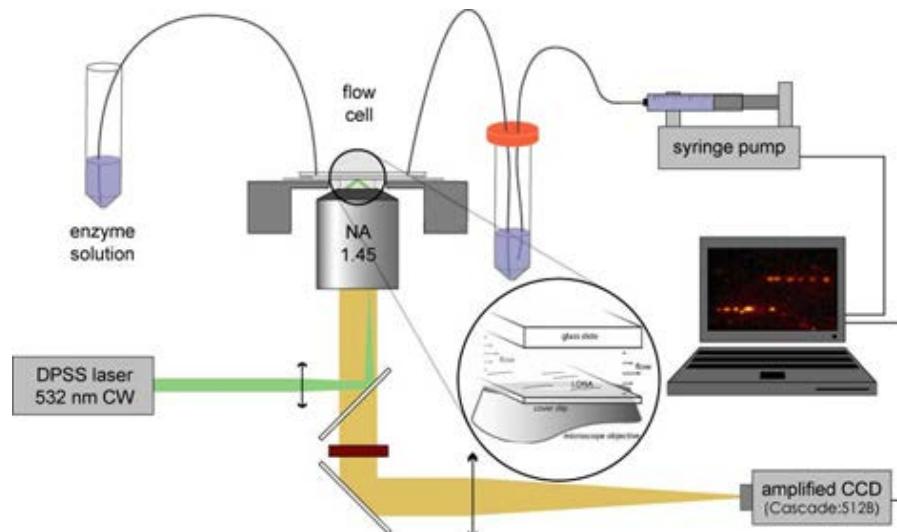
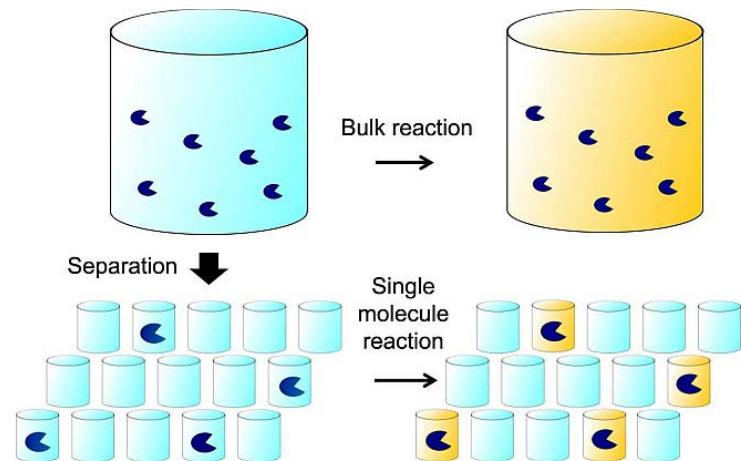


Lecture #7: Single Molecule Enzymology

- (1) Types of single enzyme movies
- (2) Advantages of single molecule studies
- (3) Applications of single molecule studies
- (4) Following enzymes in real time
 - (a) ATP synthase with tethered actin
 - (b) ATP synthase
 - (c) Myosin V
 - (d) Kinesin motor protein on microtubule
 - (e) Single molecule microscope
 - (f) Single molecule studies of cholesterol oxidase
 - (g) β -galactosidase: a model Michaelis-Menten enzyme

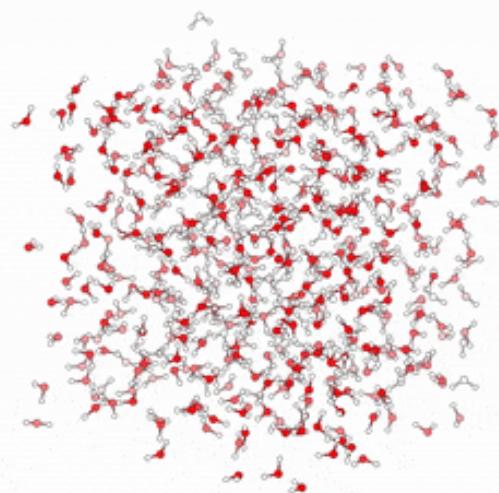
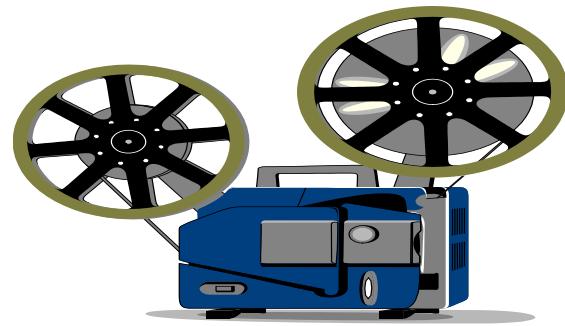


Lecture #7: Single Molecule Enzymology

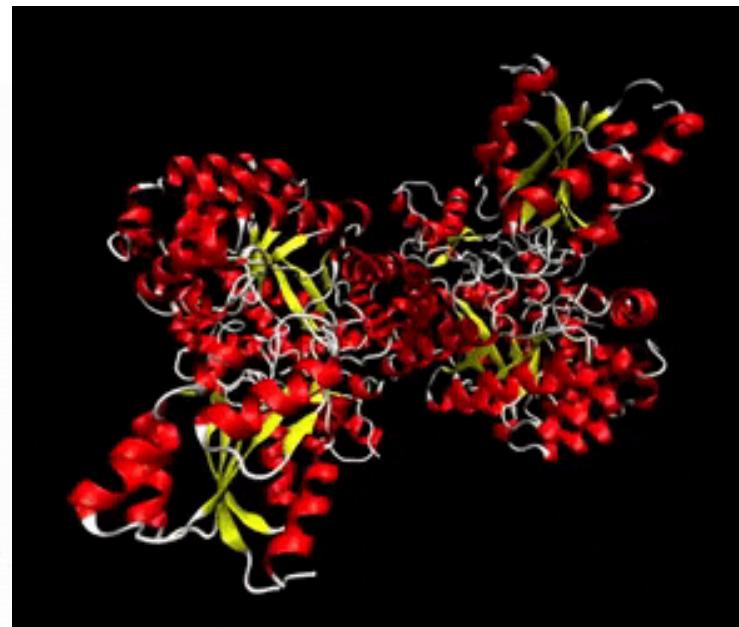
(1) Movies of Single Enzymes

Molecular dynamics simulations (MDS)

