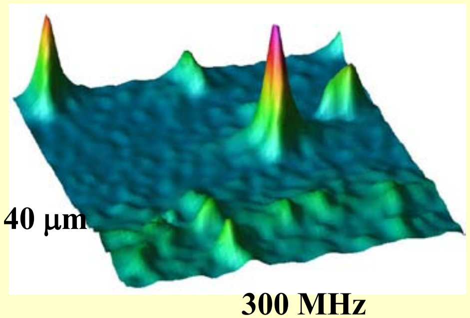
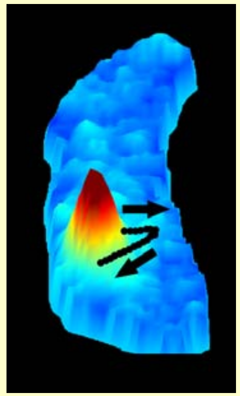
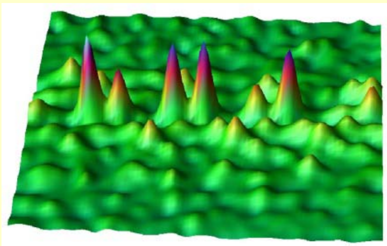
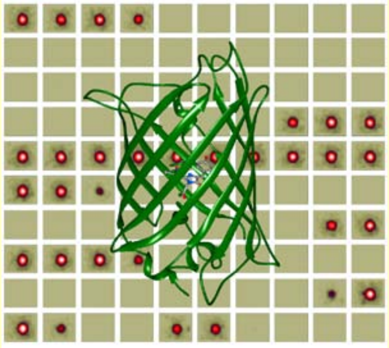
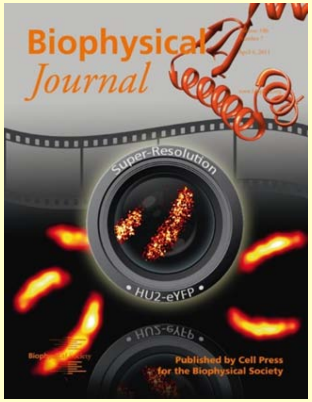
Single-Molecule Spectroscopy, Imaging, and Photocontrol: Foundations for Super-Resolution Microscopy

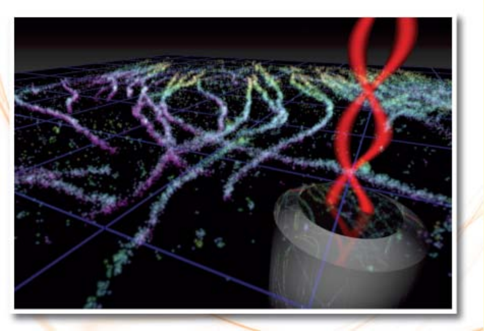


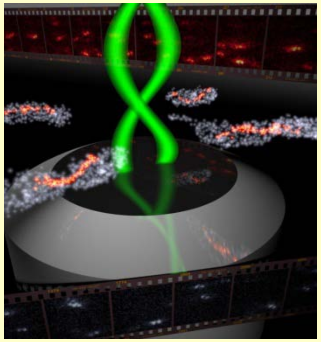












W. E. Moerner
Harry S. Mosher Professor of Chemistry and Professor, by courtesy, of Applied Physics
BioX, Biophysics, MIPS, and ChEM-H Programs, Stanford University

Nobel Prize in Chemistry Lecture

Stockholm, Sweden, 8 December 2014

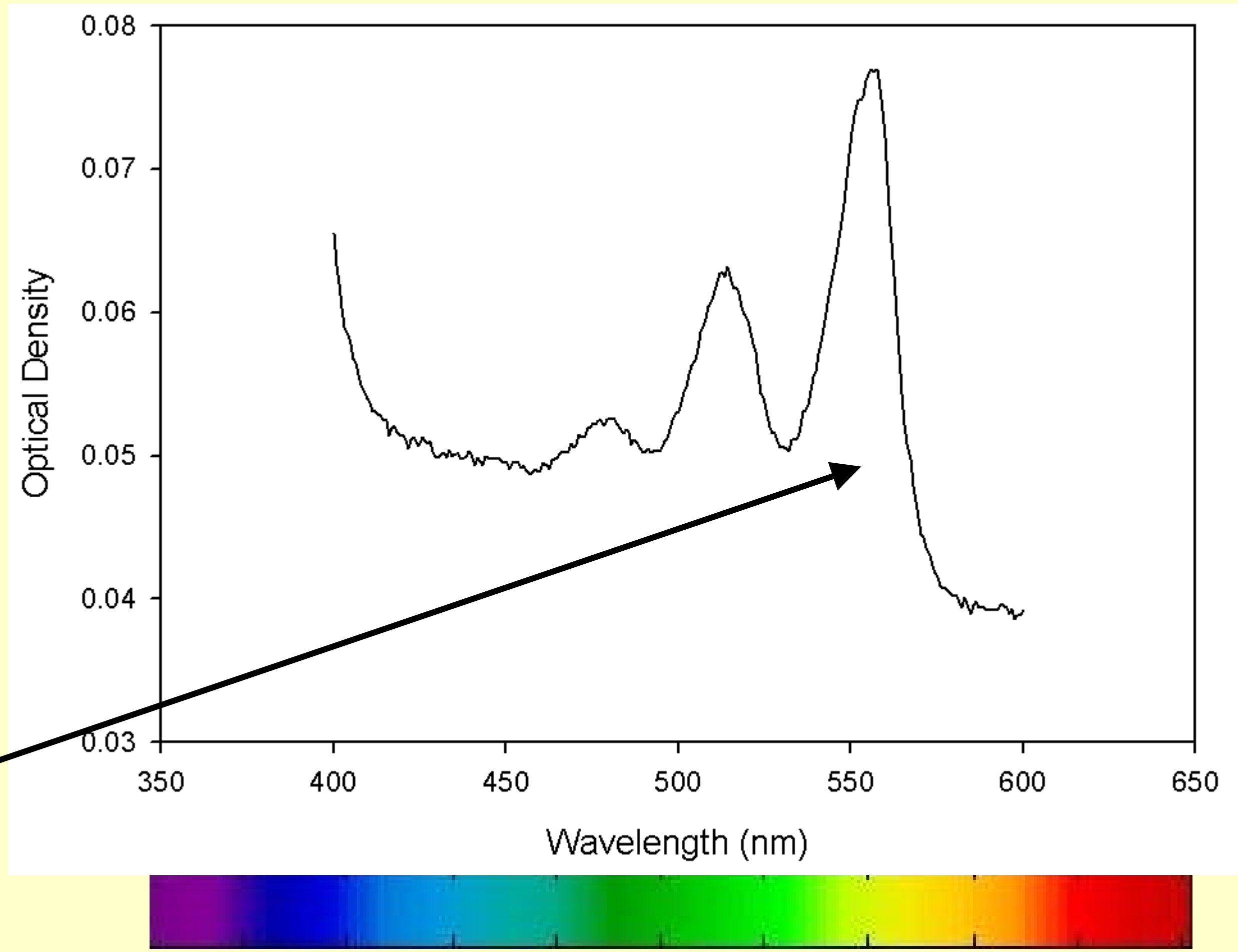
Erwin R. J. A. Schrödinger

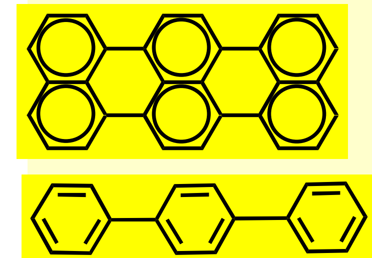
"…we *never* experiment with just *one* electron or atom or (small) molecule. In thought-experiments we sometimes assume that we do; this invariably entails ridiculous consequences…In the first place it is fair to state that we are not *experimenting* with single particles, any more than we can raise Ichthyosauria in the zoo.

"Are There Quantum Jumps? Pt. II" British J. for the Philosophy of Science **3**, 233-242 (1952).

Optical Spectroscopy of Molecules in Solids

Spectrum (absorption vs. wavelength, or color) at room T





Terrylene in p-Terphenyl

Now lets expand the color scale by 25x here, and cool to low T

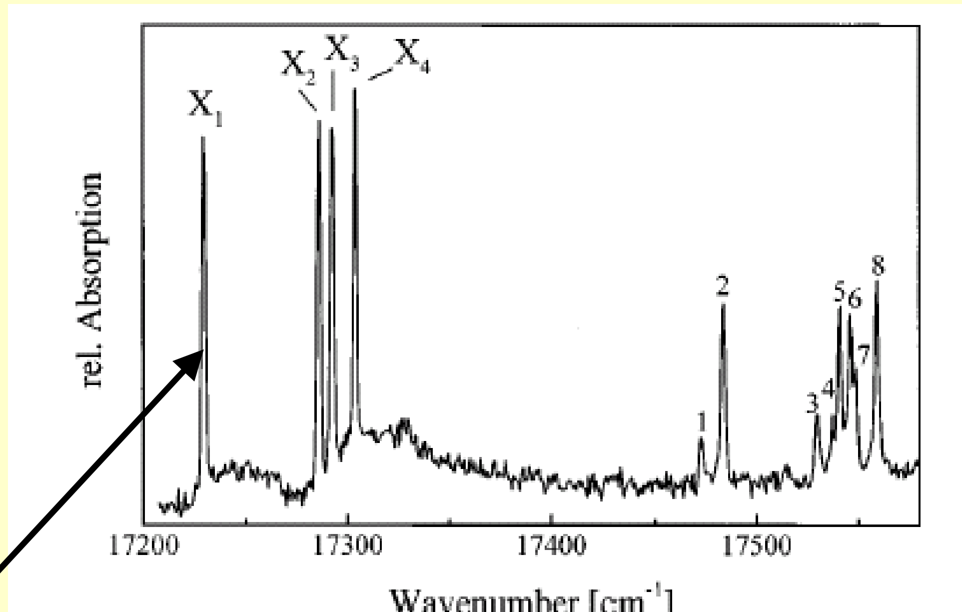
860 THz

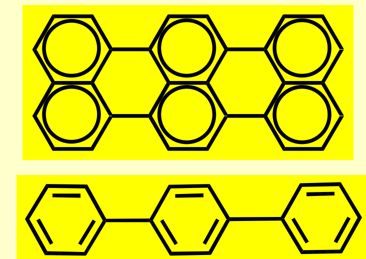
460 THz=460x10^{12} cps

Frequency=(c/wavelength)

Optical Spectroscopy of Molecules in Solids

Spectrum (absorption vs. frequency, or color) at low T (2K)





Terrylene in p-Terphenyl

Now blow up the scale here by another 1000X !

