Lecture 22: Single-molecule Dynamics: *rotation, diffusion, energy transfer, and powerful application in biological systems*

- Why and how to study molecule dynamics: rotation and diffusion. <u>2 research cases.</u>

- Single-molecule fluorescence resonance energy transfer (FRET) and *application in protein dynamic structural imaging*.

- Imaging of long-range molecule diffusion: monitoring virus attacking of a living cell, and implication for drug delivery.

Part 1:
Imaging Molecule dynamics

Help reveal the basic properties beyond the ensemble average.

Molecule properties measured at single-molecule level

- <u>Optical properties</u>: excited states (formation and decay), fluorescence wavelength and quantum yield and dependence on environment (sensor); energy transfer (dependent on distance --- for protein conformation detection).
- <u>Electronic properties</u>: electron transfer (electronic devices) and separation (photovoltaic), binding recognition (changing fluorescence properties via redox processes).
- Molecule dynamics: rotation and diffusion.
1. Critical for understanding and justifying the application in probing of surface properties and protein dynamic structures --- for any probing, the behavior of the probe itself should be characterized and controlled first.
2. Compared to particles (like pollen grains), diffusion of molecules are quite difficult to be measured at single-molecule level (mainly due to the small signal with respect to the fast motion), although Brownian motion has been known for long time. Fortunately, high sensitivity of fluorescence helps to image such a motion with confocal microscopy.
3. However, molecular rotation cannot be simply detected by fluorescence measurement. It should employ polarization technique --- since only polarized fluorescence depends on molecule dipole, which in turn depends on rotation. Such orientation dependence can only revealed at single-molecule imaging level. Why?

Experimental techniques for measuring molecule rotation

1. <u>NMR</u>: for both solution and solid. Advantages: high spatial resolution (atomic). Disadvantages: low sensitivity for C13, and challenging for selectivity for H1.

2. <u>Polarized fluorescence at single-molecule level</u>: offering in situ measurement of fast rotation of molecules, through milliseconds modulation of excitation polarization.

Experimental techniques for measuring molecule diffusion

1. <u>Diffusion NMR</u>: for high spatial resolution measurement, particularly powerful for bio-species. Potential challenge: diffusion range and time scale.

2. <u>Fluorescence correlation spectroscopy</u> (FCS): suitable for fast processes (micro-seconds) and large area diffusion. Draw a scheme.

3. <u>Absorption:</u> based on change of absorption coefficient upon mixing of different components (diffusion). Advantages: simple and cheap. Disadvantages: good for phase diffusion, like toluene diffusion in hexane, but not sensitive enough for molecules diffusion, particularly at low concentration.

4. <u>Single-molecule fluorescence based confocal microscopy:</u> increased sensitivity and resolution.

Case 1: imaging molecular rotation

Single Molecule Dynamics Studied by Polarization Modulation

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Polarized emission

- <u>Fixed polarization</u>: the angle of polarizer is fixed, so the excitation polarization is constant. Only the molecules with dipole parallel to the polarization can be excited, whereas the molecule with dipole perpendicular to the polarization cannot be excited. If a molecule is immobilized, the emission intensity will be somewhere between zero and the maximum (parallel). By measuring large number of molecules, one can reveal the distribution of molecule orientation. This cannot be done by the ensemble measurement, which gives only averaged result that does not depend on the polarization angle. Draw a scheme.

- <u>Modulated polarization</u>: the polarization angle changes with time (here milliseconds).
1. If the molecule is immobilized, the intensity will be modulated with the polarization following, $I = a\cos^2(x-b) + c$, where a is the maximum intensity, x is the polarization angle, b is the in plane dipole angle, is the background correction.
2. If the molecule is mobile --- rotating: the intensity will show a irregular behavior.
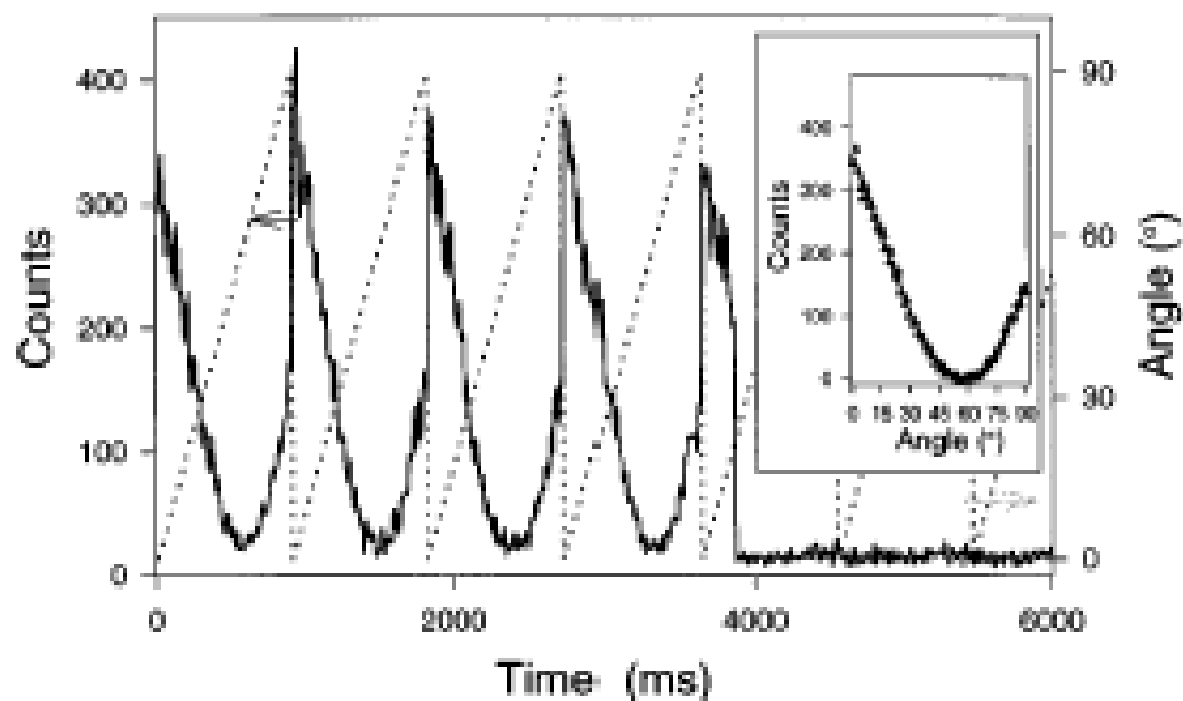
FIG. 1. Fluorescence time trace of a single TR molecule (solid line) and instantaneous excitation polarization angle (dotted line). Inset shows the averaged signal of the first four periods (filled triangles), and a fit to $a \cos^2(x - b) + c$ (solid gray line).

