Lecture 17:
Single-Molecule Imaging and Spectroscopy by NSOM

- How does single-molecule measurement work?
- Why at single-molecule level?
- Broad applications of single-molecule measurement.
- NSOM imaging at single-molecule level --- high spatial resolution (up to 10 nm).

Robert Dunn, Chem. Rev. 1999, vol.99, 2891-2927.

*A lot more in a specific issue of **single-molecule spectroscopy**, July 2005, **Acct. Chem. Res**.*

Vol. 38, Issue 07
July 19, 2005 Cover

The Power of One !

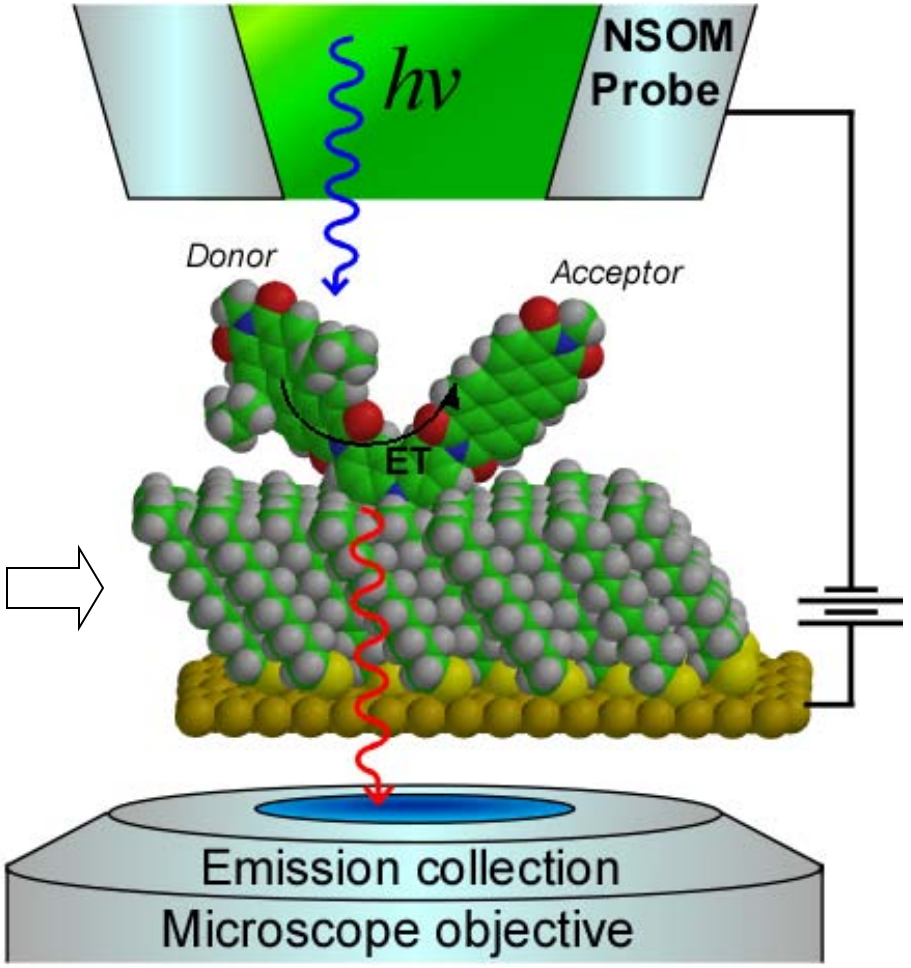
Special Issue on Single-Molecule Spectroscopy

Brief Overview of Single-Molecule Imaging and Spectroscopy

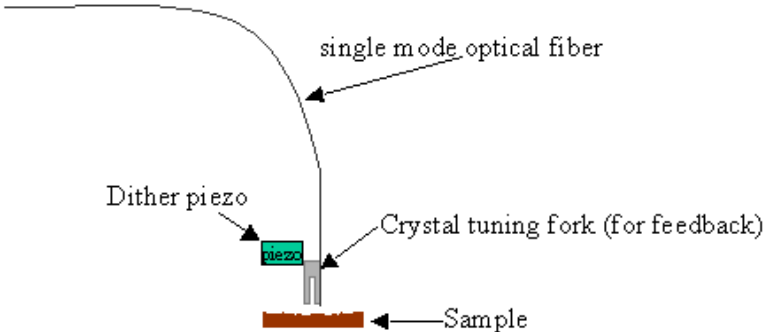
- Single-molecule spectroscopy (SMS) was initiated and pioneered by Morener and others in late 1980's, and promoted and motivated by the high resolution investigation of NSOM by Betzig and others in 1993 and 1994.
- The ultimate degree of sensitivity for detecting local structure, dynamics, chemical reactions, and physical processes.
- SMS is operated under ambient conditions.
- Taking the advantages of high sensitivity of fluorescence detection.
- NSOM based SMS provides the capability for local field modulation (bias between tip and sample substrate) to mimic the field-effect transistor (FET).

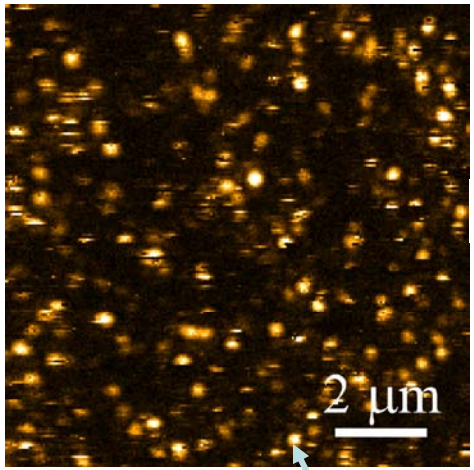
Single-molecule calibration of a molecular transistor

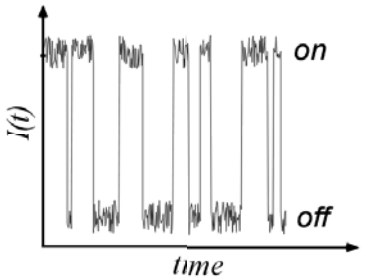
Adjust the linker to modulate the electric field effect on charge transfer.



Single Molecule Imaging

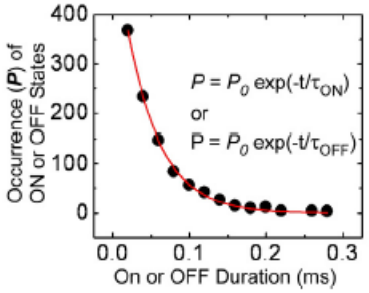






For PTCDI molecules, $\Phi(T^3) \sim 0$ and $\tau(T^3) < 1$ ms; "*off*" time is due to charge transfer state.

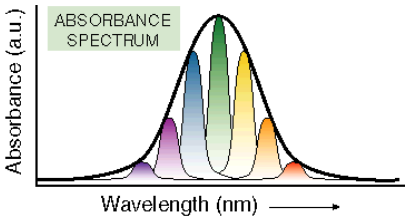
$$\frac{1}{\tau_{on}} = k_{exc}\frac{k_{ic}+k_{isc}+k_{et}}{k_r+k_{ic}-k_{isc}+k_{et}}; \quad \frac{1}{\tau_{off}} = k_b$$



Focus on one

Advantages of Single-Molecule Spectroscopy

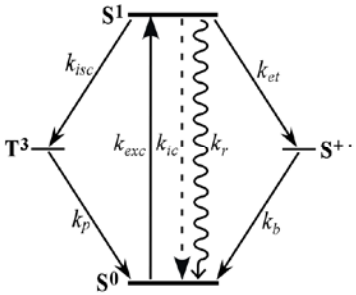
(high resolution and more detailed information)



A distribution contains more information than the average value alone!

- Remove ensemble averaging of bulk phase measurement: creating a frequency histogram of the actual distribution of values for an experimental parameter (wavelength or intensity)
  --- the probability distribution function.
  Examples: surface probing, enzyme or protein labeling.

- Reveal and diagnose the events of extremely low probability: repeatedly excitation of one molecule or measuring hundreds of different molecules. Statistics provides occurrence probability distribution as a function of structures or local environments.
  Examples: slow charge transfer (see next slide).



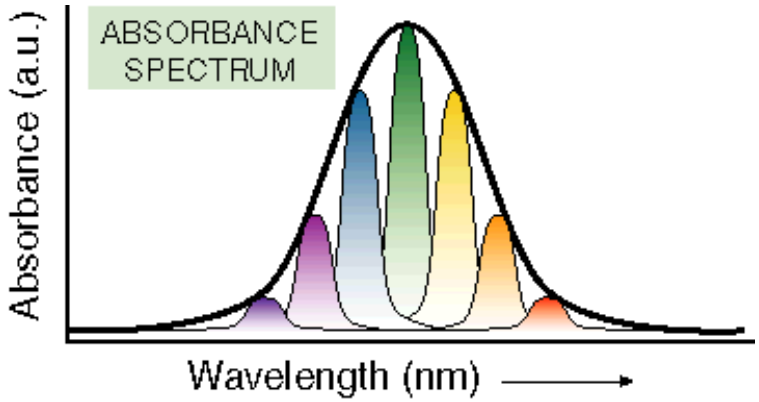
When $k_{et} << k_{isc} + k_{ic} + k_r$ It is impossible to measure k_{et} *via* bulk phase methods.

- Remove the need for synchronization of many single molecules undergoing a time-dependent process (e.g. charge or energy transfer).
  Examples: an enzyme in several catalytic state, multiple-chromophore supramolecules, proteins in different conformations. (see the attached slide).

- Monitor quantum-size effect of semiconductor nanoparticles in combination with NSOM technique: direct correlation between optical properties and particle size. Remove the tough requirement for narrow size distribution, which is particularly difficult for organic nanoparticle synthesis.

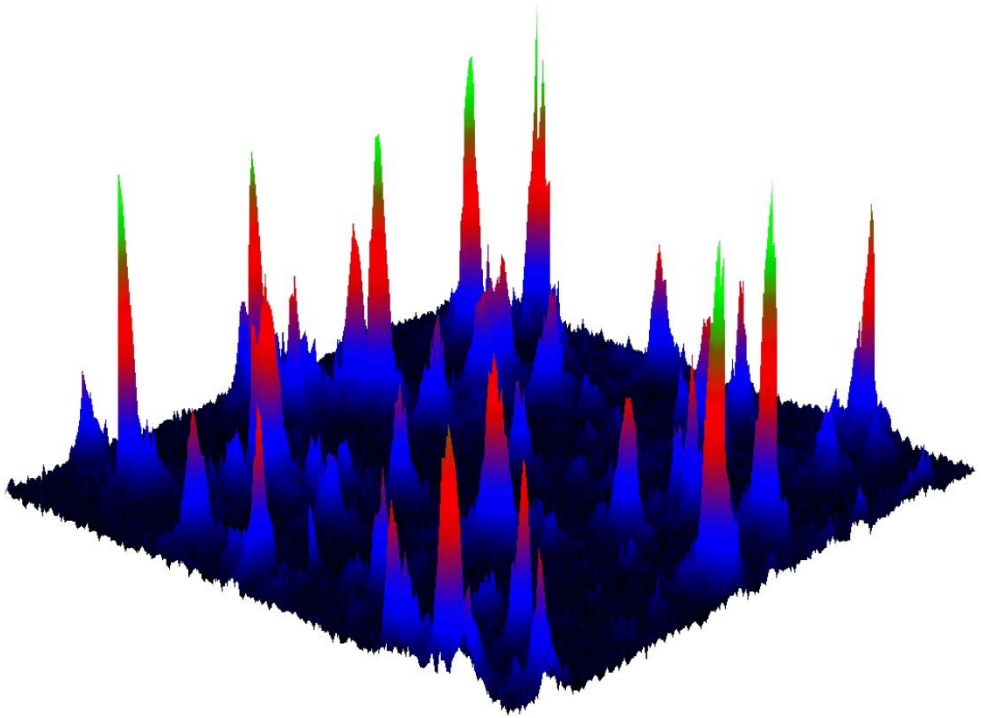
- and more ...

Single-Molecule Imaging: *revealing more detailed information*



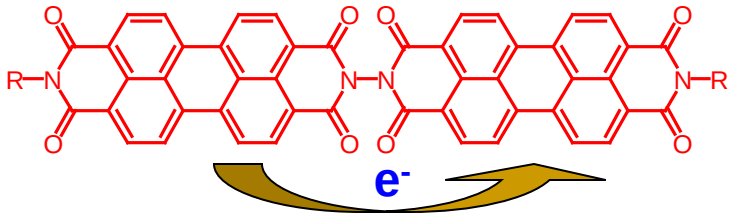
A distribution contains more information than the average value alone!