581 nm

516 THz

569 nm

527 THz

Frequency=(c/wavelength)

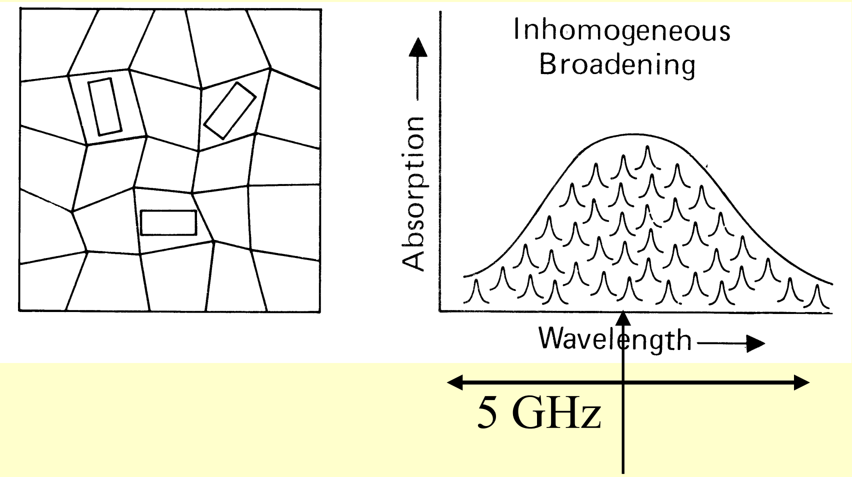
Data: S. Kummer, … Th. Basché, JChemPhys 1997

Moerner Group

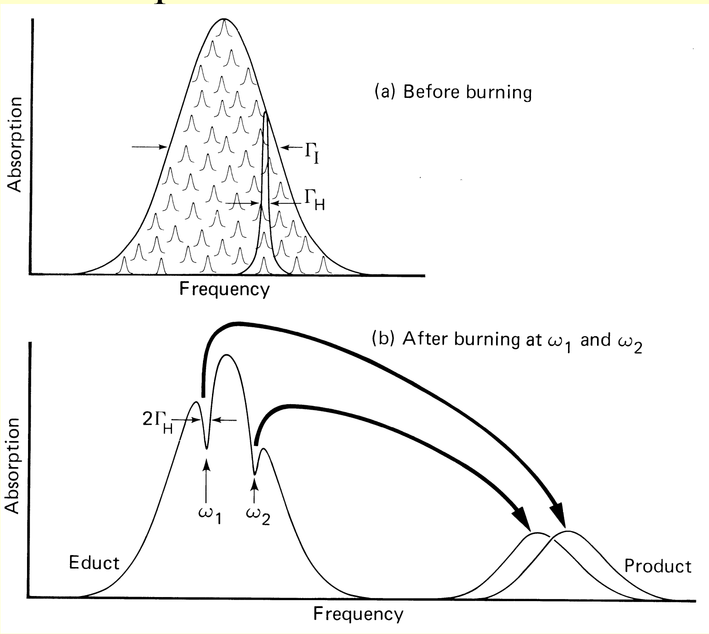
Early Steps Toward Single-Molecule Spectroscopy

Crucial concepts circa 1985 at IBM Research:

- Zero-phonon, purely electronic transitions of fairly flat, rigid molecules in solids at low T become extremely narrow
- An inhomogeneous line profile occurs due to tiny variations in local environments



- To get homogeneous widths: need photon echoes, … or
- Spectral hole-burning: laser-induced changes make a dip or “hole” at chosen laser colors

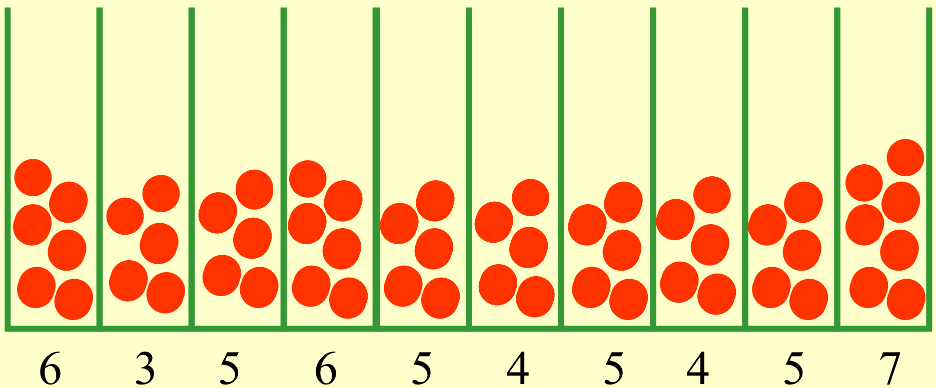


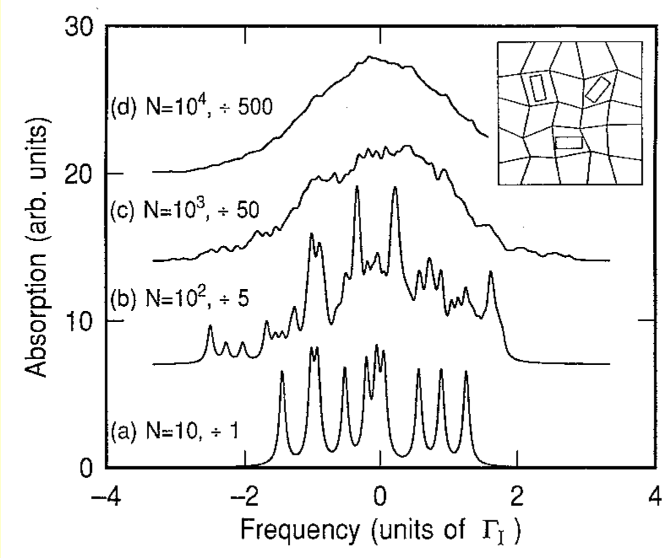
What are the ultimate limits to “frequency domain”optical storage by spectral hole-burning?

Fundamental Question: Is there a “spectral noise” that results from statistical number fluctuations or the discreteness of individual molecules? – this would define the smallest possible spectral hole that can be detected.

How might statistics appear in spectroscopy?

Suppose you have 10 boxes, and throw 50 balls at these boxes with all boxes being equally likely.



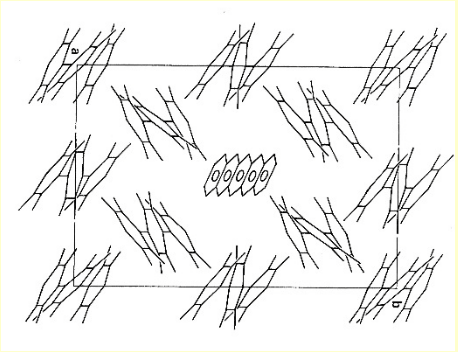


This is the well-known number fluctuation effect, scaling as √N, that arises from sampling.

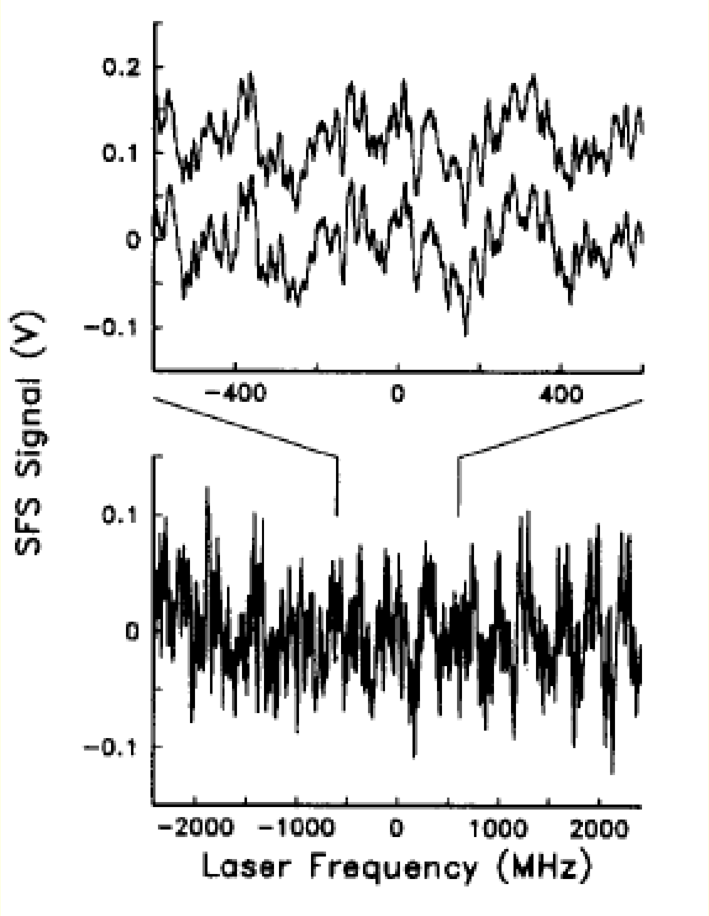
Key Idea: Now think of the horizontal axis as optical frequency (or wavelength). Each box is a bin of width Δν, the homogeneous width of an optical absorption line. Then the resulting spectrum should have a spectral roughness or fine structure scaling as √N, which arises from the discreteness of the individual molecules!

