CHAPTER 2

EXPERIMENTAL METHODS

**2.1 Materials**

The two principal fluorescent conjugated polymers used to prepare conjugated polymer nanoparticles (CPNs) in these studies were poly[(9,9-dioctylfluorenyl-2,7-diyl)-*co*-(1,4-benzo-{2,1',3}-thiadiazole)] (PFBT, MW 10,000, polydispersity 1.7), and poly[2-methoxy-5-(2'-ethyl-hexyloxy)-1,4-phenylene vinylene] (MEH-PPV, average MW 200,000, polydispersity 4.0), and were purchased from ADS Dyes, Inc. (Quebec, Canada). The fluorescent dye perylene red (Exalite 613) was purchased from Exciton (Dayton, OH). Polystyrene microspheres, and the fluorescent dye fluorescein were purchased from Life Technologies (Invitrogen, Grand Island, NY). The fluorescent dye Lucifer Yellow CH dipotassium salt (LY, 1 mg/mL in water), 3-aminopropyl-trimethoxysilane (APS, 97%), sodium hydroxide (SigmaUltra, minimum 98%), and solvents tetrahydrofuran (THF, anhydrous, inhibitor-free, 99.9%), and ethanol (200 proof, anhydrous, ≥99.5%) were purchased from Sigma-Aldrich (Milwaukee, WI). All materials were used without further purification.

**2.2** **Nanoparticle Preparation**

The method used to prepare fluorescent conjugated polymer nanoparticles is based on a previously described nano-precipitation method,{Szymanski, 2005 #42} adapted from Kurokawa and co-workers.{Kurokawa, 2001 #144} A stock solution of a given conjugated polymer was prepared at a concentration of 1000 ppm (mg/kg) in THF. The polymers were sufficiently dissolved by gentle agitation of the solution at room temperature. For these experiments, the precursor solution to the nanoparticle suspension was prepared by diluting an aliquot of stock solution to a concentration of 20 ppm. For doped samples, dopant fluorescent dyes or polymers were added into the solution in their respective amounts prior to the addition of the diluting solvent such that their concentration in the diluted precursor solution was 0.1%–10% by weight, relative to the polymer concentration (further details given in Chapter 3). The resulting solutions were agitated gently to ensure solution homogeneity. A 2 mL aliquot of precursor solution was then rapidly injected via micropipette into 8 mL of deionized water under bath sonication at a frequency of 40 kHz and room temperature for ~30 seconds.

Conjugated polymers respond in similar, yet different ways to changes in solvent environment depending on the rate of incorporation of polymer from a good solvent into a poor solvent. It is thermodynamically favorable for a hydrophobic, long-chained molecule like a conjugated polymer to aggregate with itself in water (a poor solvent). By first dissolving the polymer into a good solvent that is also water-miscible such as THF, it is much easier to incorporate hydrophobic polymers (or other hydrophobic molecules such as small-molecule fluorescent dyes) into a poor, mostly aqueous solvent. Adding the dissolved polymer into a poor solvent drop-wise results in substantially increased aggregation of the polymer. Indeed, slow mixing of a polymer solution into a poor solvent is a commonly utilized method of polymer purification.{Colborne, 1955 #145} This can be confirmed with our polymer samples, as vacuum filtration of a solution in which dissolved polymer is added drop-wise to rapidly mixing water results in the loss of >80% the polymer during the subsequent filtration step as confirmed by UV-Vis. Alternatively, fast injection of the precursor THF solution into rapidly mixing water results in local polymer chain collapse as a result of hydrophobic interactions, polymer-water interfacial tension, and surface free energy effects.{Yang, 2005 #55} Under these conditions, chain collapse occurs at a faster rate that does not allow for flocculation to occur before a given sample of polymer collapses and is moved from its local environment into the bulk solution. Thus, formation of nanoparticles (i.e. nanoscale aggregates comprised of few polymer chains) is the kinetically favored product of rapidly mixing a dissolved polymer into a poor solvent. After nanoparticle formation occurs, the collapsed chain conformation is retained as the remaining THF is removed, resulting in an aqueous colloidal suspension of conjugated polymer nanoparticles. The size of the nanoparticles can be adjusted by increasing or decreasing the concentration of the precursor solution (e.g. increasing the precursor concentration yields a concomitant increase in nanoparticle size).{Wu, 2007 #54} Typical particle sizes range from 4-30 nm, which is verified by atomic force microscope (AFM) image analysis.{Groff, 2013 #60}{Wang, 2014 #212}{Szymanski, 2005 #42}

The process by which THF was removed from the samples has been refined from our previous partial vacuum evaporation procedure. THF was removed by placing the nanoparticle samples in a vacuum oven at room temperature under nitrogen flow for 8-10 hours in order to remove enough THF to prevent bumping during the subsequent partial vacuum evaporation step. Nitrogen flow was ceased and samples were evaporated under vacuum for 6-7 hours at a temperature of ~40 °C to remove most of the remaining THF. The total volume of liquid was reduced by ~60% during the evaporation process, which was confirmed to be sufficient by an iterated Raoult’s Law calculation, the results of which yield that <1% THF exists at ~40% volume loss. No residual THF odor was detected in the samples. Additionally, further evaporation yielded no further change in the fluorescence spectrum or quantum yield. The samples were subsequently vacuum filtered through a glass fiber prefilter and a 0.1 µm PVDF membrane filter in order to remove larger aggregates. The resulting suspensions are clear (not turbid) and stable, showing no signs of aggregation for months.