Fig 1

- From the fit, the in-plane dipole orientation can be extracted with an accuracy of 0.2 $^{\circ}$.

- In this case, the molecule used is DNA (20 base pairs)---C6 linker---fluorophore (Texas red or TMR). Out of 200 molecules investigated, 97% showed stationary dipoles.
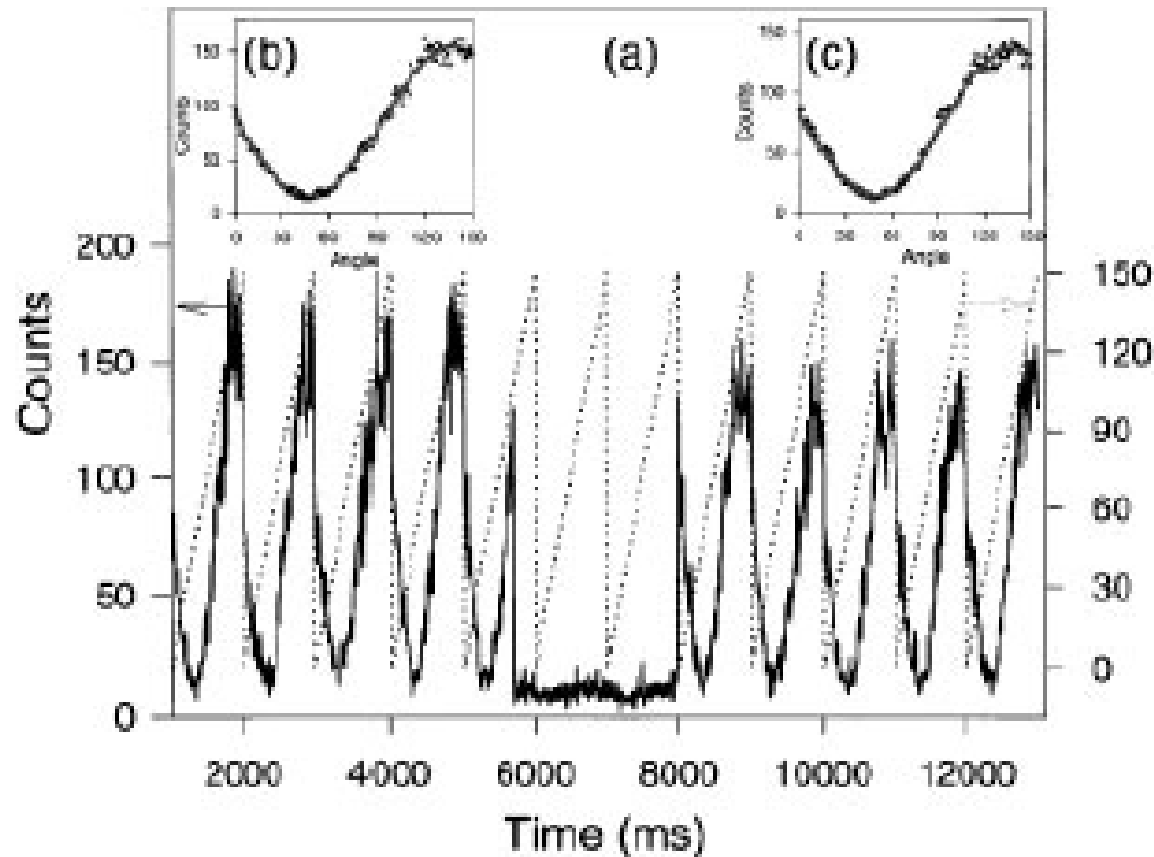
FIG. 2. (a) A transition to a dark state (2.3 s long) of a TMR molecule. The molecule photobleached after 51 s (not shown). The molecule maintained the same dipole orientation [from fits in insets (b) and (c)] throughout the transition to the dark state.

Fig 2

- A dark state was observed, indicating either a rotation or transition into a non-emitting state.

- From the fit, the in-plane dipole orientation before (-42.8 ^{o} ± 0.8 ^{o}) and after ((-41.3 ^{o} ± 0.8 ^{o}) the dark state are the same --- *the dark state is not due to rotation*.