Statistical Fine Structure in an Inhomogeneously Broadened Line

WEM and T. P. Carter, *Phys. Rev. Lett*. **59**, 2705 (1987)



pentacene in p-terphenyl crystal, 1.4K



- Number of molecules per $\Delta\nu_H$: N
- Fluctuations in N should scale as $\sqrt{N}$
- Call this Statistical Fine Structure (SFS)
- Detection achieved with FM spectroscopy since it measures $\Delta\alpha$ on the 100 MHz scale, or α(upper)-α(lower)

SFS arises directly from the discreteness of the individual molecules.

The single-molecule limit is within reach!

T. P. Carter and WEM, *J. Chem. Phys*. **89**, 1768 (1988)

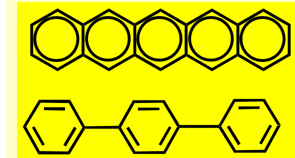
Detecting Single-Molecule Absorption

VOLUME 62, NUMBER 21 PHYSICAL REVIEW LETTERS 22 MAY 1989

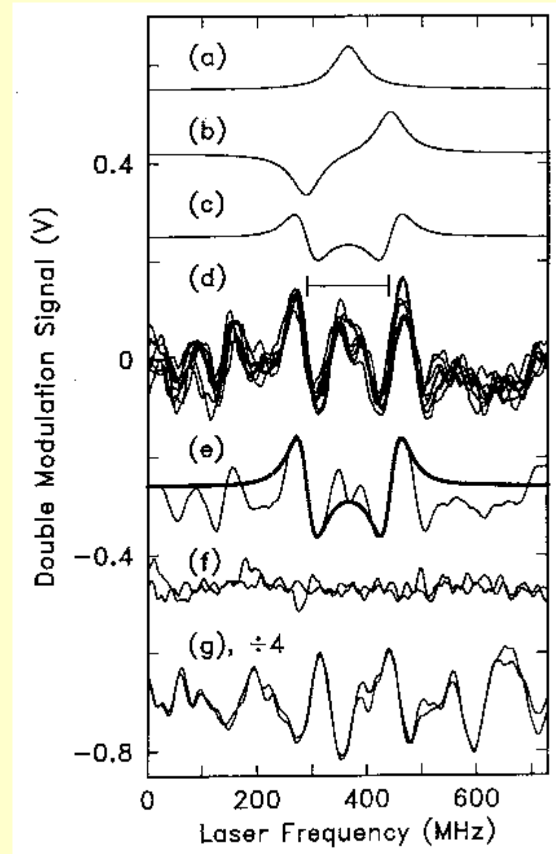
Optical Detection and Spectroscopy of Single Molecules in a Solid

W. E. Moerner and L. Kador[a]
IBM Research Division, Almaden Research Center, San Jose, California 95120
(Received 17 March 1989)

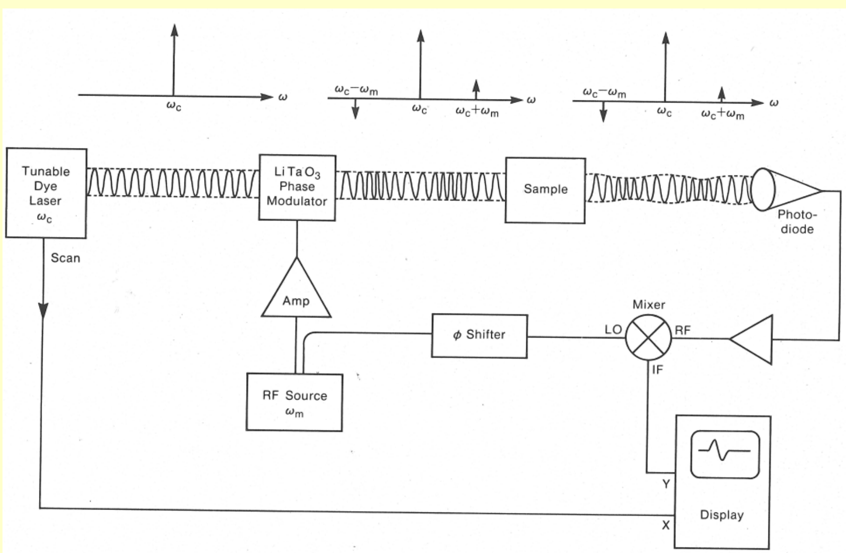
PRL **62**, 2535 (May 1989)



- Pentacene in crystalline *p*-terphenyl, 1.8 K, 593 nm
- Laser FM absorption spectroscopy with Stark (E-field) or ultrasonic (strain field) secondary modulation
- Insensitive to scattering from sample
- Limited by laser shot noise (and out-of-focus molecules from relatively thick cleaved crystal)
- Challenge: focused laser intensity had to be kept low
- Proof-of-principle: single molecules can be optically detected; pentacene/*p*-terphenyl is a useful model system

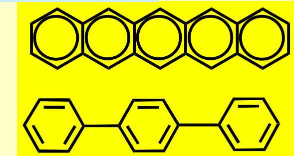


FM: G. Bjorklund, Opt. Lett. **5**, 15 (1980)



Like FM Radio at 506 THz!

Detecting Single-Molecule Absorption from Emitted Fluorescence



VOLUME 65, NUMBER 21 PHYSICAL REVIEW LETTERS 19 NOVEMBER 1990

Single Pentacene Molecules Detected by Fluorescence Excitation in a *p*-Terphenyl Crystal

M. Orrit and J. Bernard

Centre de Physique Moléculaire Optique et Herztienne, Centre National de la Recherche Scientific et Université de Bordeaux I, 351, Cours de la Libération, F-33405 Talence CEDEX, France

(Received 9 July 1990)

- Used the pentacene/*p*-terphenyl model system
- Detected absorption by measuring emitted fluorescence
- Sensitive to scattering from sample, so careful sample growth required – crystal clear sublimed flakes
- Limited by Rayleigh and Raman scattering background signals, but produced higher SNR for equal bandwidth

PRL **65**, 2716 (November 1990)

An application of Laser-Induced Fluorescence
(LIF in molecular gas - R.N. Zare, 1968)

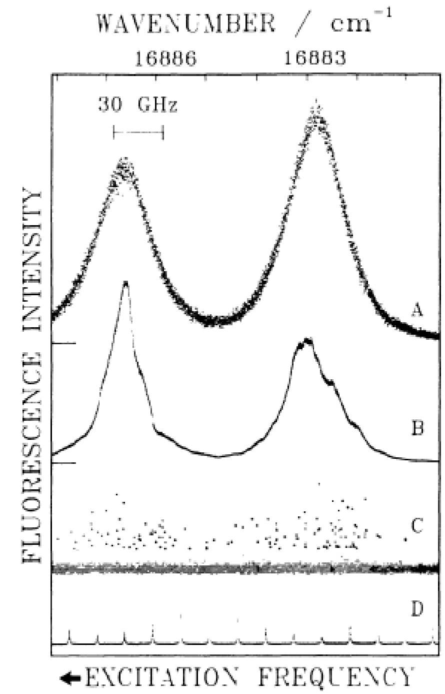
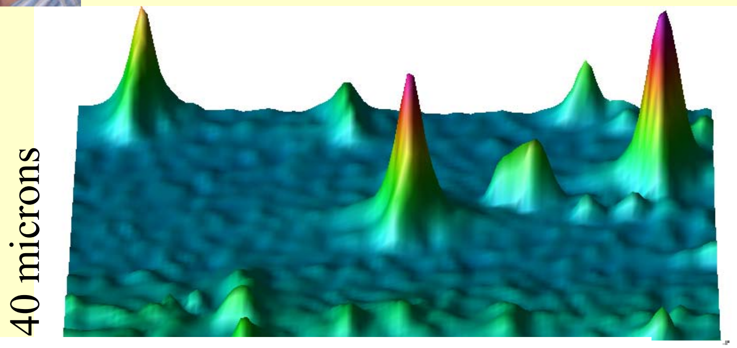


FIG. 1. O_1,O_2 region of the fluorescence excitation spectra of pentacene in different *p*-terphenyl crystals. Curve *A*, thick melt-grown crystal showing the Gaussian inhomogeneous bands. *B*, sublimation flake presenting narrower bands and substructure presumably due to cooling-induced defects. *C*, spectrum of a very small volume of a sublimation flake. The dots are the narrow excitation peaks of individual molecules. *D*, calibration spectrum of an etalon.

