Fast Tracking of Single Conjugated Polymer Nanoparticles with High Spatial Resolution

Teeranan Nongnual
Clemson University, teeranan.n@gmail.com

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FAST TRACKING OF SINGLE CONJUGATED POLYMER NANOPARTICLES
WITH HIGH SPATIAL RESOLUTION

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemistry

by
Teeranan Nongnual
August 2017

Accepted by:
Jason McNeill, Committee Chair
Andrew Tennyson
Brian Dominy
Leah Casabianca
ABSTRACT

Highly fluorescent and photostable conjugated polymer nanoparticles that freely diffuse in glycerol/water mixtures were individually tracked at an acquisition rate up to 1 kHz. The average bright fluorescence emission of about 15000 photons per particle per millisecond exposure time (~500 photons detected by sCMOS camera) yields a theoretical localization uncertainty of 10 nm per frame along lateral plane. Axial positional trajectories for 3D particle tracking were determined by defocused imaging, which evaluates the width of fluorescence spot at different displaced focal planes, yielding an axial resolution of 20 nm. The diffusion coefficient of nanoparticles in solution was measured by using the mean squared displacement, which agrees well with the Stokes-Einstein equation according to given the experimental solution viscosity and independently determined particle size. Furthermore, a high-resolution optical image of porous agarose gel was constructed by using particle tracking in order to characterize the structure of nanopores and determine the diffusion dynamics inside the pores and channels. The position trajectories consisting of confined diffusion of a particle in individual pores were analyzed by position histogram and mean squared displacement methods, yielding pore size distribution and diffusion coefficient of single particles. Our findings indicate that conjugated polymer nanoparticles, which exhibit higher emission rates and higher absorption cross sections as compared to typical results for single organic dye molecules and quantum dots, could be effectively used to investigate the dynamic behavior of individual small biomolecules or motor proteins with high
spatiotemporal resolution. The information of particle dynamics with anomalous diffusion could be useful for the study of cellular function, particle trafficking, membrane dynamic, and drug molecule delivery.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Diffraction limit in conventional light microscopy</td>
<td>2</td>
</tr>
<tr>
<td>Basic concepts in fluorescence spectroscopy</td>
<td>5</td>
</tr>
<tr>
<td>Conventional fluorescent molecules and nanoparticles</td>
<td>9</td>
</tr>
<tr>
<td>Conjugated polymers and conjugated polymer nanoparticles</td>
<td>11</td>
</tr>
<tr>
<td>Single-molecule spectroscopy</td>
<td>16</td>
</tr>
<tr>
<td>Particle tracking</td>
<td>18</td>
</tr>
<tr>
<td>Single-molecule localization microscopy</td>
<td>21</td>
</tr>
<tr>
<td>Measurements of particle diffusion</td>
<td>24</td>
</tr>
<tr>
<td>Research motivation</td>
<td>30</td>
</tr>
<tr>
<td>II. EXPERIMENTAL METHOD</td>
<td>33</td>
</tr>
<tr>
<td>Materials</td>
<td>33</td>
</tr>
<tr>
<td>Preparation of nanoparticles</td>
<td>33</td>
</tr>
<tr>
<td>Particle size and bulk spectroscopy measurements</td>
<td>35</td>
</tr>
<tr>
<td>Single-nanoparticle spectroscopy measurements</td>
<td>40</td>
</tr>
<tr>
<td>Particle-tracking measurements in solution and agarose gel</td>
<td>46</td>
</tr>
<tr>
<td>Excitation intensity</td>
<td>49</td>
</tr>
<tr>
<td>Collection efficiency</td>
<td>51</td>
</tr>
<tr>
<td>Estimation of number of emitted photons</td>
<td>54</td>
</tr>
<tr>
<td>Gain factor</td>
<td>55</td>
</tr>
<tr>
<td>Noise in fluorescence spectroscopy and digital imaging</td>
<td>59</td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>63</td>
</tr>
</tbody>
</table>
Table of Contents (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III. PHOTOPHYSICAL PROPERTIES OF CPNS</td>
<td>70</td>
</tr>
<tr>
<td>Nanoparticle size and morphology</td>
<td>70</td>
</tr>
<tr>
<td>UV-vis absorption and fluorescence spectra from bulk spectroscopy</td>
<td>72</td>
</tr>
<tr>
<td>Fluorescence spectroscopy of single nanoparticles</td>
<td>75</td>
</tr>
<tr>
<td>IV. TRACKING OF CPNS IN SOLUTION</td>
<td>87</td>
</tr>
<tr>
<td>Single particle localization</td>
<td>89</td>
</tr>
<tr>
<td>Localization uncertainty of immobilized particles</td>
<td>92</td>
</tr>
<tr>
<td>Diffusion coefficient</td>
<td>96</td>
</tr>
<tr>
<td>Lateral diffusion</td>
<td>97</td>
</tr>
<tr>
<td>Variance in MSD calculations</td>
<td>104</td>
</tr>
<tr>
<td>Localization uncertainty of moving particles</td>
<td>105</td>
</tr>
<tr>
<td>Axial diffusion</td>
<td>112</td>
</tr>
<tr>
<td>V. TRACKING OF CPNS IN AGAROSE GEL</td>
<td>120</td>
</tr>
<tr>
<td>Random-walk simulations of confined diffusion</td>
<td>121</td>
</tr>
<tr>
<td>Particle tracking of single PFBT nanoparticles in agarose gel</td>
<td>133</td>
</tr>
<tr>
<td>VI. CONCLUSIONS AND FUTURE WORK</td>
<td>143</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>154</td>
</tr>
<tr>
<td>A: Additional figures</td>
<td>155</td>
</tr>
<tr>
<td>B: Selected custom computational scripts</td>
<td>157</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>163</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Experimental diffusion coefficients calculated by using MSD method along lateral plane</td>
<td>103</td>
</tr>
<tr>
<td>4.2</td>
<td>Effect of particle motion with distorted point-spread function and increased localization uncertainty</td>
<td>109</td>
</tr>
<tr>
<td>4.3</td>
<td>Tracking offset with particle motion effect</td>
<td>111</td>
</tr>
<tr>
<td>5.1</td>
<td>Fluorescence intensity and fwhm of particle with confined diffusion</td>
<td>137</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic illustration of the absorption intensity of particle suspension in solution, and the decrease in intensity related to absorption cross section of particle</td>
<td>7</td>
</tr>
<tr>
<td>1.2</td>
<td>Particle tracking with resulting particle trajectory constructed from particle localization of each consecutive images</td>
<td>20</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic illustration of single-molecule localization by fitting the fluorescence image of a single fluorophore to 2D Gaussian function to obtain the center position</td>
<td>23</td>
</tr>
<tr>
<td>1.4</td>
<td>Relationship of MSD and lag time of various types of diffusion</td>
<td>29</td>
</tr>
<tr>
<td>2.1</td>
<td>Chemical structure of PFBT conjugated polymer</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Conjugated polymer nanoparticle preparation process by nano-precipitation method</td>
<td>35</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematic illustration of wide-field single particle imaging microscopy</td>
<td>43</td>
</tr>
<tr>
<td>2.4</td>
<td>Schematic illustration of laser excitation and fluorescence emission of immobilized single nanoparticle</td>
<td>44</td>
</tr>
<tr>
<td>2.5</td>
<td>Fluorescence intensity of a particle shows the first frames at the beginning after the laser is unblocked</td>
<td>45</td>
</tr>
<tr>
<td>2.6</td>
<td>Schematic illustration of laser excitation and fluorescence emission of single nanoparticle in solution or in agarose gel</td>
<td>48</td>
</tr>
<tr>
<td>2.7</td>
<td>Experimental image of a laser spot and corresponding simulated Gaussian profile image with fwhm of 3 μm and maximum excitation intensity of 110 W/cm²</td>
<td>51</td>
</tr>
<tr>
<td>2.8</td>
<td>Schematic illustration of collection solid angle from a scattering fluorescence emission as a fraction of total solid angle in the units of steradians</td>
<td>53</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9</td>
<td>Illustration of the various factors determining signal level in a typical sCMOS camera and inverted fluorescence microscope</td>
<td>55</td>
</tr>
<tr>
<td>2.10</td>
<td>Image of a flat-profile illumination, histogram of consecutive signals in one pixel over 600 images, and linear relationship of signal mean and variance with the gain factor as the slope</td>
<td>58</td>
</tr>
<tr>
<td>2.11</td>
<td>Poisson distribution of photoelectrons from incident photons converted by a camera sensor pixel</td>
<td>60</td>
</tr>
<tr>
<td>2.12</td>
<td>Difference per pixel of two dark frames with the standard deviation related to the readout noise</td>
<td>62</td>
</tr>
<tr>
<td>2.13</td>
<td>Photoelectron intensity of a flat-profile illumination along with the deviation boundary related to photon noise and corresponding histogram in one camera pixel, and over an area of 25 pixels</td>
<td>65</td>
</tr>
<tr>
<td>2.14</td>
<td>Fluorescence image of an immobilized single PFBT nanoparticle using an excitation intensity at 110 W/cm² with a framerate of 1 kHz, and corresponding photoelectron intensity with the mean and statistical boundary related to the deviation due to photon noise</td>
<td>67</td>
</tr>
<tr>
<td>2.15</td>
<td>Fluorescence intensity of a PFBT nanoparticle using an excitation intensity at 540 W/cm² with a framerate of 100 Hz</td>
<td>69</td>
</tr>
<tr>
<td>3.1</td>
<td>AFM image of PFBT nanoparticles, histogram of PFBT nanoparticle height, and particle size distribution determined by DLS method</td>
<td>71</td>
</tr>
<tr>
<td>3.2</td>
<td>Absorption and fluorescence ($\lambda_{ex} = 473$ nm) spectra of PFBT nanoparticles in water, and the decrease of absorbance of PFBT nanoparticles in water before and after filtration through a 0.1-µm membrane filter</td>
<td>73</td>
</tr>
<tr>
<td>3.3</td>
<td>Linear relationship of integrated fluorescence intensity and absorbance at different concentrations of fluorescein and PFBT, and quantum yield determination by the comparative method</td>
<td>75</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>Fluorescence image of single PFBT nanoparticles immobilized on a coverslip using an excitation intensity at 110 W/cm², the fluorescence intensity of weak spots was scaled up to differentiate individual nanoparticles and aggregations, the fluorescence intensity of selected individual nanoparticles, and corresponding emission rate histogram of single PFBT nanoparticles ................................................</td>
<td>78</td>
</tr>
<tr>
<td>3.5</td>
<td>Typical energy level scheme for single-molecular spectroscopy with absorption, fluorescence, inter-system crossing, and phosphorescence processes ..................................................................................................................</td>
<td>82</td>
</tr>
<tr>
<td>3.6</td>
<td>Fluorescence saturation of single PFBT plotted with a series of different laser excitation intensities ranging from 20 to 1100 W/cm² ................................................</td>
<td>82</td>
</tr>
<tr>
<td>3.7</td>
<td>Fluorescence intensity decay by photobleaching of immobilized single PFBT nanoparticle using an excitation intensity at 540 W/cm² with a framerate of 20 Hz fits to the exponential decay function, and total photons emitted per particle by the integration of intensity ........</td>
<td>85</td>
</tr>
<tr>
<td>4.1</td>
<td>Raw fluorescence image of a PFBT nanoparticle, normalized image by using a Fourier filter, single particle localization using a square image corresponding to the raw image fitted to 2D Gaussian function, and particle trajectory constructed from the centroids in subsequent images ..................................................................................................</td>
<td>91</td>
</tr>
<tr>
<td>4.2</td>
<td>Fluorescence image of immobilized PFBT nanoparticles using an excitation intensity at 540 W/cm² with a framerate of 1 kHz, particle localization after the vibration correction, MSD&lt;sub&gt;x&lt;/sub&gt;&lt;sub&gt;y&lt;/sub&gt; at early lag time related to localization uncertainty of an immobilized particle, position histogram with a mesh size of 10 × 10 nm², and fluorescence intensity with localization uncertainty of 10 nm ........</td>
<td>95</td>
</tr>
<tr>
<td>4.3</td>
<td>Fluorescence image and two-dimensional trajectory of a PFBT nanoparticle in 38% glycerol/water solution at a framerate of 1 kHz, fluorescence emission intensity and the fwhm of the particle plotted with detection time, diffusion coefficient calculated by fitting lateral MSD, and histogram of lateral diffusion coefficients.................</td>
<td>99</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>Two-dimensional trajectories and MSD plots of selected single PFBT nanoparticles in various glycerol/water ratios by using different framerates, the corresponding diffusion coefficients calculated by fitting MSD to the free diffusion equation</td>
<td>102</td>
</tr>
<tr>
<td>4.5</td>
<td>Diffusion coefficients determined by MSD_{xy} method normalized to the values at maximum number of frame, versus number of frame of segmented trajectories of single PFBT nanoparticles in various glycerol/water ratios by using different framerates</td>
<td>105</td>
</tr>
<tr>
<td>4.6</td>
<td>Fluorescence images of a diffusing PFBT nanoparticle in 38% glycerol/water solution at different framerates</td>
<td>106</td>
</tr>
<tr>
<td>4.7</td>
<td>Influence of localization precision on the analysis of single particle trajectories in the case of Brownian motion</td>
<td>110</td>
</tr>
<tr>
<td>4.8</td>
<td>Synchronization of piezoelectric stage axial translation with fluorescence imaging</td>
<td>114</td>
</tr>
<tr>
<td>4.9</td>
<td>Fluorescence spot of a PFBT nanoparticle at focal plane and at 2 µm from the focal plane, the fwhm versus axial position by scanning an immobilized PFBT nanoparticle in two opposite directions, and axial localization error by the defocus imaging method</td>
<td>116</td>
</tr>
<tr>
<td>4.10</td>
<td>The fluorescence emission intensity and the fwhm of a PFBT nanoparticle in 90% glycerol/water solution at a framerate of 1 kHz, axial position of the particle calculated by defocused imaging along with lateral position, and MSD plots</td>
<td>119</td>
</tr>
<tr>
<td>5.1</td>
<td>Particle trajectory simulated with free diffusion related to ( D = 1 , \mu m^2/s ) with a framerate of 1 kHz for 5000 frames, trajectory restriction of particle moving and reflecting to the wall of a sphere with 250 nm diameter, and simulated trajectory of confined diffusion generated with several individual pores</td>
<td>123</td>
</tr>
<tr>
<td>5.2</td>
<td>3D random-walk simulations with diffusion coefficient of 1 ( \mu m^2/s ), framerate at 200 Hz and number of frame of 2000, corresponding 2D particle trajectories with confined diffusion inside single pores separated by short free diffusion, and particle histogram of the trajectories with separated and overlapped pores</td>
<td>124</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>Simulated particle positions in a spherical pore with 250 nm in diameter and an ellipsoid pore with a rotating angle of 1 radian along lateral plane, and statistical boundary $4\sigma$</td>
<td>126</td>
</tr>
<tr>
<td>5.4</td>
<td>Pore size estimated by using position histogram of simulated confined trajectories at the $4\sigma$ statistical boundary using various numbers of frame</td>
<td>127</td>
</tr>
<tr>
<td>5.5</td>
<td>3D random-walk simulations with diffusion coefficient of 1 µm$^2$/s, framerate at 1 kHz and number of frame of 5000, corresponding 2D particle trajectories, particle histogram, and the MSD of pore 4 and 5 fitted to confined diffusion</td>
<td>129</td>
</tr>
<tr>
<td>5.6</td>
<td>Sketch of reflective boundary effect with the observed particle displacement per frame shorter than the actual displacement</td>
<td>130</td>
</tr>
<tr>
<td>5.7</td>
<td>Observed diffusion coefficients of confined simulations with various input diffusion coefficients in a confined sphere using a framerate of 1 kHz and 200 Hz</td>
<td>132</td>
</tr>
<tr>
<td>5.8</td>
<td>Two-dimensional particle trajectory of selected particle in agarose gel at a framerate of 200 Hz, corresponding particle histogram, MSD plot fitted to anomalous subdiffusion, and fluorescence intensity of the particle</td>
<td>135</td>
</tr>
<tr>
<td>5.9</td>
<td>Partial two-dimensional trajectory of Figure 5.8 and position histogram of pore 7 and 8, and fluorescence intensity and fwhm of the particle moving from pore 8 to 7</td>
<td>137</td>
</tr>
<tr>
<td>5.10</td>
<td>Two-dimensional particle trajectory of selected particle in agarose gel at a framerate of 1 kHz, corresponding particle histogram, MSD of pore 9 fitted to confined diffusion, and fluorescence intensity of the particle</td>
<td>139</td>
</tr>
<tr>
<td>5.11</td>
<td>Histogram of pore size determined by position histogram</td>
<td>141</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>High-speed tracking at 1-kHz framerate of CPNs with anomalous subdiffusion in agarose gel, porous structure and particle dynamics analyzed by using position histogram and MSD methods</td>
<td>145</td>
</tr>
<tr>
<td>A-1</td>
<td>Transmission efficiency of a 500-nm dichroic mirror</td>
<td>155</td>
</tr>
<tr>
<td>A-2</td>
<td>Transmission efficiency of typical achromat objectives with 100× and 1.25 NA (Olympus)</td>
<td>156</td>
</tr>
<tr>
<td>A-3</td>
<td>Distance between two coverslips determined from the focused fwhm of two PFBT particles immobilized on each coverslips</td>
<td>156</td>
</tr>
<tr>
<td>B-1</td>
<td>Matlab script for controlling a piezoelectric movement in z direction by generating a staircase voltage by a DAQ device</td>
<td>157</td>
</tr>
<tr>
<td>B-2</td>
<td>Matlab script for random-walk simulations and MSD calculations</td>
<td>159</td>
</tr>
<tr>
<td>B-3</td>
<td>Matlab script for confined diffusion simulations and MSD calculations</td>
<td>160</td>
</tr>
</tbody>
</table>
CHAPTER ONE
INTRODUCTION

Fluorescence microscopy has become an essential technique for subcellular structural imaging, and measuring molecular function and dynamic behavior of nanoscale biomolecules. Localization of single molecules by an analysis of the fluorescence spot yields high resolution images up to several nanometers per integration time. This method is one of the techniques known as super-resolved fluorescence microscopy or super-resolution microscopy, which brings optical microscopy into nanoscale resolution breaking the diffraction limit found in conventional light microscopy. Due to the state-of-the-art discovery and significant development, the Nobel Prize in Chemistry was awarded to Eric Betzig, W.E. Moerner and Stefan Hell in 2014. The super-resolution microscopy has been widely applied to achieve better understanding in biological studies, for instance, membrane dynamics,\textsuperscript{1–3} motor protein kinetics,\textsuperscript{4,5} or uptake pathway of pharmaceutical nanoparticles.\textsuperscript{6}

To further improve the spatiotemporal resolution in structural imaging or particle tracking, more highly luminescent and photostable nanoparticles are the key development. Fluorescent particles could be attached to a small biomolecule; however, the particle size should be within a few tens of nanometer in diameter with minimal or acceptable effect on the motion of the molecule of interest. Particle tracking obtained from video imaging of single molecules could describe such important information of cellular processes and structures. Conjugated polymer nanoparticles are the focus of this
work due to their excellent photophysical properties such as high brightness, large absorption cross section with high emission rate, and low saturated excitation intensity. These nanoparticles could be effectively used to investigate the dynamic behavior of individual small biomolecules or motor proteins with high spatiotemporal resolution in order to help achieve a better understanding of cellular function, particle trafficking, and membrane dynamics.

1.1 Diffraction limit in conventional light microscopy

Since the first reported observations in biology by using invented single-lens and compound microscopes in the seventeenth century, manufacturing breakthroughs and technological developments have led to significantly advanced microscope designs with improved image quality. However, microscopes with glass-based objectives are hindered by a limit in optical resolution related to the diffraction of visible light wavefronts as they pass through the circular aperture at the rear focal plane of the objective. Thus, the highest achievable point-to-point resolution that can be obtained with a conventional optical microscope is governed by fundamental laws in physical optics. The resolution cannot be easily improved by rational alterations in objective lens or aperture design. These resolution limitations are often referred to as the diffraction limit or barrier, which restricts the ability of optical instruments to distinguish between two objects separated by a lateral distance less than approximately half the wavelength of light used to image the specimen.
The process of diffraction involves the spreading of light waves when they interact with the complicated structures of a sample. The image observed in the microscope are composed of highly overlapping wavefronts of multiple point sources of light. Thus, the microscope diffraction barrier could be discussed by considering a single point source of light through the optical elements. The transmitted light or fluorescence emission becomes diffracted and produces an image of the point source broadened into a diffraction pattern. The image of a point emitter is known as point-spread function (PSF) that is referred to as an Airy disk named after the British astronomer George Airy. By using a high objective magnification, the diffraction pattern of the point object is observed to consist of a central spot surrounded by a series of diffraction rings in which the inner rings are brighter than the outer. The resolution was firstly defined by the radius of the diffraction Airy disk in the lateral image plane, proposed by the German physicist Ernst Abbe following the equation,

\[
\text{Abbe resolution}_{xy} = \frac{\lambda}{2 \cdot \text{NA}},
\]

where \( \lambda \) is the wavelength of fluorescence emission. The objective numerical aperture \( \text{NA} = n \cdot \sin(\theta) \) is defined by the refractive index \( n \) of the imaging medium (usually air, water, or oil) and the aperture angle \( \theta \). The equation was later refined by the British physicist Lord Rayleigh to quantitate the measure of separation necessary between two point-like objects in order to distinguish them as individual emitters. The Rayleigh criterion defines the resolution of two point sources observed in the microscope of which the central spot from one overlaps with the first dark ring from the other point source.
The two point emitters can be resolved if the distance between their point-spread functions is greater than the Rayleigh criterion,

\[
\text{Rayleigh criterion}_{xy} = \frac{0.61 \cdot \lambda}{\text{NA}.}
\] (2)

The difference between the two definitions in resolution above for two distinct objects is small in practice. Under typical fluorescence emission wavelength at about 500 nm, lateral resolution is about 250 nm by using a high numerical aperture objective of 1.25.

The PSF geometry is elongated in axial dimension due to the non-symmetrical wavefront that emerges from the microscope objective. The axial resolution in optical microscopy can be calculated by the equation,

\[
\text{Abbe resolution}_z = \frac{2 \cdot \lambda}{\text{NA}^2}.
\] (3)

This results about 500 nm in axial direction that is worse than in lateral plane. There are a few criterions for such resolution limits: the Rayleigh and Abbe criterions, as described above, as well as the Sparrow criterion. The Sparrow is used more often in astronomy, while the Rayleigh and Abbe criterions are more conventional in optical microscopy.

The diffraction limit in optical microscopy challenged the advancement in biological studies in the past decades. The structural imaging and dynamic behavior of small biomolecules such as cellular organelle or proteins could not be resolved by using conventional confocal and wide-field fluorescence microscopy. Recently, the diffraction barrier has been overcome by using fluorescence techniques that drastically improve the spatial resolution to tens of nanometer or less per image.
1.2 Basic concepts in fluorescence spectroscopy

When molecules containing π electrons are exposed to an illumination source typically in the wavelength range of ultraviolet to visible light, some of the light energy that match a possible electronic transition within the molecule will be absorbed. The π electrons are then promoted from the ground state $S_0$ to an excited state $S_1 (\pi^*)$. The absorption property of the molecule can be determined from bulk measurements.