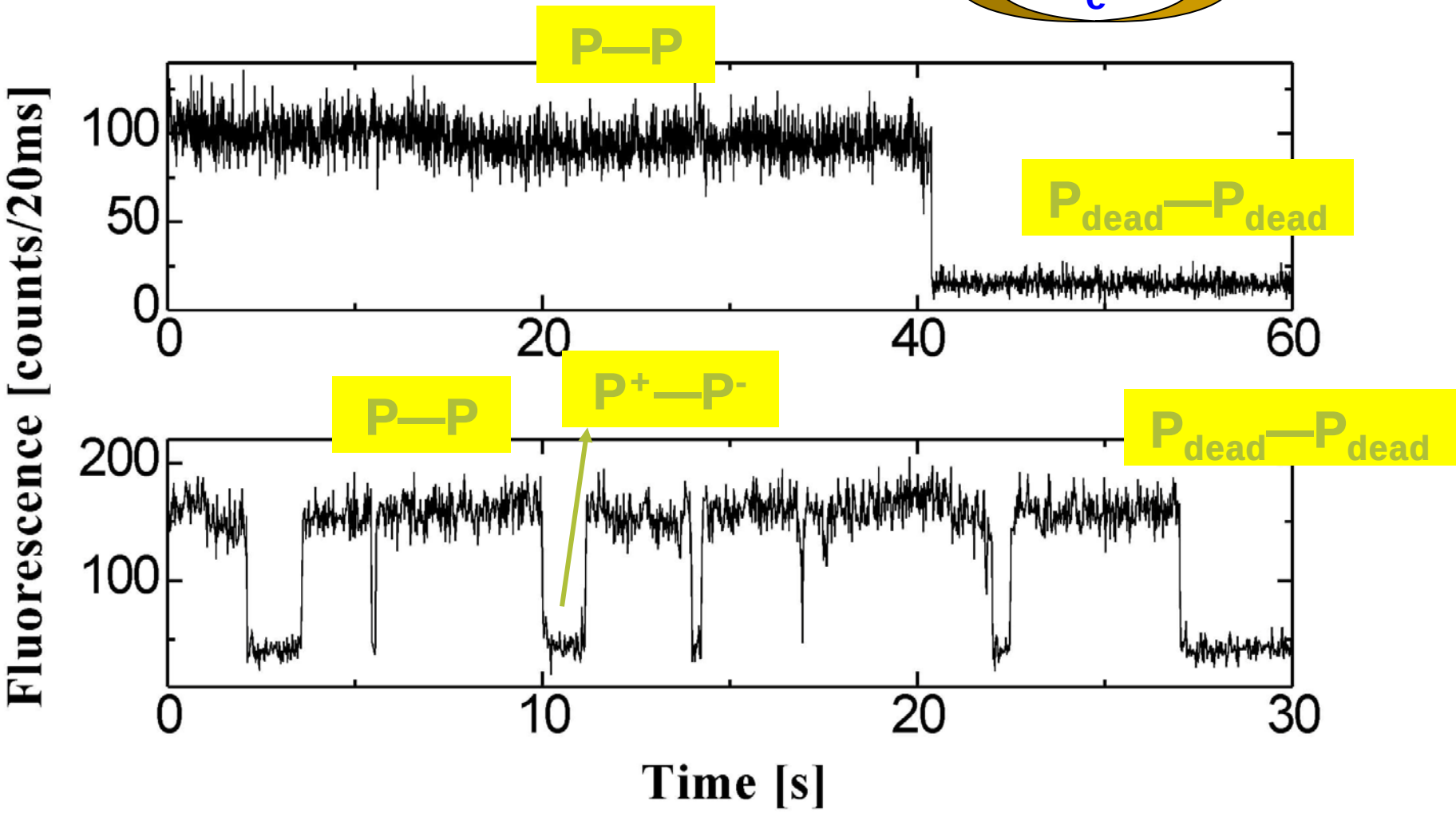
Single-Molecule Imaging: *heterogeneous distribution of intensity*



Emission intensity is often used for probing local environments!

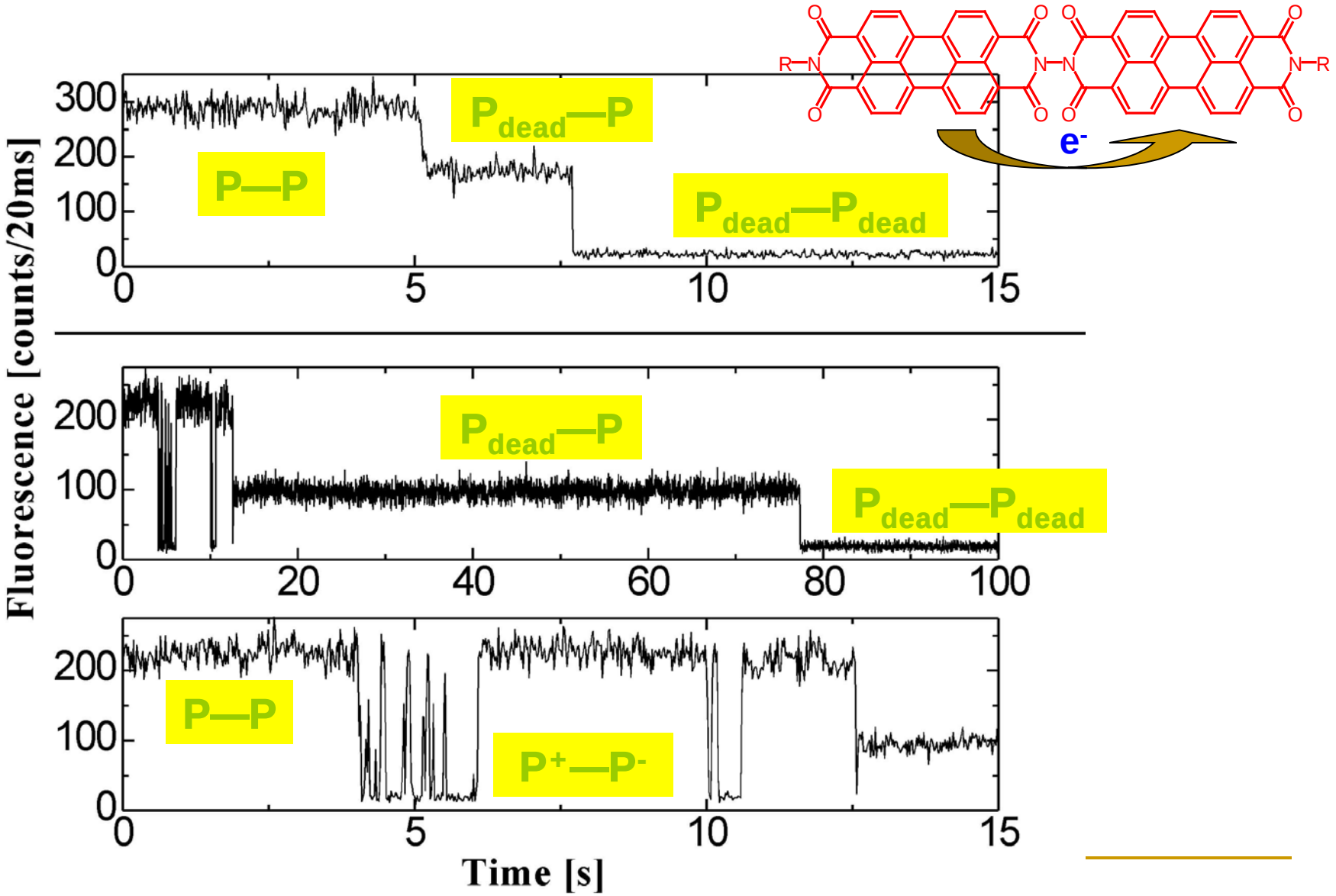
Single Molecule Imaging: *revealing rare events.*





Zang & Adams et al. *J. Am. Chem. Soc*. **126** (2004) 16126 -16133.

Single Molecule Imaging: *revealing rare events.*



Zang & Adams et al. *J. Am. Chem. Soc*. **126** (2004) 16126 -16133.

Some Publications in 2003 Science: single-molecule imaging of **proteins**

Single-Molecule Measurement of Protein Folding Kinetics

Everett A. Lipman,[1*†] Benjamin Schuler,[1,2*] Olgica Bakajin,[3] William A. Eaton[1‡]

VOL 301 10 AUGUST 2003, 1233

Protein Conformational Dynamics Probed by Single-Molecule Electron Transfer

Haw Yang,[1*] Guobin Luo,[1] Pallop Karnchanaphanurach,[1] Tai-Man Louie,[2] Ivan Rech,[3] Sergio Cova,[3] Luying Xun,[2] X. Sunney Xie[1†]

VOL 302 10 OCTOBER 2003, 262

Some Publications in 2003 Science: single-molecule imaging of **enzymes**

Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization

Ahmet Yildiz,[1] Joseph N. Forkey,[3] Sean A. McKinney,[1,2] Taekjip Ha,[1,2] Yale E. Goldman,[3] Paul R. Selvin[1,2]*

VOL 300 27 JUNE 2003, 2061

Single-Molecule Kinetics of λ Exonuclease Reveal Base Dependence and Dynamic Disorder

Antoine M. van Oijen,[1] Paul C. Blainey,[1] Donald J. Crampton,[2] Charles C. Richardson,[2] Tom Ellenberger,[2] X. Sunney Xie[1]*

VOL 301 29 AUGUST 2003, 1235

Kinesin Moves by an Asymmetric Hand-Over-Hand Mechanism

Charles L. Asbury,[1] Adrian N. Fehr,[2] Steven M. Block[1,2]*

VOL 302, 19 DECEMBER 2003, 2130

4 major papers from 1 lab in 1 year: ***all about single-molecule***

Vol 439|2 February 2006|doi:10.1038/nature04317 nature

LETTERS

DNA primase acts as a molecular brake in DNA replication

Jong-Bong Lee[1], Richard K. Hite[2], Samir M. Hamdan[1], X. Sunney Xie[3], Charles C. Richardson[1] & Antoine M. van Oijen[1]

Nature, VOL 439, 2 Feb. 2006, p621

nature Vol 440|16 March 2006|doi:10.1038/nature04599

LETTERS

Stochastic protein expression in individual cells at the single molecule level

Long Cai[1]*, Nir Friedman[1]* & X. Sunney Xie[1]

Nature, VOL 440, 16 March 2006, p358

REPORTS

Probing Gene Expression in Live Cells, One Protein Molecule at a Time

Ji Yu,[1]* Jie Xiao,[1]* Xiaojia Ren,[1] Kaiqin Lao,[2] X. Sunney Xie[1]†

We directly observed real-time production of single protein molecules in individual *Escherichia coli* cells. A fusion protein of a fast-maturing yellow fluorescent protein (YFP) and a membrane-targeting peptide was expressed under a repressed condition. The membrane-localized YFP can be

Science, VOL 311, 17 March 2006, p1600

PERSPECTIVE

Living Cells as Test Tubes

X. Sunney Xie,* Ji Yu, Wei Yuan Yang

The combination of specific probes and advanced optical microscopy now allows quantitative probing of biochemical reactions in living cells. On selected systems, one can detect and track a particular protein with single-molecule sensitivity, nanometer spatial precision, and millisecond time resolution. Metabolites, usually difficult to detect, can be imaged and monitored in living cells with coherent anti-Stokes Raman scattering microscopy. Here, we describe the application of these techniques in studying gene expression, active transport, and lipid metabolism.

Much of our quantitative understanding ... In an individual cell, gene expression is a

Science, VOL 312, 16 April 2006, p228

Typical Applications of Single-Molecule Measurement

- **Basic properties of molecules:** chemical reactivity, dynamics, optical sensitivity (switching/intensity, probing/wavelength), electrical properties (charge transfer). The modulation of properties can be due to the molecular structure itself, the molecule orientation, or the interaction (location) with the local environment. *Three slides* (see research by van Hulst, Robert Dunn, W. E, Moerner, Dan Higgins.)

- **Molecular electronic devices:** contact problem (particularly for a multiple-molecule device), requiring an alternative way to justify the promise --- *photodriven charge transfer.* PET rate can be correlated to the conductivity via theories. (see research by Paul Barbara, David Adams, De Schryver.)

- **Protein conformation and dynamics:** protein probing with attached fluorophores. Heterogeneity of localization of fluorophore on proteins and the heterogeneity of protein conformation distribution. (see research by Shimon Weiss, X. Sunney Xie, Robert Dickson, William A. Eaton, Taekjip Ha.)

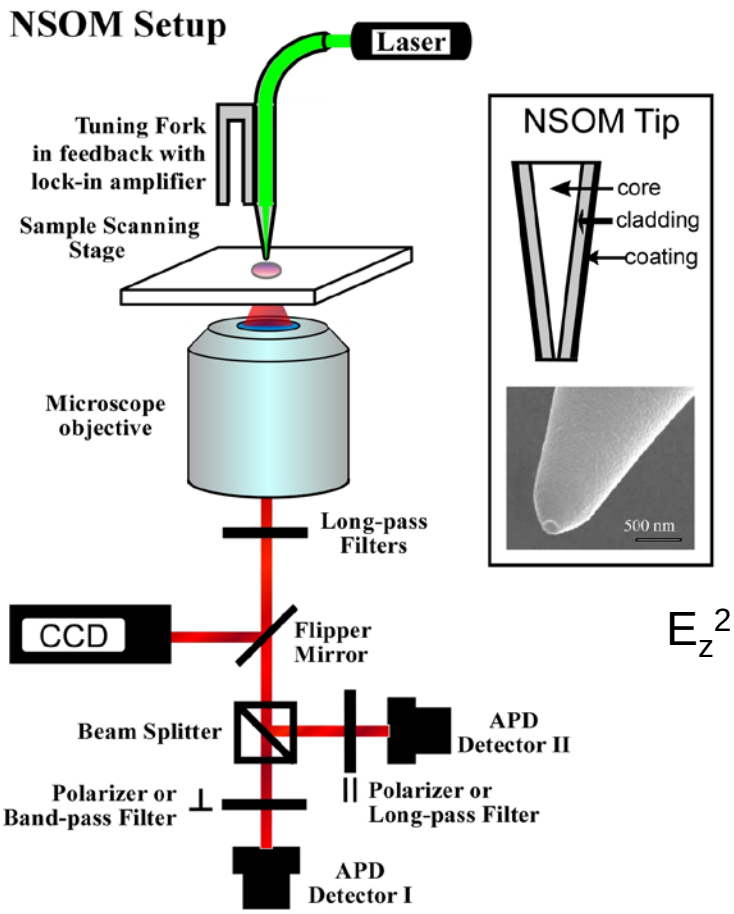
- **Enzyme catalysis:** removing the need for synchronization, and thus revealing the multiple active states of an enzyme. (see research by Xiaowei Zhuang, Steve Chu, X. Sunney Xie.)