Single-Molecule Imaging and Spectroscopy

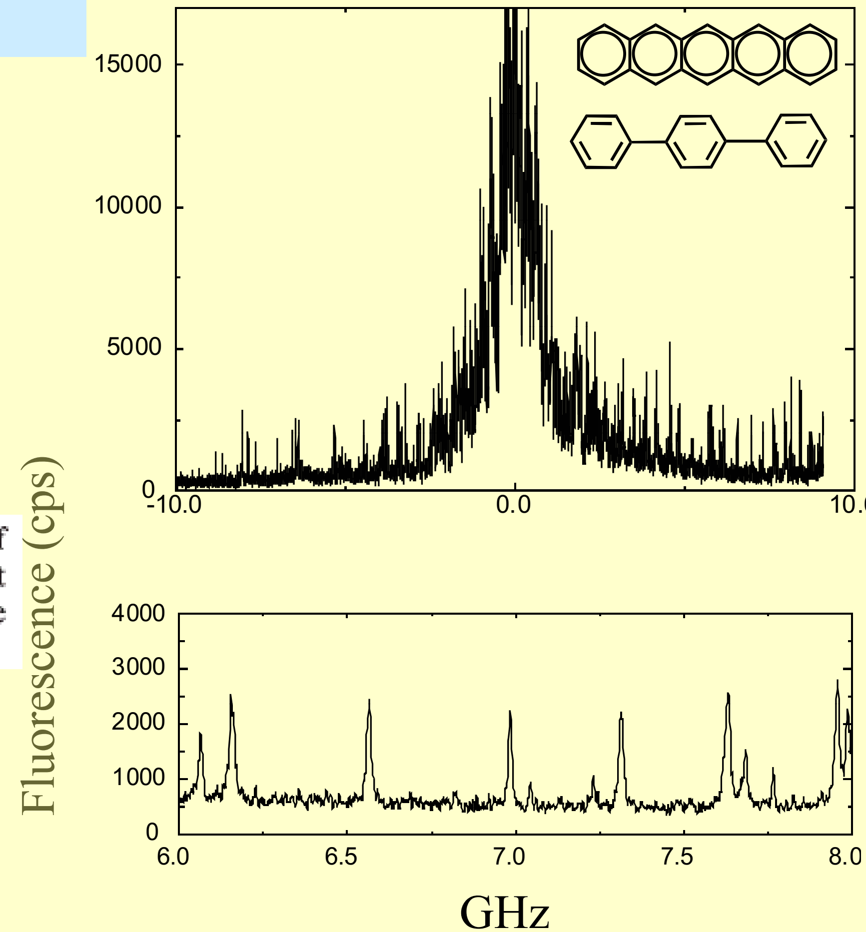
W. P. Ambrose, WEM, Nature **349**, 225 (1991)

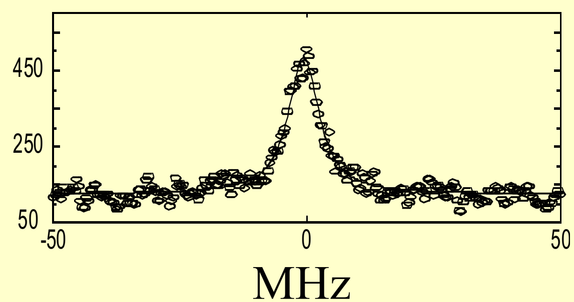


The width of the peaks along the position axis (FWHM = 5 μm) is a direct measure of the intensity profile of the focused laser beam in the crystal.

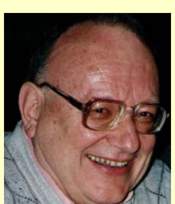
Spatial scan: SM measures laser spot size with nm probe!
Freq. scan: Second dimension selects one molecule from many in the same focal volume

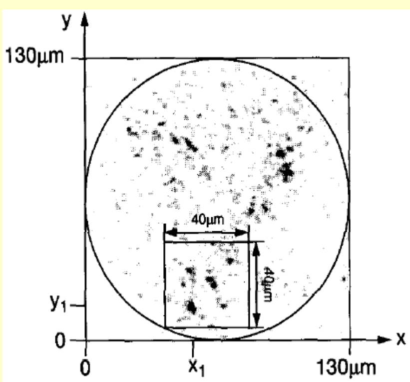
0 GHz ≡ 592 nm ≡ 506 THz





Ultralow intensity: (1.8 mW/cm^2)
Lifetime-limited width: 7.6 MHz Wow!



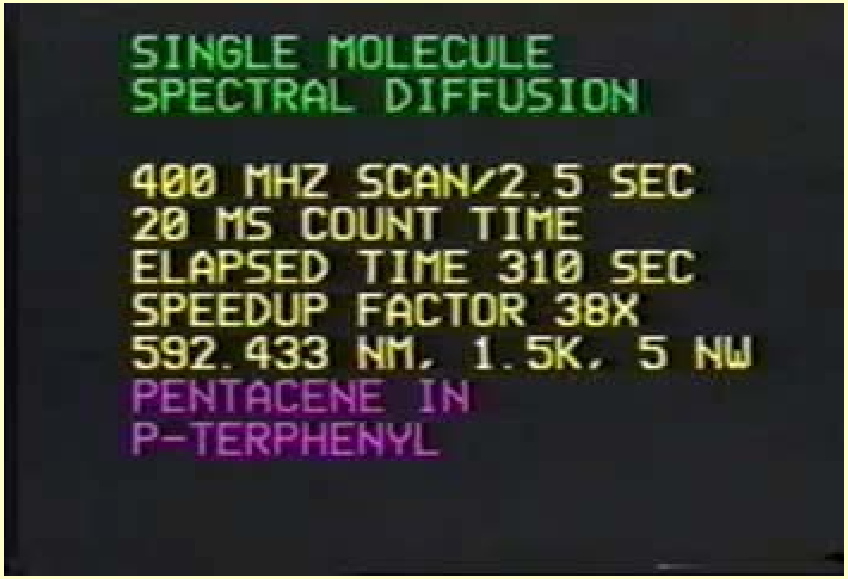


Widefield 2D Microscopy

Güttler,…Wild, ChemPhysLett **217**, 393 (1994)

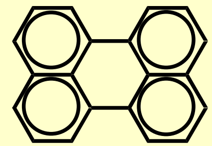
Some of the Surprises from Single Molecules!

Fluorescence excitation spectrum during repetitive scanning of cw dye laser over 400 MHz at 506 THz:



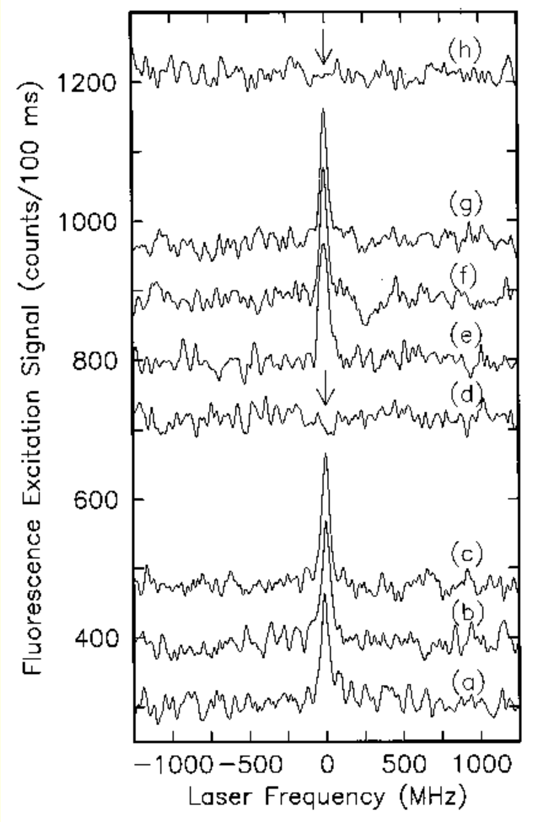
Molecule spontaneously jumps in frequency space due to nearby host dynamics!

W. P. Ambrose, WEM, Nature **349**, 225 (1991)
Theory: P. Reilly, J. Skinner, PRL **71**, 4257 (1993)
T-dep: A. Zumbusch, M.Orrit, PRL **70**, 3584 (1993)



in $(CH_2\text{-}CH_2\text{-})_n$

Optically induced spectral shifts!
Poisson kinetics observed



Th. Basché and WEM, Nature **355**, 335 (1992)

Motivations and Impact: Single-Molecule Spectroscopy and Optical Imaging in Complex Systems

Remove Ensemble Averaging

- **Explore heterogeneity**: are the various copies identical in behavior, or are they different?
- **Follow state changes in time,** especially in biological processes and complex materials
- Test theoretical understanding of stochastic behavior

Image/Detect nm-Scale Interactions

- Single molecule as a nm-sized reporter and nanometer-sized light source
- Distance rulers by FRET, TJ Ha et al. (1996)
- Probe local fields in nanophotonic structures
- **Super-resolution imaging**

Commercial: Sequence DNA, Imaging

- PacBio sequencing with ZMW's, …

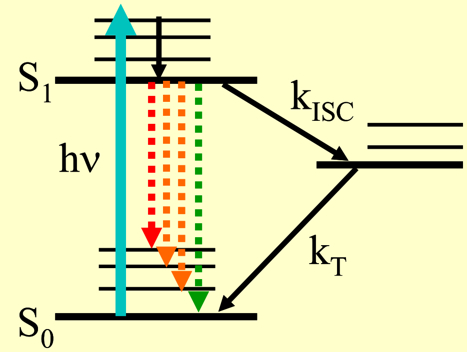
…single-photon sources, …

- Super-resolution Microscopes

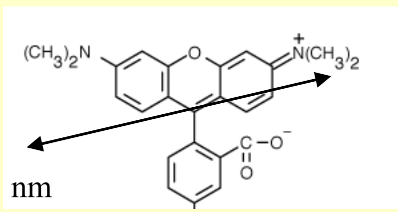
Room Temperature: Milestones of Single-Molecule Detection and Imaging

Solution: Correlation functions	Fluorescence Correlation Spectroscopy: Magde, Elson, Webb (1972, 1974); Ehrenberg, Rigler (1974); Pecora (1976); …
	Autocorrelation (FCS) from 1 fluorophore or less in the volume: Rigler, Widengren, BioScience (1990)
Solution: Single bursts	Multichromophore emitter bursts (phycoerythrin): Peck, Stryer, Glaser Mathies PNAS **86**, 4087 (1989)
	Single bursts from 1 fluorophore: Shera, Seitzinger, Davis, Keller, Soper, Chem.Phys.Lett. **174**, 553 (1990); Nie, Zare, Science (1994);…
Solution and surface	Single antibody with multiple (~80-100) labels: T. Hirschfeld, Appl. Opt. **15**, 2695 (1976)
Near-Field NSOM, SNOM	Imaging a single fluorophore: Betzig and Chicester, Science **262**, 1422 (1993); Ambrose,…, Keller PRL **72**, 160 (1994); Xie and Dunn, Science **265**, 361 (1994)
Confocal image	Macklin, Trautman, Harris, Brus, Science **272**, 255 (1996); …
Widefield, single fluorophore	*In vitro*, myosin on actin: Funatsu,…Yanagida, Nature **374**, 555 (1995).
	Cell membrane, single-lipid tracking with super-localization, Schmidt, Schütz, …Schindler, PNAS **93**, 2926 (1996).