Schematic illustration of particle suspension in solution and absorption intensity is shown in Figure 1.1. The Beer-Lambert law and absorption cross section can be derived from the probability of the light intensity decreased by the absorption of particles in small volume and the fraction of total absorption area per total area as,

$$\frac{\text{Absorbed intensity}}{\text{Scattered out}} = \frac{\text{Absorption area}}{\text{Total area}},$$

so

$$\frac{dl}{I_i} = \frac{\sigma n}{a}, \quad (4)$$

where $dl$ is the intensity absorbed by particles in the small volume, $I_i$ is the entering intensity. $\sigma$ is absorption cross section per nanoparticle in units cm$^2$, and $n$ is number of particles in the slice, thus $\sigma n$ is total fractional area for all molecules in the block where light gets absorbed. $a$ is total area of the slice in cm$^2$. The number of particle in the slice can be calculated by using particle concentration as $n = cal \cdot N_A / 1000$, where $c$ is concentration of particle in solution in molar, $l$ is path length in cm, and $N_A$ is Avogadro’s number. Substitution of this equation to Equation (4), followed by the integration yields
\[ -\ln\left(\frac{I}{I_0}\right) = \left(\frac{\sigma N_A}{1000}\right) \cdot cl, \]

determining that can be converted to the logarithm with base 10 as,

\[ -\log\left(\frac{I}{I_0}\right) = \left(\frac{\sigma N_A \log e}{1000}\right) \cdot cl. \]

The absorbance \( (A) \) is determined by measuring the amount of light that a sample absorbs related to the transmittance \( T \equiv \frac{I}{I_0} \) so that \( A = -\log T = -\log(\frac{I}{I_0}) \). Thus, the Beer-Lambert law is expressed by

\[ A = \varepsilon cl, \quad (5) \]

where \( \varepsilon \) is molar absorptivity per polymer nanoparticle in M\(^{-1}\) cm\(^{-1}\) defined as

\[ \sigma = \frac{2303\varepsilon}{N_A}. \quad (6) \]

The fluorescence emission rate of a particle can be maximized by increasing the probability that a single molecule can capture photons from the incident laser beam. High absorption cross section refers to the ability of molecule or particle to efficiently absorb the photons from the incident light beam, therefore developing a molecule or particle with high absorption cross section is important to improve the detection spatial resolution in single molecules.
After the molecule or particle is excited to excited state (S₁) by the absorption in specific area of molecule, it can undergo various photophysical processes, such as fluorescence, phosphorescence, energy transfer, and vibration relaxation to a lower energy level. Fluorescence occurs when molecule returns from the excited state (S₁) to ground state (S₀) through a radiative decay process with photon emission.

The fluorescence quantum efficiency of fluorophores is characterized by fluorescence quantum yield (\(\phi_f\)), which is defined as the ratio of number of emitted photons to number of absorbed photons. Fluorescence quantum yield can be expressed with the rate constants in the radiative and non-radiative relaxation mechanism as,
where $k_r$ is radiative rate in fluorescence emission. $k_{nr}$ is non-radiative rate, which is the sum of all non-radiative relaxation processes: intersystem crossing rate $k_{isc}$, internal conversion rate $k_{ic}$, photobleaching rate $k_{b}$, and energy transfer rate $k_{et}$. For typical fluorophores, $k_r + k_{ic}$ is much higher than $k_{isc} + k_{b}$, and $k_r$ is similar to or ideally higher than $k_{ic}$ so that the quantum yield for most useful fluorescent labelling molecules ranges from a few percent to nearly 100% such as those in rhodamine 6G,\textsuperscript{7} and coumarin 461 derivatives.\textsuperscript{8} The fluorescence quantum yield can be experimentally measured by relative or absolute determinations using a conventional fluorescence spectrometer commonly with fluorescein as standard, or photoacoustic calorimetry.\textsuperscript{9}

The fluorescence lifetime ($\tau_f$) of fluorophores is the average time that the molecule spends in the excited state before returning to the ground state. It can be expressed with the rate constants of excited state depleting processes by the equation,

$$\tau_f = \frac{1}{k_r + k_{nr}}.$$  

The number of excitation and fluorescence emission cycles per unit time is decreased for molecules that have a long fluorescence lifetime. This can limit the saturated emission rate, which is important for some demanding applications such as high-speed imaging or tracking. Thus, fluorophores with lifetimes in the range of picoseconds to several nanoseconds and high fluorescence quantum yields are preferable. The fluorescence lifetime can be calculated from the experimental fluorescence decay curve measured by time-correlated single photon counting method.\textsuperscript{10} From Equations (7) and (8), the
radiative rate constant can be estimated by \( k_r = \frac{\phi_f}{\tau_f} \). Typical conjugated polymer nanoparticles exhibit a fluorescence radiative rate of \( 10^8 - 10^9 \text{ s}^{-1} \) similar to or somewhat higher than that of typical fluorescent dyes, while single quantum dots emit at rates about 2 orders of magnitude lower.\(^{11}\)

### 1.3 Conventional fluorescent molecules and nanoparticles

Fluorescent molecules commonly used in biological applications such as particle tracking and super-resolution imaging consist of three main categories: organic dyes, quantum dots, and fluorescent proteins. Organic fluorescent dyes, such as Alexa dyes\(^{12}\) and cyanine derivatives\(^{13}\) are typically small in size of about 0.5 nm.\(^{14}\) Although the advantages of those conventional organic dyes, such as well-studied photophysical properties and their commercial availability, are convenient for researchers, their applications in single molecule analysis is limited due to the low signal level and rapid photobleaching.\(^{14}\) In order to improve their photostability, small dye molecules were loaded into a microscale particle such as polystyrene beads\(^{15}\) and mesoporous silica nanoparticles.\(^{16,17}\) Those dye-loaded particles are brighter and more photostable compared to unprotected organic dye molecules. However, the dye loading concentration is limited in order to minimize the dye aggregation and self-quenching problem. In addition, the size of typical dye-loaded beads should be controlled to be within tens of nanometer in diameter in order to minimize the particle size effect found in drug delivery systems or cell uptake mechanisms such as liposomes.\(^{18,19}\)
Colloidal semiconductor quantum dots (QDs) such as CdSe, ZnSe and CdTe are another category of fluorescent probes commonly used in single-molecule imaging applications.\textsuperscript{20,21} They have tunable sizes typically about 4 – 8 nm in diameter, narrow and symmetric emission bandwidth of about 30 – 90 nm,\textsuperscript{14} and size dependent emission wavelengths or quantum size effect related to their name, which makes them a favorable type of fluorophores. However, to increase the stability and biocompatibility, QDs typically require an inorganic shell and an encapsulation layer, which would increase the particle size to 20 – 30 nm. In addition, the blinking behavior of QDs is not preferable in particle tracking and could reduce the saturated emission rate to a few kHz or tens of kHz.\textsuperscript{22}

Fluorescent proteins such as green fluorescent proteins (GFPs) are the most frequently used labeling methods for imaging of live cells.\textsuperscript{23,24} Typical fluorescent proteins with molecular weight of 25 – 30 kDa are composed of hundreds of amino acids. Fluorescent proteins can be genetically coded to target specific biomolecules with high selectivity, and have desirable biocompatibility for living cells.\textsuperscript{25} However, their relatively low brightness and reduced photostability compared to small molecule fluorophores are disadvantages for long-term detection, such as particle tracking applications. Due to the limitations of current probes and the rapid expansion of fluorescence microscopy applications, the development of brighter and more photostable fluorescent labels is still desirable for advanced fluorescence imaging applications.
1.4 Conjugated polymers and conjugated polymer nanoparticles

The semiconducting properties of conjugated polymers were first observed in 1977 by Heeger, MacDiarmid and Shirakawa, and later they were awarded the Nobel Prize in Chemistry in 2000 for their discovery and development of conductive polymers.26 It was found that polyacetylene films increase their conductivity tremendously when they are exposed to iodine vapor, from a conductivity value at the lower end of the semiconducting range up to values comparable to metals with the increase of seventeen orders of magnitude.27 Since then, their fluorescent and semiconducting properties have generated interest in optoelectronic applications such as polymer light-emitting devices, displays and photovoltaic cells because of their light weight and flexibility.27,28

1) π-conjugated systems

Conjugated polymers are a special class of polymers, which contain alternating single and double bonds along the polymer backbone. Polyfluorenes (PFs), polyphenylene vinylenes (PPVs), polythiophenes (PTs) and polyphenyl ethynylenes (PPEs) are commonly used as conjugated polymer backbones. The π electrons in conjugated polymers can be delocalized along the length of polymer backbone,29 which makes conjugated polymers behave as organic semiconductors. The π (bonding) and π* (antibonding) orbitals form delocalized valence and conduction wavefunctions, which support mobile charge carriers. For conjugated polymers, structural disorder, such as kinks, bends, or twisting in the polymer chain as well as electron correlation effects.30
thermal disorder, and Peierls or dimerization distortion (resulting from electron-phonon coupling),\textsuperscript{31} will break the $\pi$-conjugation along the polymer backbone and lead to a typical conjugation length of 4 – 10 monomer units.\textsuperscript{32} For the case of thermally-induced disorder, according to Hückel theory, the relationship between HOMO-LUMO energy gap ($\Delta E$) related to bonding and antibonding energy levels, and number of $\pi$-electrons ($n$) is expressed by the equation,

$$\Delta E = -4\beta \sin \frac{\pi}{2(n+1)},$$

(9)

where $\beta$ is the Hückel parameter with a literature value of $-3.36$ eV per molecule.\textsuperscript{33} If we assume there are 10 delocalized $\pi$-electrons in each monomer unit, thermal energy ($k_BT$) per monomer unit at room temperature (298 K) of $\sim 0.26$ eV is obtained. Thus, thermal disorder-induced conjugation length will have $\sim 80$ delocalized $\pi$-electrons or 8 monomer units. Since the energy gap of conjugated polymers is related to number of delocalized $\pi$-electrons, the conjugation length dictates the photophysical properties of the polymer, such as absorption and emission spectra. For conjugated polymers, the energy gap is in the range of 1.5 to 3 eV,\textsuperscript{34} corresponding to the absorbance range of UV-visible light and near infrared, similar to those of typical inorganic semiconductors such as CdSe cluster (2.4 eV).\textsuperscript{35} In addition, the chemical modification and the inclusion of heteroatoms within or directly bonded to the backbone, such as O, N, or S, allow tuning of photophysical and chemical properties. For example, electrochemical potentials of cyano-substituted PPV (CN-PPV) were negatively shifted by 0.6 V as a result of the electron withdrawing effect of cyano side group.\textsuperscript{36} More importantly, heteroatoms have non-bonding electrons (n)
that give rise to n-\(\pi^*\) transitions, which are often much lower in energy than \(\pi-\pi^*\) transitions. Thus, most attempts to tune the bandgap of conjugated polymers focus on varying heteroatoms rather than increasing the conjugation length, which is more difficult to control. Conjugated polymers can be modified by attaching sidechains, such as alkyl groups, \((\text{CH}_2)_n\text{CH}_3\), to the phenylene rings, resulting in high solubility in desired solvents and tunable absorption, emission and charge transport.\(^{37,38}\)

### 2) Applications of conjugated polymers

Semiconducting \(\pi\)-conjugated polymers are optically and electrically active materials with many applications, ranging from electronic devices\(^{39,40}\) and sensor\(^{41,42}\) to tissue engineering.\(^{43}\) Electroluminescence from conjugated polymers was first reported by Burroughes and coworkers in 1990,\(^{29}\) using PPV as the single semiconductor layer between metallic electrodes in light-emitting devices (LEDs). PPV has a band gap of about 2.5 eV and emits yellow-green light. Indium-tin oxide (ITO) layer functions as a transparent positive electrode, and allows the light generated within the diode or polymer to leave the device. LED operation is achieved when the diode is biased sufficiently to achieve injection of positive and negative charge carriers from opposite electrodes. Photon emission is generated by the capture of oppositely charged carriers within the region of the polymer layer. The energy gap of conjugated polymers can be modified by using functionalized polymer such as cyano-derivatives of PPV,\(^{44}\) resulting in various emission colors of the LED.
Another application of semiconducting polymers is as photovoltaic diodes or polymer solar cells. Photovoltaics are semiconducting materials that absorb light at energies above the band gap, leading to the separation of positive and negative charge carriers. These charges are collected at opposite electrodes, resulting in a photocurrent. A pair of conjugated polymers with different electron affinities becomes energetically favorable for an electron to transfer from one molecule (donor) to another (acceptor). CN-PPV and its derivatives have been used as an electron acceptor due to the decreased energy levels of valence and conduction bands caused by cyano side group. Meanwhile, MEH-PPV has been used as an electron donor due to the increased energy levels caused by the electron-donating effect of the methoxy groups. A heterojunction made from CN-PPV and MEH-PPV therefore causes excitons to split, with the holes moving into the MEH-PPV and the electrons moving into the CN-PPV. With appropriate collection of the generated charges, this system forms an efficient photodiode. Recent developments in blended heterojunction polymers such as poly(benzothiadiazole) derivative PDTP-DFBT as a donor polymer with a low bandgap of 1.38 eV and fullerene PCBM as an acceptor result in power conversion efficiency higher than 10%.

3) Conjugated polymer nanoparticles

The conjugated polymer chains can be formed into nanoparticle by using nano-precipitation, mini-emulsion, or self-assembly methods, maintaining excellent photophysical properties of conjugated polymers such as high molar absorptivity and high emission rate. The nano-precipitation method is a modification of the reprecipitation
method, where conjugated polymers dissolved in a good solvent are rapidly added to an excess of poor solvent under sonication. Conjugated polymer nanoparticles (CPNs) as known as polymer dots are another promising category of fluorescent probes in biomedical analysis and other advanced fluorescence imaging and analysis applications, because of their small sizes, tunable emission wavelengths, high absorption coefficients, and excellent fluorescence efficiencies. Due to these advantages, CPNs have already been demonstrated in a number of fluorescence-based applications, such as multiphoton fluorescence imaging, single nanoparticle sensors, photoswitching nanoparticles, photodynamic therapy, and particle tracking.

We have developed brightly fluorescent conjugated polymer nanoparticles consisting of one or more π-conjugated polymer molecules. CPNs can be prepared with diameters ranging from 5 to 50 nm achieving high fluorescence brightness to volume ratio. Absorption cross section can be obtained higher than $10^{-13}$ cm$^2$ per particle based on ~300 polymer molecules in a nanoparticle and saturated emission rate of ~10$^8$ photons per particle per second have been observed for CPNs with 22 nm in diameter under a blue excitation. Typical fluorescent dyes exhibit much lower absorption cross section about $10^{-17}$ to $10^{-16}$ cm$^2$ per molecule, meanwhile quantum dots exhibit about $10^{-15}$ to $10^{-14}$ cm$^2$ per particle. Hence, the absorption cross section of CPNs is 10 – 100 times larger than that of QDs and about 3 – 4 orders of magnitude larger than typical organic fluorescent dyes. CPNs also exhibit a fluorescent emission rate about 10 times higher than that of QDs. Due to the brightness and photostability of CPNs, previous reported results of our lab show that three-dimensional tracking of single 15-nm
diameter particles at an acquisition rate of 50 Hz has shown the Brownian motion of the particles in >98% glycerol/water solution. The tracking uncertainty was less than 4 nm per frame in the lateral plane while the third dimension was determined by using defocused imaging with ~20 nm uncertainty. Moreover, the motion of individual charge carriers in CPNs was tracked by single polaron tracking microscopy. The charge carrier is related to the generation of a dark spot in the nanoparticle by superquenching.

1.5 Single-molecule spectroscopy

Single-molecule spectroscopy (SMS) allows exactly one molecule in a condensed phase sample to be observed by using tunable optical radiation. To probe the molecule, a laser beam is used to pump an electronic transition of the single molecule resonant with the optical wavelength, and the resulting absorption is detected by fluorescence excitation. Typical ensemble measurements such as fluorescence recovery after photobleaching yield the average value of an experimental parameter (i.e., diffusion coefficient) for a large number of molecules. In contrast, SMS completely removes the ensemble averaging, thus the resulting information is more useful than the average value.

SMS is accomplished by two basic steps: only one molecule is in resonance in the volume probed by the laser, and a signal-to-noise ratio for the single-molecule signal is greater than unity for a reasonable averaging time. Detecting SMS fluorescence requires a device that can detect single photon arrivals with minimal dark noise. There are two classes of detectors for SMS experiments: single-element detectors (i.e., avalanche photodiodes) or two-dimensional array detectors (i.e., CCD). Several microscopic
configurations with photon detector have been demonstrated to satisfy the basic requirements for SMS. Microscopic techniques include scanning methods such as near-field scanning optical microscopy (NSOM) and confocal microscopy, and wide-field methods such as total internal reflection and epifluorescence microscopy. For NSOM, a laser spot smaller than the diffraction limit is generated by using an aperture with a diameter much smaller than the wavelength of light. The emitted light that propagates through this small hole is then detected with the spatial resolution related to the sub-wavelength aperture and the axial distance from this aperture to the sample. NSOM techniques operating in the fluorescence mode have been applied for the molecular imaging, such as biomolecules in cellular membrane, and fluorescent nanoparticles on a film. For the wide-field methods, an epifluorescence SMS microscope is an example in this field that can be constructed from commonly available commercial microscopes. An illumination beam is directed to the back aperture of the microscope objective, creating a laser spot with appropriate diameter typically about 3 microns. Fluorescence emission is collected through the same objective and directed to a CCD camera. One of the applications of wide-field SMS is the study of motion of biomolecules in the membrane of single cells.

A key challenge in SMS is to obtain sufficiently high signal levels to obtain useful information. It is often a photon-starved technique, due to the typically low emission rates of fluorophores under practical conditions, as well as the background from autofluorescence and leakage of excitation light through the optical filters. Typically, many otherwise useful fluorophores are not sufficiently bright or photostable for single-
molecule spectroscopy or related methods such as molecule or particle tracking. As such, the fluorescence brightness of fluorophores is an important and fundamental characteristic for single-molecule methods.

1.6 Particle tracking

Particle tracking has been developed to determine the spatial trajectories of such individual nanoparticles with typical motion in submicron per second. A particle-tracking experiment begins with video recording a time series of images of isolated single molecules or fluorescently labeled particles as shown in Figure 1.2. Subsequently, each individual particle that stands out from the background according to certain criteria is identified in every image. The particle coordinates are estimated by several localization methods, such as cross-correlation of subsequent images, calculating the center-of-mass of the object of interest, and directly fitting Gaussian curves to the intensity profile. For point sources, direct Gaussian fit to the intensity distribution is the superior algorithm in terms of both accuracy and precision. Thus, this method is the best algorithm for tracking single fluorophores. The trajectories of the individual particles are then constructed by linking local nearest neighbors from consecutive particle localizations. Particle tracking from fluorescence image data could be automatically performed by computational algorithms implemented in various available software. The particle trajectories could describe important information about biomolecular processes, subcellular structures or material morphology, for instance, membrane dynamics.
motor protein kinetics,\textsuperscript{4,5} drug and gene carriers,\textsuperscript{71,72} porous material structure,\textsuperscript{73,74} and catalytic activities of biological enzymes.\textsuperscript{75}  

Fluorescent molecules have been used to attach bio-macromolecules of interest such as QDs or dyes conjugated to proteins,\textsuperscript{76–78} actin filament,\textsuperscript{79} RNA,\textsuperscript{80} or membrane lipid\textsuperscript{81,82} resulting in nanoscale particle trajectories obtained by molecule localization. Particle tracking data analysis can involve in sorting trajectories or segments of trajectories into various modes of motion and to find the distribution of quantities characterizing the motion, such as the diffusion coefficient, velocity, anomalous diffusion exponent, and confinement length. However, during a measurement, typical QDs and dyes may disappear as a result of photoblinking or photobleaching, resulting in discontinuous particle trajectories, which might lose important information of particle motion.\textsuperscript{78,82}
Figure 1.2 Particle tracking with resulting particle trajectory constructed from particle localization of each consecutive images.
1.7 Single-molecule localization microscopy

In fluorescence microscopy, the image of a point emitter is shown in a point-spread function of Airy disk due to the diffraction limit of light by directing through microscope objective. Within recent years, different far-field microscopic approaches have been introduced that have found a way to bypass the diffraction limit exploiting concepts to distinguish fluorescence emission of fluorophores and thus localize their positions individually. The common approaches of single-molecule localization based methods resolve the individual components by temporally modulating the emission of individual fluorescent molecules by photoactivation, photoconversion, or photoswitching, as used in photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM).

Single-molecule localization microscopy is achieved by temporarily separating fluorescent emitters in focal volume using different methods as discussed above. To find the position of an isolated molecule, the point-spread function in an array of 2D fluorescence intensity of the molecule can be fitted to various models such as Richards-Wolf model,

\[ I(x,y) = I_0 \cdot \exp \left[ -\frac{(x - \mu_x)^2 + (y - \mu_y)^2}{2s^2} \right] + C, \]  

(10)
where $I$ is fluorescence intensity in counts, $\mu_x$ and $\mu_y$ are particle centroid coordinate, $s^2$ is the variance related to the width of fluorescence spot, and $c$ is offset. The schematic process to find particle position or centroid is shown in Figure 1.3. In the shot-noise or photon counting limit, the localization uncertainty ($\sigma$) is related to the particle brightness following the equation $\sigma = s / \sqrt{N}$, where $N$ is number of detected photons per particle. The details of localization uncertainty calculations will be discussed in the chapter 4. For typical CPNs, the localization uncertainty of about 0.3 nm per frame was obtained at $N = 2 \times 10^5$ photons per particle per image at an acquisition rate of 50 Hz. At a kilohertz framerate, CPNs exhibit ~500 detected photons per frame in this work, yielding a localization uncertainty of 10 nm per frame along lateral plane, while the localization uncertainty of typical QDs was obtained at about 30 nm per frame. The localization uncertainty indicates that super-resolution imaging using single-molecule localization dramatically improves the spatial resolution of conventional light microscopy of ~250 nm, by 2 – 3 orders of magnitude.
Figure 1.3 Schematic illustration of single-molecule localization by fitting 2D fluorescence image of a single fluorophore to 2D Gaussian function to obtain the center position.
1.8 Measurements of particle diffusion

The motion of molecules can be measured by using fluorescence microscopy techniques, such as fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). FRAP typically gives the averaged dynamic properties of a large number of molecules detected in sample volume, while FCS averages the dynamic behavior of a small number of molecules within observation volume. FRAP technique begins by using fluorescent probes attached to a biomolecule of interest, for example membrane proteins labeled with coumarin dye molecules, and GFP-tagged nuclear or membrane proteins. A fluorescent image is obtained by using an excitation light at a wavelength related to the absorption of fluorophores. Then a light pulse is directed to a small area of the sample, resulting the fluorophores in this region receiving high intensity illumination. This causes the fluorophores photobleached shown as a dark spot in the image. As particle motion proceeds, fluorescent probes will diffuse throughout the sample and replace the bleached probes while the bleached area is monitored. When motion due to active transport or unidirectional flow can be neglected, particle mobility is due to Brownian motion. The mobility is expressed as the diffusion constant $D$, which can be calculated from the equation, \[ D = \frac{\omega^2 \gamma}{4\tau_D}, \] where $\omega$ is defined as the radius of the focused Gaussian-profile laser beam at the $e^{-2}$ intensity, $\gamma$ is a correction factor for the fraction of bleaching, and $\tau$ is the diffusion time.
Thus, the resulting diffusion coefficient is averaged from the mobility of ensemble molecules.

FRAP has been widely used to study the lateral diffusion of membrane-associated proteins; however, the bulk diffusion coefficients from this method cannot be used to distinguish individual molecular motion in such anomalous diffusion that is typically found in diffusive behavior of cellular proteins. Membrane proteins and biomolecules in a cellular context tend to exhibit in different motion due to their specific function and the presence of various subcellular structures. Proposed models have been developed to explain the anomalous diffusion of membrane proteins including diffusion with static obstacles such as immobilized proteins,\textsuperscript{91} binding to obstacles,\textsuperscript{92} interaction with lipid rafts,\textsuperscript{93} and molecular crowding.\textsuperscript{94} These models are evident in the deviations of mean squared displacement from the linear dependence on lag time due to conventional Brownian motion. To achieve better understanding of the anomalous diffusion of membrane proteins and high-mobility biomolecules in cellular cytoplasm, single-molecule spectroscopy could provide crucial information that could not be obtained with a bulk sample.\textsuperscript{95,96} For example, the results from FRAP shown that the diffusion coefficient of E-cadherin receptor in plasma membrane is $3.4 \times 10^{-11}$ cm$^2$/s under the effect of concentrated calcium ions.\textsuperscript{97} On the other hand, particle tracking using gold nanoparticles as the probe can distinguish particles undergoing different diffusion modes, excluding particles in the stationary mode. The resulting diffusion coefficients from particle tracking based on the plot of mean squared displacement against lag time are $6.5 \times 10^{-11}$ and $5.5 \times 10^{-11}$ cm$^2$/s for particles undergoing free and confined diffusions,
respectively. In addition, the confined length of 300 – 600 nm was obtained from tracked particles with confined diffusion mode. From the particle tracking results in different calcium ion concentrations, it was found that calcium-induced differentiation decreases the percentages of the receptor molecules in the directed diffusion and the stationary modes by ~50%, related to the receptors bound to the cytoskeleton. The comparison of the results from the ensemble measurement and the particle tracking based on single particle localization shows the advantage of particle tracking method providing more useful information of particle mobility and cellular structure.