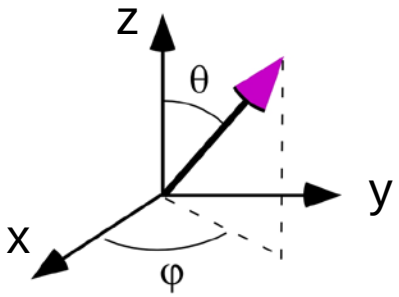
- **Single-virus dynamics:** the interaction with living cells (see research by Xiaowei Zhuang).

- **DNA conductivity** --- one of the huge debates. Huge heterogeneity of DNA configuration (folding, kinks, etc.) and defects.

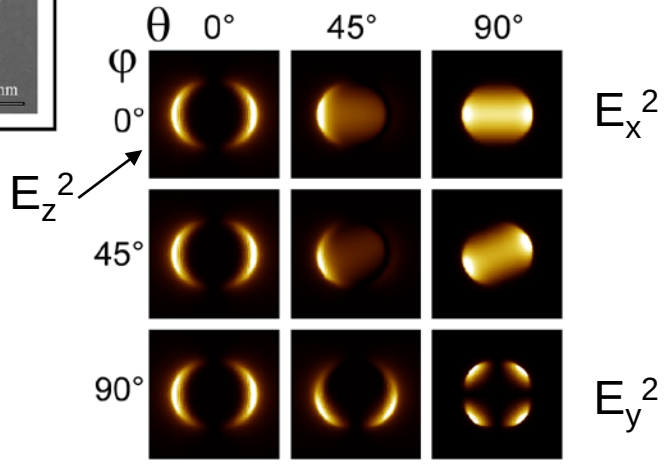
- **Nanocrystals:** inorganic vs. organic. (see research by Shuming Nie)

NSOM imaging of molecular orientation





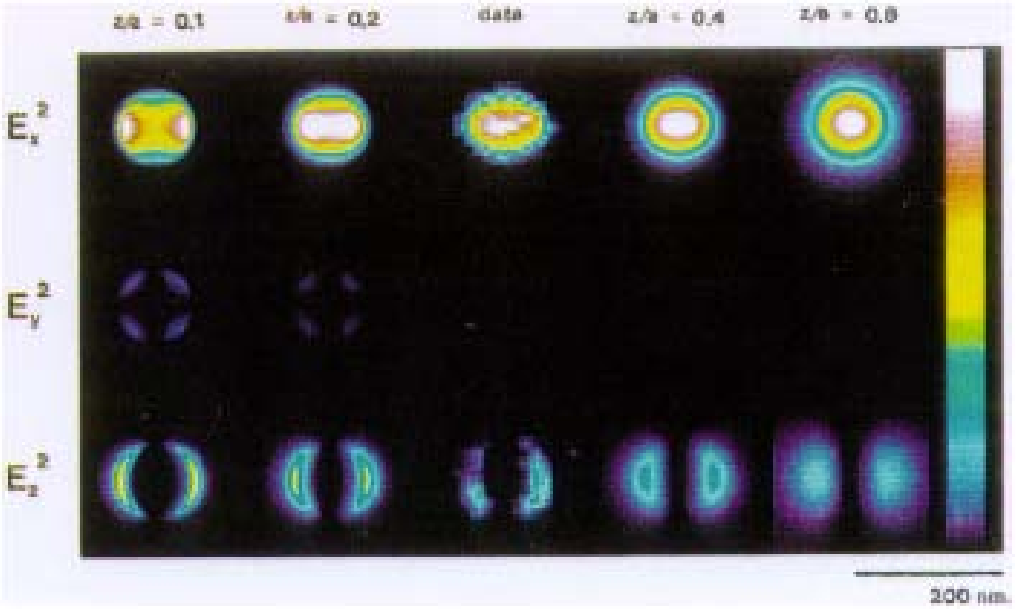
NSOM Fluorescence Imaging Patterns at Different Molecule Dipole Orientations.



Single Molecules Observed by Near-Field Scanning Optical Microscopy

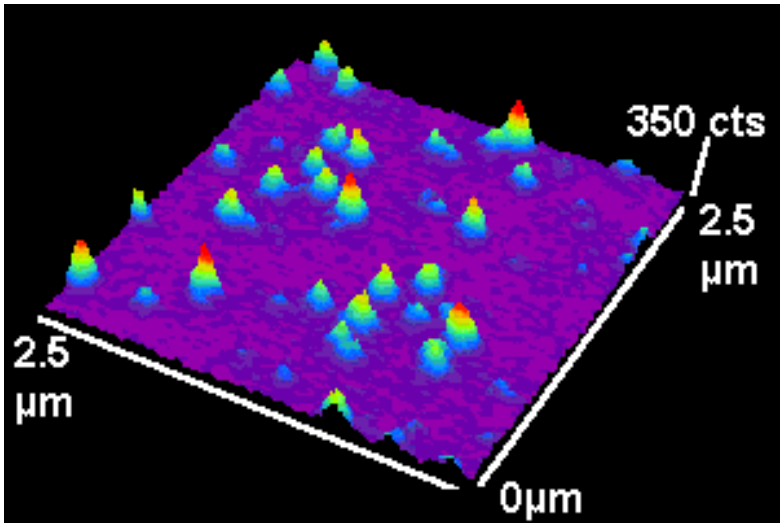
Eric Betzig and Robert J. Chichester

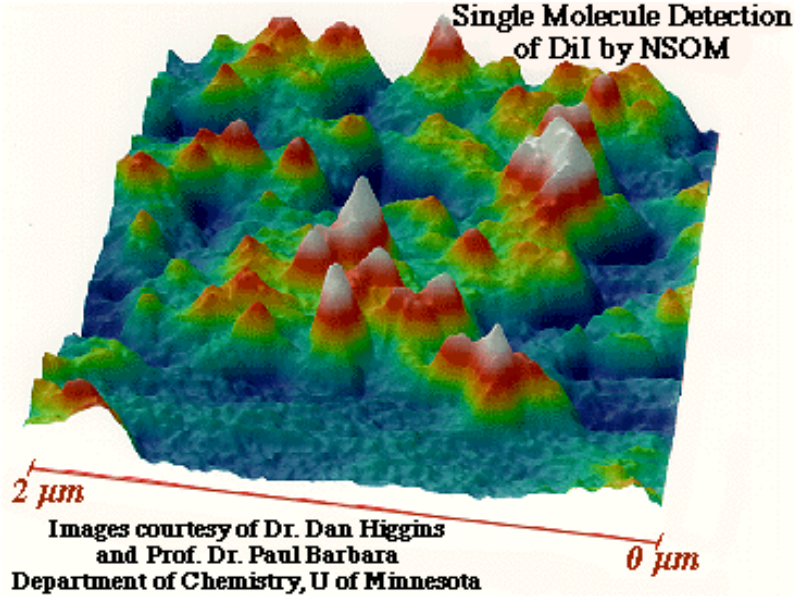
Science, 1993, vol.262, p1422.



Squared components of the electric field compared with observed fluorescence profiles *(central column)* for two different molecules.

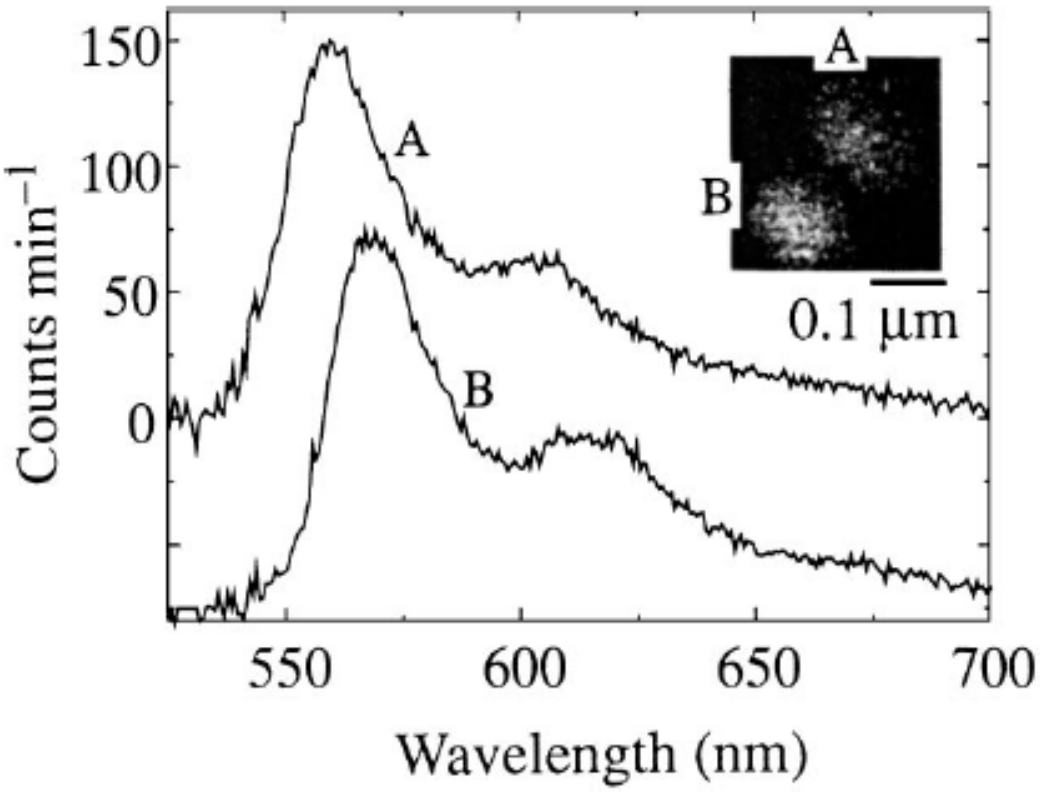
Single-Molecules Embedded in Polymer Films: *NSOM imaging reveals the local environmental effects*





Near-field spectroscopy of single-molecule at room temperature

Betzig and Brus, et al. ***Nature,*** 1994, vol.262, p1422.



Two same molecules on the same surface show different emission wavelengths.

Near-field spectroscopy of single-molecule at room temperature

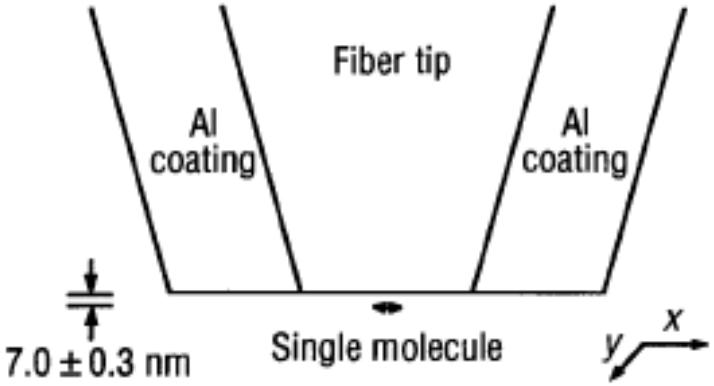
Betzig and Brus, et al. ***Nature,*** 1994, vol.262, p1422.

- Emission spectra of single molecules are narrower, relative to the ensemble spectra obtained in solutions.
- Emission of a single molecule shifts ± 8 nm --- inhomogeneous local environments.
- Single molecules also exhibit time-dependent shift of up to 10 nm --- *different environment effect at excited states.*

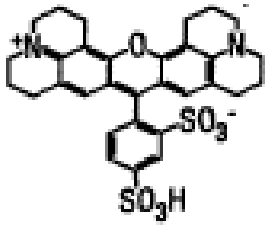
Probing Single Molecule Dynamics

X. Sunney Xie* and Robert C. Dunn

Science, 1994, vol.265, p361.