Detecting Single-Molecule Absorption from Emitted Fluorescence at Room T

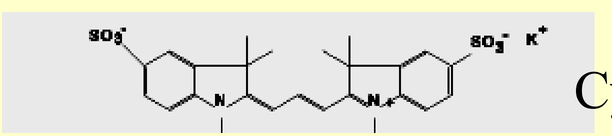


- Typical organic fluorophore labels are only ~1 nm in size, fluorescent proteins ~3-4 nm
- Light pumps electronic transitions of the molecule
- Signal indirectly reports on local nanoenvironment because only one molecule is pumped and measured, if backgrounds are low and molecule emits light efficiently

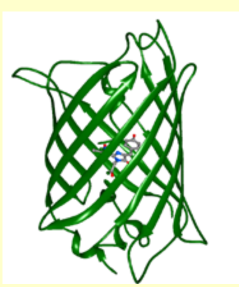


~1 nm

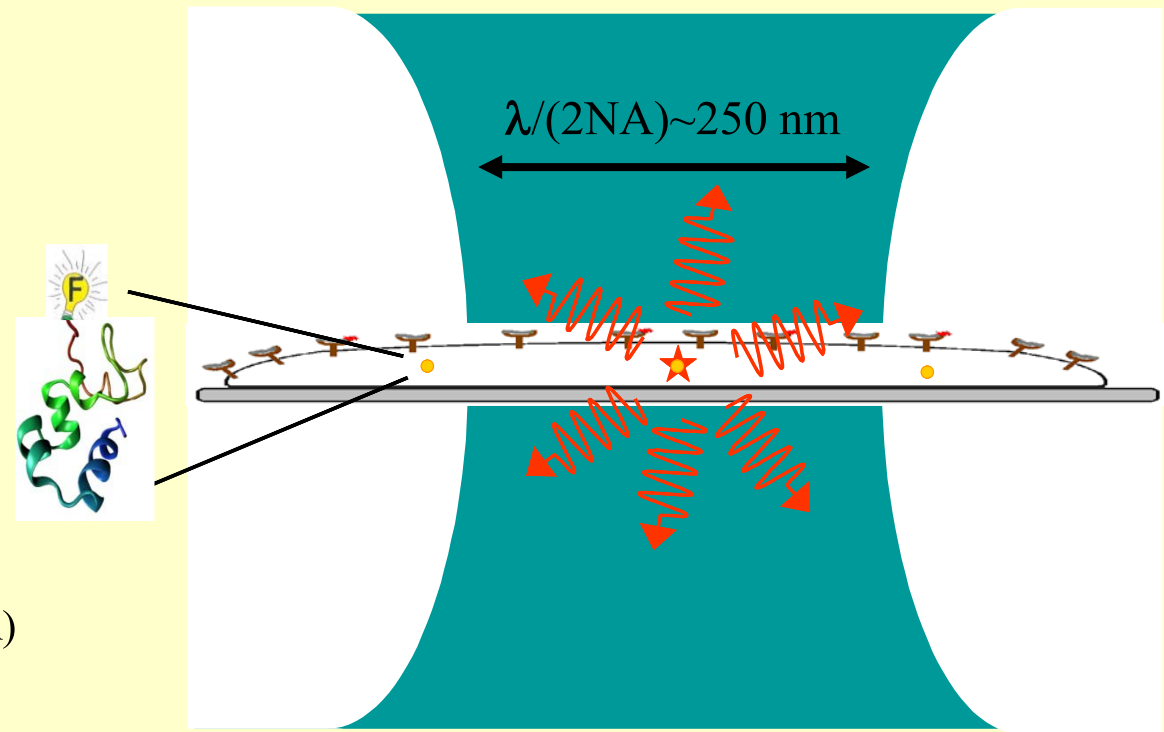
TMR



Cy3



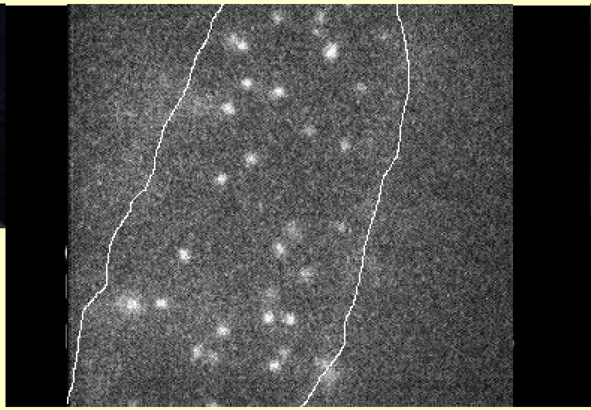
GFP, FPs
(~ 3nm x 4 nm)



Single-Molecule Imaging and Tracking Examples – Much to Learn from Isolated Single Molecules!

Immune proteins in membrane of a live CHO cell

Vrljic, Nishimura, McConnell, WEM, Biophys. J. (2002)



Super-Resolution Microscopy with Single Molecules

How can we use single-molecule labels to surpass Abbé's optical diffraction limit, a fundamental physical effect in the far-field?

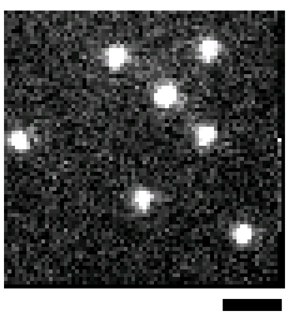
Single-molecule imaging + 2 key ideas

Key Idea #1: Super-Localization

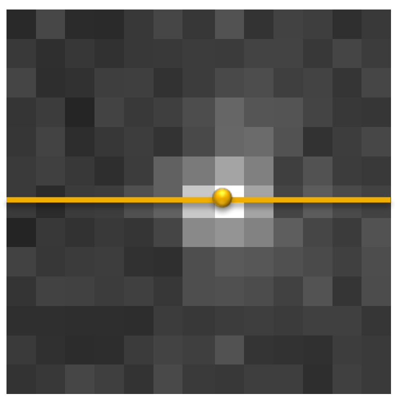
cinder cone, bar 120×10^9 nm

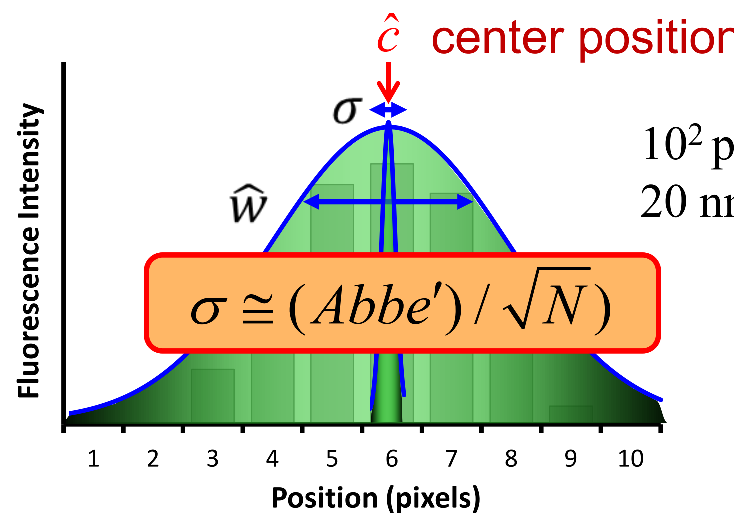
can easily find peak position to much better precision than the width

Find the <u>position</u> of the emitter by fitting the shape of the single-molecule image



1 µm





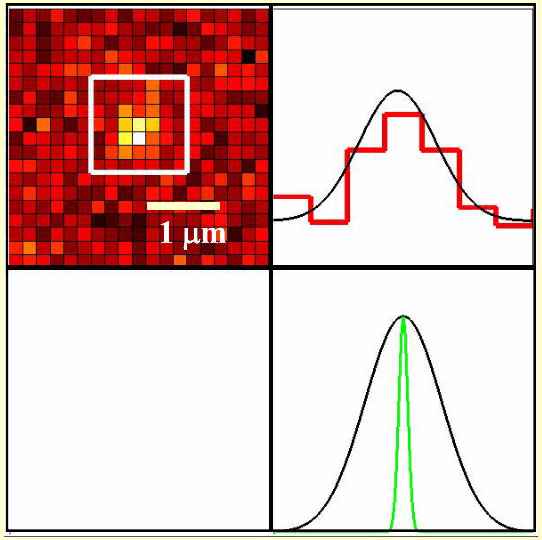
10^2 photons:
20 nm prec.

