The simultaneously acquired spectrum and lifetime of fluorescence from the larger peak are shown.

To enable the full range of measurements possible with our detection system in the microfluidic channel required modifications of the standard microfluidic chips commonly used at Sandia. In particular it was necessary to design microfluidic chips with 170 micron thick bases so that high numerical aperture oil immersion microscope objectives could be used to excite the sample and collect fluorescence. With the high collection efficiency of these objectives we can obtain excellent quality fluorescence correlation measurements inside the channel. Thus, we are now able to separate components in a sample by electrophoresis, measure the spectrum and spectrally resolved fluorescence lifetime of each component and also use fluorescence correlation spectroscopy to measure diffusion constants of the components.

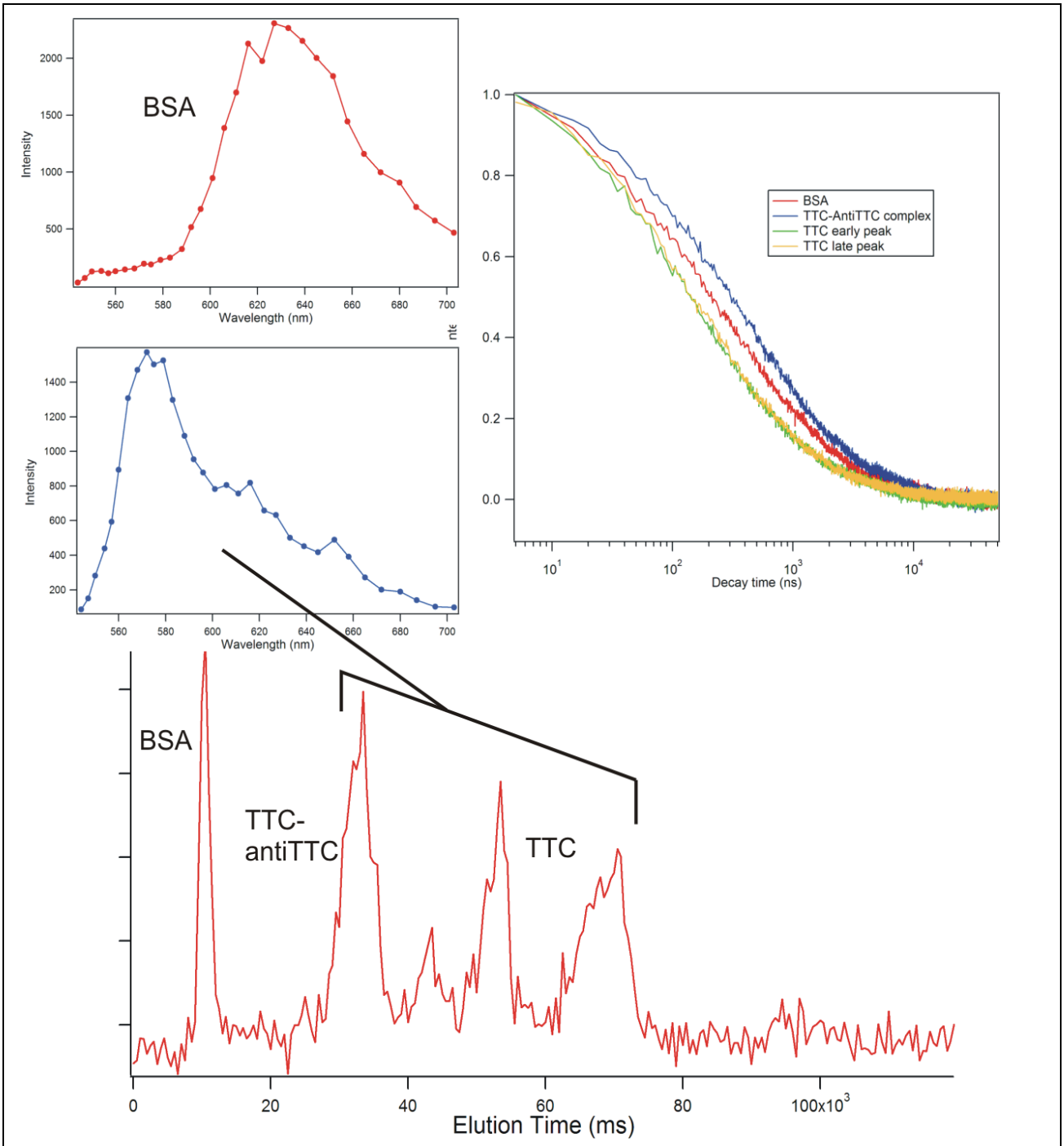

Figure 6. Electrophoresis results. The bottom plot shows the fluorescence intensity as a function of elution time for the BSA, TTC, anti-TTC mixture. The BSA spectrum corresponds to Alexa Fluor 594 while all of the TTC containing species show the Cy3 spectrum. The fluorescence correlation curves at the top right distinguish the slowly diffusing antibody complex from the TTC. Different charge species of TTC show identical diffusion times.

We have developed this capability with mixtures of bovine serum albumen (BSA), tetanus toxin C (TTC) fragment and the TTC antibody. The BSA is commercially labeled with

Alexa Fluor 594 (Invitrogen). The TTC (Roche Applied Science) is a recombinant type and thus non-toxic. We labeled the TTC with Cy3 maleimide dye (GE Health Sciences). As the TTC has four cysteines the labeling with the maleimide modified dye results in a distribution of labeled TTC with different degrees of labeling. The mixture of BSA, TTC and the anti-TTC in approximately ten fold excess is placed in the sample reservoir of the separation chip. Voltage is applied to the sample introduction channel until it is filled. The separation voltage is then applied across the separation channel. The separations were done in open as opposed the gel filled channels. The detection system can record the fluorescence spectrum and of each species detected. A great advantage of the method is that the separation voltage can be turned off to stop the flow giving time to obtain fluorescence correlation data with excellent signal to noise. Another interesting feature of the approach is that the flow velocity can also be measure by the distortion of the fluorescence correlation curves when they are taken with the separation voltage applied.

The sample system that we worked on clearly illustrates the utility of these measurements. The BSA serves as a marker and elutes rapidly due to its high charge. It has an intermediate size so its diffusion rate lies between the TTC and the antibody complex. The antibody complex is large but also elutes fairly rapidly due to its charge. However, its large size leads to the slowest diffusion rate. The small TTC protein elutes at the longest time and shows multiple peaks. There are several possible origins for the multiple free TTC peaks. One possibility is aggregation of the protein. However, the fluorescence correlation data rule this out because the peaks exhibit identical diffusion properties. Thus, the multiple TTC peaks correspond to species of the same size. The differences in their elution times must be due to different charge species either from the labeling or an inhomogeneous protein sample. In this case the ability to measure multiple fluorescence properties in addition to the electrophoretic separation of the sample provides evidence needed to identify the observed peaks and therefore increases confidence in the determination of the sample composition.

Conclusions

This work on this project has resulted in the development of the time-stamped, time and spectrally resolved single molecule microscope. This is a unique new tool for single molecule spectroscopy. It has been applied to directly time resolve the energy transfer in a single particle FRET system. We have also developed a powerful new fluorescence detection system for microfluidic applications. This detection system can measure many fluorescence properties of analytes to provide higher confidence identifications and to enhance capabilities for multiplex detection. We have made measurements in electrophoresis systems of the elution time, fluorescence spectra and lifetimes and diffusion properties of separated components of complex mixtures. This work has provided powerful new capabilities for Sandia and several of them are already in use for newly funded projects.

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