Sulforhodamine 101



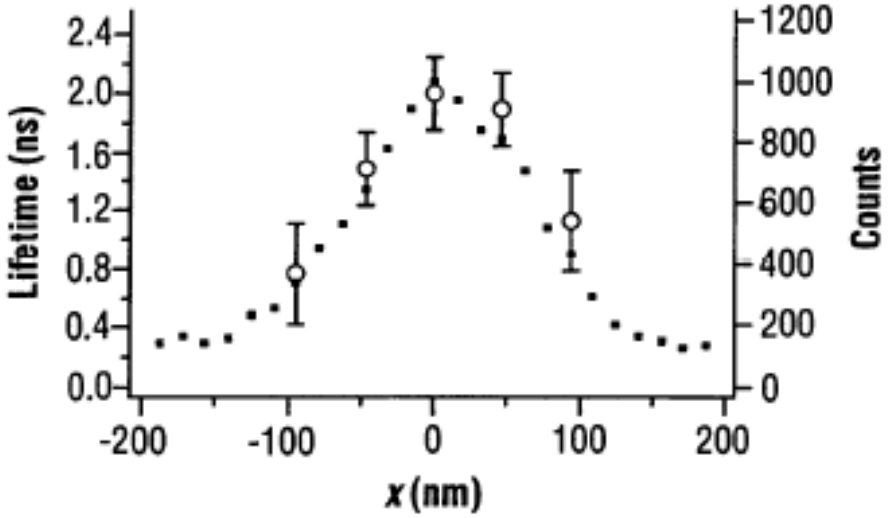
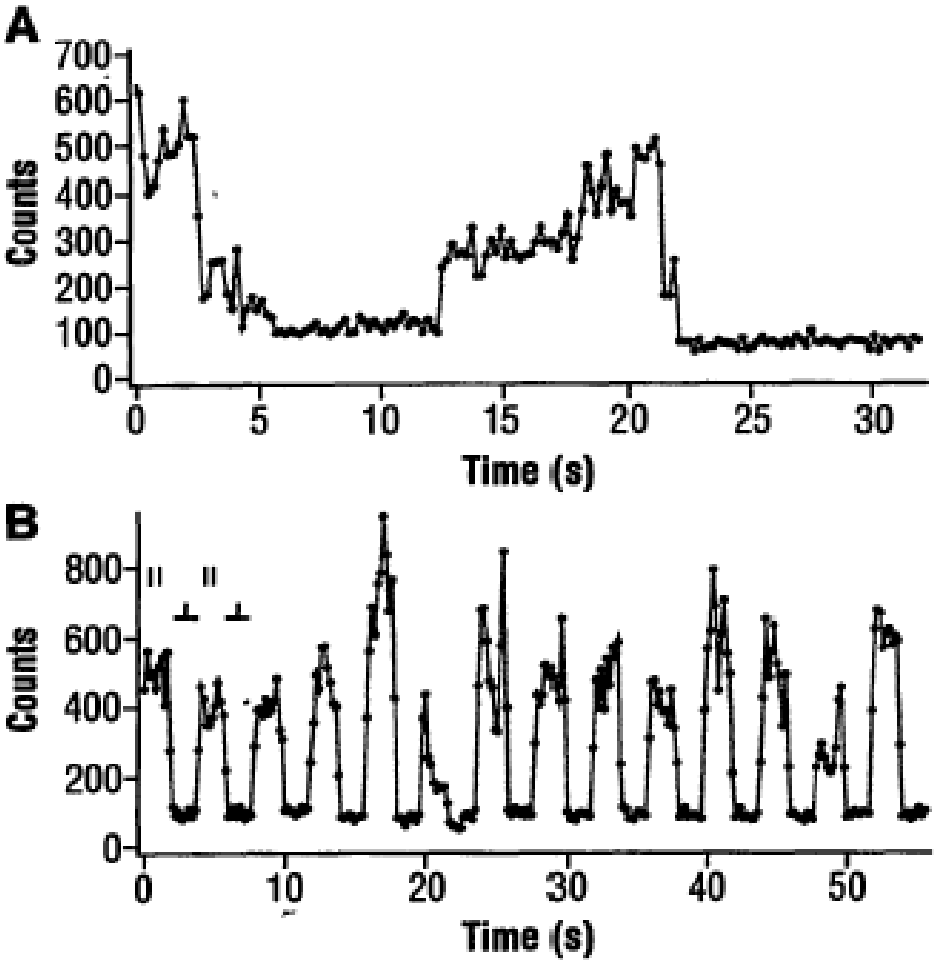


Fig. 5. Plot of the lifetime (open circles) and intensity (dots) (FWHM, 125 nm) as a function of *x* distance across the emission feature of a single molecule, extracted from the movie shown in Fig. 4. Above is a schematic of the near-field probe with its dimensions drawn on scale with the plot below. There is a substantial decrease in the fluorescence lifetime of the molecule near the sides of the fiber tip because of the quenching by the aluminum coating.

Probing Single Molecule Dynamics

X. Sunney Xie* and Robert C. Dunn

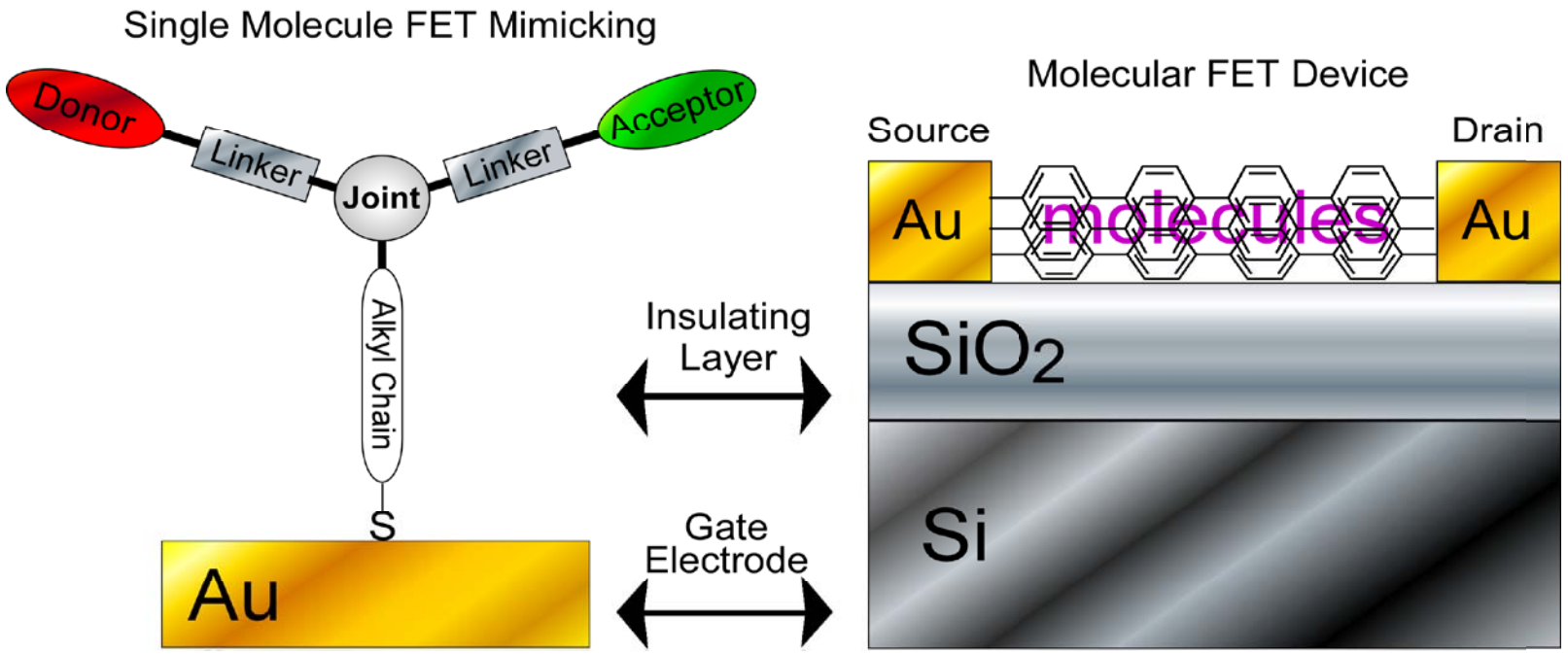
Science, 1994, vol.265, p361.



- Emission modulation by polarization.
- NSOM takes most of advantages of optical microscopes --- highly tunable with polarization.
- High sensitivity of detection by APD.

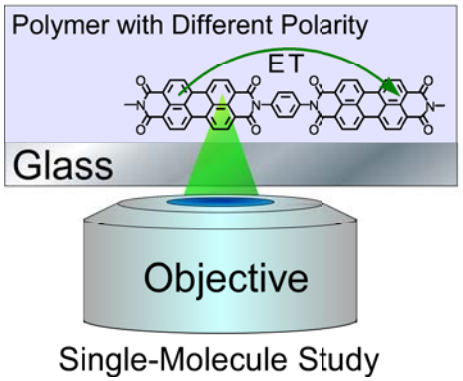
Fabrication and Evaluation of Molecular Devices:

2). Single Molecule Approach with Photochemical Methods

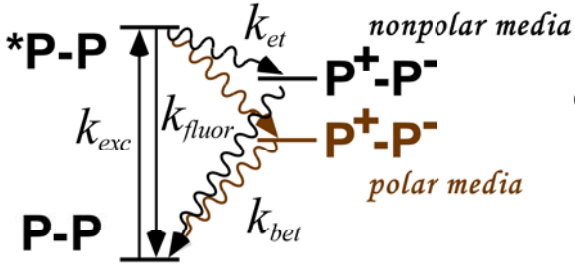


Modulation of Intramolecular Charge Transfer:

Solvent Polarity vs. Electric Field

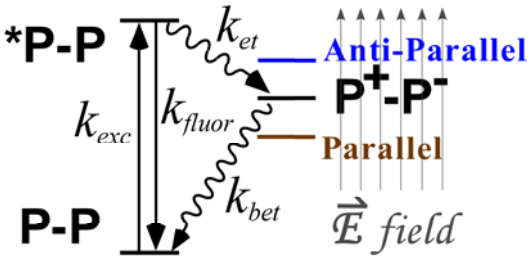


Single-Molecule Study



Chemistry
(solvent effect, molecular structure)

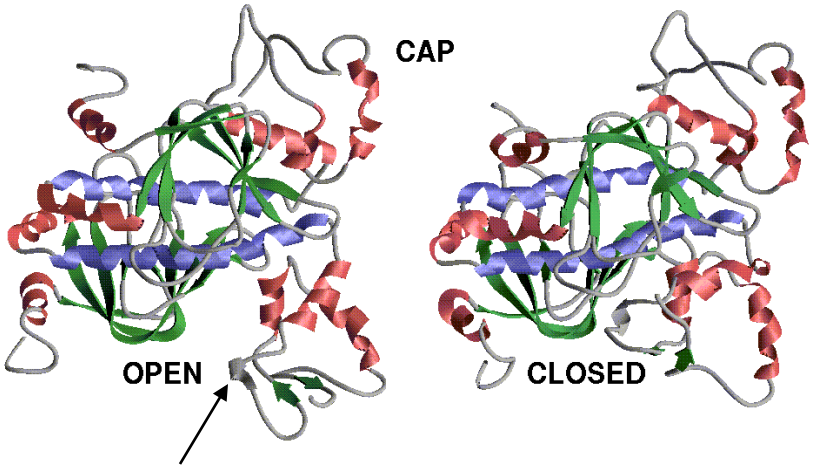
Complementary
Investigations

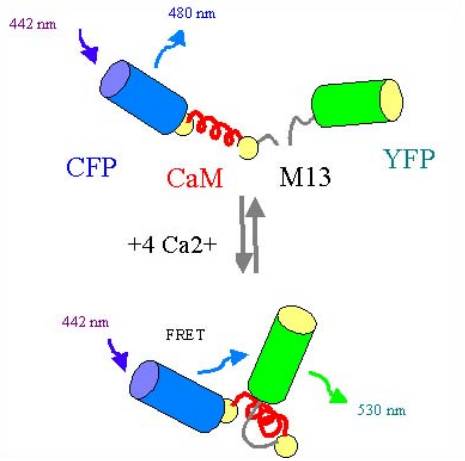


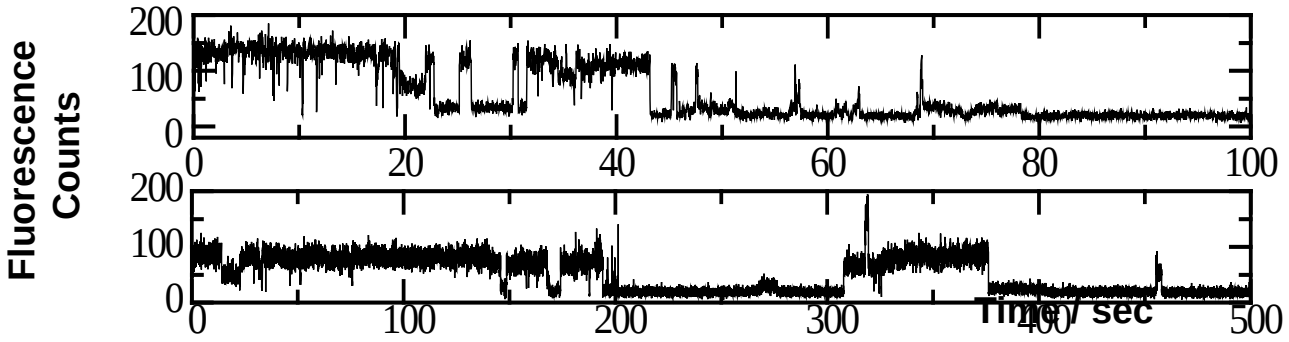
Physics
(nanoelectrode measurement)

Zang & Adams et al. *J. Am. Chem. Soc*. **126** (2004) 16126 -16133.