Fig 3

red emission

Blue emission

dipole orientation

Intensity ratio:
Blue/Red
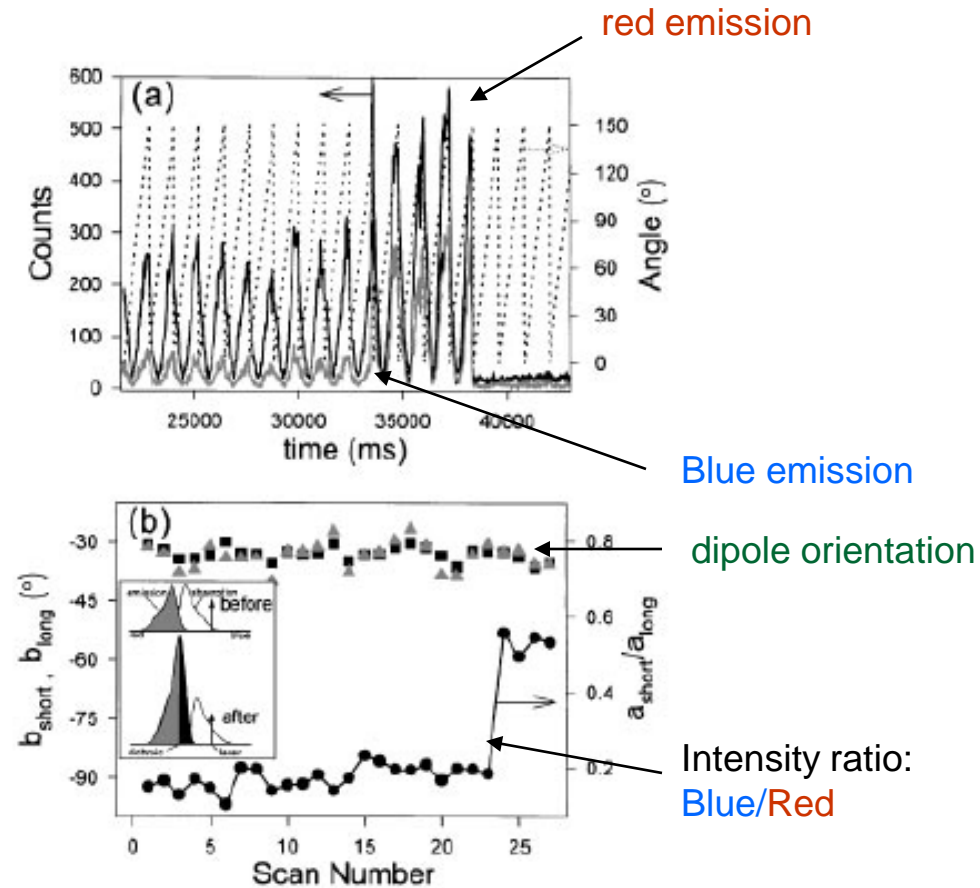
FIG. 3. (a) A spectral jump toward the blue of a TR molecule. Black line is the signal on the long-wavelength channel, and gray line is the signal on the short-wavelength channel. (Data from scan #1 to #14 are not shown.) (b) The data for each channel are fit for each polarization modulation period. The ratio a_{short}/a_{long} (solid black line, filled circles) and the phases of both signals, b_{long} and b_{short}, are plotted in black squares and gray triangles, respectively.

Fig 3

- A sudden increase intensity: due to spectral change (shift of absorption upon continuous irradiation) or molecule rotation?

- Spectral change: the dipole angle does not change.

- The constant dipole from the fitting suggests a spectral change.

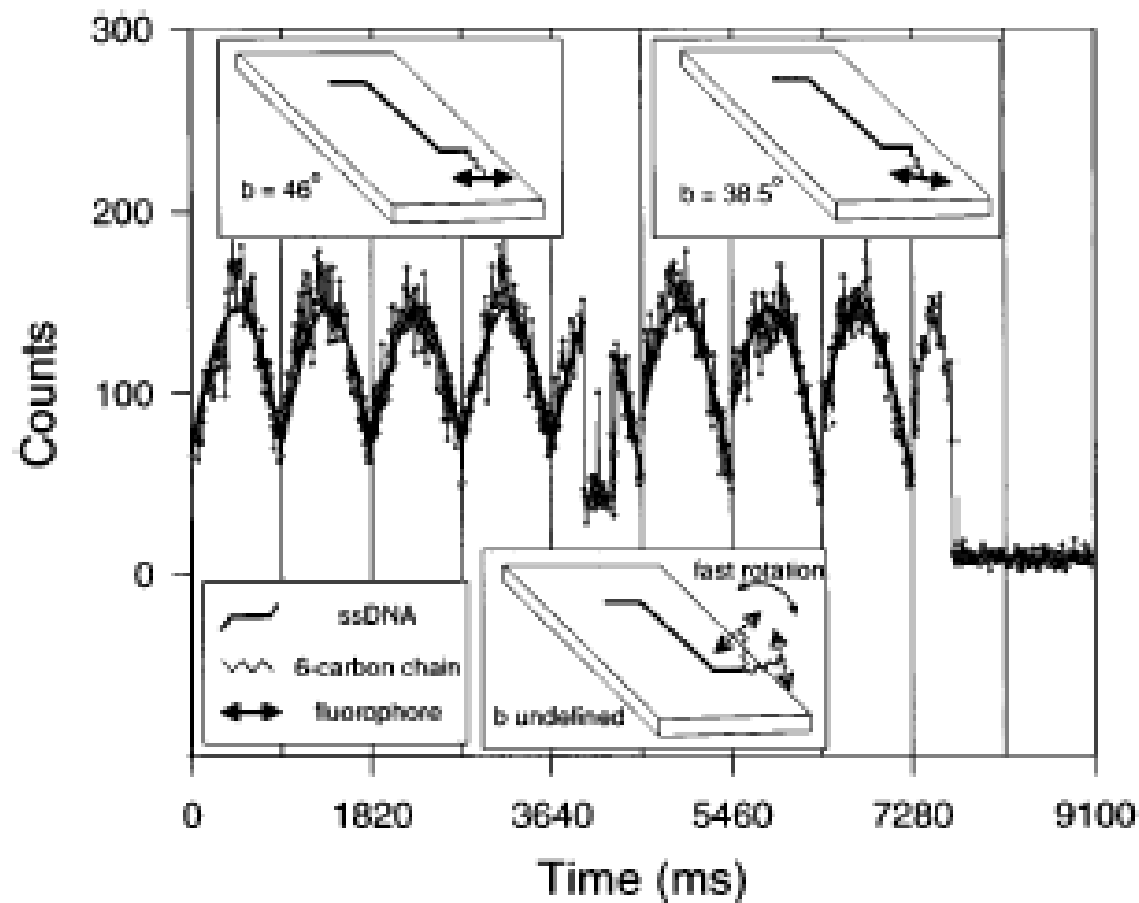- This is consistent with the increase in the intensity at short wavelengths.

