Determining 3D Orientations of Single Molecules

INTRODUCTION

Single molecule spectroscopy has become an increasingly useful and important technique for measuring various phenomena at the nanometer scale. The power of single molecule spectroscopy comes from its ability to access information otherwise lost in ensemble averaging. Ensemble averaged measurements give the mean of a number of molecules, each with potentially different reaction pathways and time-varying fluctuations. In other words, one is limited to an overview or summary of a system. In contrast, single molecule spectroscopy provides access to local information about physical, chemical, and biological processes.

In this study, we analyze the orientation of single dye molecules by raster scanning a sample to map out the fluorescence emission and resultant absorption dipole. This orientation information can be used in combination with knowledge of the dye molecule to probe the local environment. Specifically, orientational information is important in biological studies where an understanding of a molecule’s local environment is critical to understanding its behavior. Since many biological molecules have small absorption cross-sections, fluorophores can be bound to particular sites to aid in analysis. Many photostable fluorophores exist and have been well documented.

THEORY

The starting point for any discussion of single molecule spectroscopy is the absorption and emission characteristics of a dipole. Specifically, we consider the dipole emission near a planar interface, since the molecule of interest is on a glass coverslip, under a thin layer of poly (methyl methacrylate), (PMMA). Work on the various emission patterns of a dipole corresponding to its various orientations was developed by Sommerfeld and others in the early twentieth century. From this work, we can calculate the expected radiation patterns corresponding to various orientations. The fluorescence intensity is proportional to the projection of the absorption dipole of the molecule onto the electric field vector of the focused laser beam. The fluorescence rate $R$ in the focus is given by

$$R(r) = c |d \cdot E(r)|^2$$

Where $d$ is the unit vector corresponding to the dipole orientation and $E(r)$ is the electric field vector in the focus. Since we know the E-field, a measure of the intensity of the fluorescence emission is proportional to the orientation of the dipole. Using scanning confocal microscopy, the fluorescence intensity emitted by the dipole is collected by an avalanche photodiode. The emission pattern can then be correlated with calculated values of the orientation of the absorption dipole. The resulting intensity distribution of the fluorescence is described by

$$I(r, \phi, \Theta, \Phi) \propto \frac{1}{\cos \Theta} (E_p^* E_p + E_s^* E_s)$$

Where $r$ is the distance from the center of the molecule in the image plane, $\phi$ is the azimuthal angle in the image plane, $\Theta$ is the polar angle of the dipole, and $\Phi$ is the azimuthal angle of the dipole. $E_p$ and $E_s$ refer to the p- and s- polarizations of the electric field. $E_p$ and $E_s$ for a dipole below a surface are given by

$$E_p = c_1(\theta) \cos \Theta \sin \theta + c_2(\theta) \sin \Theta \cos \theta \cos (\phi - \Phi)$$

$$E_s = c_3(\theta) \sin \Theta \sin (\phi - \Phi)$$

where $c_1$, $c_2$, and $c_3$ or given by

$$c_1 = -\frac{1}{2} (\cos \Theta + \sin \Theta \cos \phi)$$

$$c_2 = \frac{1}{2} (\cos \Theta - \sin \Theta \cos \phi)$$

$$c_3 = \frac{1}{2} (\sin \Theta \cos \phi)$$
\[ c_1(\theta) = \Pi^{-1}(\theta) + r^p(\theta)\Pi(\theta) \]
\[ c_2 = \Pi^{-1}(\theta) - r^p(\theta)\Pi(\theta) \]
\[ c_2 = -[\Pi^{-1}(\theta) + r^s(\theta)\Pi(\theta)] \]
\[ \Pi(\theta) = \exp(-ikn_2 \cos(\theta\delta)) \]  

where \( n_2 \) is the index in which the dye molecule is embedded, \( r^s \) and \( r^p \) are the Fresnel reflection coefficients corresponding to the s- and p- polarizations, and \( k \) is the vacuum wave vector.  

For a given dipole orientation and electric field polarization, fluorescence emission patterns have been calculated. As stated above, the fluorescence pattern observed by scanning over the sample is related to the projection of the electric field onto the dipole. This can be seen in (figure 1) for x- and y- polarizations. Note the change in fluorescence pattern and intensity for various polar and azimuthal angles.  