- (MDS) can provide movies of single enzyme molecules
 - computer simulation method
- only run for a few **nanoseconds**



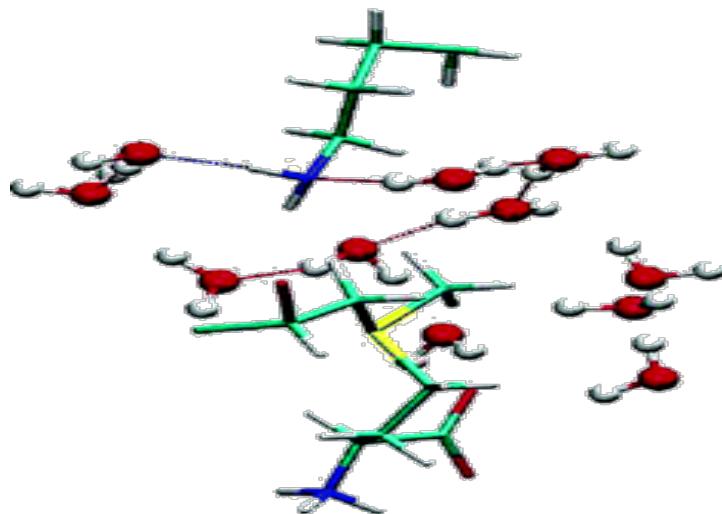
MDS of H_2O



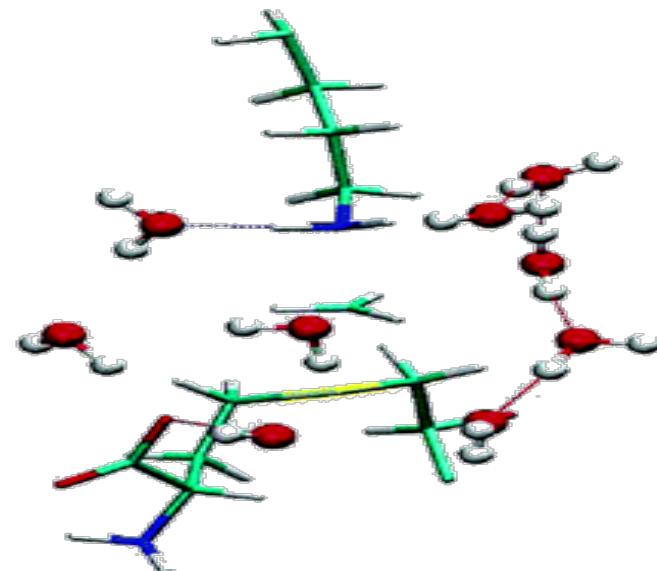
MDS of a protein in solution

***Ab Initio* Quantum Mechanical/Molecular Mechanical Molecular Dynamics Simulation of Enzyme Catalysis: The Case of Histone Lysine Methyltransferase**

- Y. Zhang performed quantum mechanical/molecular dynamics simulation of enzyme catalysis for histone-lysine methyltransferase
 - computer simulation of the formation of the transition state for the reaction
 - energetics of reaction trajectory agreed well with experimental data



Pre-reaction state

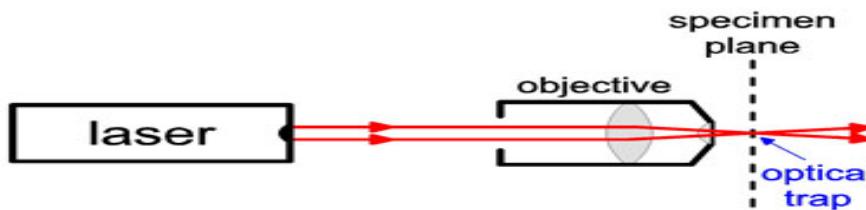


Transition state

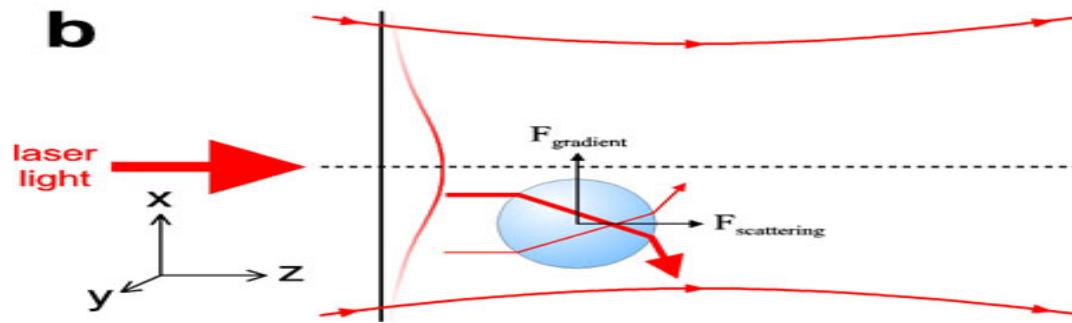
- AFM (atomic force microscopy), optical tweezers, and fluorescence microscopy can produce movies based on experimental data
 - millisec to sec time scale

Optical Tweezers

a



b

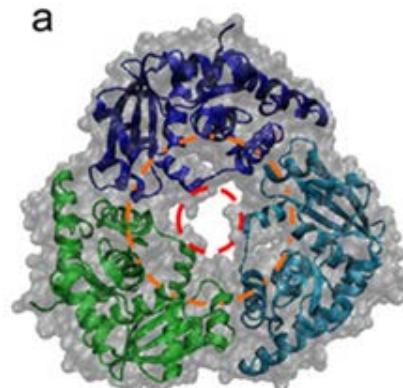


- Trap beads, viruses, bacteria, organelles,
- DNA strands, mammalian cells, metal particles

- lower resolution than MDS but can track slower motions in single molecules
- these motions would be lost in the ensemble-averaged experiments
 - enzyme motions and reaction steps for a few enzymes (nuclease, β -galactosidase) have been monitored optically **in real time!**

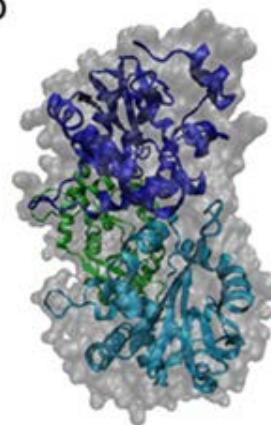
Single-molecule FRET for λ -nuclease activity