Fig 4

FIG. 4. A 7.5° in-plane rotational jump of a TR molecule. Solid line is the fit to the data before and after the interruption. Vertical lines separate each modulation period from 0 to 90 degrees. Insets show a model for the rotational jump.

Fig 4

- 5 out of more than 200 molecules observed showed molecule rotation.

- Only phase changes, the maximum intensity does not change due to the full angle modulation of the polarization.

Single-molecule rotation imaged as movie clips in various time intervals
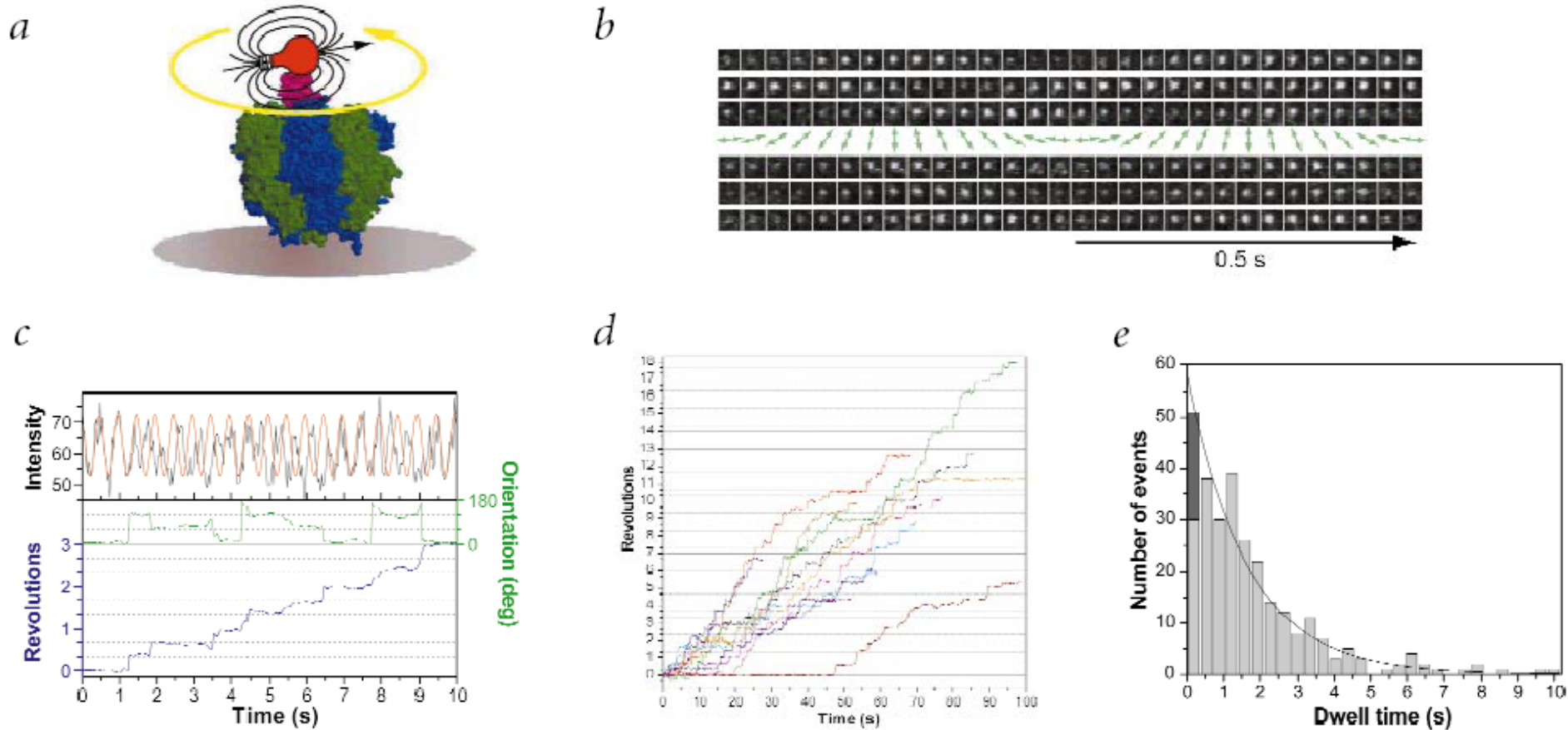
Fig. 4 Single molecule dipole rotation. **a**, A cartoon of the F_1-ATPase motor with a single fluorescent probe attached to its rotor. **b**, Two sequential fluorescence images (33 ms intervals) of single Cy3-F_1-ATPase molecules. The direction of the modulated excitation polarization is shown by green arrows. **c**, Time trajectories of the fluorescence intensity (black, top) and calculated fluorophore angle between 0° and 180° (green, bottom). The accumulated rotation angle (blue, bottom) was obtained by assuming that all steps were counterclockwise. **d**, Several time trajectories showing stepwise rotation of the rotor. Different lines represent trajectories of different fluorophores. **e**, Distribution of dwell times between steps. (Figure modified from ref. 48 with permission.)

