Speaker: Christi Gwyn
Date: April 12, 1999
Time: 4 p.m.
Place: Rm. 2102
Advisor: Dr. Crooks

Single-Molecule Detection

In 1976, Hirschfeld reported the first example of single molecule detection using a single polyethyleneimine molecule (MW 20000) tagged with 80 to 100 fluoresceine molecules and bound to one molecule of γ globulin.[1] Since this accomplishment, many techniques have been employed to detect single molecules. The consequence of such a feat touches different areas, from DNA fragment sequencing and sizing to the study of the photochemistry of single molecules.

Why is single-molecule detection important? It represents the ultimate in analytical sensitivity and accuracy. No measurement can be more accurate than when it counts individual molecules.[2] By studying the behavior and properties of single molecules, one can remove the typical ensemble averaging that occurs in bulk samples, thus obtaining a distribution of the behavior of single molecules. The chemical and physical behavior of single molecules is an unexplored territory, which opens the door for discovering new phenomena. In some cases, the single molecule itself serves as a probe of its local environment ("nanoenvironment").[3]

Before studying the different techniques for single-molecule detection (SMD), one must build a foundation of criteria for single molecules and SMD. First, the molecule must have some property that makes it "detectable." Single-molecule studies are usually not performed on molecules such as ethanol or even long-chain polymers. Rather, SMD is performed on molecules that fluoresce or are electrochemically active. SMD is also done on products of electron-transfer reactions that emit a photon upon relaxation.

Second, the volume being probed and the concentration of the sample must be very small to ensure the presence of only one molecule. One must span many orders of magnitude to go from Avogadro's number of molecules to a single molecule, 1.66×10^{-24} mol or 1 yoctomole.[3]

Third, in some experiments, statistical analysis must be used because of high background signals, diffusion of the individual molecules in and out of the probe volume, and photobleaching during fluorescence excitation.[4]

Single-molecule detection is a very large field in terms of techniques used. I will discuss several representative experiments that are interesting in terms of detection and results: fluorescence-based methods on solids and liquids, scanning electrochemical microscopy, and electrogenerated chemiluminescence.

In fluorescence experiments, an excitation laser causes excitation of a molecule from the singlet ground state S_0 to the first excited state S_1 (Figure 1).[5] This transition occurs when the laser is tuned to the absorption maximum that is required for the S_0 to S_1 transition. The relaxation of the molecule from high-lying vibrational levels to low-lying levels in the first excited state is non-radiative, but the decay from these low-lying vibrational levels to the ground state is radiative, resulting in fluorescent emission.

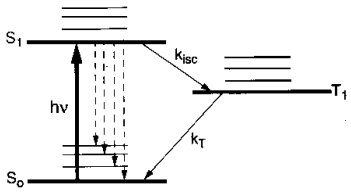

Figure 1. Schematic of electronic energy-level structure.[5]

In single-molecule spectroscopy (SMS), the single molecule is considered to be a guest in a solid host, and only this molecule is in resonance in the probe volume. Again, reduction of the probe volume is important. By focusing on a small area, having a diameter of a few micrometers, which is close to the diffraction limit, and using a small sample thickness of 3 to 10 μm, one can approach 11 to 12 orders of magnitude of reduction in the number of molecules in resonance. The amount of reduction achieved depends on the concentration of the sample, the temperature at which the experiment is being performed, and the host itself.

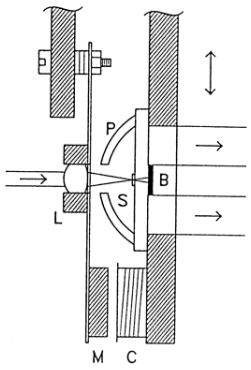

Figure 2. Lens-parabola experimental setup. L = lens, S = sample, B = beam block, P = paraboloid, M = magnet, C = coil electro-magnet.[3]

One experimental setup used for SMS is the lens-parabola (Figure 2).[3] A small lens is used to focus the light from the excitation laser onto the sample. The beam passes through the sample and the transparent host, and then the fluorescence is captured by the paraboloid and aimed out of the cryostat. A beam block stops any transmission from the pumping laser. This setup can be immersed in liquid He for low temperature experiments. Other setups are also available for SMS experiments.[3,5]

One host-guest system that has been studied is pentacene in ρ-terphenyl. The lens-parabola setup was used at 1.5 K to obtain a high-resolution spectrum containing statistical fine structure (Figure 3)[3], where each narrow peak in the absorption spectrum corresponds to a single molecule of pentacene.