Tracking trajectories of individual particles constructed from the particle position in each frame are commonly represented by the mean squared displacement (MSD) in one dimension as,

$$\text{MSD}_{1D}(\tau) = \langle (x(\tau) - x_0)^2 \rangle, \quad (12)$$

where $x$ is particle position in one dimension, $x_0$ is the reference position, and $\tau$ is lag time. This equation can also be written by

$$\text{MSD}_{1D}(i) = \frac{1}{N-i} \sum_{j=1}^{N-i} (x_{i+j} - x_j)^2, \quad (13)$$

where $N$ is the number of particle positions to be averaged. The MSD in three dimension is calculated by the linear combination of the MSD along each dimension, following the equation,

$$\text{MSD}_{3D}(\tau) = \langle (x(\tau) - x_0)^2 \rangle + \langle (y(\tau) - y_0)^2 \rangle + \langle (z(\tau) - z_0)^2 \rangle, \quad (14)$$

revealing four basic motion types: free, confined, directed, or anomalous diffusion as shown in Figure 1.4. Free diffusion also known as Brownian diffusion takes place when
particle movements occur completely unrestricted by the thermodynamic driving force. According to Einstein’s theory of Brownian motion, the MSD increases linearly with lag time, as given by the expression,

\[ \text{MSD}(\tau) = 2dD\tau, \quad (15) \]

where \( d \) is dimensionality of the system, and \( D \) is diffusion coefficient. Free diffusion is a simple particle motion, which can be found in particle motion in a stationary fluid\(^98\) or as a part of biomolecule motion undergoing the combination of free and confined diffusion.\(^99\)-\(^101\) Directed motion is an active process such as a flow motion and can be clearly observed when small molecules or vesicles are transported along actin filaments inside living cells.\(^102\) The MSD of directed diffusion can be expressed by the combination of free diffusion as shown in Equation (15) and the flow velocity, as given by the equation,

\[ \text{MSD}(\tau) = 2dD\tau + (V\tau)^2, \quad (16) \]

where \( V \) is drift velocity. Confined diffusion is observable for particles trapped in confined space, where the MSD exhibits a flat profile at a late lag time as,

\[ \text{MSD}(\tau) = \theta^2 \left[ 1 - A \cdot \exp\left( \frac{-2dD\tau}{\theta^2} \right) \right], \quad (17) \]

where \( \theta \) is confined radius, and \( A \) is constant. Anomalous diffusion particularly subdiffusion as the combination of free and confined diffusions is commonly found in diffusive behavior of biomolecules. Typical biomolecules such as proteins can undergo partially confined motion to complete their specific functions, or free diffusion in cellular context or local environment. The MSD of anomalous subdiffusion follows the equation,
Where $\alpha < 1$. Although the FRAP data could be fitted well by the anomalous diffusion equation, particle tracking is more sensitive to anomalous diffusion than FRAP because in particle tracking every tracer is tested for anomalous diffusion individually, but FRAP averages over many tracers, some of which may be diffusing normally, and others anomalously. Moreover, the parameter $\alpha$ is not useful to explain particle dynamics unless particle trajectories can be split in small fractions of free and confined diffusions, which can be obtained only by particle tracking technique. For example, particle tracking of neurotransmitter receptors labeled by quantum dots has shown free diffusion with diffusion coefficient of 0.1 $\mu$m$^2$/s due to the receptors rapidly diffusing in neuronal plasma membrane. Meanwhile, a fraction of the receptors interacts with protein gephyrin, resulting in confined diffusion with diffusion coefficient of 0.02 $\mu$m$^2$/s.
Figure 1.4 The relationship of MSD and lag time of various types of diffusion.
1.9 Research motivation

Previous studies have demonstrated that membrane proteins and biomolecules in a cellular context tend to exhibit in different motion due to their specific function and the presence of various subcellular structures. To achieve better understanding of this anomalous diffusion of membrane proteins and high-mobility biomolecules in cellular cytoplasm, particle tracking should be performed at high temporal resolution. The spatial resolution that is limited due to low particle brightness at short integration time should be improved. A variety of nanoscale particles have been used in tracking experiments in order to improve spatiotemporal resolution including colloidal particles in solution such as light scattering metal nanoparticles,\textsuperscript{97,103} single organic dyes,\textsuperscript{3} semiconducting quantum dots,\textsuperscript{20,76,78} or single fluorescent proteins,\textsuperscript{104} or these particles attaching to biomolecule of interest such as protein,\textsuperscript{105} DNA or virus.\textsuperscript{106} Although an individual lipid-conjugated QD can be detectable on the cell surface at high acquisition rate up to 1 kHz,\textsuperscript{107} the fluorescence brightness is limited by saturation effects and photoblinking, thus limiting the spatial resolution. Typical scattering metal nanoparticles exhibit small signal-to-background ratio and hydrodynamic drag of particles places an effective limit on temporal resolution because they are typically 50 nm in diameter or larger. Hence, a more highly luminescent and photostable nanoparticle could improve the resolution of particle tracking, while particle size should be within a few tens of nanometer in diameter in order to efficiently bind to a small biomolecule with minimal or acceptable effect on the motion of the molecule of interest.
In this work, we study the particle tracking of single CPNs by using high-framerate fluorescence imaging with low per-frame tracking uncertainty. The particles are freely diffusing in glycerol/water mixtures at the viscosity up to that of human blood at body temperature. High-speed video imaging at a kilohertz framerate can observe the particle displacement with diffusion coefficient ranging from 0.026 to 5.0 µm²/s. The average bright fluorescence emission of about 15000 photons per particle per millisecond exposure time yields a localization uncertainty of 10 nm per frame along the lateral plane. Particle trajectories in the third dimension were obtained by defocus imaging, which estimates the axial position of a defocused particle from the fluorescence spot width. In order to validate the tracking method, the diffusion coefficients obtained from MSD were compared to the expected values from theoretical Stokes-Einstein equation based on particle size and viscosity of the mixtures. Furthermore, the particle tracking in a porous gel has been performed to characterize the structure of nanopores and determine the diffusion dynamics inside the pores and channels. The pore size was determined by the boundary of position histogram corresponding to a particle trajectory that consists of several confined diffusions in individual pores. At a kilohertz framerate, the MSD of segmented particle trajectories with sufficient number of frame that is typically higher than 200 frames was fitted to the confined diffusion equation, obtaining an estimated pore size and diffusion coefficient of the particle inside the pore. The data analyses of pore size and particle dynamics by using specific boundary of position histogram and the MSD of confined diffusion were validated by performing random-walk simulations with experimental parameters. The results indicate that conjugated polymer nanoparticles are
promising for measuring the complex motion of individual small biomolecules in a complex, nanostructured environment, such as on a membrane or in organelles, with high spatial and temporal resolution.
CHAPTER TWO
EXPERIMENTAL METHOD

2.1 Materials

The copolymer poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-{2,1’,3}-thiadiazole)] (PFBT, MW 10,000, polydispersity 1.7) was purchased from ADS Dyes, Inc. (Quebec, Canada). Fluorescein (reference standard) was purchased from Life Technologies (Invitrogen). The solvent tetrahydrofuran (THF, anhydrous, 99.9%), sodium hydroxide (NaOH, SigmaUltra, 98%), and (3-aminopropyl)-trimethoxysilane (APTMS or APS, 97%) were purchased from Sigma-Aldrich (Milwaukee, WI). Glycerol (99.95%) was purchased from Alfa Aesar (Ward Hill, MA). Agarose powder was purchased from Acros Organics (New Jersey). All chemicals were used as provided without further purification.

Figure 2.1 Chemical structure of PFBT conjugated polymer

2.2 Preparation of nanoparticles

Conjugated polymer nanoparticles were prepared by a nano-precipitation procedure as described previously. This method has been modified from the
reprecipitation procedure developed by Kurokawa and co-workers,\textsuperscript{109} and it is chosen because of its simple procedure and good reproducibility. The reprecipitation method starts with dissolving hydrophobic conjugated polymers in a water-miscible solvent such as THF, and quickly injecting the polymer solution into deionized water. The rapid change of the polymer solubility in mixed solvent results in polymer chain collapse, leading to the formation of polymer nanoparticles. Then, THF solvent is removed, yielding a clear aqueous nanoparticle suspension. During the reprecipitation process, polymer aggregation forms small nanoparticles, and large aggregates. However, the combination of low precursor concentration with rapid mixing rate disfavors the aggregation and favors smaller nanoparticle creation. Higher polymer concentrations lead to larger nanoparticle sizes and a larger fraction of polymer lost by filtration of larger nanoparticle sizes.

For a PFBT nanoparticle preparation, 1000 ppm stock solution of PFBT in THF was prepared by dissolving PFBT in THF by stirring and the resulting solution was then diluted to 20 ppm. It should be noted that employing a lower concentration of conjugated polymer in THF will result in smaller nanoparticles of about 10 nm in diameter as described previously.\textsuperscript{110} After that, 2 mL of the solution was rapidly injected into 8 mL of water under mild sonication. The THF solvent was then removed under a vacuum pressure at \textasciitilde 5 psi with N\textsubscript{2} flow, and the nanoparticle suspension was filtered through a 0.1 \textmu m membrane filter (Millipore) to remove aggregates. Only 9\% of the polymer was removed by filtration, as determined by the decrease of the absorbance, indicating that
most of the polymer molecules formed nanoparticles. The resulting CPN solution was clear and stable for several months with no changes in spectroscopic characteristics.

Figure 2.2 Conjugated polymer nanoparticle preparation process by nano-precipitation method

2.3 Particle size and bulk spectroscopy measurements

1) Atomic force microscopy

Atomic force microscopy (AFM) was used to characterize the size distribution of prepared nanoparticles in this work. The samples were prepared on standard glass coverslips (Fisher Scientific) with the dimension of 25 × 25 mm² and the thickness of 0.12 mm. Those coverslips were cleaned in a bath of the solution of Nochromix (Godax Laboratories) in concentrated sulfuric acid for 1 hour. Then the coverslips were silanized
by functionalizing the surface with organofunctional alkoxy silane molecules. An amount of 50 μL of 5 mM freshly prepared (3-aminopropyl)-trimethoxysilane (APS) in ethanol solution was dropped onto the coverslip and left for about 2 min, followed by rinsing with deionized water. The coverslip was then submerged into a diluted nanoparticle suspension for an hour. Excess CPNs that are not adsorbed on the surface were removed with deionized water and the coverslip was dried with nitrogen gas.

AFM is one of the scanning microscopy methods that is widely used to characterize the surface properties of a material by scanning the surface with a sharp tip. The sharp tip with a tip radius of curvature on the order of nanometers is located at one end of a spring-like cantilever, which is typically silicon or silicon nitride. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever. AFM can be operated in a number of modes, depending on the application. In general, possible imaging modes are divided into contact modes and a variety of intermittent-contact modes where the cantilever is vibrated or oscillated at a given frequency. Intermittent mode was employed for the AFM scanning in this work, since it is typically suitable for samples that are weakly adhered to the surface. The intermittent mode scanning is performed by oscillating the cantilever in the z direction at or near the cantilever resonant frequency in the range of 70 – 200 kHz by using a piezoelectric ceramic. Topographic image of the surface is acquired by measuring the deflection of cantilever while operating a raster scan of the surface. The beam-deflection measurement uses laser light from a solid-state diode reflected off the back of the cantilever and collected by a position-sensitive detector. Frequency shift of the cantilever
from the original resonance frequency caused by uneven surface below the tip results in one photodiode collecting more light than the other photodiode. The cantilever oscillation amplitude is kept constant by moving the probe upward or downward in z direction during the raster scan of the sample surface along the xy direction. The movement is controlled by adjusting the tip distance from the surface through the feedback controller, called proportional-integral-derivative controller (PID). The topographic image of the scanned surface is assembled with a series of scanning lines while the piezo moves the tip along the sample.

In this work, isolated particles immobilized on a silanized coverslip were imaged with an Ambios Q250 multimode AFM in the intermittent mode. The scan range of the instrument is 40 μm in the xy direction, and 5.7 μm in the z direction. The scanning parameters: a scanning area of 5 × 5 μm² with 500 lines per scan, and a scan rate of 0.5 Hz with a pixel resolution of 10 nm, were employed in this study. The tip used in this work is Q-WM190 (NanoAndMore, Watsonville, CA) along with the intermittent mode with the mean resonance frequency of 190 kHz. The general guidelines for setting PID parameters are: to image flat surfaces with low scan rates over small area, low Integral and Proportional gains are adequate; conversely, higher gains are needed for rough surfaces at fasting scanning with large scan area. The typical PID parameters employed in this work were Integral at 300, Proportional at 450 and Derivative at 0. The setpoint was adjusted to obtain a damping amplitude of ~50%. Blank silanized coverslip was scanned as a control sample. For typical AFM images, the density of particles is about 10 particles per μm². Since the particles are presumably spherical, the particle height was taken as
particle diameter due to the higher vertical resolution more reliable than the lateral resolution that is also affected by the tip convolution broadening the image of small particles in lateral plane.

2) Dynamic light scattering

The size distribution of prepared nanoparticles in solution was also determined by dynamic light scattering (DLS) based on the fluctuation of light intensity scattered by particles in a small volume. Polarized laser is directed to a sample and scatters in all directions due to the Rayleigh scattering such that the particle size is one tenth smaller than the wavelength of the laser. At a fixed scattering angle deviated from the laser beam, a fraction of scattered light is detected by a photomultiplier that converts a fluctuation of intensity into voltage. Particles in solution are assumed to undergo Brownian motion related to thermally-driven collisions of solvent molecules with the relevant particles. From Stokes-Einstein equation, smaller particles will move faster in solution, resulting the intensity to fluctuate more rapidly than the large particles. The detected intensity is measured over time and the autocorrelation of an intensity trajectory is defined by the expression,

\[ g(\tau) = \langle I(t) I(t+\tau) \rangle, \]  

(19)

where \( g \) is the autocorrelation function, \( \tau \) is lag time, and \( I \) is the intensity. The autocorrelation curve usually decays starting from zero lag time, and faster dynamics due to smaller particles lead to faster decorrelation of scattered intensity trace. Different mathematical approaches can be employed to determine the hydrodynamic radius of
particles related to the diffusion coefficient ($D$) defined in the Stokes-Einstein equation. In case of monodisperse samples, it is possible to calculate the normalized autocorrelation curve into a single exponential form,

$$g_{(\tau)} = \exp(-q^2 D \tau),$$

(20)

where $q$ is the wave vector, $D$ is diffusion coefficient, and $\tau$ is lag time. In most cases, samples are polydisperse, thus the autocorrelation function is a sum of the exponential decays corresponding to each of the species in the population.

In this work, the size distribution of nanoparticles in solution was determined by dynamic light scattering using a NanoBrook Omni (Brookhaven Instruments Corporation, NY) equipped with a 640-nm laser and an avalanche photodiode detector at the detection angle of 173°. The size range of the instrument is 0.3 nm to 10 µm in diameter depending on refractive index and concentration. The results from the ensemble measurements by DLS show percentage number of particles at various hydrodynamic radius, which are similar to those obtained by AFM.

3) **UV-vis and fluorescence spectroscopy**

The UV-vis absorption spectra were collected by using Shimadzu UV-2101PC scanning spectrophotometer. The sample was diluted to the concentrations that are in linear relationship with absorbance, and filled in standard 1-cm cuvettes. The instrument is equipped with two light sources, which allows the scanning wavelength of absorbance from 350 – 700 nm. A photomultiplier tube is employed as the detector in the system for sensitive absorbance measurements. The molar absorptivity ($\varepsilon$) of polymer in solution
was calculated by using the Beer-Lambert law. Then the molar absorptivity of nanoparticles can be calculated from the estimated number of polymer molecules per particle related to the particle size as determined by AFM. The calculation details will be discussed in the chapter 3.

The fluorescence emission spectra were obtained with a commercial fluorometer (Quantamaster, PTI, Inc.). The instrument has a xenon arc lamp as the excitation source. The monochromators for excitation and emission have 1/4 m focal length with grating of 1200 grooves per mm. The detector employed in the instrument is a photomultiplier tube (PMT, model 814) in photon counting mode. All the slit widths used in this work are 0.5 mm, yielding a wavelength resolution of 2 nm (4 nm/mm for a 1200 grooves/mm grating, 1/4 m focal length). To measure the quantum yield of nanoparticles, fluorescence spectra of 70 µL of nanoparticle suspension in a small 3-mm cuvette were recorded by using a custom fluorometer. A 473-nm laser beam with a laser power of 1 mW from a diode-pumped solid-state continuous wave blue laser was employed as the excitation source with the beam diameter of about 2 – 3 mm. CCD spectrograph (1/8 m monochromator, Acton; Spec-10 CCD, Princeton Instruments) was used as detector.

2.4 Single-nanoparticle spectroscopy measurements

For single-nanoparticle measurements, a sample coverslip was prepared by using the same method as used for AFM as described above. For typical single-nanoparticle images, the density of particles is ~0.5 per µm², lower than those for AFM, in order to separate the fluorescence spot of point-spread function of each particle. Measurements
were performed on a custom epifluorescence microscope as shown in Figure 2.3. The 445-nm excitation laser beam was guided through a liquid crystal noise eater (LCC3111L, Thorlabs) in order to reduce laser intensity noise achieving amplitude stabilization of within 0.05% and was then coupled via an optic fiber with ~10% coupling efficiency, to the rear epi port of an inverted fluorescence microscope (Olympus IX-71). A 500-nm dichroic (500DCLP, Chroma) was used to direct the laser beam into a high numerical aperture objective (Olympus Ach, 100×, 1.25 NA, 0.13 mm working distance, Oil) with ~90% transmission efficiency at the laser wavelength. The laser excitation at the sample plane exhibits a Gaussian profile with the full width at half maximum (fwhm) of ~3 μm, and the typical laser intensities were employed in a range from 20 to 1100 W/cm² in the center of the laser spot, depending on the experiment. The excitation intensities were estimated by converting a laser power in watt measured by a laser power meter (1918-R, Newport) with ~98% reflection efficiency of the dichroic mirror at the laser wavelength and transmission efficiency of the microscope objective, and fitting to a Gaussian profile with known fwhm of the laser spot. An xyz piezoelectric scanning stage (P-517.3CL, Polytec PI) was translated in xy plane to center a particle in the laser beam and in z axis to bring the particles into focus. The stage was operated by a PZT feedback controller (E-509.C3A, Polytec PI) and a voltage amplifier (10×, E-503.00, Polytec PI). An input voltage for positioning the stage along xy plane and z direction was generated by data acquisition devices: (BNC-2090, National Instruments) and (NI USB-6008, National Instruments) with custom scripts written for LabVIEW (National Instruments) and Matlab (Mathworks, Natick), respectively.
The schematic illustration in Figure 2.3 shows that fluorescence emission from the nanoparticles was collected by the same objective. The illumination residual was filtered out through a specific cutoff 500-nm long-pass filter. The fluorescence emission was then focused onto an sCMOS camera (Neo sCMOS, Andor) with the detector plane placed approximately 10 cm from the side camera port. The fluorescence images in 16-bit integers were collected with the Solis software provided by Andor Technology. The pixel resolution of the detector was determined by imaging a spherical object with diameter of 70 µm fixed on a calibration slide with white-light illumination resulting in a pixel resolution of 65.85 nm, similar to the estimated pixel resolution of 65 nm based on the actual pixel size of 6.5 × 6.5 µm² and the objective magnification of 100×. An overall microscope fluorescence collection efficiency was estimated from the microscope objective collection efficiency, transmission efficiency of the dichroic mirror, and filter transmission efficiency, resulting 3% total collection efficiency similar to our previous value determined by using nile red loaded polystyrene spheres (Invitrogen) as standards. The rolling shutter mode of the camera at 560 MHz pixel readout rate with readout noise of 1.7 electrons per pixel was used for all experiments including high-speed particle tracking measurements. Temperature of the camera sensor was cooled to −30 °C suppressing dark current to 0.015 electrons per pixel per second that the dark noise is negligible comparing to typical fluorescence signal and readout noise.
Figure 2.3 Schematic illustration of wide-field single particle imaging microscopy
Figure 2.4 Schematic illustration of laser excitation and fluorescence emission of immobilized single nanoparticle
For single-nanoparticle fluorescence saturation measurements, a series of excitation intensities: 20, 50, 110, 220, 540 and 1100 W/cm², was used to excite the sample, and the corresponding fluorescence images were recorded. Short acquisition duration of 100 frames at an acquisition time of 0.1 s per frame was employed to minimize photobleaching. The fluorescence intensity was calculated by averaging emitted photons of a particle over the first 10 frames with photobleaching under 10%. To ensure these frames are at the beginning of the emission, the laser beam was unblocked after the detector had started for a few seconds as shown in Figure 2.5. The frame number was converted into time by the real exposure time for each frame counted by using internal trigger mode. The emission rate or number of photons emitted per second per particle was calculated by based on the number of photons detected over the exposure time and the overall microscope efficiency discussed above. The saturation emission rate and saturation excitation intensity were obtained by fitting the experimental results to the saturation equation.

Figure 2.5 Fluorescence intensity of a particle shows the first frames at the beginning after the laser is unblocked.
For typical photobleaching kinetics measurements, an acquisition time of 20 Hz with total acquisition duration of \(~60\) s was employed with an excitation intensity at 1.1 kW/cm\(^2\) to ensure more than 80% photobleaching by the end of the acquisition. The fluorescence intensity decay kinetics by photobleaching for a particle was obtained by fitting the number of photons per frame vs. time to an exponential decay function. The total number of photons emitted prior to photobleaching or death number for a given particle was calculated by integrating the emitted photons over all frames.

2.5 Particle-tracking measurements in solution and agarose gel

Particle tracking in solution was studied on PFBT nanoparticles undergoing Brownian motion in various ratios of homogeneous glycerol and water solution: 98, 90, 80 and 38 percent by volume of glycerol/water at room temperature of 23 °C under nitrogen atmosphere. A small droplet \(~5\) µL of glycerol/water solution containing \(~0.1\) nM of 22 nm diameter PFBT nanoparticles was sandwiched between two coverslips. The sample was left at microscope stage for 30 minutes in order to eliminate the flow motion of particles in solution from squeezing the droplet by the coverslips. After the solution was in equilibrium condition without an observable outside force, an \(xyz\) piezoelectric scanning stage was translated in \(xy\) plane to center a particle in the laser beam and in \(z\) axis to bring the particles into focus at the middle of the gap of coverslips. Detection framerates at 100 Hz and 1 kHz of sCMOS were used with excitation intensities at 110 and 540 W/cm\(^2\), respectively. Particle trajectories in the \(xy\) plane were determined by
fitting the fluorescence intensity of the particle in each frame to a 2D Gaussian, yielding the particle position and the width of point-spread function.

For the particle tracking in agarose gel, a small droplet ~30 µL of the solution of PFBT nanoparticle with 25% glycerol (v/v) and 4% agarose (w/v) in water heated to 90 °C was sandwiched between two coverslips and the agarose gelled at the room temperature for an hour at microscope stage. Detection framerates at 200 Hz and 1 kHz of sCMOS camera with an excitation intensity at 540 W/cm² at the center of Gaussian profile with fwhm ~3 μm were employed for nanoparticle tracking for 2000 and 5000 consecutive images, respectively.
Figure 2.6 Schematic illustration of laser excitation and fluorescence emission of single nanoparticle in solution or in agarose gel
2.6 Excitation intensity determination

The 445-nm excitation laser beam at an excitation power ($P_0$) mW measured by a laser power meter (1918-R, Newport) was guided through a noise eater (LCC3111L, Thorlabs) achieving amplitude stabilization of within 0.05%. The laser beam was then coupled via an optic fiber with 10% coupling efficiency estimated by the decreased intensity after the fiber, resulting the laser power of $P_m = 0.1P_0$ mW at the rear epi port of microscope. The laser beam was reflected by a 500-nm dichroic (500DCLP, Chroma) with 98% reflective efficiency at the excitation wavelength and passes through a high numerical aperture objective (Olympus Ach, 100×, 1.25 NA, Oil) with ~10% transmission efficiency (estimated by those of typical achromat 100×, 1.25 NA objectives, Olympus, see Appendix) to a coverslip. To determine the excitation intensity ($I_s$) in watts per area of the laser beam at a sample coverslip, the laser power at the sample $P_s = 0.1 \times 0.98P_m \approx 0.1P_m$ and spot size or fwhm of the laser beam will be determined.