Single-Molecule Probing of Protein Structure







Journal of Microscopy, Vol. 202, Pt 2, May 2001, pp. 362–364.
Received 28 August 2000; accepted 11 October 2000

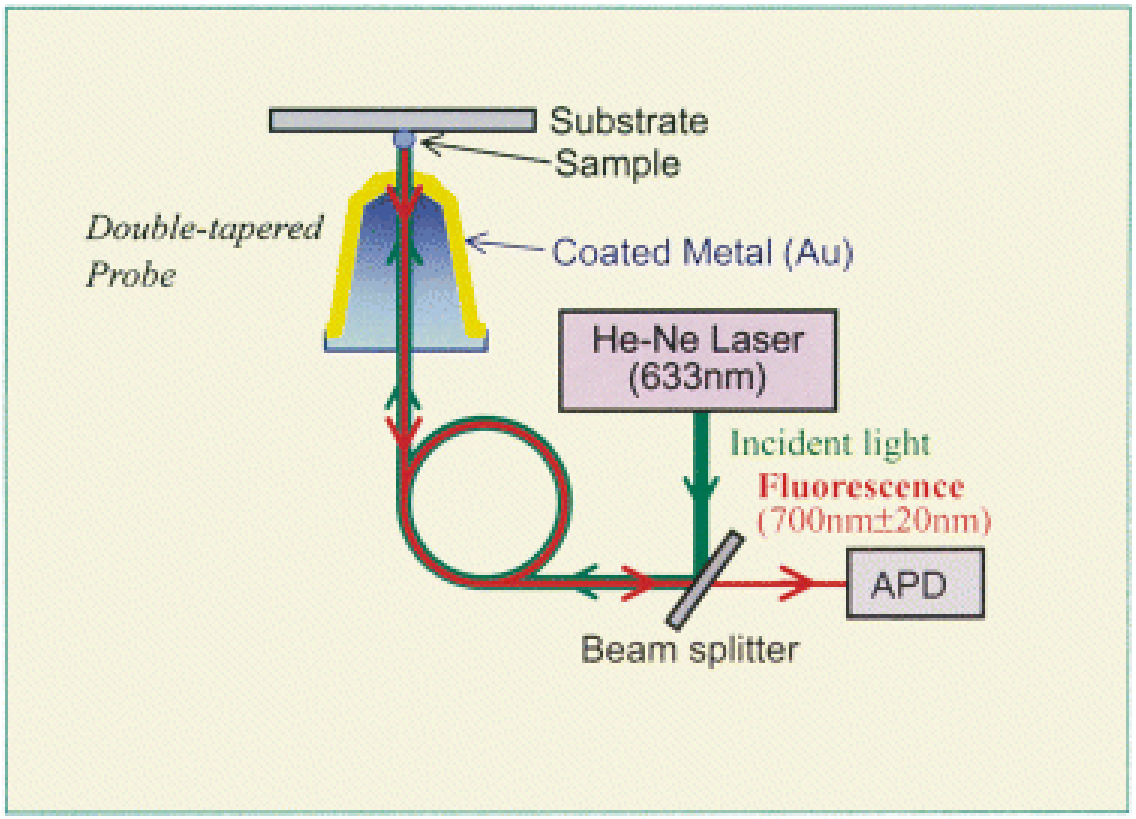
Near-field fluorescence imaging of single molecules with a resolution in the range of 10 nm

N. HOSAKA AND T. SAIKI*
Kanagawa Academy of Science and Technology, 408 KSP-East, 3-2-1 Sakado, Takatsu-ku, Kawasaki, Kanagawa 213-0012, Japan
**The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

Outline

- A NSOM system based on operation mode of illumination-collection through the same aperture probe.
- Fluorescence images of single dye molecules were obtained with a spatial resolution of 15 nm, which is smaller than the diameter of the aperture (20 nm) of the probe employed.
- Such super-resolution may be attributable to non-radiative energy transfer from the molecules to the coated metal of the probe since the resolution obtained in the case of conventional NSOM is limited to 30 - 50 nm due to penetration of light into the metal.

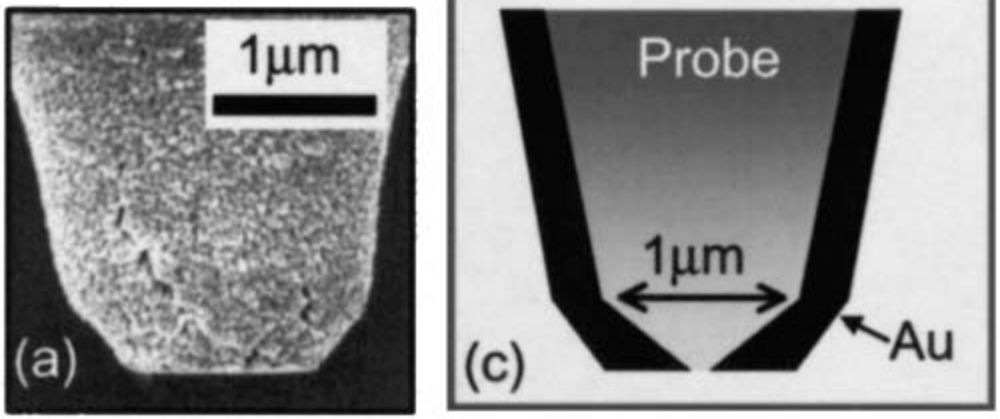
Hosaka, J.Microscopy, 2001, Vol 202, p362



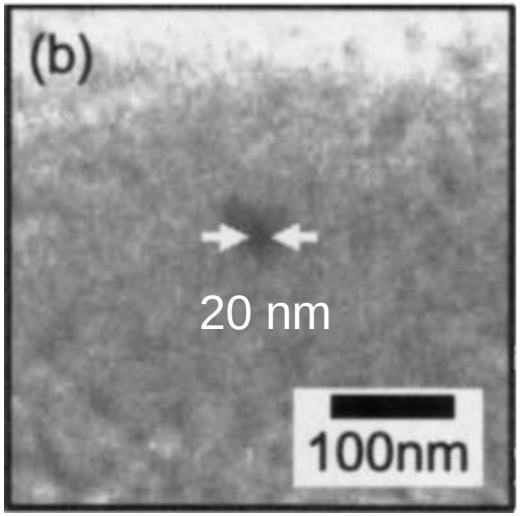
A NSOM of illumination-collection mode

Hosaka, J.Microscopy, 2001, Vol 202, p362

a side view



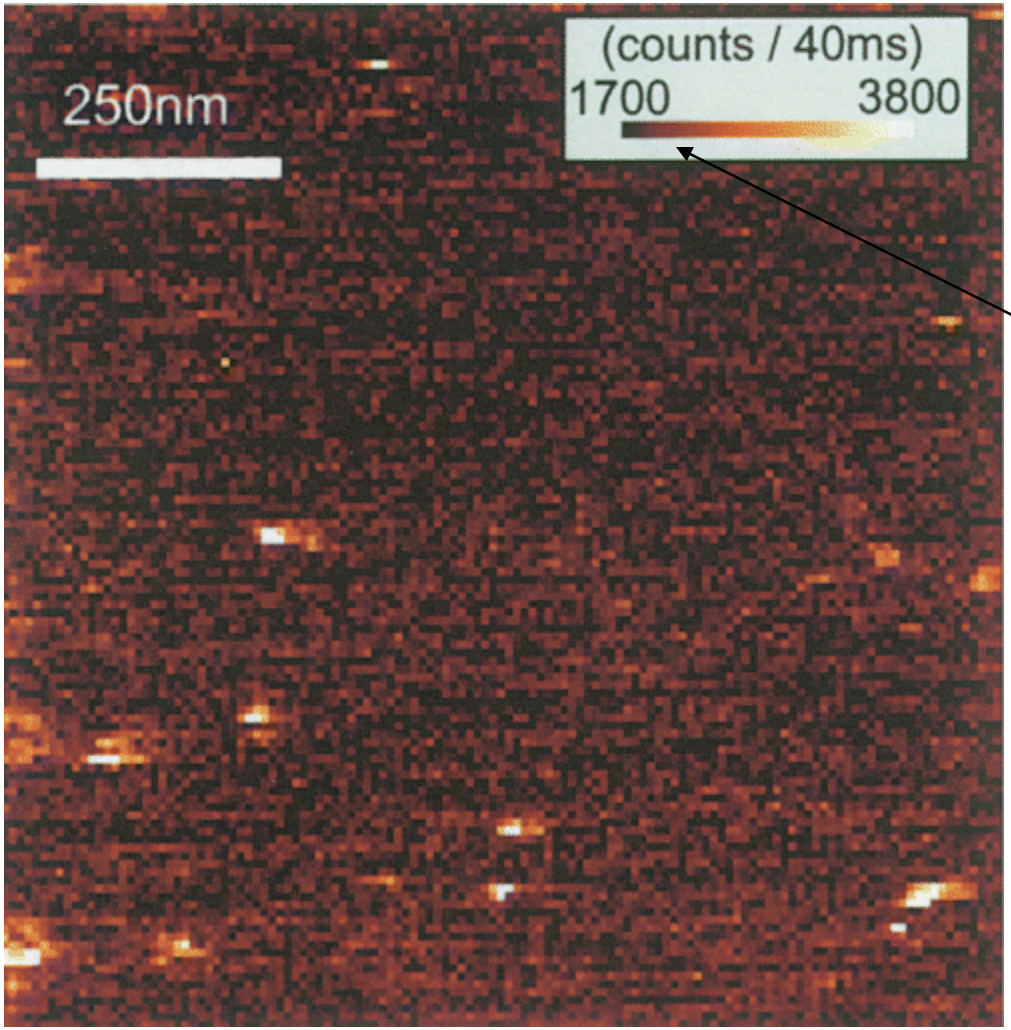
cross-sectional illustration



an overhead view

SEM images of an aperture probe

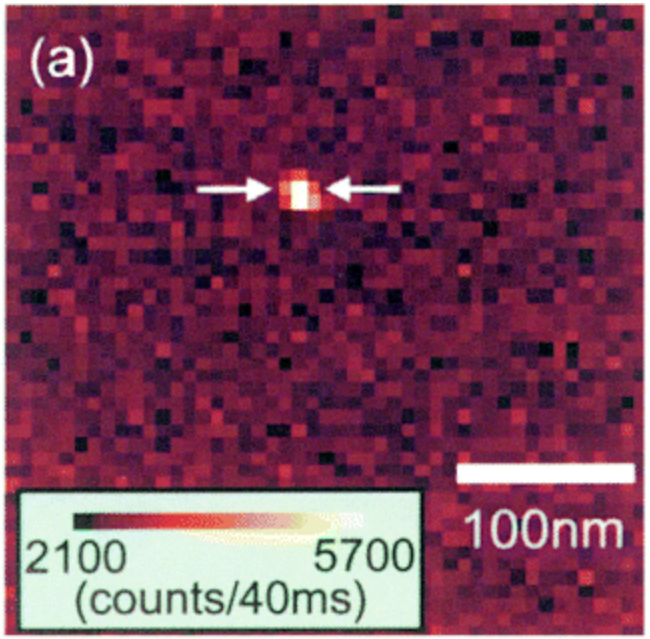
Hosaka, J.Microscopy, 2001, Vol 202, p362

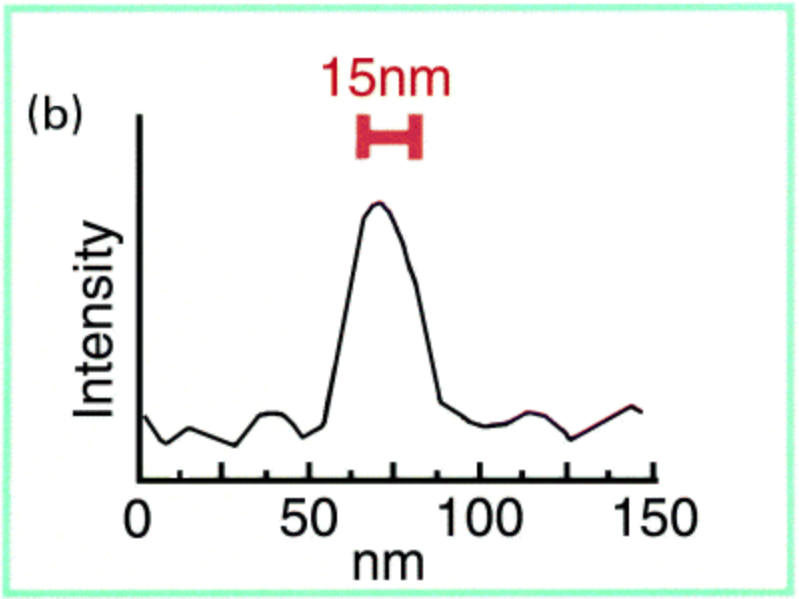


High background

A fluorescence image of single dye molecules in a 1 X 1 μm^2 area.

Hosaka, J.Microscopy, 2001, Vol 202, p362





Push the NSOM

resolution to the limit.