**2.3 Characterization Methods**

Several techniques are employed in order to characterize our nanoparticle samples. These include atomic force microscopy (AFM), UV-Vis spectroscopy, steady-state fluorescence spectroscopy, and fluorescence quantum yield measurement.

**2.3.1 Atomic Force Microscopy (AFM)**

AFM is a subtype of scanning probe microscopy, in which the surface topography of a sample is analyzed using a sharp probe tip affixed to a reflective cantilever spring. In all modes of AFM imaging, the tip is raster-scanned across a sample that is either immobilized or patterned onto a substrate. A laser is reflected off of the back of the cantilever and onto a detector (e.g. a quadrant photodiode). The laser signal is then monitored in order to track changes in the cantilever as the tip interacts with the sample. The typical properties that are tracked are either cantilever deflection, or the amplitude or frequency of near-resonant cantilever vibrations, depending on the mode of operation, discussed in detail below. The image is produced line-by-line by translating the changes in probe height into a topographic image of the sample.{Binnig, 1986 #213}{Rugar, 1990 #214}{Giessibl, 2003 #215}

Depending on the properties of sample to be studied, different operating modes may be chosen. The simplest mode of AFM operation is contact mode (sometimes referred to as static AFM).{Giessibl, 2003 #215} In contact mode, the sample is brought into direct contact with the tip, which is affixed to a cantilever with a low spring constant (0.1-5 N/m, typ.), causing deflection of the cantilever. As the tip is scanned across a sample, the cantilever deflection (and thus the force on the tip) is kept constant by raising or lowering the tip in response to changes in surface topography. Contact mode is best suited for harder samples, where the interatomic spring constants (~10 N/m) are greater than the spring constant of the cantilever material, thus preventing deformation of the sample by the AFM. Typical contact mode (repulsive) forces range from ~0.01 nN (requires solvent submerged samples) or ~1-100 nN (in air).{Rugar, 1990 #214} An inherent difficulty with contact mode AFM imaging arises from capillary forces due to liquid layers adhered to the surface of a sample under ambient conditions. This leads to substantial attractive forces (~100 nN) on the probe that lead to the probe suddenly jumping into contact with the sample through the liquid layer, or “snapping-in,” which can damage samples in some cases.{Giessibl, 2003 #215}

Non-contact mode is one of two dynamic AFM modes designed to circumvent cantilever adhesion, as well as the difficulties related to soft samples. In this mode, a stiff cantilever (20-100 N/m) is vibrated at or close to the resonant frequency of the cantilever material (70-200 kHz,), and at constant amplitude (~30 nm) by a piezoelectric element on the AFM tip mount. As the cantilever is brought closer to the sample, attractive, long-range forces on the order of pN (e.g. Van der Waals forces) dampen the oscillation of the cantilever and cause a slight shift in the phase of the cantilever oscillations, which shifts the effective resonance frequency (thus, this mode of AFM is sometimes referred to as frequency modulation AFM). The tip-sample distance that yields this dampening and frequency shift is held constant (~5-10 nm) as the probe is rastered across the sample. This method is non-destructive, since the tip never makes contact with the sample. However, non-contact mode is still prone to “snap-in” if the response time of the z-height electronics is slow, which can reduce scan speeds and cause artifacts in topographic images if tip adhesion occurs.{Martin, 1987 #217}

Intermittent contact (tapping or AC) mode AFM is another dynamic AFM mode, which shares similar probe stiffness and oscillation frequency characteristics to non-contact AFM. The key differences of tapping mode AFM are as follows. Once the probe is moved close enough to the sample in order to make periodic contacts, the cantilever oscillations are dampened. The amount of oscillation dampening is maintained at a set-point value, which is related to the amount of tip-sample contact force (lower set-points correspond to greater vibration dampening, and hence, higher contact force). Thus, rather than controlling z-height by maintaining a resonance frequency shift, the vibration amplitude of the cantilever is monitored and the z-height is adjusted to maintain the dampened amplitude (as such, this mode is sometimes referred to as amplitude modulation AFM). Another key difference in tapping mode operation is that the cantilever is vibrated at a higher oscillation amplitude (~100 nm, typ.), which allows the probe to overcome adhesion forces, electrostatic forces, and friction between the tip and sample.{Sulchek, 2002 #218} In addition, tip-sample contact forces in tapping mode AFM can be as low as 0.1 nN in air, which is greatly reduced as compared to contact mode under ambient conditions, making this method preferable for soft samples that are easily deformed or damaged by contact mode operation, or for weakly bound samples that would be easily swept off of the substrate by lateral forces in contact mode.{Zhong, 1993 #216}{Sulchek, 2002 #218}

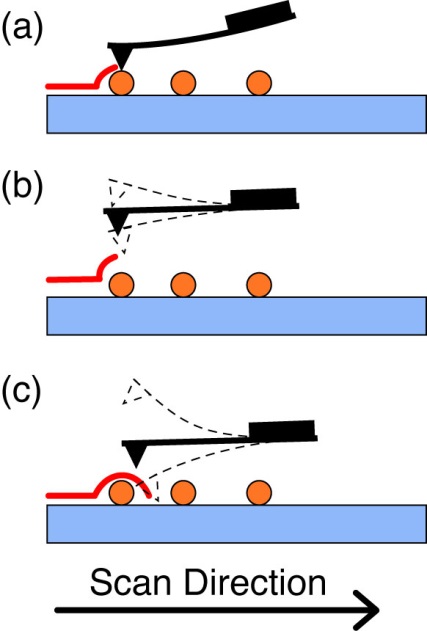


Fig. 2.1. Simplified illustration of a line scan using the various modes of AFM imaging, including (a) contact mode, (b) non-contact mode, and (c) intermittent contact mode.