The laser beam was mostly reflected back to the objective by using an opaque material marked on the coverslip. Partial amount of the reflected laser beam can pass through the dichroic mirror to the detector but the fwhm of the laser beam was not significantly affected by the change in laser power. This can be explained by sufficient number of statistical samples or counts that will not affect the variance. Thus, the 2D Gaussian profile of the excitation intensity ($I_s$) at the sample is in the expression,

$$I_s = \frac{P_s}{2\pi\sigma_z^2} \exp \left[ -\frac{x^2 + y^2}{2\sigma_z^2} \right], \quad (21)$$
where $P_s$ is the laser power at the sample $\approx 0.1P_m$, $\sigma$ or standard deviation related to laser size by $\sigma = \text{fwhm}/2.35$. The fwhm was manually estimated by the distance from the maximum of laser spot to its half intensities in $x\ y$ directions. From this equation, the maximum excitation intensity ($I_{\text{max}}$) located in the middle of laser spot can be calculated by using the equation as,

$$I_{\text{max}} = \frac{P_s}{2\pi\sigma^2}. \quad (22)$$

A typical fwhm of laser spot used in this work is about 3 $\mu$m, which can be manually adjusted by using an optic translator in $z$ direction mounted at one end of the optic fiber. Figure 2.7 shows a laser power $P_s = 10 \mu$W generating a Gaussian profile with fwhm = 3 $\mu$m. The maximum excitation intensity $I_{\text{max}} = 110$ W/cm$^2$ will be reported in this case. It should be noted that the size of laser spot can be adjusted by an $x\ y\ z$ translation that mounts to one end of the fiber optic. However, the laser spot diameter was fixed in all experiments for consistency.
Figure 2.7 (a) Selected experimental image of a laser spot. (b) Simulated Gaussian profile image of corresponding laser spot with fwhm of 3 µm and maximum excitation intensity of 110 W/cm².

2.7 Collection efficiency

Total collection efficiency ($C_{tot}$) of fluorescence signal directed to the microscope used in this work was determined for the accurate calculations of total emitted photons of a particle. The $C_{tot}$ depends on collection efficiency of solid angle of an objective lens, and transmission efficiencies of the objective, dichroic mirror, reflecting mirror and long-pass filter at signal wavelength, therefore $C_{tot} = C_s \times (C_{trans})_i \times (C_{refl})_i$. A fluorescent particle as a point light emitter scatters fluorescence signal in all directions. Only a small fraction of total solid angle of a sphere of $4\pi$ steradians can direct into an objective lens as shown in Figure 2.8. The maximal half-angle of the cone of light that can enter the lens $\theta$ can be calculated by Snell’s law,
\[ \theta = \arcsin \left( \frac{\text{NA}}{n} \right), \]  

where NA is numerical of the lens = 1.25, and \( n \) is refractive index of medium = 1.5150 (Immersion oil, Type DF, Cargille labs, NJ). The paraboloid fraction or collection solid angle can be determined by the solid angle of a cone, whose cross-section subtends the angle \( 2\theta \), that is \( 2\pi(1 - \cos \theta) \) steradians. Thus, the collection efficiency of solid angle \( (C_s) \) of a scattering fluorescence signal by a microscope objective can be calculated by the ratio of the collection solid angle and the total solid angle so:

\[ C_s = \frac{2\pi(1 - \cos \theta)}{4\pi}. \]  

Substitute Equation (23) in to Equation (24), so:

\[ C_s = \frac{1 - \cos \left[ \arcsin \left( \frac{\text{NA}}{n} \right) \right]}{2}. \]  

This can be simplified to:

\[ C_s = \frac{1 - \sqrt{1 - \left( \frac{\text{NA}}{n} \right)^2}}{2}, \]  

which indicates that the collection efficiency increases with \( \text{NA}^2 \). The resulting \( C_s \) in this work is 0.22, showing a typical collection limitation of wide-field epifluorescence microscope due to an objective lens. From this equation, the collection efficiency can be improved by using an objective lens with higher numerical aperture; however more optical elements could lower the transmission efficiency of the objective. Other techniques such as fluorescence detection on a mirror or 4Pi microscopy with two
objectives can significantly improve the collection efficiency but the microscope setups and data analyses are complicated.\textsuperscript{111}

The objective lens, dichroic mirror and long-pass filter can transmit the fluorescence emission at specific fluorescence wavelength of 545 nm with their transmission efficiency of about 0.90, 0.90 and 0.80, respectively. A reflecting mirror placed inside the microscope can reflect the emission with a reflection efficiency about 0.90. Therefore, theoretical total collection efficiency ($C_{\text{tot}}$) is about 13%. Moreover, there are other sources of uncertainty reducing the efficiency such as imaging depth, scattering length, scattering anisotropy,\textsuperscript{112,113} or spherical aberration caused by objective lens,\textsuperscript{114} Thus, the total collection efficiency is decreased to about $3 \sim 10\%$ from the microscope setup in this work. This was determined by using nile red loaded polystyrene spheres (Invitrogen) as standards. For the experiments of particle tracking in solution and in agarose gel, fluorescence signal can be significantly decreased by scattering by the solvents and agarose framework.

Figure 2.8 Schematic illustration of collection solid angle from a scattering fluorescence emission, as a fraction of total solid angle in the units of steradians.
2.8 Estimation of number of emitted photons

The estimation of number of emitted photons from fluorescence emission of a particle through typical microscope and sCMOS camera is shown in Figure 2.9. Emitted photons from a particle placed on a coverslip spread out in all directions of which partial amount is collected by the microscope objective and passes through dichroic mirror, and long-pass filter. The number of emitted photons per frame per particle \( N \) is determined by the expression, \( N = N_d / C \), where \( N_d \) is number of detected photons per frame, and \( C \) is total collection efficiency of microscope = 0.03. Then the detected photons are converted to photoelectrons (e\(^-\)) on an array of silicon wafer sensor in the camera with the conversion efficiency called quantum efficiency \( (Q) \), so \( N_d = N_e / Q \). These photoelectrons are transferred to an amplifier generating analog voltages, and then converted by an Analog-to-Digital converter (A/D converter) to intensity \( (I) \) in Analogue-to-Digital Units (ADU) or counts. This step shows the ratio of the initial number of electrons to the final number of counts reported by camera software, which is called the gain factor \( (g) \) of the camera (gain is typically adjustable, allowing the user to optimize for low-light or high-light conditions). Therefore, the number of detected photons per frame \( (N_d) \) for a given particle is calculated by using the equation, \( N_d = N_e / Q = I \times g / Q \), where \( I \) is the intensity in counts of isolated fluorescence spot subtracted by the offset, \( g \) is gain factor of the camera = 0.6 electrons/count, and \( Q \) is quantum efficiency of sCMOS camera = 0.57 electrons created per incident photon. These expressions above can be written in an equation for photon counting calculations used in this work as,
\[ N = \frac{N_d}{C} = \frac{N_e}{Q \cdot C} = \frac{I \cdot g}{Q \cdot C}, \]  

where \( N \) is number of emitted photons per frame per particle, \( N_d \) is number of detected photons per frame, \( N_e \) is number of photoelectrons per frame, \( C \) is total collection efficiency of microscope = 0.03, \( I \) is intensity in counts, \( g \) is gain factor of the camera = 0.6 e\(^{-}/\)count, and \( Q \) is quantum efficiency of sCMOS camera = 0.57 electrons created per incident photon. These calculations were also used for determining the number of detected photons per pixel following Poisson distribution with corresponding photon noise as discussed in the chapter 3.

![Diagram](image)

Figure 2.9 Illustration of the various factors determining signal level in a typical sCMOS camera and inverted fluorescence microscope

### 2.9 Gain factor

The gain factor of sCMOS camera commonly in the unit of electrons per count refers to the ratio of initial number of electrons before transferred to amplifier and A/D converter, to the final number of counts reported by camera software. For Neo sCMOS camera used in this work, the gain is adjustable via the amplifier settings with a high gain
of 0.6 e'/count or a low gain of 1.6 e'/count by using dual amplifiers. It should be noted that the unit of gain factor can be defined as ‘e'/count’ or ‘count/e’ elsewhere. However, the unit of e'/count is only used in this work for consistency. To ensure the values stated in hardware guide book, the gain factor was determined by using a series of illumination intensities of 455-nm LED (M455L2-C1, Thorlabs) in a flat profile without long-pass filter. By considering the intensity fluctuation of ‘one pixel’ in the flat-profile image for 600 frames, the mean of signal ($\mu$) and its variance ($\sigma^2$) of the intensity per pixel were obtained. The units in counts and in photoelectrons of the mean and variance are related as shown in Equation (27) as,

$$\mu_{Ne} = \mu_{Ne} / g,$$  \hspace{1cm} (28)

and

$$\sigma^2_{Ne} = \sigma^2_{Ne} / g^2,$$  \hspace{1cm} (29)

where $g$ is the gain factor of the camera in e'/count. The subscriptions $c$ and $e$ refer to the units in counts and photoelectrons, respectively. The detected photons per pixel converted to photoelectrons by the sensor obey Poisson distribution of the photon counting as,

$$\mu_{Ne} = \sigma^2_{Ne},$$  \hspace{1cm} (30)

which will be described in the next chapter. Substituting Equation (30) into Equation (28) and divided by Equation (29), we get:

$$g = \frac{\mu_{Ne}}{\sigma^2_{Ne}}.$$  \hspace{1cm} (31)
This equation shows that the gain factor can be determined as the slope of the linear relationship between the mean of signal and its variance of the intensity of a pixel. Although the flat-profile LED light source was used, all images except the first image were normalized by the fraction of the mean of each image and the mean of the first image. This can correct each image to the same average intensity avoiding flat field variation by the illumination. Each pixel in a small image area of $10 \times 10$ pixel$^2$ yields mean and variance of an illumination intensity over entire frames as shown in Figure 2.10a-b, resulting in 100 data points at one flat-field intensity. The pixel size of the image is 65 nm. Figure 2.10c shows the linear fitting of the means and variances per pixel of a series of five illumination intensities, resulting the gain factor of the camera $= 0.62 \text{ e}^{-/\text{count}}$ that agrees well with those stated in hardware specifications. The $y$-intercept from the fitting of 37 counts could not be accurately converted to readout noise by using known gain factor due to large uncertainty of the small quantity of readout noise compared to large signal means in the plot. Instead, two-bias method with two dark frames taken in succession was used to determine accurate readout noise as described in the next chapter.
Figure 2.10 (a) Image of a flat-profile illumination. (b) Histogram of consecutive signals in one pixel over 600 images. (c) Linear relationship of signal mean and variance with the gain factor as the slope.
2.10 Noise in fluorescence spectroscopy and digital imaging

The noise in optical imaging consists of undesirable signal components arising in the electronic system, and inherent natural variation of the incident photon flux. The three primary sources of noise in a CCD imaging system are photon noise, dark noise, and read noise, all of which should be considered in the signal-to-noise calculation. The units of photoelectrons or electrons will be used in this section for consistency.

1) Photon noise

Photons incident on the CCD convert to photoelectrons within the silicon layer sensor of the device. These photoelectrons constitute the signal but also carry statistical variation in the photon arrival rate at a given point. Photon noise, also known as shot noise, refers to the inherent natural variation of the incident photon flux \( N \) in photons per pixel per second. The number of photoelectrons \( N_e \) collected by a CCD pixel as shown in Figure 2.11 can be calculated by the relationship

\[
N_e = N Q t
\]

where \( N \) is the incident photon flux, \( Q \) is quantum efficiency and \( t \) is integration time. The photon flux is independent of time. Each event of the photon counting is also independent, i.e., the occurrence of one event does not make the next event more or less likely to happen. Thus, the collection of numbers of photoelectrons exhibits a Poisson distribution, which indicates a relationship between the mean and variance by

\[
\mu_{N_e} = \sigma_{N_e}^2,
\]

where \( \mu_{N_e} \) and \( \sigma_{N_e}^2 \) are the mean and variance of photoelectron \( N_e \), respectively. Thus, the signal = \( \mu_{N_e} \) and the photon noise is the standard deviation of the signal that photon
noise $\sigma_{Ne} = \sqrt{\mu_{Ne}}$. In addition, it should be noted that photon noise cannot be reduced via camera design; however, improving quantum efficiency of the camera sensor could significantly increase fluorescence signal (up to the limit of 100%).

![Figure 2.11 Poisson distribution of photoelectrons from incident photons converted by a camera sensor pixel.](image)

**2) Dark noise**

Dark noise ($\sigma_D$) arises from the statistical variation of thermally generated electrons within the silicon structure of the CCD, which is independent of photon-induced signal, but highly dependent on device temperature. Dark current describes the rate of generation of thermal electrons at a given CCD temperature. In comparison with photon noise, dark noise also exhibits a Poisson distribution, which is equivalent to the square root of the number of thermal electrons generated within a given exposure time. Cooling the CCD can significantly reduce the dark current, and in practice, high-performance cameras are usually cooled to a temperature at which dark current is negligible over a typical exposure interval. It should be noted, however, that cooling also reduces the detection quantum efficiency, so there is often a tradeoff. The sCMOS
camera used in this work has been designed with a high performance scientific sensor housed in a sealed vacuum head to avoid condensed moisture and other gas contaminants. The sensor will be cooled down to −30°C by using a deep thermoelectric cooling system. At this operating temperature, the generated dark current was extremely low as 1 electron rms/pixel/min, lower than those of typical CCD cameras. Thus, the dark noise is $\sigma_D = (\mu_D)^{1/2}$, where $\mu_D = \text{dark current rms} \times \text{integration time}$. Typically, the dark noise is much lower than the photon and readout noise components, and therefore the dark noise is neglected in the noise determination in this work.

3) Readout noise

Electronic noise sources inherent to the camera system and the CCD also introduce uncertainty in the measured signal. Collectively, these noise components are referred to as readout noise ($\sigma_R$) and represent the error introduced during the process of quantifying the electronic signal on the CCD. The major component of read noise arises from the on-chip preamplifier, and this noise is added uniformly to every image pixel (in the case of CCD detectors). High-performance camera systems utilize design enhancements that greatly reduce the significance of read noise. The readout noise of the sCMOS camera used in this work is 1.3 (median) or 1.7 (rms) electrons per min, using the rolling shutter mode at a readout speed of 560 MHz. To verify the manufacturer values stated in hardware guide book, the readout noise was determined by using any two dark frames (also called as two-bias method). The difference between two dark frames of
each pixel should yield the mean of zero electrons and the readout noise is the averaged standard deviation divided by 1.414.

A dark image of $512 \times 512$ pixel squared without an illumination was taken at 10 Hz for 100 frames by using rolling shutter mode at a readout rate of 560 MHz. The units in counts per pixel were converted to electrons per pixel by using the gain factor of $0.6 \, \text{e}^-/\text{count}$. The difference between any two images was calculated by subtraction of each pixel. The mean and standard deviation can be then obtained by the distribution as shown in Figure 2.12. By considering 4950 image differentiations of entire 100 frames, the mean is $0.00010$ electrons that is likely zero mean as expected. The readout noise is $1.66$ electrons, which agrees well with the manufacturer value of 1.7 electrons.

![Figure 2.12 Difference per pixel of two dark frames with the standard deviation related to the readout noise.](image)
2.11 Signal-to-noise ratio determination

The figure of merit that dictates the ultimate performance of a system is signal-to-noise ratio (SNR), which describes the quality of an electronic measurement. In CCD imaging, the SNR value represents the relative magnitude of the measured light signal compared to the uncertainty in that signal on a per-pixel basis. Because a CCD sensor collects charge over an array of discrete physical locations, the SNR is the ratio of the measured signal to the combined noise (frame-to-frame) at that pixel.

1) SNR of a flat-profile and stable illumination

To determine SNR ratio in an experiment, an image of a flat-profile and stable illumination was studied as a simple case, in which the intensity fluctuation by typical photoblinking or photobleaching of a fluorescent nanoparticle are neglected. An illumination with a moderate intensity at 455 nm wavelength in a flat profile was imaged at a framerate of 10 Hz for 600 frames. By considering the intensity fluctuation of ‘one pixel’ over 600 images as shown in Figure 2.13a-b, the mean is $2.63 \times 10^3$ photoelectrons and the variance is $2.68 \times 10^3$ photoelectrons squared. This agrees well with the relationship shown in Eq. (32) as expected by Poisson distribution. In addition, the fluorescence fluctuation due to photon noise can be plotted with the 99.7% statistical boundary of $3\sigma = \pm 150$ photons as shown in dotted lines.

Integration of the intensity of all pixels over a small image area should statistically hold the relationship of the mean and variance over entire frames. By considering the same image, the intensity of a small area of $5 \times 5$ pixels squared was
determined over entire frames. Figure 2.13c-d shows that the mean is $6.54 \times 10^4$
photoelectrons, which is close to the variance of $6.28 \times 10^4$ photoelectrons squared that
indicates the summation of variance over 25 independent pixels. Thus, summing the
signal over an area of interest will result in the relationship that the mean is equal to the
variance. This integration of the signal could apply to an image of single fluorescence
spot of a nanoparticle.

The SNR can be calculated from the signal mean and the summation of all noises
generated in fluorescence imaging system as,

$$\text{SNR} = \frac{\mu_{Ne}}{\sqrt{\sigma_{Ne}^2 + \sigma_R^2 + \sigma_D^2}}, \quad \text{(33)}$$

where $\mu_{Ne}$ and $\sigma_{Ne}$ are the mean and variance of photoelectron ($N_e$), respectively. $\sigma_R$ is
readout noise = 1.7 electrons rms/pixel. $\sigma_D$ is dark noise = (1 electron rms /pixel/min $\times$
0.1 s)$^{1/2}$ = 0.04 electrons/pixel, based on a framerate of 10 Hz, which equals to only ~2% of the
readout noise. The signals obtained from both cases in a flat-profile illumination
are much larger than those in readout noise and dark noise combined. Thus, the readout
noise and dark noise can be neglected and the SNR can be estimated by the equation,

$$\text{SNR} = \frac{\mu_{Ne}}{\sigma_{Ne}}. \quad \text{(34)}$$

Substitution Eq. (32) in Eq. (34) so,

$$\text{SNR} = \sqrt{\mu_{Ne}}. \quad \text{(35)}$$

The SNR from both cases can be easily obtained of about 50 and 250, respectively.
Figure 2.13 Photoelectron intensity of a flat-profile illumination and corresponding histogram in: (a-b) one camera pixel, and (c-d) an area of 25 pixels. The deviation related to photon noise of $\mu_{Ne}^{1/2}$ is shown in dotted lines, $\mu_{Ne}$ in the middle, with photon noise statistical boundary of $\mu_{Ne} \pm 3\mu_{Ne}^{1/2}$.

2) SNR of a fluorescent nanoparticle

An image of a flat-profile and stable illumination has clearly shown the intensity fluctuation mainly due to photon noise, thus a simple calculation of the SNR was described previously. In a more complex case such as fluorescence intensity of
nanoparticle, typical photoblinking and photobleaching could cause additional intensity fluctuations that contribute to the noise. Figure 2.14 shows a fluorescence image of an immobilized single PFBT nanoparticle using an excitation intensity at 110 W/cm² with a framerate of 1 kHz. Photoelectron intensity converted by using the gain factor of 0.6 e⁻/count shows that fast fluorescence decay occurs at the early time. In addition, the intensity fluctuation is caused by quantum jumps between states of different multiplicity or trapping of charge carriers. This results in the non-linear mean $\mu_{Ne}$ of the intensity as shown in the figure inset. The estimated photon noise is $\mu_{Ne}^{1/2} \approx 50$ photoelectrons, shown in the inset as the 99.7% statistical boundary ($\pm 3\sigma$) lines with $\pm 150$ photoelectrons. Hence, the SNR is about 50 with the noise mainly due to photon noise.
Figure 2.14 (a) Fluorescence image of an immobilized single PFBT nanoparticle using an excitation intensity at 110 W/cm² with a framerate of 1 kHz. Pixel size is 65 nm. (b) Corresponding photoelectron intensity and the inset (a portion of the intensity from time 1.45 to 1.50 s) showing the mean $\mu_{Ne}$ as dotted trendline in the middle, estimated by using a local regression smoothing function. The statistical boundary lines of $\mu_{Ne} \pm 3\mu_{Ne}^{1/2}$ corresponding to the mean line are related to the deviation due to photon noise.

In a low signal case, such as a high-speed framerate at 1 kHz, the fluorescence intensity yields about 500 counts per particle per 1 ms (~300 photoelectrons). The readout noise should be included in the calculation. Thus, the SNR = 300 / $[300 + (11^2 \times 1.7^2)]^{1/2} \approx 12$, based on the integration area of 11 × 11 pixels squared of the fluorescence spot, and the readout noise of 1.7 electrons/pixel. The readout noise decreases the SNR by 30% in this case comparing to the calculation with only photon noise considered. The SNR can be improved by reducing number of readout pixels using a pixel binning, which combines an array of pixels into one readout. A binning of 2 × 2 pixel array in a CCD camera can reduce the readout noise by a factor of 4, thus improving the SNR by only
~10% related to readout noise. In the case of CMOS devices, the charge-to-voltage conversion is done in each pixel, therefore the readout noise is applied per pixel. Instead of N-fold benefit it only becomes $N^{1/2}$.

It should be noted that a pixel binning yields an image with larger pixel sizes, thus resulting in a reduced localization precision. Typically, a sampling interval equal to the Nyquist criterion, twice the highest spatial frequency of the specimen, is required to accurately preserve the spatial resolution in the resulting digital image. If sampling occurs at a pixel above that required by the Nyquist criterion, details with high spatial frequency will not be accurately represented in the digital image. The Abbe limit of resolution for optical images is approximately 250 nm, meaning that a digitizer must be capable of sampling at a pixel resolution of 125 nm or less.

3) SNR in the units of counts and photons

It is worth mentioning the photon noise estimation of the fluorescence intensity in the units of counts or emitted photons per particle that are typically used in the next chapters. Photoelectrons $N_e$ can be converted to counts $I$ by using Eq. (27). This conversion changes the mean $\mu_{N_e} = \mu_c g$, and variance $\sigma_{N_e}^2 = \sigma_c^2 g^2$, where the subscriptions $N_e$ and $c$ refer to the units in photoelectrons and counts, respectively, and $g$ is the gain factor. Considering the mean-variance relationship in the Poisson distribution in Eq. (32), the mean of fluorescence intensity in counts is related to the variance and gain as $\mu_c = \sigma_c^2 g$. In other words, the standard deviation of intensity in counts due to photon noise is equal to $(\mu_c / g)^{1/2}$. Thus, the SNR is $(\mu_c g)^{1/2}$.
The standard deviation of fluorescence intensity in the units of emitted photons can also be determined. From Equations (27) and (30),

\[ \sigma_N = \sqrt{\frac{\mu_N}{QC}}, \]  

(36)

where \( C \) is total collection efficiency of microscope = 0.03, and \( Q \) is quantum efficiency of sCMOS camera = 0.57 electrons created per incident photon. Figure 2.15 shows fluorescence intensity of a PFBT nanoparticle using an excitation intensity at 540 W/cm\(^2\) with a framerate of 100 Hz. The short total acquisition time is considered to neglect the photobleaching that can be observed in a longer period. The mean of total number of photons emitted per particle is about \( 2.5 \times 10^4 \) photons. The deviation can be estimated from the mean of photons emitted following the equation above, resulting 99.7% statistical boundary of \( \pm 3\sigma_N = \pm 3.6 \times 10^3 \) photons of the mean. Thus, the intensity of \( 2.5 \times 10^4 \) photons emitted per particle should yield in the range of about 2 to \( 3 \times 10^4 \) photons due to photon noise. In addition, the SNR in this case is \((\mu_N QC)^{1/2} = 20\).