Hosaka, J.Microscopy, 2001, Vol 202, p362

J. Phys. Chem. A **1999,** *103,* 11264−11270

Near-Field Scanning Optical Microscopy of Single Fluorescent Dendritic Molecules

Joost A. Veerman,[†] Stefano A. Levi,[‡] Frank C. J. M. van Veggel,[‡] David N. Reinhoudt,*[,‡] and Niek F. van Hulst*[,†]

Applied Optics Group, and Laboratory of Supramolecular Chemistry and Technology, MESA+ Research Institute, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

Research Outline

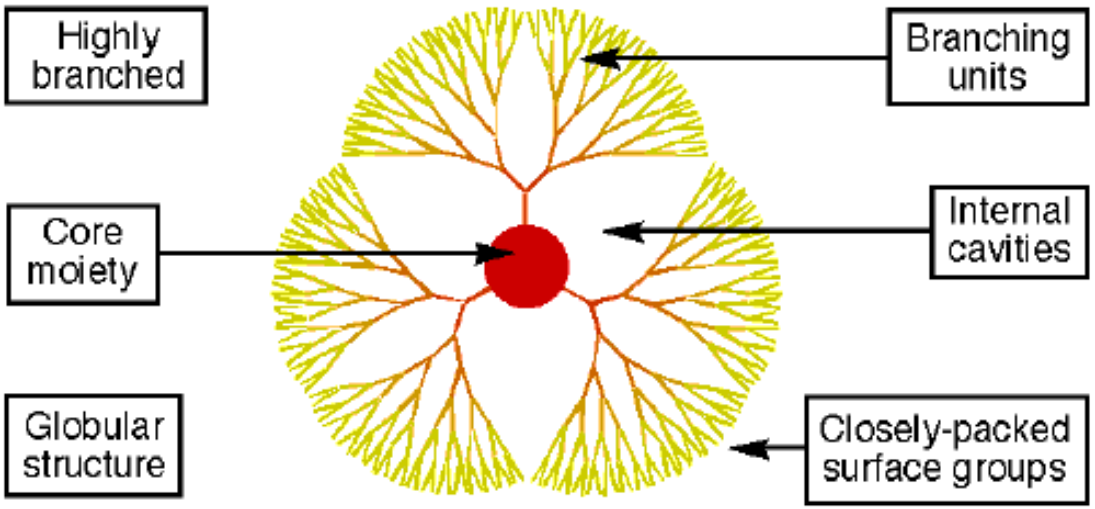
- Dendritic molecules are regular hyperbranched polymers that originate from a core by means of repetitive reactions.
- With dimensions from few to hundreds of nanometers, they are ideal "molecular platforms" to attach functional groups in well-defined three-dimensional architectures.

In this study:

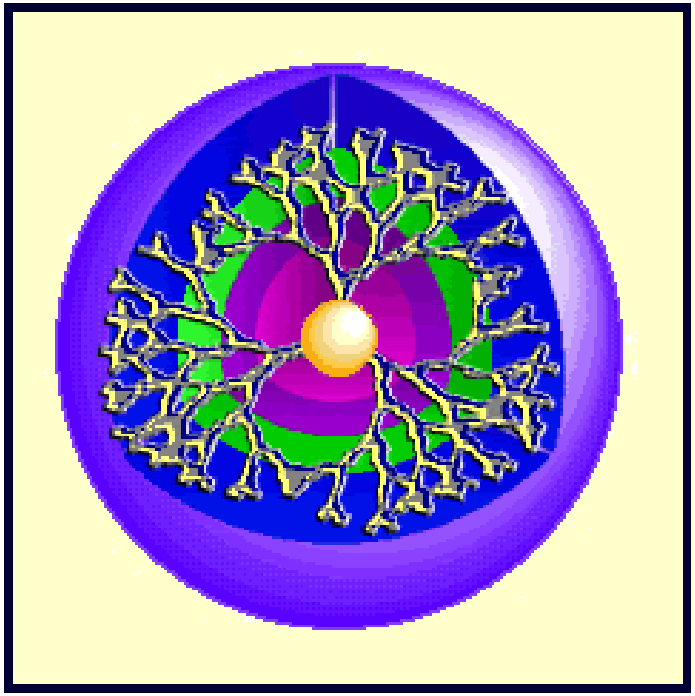
- Individual dendritic molecules adsorbed on glass containing a single fluorescent rhodamine B core have been observed with NSOM;
- height and fluorescence images were obtained simultaneously.
- The dendritic assemblies can be discriminated from free fluorescent cores based on accurate simultaneous mapping of both the fluorescent core and the surrounding dendritic shell. *See the slide*.
- The full three-dimensional orientation of each individual fluorescent core can be resolved.
- Most dendritic structures exhibited rotational motion of the fluorescent core on a ms time scale, revealing intramolecular conformational dynamics.

Veerman, JPC-A, 1999, vol. 103, p11264

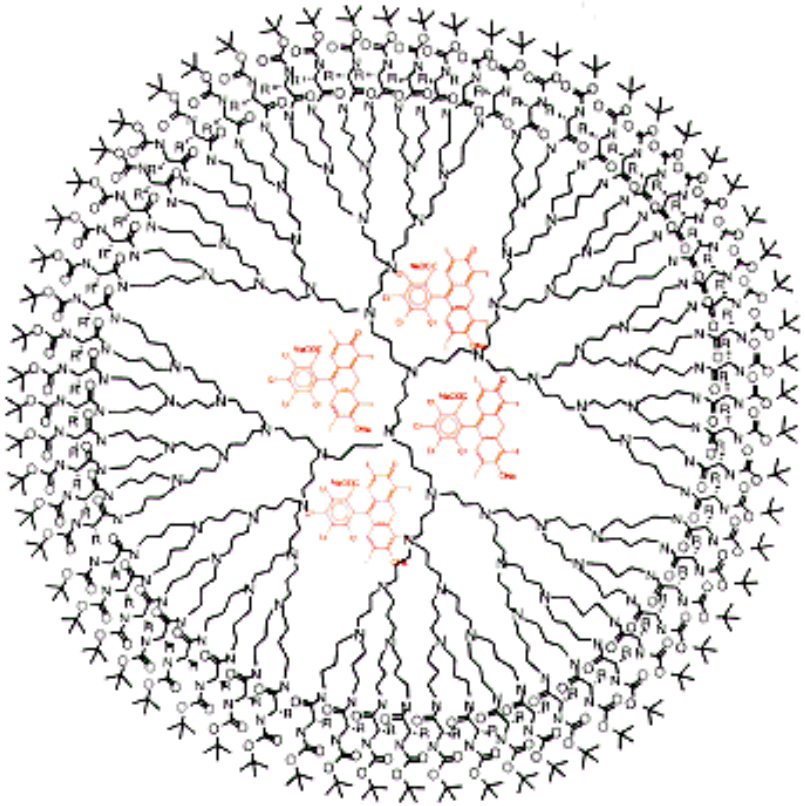
The Dendritic Structure



3-D structure of dendrimer

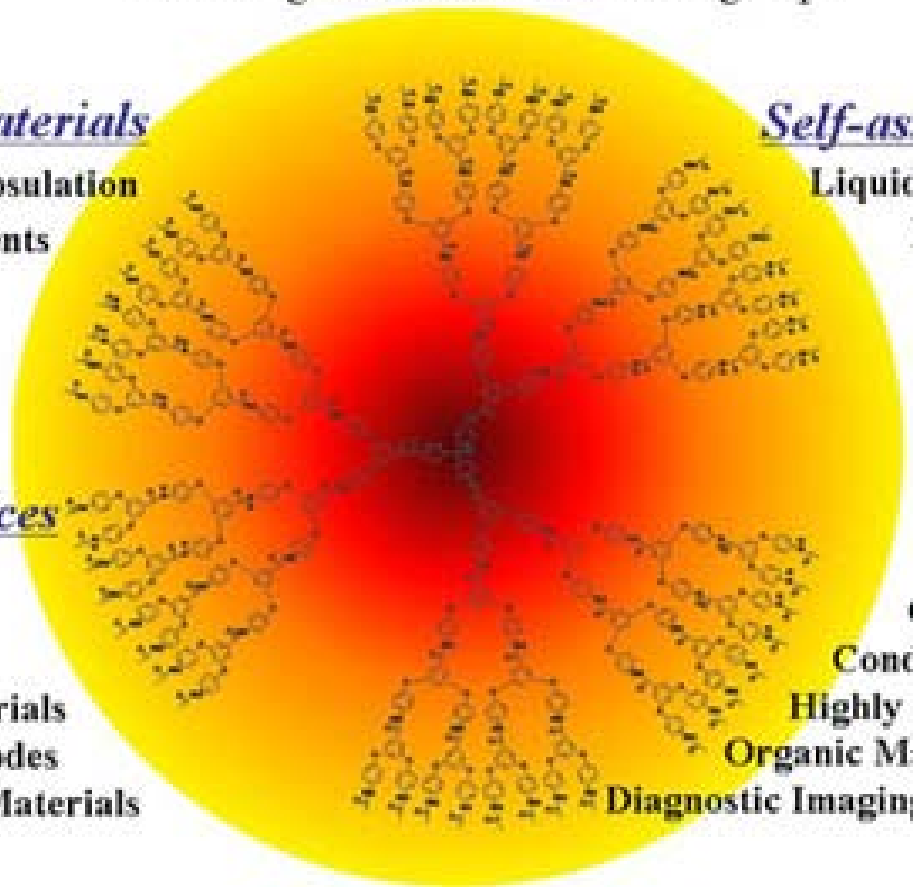


A typical dendrimer with fluorophore core



Dendrimer

Dendrimer : well-defined, highly branched, three-dimensional macromolecule with a large number of functional groups



Biomedical Materials

Micelles and encapsulation
Drug Delivery Agents
Immunoassays

Self-assembly

Liquid Crystal
LB layer

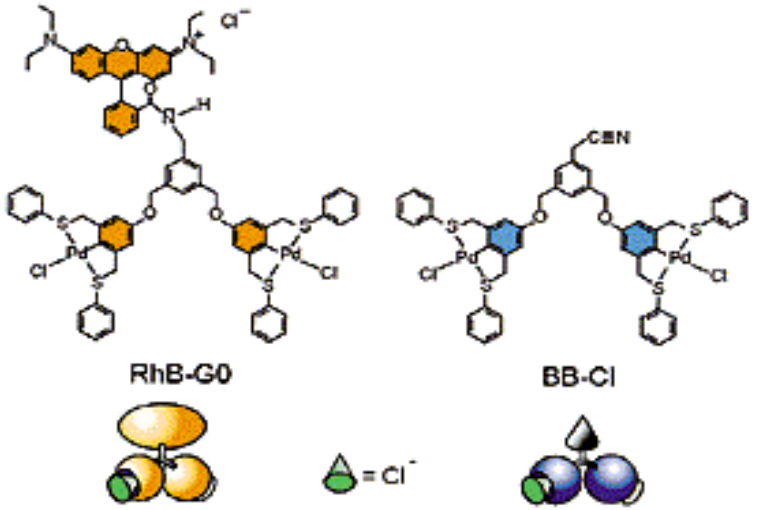
Photonic Devices

Sensor
Photoresists
Nonlinear Optics
UV-Curable Materials
Light Emitting Diodes
Light-harvesting Materials

Others

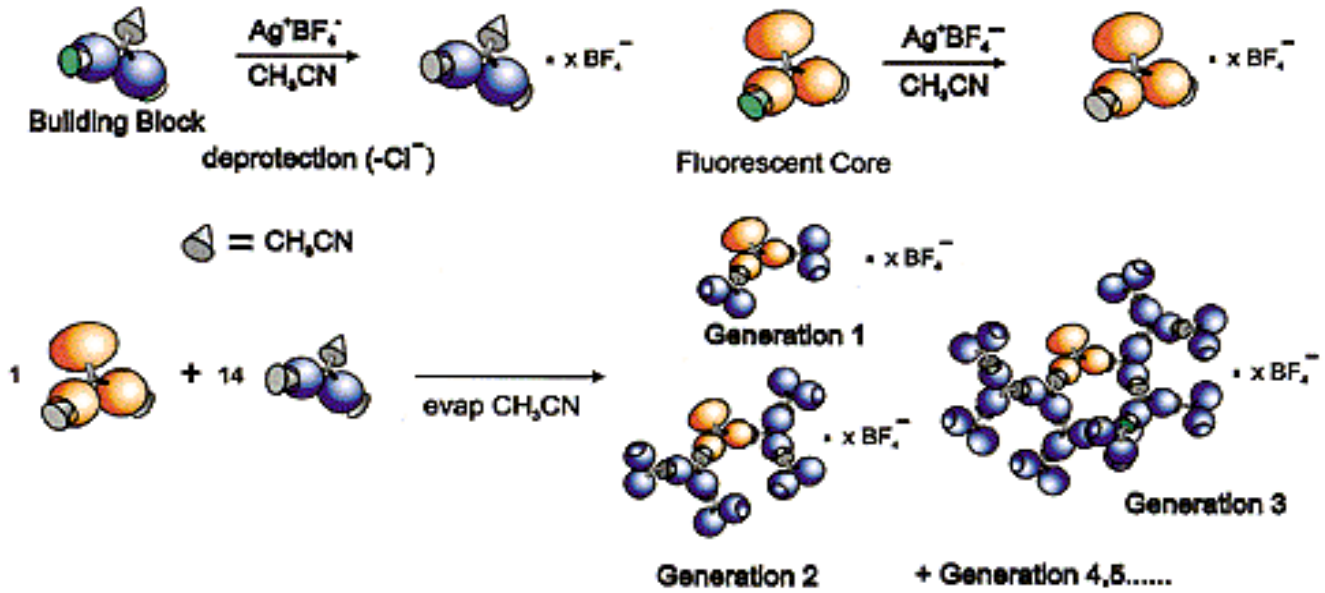
Chromatography
Conducting Materials
Highly effective Catalyst
Organic Magnetic Materials
Diagnostic Imaging Contrast Agent