Nanoparticle size distributions are determined with an Ambios Q250 multimode AFM in tapping mode. AFM samples were prepared by a dip-casting procedure. First, a cleaned, dry glass coverslip was amine-functionalized with 70 µL of freshly prepared 5×10-4 M APS in anhydrous ethanol. The solution was left on the coverslip for 3 minutes, followed by rinsing the coverslip with deionized water, and blowing it dry with nitrogen. An aliquot of the nanoparticle suspension was diluted by 30-40 times in a small beaker, and the coverslip was submerged into the diluted suspension with the functionalized side up for 40 minutes. The coverslip was then carefully removed from the solution and allowed to dry completely in a vacuum oven. To prepare the sample for AFM use, the coverslip was fixed to a glass slide with thin strips of tape. Particle sizes are determined by analyzing the peak z-heights of each viable particle in the image (i.e. ignoring aggregated particle clusters) and constructing a histogram to determine the mean and standard deviation of the particle sizes. Each scan was performed on a 2 µm by 2 µm scan region at a scan rate of 0.5 Hz (lines/s), with scan resolution of 500 lines per image.

There are several factors that impact the quality of an AFM image, including tip wear or breakage, as well as improper tuning of the PID (Proportional, Integral, and Differential) feedback loop. It is not possible to extract accurate particle size information from the diameters of the particles in the x-y plane due to tip convolution effects between the sample surface and the tip, which distort the image in the x-y plane (convolution is discussed in detail in section 2.4.2). The sharper the tip is, the smaller this effect is. As the tip wears and becomes blunted, this convolution effect is exacerbated, making the particles appear larger in the x-y plane. If foreign matter (e.g. dust, dirt) is stuck to the tip, or the tip is broken as a result of crashing the tip into the sample substrate, this can yield false duplicate particles in the image (for every particle in the image). Improper setting of the PID feedback loop can result in cantilever hysteresis, in which the change in z-height needed to restore the cantilever oscillations to the set point value is overshot (or undershot) compared to the actual change required to restore the oscillations to the set point value. This translates into improper z-height values, and the potential to have the probe tip either scratch the sample or crash into the sample or substrate, damaging the probe tip.

Sample standards are utilized to validate lateral and vertical resolution of the AFM. The standards are either gratings with known height and spacing, or particles of known diameter. Lateral resolution is limited by the sharpness of the AFM tip since the observed image in AFM is the convolution of the tip with the sample. The probes used with our AFM (NSC16) have a tip width of 8 nm, as supplied by the manufacturer. Vertical resolution (often referred to as “Z noise”) is determined by oscillating the cantilever with the x-y scanning electronics disabled and the tip engaged with a sample, then determining the standard deviation of the cantilever oscillations. The RMS noise of the AFM in the z-direction was measured using the aforementioned process, yielding a vertical resolution of 1.76 angstroms. Proper adjustment of the vibration isolation platform, and reduction of air currents and acoustic noise is required to maintain RMS noise below acceptable levels.

**2.3.2 UV-Vis/Absorption and Fluorescence Spectroscopy**

UV-Vis absorption spectra were collected on a Shimadzu UV2101PC scanning spectrophotometer using 1 cm quartz cuvettes. The absorption spectra were used to determine the nanoparticle concentration and peak absorption wavelength corresponding to the HOMO-LUMO electronic transition. For fluorescence measurements, concentrated nanoparticle samples (peak absorbance between 0.2–0.4) were diluted to yield a peak absorbance of ~0.1 for collection of fluorescence spectra or fluorescence lifetime measurement. Extinction coefficients for each polymer in THF were calculated by rearranging Beer’s Law to give , where ** is the extinction coefficient of the polymer at a given wavelength, *A* is the absorbance of the sample, *l* is the sample path length (typically 1 cm) and *c* is the molar concentration of polymer. To calculate the nanoparticle extinction coefficient, the number of polymer molecules per nanoparticle *Nnp* is determined from the mean nanoparticle volume (determined from the mean radius from AFM measurements) and the polymer molecular weight, using the assumption that the polymer density is ~1 g/cm3. This is then multiplied by the extinction coefficient for the polymer in THF yielding . Samples were diluted to an absorbance of ~0.05 for fluorescence quantum yield measurement. Steady-state fluorescence spectra were collected using a commercial fluorescence spectrometer (Quantamaster, Photon Technology International, Inc.) with 1 cm cuvettes. Samples were diluted to an absorbance of ~0.1 AU or less at the relevant excitation wavelengths. To avoid saturation of the photomultiplier tube (PMT) detector, all slit widths were kept at 0.50 mm. Multiplying the slit width by the reciprocal linear dispersion of the system (4 nm/mm for a 1200 grooves/mm grating) yields a wavelength resolution of 2 nm. The acquisition time was set to 1 s/nm to overcome noise in the spectra evident at lower acquisition times. The signal-to-noise (SNR) ratio for this instrument under the aforementioned conditions was measured to be ~10,000:1 using methods described by the manufacturer.