Figure 2.15 Fluorescence intensity in the units of emitted photons per frame of a PFBT nanoparticle using an excitation intensity at 540 W/cm\(^2\) with a framerate of 200 Hz.
3.1 Nanoparticle size and morphology

PFBT conjugated polymer nanoparticles were immobilized on a glass coverslip for analysis of particle morphology and size distribution by AFM technique. An AFM image and corresponding particle height histogram are shown in Figure 3.1a-b. The resulting particle size by using AFM is $22 \pm 6 \text{ nm} \ (n = 366)$ in diameter. The hydrodynamic diameter distribution of nanoparticle suspension in water at 25 °C measured by DLS is shown in Figure 3.1c as the average of 5 runs in a unit of percentage number of particle. The resulting hydrodynamic particle size by using DLS is $23 \pm 7 \text{ nm} \ (n = 5)$, which is slightly larger than the AFM results, but consistent with the slightly larger particle radius typically observed in DLS due to the solvation shell and surface irregularities.
Figure 3.1 (a) AFM image of PFBT nanoparticles and (b) the corresponding histogram of PFBT nanoparticle height. (c) Particle size distribution determined by DLS method.
3.2 UV-vis absorption and fluorescence spectra from bulk spectroscopy

PFBT was chosen in this study because of its high photostability and high brightness for fluorescence microscopy, which shows high absorptivity of polymer in THF with molar absorptivity of $5.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ per polymer molecule for a molecular weight of 10,000. This is based on Equation (5) with the absorbance $A = 0.491$ at $\lambda_{\text{max}} = 450$ nm, and polymer concentration $c = 8.9 \times 10^{-7} \text{ M}$. The molar absorptivity per molecule can be converted to the molar absorptivity of nanoparticle of $1.9 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$. The conversion is based on the estimated number of polymer molecules in a nanoparticle ($n_p$) as

$$n_p = \frac{4}{3} \pi r^3 \cdot \rho \cdot \frac{N_A}{MW} = 335,$$

where $r$ is particle radius = 11 nm determined by AFM, and particle density $\rho$ is about 1 g/cm$^3$, $N_A$ is Avogadro’s number, and MW is molecular weight. From Equation (6), the absorptivity yields high absorption cross section $\sigma_a = 7.1 \times 10^{-13} \text{ cm}^2$ per nanoparticle.

The optical penetration depth of the particle was considered to determine whether all polymer molecules in a nanoparticle can efficiently absorb light. The intensity $I$ decays exponentially with depth $z$ according to the Beer–Lambert law,

$$I(z) = I_0 \exp(-\alpha z),$$

where $\alpha$ is a function of wavelength and temperature. The optical penetration depth $\delta$ is defined as $\delta = 1/\alpha$, which is the depth at which the intensity of the transmitted light drops to $1/e$ of its initial value at the surface. In the case of a silicon wafer undergoing a 458-nm illumination of which the wavelength is close to that used in this work, the penetration
depth in silicon is about 300 nm. This clearly indicates that all polymer molecules in a particle with 22 nm in diameter much smaller than the penetration depth can efficiently absorb an illumination at the wavelength of 445 nm.

Figure 3.2a shows that the peaks of absorption and fluorescence emission spectra of PFBT polymers in THF are located at 450 and 545 nm, respectively. Figure 3.2b shows that the absorbance was decreased by 9% after a filtration of nanoparticle suspension through a 0.1 µm membrane filter. This indicates that most of the polymer molecules formed nanoparticles.

Figure 3.2 (a) Absorption (blue) and fluorescence (λ_{ex} = 473 nm, green) spectra of PFBT nanoparticles in water. (b) The absorbance of PFBT nanoparticles in water before (red) and after (blue) filtration through a 0.1-µm membrane filter decreases by 9% corresponding to the amount of polymer removed.
The quantum yield ($\phi$) of PFBT nanoparticle in water was determined by using the comparative method,\textsuperscript{117} which involves the use of a well-characterized standard sample with known quantum yield and applying the formula,

$$\phi_f = \frac{F_i f_i n_i^2}{F_s f_s n_s^2} \phi_s,$$

where $F$ is the area under the corrected fluorescence emission curve, $f$ is the absorption factor that is the absorption fraction of the light impinging on the sample ($f_x = 1 - 10^{-A}$, where $A$ is the absorbance at the excitation wavelength of 470 nm), $n_i$ and $n_s$ are refractive index of water. Subscripts $s$ and $i$ refer to standard and PFBT, respectively. Fluorescein in 0.01 M NaOH was used as the quantum yield standard with $\phi_s$ of 0.91 at excitation wavelength of 470 nm.\textsuperscript{118} For consistency, a 473-nm excitation was employed as excitation wavelength in the fluorescence quantum yield measurement of all samples in this work including the standard of fluorescein in aqueous NaOH at pH = 12. The fluorescence intensity and absorbance ratios were determined by the gradient of several concentrations as shown in Figure 3.3. By considering the equation above, the quantum yield of PFBT can be calculated by the linear relationship from the plot as $\phi_f = \phi_s / \text{slope}$. Thus the resulting quantum yield of PFBT nanoparticle is 0.14, which agrees well with our previous experimental results.\textsuperscript{58}
Figure 3.3 (a) Linear relationship of integrated fluorescence intensity and absorbance at different concentrations of fluorescein and PFBT. (b) Quantum yield determination by the comparative method with the fluorescence emission ($F$) and absorption factor ($f$) plot of fluorescein standard ($s$) and PFBT nanoparticle ($i$).

### 3.3 Fluorescence spectroscopy of single nanoparticles

Fluorescence spectroscopy of single nanoparticles immobilized on a coverslip was studied by using an inverted fluorescence microscope equipped with an sCMOS camera. Emission rate of particle or number of photons emitted per second was determined, which depends on excitation intensity of an illumination. This value can be compared with theoretical prediction that the emission rate depends on absorption cross section of particle, quantum yield and excitation intensity. Saturated emission rate and saturated excitation intensity were then calculated by using the triplet saturation equation. These could be useful for laser power adjustment with proper excitation intensity obtaining possible emission signal in further tracking experiments. Spatial resolution can
be significantly increased with higher emission intensity. Fluorescence decay as a result of photobleaching was then determined and total number of photons emitted per particle was also calculated.

1) Emission rate

Single nanoparticles of PFBT were deposited on a silanized coverslip with an approximate particle density of 1 particle per 1 µm². The fluorescence intensity was determined by using a 445-nm laser as the excitation wavelength at a series of different laser intensities ranging from 20 to 1100 W/cm². Framerate of 10 images per second was employed by using sCMOS camera acquiring 600 frames or 60 seconds. The samples were placed under nitrogen atmosphere in order to suppress photobleaching. To calculate the average of emission rate, fluorescence images of several hundreds of individual nanoparticles were manually extracted from imaging data at each laser intensity using a custom script. Bright fluorescence spots of individual particles were selected, which were differentiated from those of the aggregates of which the fwhm is larger than 320 nm. Several weak single spots in each image were also included by using the logarithm scale of fluorescence intensity to help in identifying dim particles. The fluorescence intensity was calculated by averaging detected photons of a particle over the first 10 frames. The photon emission per particle was then calculated by taking into account of the sCMOS gain parameter and an estimated overall detection efficiency of 3% for the microscope as shown in Equation (27). Selected fluorescence image is shown in Figure 3.4 using a moderate excitation intensity at 110 W/cm² at a framerate of 10 Hz. Emission rate of all
individual particles including those of weak spots in tens of fluorescence images yields
the mean and its standard error of $6.7 \times 10^6 \pm 0.3$ photons/s ($n = 427$). In addition, the
fluorescence decay plot of selected particles shows small photobleaching at this moderate
illumination under nitrogen atmosphere.
Figure 3.4 (a) Fluorescence image of single PFBT nanoparticles immobilized on a coverslip using an excitation intensity at 110 W/cm². (b) The fluorescence intensity of weak spots was scaled up to differentiate individual nanoparticles and aggregations. (c) The fluorescence intensity of selected individual nanoparticles. (d) Corresponding emission rate histogram of single PFBT nanoparticles.
The emission rate ($R$) of particle can be theoretically estimated by considering the number of photons absorbed by a particle per second ($R_A$) and quantum yield ($\phi_f$) as,

$$R = R_A \cdot \phi_f,$$

(38)

where the rate of photon absorption can be calculated by converting illumination intensity to number of photons using Planck–Einstein relation equation so

$$R_A = \frac{I_{ex} \cdot \sigma}{E},$$

(39)

therefore,

$$R = \frac{I_{ex} \cdot \sigma \cdot \phi_f}{hc / \lambda},$$

(40)

where $I_{ex}$ is average excitation intensity, $\sigma$ is absorption cross section per particle, $\phi_f$ is quantum yield, $E$ is particulate photon energy, $h$ is Planck constant, $c$ is speed of light, and $\lambda$ is excitation wavelength. This equation shows linear relationship of theoretical emission rate versus excitation intensity. To compare with the experimental moderate excitation intensity with the mean of emission rate of $6.7 \times 10^6$ photons/s at a moderate excitation intensity of 110 W/cm$^2$ as discussed previously, $I_{ex}$ can be estimated over an image at about half of the maximum intensity. Thus,

$$R = \frac{55 \text{ W/cm}^2 \cdot 7.1 \times 10^{-13} \text{ cm}^2 \cdot 0.14}{\left(6.626 \times 10^{-34} \text{ Js} \cdot 3 \times 10^{18} \text{ nm s}^{-1} / 445 \text{ nm}\right)},$$

(41)

resulting theoretical emission rate of PFBT particle of $1.2 \times 10^7$ photons/s. This ideal emission rate is larger by about a factor of two than the experimental value of $6.7 \times 10^6$
photons/s at the same excitation intensity shown above, mainly due to emission saturation of the particle. The emission saturation limits emission intensity particularly at the emission rate higher than half of saturated emission rate, which will now be described.

2) Saturated emission rate and saturated intensity

The estimated emission rate per molecule in Equation (40) shows the ideal number of photons emitted, but several effects act to limit the photon emission such as optical saturation and photobleaching. The saturation phenomenon observed in CPNs is complex, involving exciton diffusion, energy transfer, and quenching by photogenerated hole polarons.\textsuperscript{51} Fluorescence saturation, a phenomenon in which fluorescence intensity plateaus as the excitation intensity increases, is a key process limiting the emission rate of fluorophores. Saturation phenomena can also be exploited for super-resolution imaging.\textsuperscript{119}

As laser power is increased in intensity resulting in increasing in number of photons absorbed per molecule, more photons are emitted per second as long as the optical transition is not saturated. When saturation occurs, the absorption cross section from the molecule decreases, and further increases in laser power generate more background photons rather than signal photons. The characteristic saturation intensity defines as the laser intensity corresponding to the absorption cross section reduced by half is given by,

$$\sigma_s = \frac{\sigma}{1 + I_e / I_s},$$

(42)
where \( \sigma \) is absorption cross section per particle, \( I_e \) is excitation intensity. Subscription \( s \) refers to saturation. The saturation intensity depends upon further details of the energy level structure of the molecule.

Figure 3.5 shows three-level energy diagram, in which the intersystem crossing process to a long-lived triplet state associates with a bottleneck model, limiting photon absorption. By solving the three-level rate equations, the fluorescence emission rate \((R)\) for a single molecule can be expressed in the triplet saturation equation,

\[
R = R_s \frac{I_e / I_s}{1 + I_e / I_s},
\]  

where \( R_s \) is saturated emission rate, \( I_e \) is excitation intensity and \( I_s \) is saturated intensity. Figure 3.6 shows the mean and its standard error of emission rate of PFBT nanoparticles that is in nonlinear response to excitation intensities ranging from 20 to 1100 W/cm\(^2\). The resulting \( R_s = 2.4 \times 10^7 \) photons/s, which is larger than the saturated emission rate of most dyes, and the average \( I_s = 300 \) W/cm\(^2\) is much lower than that of typical dyes.
Figure 3.5 Typical energy level scheme for single-molecular spectroscopy with absorption, fluorescence, inter-system crossing, and phosphorescence processes.

Figure 3.6 Fluorescence saturation of single PFBT plotted with a series of different laser excitation intensities ranging from 20 to 1100 W/cm².
3) Photobleaching and photon number

Photobleaching is a typical phenomenon of almost all fluorescent molecules or particles that their emitted fluorescence fades during observation. The lack of signal limits the localization precision in fluorescent imaging and particle tracking experiments. Photobleaching involves a photochemical modification resulting in the irreversible loss of its ability to fluoresce. When fluorescent molecules are illuminated at a certain wavelength, there is a shift from the ground state ($S_0$) to the singlet-excited state ($S_1$). The excess energy can then be dissipated by the emission of fluorescence, or in intersystem crossing process to the excited triplet state ($T_1$). A molecule in $T_1$ state can undergo a permanent structural change, which is caused by interactions between excited fluorophores and molecular oxygen $^3O_2$ in the sample media. Reactive oxygen species such as excited singlet oxygen $^1O_2$ and superoxide radical $O_2^-$ are generated via redox reactions. Degassing and saturating with nitrogen air can minimize oxygen dissolved in sample, suppressing photobleaching process. Antifade reagents such as Trolox added to sample with deoxygenated buffer can efficiently remove dissolved oxygen in solution and quench the molecule in triplet state, enhancing photostability of fluorescent molecule.121

Figure 3.7b shows the fluorescence intensity of immobilized single PFBT nanoparticle under nitrogen air without an antifade, thus the intensity decay is expected. An excitation intensity at 540 W/cm² with a framerate of 20 Hz was used. The intensity decay was fitted to the exponential decay function, $y = A \exp(-x / \tau)$, yielding $A = 0.82$ and $\tau = 41.4$. At lower excitation intensity, slower intensity decay of particle with smaller
τ is expected due to the particle in triplet state less likely to occur. Therefore, the fluorescence decay functions between particles are comparable at the same excitation intensity. This is useful for particle tracking experiment analysis whether the fluorescence decay is mainly caused by photobleaching or the particle movement away from focal plane.

The total number of photons emitted by single nanoparticles over their lifetime was estimated by the integration of the intensity over each fluorescence spot (also called as the photon number). Figure 3.7b shows the fluorescence intensity decay with an excitation intensity at 540 W/cm² and a framerate of 20 Hz. Figure 3.7c shows the histogram of photon number with the mean of 1.1 ±1.1 × 10⁹ total photons emitted per particle (n = 489). The photon number of undoped PFBT nanoparticles is consistent with the number reported previously.¹¹ At high framerate of 1 kHz, the typical number of photons emitted per particle per frame is expected to be in the ranges between 10⁵ and 10⁶ for several hundred consecutive images. The particle brightness and photostability of CPNs show that spatiotemporal resolution of particle tracking at high framerates could be significantly improved with low tracking uncertainty.
Figure 3.7 (a-b) Fluorescence intensity decay by photobleaching of immobilized single PFBT nanoparticle using an excitation intensity at 540 W/cm$^2$ with a framerate of 20 Hz fits to the exponential decay function, $y = A \exp(-x / \tau)$, yielding $A = 0.82$ and $\tau = 41.4$. (c) Integration of the intensity over each fluorescence spot yields an average of $1.1 \pm 1.1 \times 10^9$ total photons emitted per particle ($n = 489$).
In conclusion, PFBT conjugated polymer nanoparticles were prepared and characterized by using AFM and DLS. From bulk measurements, UV-vis absorption and fluorescence spectra indicate high absorption cross section and moderate quantum yield of particles. From single molecule experiments, high saturated emission rate and low saturated excitation intensity exhibit high fluorescence brightness and good photostability. Thus, signal-to-noise ratio could be significantly improved in single nanoparticle imaging experiments with CPNs due to their excellent properties, as compared to limited photophysical properties of typical dyes or quantum dots. The results also suggest that CPNs are highly capable for advanced fluorescence-based imaging applications such as fast particle tracking that will be focused in the next chapter.
CHAPTER FOUR
TRACKING OF CPNS IN SOLUTION

Particle tracking is of key importance for quantitative analysis of cellular biomolecule dynamics from time-lapse microscopy imaging. A particle may be anything from a single molecule to a macromolecule, protein, organelle, virus or microsphere. Previous studies have demonstrated that proteins and biomolecules in a cellular context tend to exhibit in different motion due to their specific function and the presence of various subcellular structures. To achieve better understanding of these particle dynamics, particle tracking should be performed at high temporal resolution. The spatial resolution that is limited due to low particle brightness at short integration time should be improved.

In this chapter, high-speed particle tracking was studied on fluorescent PFBT nanoparticles, which exhibit high particle brightness resulting high spatial resolution. The particles undergo Brownian motion in various ratios of homogeneous glycerol and water solution; 98, 90, 80 and 38 percent by volume of glycerol/water at room temperature of 23 °C under nitrogen atmosphere. The ratios of glycerol/water mixture were selected as a simple and standard system for validating the overall particle tracking setup. The 38% solution imitates the viscosity of human blood at body temperature and the displacement of PFBT nanoparticles in this solution is close to the mean displacement of proteins in a prokaryotic cytoplasm.\(^{122}\)
A small droplet ~5 µL of glycerol/water solution containing ~0.1 nM (equivalent to ~3 × 10^8 particles) of 22 nm diameter PFBT nanoparticles was sandwiched between two coverslips with a gap of 60 µm as shown in Figure 2.6. The sample was left on the microscope stage for 30 minutes in order to eliminate the flow motion of particles in solution from pressing the coverslips. After the solution was in equilibrium condition without an observable outside force, an xyz piezoelectric scanning stage was translated in xy plane to center a particle in the laser beam and in z axis to bring the particles into focus at the middle of the gap of coverslips. The depth of field about 3 µm, determined by the relationship of fwhm of fluorescence spot of a particle immobilized on coverslip and axial translation as shown in the appendix, is much smaller than the gap between coverslips. Therefore, frictional force and hydrodynamic drag between particles and coverslip surface can be avoided in the particle tracking in solution. In addition, a few particles in solution could be detected in a typical image based on the focal volume and particle concentration.

A detection framerate at 100 Hz with an excitation intensity at 110 W/cm² was used only for high-viscosity solutions; 98 and 90%, where the nanoparticles are moving slowly enough to remain in focus for 1000 consecutive images, which equal 10 seconds in total. At these high-viscosity solutions, a fast framerate at 1 kHz with excitation intensity at 540 W/cm² was also used for comparison at the same consecutive images with shorter total detection time of 1 second. For low-viscosity solutions of 80 and 38%, in which the nanoparticles move 3 and 14 times faster in velocity than those in 98%, the framerate at 100 Hz cannot be efficiently used due to the nanoparticles always moving
out of focal volume at a short time particularly in axial direction of the depth of field. On the other hand, the fast framerate at 1 kHz can be used to track the fast movement of nanoparticles in the 80 and 38% solutions for 500 and 200 images in maximum, respectively, with insignificant motion blur effect of the particle.

4.1 Single particle localization

Particle trajectories are constructed by connecting single particle positions in each succeeding images. Firstly, a fluorescence image as a representative shown in Figure 4.1a was normalized by using a Fourier filter with optimal frequency cutoffs. Fluorescence spot in the resulting image was clear with reduced background noise as shown in Figure 4.1b. Individual nanoparticle was then roughly located at the pixel with maximum intensity of the associated fluorescence spot. The nearest intensity peak in subsequent frames generated an approximate $xy$ trajectory of the particle.

The trajectory was calculated to find the centroid by considering a small area over the fluorescence spot in the raw image. Square fluorescence image of $11 \times 11$ pixel$^2$ (about $0.7 \times 0.7$ µm$^2$) with the approximate $xy$ position at the center as shown in Figure 4.1c was selected. The Airy point-spread function can be approximated by fitting to 2D circular Gaussian function,

$$I_{(x,y)} = I_0 \cdot \exp \left[ -\frac{(x-\mu_x)^2 + (y-\mu_y)^2}{2s^2} \right] + c,$$

where $I$ is fluorescence intensity in counts, $\mu_x$ and $\mu_y$ are particle centroid coordinate, $s^2$ is the variance related to the width of fluorescence spot, and $c$ is offset. The square image of
0.7 µm fully covers the fluorescence spot with typical fwhm of 320 nm as shown in the raw image. The single particle localization yields the exact centroid in each frame with a localization error much lower than the diffraction limit. The 2D particle trajectory corresponding to the exact centroids was then used to calculate experimental diffusion coefficient by using mean squared displacement method.
Figure 4.1 (a) Raw fluorescence image of a PFBT nanoparticle. (b) Corresponding image by using a Fourier filter. (c) Single particle localization using square image corresponding to the raw image (black dots as the intensity in each pixel) fitted to 2D Gaussian function (surface plot). (d) Particle trajectory constructed from exact centroids in subsequent images.
4.2 Localization uncertainty of immobilized particles

The localization uncertainty along lateral plane arises in the position measurements of immobilized particles, which is due to the standard deviation of the point-spread function combining with photon counting noise and the focal characteristics of the imaging setup. The localization uncertainty per frame $\sigma$ is given by the expression,\textsuperscript{123}

$$\sigma = \sqrt{\frac{s^2}{N} + \frac{a^2}{12N} + \frac{8\pi s^4 b^2}{(aN)^2}},$$

(45)

where $s$ is the width of point-spread function, which is equal to fwhm of the single particle fluorescence spot divided by $2\sqrt{2\ln 2}$ or 2.355. Typical fwhm is 320 nm as determined in Equation (57). $a$ is pixel size = 65 nm/pixel. $b$ is background noise related to the readout noise of 1.7 e$^{-}$ per pixel equivalent to ~3 counts. The background noise due to quantization noise can be neglected due to high gain factor of 0.6 e$^{-}$/count used in this work. $N$ is number of detected photons per particle per frame. Three terms in the above expression refer to photon noise, pixelation noise and background noise, respectively. For the case of high fluorescence signal per frame with $N$ larger than 10$^5$ photons per particle, the photon noise mainly contributes more than 90% in the localization uncertainty simplified as,

$$\sigma = \frac{s}{\sqrt{N}},$$

(46)

for the case where the pixilation and background noise can be neglected. For example, the localization uncertainty of about 0.3 nm per frame was obtained at $N = 2 \times 10^5$
photons per particle per image at an acquisition rate of 50 Hz.\textsuperscript{56} This indicates that super-resolution imaging using single molecule localization dramatically improves the spatial resolution of conventional light microscopy by 1 – 3 orders of magnitude.

High-speed imaging could significantly decrease number of photons detected per particle with a short integration, thus the localization uncertainty of a moderate fluorescence signal per frame is then considered. Based on the average number of detected photon per frame per particle in this work $N = 500$ (about 15000 emitted photons) at 1 kHz framerate, an expected localization uncertainty of 10.4 nm per frame was obtained by using Equation (45). The photon noise and background noise in the above expression mostly contribute in the expected tracking uncertainty of 34 and 65\%, respectively, which are expected in high-speed tracking experiments. The second term contributes less than 1\% indicating that the pixelation noise can be neglected due to proper pixel size. The expected tracking uncertainty is consistent with the experimental position deviation of a stationary particle at similar particle brightness as shown in Figure 4.2. The trajectories of two immobilized particles in the same set of images indicate highly correlated motion, likely due to the vibration of the imaging apparatus. The vibration was removed by subtracting the position fluctuations of one particle from the other particle. The localization uncertainty was estimated by using MSD equation\textsuperscript{124} with the corrected trajectory of stationary particle as,

$$\text{MSD}_{xy} = 2d\sigma^2,$$  \hspace{1cm} (47)

where $d$ is dimensionality of the system, resulting $\sigma = 12.5$ nm. In addition, the localization uncertainty can be also estimated by the position histogram with a mesh size
of $10 \times 10 \text{ nm}^2$ optimal for this case. The histogram fitted to a 2D circular Gaussian
distribution yields an estimated $\sigma_{xy}$ of 12.7 nm per frame, where $\sigma_{xy}^2 = \sigma_x \sigma_y$. The small
discrepancy between the expected and experimental localization uncertainty might be due
to additional background noise from the effect of autofluorescence and nearby particles.