(Figure 1) Expected patterns for a single Dil molecule overcoated with PMMA. Copied (w/o permission) from M. Andreas Lieb’s poster presentation.

An important note about the dye molecules being used is photostability. Photobleaching occurs from various photochemical processes, many of which result from binding with oxygen molecules. For orientational imaging, molecules must be photostable for greater than the average number of excitations expected, since the sample is subjected to high irradiances and raster scanned.  

**EXPERIMENT**  
The laser used in this experiment is a frequency doubled Nd:YAG with a wavelength of 532 nm. The laser’s polarization is initially set by passing it through a Glan Thompson type linear polarizer. This type of polarizer is a good choice mainly because of its large acceptance angle, but also because of its large extinction ratio, and its large transmission efficiency. Then x- and y-linear polarizations are set by rotating a half wave plate. The microscope is a Nikon Eclipse TE 300, and it is used in inverted epi illumination. The magnification of the objective used in this experiment is 100x and the numerical aperture is 1.4. The laser line becomes incident upon a clean up bandpass filter centered at 532 nm with a spectral width of 10 nm. This is attached to a dichroic beamsplitter which is designed for the 532 laser line. The emission filter used in this setup is a 550 nm long pass filter. The light then encounters a dichroic beam splitter (645DCXR) and is then sent through a green bandpass filter (HQ590/75nm).  

The sample is placed onto a clean glass coverslip, which is mounted on a closed-loop piezoelectric scanner with nanometer precision. The emitted fluorescence from the sample is collected using the same objective that is used for illumination. An image is formed by raster scanning the sample through the laser focus and then collecting the emitted photons using a single-photon counting APD. With the
assumption of a constant absorption cross section and quantum yield the orientation of the absorption dipole can be found by measuring the excitation rate at each position (x, y) of the sample.

![Experimental Setup](image)

(Figure 2) Experimental Setup for Absorption Dipole Measurement

The samples were made “by spincasting 10 μL of a 1 nM solution of 1,1'-dioactadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate [DiIC18(3)] in methanol on a clean glass coverslip and by overcoating it with a thin film (10-30 nm) of poly(methyl methacrylate) (PMMA) to improve the photostability of the dye.”

**DISCUSSION**

The sample was raster scanned through the laser focus in order to map out the absorption dipoles. The figure below shows molecules which were scanned using x-polarized light on the left and y-polarized light on the right. We have labeled 6 molecules which were found in both images and we have fit their absorption dipoles using the theory which was previously discussed.
The fitted molecules are shown below. The values obtained for both the x- and the y- polarizations are shown to be in good agreement. As can be seen for molecule 1 the orientation of the absorption dipole is mostly perpendicular to the plane of the glass coverslip and the value for the phi angle is very small.

Most of the other molecules absorption dipoles are oriented such that they are nearly in the plane of the glass. This trend may be explained by the effects of the PMMA, as it may be that the PMMA tends to more often than not pull the molecules into the plane of the glass. When the molecule lies in the plane of the coverslip and is oriented along the direction of the incident polarization a maximum of intensity is observed. This is most clearly evident for molecule number 4 in which the molecule appears to lies nearly in the plane of the coverslip and along the y-direction. For molecules which lie in the plane of the glass and have absorption dipoles perpendicular to the incident polarization, a clover leaf pattern may be seen which is weak in intensity.

The cloverleaf pattern is difficult to fit because the intensity is low enough that there may be signal to noise issues. One source of noise is adjacent molecules, and if a clover leaf pattern appears to close to neighboring molecules the pattern will be ill resolved. Therefore imaging a molecule which is not well imaged with a given polarization may be imaged to a high degree of precision with the perpendicular polarization. There may be some degree of rotational diffusion between measurements and the effect of the laser focus on the single molecule may also account for some rotation of the molecule between measurements. However, we have demonstrated that single molecules are dipole absorbers through direct imaging of their absorption patterns. This experiment shows how easily one may measure such patterns,
and it also shows how nicely the measured absorption patterns may be fit to the theory. The fact that the measurements between x- and y-linear polarizations show slightly different orientations may be explained by rotational diffusion of the molecules between measurements, which may have been anywhere between 20 minutes to an hour depending on how many regions were scanned before the polarization was rotated. There may also be issues due to the fact that there is error in the x- and y-polarizations, and this may also account for some of the experimental error.

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REFERENCES