It is important to ensure sufficient dilution of fluorescent samples in order to avoid the inner filter effect, in which a photon emitted by one molecule is reabsorbed or scattered by another molecule in close proximity. An important note about the inner filter effect is that it is not limited by sample concentration alone in CPNs. Particle size must also be considered in this case, since it is possible to prepare nanoparticles with diameters that exceed the optical penetration depth of the material. The optical penetration depth (or optical skin depth) of a material can be determined by , where **(**) is the absorption coefficient of the material at wavelength **, *A*(**) is the absorbance at wavelength **, and *d* is the thickness of the material. Optical penetration depths range from tens of nanometers to several microns, depending on the material. Typical values for conjugated polymers range from ~40 nm to ~400 nm.{Yu, 2009 #146}{Bazani, 2009 #147}{Snaith, 2004 #165} Since CPNs are comprised of multiple closely-spaced chromophores, it is possible for a photon to be emitted from a chromophore on one end of a large particle, transmit through the particle, and be reabsorbed by another chromophore at the opposite end. This is one of the reasons why vacuum filtration is used to remove larger aggregates from the nanoparticle suspensions prior to measuring their fluorescence.

**2.3.3 Fluorescence Quantum Yield**

Fluorescence (or photoluminescence) quantum yield is a measure of the fraction of photon absorption events that result in emission of a fluorophore (expressed as a ratio between 0 and 1 or as a percentage). Fluorescence quantum yield measurement provides a quantitative comparison of the quantum efficiency of fluorescence between fluorophores. In terms of rates, the fluorescence quantum yield is given by . Where  is the fluorescence quantum yield, *kr* is the radiative rate and *knr* is the non-radiative rate (*knr* encompasses all other processes that do not contribute to the fluorescence emission, e.g. non-radiative decay by internal conversion, energy transfer pathways, inter-system crossing to form triplet states, polaron formation, etc.).

The fluorescence (photoluminescence) quantum yield (QY or PLQY) of the CPN samples was determined in several steps. First, an appropriate standard fluorophore must be selected with an absorption and emission maximum similar to the conjugated polymers under study. In this work, the standard fluorophores used were fluorescein dissolved in 0.01 M sodium hydroxide (note: it is important to check the pH of the solution to ensure it is above 10 to ensure that only the fluorescein dianion exists in solution,{Sjöback, 1995 #40} since NaOH and solutions thereof tend to absorb ambient CO2), and Lucifer Yellow CH dissolved in water (LY). A common absorption wavelength was selected between the sample and standard (473 nm for fluorescein, 450 nm for LY), and solutions were prepared at an absorbance of ~0.05 AU at the common absorption wavelength. Emission spectra were collected at the common excitation wavelength, and the fluorescence quantum yield is calculated by the expression

, (2.1)



where, *F*,*s* is the fluorescence quantum yield of the standards (0.92 for fluorescein in 0.01 M NaOH and 0.21 for LY),{Weber, 1957 #47}{Stewart, 1981 #93} *F,x* is the fluorescence quantum yield of the sample, *A* is the absorbance, *I* is the integrated fluorescence intensity, and *n* is the refractive index of the solvent. It is important to note that a solvent blank must be measured for both absorbance and fluorescence measurements in order to properly apply baseline correction to the spectra when calculating the fluorescence quantum yield.

**2.4 Time-Resolved Fluorescence Spectroscopy**

Time-resolved fluorescence spectroscopy provides a window into many physical processes that are not readily apparent from steady-state fluorescence measurements alone. Measurement of the mean fluorescence lifetime of an ensemble of fluorophores gives the average amount of time that a given fluorophore spends in its excited electronic state following photon absorption, which can be used as a means to determine how various physical processes affect the excited state. The fluorescence lifetime is related to the rate of depopulation of the excited state () by , where is the fluorescence lifetime. When both lifetime and fluorescence quantum yield are known, it is possible to separate out the radiative and non-radiative rates. For complex systems in which two or more species interact, lifetime measurement elucidates how intermolecular interactions (e.g. intermolecular collisions, FRET, charge transfer, etc.) affect the excited state of a fluorophore. For example, if fluorescence quenching is observed in the steady-state measurements, lifetime measurements will indicate whether the observed quenching is static or dynamic, which provides insight into the physical processes that cause fluorescence quenching. Static quenching does not affect the fluorescence lifetime because this type of quenching is often the result of formation of a ground state non-fluorescent complex with another molecule. Photon absorption by the complex results in no emission events from the complexed fluorophores, thus reducing the fluorescence intensity of the ensemble, but not the lifetime, since the excited states of unbound fluorophores remain unchanged. Dynamic quenching typically reduces the lifetime of the quenched fluorophore, since dynamic quenching only occurs when the fluorophore is already in the excited state (e.g., quenching by molecular oxygen, FRET), thus reducing the mean lifetime of the quenched fluorophores by depopulating the longer-lived excited state. One means of describing fluorescence quenching is via the Stern-Volmer equation, given by:

, (2.2)

where *F*0 is the initial fluorescence intensity in the absence of quenchers, *F* is the fluorescence intensity in the presence of quenchers, is the Stern-Volmer quenching constant, and [*Q*] is the concentration of the quenching species. takes on different definitions, depending on the type of quenching in the system. The following derivation of Equation 2.2 is originally for a simple collisional quenching process; however, other quenching mechanisms such as FRET are observed to follow Stern-Volmer-type quenching. For dynamic quenching, is derived using the rate equations describing emission with and without quenching at steady state,

, (2.3)

and

, (2.4)

where the asterisk on [*F*] is used to denote an excited fluorophore, and *kq* is the quenching rate constant. Division of 2.3 by 2.4 yields

. (2.5)

Thus, for dynamic quenching, , where and correspond to the unquenched and quenched fluorescence lifetimes, respectively. For static quenching, is related to the concentrations of complexed fluorophore , non-complexed fluorophore (with initial concentration [*F*]0) and quencher by