(a)



Fluorescent core unit (RhB-G0) and building blocks (BB-Cl);

(b)



Assembly of the dendritic molecules

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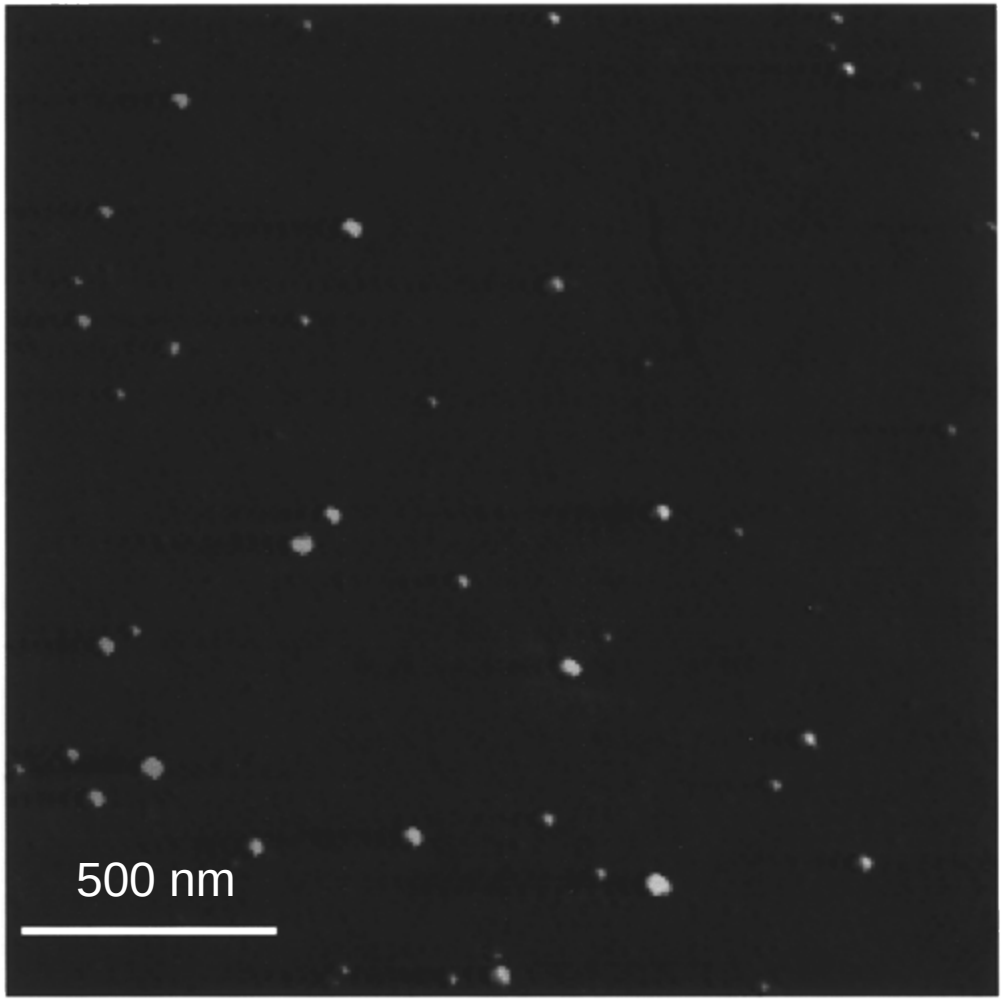

AFM image (2 × 2 μm^2) showing dispersed spheres corresponding to individual dendritic molecules adsorbed on a glass surface, with a mean height of 7.5 ± 0.5 nm and a mean width of 21 ± 1 nm.

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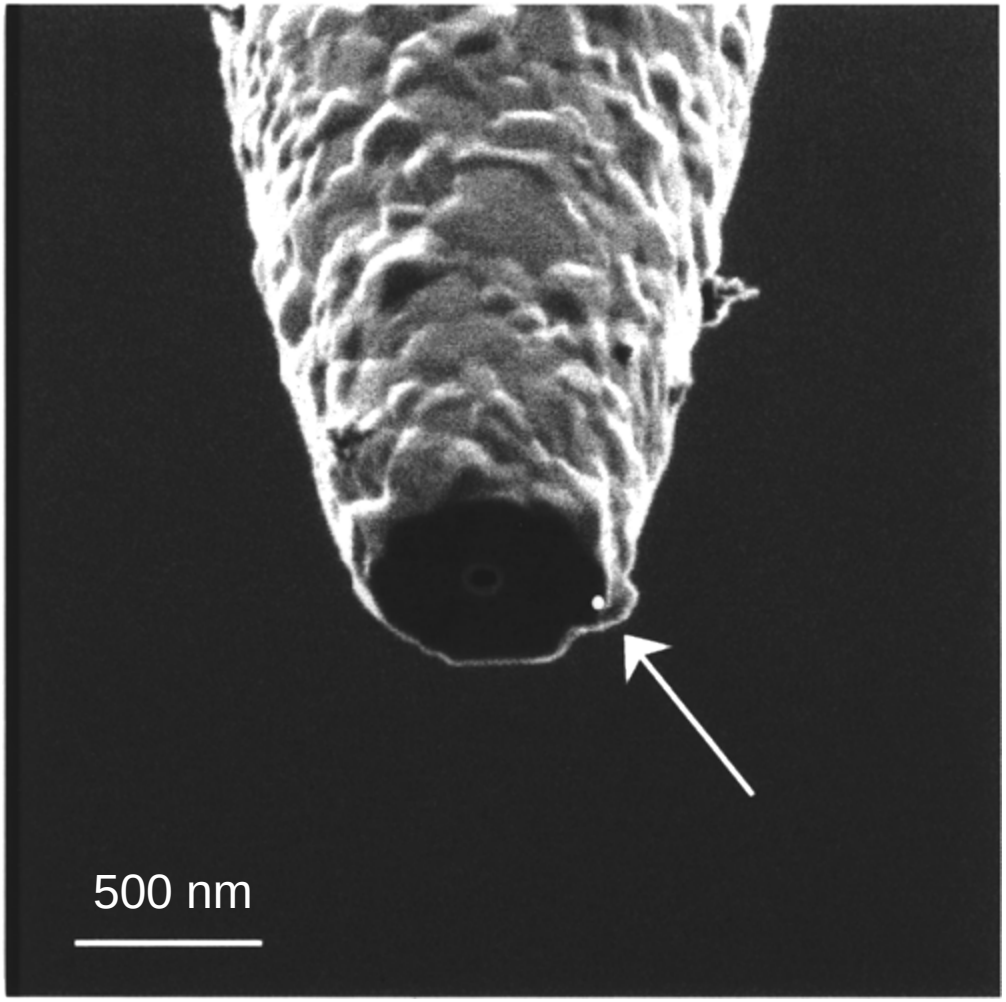

Focused ion beam (FIB) image recorded after side-on milling of the 70 nm aperture NSOM probe that was used for the measurements. The white dot indicates the position of the shear-force sensing local protrusion at the edge of the end-face.

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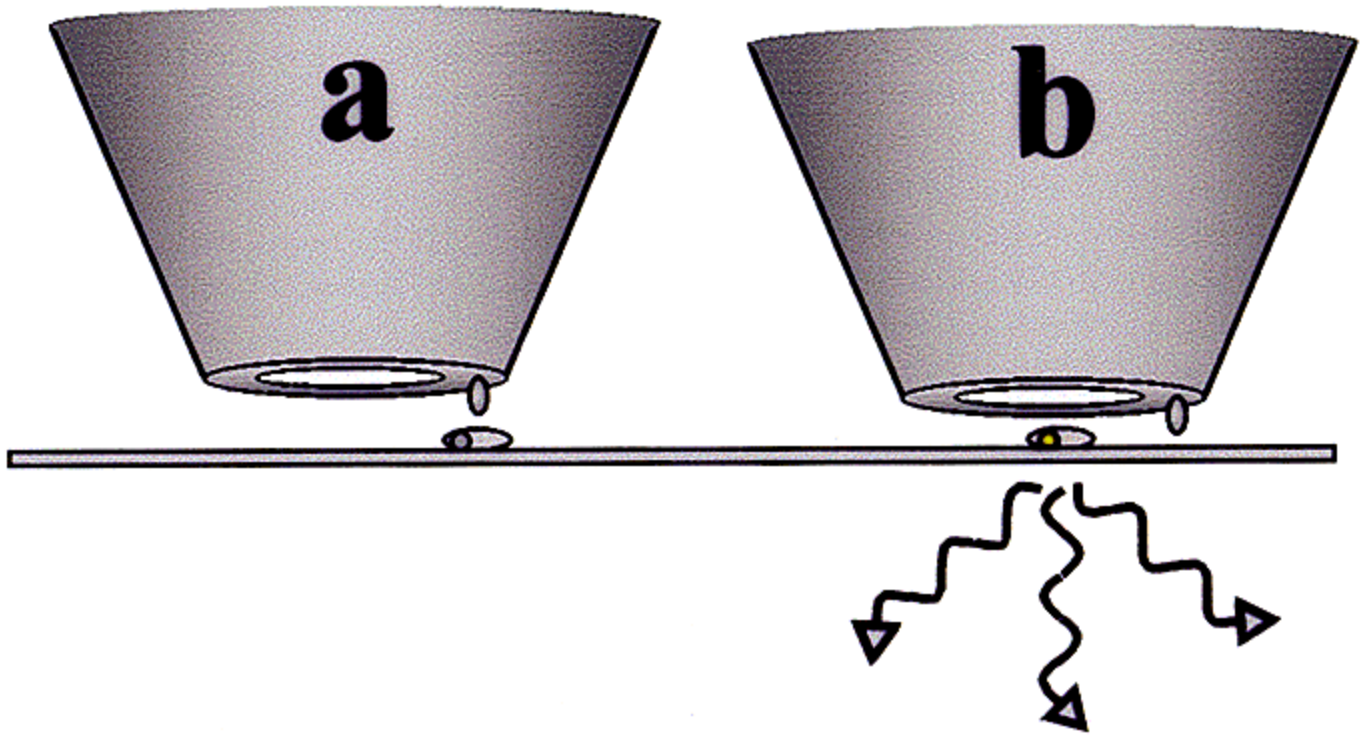

(a) Schematic view of the height tracking of an adsorbed dendritic molecule by a local protrusion on the NSOM probe;

(b) the optical imaging of the fluorescent core-unit by the aperture.

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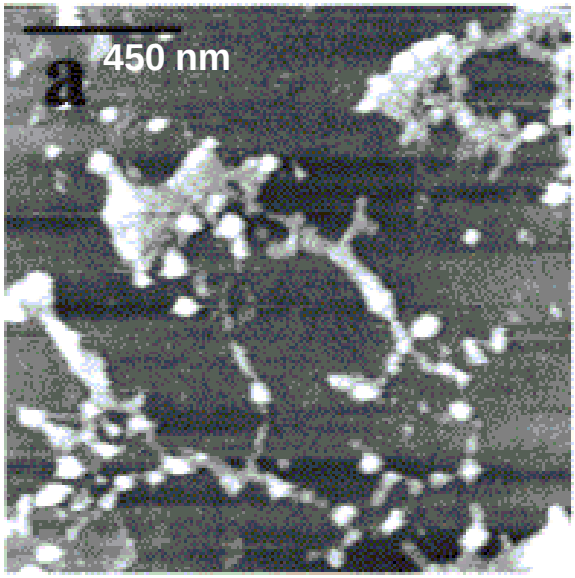

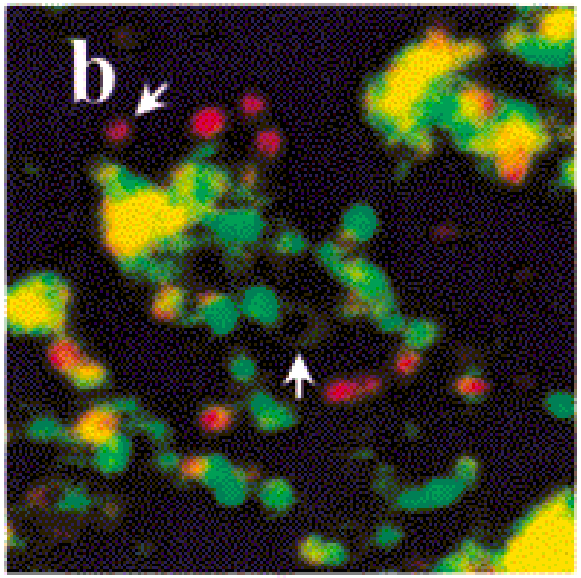

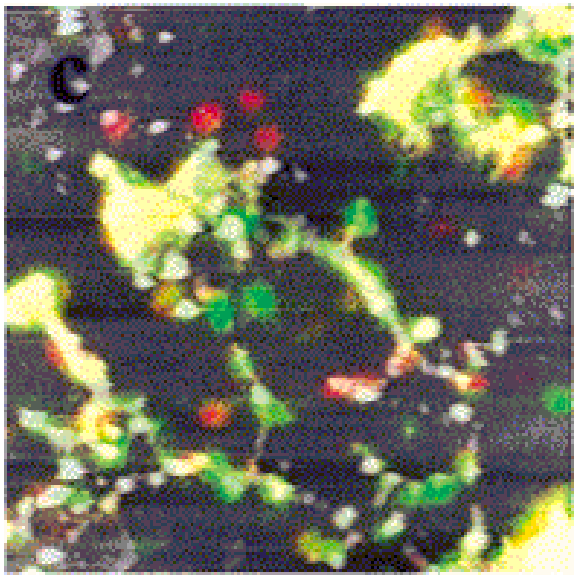

NSOM shear-force image (1.65 × 1.65 μm^2) showing both isolated and clustered dendritic molecules on a glass surface. Height of spheres: 5-7 nm.