The localization uncertainty mainly depends on number of detected photons per
frame. Typical photobleaching that decreases number of photons by a factor of $i$ could
increase the uncertainty by $i^{1/2}$. Thus, the photobleaching during fluorescence imaging
should be suppressed by using nitrogen atmosphere covering the sample. The excitation
intensity should be carefully selected corresponding to; an emission rate of the particle
with adequate signal for an experiment, and acceptable photobleaching.
Figure 4.2 (a) Fluorescence image of immobilized PFBT nanoparticles using an excitation intensity at 540 W/cm² with a framerate of 1 kHz for 0.6 s. The image shows summed intensity over entire frames. (b) Particle localization of particle 1 after the vibration correction related to particle 2. (c) MSD_{xy} of particle 1 at early lag time from 0 to 0.10 s is related to localization uncertainty of an immobilized particle that MSD_{xy} = 4\sigma^2, resulting \sigma = 12.5 nm. (d) Position histogram of particle 1 with a mesh size of 10 × 10 nm². (e) Fluorescence intensity of particle 1 with the mean intensity of ~550 detected photons per frame, yielding a theoretical localization uncertainty of 10 nm.
4.3 Diffusion coefficient

Membrane proteins and biomolecules in a cellular context tend to exhibit different translational behavior due to their specific function, localization, and the presence of various subcellular structures. Tracking trajectories of individual particles at high spatiotemporal resolution could be an important indication of subcellular structure and the function of the biomolecule within the context of the cell or organelle. Diffusion coefficient is a simple numerical expression as a magnitude of the molar flux due to molecular diffusion through a surface per unit concentration gradient. For particle diffusion in a liquid at a uniform temperature, the theoretical diffusion coefficient of the particle can be calculated from the Stokes-Einstein equation,

\[ D = \frac{k_B T}{6\pi \eta r}, \]  

where \( D \) is diffusion coefficient in m\(^2\)/s. \( k_B \) is Boltzmann’s constant = 1.38 × 10\(^{23}\) J/K. \( T \) is absolute temperature in K. \( r \) is hydrodynamic radius of the particle in nm. \( \eta \) is dynamic viscosity in Ns/m\(^2\) calculated by an empirical formula related to the different volume ratios of glycerol and water in the mixture at given temperature,\(^{125}\)

\[ \eta = \eta_g \exp \left[ \ln \left( \frac{\eta_w}{\eta_g} \right) \alpha \right], \]

where \( \eta_g \) and \( \eta_w \) are dynamic viscosity of glycerol and water, respectively, which are an exponential function of temperature. \( \alpha \) is the weighting factor related to glycerol concentration in mass fraction in the solution. It should be noted that a small change in temperature of ±3 °C from room temperature could increase the diffusion coefficient of
particle by larger than 10%. This elaborate calculation of a diffusion coefficient at specific temperature could be efficiently used for precisely validating the tracking method by the comparison of the expected diffusion coefficients and those from the experiments.

4.4 Lateral diffusion

Since the fluorescence image of particles diffusing in solution was acquired in two-dimensional plane, particle trajectories from resolved localizations represent particle motion in lateral or $xy$ plane. Fluorescence image and two-dimensional trajectory of a selected PFBT nanoparticle in 38% glycerol/water solution observed for 0.2 s are shown in Figure 4.3a-b. Fluorescence intensity in Figure 4.3c shows that number of emitted photons of about 15000, related to 500 detected photons per frame corresponding to a tracking uncertainty of ~10 nm per frame. The standard deviation of fluorescence intensity due to photon noise is about 1000 photons estimated by using Equation (36), which agrees well with the intensity fluctuation within ±3000 photons. In addition, the fluctuation of fwhm and the intensity change particularly at 0.9 s indicates that the particle might be moving near or pass the focal plane along $z$ direction.

The lateral trajectory was then converted to mean-squared displacement in $xy$ plane (MSD$_{xy}$) at different lag times. The experimental diffusion coefficient was determined by fitting the MSD$_{xy}$ to the free diffusion equation,

\[
\text{MSD}_{xy}(\tau) = \left\langle (x(\tau) - x_0)^2 \right\rangle + \left\langle (y(\tau) - y_0)^2 \right\rangle = 2dD\tau + (V\tau)^2 + \text{MSD}_0,
\] (50)
where $x$ and $y$ are particle position coordinate and the displacement was in $\mu$m. $d$ is number of dimension. $D$ is diffusion coefficient. $\tau$ is lag time in second. $V$ is drift velocity. $\text{MSD}_0$ is tracking offset related to the localization uncertainty.$^{126}$ The drift velocity $V$ related to the flow motion of liquid was not observed in this experiment because it was eliminated as described above. Only the MSD points at the first 50 lag times were included in the fit because the points at large lag times are less averaged resulting in statistical fluctuations from linearity.

The MSD$_{xy}$ plot of the particle in Figure 4.3d was fitted to the free diffusion equation yielding lateral diffusion coefficient $D_{xy} = 4.97 \, \mu\text{m}^2/\text{s}$. The histogram of the lateral diffusion coefficients of all observed particles shows a normal distribution with the mean of $D_{xy} = 4.92 \pm 0.88 \, \mu\text{m}^2/\text{s}$ ($n = 68$). This agrees well with the theoretical $D$ of the particle in 38% solution calculated from Equation (48) of 5.0 $\mu\text{m}^2/\text{s}$. 
Figure 4.3 (a) Fluorescence image and (b) two-dimensional trajectory of a PFBT nanoparticle in 38% glycerol/water solution at a framerate of 1 kHz for 0.2 s. (c) The fluorescence emission intensity and the fwhm of the particle plotted with detection time. (d) The $D$ can be calculated by fitting lateral MSD resulting $D_x = 4.62$, $D_y = 5.32$, and $D_{xy} = 4.97 \, \mu m^2/s$. (Theoretical $D = 5.0 \, \mu m^2/s$ calculated from Stokes-Einstein equation.) (e) Histogram of the $D_{xy}$ of nanoparticles with the mean of $4.84 \pm 0.77 \, \mu m^2/s$ ($n = 68$).
The 2D trajectory and MSD plot of a representative PFBT nanoparticle in each solution ratio are shown in Figure 4.4. The coordinate boundary of particle trajectory agrees well with an expected distance of particle undergoing free diffusion in solution. For example, a PFBT nanoparticle traverses in 80% glycerol/water solution with an expected distance of $\sqrt{6Dt} = 0.9 \, \mu m$ after $t = 0.5 \, s$. The mean of experimental $D_{xy}$ calculated by the MSD method is shown in Table 4.1. The results indicate that experimental diffusion coefficients agree well with theoretical values calculated by using Stokes-Einstein equation with known particle diameter. The results clearly validate the lateral particle tracking including that of fast particle mobility in 38% solution using the high-speed framerate.
Figure 4.4 Two-dimensional trajectories and MSD plots of selected single PFBT nanoparticles in various glycerol/water ratios by using different framerates; (a) 98% at 100 Hz for 10 s, (b) 98% at 1 kHz for 1 s, (c) 90% at 100 Hz for 10 s, (d) 90% at 1 kHz for 1 s, and (e) 80% at 1 kHz for 0.5 s. The corresponding diffusion coefficients were calculated by fitting MSD to the free diffusion equation.
Table 4.1 Experimental parameters of single PFBT nanoparticles in glycerol/water solutions at 23°C. Theoretical diffusion coefficients were calculated by using Stokes-Einstein equation with the particle diameter of 22 nm. Experimental diffusion coefficients were calculated by using MSD method along xy plane and averaged over number of particle (n).

<table>
<thead>
<tr>
<th>Glycerol/water (% v/v)</th>
<th>Viscosity (Ns/m²)</th>
<th>Framerate (kHz)</th>
<th>Number of frame (frame)</th>
<th>D_{xy} (µm²/s)</th>
<th>Theoretical</th>
<th>Experimental (Mean ± S.D.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>0.772</td>
<td>0.1</td>
<td>1000</td>
<td>0.028</td>
<td>0.029 ± 0.007</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1000</td>
<td>0.030 ± 0.006</td>
<td>77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.241</td>
<td>0.1</td>
<td>1000</td>
<td>0.082</td>
<td>0.077 ± 0.012</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1000</td>
<td>0.082 ± 0.016</td>
<td>119</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>0.075</td>
<td>1</td>
<td>500</td>
<td>0.26</td>
<td>0.25 ± 0.04</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>0.004</td>
<td>1</td>
<td>200</td>
<td>5.0</td>
<td>4.92 ± 0.88</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>


4.5 Variance in MSD calculations

Particle diffusion is a stochastic process that the calculations of mean-square displacement (MSD) and the diffusion coefficient will have theoretically expected statistical variances with finite particle positions from precise measurements. The length of particle trajectories is limited in some particle tracking experiments due to dark states of the particle by photoblinking, or particle travelling out of focal volume. The theoretical relative error $\xi$ is a boundary related to the variance of MSD, which is in a linear relationship with the diffusion coefficient. The $\xi$ can be obtained from the expression,\textsuperscript{127}

$$\xi = \pm \sqrt{\frac{0.67}{F - 1}},$$

(51)

where $F$ is number of frames. At the smallest $F = 200$ for the particle tracking in 38% glycerol/water solution, a relative error of less than 6 percent is expected. This agrees well with the experimental value with the relative error of the diffusion coefficients of $\sim$4 percent.

Owing to the limitation of focal detection volume of $10 \times 10 \times 3$ µm$^3$ according to the area of imaging ($10 \times 10$ µm$^2$) and the depth of field (3 µm), the nanoparticles with highest $D$ at 5.0 µm$^2$/s in this work would have traversed along the $z$ direction within $\Delta t = (3$ µm$)^2 / (6 \times D) = 300$ ms. This corresponds to 300 frames at 1 kHz framerate that is close to 200 frames in the experiment due to random starting points of particles in the focal volume. Hence, the optimal length of $F$ was determined to efficiently calculate the lateral MSD and the diffusion coefficient. The diffusion coefficients were normalized to those at maximum number of frame shown in Figure 4.5. The experimental results show
that only 200 consecutive frames are sufficient for particle tracking in all solution ratios with an error within 10%. In addition, it should be noted for a further study that increasing the length of $F$ by extending the axial detection range could improve the MSD analysis by using multiple focal planes or feedback focusing method. It should also be noted that for many important systems of interest (e.g., in cells) the particles are confined, therefore particle escape would not be an issue for these systems.

Figure 4.5 Diffusion coefficients determined by MSD$_{xy}$ method normalized to the values at maximum number of frame, versus number of frame of segmented trajectories of single PFBT nanoparticles in various glycerol/water ratios by using different framerates.

4.6 Localization uncertainty of moving particles

Fluorescence spot of a stationary emitter has been clearly fitted by a Gaussian profile yielding particle localization with known error mainly due to photon noise. However, translational movement is commonly found in live sample and expected in particle tracking experiments. Localization accuracy is significantly affected when the
distance traveled by the particle during camera exposure reaches the same order of magnitude as that of the PSF width.

Figure 4.6 Fluorescence images of a diffusing PFBT nanoparticle in 38% glycerol/water solution at different framerates; (a) 1 kHz, and (b) 100 Hz.

Figure 4.6 shows two fluorescence images of a diffusing PFBT nanoparticle in 38% glycerol/water solution with $D = 5.0 \, \mu \text{m}^2/\text{s}$ at different framerates; 1 kHz and 100 Hz. At the exposure time of 1 ms per frame, the particle has traveled about 170 nm per frame that is smaller than the PSF width of 320 nm. This causes the fluorescence spot present in a circular shape similar to that of immobilized particle. The particle localization can be precisely determined by a 2D Gaussian function with known uncertainty as discussed earlier. On the other hand, the fluorescence spot under the longer exposure time of 10 ms per frame is distorted due to the traveled distance is 550 nm larger than the PSF width.
The distortion of object image is commonly found in photography when the object moves faster related to the shutter speed of a camera. This effect is known as motion blur that can be observed in particle tracking experiments. Fitting a distorted PSF with a 2D Gaussian function becomes inaccurate and imprecise particle localization. The localization uncertainty including the motion blur effect can be calculated by the expression,\(^{126}\)

\[ \sigma_m = \frac{s}{\sqrt{N}}, \sqrt{1 + \frac{Dt_e}{s^2}}, \] \hspace{1cm} (52)

where \(\sigma_m\) is localization uncertainty of mobilizing particles. \(s\) is the width of point-spread function. \(N\) is number of photons detected per particle per frame. \(D\) is diffusion coefficient and \(t_e\) is exposure time. This equation shows that localization uncertainty mainly depends on number of detected photons present in the first term. Moreover, the root-mean-squared displacement (rmsd) of particle per frame larger than the spot width could significantly affect the uncertainty as shown in the second term.

To choose a proper framerate for particle-tracking experiments, an expected rmsd of particles should be less than the spot width of about 320 nm at the focal plane. Root-mean-squared displacement (rmsd) per frame can be calculated from the MSD at the first lag time as,

\[ \text{rmsd} = \sqrt{\text{MSD}_{(i)}} = \left\langle (x_{(i+1)} - x_{(i)})^2 \right\rangle + \left\langle (y_{(i+1)} - y_{(i)})^2 \right\rangle. \] \hspace{1cm} (53)

For particles undergoing free diffusion following the MSD in Equation (50), the rmsd per frame is given by,
\[ \text{rmsd} = \sqrt{2dD\tau_1} . \]

The motion blur effect should also be reasonably low roughly not more than 30% related to the localization error of immobilized particles at the same particle brightness, so \( \frac{\sigma_m}{\sigma} \leq 1.30 \). In addition, a framerate should not be so fast that particles with low mobility would not be observed due to the rmsd per frame being less than the localization uncertainty. Table 4.2 shows two framerates chosen in this work. At the framerate of 1 kHz, particle tracking with single particle localizations is practical for the particle motion with diffusion coefficients observed in this work. The reason is that the particle rmsd per frame is larger than the localization uncertainty that is about 10 nm, and smaller than the spot width. The motion blur effect as determined by \( \frac{\sigma_m}{\sigma} \) is also less than 13% corresponding to the uncertainty.

The motion blur can significantly affect the localization uncertainty at a framerate of 100 Hz. The fluorescence spot is distorted due to the rmsd of particles larger than 320 nm, resulting in the uncertainty increasing by 92%. This effect has been observed elsewhere, for example, single molecules with \( D \) of 5.8 \( \mu \text{m}^2/\text{s} \) or higher cannot be tracked by using a framerate of 65 Hz.\textsuperscript{79} The particle motion could increase the particle localization error by more than a factor of 2.
Table 4.2 The effect of particle motion with distorted point-spread function and increased localization uncertainty.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>$D$ (µm²/s)</th>
<th>Framerate (kHz)</th>
<th>$\sigma_m / \sigma$</th>
<th>rmsd (nm/frame)</th>
<th>Localization precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>This work</td>
<td>0.026</td>
<td>1</td>
<td>1.001</td>
<td>12</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>0.082</td>
<td>1</td>
<td>1.002</td>
<td>22</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>0.26</td>
<td>1</td>
<td>1.01</td>
<td>39</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>1</td>
<td>1.13</td>
<td>170</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.1</td>
<td>1.92</td>
<td>550</td>
<td>Poor</td>
</tr>
<tr>
<td>Kim and Moerner⁷⁹</td>
<td>1.0</td>
<td>0.065</td>
<td>1.35</td>
<td>300</td>
<td>Fair</td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>0.065</td>
<td>2.42</td>
<td>730</td>
<td>Poor</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td>0.065</td>
<td>2.72</td>
<td>840</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Particle trajectories constructed by measured particle localization in each image are usually analyzed by the MSD method to obtain information about the particle mobility. Thus, localization precision and accuracy of particles are important for correct trajectory analysis. In the case of tracking of particles undergoing Brownian motion, the localization error due to particle motion in each frame leads to somewhat less precise in the estimated particle trajectories as shown in Figure 4.7. Boundary of localization error in each frame is shown in red circles, in which particle localization is estimated as shown in red dots.
Figure 4.7 Influence of localization precision on the analysis of single particle trajectories in the case of Brownian motion. The observed trajectory (red line) consisting of estimated particle positions (red dots) is different from the actual trajectory (blue line) consisting of actual particle positions (blue dots) because of the limited localization precision (symbolized by the open red circles). The observed mean-square displacements (MSD) of the observed trajectory as a function of the time lags in the trajectory can be modeled by a linear function (dotted red line) with an offset value that is related to the localization precision.

The MSD$_0$ or tracking offset is calculated by the localization uncertainty of stationary particle as described in Equation (47) and the uncertainty due to particle motion in the expression,\textsuperscript{126,129,130}

\[
\text{MSD}_0 = 2d\sigma^2_m - \frac{2}{3}dDt_e, \quad \text{(55)}
\]
where $d$ is dimensionality of the system, $\sigma_m$ is localization uncertainty of mobilizing particles, $D$ is diffusion coefficient, and $t_E$ is exposure time. The first term introduces a positive offset in the MSD curve, while including the second term as known as particle dynamic localization error with significant particle displacement per frame could result a negative offset. Table 4.3 shows theoretical MSD$_0$ calculated by using Equation (55) with estimated diffusion coefficients as determined by Equation (48). These show the same trend and agree with the offsets determined from the MSD plots of particle tracking experiments.

Table 4.3 Tracking offset (MSD$_0$) with the particle motion effect. Theoretical values were calculated by using the estimated diffusion coefficients with an exposure time of 1 ms.

<table>
<thead>
<tr>
<th>$D$ (µm$^2$/s)</th>
<th>Theoretical MSD$_0$ (µm$^2$)</th>
<th>Experimental MSD$_0$ (µm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.026</td>
<td>0.0006</td>
<td>0.0013</td>
</tr>
<tr>
<td>0.082</td>
<td>0.0005</td>
<td>0.0009</td>
</tr>
<tr>
<td>0.26</td>
<td>0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>5.0</td>
<td>$-0.0092$</td>
<td>$-0.0390$</td>
</tr>
</tbody>
</table>

Particle tracking by using single particle localizations could be potentially useful to study the motion of nanoscale biomolecules. However, an acquisition rate should be properly selected to observe the motion of particles of interest in order to minimize the motion blur of particles. High-speed framerate at 1 kHz can be used to track particles or biomolecules of interest with low particle motion effect, for example, the average rotational velocity of a bacterial flagellum, the transport of cargo along actin filaments, or
proteins in complex media undergoing anomalous diffusion. However, the size of PSF could be distorted by using a framerate much slower than the particle displacement in each frame, resulting a significant localization uncertainty related to a negative offset in MSD plots. The offset constraint would result in a biased estimation of $D$ by forcing a negative intercept in the fitting to Equation (50).

4.7 Axial diffusion

Since the fluorescence imaging in this work has been performed by using one camera, stacked images of fluorescence spots can directly provide particle displacements only in the lateral plane by fitting to 2D Gaussian function. Although determination of particle motion in $z$ direction could not follow this procedure, the width or diffraction pattern of fluorescence spots could be used to determine the axial position of particles. Defocusing is a simple method to calculate axial position of particles by the relationship between the width of the defocused point spread function and nanoparticle axial position, which was previously described and firstly used for CPNs by our group.

The relationship between the spot width and axial position was calibrated by using immobilized PFBT particles. The axial position of immobilized PFBT nanoparticles was obtained by continuously displacing an $xyz$ piezoelectric stage along $z$ direction in a staircase step of 20 nm per 50 ms for several microns, whereas the sCMOS camera was imaging at a framerate of 50 Hz giving the spot width determined by 2D Gaussian fitting. The displacement of the piezoelectric stage was controlled by a custom Matlab script for NI-DAQ 6008 device as shown in the Appendix. The device generated
an output voltage with a step of 2 mV per 50 ms, related to the conversion of 10 µm of the stage displaced in z direction per input voltage difference of 1 V (1 µm/V of the piezoelectric stage with 10× voltage amplifier). The device latency of generating an output voltage and a sleep command for remaining milliseconds of the interval were tracked by the system clock, yielding latency 1.52 ms and timing inaccuracy of 1.57 µs per step. Moreover, the displacement of piezoelectric stage along z direction was synchronized with the camera imaging by setting two large voltage jumps from zero to 0.2 V and vice versa. These generated a rapid z translation of 2 µm resulting in sharp difference in fluorescence intensity and fwhm as shown in Figure 4.8. These differences were located by using a differencing function and then marked as a starting and ending point of the axial translation. The time difference between these marks is 45.0566 s determined by the camera exposure time of imaging. This agrees well with 902 steps of output voltage that equals to 45.05 s in total. The results show that axial translation of the piezoelectric stage by using a custom staircase voltage was well synchronized with the fluorescence imaging.
Figure 4.8 Synchronization of piezo stage axial translation with fluorescence imaging.

Figure 4.9a-b show a fluorescence spot at focal plane and at 2 µm from the focal plane with spot width of 317 and 396 nm, respectively. Scanning the spot fwhm of an immobilized PFBT particle at different axial position of about 6 µm around focal plane yields a relationship of fwhm and axial position. The fwhm of fluorescent spot as a function of axial position $z$, in unit of nanometers, was centered to $y$ axis and fitted to an equation,\cite{101}

$$W^2(z) = \left[ \frac{W_F \cdot z}{z_{dof}} \right]^2 + W_F^2,$$

(56)

where $W$ is fwhm of fluorescence spot, $W_F$ is fwhm of the spot at focal plane, $z$ is axial distance, and $z_{dof}$ is depth of field. The fit is shown as black line in Figure 4.9c resulting $W_F$ is 320 nm and the depth of field is 2.8 µm corresponding to Figure 2.6. Equation (56) can be estimated by a simple quadratic equation,

$$W(z) = a z^2 + W_F,$$

(57)
yielding a good fit for the parameters $a = 2.0 \pm 0.21 \times 10^{-5}$ and focus fwhm $W_F = 320 \pm 18$ nm ($n = 44$) as shown in green. The fitting shows root-mean-square error of $\sim 8$ nm due to the fluctuation in fluorescence intensity associated with photon noise. The $W_F$ refers to the width of point-spread function at focal plane related to sigma ($\sigma$) in Equation (45). The width can be also used to set criteria for axial particle tracking such that fwhm should not higher than about 500 nm, which implies that a particle moves within the focal volume.

Particle axial location can be determined from the spot width by using Equation (57). However, a small difference in the spot width of a particle near focal plane due to particle motion or fluorescence intensity fluctuation could exhibit large axial localization error as shown in Figure 4.9d. The plot was estimated by the change of $z$ distance corresponding to the difference of spot widths, related to Equation (57). The localization error is clearly higher than 100 nm per frame when a particle moves within $\pm 0.5$ $\mu$m from focal plane. Thus, particles in solution with displacement per frame of less than the axial localization error cannot be tracked in axial direction (see Table 4.2).
Figure 4.9 (a-b) Fluorescence spot of a PFBT nanoparticle at focal plane and at 2 μm from the focal plane, respectively. (c) The fwhm versus axial position by scanning an immobilized PFBT nanoparticle in two opposite directions as shown in blue and red dots, with the fitting in black and green. (d) Axial localization error by the defocus imaging method.
In order to avoid large position deviation near focal plane, defocused imaging should be analyzed when particles are not within ±0.5 µm from the focal plane. It was expected that only defocused imaging section with spot fwhm from 350 to 400 nm can be used in Equation (57), giving the displacement in z direction of ~0.8 µm related to axial spatial resolution of 20 nm. In fact, the PFBT nanoparticles in 38% solution have rapidly traversed beyond 2.5 µm in 200 ms of the total detection time. This can be observed in the selected nanoparticle as discussed above moving near or pass the focal plane, which can be seen at 0.08 s according to photon intensity in Figure 4.3c. Therefore, the axial particle movement in 38% glycerol/water solution cannot be precisely calculated through the quadratic equation.