Summary: WEM, J. Microscopy **246**, 213-220 (2012)

Some Early Examples of Super-Localization in Biological Imaging

Find centroid of large fluorescent object	LDL (Low-Density Lipoprotein particles with many labels) on cell surface: Barak, Webb, J. Cell Biol 95, 846 (1982)
	Tracking kinesin motor-driven 190 nm bead with few nanometer precision: Gelles, Schnapp, Sheetz, Nature **331**, 450 (1988)
Find position of single fluorophore	Cell membrane, single-lipid tracking to 30 nm precision: Schmidt, Schütz, …Schindler, PNAS **93**, 2926 (1996).
	Single virus particle on HeLa Cell to 40 nm precision: Seisenberger,…Bräuchle, Science **294**, 1929 (2001); …

If all photons come from the same nm-sized object, we can truly extract nanometer-scale position information, and follow the trajectory in time!



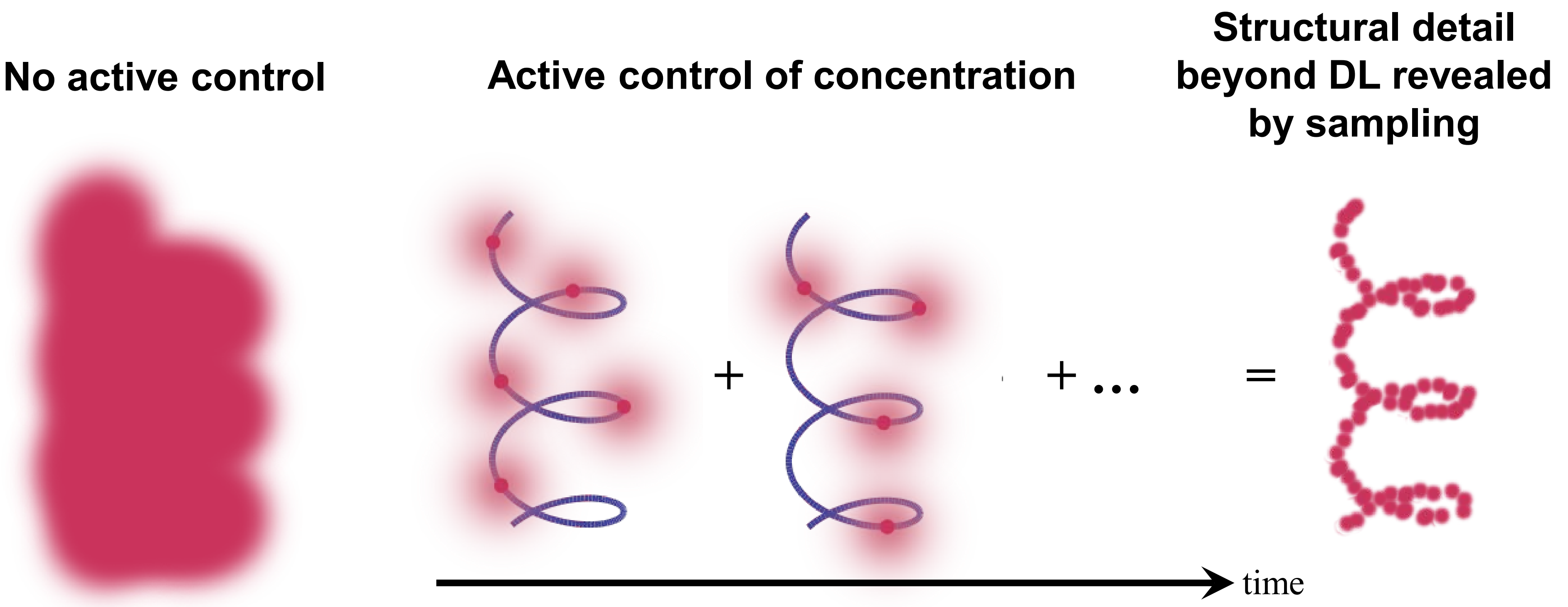
Simulation courtesy Lucien Weiss

Toward Super-Resolution with Single-Molecule Emitters

Key proposal	Use some additional control variable to separate DL spots in spatial dimension – spectral tunability suggested: E. Betzig, Opt. Lett. **20**, 237 (1995)
Low T	Spectral tunability used to achieve 3D super-resolution: A. van Oijen, J. Koehler, J. Schmidt, Mueller, Brakenhoff, Chem. Phys. Lett. **292**, 183 (1998) – 40 nm lateral, 100 nm axial for several single molecules
Room T	Distinguish two dyes by fluorescence lifetime: Heilemann,…,Sauer, Anal. Chem. **74**, 3511 (2002).
	Use photobleaching of overlapping fluors: SHRImP: Gordon, Ha, Selvin, PNAS **101**, 6462 (2004); NALMS: Qu, Wu, Mets, Scherer, PNAS **101**, 11298 (2004)
	Two differently colored probes: SHREC: Churchman,…,Spudich, PNAS **102**, 1419 (2005)
	Blinking of semiconductor quantum dots: Lidke, Rieger, Jovin, Heintzmann, Opt. Exp. **13**, 7052 (2005)

Acronyms, see WEM, PNAS **104**, 12596 (2007)

Key idea #2: Active control of emitter concentration, sequential imaging



PALM (4/2006) E. Betzig/H. Hess

STORM X. Zhuang

F-PALM S. Hess

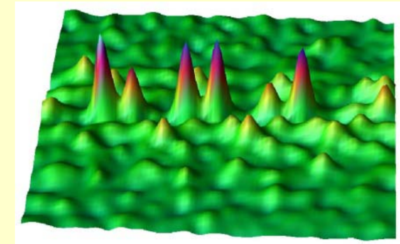
(also: PAINT (Hochstrasser), dSTORM (Sauer), YFP reactivation, GSDIM (Hell), BLINK (Tinnefeld), SPDM (Cremer)…)

Mechanism-Independent: **Single-Molecule Active Control Microscopy: "SMACM"**

1997: Imaging, Blinking, and Photorecovery for Single EYFP

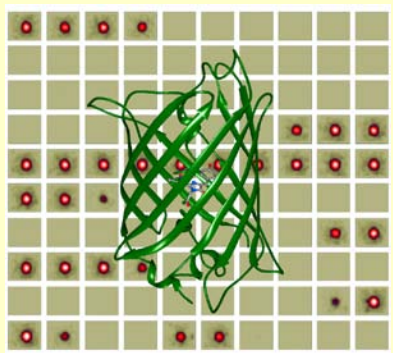
(Enhanced Yellow Fluorescent Protein, a GFP variant)

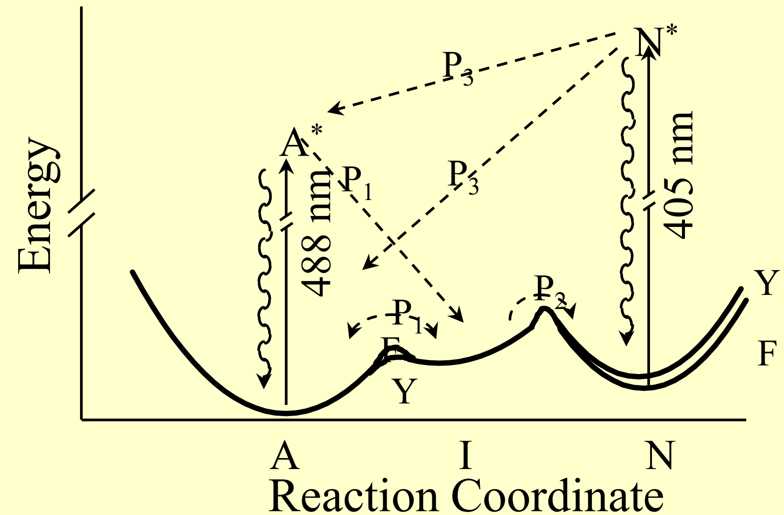
R. M. Dickson, A. B. Cubitt, R. Y. Tsien, and W. E. Moerner, Nature 388, 355 (1997), U. S. Patent 6,046,925.