Adachi, K. *et al. Proc. Natl. Acad. Sci. USA* **97**, 7243–7247 (2000).

Case 2: the first report on single-molecule diffusion

Imaging of single molecule diffusion

(fluorescence microscopy/single dye detection/time-resolved imaging/quantal fluorescence/lipid bilayers)

TH. SCHMIDT, G. J. SCHÜTZ, W. BAUMGARTNER, H. J. GRUBER, AND H. SCHINDLER

Institute for Biophysics, University of Linz, 4040 Linz, Austria
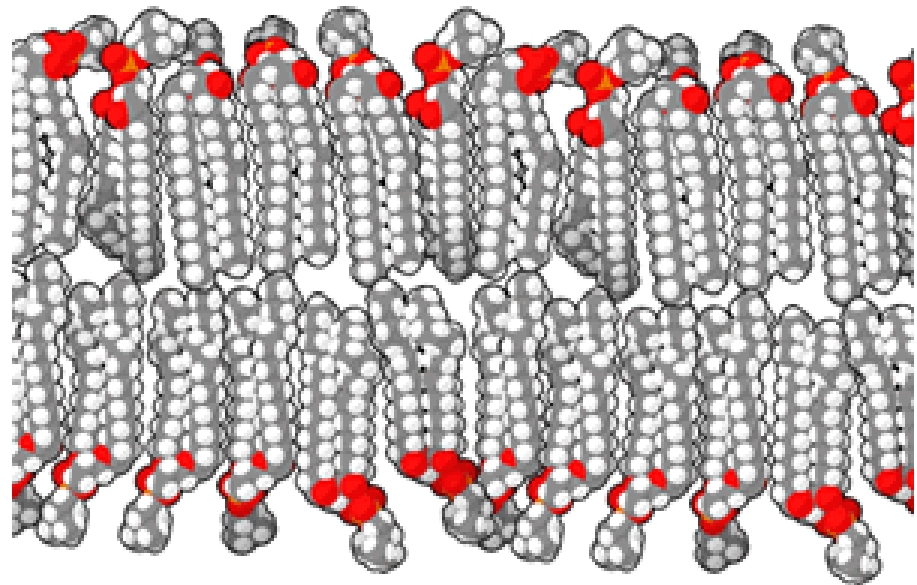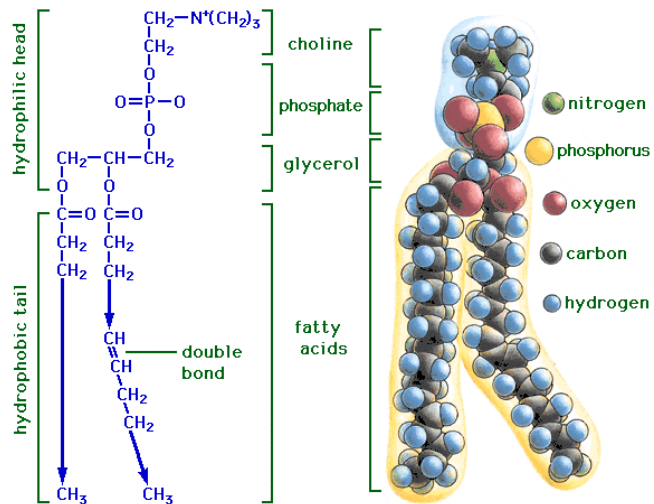
Experimental details

- Fluorescence labeled lipid embedded in fluid lipid membranes.

- Detector: LN cooled CCD.

- Objective: Zeiss, 100X, NA 1.3.