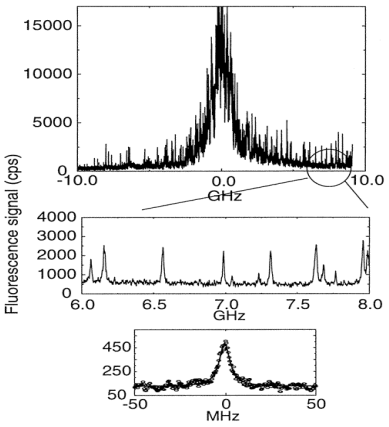

Figure 3. Fluorescence excitation spectra of pentacene in ρ-terphenyl at 1.5 K.[3]

One example of fluorescence SMD in liquids is laser-induced fluorescence coupled with flow cytometry, in which sheath fluid is used to hydrodynamically focus the sample into a small stream (Figure 4)[6]. By focusing the sample, one can narrow the probe volume and minimize the contribution of the flow walls to scattered light that results from excitation of the sample. Here a laser beam is focused to a circular spot at the center of the flow cell, and the fluorescence is collected at a 90° angle to the flow and laser using a micro-scope objective. The detection volume is defined by the long axis of a slit that runs parallel to the flow stream in the image plane of the collection objective. Photomultiplier tubes (PMTs) and silicon avalanche photodiodes (SAPDs) serve as detectors.

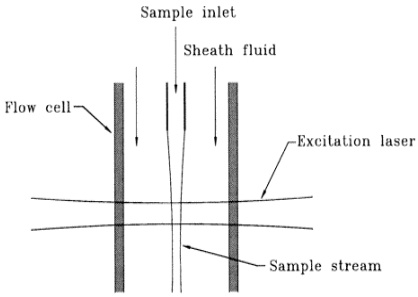

Figure 4. Sheath flow apparatus for hydrodynamic focusing.[6]

One particular experiment was conducted on TRITC, a fluorochrome, and TRITC-dUTP (TRITC-labeled nucleotide). Fluorescence was produced on a 1 x 10^{-6} M TRITC solution in salt buffer using a mode-locked dye laser at 554 nm. Figure 5 shows a spectrum of fluorescence

bursts from individual TRITC molecules crossing the probe volume, where each peak is a TRITC molecule. Photon burst size distribution of TRITC was compiled for 64 seconds of data and compared to the data generated by Monte Carlo simulation. The simulation was used to account for photobleaching, optical saturation, diffusion of the TRITC molecules, and the spatial variations in the intensity of the excitation laser and the light gathering efficiency of the optics.[6] The burst size distribution from the simulation and the experiment were in good agreement, hence single molecules were detected. This method of detection has been used for DNA fragment sequencing and sizing.[6]

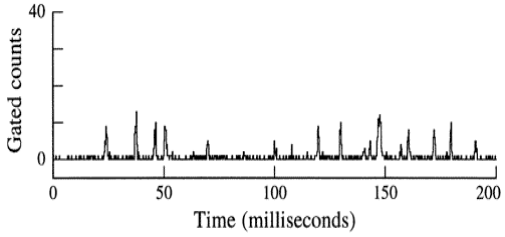

Figure 5. Fluorescence spectra of TRITC at an electro-kinetic drive voltage of 1.50 V.[6]

A completely different method of single molecule detection uses scanning electro-chemical microscopy (SECM), allowing the detection of molecules that do not fluoresce, but are electrochemically active. In SECM on single molecules, a current is measured as a result of an electron transfer reaction with individual molecules as a tip moves toward or away from a surface. The tip is a recessed disk-shaped electrode of Pt-Ir in an insulating sheath of polyethylene or Apiezon wax (Figure 6).[7] The current obtained from a single electron transfer reaction is very small and cannot be measured, unless the current is amplified by making the surface conductive. Now, the species that is reduced at the tip is oxidized at the surface, then reduced again at the tip, in a process called positive feedback.

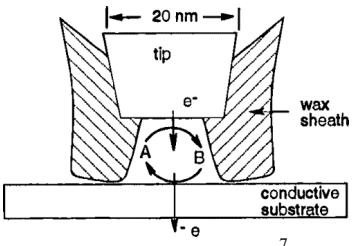

Figure 6. SECM tip.[7]

In one such experiment, a 7-nm radius SECM tip was placed at 0.55 V versus SCE at 10 nm from an indium-tin oxide (ITO) substrate at -0.3 V. A solution of 2 mM [(trimethyl-ammonio)methyl] ferrocene ($CpFeCpTMA^+$) in 2.0 M $NaNO_3$ was oxidized at the tip to a

ferrocenium species that was then reduced at the ITO substrate. The resulting fluctuations in tip current over time correspond to zero, one, or two molecules (Figure 7).[7]

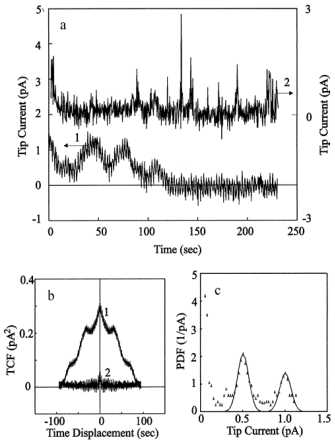

Figure 7. a) Tip current vs. time. Curve 1: 2 mM $CpFeCpTMA^+$ in 2.0 M $NaNO_3$ with d = 10 nm as described in the text. Curve 2: 2.0 M $NaNO_3$ for d within tunneling range. b) Time correlation function. c) Probability density function for curve 1.[7]

One way to describe the data is by the time correlation function (TCF), which is a measure of the time-related properties in data that are separated by fixed time delays, such as current fluctuations. TCF indicates that several fluctuations occur at frequencies of the order of a few tenths of a hertz.[7] The probability density function (PDF) is the probability that the data will be within a certain range, or current, at any point in time. The two gaussian peaks are the most probable tip currents, 0.5 pA apart, for the given experiment. The results show that single molecules do contribute to the current for the experimental parameters used.