, (2.6)

which is rearranged to give Eq. 2.2. If quenching is described by a combination of static and dynamic quenching, then plotting vs. [*Q*] will yield positive deviations from the linear Stern-Volmer relations given above. Instead, vs. [*Q*] is accurately described by multiplying the individual Stern-Volmer equations corresponding to static and dynamic quenching.{Lakowicz, 2006 #26} In addition, high energy transfer rates (large *kq*) can result in positive deviations from Eq. 2.6 despite resulting solely from dynamic quenching (e.g., in blended CPNs that can have multiple quenchers doped within the nanoparticle at high doping levels), which requires alternative methods to describe quenching (e.g. Poisson statistical distribution of quenchers in CPNs, discussed in Chapter 3).{Wang, 2014 #212}

**2.4.1 Overview of Time-Resolved Fluorescence Instrumentation**

There are various types of time-resolved fluorescence setups that vary in their complexity, cost, and effective time resolution. Lifetime methods with slower time resolution (e.g. using pulsed LEDs/lamps, with temporal width of a few ns, coupled with less sophisticated electronics) are less expensive and less complex than many setups with time resolution below 1 ns or faster, and are useful when the dynamics to be measured are slow (e.g. triplet dynamics, dynamics of conventional fluorescent dyes). The methods that afford the best time resolution of time-resolved fluorescence spectroscopy (sub-picosecond time resolution) typically utilize amplified ultrafast lasers at lower repetition rates (kHz repetition rates) as excitation sources (making these methods the most expensive). In the aforementioned systems, emission is detected via non-linear optical processes using intense laser pulses to create an optical gate (e.g., via Kerr gating, or by frequency mixing of the fundamental laser pulse with the sample emission in a non-linear crystal medium as in fluorescence upconversion),{Arzhantsev, 2005 #241}{Kahlow, 1988 #242} or by monitoring changes in absorbance of a sample after excitation using a pump-probe type method (as in transient absorption spectroscopy).{Collini, 2009 #63} The intensity decay is then constructed point-by-point by delaying the fundamental pulse by some known amount using an optical delay line (i.e., a pair of mirrored surfaces on a translation stage), and the changes in intensity of a given non-linear phenomenon (e.g. intensity of a sum-frequency signal vs. delay time) are measured to construct the decay trace. The time resolution of these setups is determined by the pulse width of the fundamental laser (typically a few hundred femtoseconds). In addition to measuring lifetimes of emissive states, methods such as transient absorption are also useful for probing dark states that do not contribute to an emission signal, but possess transient spectroscopic signatures in the absorption spectrum (e.g. measuring the lifetimes of charge-transfer states).{Hsu, 1994 #240}

One of the more common methods of time-resolved fluorescence spectroscopy that balances cost, complexity, acquisition time and effective time resolution is Time-Correlated Single Photon Counting (TCSPC) spectroscopy. The mechanism of operation is described as follows. A pulsed light source is utilized as an excitation source. The temporal width of the excitation pulse depends on the source used and the dynamics to be measured (Pulsed LEDs or lasers such as N2 lasers have ns pulsewidths, dye lasers have pulsewidths down to a few ps, and ultrafast Ti:Sapphire lasers offer fs pulsewidths). The pulses are split, where one pulse is used to excite the sample, resulting in fluorescence photons that are incident on a single-photon counting detector (e.g. an avalanche photodiode or APD) and the other pulse travels to another fast detector (e.g. a PIN diode). The relative timing of the pulses is determined by specialized electronics. Here we describe one approach based on fast NIM (Nuclear Instrument Module) electronics originally developed for use in particle physics and in neutron and gamma detectors. Both detector signals are used generate timing pulses in a timing discriminator, such as a threshold discriminator (TD), or a constant-fraction discriminator (CFD). A threshold discriminator outputs a timing pulse whenever the input voltage from a pulse crosses a certain threshold voltage. A disadvantage of this type of discriminator is that timing pulses are output at different times for pulses with varying amplitudes but equivalent arrival times, thus increasing the timing uncertainty. This timing uncertainty is remedied using a constant fraction discriminator. CFDs operate by splitting the voltage signal by a constant fraction, inverting, and delaying the split voltage signal by some constant amount of time, then recombining the signal. The time at which the voltage crosses zero is used to generate an output pulse. CFDs greatly reduce timing uncertainty due to differences in input signal amplitudes. The timing pulses from the PIN and APD are then sent to a time-to-amplitude converter (TAC), where the time between the arrival of the two pulses is measured by an analog linear voltage ramp that starts when one timing pulse (from the PIN) arrives and stops when the other timing pulse (from the APD) arrives. The analog signal from the TAC is passed to an analog-to-digital converter (ADC), where the analog voltage is converted into a bin number and stored in the memory of a multi-channel analyzer (MCA). Many photon arrival times are measured, and a histogram of photon arrival times is constructed, resulting in a fluorescence intensity decay trace convolved with the instrument response function (IRF). Fitting procedures (discussed in detail in section 2.4.3) are then employed to separate the intensity decay signal from the IRF signal and determine the time constants from the intensity decay. The specific components of our TCSPC setup and experimental details are discussed in the next section.