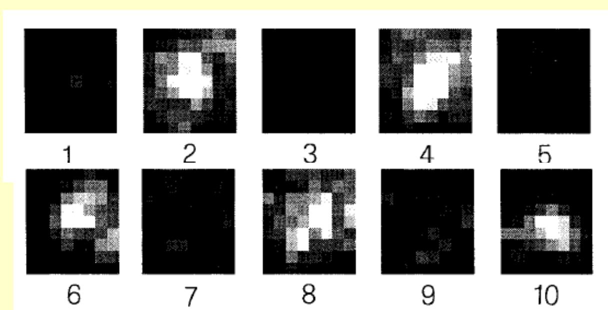
Imaging of single S65G/S72A/T203Y variants in a gel showed blinking and switching, i.e., thermal AND light-induced recovery from a metastable dark state

Blinking (488 nm, 100 ms)





Switching: 405 nm causes recovery of yellow emission for one single molecule



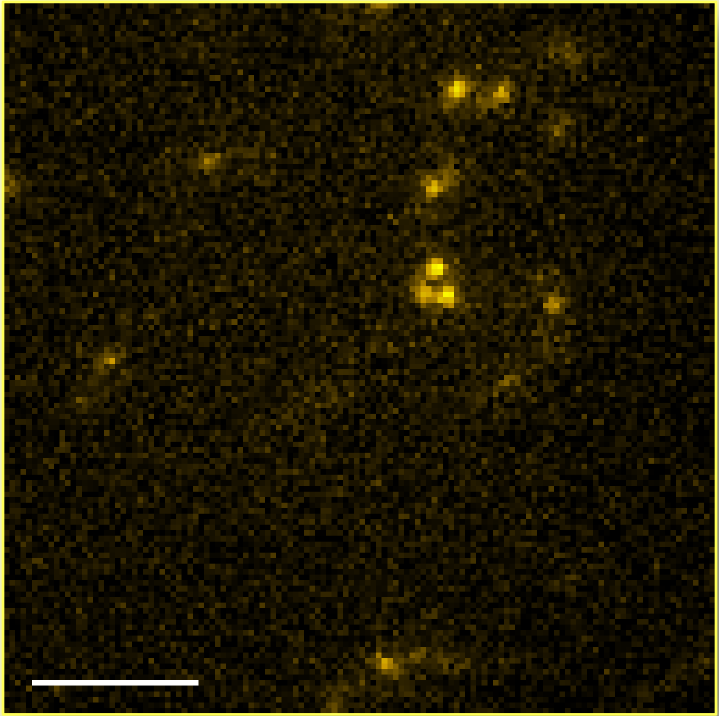
Further development of switchable FPs:
PA-GFP: Patterson, Lippincott-Schwartz (2002)
DRONPA: Ando, Miyawaki (2004)
…

EYFP immobilized in polyacrylamide gels

Likely photoinduced *E*,*Z*-isomerization of the GFP chromophore

What the data look like….

eYFP fusions to a bacterial protein blinking in *Caulobacter crescentus* cells



5µm Scale bar, 10ms per frame, movie slowed down 3X

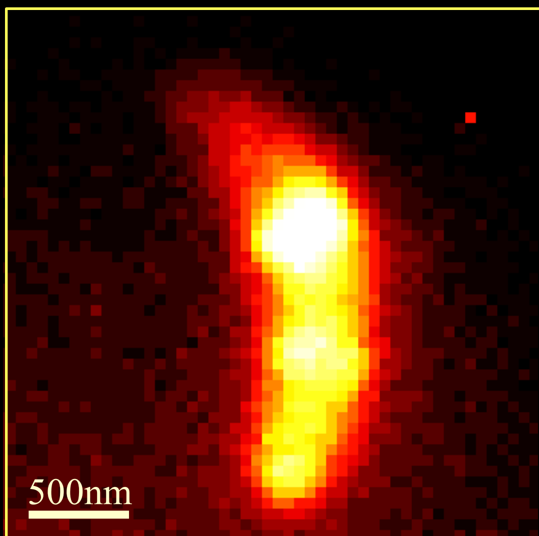
eYFP blinking and photocontrol:
R. M. Dickson, A. B. Cubitt, R. Y. Tsien, and W. E. Moerner, Nature 388, 355 (1997).

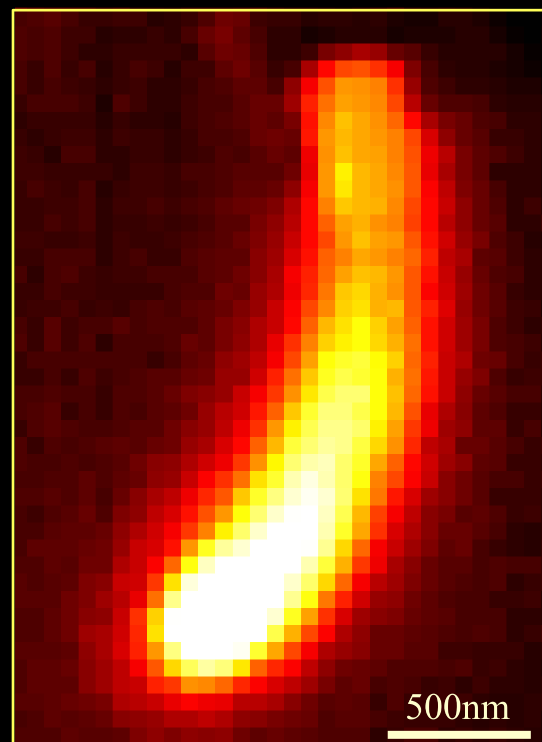
Examples: Diffraction-Limited Imaging *vs.* SR

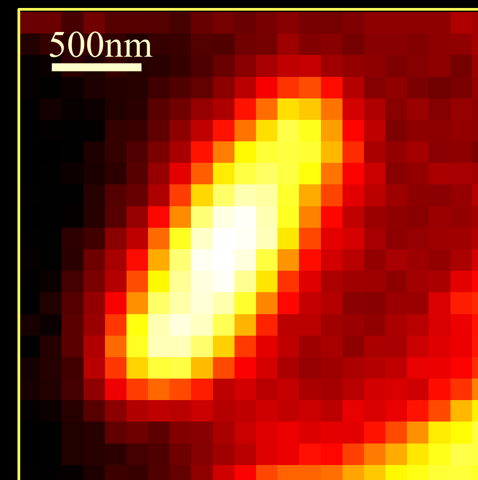
(Collaboration with Lucy Shapiro lab, Stanford)

Epi-fluorescence images, of 3 different eYFP fusions in *Caulobacter crescentus*

MreB | ParA | HU

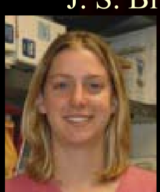


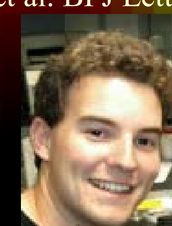




Lee, Thompson, et al. BPJ Lett 2011

J. S. Biteen, et al. Nature Methods (2008)

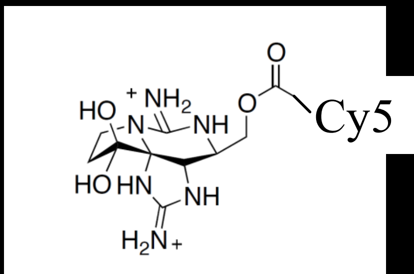




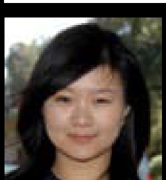
Ptacin, Lee, et al. Nature Cell Biol. (2010)

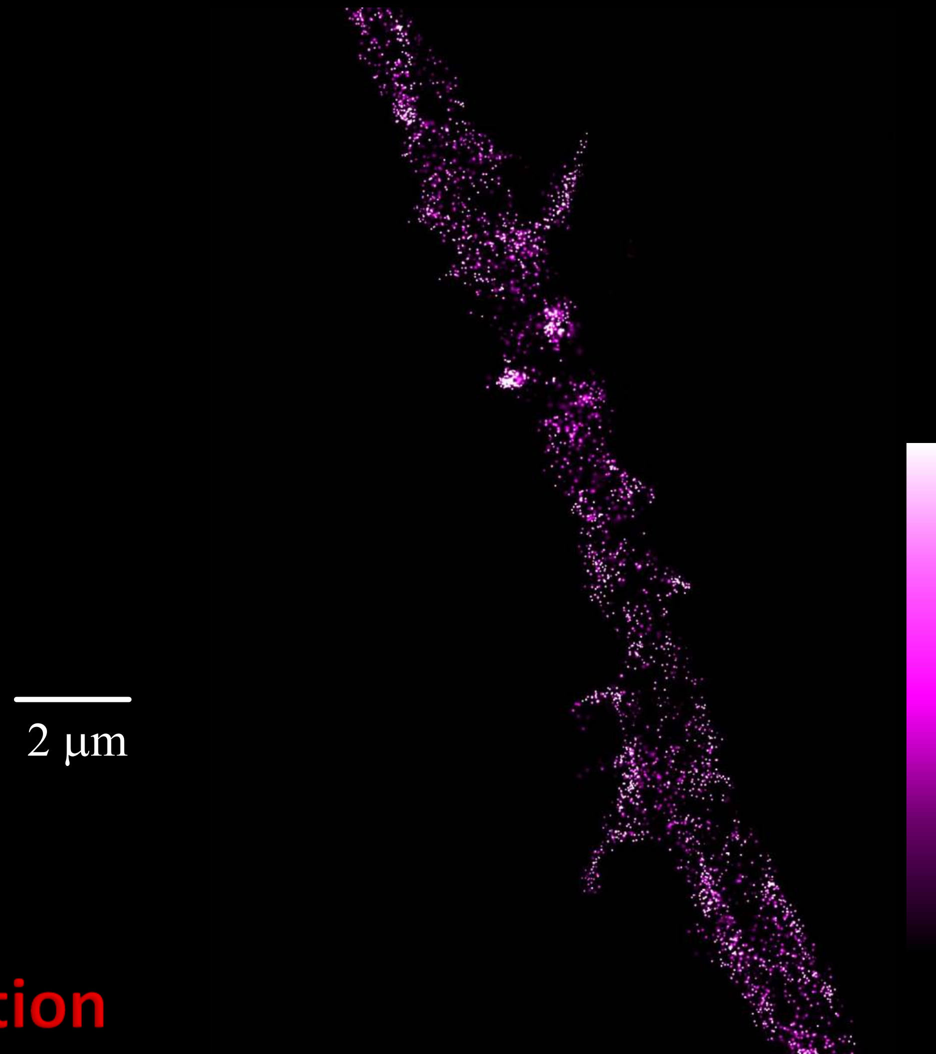
Super-Resolution (40 nm loc. precision) reveals multiple localization patterns!