Nat Chem Biol. 7, 367-374, 2011

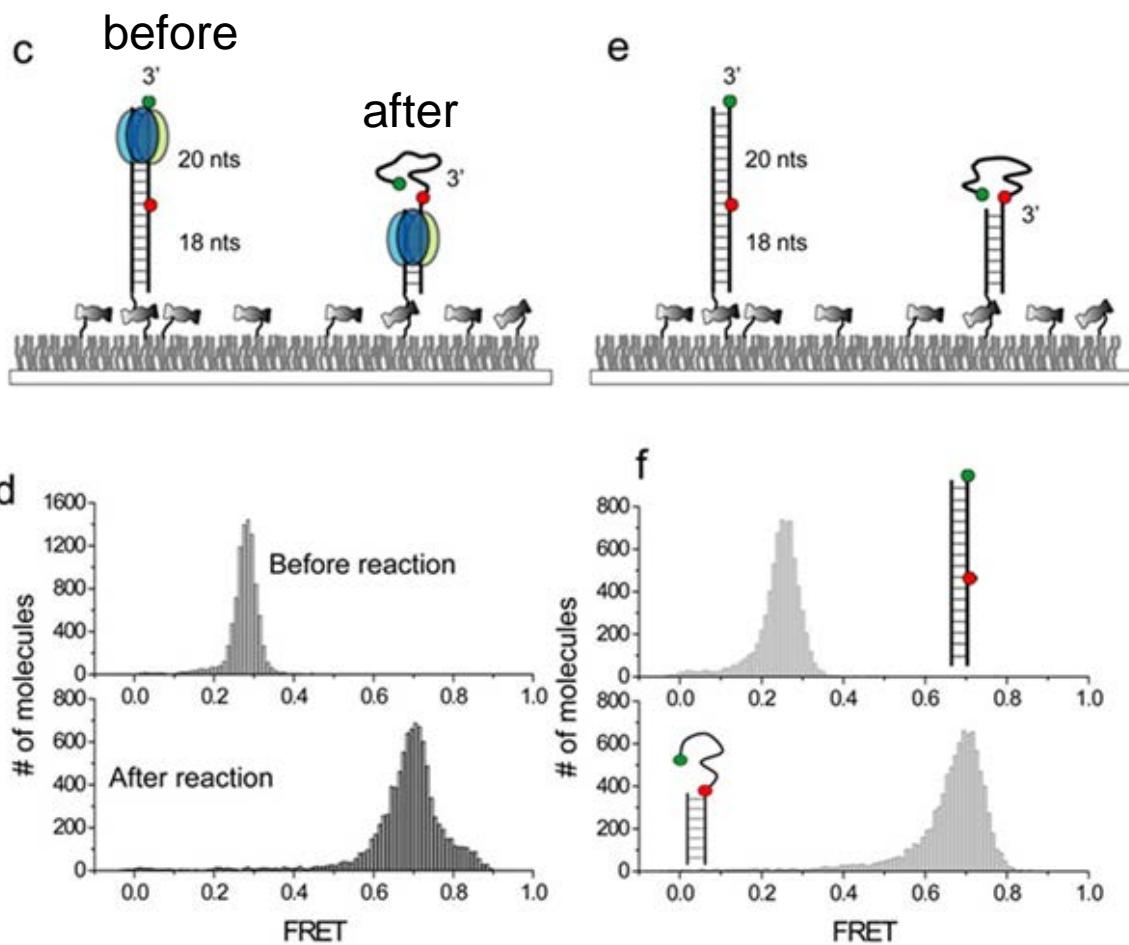


a

- λ nuclease is an enzyme that cleaves dsDNA
- dsDNA enters the outer ring (30 Å dia) with ssDNA product exiting from the inner ring (15 Å)



- enzyme converts dsDNA between the donor (green) and the acceptor (red) to ssDNA causing an increase in FRET
- Single molecule histograms before and after degradation by nuclease
- Partial duplex mimicking the degradation product constructed to estimate the FRET value after degradation
- Single molecule FRET histograms from dsDNA and partial duplex in the absence of nuclease



Nat Chem Biol. 7, 367-374, 2011

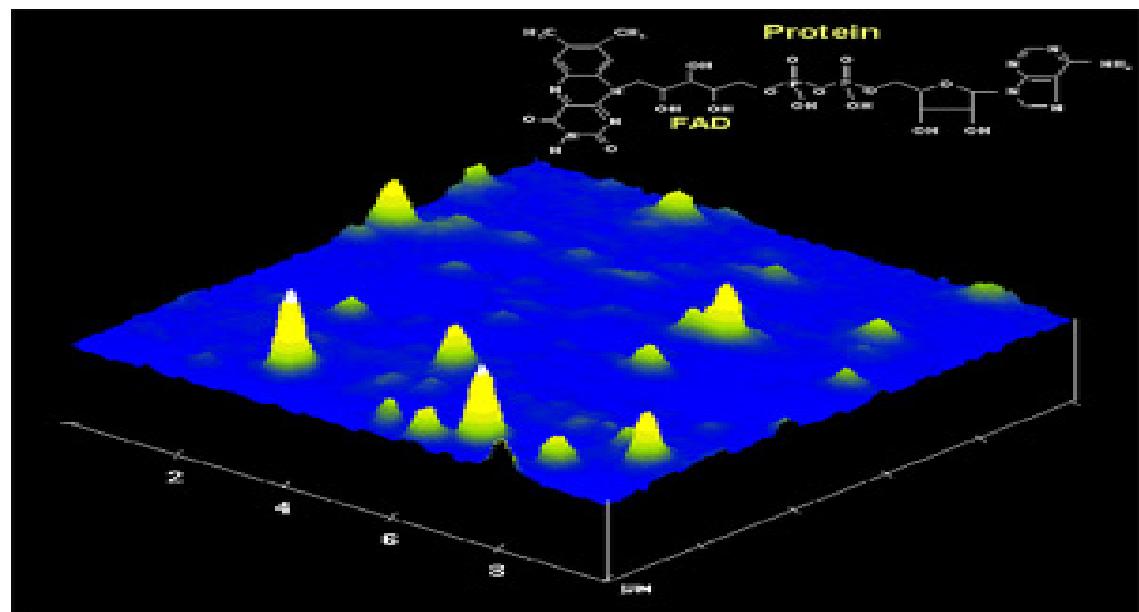
(2) Advantages of Single Molecule Studies (SMS)

- concentration of the molecule(s) becomes meaningless
 - no need to worry about [E] in sample