There are several types of single-photon detectors, such as photomultiplier tubes (PMTs), of which there are dynode chain PMTs (which convert photons into electron current via the photoelectric effect, multiplied many times over as photoelectrons bounce off of a chain of dynodes) and micro-channel plate PMTs (containing many narrow channels lined with dynode material which multiply photoelectrons as they bounce off of the walls of the channels in transit down the channel), or diode detectors such as single-photon avalanche photodiodes (SPADs or APDs, where electron current results from an avalanche of electrons following photon absorption. The avalanche is generated by impact ionization with semiconductor atoms in a reverse biased P-N junction). The key difference between each detector is in the minimum temporal resolution each type affords. APDs and MCP-PMTs afford the best time resolution at tens of picoseconds, whereas dynode chain PMTs typically afford time resolution from hundreds of picoseconds to ~1 ns.{Lakowicz, 2006 #26}

When polarizers are incorporated into a time-resolved fluorescence setup, then it becomes possible to measure the fluorescence anisotropy decay of the system, which is a measurement of the depolarization of fluorescence after being excited by a linearly polarized source. Fluorescence anisotropy can decay by either by rotation or other mechanisms (such as energy transfer), and measuring the anisotropy decay quantifies the rates of these depolarization processes. If depolarization is due to energy transfer mechanisms, it is possible to probe phenomena such as incoherent energy transfer (using either ultrafast or slower methods). When fluorescence anisotropy decay methods are taken in conjunction with fluorescence lifetime measurements, it is possible to determine the average number of energy transfer events occurring within the excited state lifetime (as discussed previously in Chapter 1). Thus, time-resolved spectroscopy can be a very versatile tool for exploring the photophysics of a fluorescent system.{Lakowicz, 2006 #26}

**2.4.2. TCSPC Setup and Experiment**

Picosecond fluorescence lifetimes were measured in air and under nitrogen using a home-built setup for time-correlated single photon counting (TCSPC) spectroscopy operating in either forward or reverse mode. Frequency doubled pulses (420 nm) from a passively (Kerr lens) mode-locked Ti:Sapphire laser (Coherent Mira 900, 840 nm pulses, ~150 fs pulsewidth, 76 MHz rep. rate) were used as the excitation source for the nanoparticle samples. The non-linear medium used for second harmonic generation (SHG/frequency doubling) was a -barium borate crystal (BBO, Type I, 100 µm thick, AR-coated, SHG range 760-840 nm). Fluorescence was collected perpendicular to the excitation source and passed through a 460 nm long pass filter, and a calcite Glan-Taylor linear polarizer (Thorlabs, GT10-A) oriented at magic angle (55°) to the vertically polarized excitation pulses. For (unpolarized) TCSPC measurement, the polarizer is oriented at 55° (magic angle) since the intensity decay collected at this orientation corresponds to the sum of the fluorescence intensity in the x, y, and z planes, given by , as previously discussed in Chapter 1.{Cross, 1984 #87} One-inch diameter, plano-convex lenses were utilized throughout the setup to focus or collimate light as necessary (75 mm focal length lens to focus on APD and 50 mm focal length lenses elsewhere, c.f. Fig 2.2). For the experiments in Chapter 3, the output of a fast PIN diode (Thorlabs, DET210) was used as the start timing pulse for a time-to-amplitude converter (TAC, Canberra Model 2145), and the output of a single photon avalanche photodiode (APD, id Quantique, id100-50) was used as the stop timing pulse. For the experiments in Chapter 4, the detector outputs are switched, in a standard reverse-mode configuration.{Schaffer, 1999 #88} The excitation power was attenuated (between ~300 µW and 1 mW, typ.) to maintain a count rate of ~400 kHz as measured at the APD (to ensure that the probability of two fluorescence photons arriving from one laser pulse is under 10%). The analog TAC output was digitized using a 13-bit multi-channel analyzer (FastComTec, MCA-3A). Before and after each measurement an instrument response function (IRF) was measured using scattered excitation light from a dilute suspension of polystyrene microspheres. The width of the IRF was determined to be ~70 ps (fwhm). Typical peak signal-to-noise ratios (SNR) for each run (limited to ~10 minutes per run, to minimize the effect of timing drift) were between 200:1-500:1 in reverse mode, and 50:1-100:1 in forward mode. The dwell time per MCA bin was determined by measuring an IRF sample and varying the delay times on the TAC until the 20 ns sample window contained two IRF peaks. The bin spacing between the IRF peaks was measured, and then used in conjunction with the measured repetition rate of the Ti:sapphire laser (a 76 MHz repetition rate corresponds to ~13 ns between laser pulses) to determine a dwell time of 2.6333 ps/bin.