Neuritic Spine-Like Structures: Na_V Channels in a Differentiated PC12 Cell



(+)-saxitoxin (STX)





High (=newest frame)

Low (=Last frame)

Super-Resolution Reconstruction, 500 ms sliding window

(H. Lee, S. Iwanaga,… J. DuBois, WEM, Chem.Bio.(2012))

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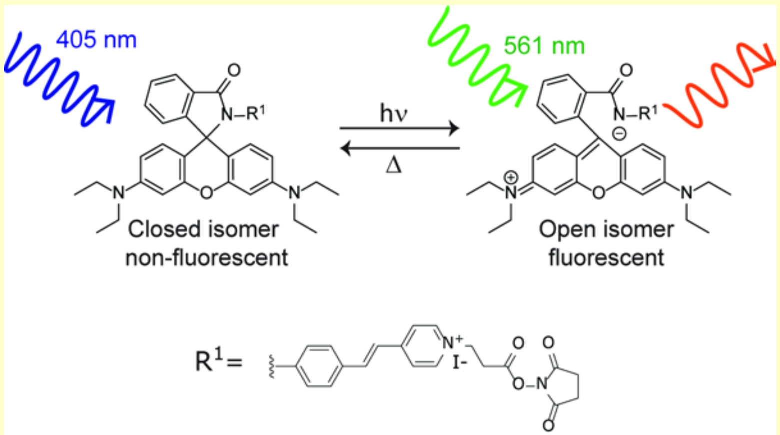
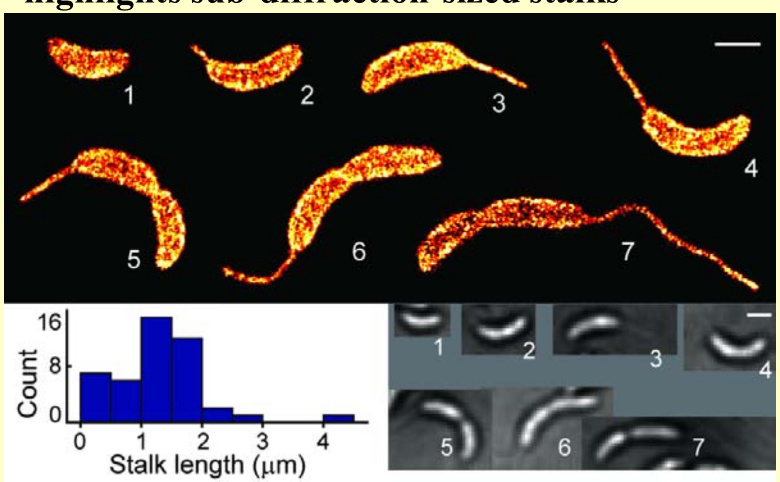
New Fluorophores, New 3D Imaging Methods

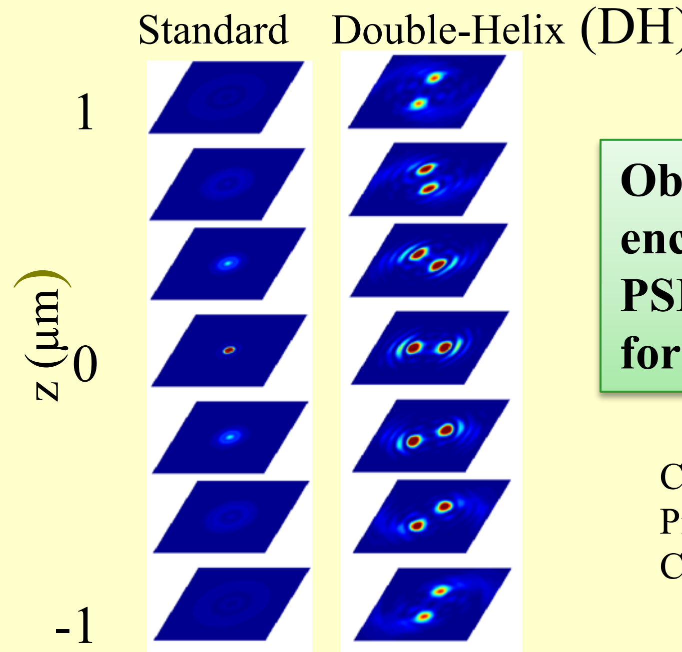
Marissa Lee, P. Rai, J. Williams, R. J. Twieg, WEM
J. Am. Chem. Soc. **136**, 14003-14006 (2014)

Optimized rhodamine spirolactams photoswitch with blue light



Labeling free amines on live bacteria surfaces highlights sub-diffraction-sized stalks





Object depth is encoded into PSF rotation for the DH-PSF

Collaboration: R. Piestun, Univ. Colorado

Impact of Single-Molecule Spectroscopy/Imaging

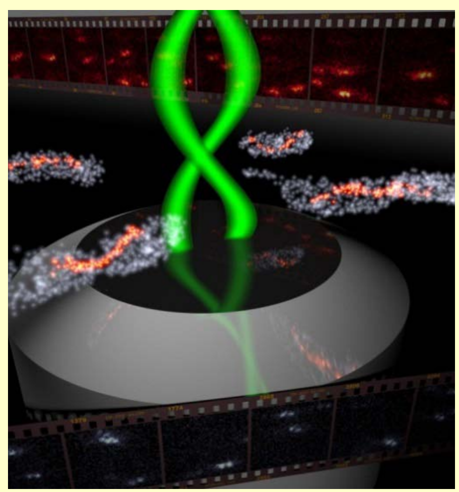
Chemistry:
spectral diffusion,
intersystem crossing,
molecular distortions,
nanoantennas, structures of
materials, photocontrol,
catalysis,...

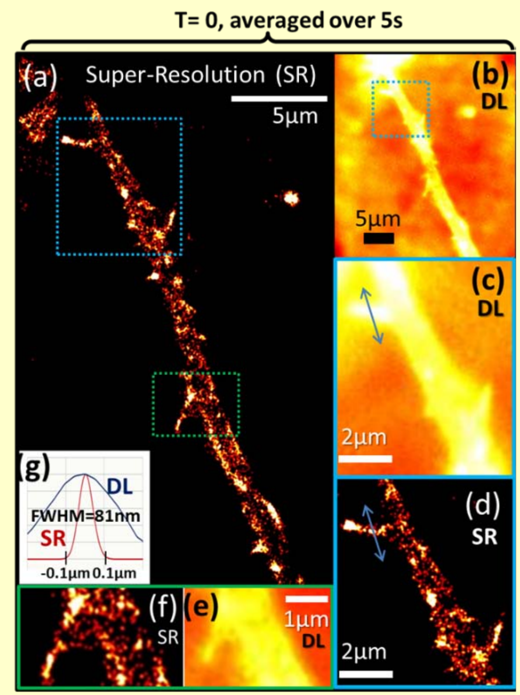
Physics:
nanoenvironments,
magnetic interactions,
diffusion, structure,
EM enhancements
quantum optics,...

Biology:
fluctuations,
enzymatic states/
mechanisms, cell biology,
folding, membrane
behavior, cellular
structures beyond the
diffraction limit …

Working at the ultimate single-molecule limit has attracted the attention of many talented scientists around the world, who continue to make seminal contributions to this field!

FRET: TJ Ha, S Weiss …
Enzymes: XS Xie, H Yang, …
RNA folding, actin bands: X Zhuang, ...
Motors, DNA processing, DNA dynamics, gene expression, nuclear pores, RNA/proteins in cells, chaperonins, viral entry, quantum optics, new labels, 3D, …





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- Dr. Alan Huston
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- Dr. Lothar Kador
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- Prof. Dr. Thomas Basché
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- Dr. Jürgen Köhler
- Prof. Stephen Ducharme
- Dr. Peggy Walsh
- Dr. John Stankus
- Dr. Scott Silence
- Dr. Constantina Poga
- Dr. Yiwei Jia

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- Ms. Courtney Thompson
- Dr. David J. Norris,
- Dr. Anders Grunnet-Jepsen
- Dr. Susanne Kummer
- Dr. Rob Dickson
- Dr. Maria Diaz-Garcia
- Mr. James Frazier
- Mr. Tim Marsh
- Ms. Julie Casperson
- Ms. Laura Neurauter
- Mr. Barry Smith

Stanford University:

- Dr. Erwin J. G. Peterman
- Dr. Arosha Goonesekera
- Dr. Sophie Brasselet
- Dr. Brahim Lounis
- Mr. Andre Leopold
- Mr. Erik Bjerneld
- Mr. Shaumo Sudhukhan
- Ms. Yeonsuk Roh
- Dr. Ueli Gubler
- Dr. Dan Wright
- Dr. Matt Paige
- Dr. Oksana Ostroverkhova
- Dr. Stephan Hess
- Dr. Marija Vrljic
- Dr. Jason Deich
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- Dr. Kallie Willets
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- Dr. Hsiao-lu Denise Lee
- Ms. Yao Yue
- Dr. Whitney Duim
- Dr. Yan Jiang
- Ms. Katie Evans
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- Dr. Andreas Gahlmann
- Dr. Steffen Sahl
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Dr. Saumya Saurabh
Dr. Quan Wang
Dr. Allison Squires
Marissa Lee
Mikael Backlund
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Adam Backer
Alex Diezmann
Hsiang-yu Yang
Colin Comerci
Camille Bayas
Josh Yoon
Maurice Lee
Petar Petrov
Jingying Yue (rotator)

S. Lord

one molecule = one guacamole

(i.e., 1 over Avocado's Number of moles, $1/N_A$ moles)

(with apologies to the memory of Amadeo Avogadro)

Moerner Group

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Washington University:

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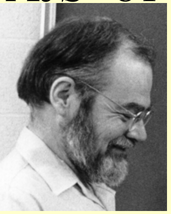
Mentors:

JGM '75 AJS '81



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