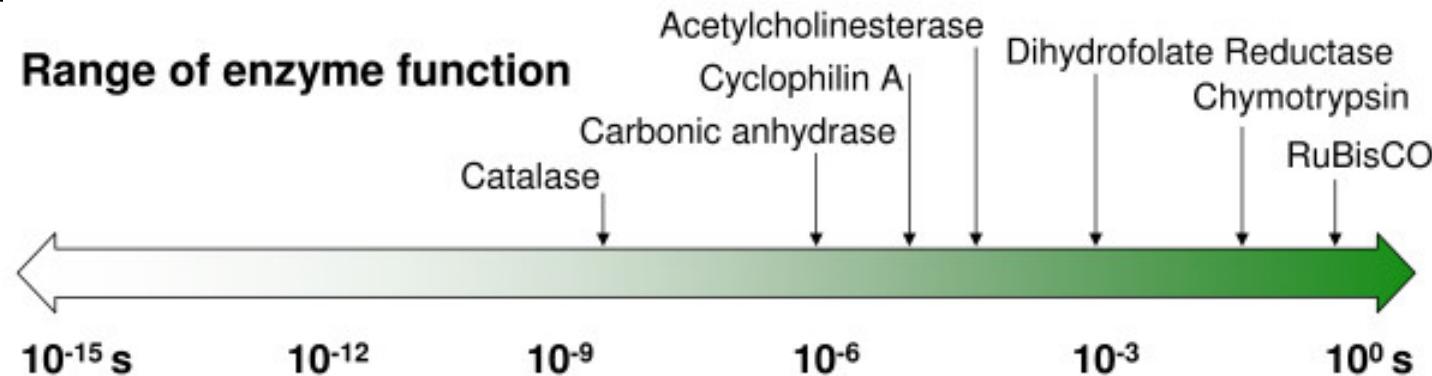
- Stochastic events of individual molecules are **not observable**
 - steady state concentrations of transient intermediates are usually too low to detect

- Allows direct observation of individual steps or Intermediates
 - grid shows individual events from an enzyme-coated grid that converts FADH to FAD (fluorescent)

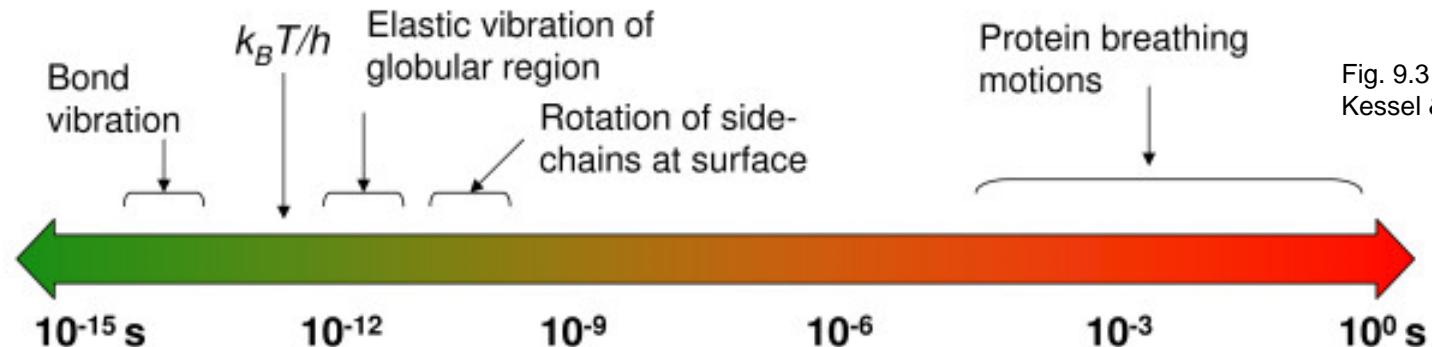


- Ensure that you have trapped/captured a single molecule on a bead or surface

- Single molecule spectroscopy can **capture** reaction intermediates
- SMS can **unravel** the conformations and dynamics of the enzyme during a single catalytic turnover
- An enzyme molecule is a dynamic entity with fluctuations spanning a vast range of time-scales



Range of protein dynamical events



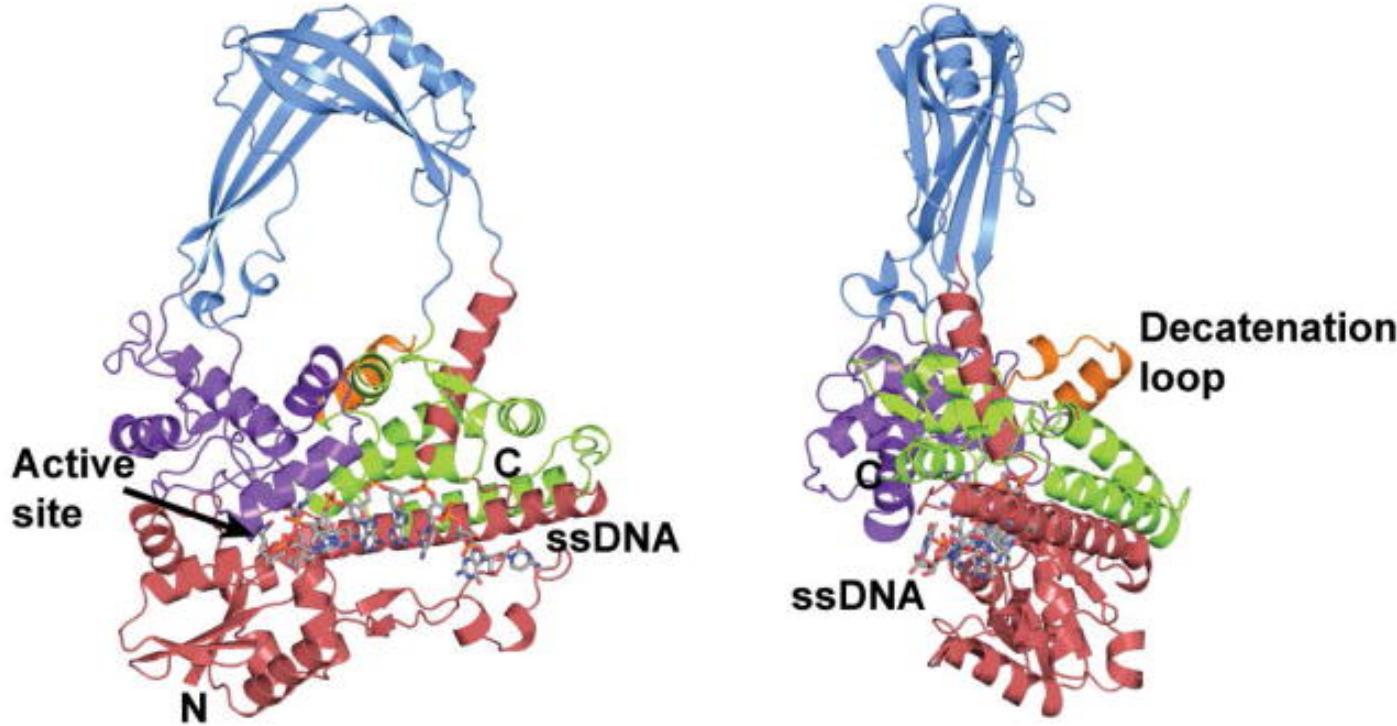
Dynamics-related enzyme mechanisms:

- Enzymes use atomic motions to promote catalysis:
 - **Facilitate ligand diffusion into active site and product release** – by ‘breathing’ motions that create tunnels on the surface
 - **Optimize substrate binding** – by induced fit motions
 - **Increase catalytic rate** – by fast motions (10^{-13} - 10^{-9} s) that optimize quantum phenomena and help to cross energy barriers

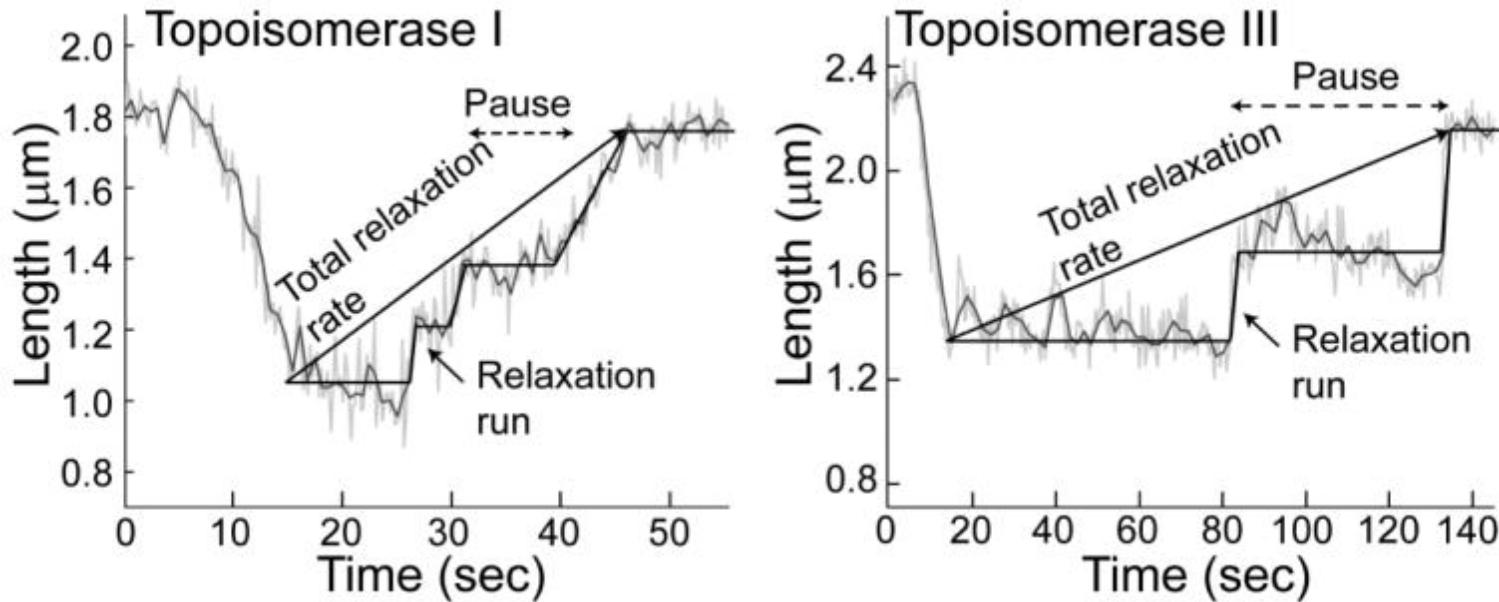
(3) Applications of SMS

Biochem. Soc. Trans. 41: 571-75 (2013)

Topoisomerases (Topo)



- Enzymes responsible for maintaining supercoiled DNA in cells
 - DNA relaxation studied by *E. coli* Topo I and III
 - TopoIII in complex with ssDNA
 - 4 domains that form toroidal shape with a central hole
 - Active site is at the interface of two of the domains



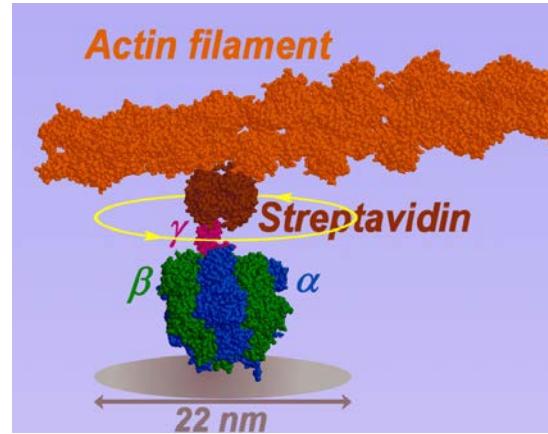
- Length of the DNA is directly related to DNA superhelical state
- Topo enzymes relax DNA via a combination of relaxation runs preceded by pauses
 - Topo I: short pauses and slow relaxation runs
 - Topo III: long pauses and fast relaxation runs

(4) Following Enzymes in Real-Time

- ATP synthase produces ATP
 - Large turbine with multiple subunits
 - F_o is in the membrane stator
 - F_1 is the enzyme turbine

(a) ATP Synthase with tethered actin

- actin filament is labelled with rhodamine dye (red)
 - linked to streptavidin
- F_1 is linked with biotin
- actin-streptavidin forms a complex with the biotin- F_1 synthase
- confocal fluorescence microscope images the actin filament
- actin spins as F_1 synthase turns to make ATP