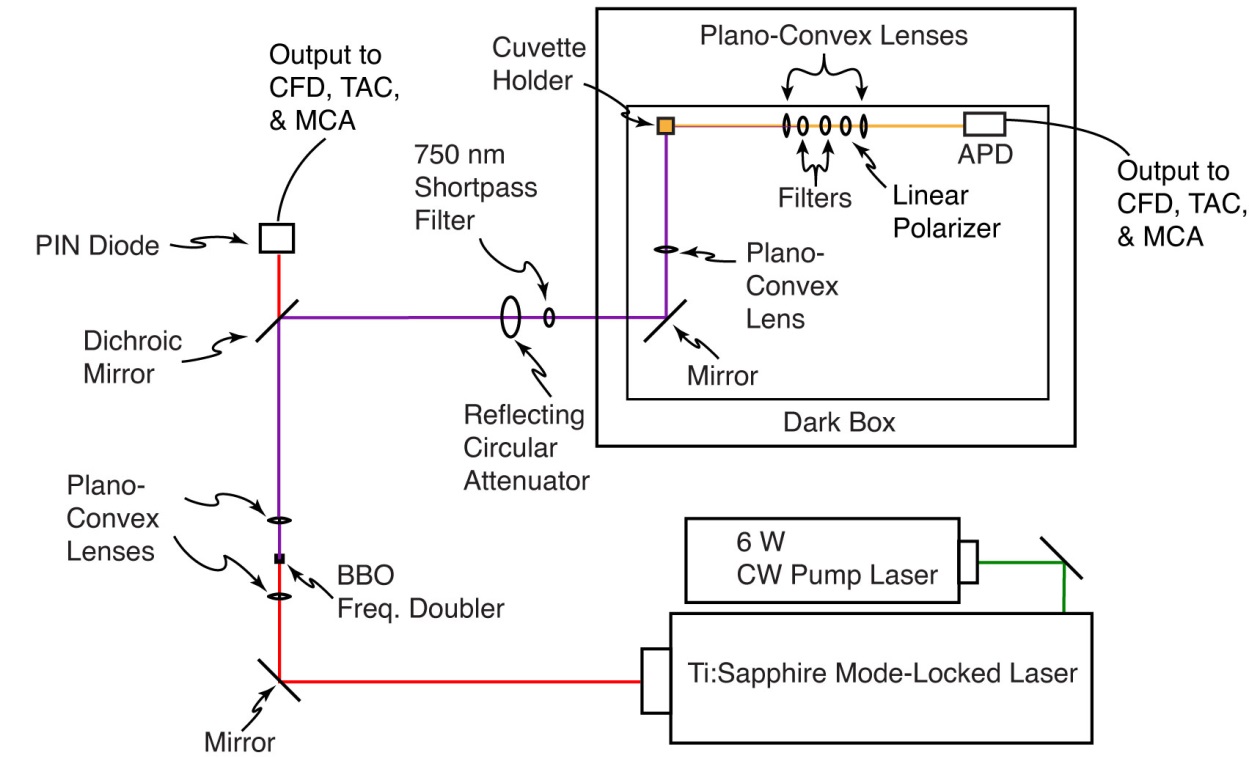
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Fig. 2.2. Time-Correlated Single Photon Counting and Fluorescence Anisotropy Decay setup.

Due to the random nature of photon arrival, the SNR is determined from the Poisson probability distribution function (PDF), given by

, (2.7)



which gives the probability of recording *n* counts in a measurement of finite duration. In the limit of an infinite number of measurements, the average value of *n* approaches the mean of the distribution, given by *N*. Given the average signal (average number of photon counts) *N* and given thatthe noise is approximated by the standard deviation of the distribution as , the signal-to-noise ratio is given by:



(2.8)



(e.g. 10,000 photon counts at the peak results in a SNR of 100:1).

**2.4.3 TCSPC Fitting Procedure**

As mentioned in the previous section, the sample trace obtained by TCSPC *S*(*t*) is a convolution of an instrument response function *IRF*(*t*) with the true fluorescence intensity decay of the sample *F*(*t*) (c.f. Fig 2.3). It will be shown below that convolution in the time domain is essentially a time-shifted overlap calculation between two functions. If one function has broader features compared to the second (e.g., if *IRF*(*t*) has large temporal width compared to the temporal features of *F*(*t*)), the narrower features of the second function are consequently broadened in the convolution of the two (this is sometimes termed “smearing”). Typically, one function is given by a signal or stream of data (Fig 2.3b, in TCSPC this is the fluorescence decay), and the other function is a peaked function that drops to zero on either side of the peak (Fig 2.3a, in TCSPC, this is given by the instrument response function of the detector). This is expressed generally by:

. (2.9)



The convolution is calculated by shifting one function in time and multiplying the two functions for each shift in time. For continuous functions, the convolution is given by:

, (2.10)



and the integral in Eq. 2.10 becomes a finite summation over the duration of the IRF for discrete functions. Another way to express Eq. 2.9 is in terms of the Fourier Transforms of *IRF*(*t*) and *F*(*t*):

, (2.11)

where ** is used to denote the frequency domain following Fourier Transformation. Thus, the convolution of two time domain functions is computed by multiplication of the respective Fourier Transforms. For discrete functions, this is calculated using Fast Fourier Transform (FFT) methods in order to compute the convolution efficiently. Thus, the convolution is calculated by computing the FFT of both *IRF*(*t*) and *F*(*t*) to give  and , multiplying them, and then computing the inverse FFT of the resulting product to return the convolution in the time domain (c.f. fig 2.3c).{Press, 2007 #221}

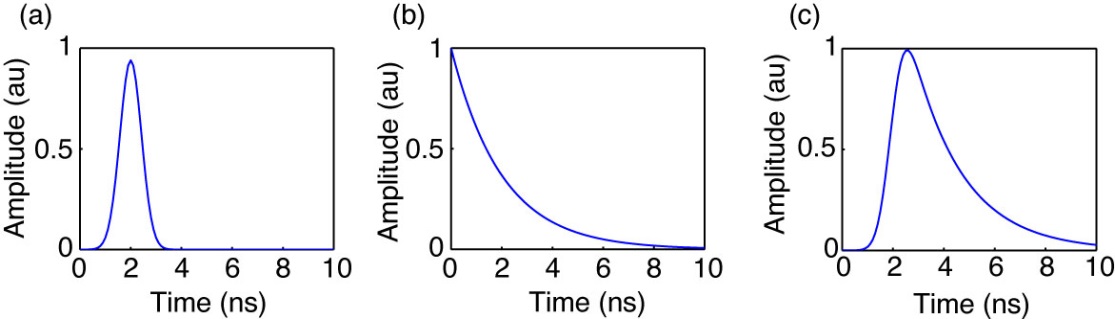
****

Fig 2.3. Example of discrete convolution in which (a) a Gaussian instrument response function with 1 ns fwhm is convolved with (b) a single exponential decay with  = 2 ns to produce (c) the convolution of (a) and (b). The result depicted in (c) exemplifies the shape of an intensity decay trace measured using TCSPC.