In electrogenerated chemiluminescence (ECL), electrochemistry at a microelectrode is used to generate radical cations and radical anions of 9,10-diphenylanthracene (DPA) by pulsing the potential. An electron transfer reaction between the two radical ions takes place to form a triplet state of the species or an excited singlet state of the species:

$DPA^{\cdot -} + DPA^{\cdot +} \equiv DPA^* + DPA.$

The excited singlet state decays to the ground state by emitting a photon that is observed when the events of one cathodic pulse are viewed (Figure 8)[8]:

$DPA^* \equiv DPA + h\nu.$

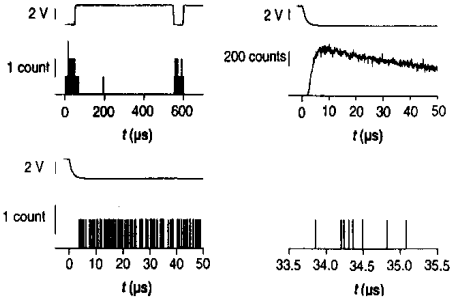

Figure 8. Single reaction events. The data was collected between 50 and 200 s. Data expanded through successive decrease in bin size from 1 s to 100 ns (top right and left) and to 5 ns (bottom left and right).[8]

The data follow Poisson distribution describing random, discrete, and independent events, which is given by $P_n(t) = e^{-\lambda t}(\lambda t)^n/n!$. The value n is the

number of cathodic pulses and λ is the mean rate of events, which is dependent on the rate of radical ion formation and their diffusion, the rate and efficiency at which they react by electron transfer, and the efficiency of photon collection. The value of λ agrees with the mean rate of photon arrival. The result is that single reactions were observed based on the time period of the measurement and the detection volume.

SMD can be accomplished in many ways using fluorescence in both solids (SMS) and liquids (LIF), electrochemistry, and chemiluminescence. Its applications are many as well. DNA fragment sequencing and sizing[6], genetic screening[9], and diagnostics[10] are important for biotechnological advances. Kinetic studies, reactivities, conformation transition studies, and single molecule reaction monitoring[11,12] are also being pursued with SMD. Molecular electronic devices[13,14] using nanotubes and microfabricated instruments[15] are of great interest as well. In terms of analytical chemistry, SMD can be applied to sensing[16], as well as the ultimate goal for analytical chemists: ultrasensitive chemical analysis.

---

[1] Hirschfeld, T. *Appl. Opt.* **1976**, *15*, 2965.
[2] Skoog, D. A.; Leary, J. J. *Principles of Instrumental Analysis*, 4th ed.; Saunders: Orlando, 1992; A1.
[3] Moerner, W. E. *Acc. Chem. Res.* **1996**, *29*, 563.
[4] Enderlein, J.; Robbins, D. L.; Ambrose, W. P.; Goodwin, P. M.; Keller, R. A. *J. Phys. Chem. B.* **1997**, *101*, 3626.
[5] *Single-Molecule Optical Detection, Imaging and Spectroscopy*; Basché, T.; Moerner, W. E.; Orrit, M.; Wild, U. P., Eds; Verlag Chemie: Munich, 1996.
[6] Goodwin, P. M.; Ambrose, W. P.; Keller, R. A. *Acc. Chem. Res.* **1996**, *29*, 607.
[7] Bard, A. J.; Fan, F. F. *Acc. Chem. Res.*, **1996**, *29*, 572.
[8] Collinson, M. A.; Wightman, R. M. *Science* **1995**, *268*, 1883.
[9] Castro, A.; Shera, E. B. *Appl. Opt.* **1995**, *34*, 3218.
[10] Eigen, M.; Rigler, R. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 5740.
[11] Funatsu, T,; Harada, Y.; Tokunaga, M.; Salto, K.; Yanagida, T. *Nature* **1995**, *374*, 555.
[12] Tan, W.; Yeung, E. S. *Anal. Chem.* **1997**, *69*, 4242.
[13] Gimzewski, J. *Physics World* **1998**, *11*, 29.
[14] Gimzewski, J. K.; Joachim, C. *Science* **1999**, *283*, 1683.
[15] Woolley, A. T.; Hadley, D.; Landre, P.; deMello, A. J.; Mathies, R. A.; Northrup, M. A. *Anal. Chem.* **1996**, *68*, 4081.
[16] Smith, C. L.; Kricka, L.; Krull, U. J. *Genetic Analysis: Biomolecular Engineering* **1995**, *12*, 33.