Simultaneously obtained fluorescence image, with ~70 nm optical resolution. Circularly polarized excitation light at 514.5 nm was used. A false-color scale indicates the polarization of the fluorescence (red, horizontal direction; green, vertical direction, yellow, non-polarized or 45º polarized). *Draw a scheme for polarized NSOM system.*

Combined shear-force (gray scale) and fluorescence (red-green color scale) image (*a* + *b*) illustrating the correlation between height and optical signals.

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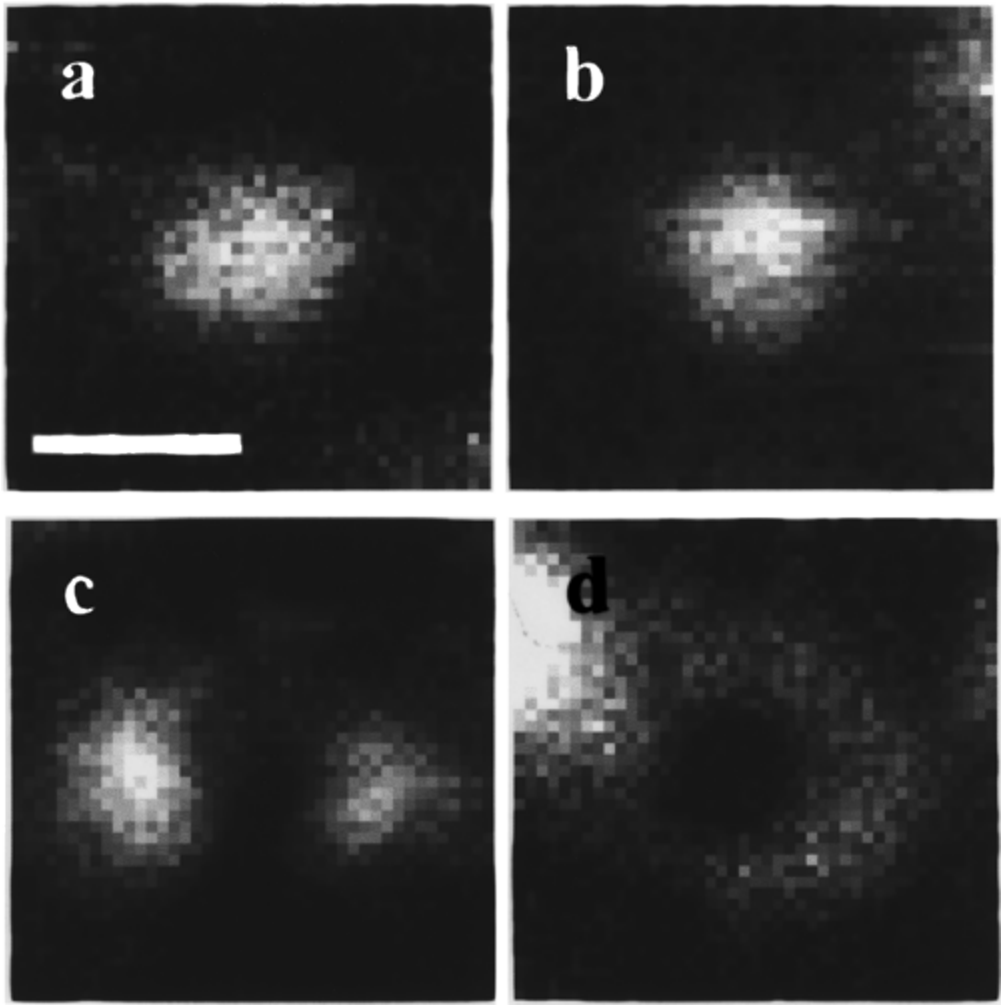
in-plane absorption horizontal dipole orientation



in-plane absorption vertical dipole orientation

out-of-plane tilted absorption dipole orientation:

Excited with circular polarized light.

out-of-plane tilted absorption dipole orientation:

Excited with linear polarized light.

Characteristic NSOM single-molecule emission patterns.

Resolution: 65 - 90 nm fwhm for the elliptical in-plane molecule patterns; 45 nm fwhm for the double-lobed and ringlike patterns.

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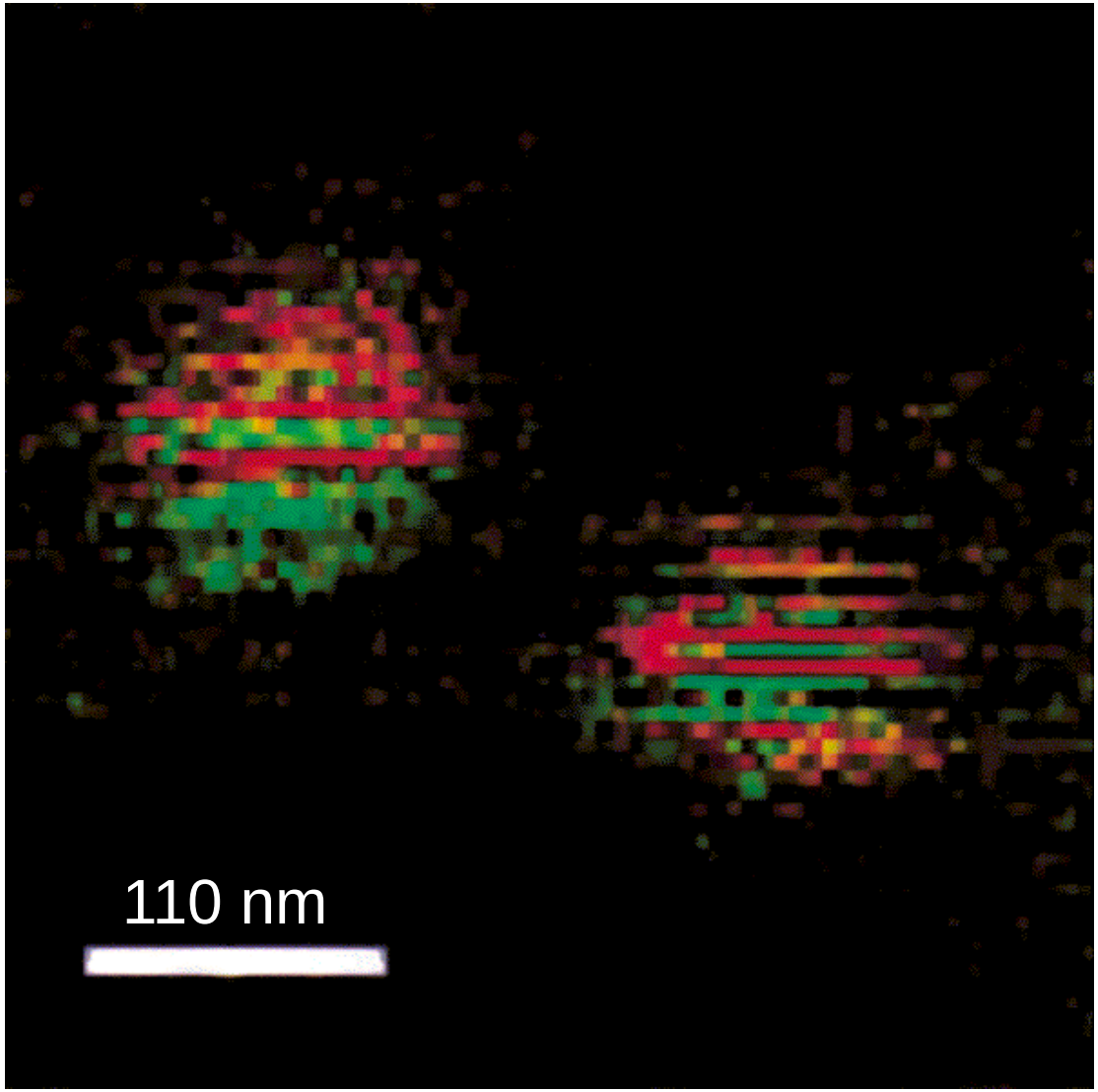
a fluorescent dendritic molecule



A smaller assembly of fluorescent core unit

Emission dipole rotational movements visualized by the red-green transitions (red, horizontal direction; green, vertical direction). Rotations occur on a ms time scale.

pixel dwell time, 5 ms; time between successive lines, 4 s.

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Comments for the next slide:

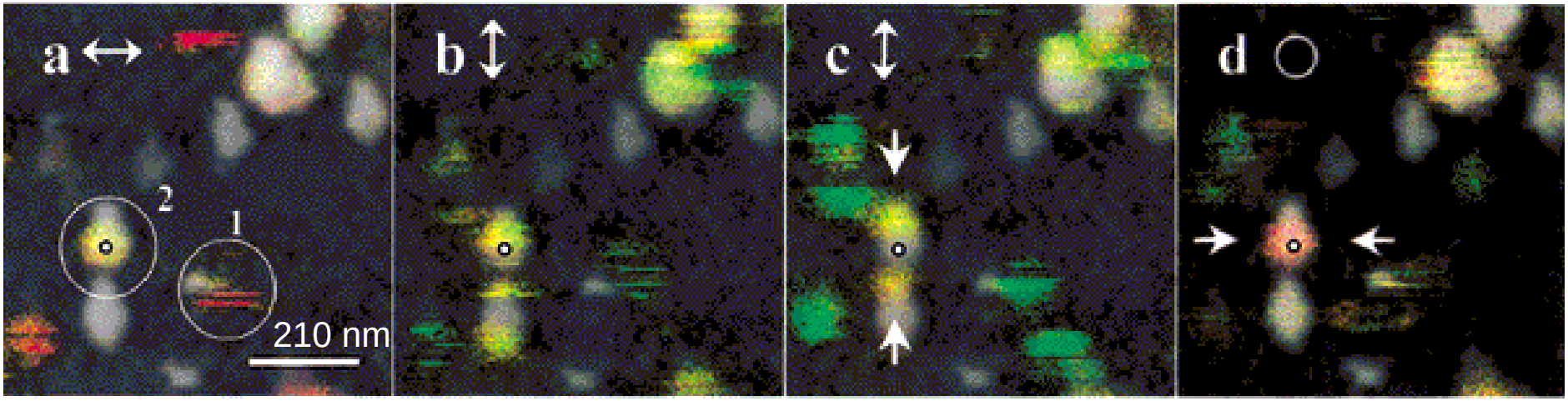
- The fluorescence of the molecule in region 1 exhibits a strong on-off behavior, due to the fast molecular rotation.
- Out-of-plane reorientations are observed for the molecule in region 2.

  a: the molecular fluorescence pattern is circular and yellow-colored, which indicates a major in-plane orientation at about 45°.

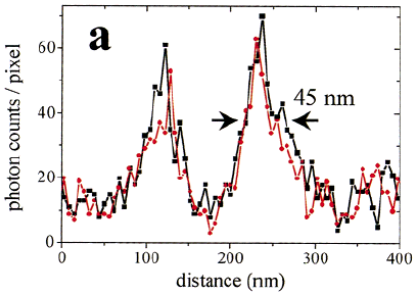
  c: it changes to a double-lobed pattern, which is characteristic for a mainly perpendicularly (out of plane) oriented dipole.

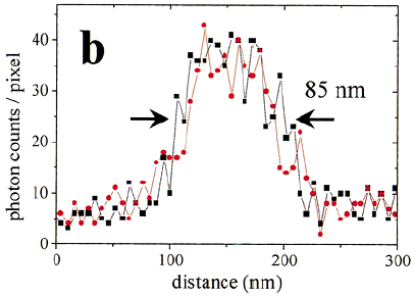
  d: it changes to a elliptical shape, which indicates an in-plane horizontal orientation along the *x*-direction.

Draw the 3 orientations on board.



2 adjacent lines.





Series of four successive combined shear-force (gray scale) and fluorescence (red-green false-color scale) images of the same area (750 × 750 nm^2) obtained with different excitation polarization conditions.

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