By measuring both *S*(*t*) and *IRF*(*t*), we have all of the information we need to determine *F*(*t*). This method of fitting works by convolving the measured IRF with a trial decay function, typically one of various exponential decay functions in this case, of the basic form , where *A*0 is the amplitude of the function at time *t*0, and  is the time constant (for a single exponential decay). Thus, 2.9 becomes



, (2.12)



where *Ssim*(*t*) is the simulated decay trace. *Ssim*(*t*) is then compared to *S*(*t*), and the quality of fit is assessed by calculating the sum of the squared residuals, given by:

. (2.13)

The parameters in *Ftrial*(*t*) are varied by random number generation between preset lower and upper bounds (the parameters are *A*0, *t*0, and for a single exponential, given above), and if the trial parameters yield a better fit (lower sum of squared residuals), the parameters are saved before moving onto the next calculation. This is repeated for thousands of iterations until the best guess of fit parameters to *F*(*t*) is found. The aforementioned random number minimization is used in conjunction with linear least-squares fitting in order to fit the linear coefficients (e.g., *A0*). After the minimization is complete, the residuals are assessed for any systematic deviations (e.g., the best fit between *Ssim*(*t*) and *S*(*t*) will yield a flat residuals trace). This method of fitting analysis affords time resolution below the fwhm of the measured IRF, with a typical lower limit in determining accurate time constants as short as ~10-20% of the IRF width (e.g. 7-14 ps for a 70 ps fwhm IRF).{O'Connor, 1979 #223}{Wahl, 2014 #222} The main reason for this limitation is due to drift in the TCSPC timing electronics (i.e., shifting of the IRF), which occurs on the time scale of a few picoseconds. As such, time constants of less than about 10-20% the width of the IRF are regarded as suspect, either indicating a poor-quality data set, poor choice of fitting function, or an experimental issue such as leakage of excitation light into the detector, excess laser noise, cabling issues, improper discriminator settings, etc.

While other fitting methods exist that would successfully minimize a single-exponential decay function such as downhill simplex or Newton-Raphson minimization methods,{Press, 2007 #221} CPN intensity decay kinetics are typically described by more complex exponential functions, such as weighted sums of exponential decays (bi-exponential or multiexponential functions), the Kolrausch-Williams-Watts (KWW or stretched exponential) function, given by

(2.14)

(where ** is a number typically between ~0.2 and 1, where ** = 1 yields a single exponential decay),{Chen, 2003 #8} or a weighted sum of a KWW function with an exponential decay (in the case of fluorescence anisotropy decay analysis, given in Chapter 4). Difficulties arise when fitting complex exponential decays using methods such as downhill simplex due to local minima encountered when minimizing Eq. 2.13, which necessitates an exhaustive, random search across the parameter space to ensure that the global minimum of Eq. 2.13 is found.

**2.4.4 Validation of the TCSPC Instrument**

There are several sources of artifacts in TCSPC, including afterpulsing in detectors, stray reflections in the apparatus, improper triggering (e.g., triggering on a ring instead of on the principal pulse), and signal reflections and nonlinearities in the timing and readout electronics. As such, precautions must be taken to ensure that data being collected in these experiments is of the highest possible quality. The instrument was carefully aligned, and baffles were employed to minimize stray light. Discriminator levels were adjusted to minimize the width of the instrument response function and minimize ringing. Proper impedance matching was employed. While there is a slight ring apparent in results obtained for samples with lifetimes greater than ~2 ns (c.f. Fig 3 in Chapter 3), we have validated the accuracy of the instrument using several standard dyes, including fluorescein in 0.01 M NaOH (τ = 4.1 ns),{Sjöback, 1995 #1313} coumarin 6 in ethanol (τ = 2.5 ns),{Sun, 2011 #1314} and perylene red in THF (τ = 5.6 ns).{Al-Kaysi, 2006 #1309} All lifetimes measured were within 10% of the literature values, indicating that the ringing, while visible, has a negligible effect on the lifetime results obtained.

**2.5 Picosecond Fluorescence Anisotropy Decay (FAD)**

Fluorescence anisotropy decay (FAD) measurements were performed using the aforementioned TCSPC setup, with the addition of a calcite Glan-Taylor linear polarizer (Thorlabs GT-10A) placed after the filter set, and before the focusing lens. For fluorescence anisotropy decay measurement, intensity decays and IRFs were collected at all three polarizer orientations. Unlike TCSPC, the excitation power is attenuated once and then held constant in the FAD experiment (rather than variably attenuated for each sample) in order to avoid systematic deviations in the anisotropy signal due to inconsistent excitation power. The excitation power is selected so that the collected emission at each polarizer orientation yields a count rate between 200 kHz and 800 KHz as measured on the APD. Typically, the strongest emission is collected when the emission polarizer is parallel to the excitation. The FAD least-squares fitting analysis is similar to the iterative convolution fitting procedure outlined in Section 2.4.3, with adaptations from a procedure given by Fleming, et al.{Cross, 1984 #87} Further detail will be given in Chapter 4.