- S/N was obtained at 28, and the positional accuracy for molecule is achieved at 30 nm, far below the diffraction limit of optical microscopes.
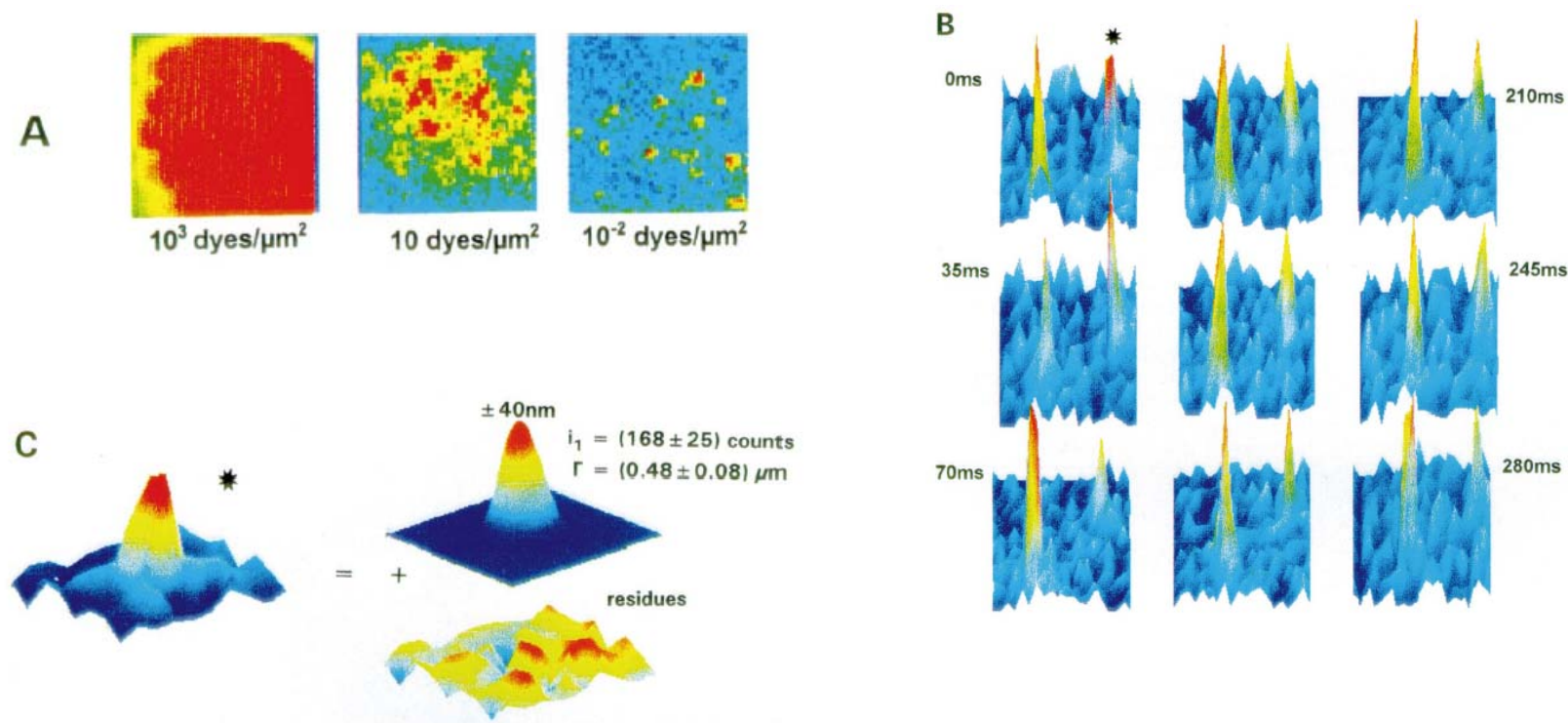
FIG. 1. Images of fluorescence-labeled lipids in a lipid membrane. The samples were illuminated for 5 ms with a Gaussian-shaped laser beam of 6.1 μm width and 57 kW/cm^2 mean excitation intensity. (A) Fluorescence images for three surface densities of labeled lipid. The images show a membrane area of 6.8 $\times$ 6.8 μm^2. Color scaling (blue = 0; red = 60 counts) is identical for the three images presented. Image intensity at 10^3 dyes/μm^2 was divided by 60. (B) Sequence of nine images of two labeled lipids observed at lowest surface density in a 5.4 $\times$ 5.4 μm^2 membrane area, taken every 35 ms. (C) Subimage of a 2.4 $\times$ 2.4 μm^2 membrane area showing the peak marked by * in B. The nonlinear least-squares fit of a two-dimensional Gaussian profile (18) to the peak yielded the indicated values for fluorescence intensity $\bar{i}_1$, width and two-dimensional position with their respective confidence limits (see *Materials and Methods*). Residues of the fit compare with background noise.