PFBT nanoparticles in 90% glycerol/water solution have traversed only 0.7 µm in 1 s of the total detection time. The defocused imaging section with fwhm from 350 to 400 nm can be seen from 0.85 to 1 s in Figure 4.10a, involving the particle movement of 0.3 µm. The resulting axial position of this section is shown with lateral trajectories in Figure 4.10b, which were used to calculate the MSD in Figure 4.10c. The resulting $D_y = 0.095 \mu m^2/s$ and $D_z = 0.120 \mu m^2/s$, agreeing with theoretical $D = 0.082 \mu m^2/s$. The small discrepancy in diffusion coefficients is caused by the limited number of frame that only 150 frames of defocused section were used and the difference in lateral and axial resolutions. In addition, the axial tracking uncertainty can be improved by using a cylindrical lens, stepwise imaging, or bifocal technique.$^{131,132}$

In conclusion, particle tracking using the localization of fluorescence spots in consecutive images has been performed to track the motion of PFBT nanoparticles
undergoing free diffusion in solution. At a framerate of 1 kHz, the nanoparticles exhibit fluorescence emission of ~15000 photons per particle per 1 ms, yielding theoretical localization uncertainty of 10 nm per frame along lateral plane. The diffusion coefficients ranging from 0.026 to 5.0 µm²/s were measured by using the MSD method, which agree well with those from the Stokes-Einstein equation according to various solution viscosities and particle size. In addition, the rmsd of particle mobility in chosen solutions is in the range of 12 – 170 nm per ms. This results insignificant motion blur effect in the particle tracking experiments, due to the rmsd smaller than the width of point-spread function of about 320 nm. Thus, the estimated diffusion coefficients indicate that precise particle localization was obtained.

The axial positional trajectories for 3D particle tracking were determined by defocused imaging, which evaluates the width of fluorescence spot at different displaced focal planes, yielding an axial resolution of 20 nm. Selected particle trajectory along the axial direction related to the change in fwhm of fluorescence spot in the range of 350 – 400 nm yields the estimated diffusion coefficient of 0.120 µm²/s, which agrees well with expected value of 0.082 µm²/s. The results suggest that CPNs are highly capable for a high-speed tracking experiment of particles undergoing anomalous diffusion in a porous material that will be focused in the next chapter.
Figure 4.10 (a) The fluorescence emission intensity and the fwhm of a PFBT nanoparticle in 90% glycerol/water solution at a framerate of 1 kHz for 1 s. (b) Axial position of the particle calculated by defocused imaging along with lateral position. (c) MSD plots with resulting $D_{xy} = 0.095 \, \mu m^2/s$ and $D_z = 0.120 \, \mu m^2/s$. (Theoretical $D = 0.082 \, \mu m^2/s$)
CHAPTER FIVE

TRACKING OF CPNS IN AGAROSE GEL

High-speed tracking of nanoparticles undergoing free diffusion in solution has been well validated by using mean-squared displacement method as shown in the previous chapter. However, membrane proteins and biomolecules in a cellular context tend to exhibit in anomalous diffusion due to their specific function and the presence of various subcellular structures. Tracking of nanoparticles with an anomalous diffusion is expected to provide such important information of particle dynamics with high spatiotemporal resolution. For complex dynamic systems such as proteins in a cellular membrane, there are multiple timescales and length scales of motion, as well as various types of heterogeneity. Particle mobility in a porous material was chosen as a simple experiment to study anomalous diffusion behavior. Random-walk simulations of confined diffusion have been performed by using the position histogram and the MSD of confined diffusion, which were used in the experimental analyses of pore size and particle dynamics. Particle tracking including at a kilohertz framerate was used to characterize the structure of nanopores and determine the diffusion dynamics inside the pores and channels in agarose gel.
5.1 Random-walk simulations of confined diffusion

Three-dimensional random-walk simulations have been performed in order to find the optimal parameters, such as mesh size, histogram boundary used in position histogram and MSD methods for characterizing the structure of nanopores and determining the diffusion dynamics of the particle inside the pores. Simulated trajectories of Brownian diffusion without considering molecular interaction were generated from (pseudo-) random positions using a custom script written in Matlab. Two sets of input parameters; root-mean-squared displacement per step (rmsd) calculated from a given diffusion coefficient of 1 µm²/s, framerate at 200 Hz and 1 kHz, and number of frame of 2000 and 5000, respectively, were defined in the simulations with no steps shorter than a given frame (microsteps) considered. The successive positions of a given trajectory were calculated according to the following algorithm:

\[
\begin{align*}
    x_{i+1} &= x_i + \Delta x_i \\
    y_{i+1} &= y_i + \Delta y_i \\
    z_{i+1} &= z_i + \Delta z_i
\end{align*}
\]

where \((x_i, y_i, z_i)\) represents a position in the observed trajectory. In Equation (58), \((\Delta x_i, \Delta y_i, \Delta z_i) \sim N(0, \text{rmsd})\), where the overall displacements from entire trajectories indicate a normal distribution with mean zero and standard deviation equal to the rmsd. The symbol “~” indicates that the random variables to the left are distributed according to the probability distribution function to the right. The default generator in Matlab was used for normally distributed random numbers. 3D trajectories of a free-diffusion particle were simulated without localization uncertainty as shown in Figure 5.1.
The simulated pores were then constructed by restricting several parts of the particle motion within a confined sphere with diameter of 250 nm mimicking the experimental average pore size of 4% agarose gel. Position coordinates of particle \( \{x_i, y_i, z_i\} \) were confined in a sphere with radius \( r \) as,

\[
x_i^2 + y_i^2 + z_i^2 \leq r^2.
\]  

For those coordinates outside the sphere, the wall of sphere acts as a reflecting barrier that turns the walker around, moving away in the opposite direction as described elsewhere.\(^{133}\) Instead of the reflection, the barrier encounter can also be considered as adsorption where a particle motion is terminated, and no-go condition where a particle predicts the next step being outside of the boundary thus chooses alternative to move within the confined space.\(^{134}\) Among these different types of boundary encounter, the reflective boundary condition is the most common implementation, which treats the individual movement after the encounter as if it was perfectly reflected in the boundary.\(^{135}\) The reflecting direction was random but the distance was equal to the remaining length, which was calculated by the intersection of a directional line and pore sphere. The script for generating a particle trajectory with free diffusion and restricting the trajectory into confined diffusion is shown in the appendix. Number of pores was adjustable by inserting a short free diffusion between any adjacent pores. The free diffusion represents a simple motion of particle in a channel and is a part of an anomalous subdiffusion.
Figure 5.1 Particle trajectory was simulated with free diffusion related to \( D = 1 \, \mu m^2/s \) with a framerate of 1 kHz for 5000 frames. Trajectory restriction of particle in black moving to the wall of a sphere with 250 nm diameter, and reflecting as shown in green. Simulated trajectory of confined diffusion was then generated with several individual pores.

1) Simulations at 200-Hz framerate

Selected resulting trajectory from the simulations at 200 Hz is shown in Figure 5.2a-b, which consists of five confined diffusions in individual spherical pores separated by a short free diffusion. The particle positions were transformed into two-dimensional histogram with a mesh size of 40 \( \times \) 40 nm\(^2\) as shown in Figure 5.2c. The mesh size was estimated by the Nyquist criterion that requires a sampling size less than half of the fwhm. The sampling size in the case of a pore size with 250 nm in diameter should be less than 63 nm.
Figure 5.2 (a) 3D random-walk simulations with input parameters; diffusion coefficient of 1 $\mu$m$^2$/s, framerate at 200 Hz and number of frame of 2000. (b) Corresponding 2D particle trajectories with confined diffusion inside single pores separated by short free diffusion. (c) Particle histogram of the trajectories shows separated and overlapped pores.
Each position histogram of a single pore was used to calculate pore size by fitting the position histogram \( h \) to a two-dimensional elliptical Gaussian function with rotating coordinates by an angle \( \theta \) as,

\[
h_{(x,y)} = A \cdot \exp\left[ -a(x-x_0)^2 + 2b(x-x_0)(y-y_0) - c(y-y_0)^2 \right], \tag{60}
\]

where

\[
a = \frac{\cos^2 \theta}{2\sigma_x^2} + \frac{\sin^2 \theta}{2\sigma_y^2},
\]

\[
b = -\frac{\sin 2\theta}{4\sigma_x^2} + \frac{\sin 2\theta}{4\sigma_y^2},
\]

and

\[
c = \frac{\sin^2 \theta}{2\sigma_x^2} + \frac{\cos^2 \theta}{2\sigma_y^2}.
\]

The elliptical Gaussian function was expected to be effectively fitted to particle position histogram in agarose pores that are in non-uniform shape. The position distribution with the maximum located at the pore centroid was observed from particle motion in a confined sphere. The pore diameters were estimated at the boundary of statistical fractions of \( 4 \times \sigma \) at a confidence level of 95\% of the Gaussian distribution. Figure 5.3a shows simulated particle positions in a spherical pore with 250 nm in diameter present in a simple circular shape \((\sigma_x = \sigma_y)\) in lateral plane. The pore diameter agrees well with an estimation by using the boundary of \( 4\sigma \). However, an ellipsoid shape could be found in agarose pores, thus the pore size was estimated by the average of the sigma in \( x \) and \( y \) rotated coordinates as shown in Figure 5.3b.
Figure 5.3 Simulated particle positions (red dots) in; (a) a spherical pore with 250 nm in diameter, (b) an ellipsoid pore with a rotating angle of 1 radian along lateral plane. The position histogram is shown in a contour surface. Statistical boundary $4\sigma$ of the corresponding position histogram is shown in solid black lines. Overlapped histogram of multiple pores that merge into a large pore or locate in the same lateral plane but in different axial positions was excluded from the analysis. The resulting diameters of pore 1 – 3 in Figure 5.2 are 258, 255 and 258 nm, respectively. The deviation of position distribution is not significantly affected at the number of frame higher than 150 frames as shown in Figure 5.4, resulting in a reliable pore size determination by using the deviation of particle positions. The position histogram of all simulated trajectories at 200 Hz yields the mean pore size of $251 \pm 13$ nm ($n = 50$), which agrees well with the input pore size of 250 nm.
Figure 5.4 Pore size was estimated by using position histogram of confined trajectories at the $4\sigma$ statistical boundary. Simulation results of normalized $4\sigma$ versus number of frame of a confined trajectory show that pore size is not significantly affected at number of frame higher than 150 frames.

2) Simulations at 1-kHz framerate

Particle trajectory from the simulations at 1 kHz with input parameters; diffusion coefficient of 1 $\mu$m$^2$/s, and number of frame of 5000 is shown in Figure 5.5. The position histogram was also used to determine the pore size of simulated trajectories with a fast framerate at 1 kHz as shown in Figure 5.5c, resulting pore diameters of individual pore 4 and 5 of 240 and 245 nm, respectively. These results show that the position histogram could efficiently determine the pore size from the particle tracking in agarose gel. In addition, using this fast framerate at 1 kHz can observe long segmented trajectories of particle in a single pore before hopping to an adjacent pore. The segmented trajectories with large number of frame; 434 frames in pore 4 and 447 frames in pore 5, were used to calculate the MSD relating to the confined diffusion in a spherical space with confined radius $\theta$ obtained from the equation,
MSD(\(\tau\)) = \theta^2 \left[ 1 - A \cdot \exp\left(\frac{-2dD\tau}{\theta^2}\right) \right], \quad (61)

where \(d\) is dimensionality of the system, \(D\) is diffusion coefficient, \(\tau\) is lag time and \(A\) is constant. The MSD method of the segmented trajectories in pore 4 and 5 yields the pore diameters of 269 and 257 nm and the diffusion coefficients of 0.62 and 1.19 \(\mu\)m\(^2\)/s, respectively. The results from the fast framerate show that the pore size can be determined by using either position histogram or the MSD of segmented trajectories with sufficient number of frame. The theoretical prediction highlights a feasible application of the fast tracking of bright CPNs for determining the morphology of a porous material such as agarose gel, which will be discussed in the next section.
Figure 5.5 (a) 3D random-walk simulations with input parameters; diffusion coefficient of 1 µm²/s, framerate at 1 kHz and number of frame of 5000. (b) Corresponding 2D particle trajectories. (c) Particle histogram of the trajectories. (d) The MSD of pore 4 and 5 fitted to confined diffusion shown in red.
Particle dynamics in a confined space represented by diffusion coefficient could not be precisely estimated by particle tracking at a given framerate due to particles encountering the boundary. Individual particles could be absorbed or reflected to the wall depending on ionic strength condition and concentration proposed in different models.\textsuperscript{136,137} The reflective boundary was used in this work due to the weak attraction interaction between nanoparticles with negative zeta potential and agarose pores. The reflection alters the observed particle displacement per frame clearly shorter than the actual displacement as shown in Figure 5.6. Therefore, the corresponding diffusion coefficient of a particle with significant number of events bouncing to the boundary is less than the average displacement of unbounded condition.\textsuperscript{134}

Confined trajectories of a particle with various input diffusion coefficients were simulated to estimate the corresponding diffusion coefficient reduced by the reflective boundary effect. Figure 5.7 shows the observed $D$ of confined simulations with input $D$ ranging from 0.25 to 12 $\mu$m$^2$/s using a framerate of 1 kHz and 200 Hz for 500 frames.
The corresponding rmsd per timestep is 38 nm to 270 nm for the 1 kHz framerate, and 270 nm to 1.9 µm for the 200 Hz framerate. The framerates were chosen in the simulations, mimicking those in the experiments. The confined sphere with 250 nm in diameter was used in the simulations mimicking the expected pore size of agarose gel. The particle trajectory confinement in an individual pore was constructed as described previously. The output $D$ was calculated by using the MSD equation of each simulated trajectory as shown in Equation (61). The identity line ($y = x$) is shown in red color, referring to the ideal analyses of accurate $D$ without such impact from the reflection effect.

By comparing the simulation results in Figure 5.7a to the identity line, it was found that simulated trajectories with input $D$ less than about 2.6 µm$^2$/s yield somewhat accurate output $D$ as shown in zone $i$. This diffusion coefficient corresponds to rmsd per frame less than half of the pore size so, $D = (0.5 \times 250 \text{ nm})^2 / 6 / 1 \text{ ms} = 2.6 \text{ µm}^2$/s. For input $D$ higher than those in the accurate zone, large number of events of particles reflected by the boundary significantly decreases the average displacement, thus large discrepancy was found in zone $ii$. In addition, there is a limit in the calculation of $D$ that the observed rmsd per frame will not be higher than the pore diameter. Thus, the maximum $D$ from the calculations in this case is $D = (250 \text{ nm})^2 / 6 / 1 \text{ ms} = 10.4 \text{ µm}^2$/s. In other words, calculating a diffusion coefficient of a confined trajectory by using the MSD equation will not result in zone $iii$.

Figure 5.7b shows confined simulations using a framerate of 200 Hz. In zone $i$, diffusion coefficient corresponding to the rmsd per frame less than half of the pore size is
\[ D = (0.5 \times 250 \text{ nm})^2 / 6 / 5 \text{ ms} = 0.5 \mu\text{m}^2/\text{s} \]. The results indicate the analyses of particle mobility:

1. Particle dynamics with rmsd per frame lower than half of the pore diameter could be precisely estimated by particle tracking.
2. For particles with rmsd per frame higher than half of the pore diameter, large number of particle reflections to the boundary significantly reduces the observed diffusion coefficient.

Simulations of confined diffusion suggest that observable diffusion coefficients should not be higher 0.5 \( \mu\text{m}^2/\text{s} \) determined by particle-tracking experiment at a framerate of 200 Hz of nanoparticles in agarose gel with pore diameter of 250 nm.

(a)  
(b)

Figure 5.7 Observed diffusion coefficients of confined simulations with various input \( D \) ranging from 0.25 to 12 \( \mu\text{m}^2/\text{s} \) in a confined sphere with 250 nm in diameter, using a framerate of (a) 1 kHz, and (b) 200 Hz. Zone i shows accurate analyses of \( D \). While the reflective boundary effect in zone ii reduces the observed \( D \).
5.2 Particle tracking of single PFBT nanoparticles in agarose gel

The porous structure of agarose gel has been characterized by particle tracking of CPNs diffusing inside the transparent material. A small droplet ∼30 µL of the solution of PFBT nanoparticle with 25% glycerol (v/v) and 4% agarose (w/v) in water heated to 90 °C was sandwiched between two coverslips and the agarose gelled at room temperature for an hour at microscope stage. At this low concentration of nanoparticles of ∼10 pM in the solution, the particles could swim inside a pore, bounce from a pore wall or diffuse through a channel. The 2D particle dynamics was observed by fluorescence imaging with the depth of field of 3 µm, revealing the particle tracking with localization uncertainty of ∼10 nm per frame along lateral plane. Camera acquisition framerates of 200 Hz and 1 kHz with an excitation intensity at 540 W/cm² at the center of Gaussian profile with fwhm ∼3 µm were employed for nanoparticle tracking for 2000 and 5000 consecutive images, respectively. These tracking experiments clearly last longer than the previous tracking of particles with free diffusion due to the confinement restricting particle motion in focal volume.

1) Particle tracking at 200-Hz framerate

Selected particle trajectory from the particle tracking at 200-Hz framerate in agarose gel and the position histogram clearly show five individual pores with channels linking in between as shown in Figure 5.8a-c. The pore diameters of pore 6 – 8 determined by position histogram method as described in the simulations are 288 nm, 258 nm and 272 nm, whereas those of pore a and b cannot be determined due to an
insufficient number of particle trajectory points in these pores. The longest segmented trajectory was found in pore 6 of only 124 frames, which is insufficient to determine the pore size and diffusion coefficient by MSD method. Although the particle moves back in to the same pore yielding several separated trajectories inside an individual pore, these trajectories could not be directly connected to extend the length of trajectory. Therefore, particle tracking should be performed at a faster framerate with larger points of particle position in a single pore.

The entire particle trajectory shows the combination of confined diffusions in individual pores separated by free diffusions along the axis of the channel. The MSD calculated from overall particle positions can be fitted to the expression for anomalous subdiffusion,

\[
\text{MSD}(\tau) = 2dD\tau^\alpha, \tag{62}
\]

where \(d\) is dimensionality of the system, \(D\) is diffusion coefficient, \(\tau\) is lag time and \(\alpha < 1\). The plot as shown in Figure 5.8d yields average \(D = 0.05 \ \mu\text{m}^2/\text{s}\) and \(\alpha = 0.59\). The average diffusion coefficient could estimate overall dynamics of the particle; however, a fast framerate should provide segmented trajectories with sufficient number of particle trajectory points. Thus, the free and confined diffusions of the segmented trajectories can be observed, providing particle dynamic behavior in a confined area of interest.
Figure 5.8 (a) Two-dimensional particle trajectory of selected particle in agarose gel at a framerate of 200 Hz. (b-c) Corresponding position histogram of the trajectory with a grid size of $40 \times 40 \text{ nm}^2$. (d) MSD plot calculated from entire trajectories and fitted to anomalous subdiffusion (e) Fluorescence intensity of the particle.
The fluorescence intensity as shown in Figure 5.8e, which is related to the fwhm of fluorescence spot and the particle axial position, suggests that the pores are in different axial positions resulting in rapid changes of the intensity, due to the particle hopping between the pores. Partial trajectory cropped from the period of 3.36 to 5.09 s as shown in Figure 5.9 clearly shows the difference in fluorescence intensity and fwhm of fluorescence spot of the particle diffusing from pore 8 to 7. The fwhm difference can be used to estimate the distance between the pores of about 45 nm along $z$ direction by using Equation (57). The distance between the pore centers is 12 nm and 504 nm along $x$ and $y$ directions, respectively, as estimated from the centroids of position histogram. The particle moves between these pores within about 20 ms, resulting in an approximate diffusion coefficient of 2.1 $\mu$m$^2$/s inside the channel.
Figure 5.9 (a) Partial two-dimensional trajectory of Figure 5.8 cropped from the period of 3.36 to 5.09 s and position histogram of pore 7 and 8. (b) Partial fluorescence intensity (black) and fwhm (red) of the particle moving from pore 8 to 7.

Table 5.1 Partial fluorescence intensity and fwhm corresponding to Figure 5.9b.

<table>
<thead>
<tr>
<th></th>
<th>Pore 8</th>
<th>Pore 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence intensity</td>
<td>$2.65 \pm 0.35 \times 10^4$ ($n = 178$)</td>
<td>$5.79 \pm 0.84 \times 10^4$ ($n = 169$)</td>
</tr>
<tr>
<td>(photons/frame)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fwhm of fluorescence spot</td>
<td>$369.4 \pm 21.8$ ($n = 178$)</td>
<td>$366.6 \pm 14.2$ ($n = 169$)</td>
</tr>
</tbody>
</table>
2) Particle tracking at 1-kHz framerate

The particle trajectory obtained by particle tracking at a fast framerate of 1 kHz as shown in Figure 5.10a exhibits the structure of agarose gel. Two individual pores labeled as pore 9 and 10 yield the pore diameters of 219 and 220 nm, respectively, determined by corresponding position histogram as shown in Figure 5.10b. The longest segmented trajectory of pore 9 has 673 frames, which can be used to calculate the MSD fitted to confined diffusion equation (61) as shown in Figure 5.10d. The resulting pore diameter of 237 nm and diffusion coefficient of 0.31 µm²/s inside the pore. The fluorescence intensity in Figure 5.10e exhibits fluctuations as expected based on Poisson noise. To determine the particle photobleaching, the normalized intensity was fitted to an exponential decay function,

\[
y = A \cdot \exp \left( -\frac{x}{\tau} \right),
\]

yielding \( A = 0.98 \) and \( \tau = 6.0 \). Comparison these results to that of an immobilized particle at the same excitation intensity as shown in Figure 3.7 might indicate that the agarose pores are in different axial locations. Thus, the decrease of intensity mainly corresponds to the particle moving away from focal plane. The particle tracking at high-speed framerate with large number of frames at short acquisition time shows more details of porous structure with various pore sizes and shapes, and particle dynamics undergoing confined or anomalous diffusion.
Figure 5.10 (a) Two-dimensional particle trajectory of selected particle in agarose gel at a framerate of 1 kHz. (b-c) Corresponding particle histogram of the trajectory with a mesh size of $40 \times 40 \text{ nm}^2$. (d) The MSD of pore 9 fitted to confined diffusion shown in red. (e) Fluorescence intensity of the particle.
Segmented particle trajectories in a single pore, of which the segment length is higher than 200 frames sufficient for MSD calculations, yield experimental diffusion coefficients ranging from 0.06 to 0.80 µm²/s (n = 12). This can be compared to the expected $D$ in the range from 5 to 8 µm²/s based on the viscosity of solution and particle size. The discrepancy is clearly due to the reflective boundary effect as described previously by the simulations of confined diffusion. Significant amount of particle reflections in an agarose pore with 250 nm in diameter reduces the rmsd per frame observed by particle tracking. Thus, the expected diffusion coefficients were limited at about 0.5 µm²/s that agrees well with the results.

The position histogram of individual pores obtained by particle tracking at both 200 Hz and 1 kHz framerates was used to determine the pore size of 4% agarose gel with the resulting mean of 245 nm with S.D. ±83 nm (n = 178) as shown in Figure 5.11. This excluded the pore diameters of less than 60 nm of 4$\sigma$, which refer to immobilized particles yielding the tracking error of less than 15 nm per frame. The experimental pore size in this work agrees well with the previous result of 243 nm with S.E.M. ±5 nm determined by AFM at the material surface. It was found that nanoparticle tracking at high framerates with high spatial resolution of bright CPNs provides such reliable information of heterogeneous pore size and confined or anomalous diffusion inside porous material. In addition, the results from the tracking of CPNs in this work yield better spatiotemporal resolution with tracking uncertainty of 10 nm per 1 ms than those of fluorescent polystyrene beads tracked in agarose gel at a 25-Hz framerate. The particle tracking of CPNs could be used to improve the diffusion analysis of a particle in nano-
patterned substrate or a complex biomolecule with anomalous diffusion in cellular membrane or in cytoplasmic matrix of a eukaryotic cell.