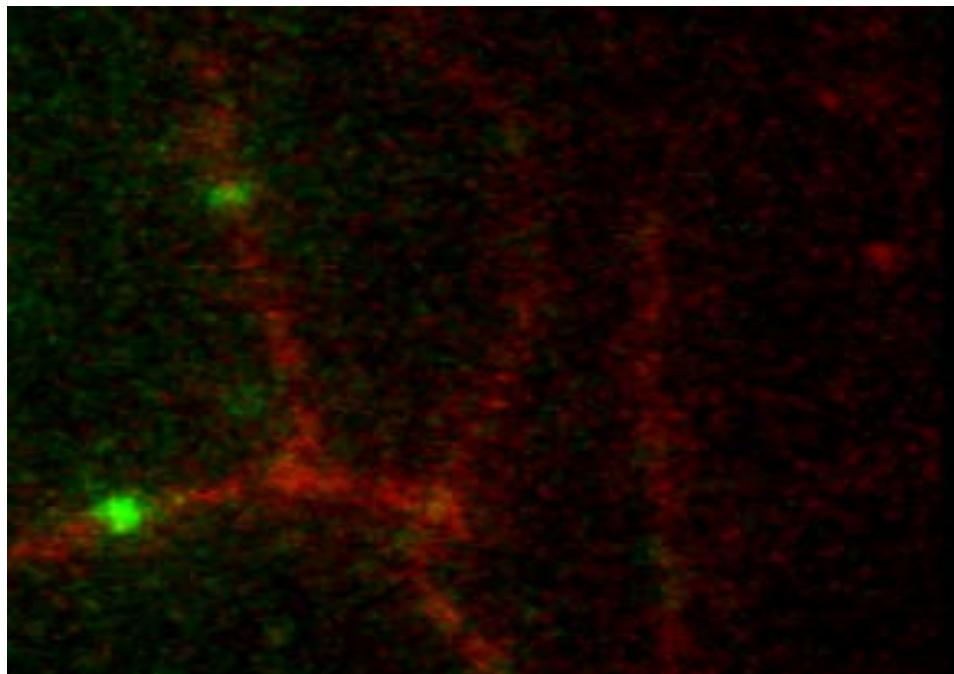
(b) ATP Synthase

<https://www.youtube.com/watch?v=GM9buhWJjlA>



(c) Myosin-V moving along an actin filament

- Myosins
 - Superfamily of motor proteins
 - ATP-dependent
 - Control actin-based motility
- Myosin-V labelled with Cyanine5 dye on its calmodulin subunit (green)
- Actin is stained red with rhodamine dye (red)
- Myosin-V walks along the filament transporting cargo (RNA, vesicles, organelles, etc) from the center of the cell to the periphery

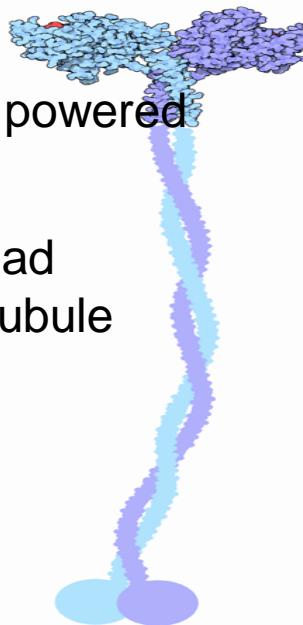


Red: Actin
Green: ~1 µg myosin-V Cy5-CaM
2 mM ATP, 2x frame rate, 10 x 10 µm

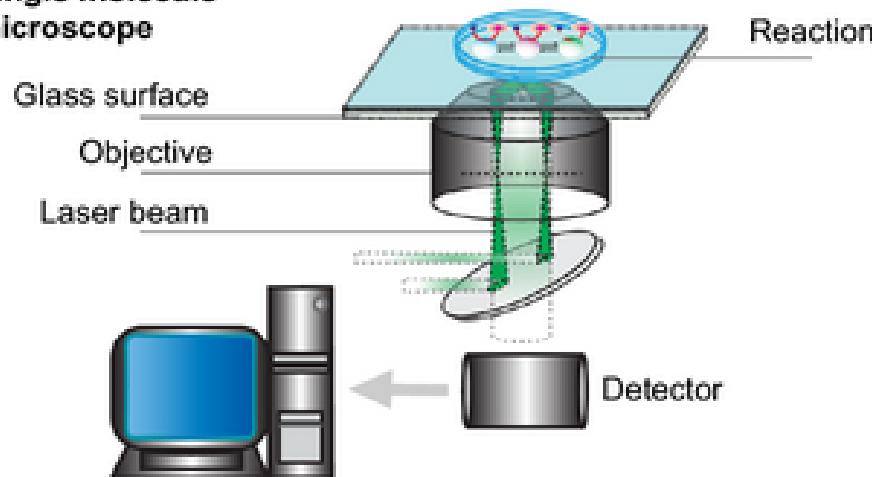
(d) Kinesin motor attached to a fluorescent bead

Contrast Microscopy

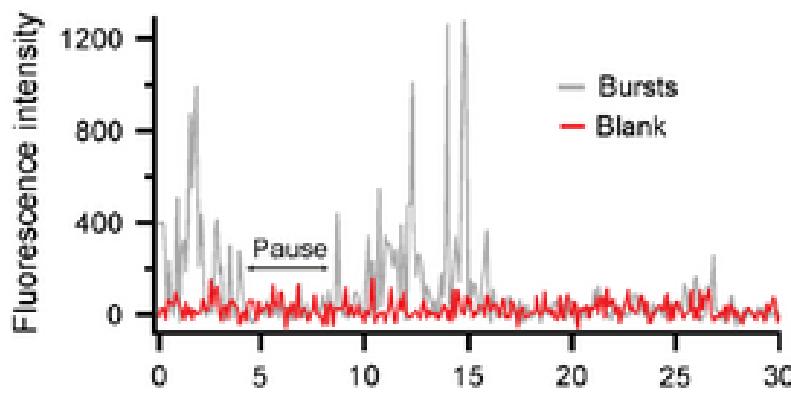
- Kinesins:motor proteins (ATPases) in eukaryotic cells
- Move along microtubule cables powered by ATP hydrolysis
- Kinesin motor with a 100 nm bead attached moving along a microtubule
- 1 mM ATP (kinesin substrate)
- 1 mM AMP-PNP (ATP analog)
 - kinesin competitive inhibitor
- inhibitor binding results in a pause in movement