Fig 1

- A mean value of (172±15) counts per dye molecule was found for six dye densities covering the range from 10 to 10^3 dyes/μm^2 --- indicating the well isolated distribution of fluorescent molecule within the lipid membranes.
- Images at this density were characterized by large fluctuations of the intensity as shown in Fig. 1 *A.*
- The sequence was obtained from consecutive illuminations of the same membrane area lasting 5 ms at 35-ms intervals.
- The two molecules undergo lateral motion (Brownian motion), with position uncertainty of 30 nm, 7 times smaller than the diffraction limit, here ~ 300 nm.
- This position accuracy allows for monitoring of the molecule movement within the time interval of 30 ms.
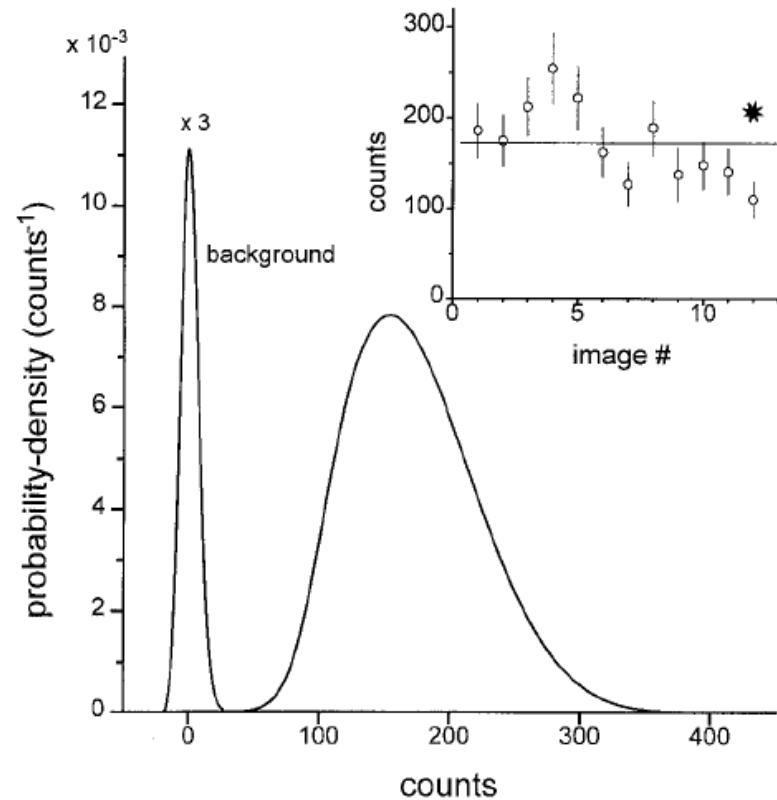
FIG. 2. Unitary fluorescence intensity of single fluorescence-labeled lipid molecules. The probability-density distribution of single molecule fluorescence intensity is shown. The probability-density distribution is calculated from data shown in the *Inset* as a superposition of normalized Gaussians, each entering at i_1 with a width given by the error bar. For comparison, the probability-density distribution of the background noise is shown. (*Inset*) Fluorescence intensity of the molecule marked by $*$ in Fig. 1B as it progressed throughout the image sequence as determined by Gaussian fits (Fig. 1C).

Fig 2

- Statistics based on repeated measurements over one molecules: averaged intensity is 154 counts, as shown in the figure.

- Statistics based on measurements over large number of different molecules: averaged intensity is 172 counts.

- The consistence indicates that the observed emission spots (peaks) is due to isolated molecules.

-
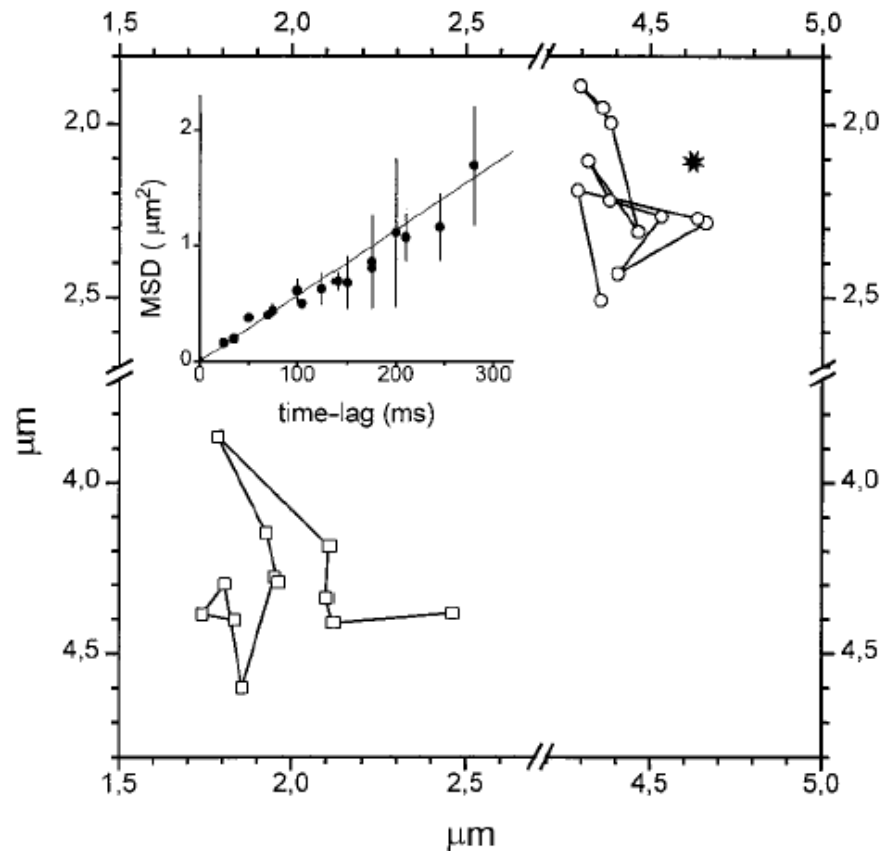
$$<r^2> = 6 \, D \, t + C$$

D: diffusion coefficient
t: time
C: constant

FIG. 3. Diffusion of single lipid molecules. Trajectories of the two-dimensional diffusion for the two lipid molecules in Fig. 1B determined from 12 successive images. (*Inset*) MSD versus time lag calculated for 531 trajectories. Observed displacements are consistent with two-dimensional Brownian diffusion of the lipids, since the data follow a linear relationship (solid line) well within their variance.

Fig 3

- Analysis of trajectories of 531 individual molecules showed that the direction of diffusion steps of simultaneously observed molecules were uncorrelated.