In summary, tracking of PFBT conjugated polymer nanoparticles was applied to study particle motion with anomalous subdiffusion. Particle tracking at up to 1 kHz of CPNs in agarose gel has been performed to characterize the structure of nanopores and determine the diffusion dynamics inside the pores and channels. The pore size was determined by the position histogram of particle trajectory that consists of several confined diffusions in individual pores. The resulting average pore diameter agrees well with the previous result determined by AFM at the agarose surface. In addition, particle tracking at 1 kHz has long particle trajectories in a single pore with number of frame sufficient for the MSD calculation, yielding the pore size and diffusion coefficient of the particle inside the pore. The estimated diffusion coefficients were obtained in the range from 0.06 to 0.80 μm²/s (n = 12), which agrees well with the expected values based on the reflective boundary effect of given particle size, solution viscosity, camera
framerate, and pore size. The position histogram and the MSD of confined trajectories were validated by random-walk simulations using experimental parameters.
CHAPTER SIX
CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

This dissertation has demonstrated the preparation and characterization of bright conjugated polymer nanoparticles. The photophysical properties have shown that the nanoparticles exhibit excellent photostability, high emission rate with high absorption cross section, and low saturation excitation intensity. These excellent features indicate that CPNs could be a promising candidate for biological imaging applications. In this work, CPNs have been used in simple particle-tracking experiments including particles undergoing free diffusion in solution and confined diffusion in agarose gel. Owing to high brightness of CPNs, particle tracking has been performed at a short integration time of such a high-speed framerate of 1 kHz. At this framerate, particles with fast mobility in solution or in agarose gel were precisely tracked with high spatiotemporal resolution. The results show that single particle spectroscopy of CPNs could be applied for structural imaging and tracking of biomolecules.

Particle tracking using the localization of fluorescence spots in consecutive images has been performed to determine the mobility of nanoparticles undergoing free diffusion in solution. A framerate at 100 Hz can be used to determine the free diffusion behavior of nanoparticles with 22 nm in diameter mobilizing in 98 and 90% of glycerol/water mixtures. However, this framerate is not practical for tracking particles in 80 and 38% solutions with lower viscosity, in which those particles jump out of a focal...
volume especially in axial direction in a short time. On the other hand, high-speed particle tracking at a high framerate of 1 kHz can observe the diffusion characteristic of particle in all given solutions. At this high temporal resolution of 1 ms, the nanoparticles exhibit fluorescence emission of ~15000 photons per particle per 1 ms, yielding theoretical localization uncertainty of 10 nm per frame along lateral plane. Because CPNs exhibit a fluorescent emission rate about 10 times higher and adsorption cross section of 10 – 100 times larger than that of QDs with similar size, the spatial resolution for tracking experiments is significantly improved by a factor of 3. The diffusion coefficients ranging from 0.026 to 5.0 µm²/s were measured by using the MSD method, which agree well with those from the Stokes-Einstein equation according to various solution viscosities and particle size. The axial positional trajectories for 3D particle tracking were determined by defocused imaging, which evaluates the width of fluorescence spot at different displaced focal planes, yielding an axial resolution of 20 nm.

Single particle spectroscopy of CPNs was then applied to study particle mobility with confined diffusion. Particle tracking at 200 Hz and 1 kHz of CPNs with anomalous subdiffusion in a porous material has been performed to characterize the structure of nanopores and determine the diffusion dynamics inside the pores and channels. The pore size was determined by the position histogram of particle trajectory that consists of several confined diffusions in individual pores. The resulting average pore diameter agrees well with the previous result determined by AFM at the agarose surface. In addition, particle tracking at 1 kHz lengthens particle trajectories in a single pore with
number of frame sufficient for the MSD calculation. It was fitted to the confined
diffusion equation, yielding the pore size and diffusion coefficient of the particle inside
the pore. The position histogram and the MSD of confined trajectories were validated by
random-walk simulations using experimental parameters. Based on these results, we
conclude that the brightness and photostability of CPNs should be useful for a broad
range of high-speed tracking applications of nanoparticles or biomolecules with
anomalous diffusion such as membrane dynamics or protein tracking, which require high
temporal resolution and nanometer spatial resolution.

Figure 6.1 High-speed tracking at 1-kHz framerate of CPNs with anomalous subdiffusion
in agarose gel. Porous structure and particle dynamics were analyzed by using position
histogram and MSD methods.
6.2 Future work

The results of particle tracking in this work could suggest several aspects that can be continued for future work. Single molecule localization and particle imaging could be used to study morphology of a transparent material and dynamics of particles in a confined vesicle or framework. In addition, axial localization accuracy can be improved by various techniques, resulting a useful information of 3D dynamics of a particle. The axial detection range could be extended for tracking biomolecules in large bacteria or a eukaryote cell.

1) Nanoparticle dynamics for super-resolution imaging

To study the morphology of a microscale particle, hundreds of fluorescent molecules have been adsorbed onto the particle surface. Localization of the adsorbed molecules by fluorescence imaging was used to construct the particle structure. For example, a 5-µm silica bead was stained with photo-switching rhodamine B derivative molecules. The fluorescence image taken by photo-activated localization microscopy yields a 3D structural image of the bead with an average lateral localization precision of single dye molecule of 10 nm at 30 Hz framerate and an axial separation of 330 nm between slices. This technique can be applied to image the structure of a porous material such as agarose gel. The pore size depends on its concentration, for instance pore size of 2% agarose gel in 0.1 M PBS is ~0.5 µm in diameter. At a moderate concentration of CPNs with average diameter of ~20 nm, the particles can disperse through the gel cavities and bind to the wall by a strong hydrodynamic interaction. The fluorescence
image of bright localized nanoparticles at a framerate of 100 Hz can illustrate an individual agarose pore with lateral resolution of better than 1 nm. The video imaging can be taken with a continuous axial translation of an $xyz$ piezoelectric stage at 10 nm per 50 ms for 5 seconds (1 µm in total). The axial position of particles can be located by finding the focused fwhm along $z$ direction through the quadratic equation, resulting in less than 10 nm axial resolution. This method will dramatically increase the accuracy of the $z$ coordinate and retain the high resolution along lateral plane.

Besides the porous materials, this technique could be applied to image nano-patterned conducting films. Their excellent properties exhibit high optical transparency and high electrical conductivity. The conducting films are widely used as electronic devices including flat panel displays and photovoltaics. Metal nanostructure networks such as copper mesh or gold grid structures have been developed to replace indium tin oxide in order to reduce material costs and lower processing temperature in mass production. The mesh structure can be fabricated by using photolithography and wet etching, which create tiny stripe or holes on a surface at a few tens of nanometers in size. Briefly, a cleansed flat substrate is covered with a photoresist by spin coating resulting in a uniform thin layer of ~0.5 µm thickness. The photoresist layer is then partially removed by exposing to a pattern of intense light. The wet etching process is employed at the unprotected part of substrate resulting the desired patterns, which can be characterized by AFM method or using optical microscope. The super-resolution image of the patterned surface can be constructed by the positioning of adsorbed CPNs on the surface as described above.
Particle tracking in homogeneous solution has been verified to be an alternative method to illustrate the agarose pore and channel. The confined dynamics of a single nanoparticle could also determine the diffusion properties inside a moving spherical structure. For example, an artificial single-bilayer phospholipid vesicle (0.1 – 3.5 µm in diameter) can be prepared by injecting aqueous buffer into phospholipid and organic solution, imitating commercial liposomes widely used in drug delivery system or small living bacteria. The movement of nanoparticles is enhanced by their confined diffusion inside the vesicle and the directed motion of the vesicle. Hence, a fast particle tracking is required to observe this fast motion and differentiate these types of motion. This could elaborately explain the carrying and releasing behaviors of various vesicles with a drug molecule of interest.

2) Multiplane imaging techniques to improve axial localization accuracy

The axial position of a particle mobilizing in a solution has been determined in this work by using defocused imaging without complicated setups that are typical for multiplane imaging methods. Defocused imaging calculates the fwhm or radius of the first or outermost diffraction pattern around the particle to determine the out-of-plane position (z) by comparing to the quadratic fwhm-z relationship of fixed particles. The limitations of this method are that negative and positive defocus cannot be distinguished by the symmetric quadratic relationship and the error in the axial location is very large especially when the particles are close to the plane of focus.
To improve the axial spatial resolution, a weak cylindrical lens ($f \approx 10$ m) was placed at the emission beam path resulting in an axial astigmatism. The lens bends the fluorescence spot into elliptical shape with its major axis reversed between $x$ and $y$ at negative and positive defocus.\textsuperscript{131} Besides, multiplane imaging techniques have been studied to increase the axial resolution by:

1. Sequentially translating an $xyz$ piezoelectric stage or an objective lens along $z$ axis using a staircase waveform generator acquiring the *staircase images* at different planes,

2. Splitting the fluorescence beam collected by an objective lens into two paths resulting in *bifocal image*.\textsuperscript{132}

A drawback of the staircase imaging method is that translating devices are typically slow with large latency time and suffer from the synchronization between their movement and the displacement of the particles. Moreover, when the specimen is being imaged at one focal plane, important events can be missed in the other planes. On the other hand, the bifocal imaging is performed by adjusting the position of an additional lens placing along one light path shifting the focal plane of the two beams relative to each other. Two images at different focus levels of the point source acquired simultaneously on different parts of the camera chip give additional information to estimate the $z$ position with better accuracy around the plane of focus. Applying the bifocal imaging with a cylindrical lens to particle-tracking experiments with the high-speed tracking at 1 kHz will split the fluorescence beam into two paths resulting in higher axial resolution, however, the lateral resolution could be slightly lowered with less signal intensity.
3) Extend the axial detection range

Since different approaches towards 3D particle tracking have been developed in recent years extending the observable spatial parameters from 2D projections to full volumes, the axial range remained mostly restricted to several microns by the depth of field of high numerical aperture objectives required for single particle detection. Our experiment shows that the high-speed single-particle tracking at 1 kHz can trace a fast diffusion with the particle displacement with $D$ up to 5.0 $\mu$m$^2$/s. However, the particles tend to move out of the limited focal volume ($10 \times 10 \times 3$ $\mu$m$^3$, according to the area of laser excitation and the depth of field) especially in the $z$ direction in a short time. Nanoparticles with diffusion coefficient $D$ would have traversed the depth of field of the detection objective of 3 $\mu$m with a probability of 99.7% within $\Delta t = (3 \mu m)^2 / (6 \times D) = 300$ ms corresponding to 300 frames at 1 kHz framerate, which can be compared to 200 frames in the experiment with random starting points of particles. This axial range limits the length of particle trajectories that obtain crucial information such as the diffusion of proteins or biomolecules in the cytoplasm of eukaryotic cell (10 – 30 $\mu$m).

Particles could be retained in the focus by; using multiple focal planes to simultaneously observe a substantial fraction of desired volume, or feedback autofocus with the translation of the $xyz$ piezoelectric stage toward the movement of a particle of interest reaching a focal boundary. Multiple focal planes not only extend the detection range (for instance, nine focal planes with 250-nm separations can cover the nuclear volume) but also improve the axial resolution as described above. However, the fluorescence beam is divided into sub-images with some signal losses by imperfect
transmission of the phase elements, resulting in lower lateral resolution. On the other hand, feedback autofocusing requires a real-time tracking for coarsely locating the particle, which can operate by a custom script written in Matlab with Andor software development kit. The feedback loop process spends time on the bus time between camera and computer, execution time of coarse positioning and the latency time of the piezo stage. Previously, particle tracking experiment of a 20-nm fluorescent bead within the nucleus of a *C. tentans* salivary gland cell nucleus shown a feedback autofocusing at 20 Hz following particles with mean $D = 0.21 \pm 0.03 \ \mu m^2/s$ for 4 min with an axial range of more than 10 µm.$^{145}$ Increasing the framerate beyond 100 Hz, piezo stage response might affect the performance of the feedback loop. However, a fast piezo-driven tilt mirror and a piezo actuator mounted to the objective combining with bifocal method can extend the lateral and axial tracking ranges to 10 µm and 100 µm, respectively.$^{146}$ This technique can trace a 200-nm particle in water with mean $D = 2.4 \pm 0.4 \ \mu m^2/s$. In addition, due to the autofocusing the particle image stays in the same small detection areas on the camera chip about 750 nm width (5 pixels), which can dramatically decrease the buffering time of the camera reaching 3.2-kHz framerate. Hence, we could apply the multiple focal plane and feedback autofocusing techniques for 3D tracking of 20-nm CPNs in solutions or a porous material with $D$ of 7 to 30 µm$^2$/s, imitating the movement of a small protein with ~30 kDa in *E. coli* cytoplasm or RNA with ~20 kDa in eukaryotic cell cytoplasm.
4) Particle tracking of biomolecules in cell

A variety of bright and photo-stable labelling techniques have been developed to exhibit sufficient contrast for single particle tracking within the cellular context in a wide range of specimen from yeast and bacteria to cultured cells, and even multicellular organisms or live animals. RNA molecules carry out widely diverse functions in different physiological processes in living cells such as transcription through the processing of nascent RNA, intranuclear trafficking, and cytoplasmic translation of messenger RNA. Single molecule imaging and particle tracking can yield further insight into the dynamics of RNA particles. For instance, particle tracking of fluorescent labelled ribosomal subunits including 30S and 50S ribosomal proteins (~20 nm in diameter) in *E. coli* (2 µm in length) revealed that translating ribosomes move much slower than free subunits and mRNA-bound translating ribosomes are excluded from the bacterial nucleoid, whereas free subunits have full access to it.\textsuperscript{147} Recent studies of mRNA molecules transporting through nuclear pore complexes embedded in the nuclear envelope of an eukaryotic cell have found that the export process took between 65 ms to several seconds depending on their size of mRNA molecules.\textsuperscript{148} The mRNAs were attached to fluorescent dye molecules yielding a localization precision of 10 nm at 50-Hz framerate. We expect to apply the bright and photostable CPNs to observe such important cellular activities in living cells including small bacteria and large eukaryotic cells with better temporal resolution of 1 ms and localization uncertainty of ~10 nm. Moreover, combining particle tracking with the bifocal technique and an additional cylindrical lens could yield better
spatial resolution along $z$ direction and extend the axial detection range required in 3D particle tracking of biomolecules in living cells.
Appendix A

Additional figures

Figure A-1 Transmission efficiency of a 500-nm dichroic (500DCLP, Chroma) mirror at different wavelength in nm. This can be used to estimate transmission or reflection efficiency at this optical window such as 98% reflection efficiency of a 445-nm excitation. Retrieved from Ref. 149.
Figure A-2 Transmission efficiency of typical achromat objectives with 100× and 1.25 NA, Olympus at different wavelength in nm. Transmission efficiency of fluorescence emission of PFBT nanoparticles at 545 nm was estimated of about 0.10 through the objective. Retrieved from Ref\textsuperscript{150}.

Figure A-3 Distance between two coverslips was determined from the focused fwhm of two PFBT particles immobilized on each coverslips. The widths were fitted to a quadratic function of axial position $z$ shown in blue and green, giving the distance between the vertex of 60 µm.
Appendix B

Selected custom computational Matlab scripts

Figure B-1 Custom Matlab script for controlling a piezoelectric movement in z direction by generating a staircase voltage by a DAQ device.

```matlab
% Staircase voltage script for NI-DAQ 6008.
% (1) 64-bit version of MATLAB with DAQ toolbox and
% (2) Latest NI-DAQmx software (15.0 or higher).

close all
% Close all windows to avoid timing problems.

%% Staircase details.
dt = 0.05;
% Step size in seconds.
Vs = 0.200:0.002:1.100;
% Voltage vector. Maximum is 5 V for the device.
V = [0 Vs Vs(end:-1:1) 0];
% Scan forward and backward with two voltage jumps for synchronization.

%% DAQ device details
devices = daq.getDevices;
s = daq.createSession('ni');
ao = addAnalogOutputChannel(s,'Dev1', 'ao0', 'Voltage');
outputSingleScan(s,0);
% Set initial output at zero volt.

%% Busy-wait timing loop parameters
sleepms = 1;
% Number of ms per sleep call. <2X smaller than dt.

%% Set up variables for keeping track of timing and number of loops
pstime = [];
% Array for keeping track of latency for each step
jitter = [];
% Keep track of timing jitter for each step

%% Start timer
tic;
%
for step=1:length(V)
  % Calculate jitter (Timing inaccuracy)
  theotime=(step-1)*dt;
  jitter(step)=toc-theotime;
```
% Switch voltage, keeping track of AO call time
% Switch voltage, keeping track of AO call time
t1 = toc;
outputSingleScan(s,V(step));
t2=toc;
pstime(step)=t2-t1;
    % Record the time required to putsample()

%% Output current cycle, step number, jitter, and voltage to
screen
fprintf(1,'Step: %i, Volts: %2.3f, Time: %.5f s, Jitter: %.5f
ms\n',step, V(step),toc, jitter(step)*1e3);

%% Secondary loop for waiting until next step
while toc < theotime+dt
    if theotime+dt-toc > sleepms*1.1
        java.lang.Thread.sleep(sleepms);
    end
end

%% Report timing results.
% Report timing results.
fprintf(1,'Timing error = %1.3f us\n',
mean(deleteoutliers(jitter,0.05))*1e6)
    %deleteoutliers.m (Brett Shoelson,2009)
fprintf(1,'DAQ latency time = %1.3f ms\n',mean(pstime)*1000)
Figure B-2 Custom Matlab script for random-walk simulations and MSD calculations.

```matlab
%% Input parameters
Din = 1.0;
% Input diffusion coefficient, Din, um^2/s
frate = 1/0.001;
% Frequency, Hz
sz = 5000;
% Size of random walk (Number of frame)
dim = 3;
% Dimension
delta = 2*dim*Din;
% MSD/sec
sigma = sqrt(delta/frate);
% RMSD/step
FA = 50;
% Number of frame to be analyzed
tsec = 0:1/frate:(FA-1)/frate;
warning off MATLAB:structOnObject
fprintf('Input: D = %1.3f um^2/s\n', Din)

%% XYZ trajectories from random-walk simulations
rng('shuffle')
XYZwalk = zeros(sz,dim);
% Compute the individual steps
SS = sigma*randn(sz,1); % Stepsize is normal with SD=sigma
DI = randn(sz,dim); % Direction is random
v = SS./sqrt(sum(DI.^2,2));
b = spdiags(v,0,sz,sz);
dx(1:sz,1:dim) = b*DI;
XYZwalk(1:sz,1:dim) = cumsum(dx(1:sz,1:dim),1);
% Each position is the sum of the previous steps

%% (MSD) Mean squared displacement method
XYZmsd = zeros([FA dim]);
for cnt = 1:FA
    tau = cnt+1;
    XYZttau = XYZwalk(tau:end,1:end);
    % XYZ(t+tau)
    XYZt = XYZwalk(1:length(XYZttau),1:end);
    % XYZ(t)
dXYZsq = (XYZttau-XYZt).^2;
% <dXYZ^2>=mean(dXYZ^2)=mean[(XYZ(t+tau)-XYZ(t))^2]
    XYZmsd(cnt,1:dim) = mean(dXYZsq,1);
end
[fitR,~] = fit(((1:FA)/frate)',(sum(XYZmsd,2)),[num2str(dim)
'2*dim*x+(V*x)^2+E'], 'startpoint', [0.5*Din,1e-10,0]);
% <dXYZ^2>=2*dim*D*t-V^2t^2+E
fitRv = struct(fitR);
Dmsd = fitRv.coeffValues{1,1};
fprintf('MSD: Dxyz = %1.3f um^2/s\n', Dmsd)
```

159
Figure B-3 Custom Matlab script for confined diffusion simulations and MSD calculations.

```matlab
%% Input parameters
Din = 1;
    % Input diffusion coefficient, Din, um^2/s
frate = 1/0.001;
    % Frequency, Hz
sz = 5000;
    % Size of random walk
dim = 3;
    % Dimension
delta = 2*dim*Din;
    % MSD/sec
sigma = sqrt(delta/frate);
    % RMSD/step
FA = 50;
    % Frame number to be analyzed
ranmsdosec = (1:FA)/frate;
Ts=(1:sz)/frate;
rsphere=0.125;
    % Confined radius
confnode=10;
    % n/2 pores
confraction=0.9;
    % Confined trajectory fraction
fprintf('RMSD=%1.3fum 6*RMSD=%1.3fum Pore=%1.3fum\n', sigma, 6*sigma, 2*rsphere)

%% XYZ trajectories from random-walk simulations
rng('shuffle')
XYZwalk = zeros(sz,dim);
    % Compute the individual steps
SS = sigma*randn(sz,1);
    % Stepsize is normal with SD=sigma
DI = randn(sz,dim);
    % Direction is random
v = SS./sqrt(sum(DI.^2,2));
b = spdiags(v,0,sz,sz);
dx(1:sz,1:dim) = b*DI;
XYZwalk(1:end,1:end,1:dim) = cumsum(dx(1:sz,1:dim),1);
    % Each position is the sum of the previous steps

XYZwalk = [XYZwalk(1:end,1)-XYZwalk(1,1) XYZwalk(1:end,2)-XYZwalk(1,2)
            XYZwalk(1:end,3)-XYZwalk(1,3)];
Xwalk = XYZwalk(1:end,1);
Ywalk = XYZwalk(1:end,2);
Zwalk = XYZwalk(1:end,3);

%% Restriction of the simulated trajectory in given pores
% Node
```

\texttt{intv = round(length(XYZwalk)/confnode);}
\texttt{FF = 1:length(XYZwalk);}

\texttt{\% Boundary}
\texttt{frameconfined = [];}
\texttt{framebounce = [];
for j = 1:1:confnode/2
  confF = FF((j-1)*2*intv+1:((j-1)*2*intv)+2*intv*confraction);
  Xmove = Xwalk(confF(1));
  Ymove = Ywalk(confF(1));
  Zmove = Zwalk(confF(1));
  Xwalk = Xwalk-Xmove;
  Ywalk = Ywalk-Ymove;
  Zwalk = Zwalk-Zmove;
  frametotal = 2*intv*confraction;
  frameconfined(j) = size(confF,2);
  framebounce(j) = 0;
  for i = confF
    if Xwalk(i)^2+Ywalk(i)^2+Zwalk(i)^2 > rsphere^2
      framebounce(j) = framebounce(j)+1;
      X1 = Xwalk(i); Y1 = Ywalk(i); Z1 = Zwalk(i);
      X0 = Xwalk(i-1); Y0 = Ywalk(i-1); Z0 = Zwalk(i-1);
      Xrand = Xwalk(i); Yrand = Ywalk(i); Zrand = Zwalk(i);
      AA = (X1-X0)^2+(Y1-Y0)^2+(Z1-Z0)^2;
      BB = 2*((X1-X0)*(X0)+(Y1-Y0)*(Y0)+(Z1-Z0)*(Z0));
      CC = (X0)^2+(Y0)^2+(Z0)^2-rsphere^2;
      TT1 = (-BB+sqrt(BB^2-4*CC*AA))/2/AA;
      TT2 = (-BB-sqrt(BB^2-4*CC*AA))/2/AA;
      TT = max([TT1 TT2]);
      Xitc = X0 + TT*(X1-X0);
      Yitc = Y0 + TT*(Y1-Y0);
      Zitc = Z0 + TT*(Z1-Z0);
      Lbnc = sqrt((X1-Xitc)^2+(Y1-Yitc)^2+(Z1-Zitc)^2);
      while Lbnc >rsphere
        Lbnc = Lbnc-rsphere;
      end
      while Xrand^2+Yrand^2+Zrand^2 > rsphere^2
        theta = 2*pi*rand(1,1);
        phi = asin(2*rand(1,1)-1);
        [Xrand,Yrand,Zrand]=sph2cart(theta,phi,Lbnc);
        Xrand = Xrand+Xitc;
        Yrand = Yrand+Yitc;
        Zrand = Zrand+Zitc;
      end
    end
  end
end
Xwalk = Xwalk+Xmove;
Ywalk = Ywalk+Ymove;
Zwalk = Zwalk+Zmove;
end

fprintf('Total frame = %1.0f, #Pore = %1.0f
',sz,confnode/2)
for m = 1:size(framebounce,2)
    fprintf('Pore %1i: #bouncing/#totalf = %1.0f/%1.0f\n', m, framebounce(m), frametotal)
end

%% Plot 3D trajectory
figure
plot3(Xwalk,Ywalk,Zwalk);
xlabel(['x (',char(181),'m)'])
ylabel(['y (',char(181),'m)'])
zielabel(['z (',char(181),'m)'])
grid on
set(gca,'dataaspectratio',[1 1 1])
REFERENCES