A
Single molecule
microscope



B
Experimental
result



(e) Single-molecule fluorescence microscope

- read out turnover of single immobilized enzymes
- convert fluorogenic substrate to product
- show bursts of activity
- TIRF (total internal reflection microscope)

-real time single molecule recordings of enzymatic turnovers as fluorogenic substrate is converted to product

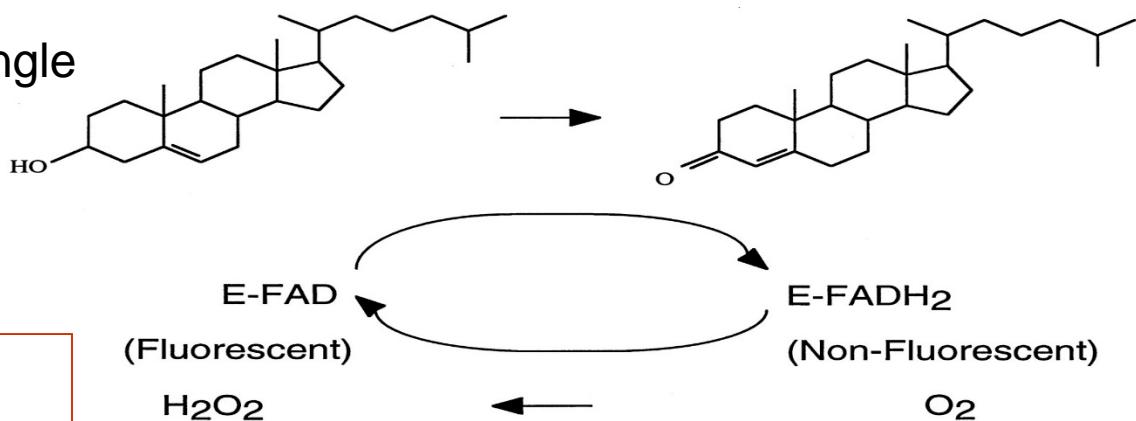
FEBS J. 281: 518-530 (2013)

(f) Single Molecule Studies of Cholesterol Oxidase

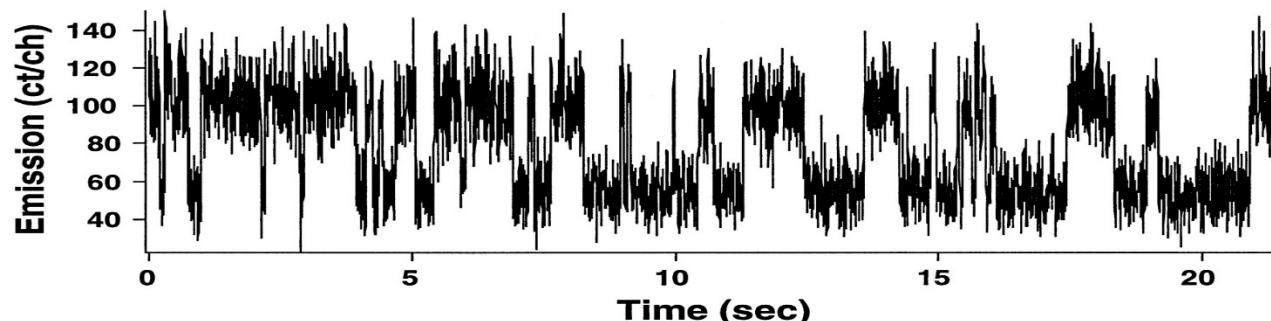
Steroids 143, 6-17 (2019); J. Biol. Chem. 274, 15967-15970 (1999)



Real-time observation of enzymatic turnovers of a single cholesterol oxidase (COx) molecule catalyzing the oxidation of cholesterol

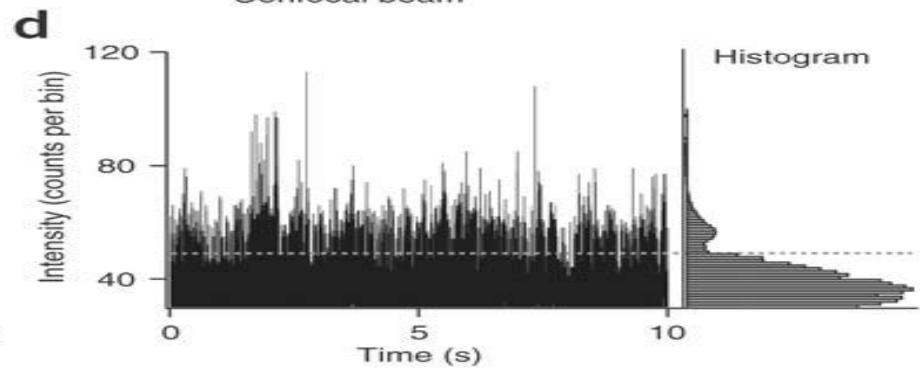
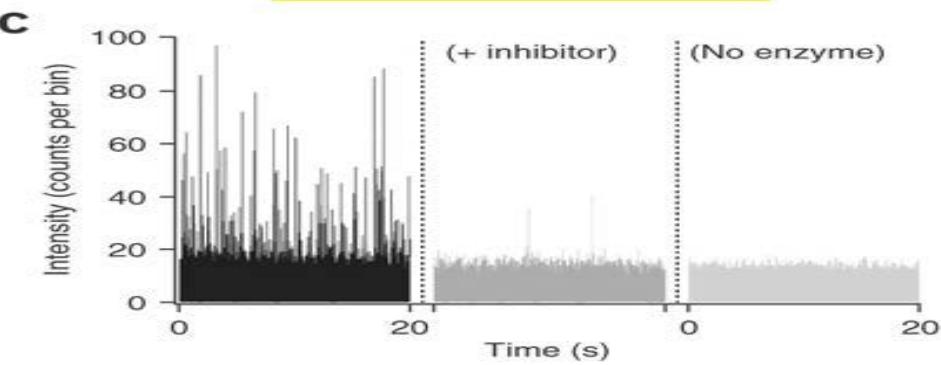
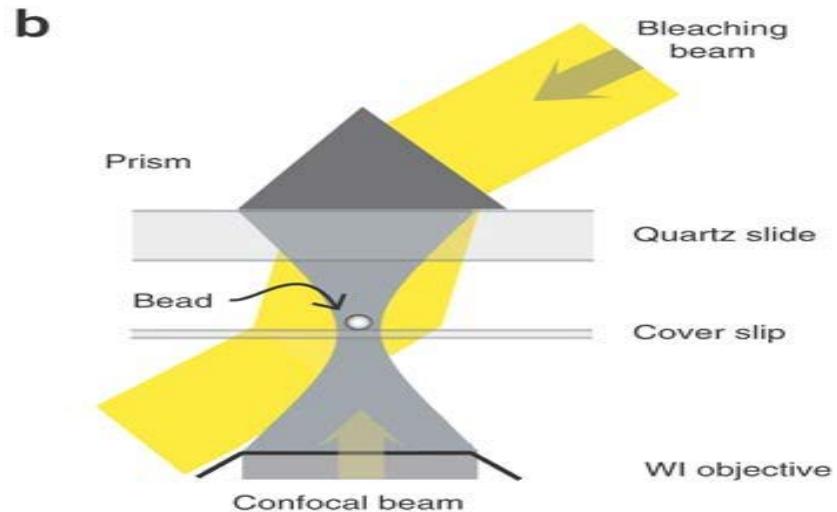
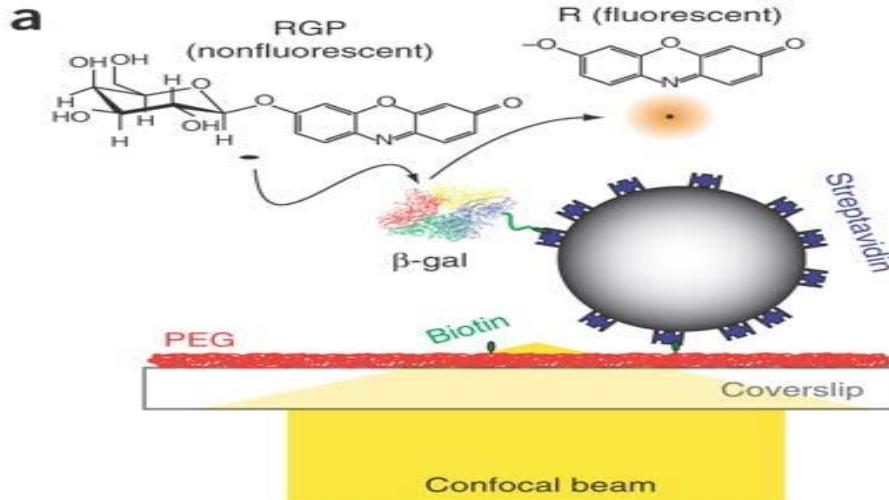


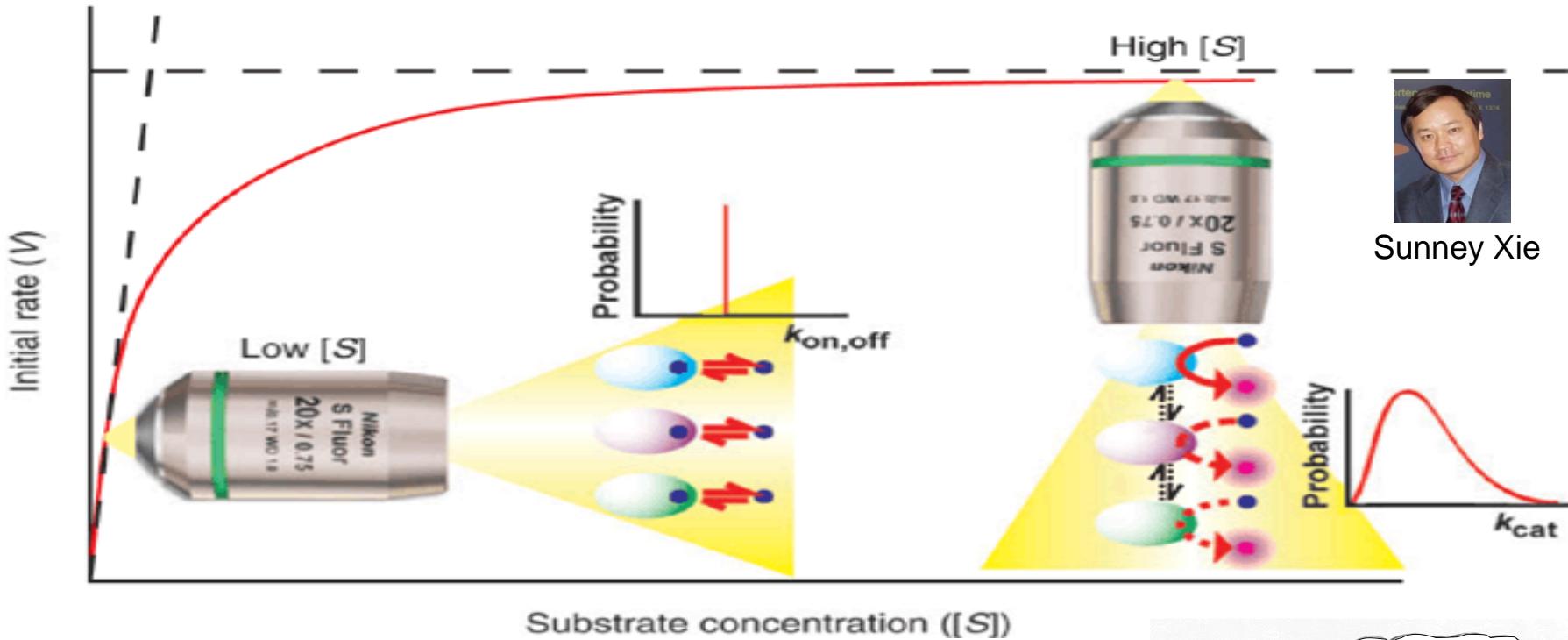
-COx is trapped in a thin agarose film



(g) β -galactosidase: a model Michaelis-Menten enzyme?

English et al., *Nat. Chem. Biol.* **2**, 87-94 (2006)





- M-M equation holds even at the single molecule level
- At high $[S]$ the waiting times between substrate turnovers of a single enzyme show asymmetric probability distribution



- Implies that rate-limiting-constant (k_{cat}) varies over time for an individual enzyme
- Molecular basis for **catalytic heterogeneity** is proposed by Sunney Xie to be the conformational isomers of the enzyme

<http://harvard.sunneyxielab.org/>

- Broad distribution of k_{cat} values suggests that large numbers of conformers with highly variable catalytic powers exist for a single enzyme molecule and **interconvert only slowly**