- The mean-square displacement (MSD), and the time lag (t_{lag}) yielded the expected linear relationship as shown in Fig. 3 (*Inset*). The lateral diffusion constant D_{lat} was estimated as 1.42 ± 0.13 x 10^{-8} cm^2/s.
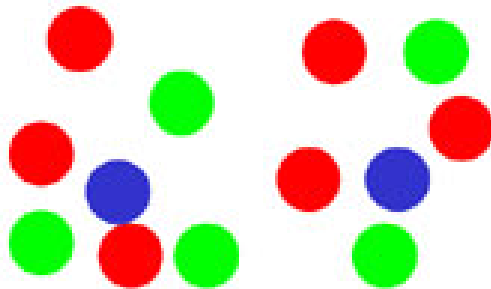
Part 2:
Imaging Protein dynamics *via*

fluorescence resonance energy transfer (FRET)

where physics and biology meet, DNA and RNA dance, enzymes wiggle and flutter, and dye molecules FRET *and ... die!*

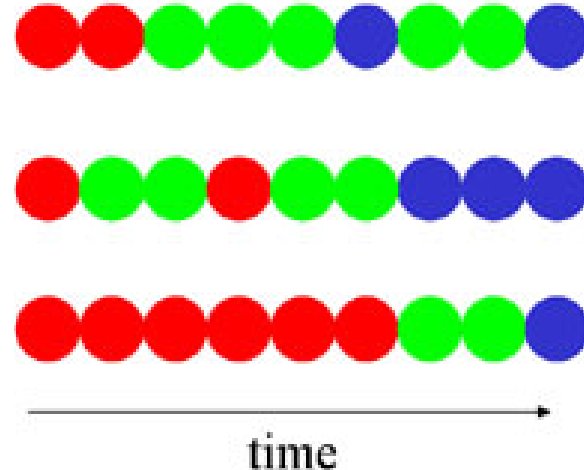# Two advantages of single-molecule imaging for biological systems

Distribution
-useful if population is heterogeneous.

Time trajectory
-useful if the dynamics is not synchronizable

time

T. Ha @ UIUC

Single molecule imaging/spectroscopy of biological systems

- **Heterogeneous:** most (if not all) biological systems are heterogeneous.

   Single molecule imaging/spectroscopy reveals distribution of molecular properties (like the colors of the balls), by looking at one molecule at a time and tabulate the result (similar to taking a census).

- **Dynamic:** most (if not all) biological systems are dynamic, keeping change in shape, conformation, composition, or position.

   Single molecule imaging/spectroscopy measures the entire time trajectories, which include multiple pathways and intermediates involved. See the last slide.

- Though elegant biochemical and structural methods are available, they don't provide with the real time measurements of conformational changes.

Typical fluorescence properties used for single molecule probing and imaging

- Fluorescence depends on local polarity --- for both wavelength and quantum yield (intensity). This is normally used for single fluorophore labeling. Fluorescence intensity fluctuation reflects conformational changes of the host system (like a protein or membrane). *Draw scheme (sigmoidal plot for intensity and spectral shift). Also see a slide next.*

- Fluorescence polarization --- revealing rotation and diffusion of molecules (see last lecture), which in turn reflecting the conformation changes of the host systems.

- **Fluorescence resonance energy transfer (FRET)** --- revealing dynamic conformational changes involving two domains, where the fluorophores are labeled. This is unique for probing the coincident structural change within proteins or other large biological systems. a typical example is the signal transducing membrane proteins functional in neuron cells, where upon a ligand binding the membrane protein experience great structure change; some domains may transform across the membrane. Such a large change can be revealed by labeling two dyes at the active domains. Draw a scheme for a later slide.

# SMS measurement based on intensity fluctuation and wavelength shift
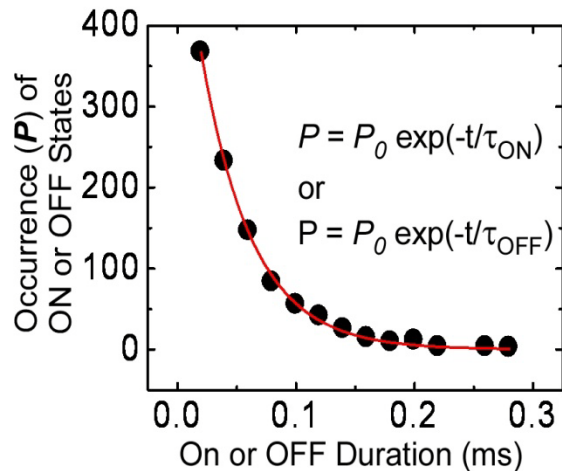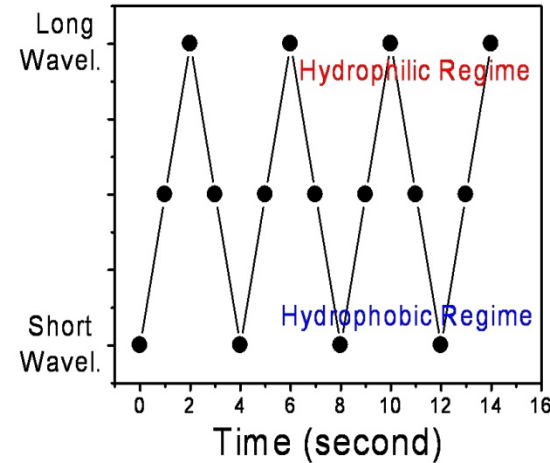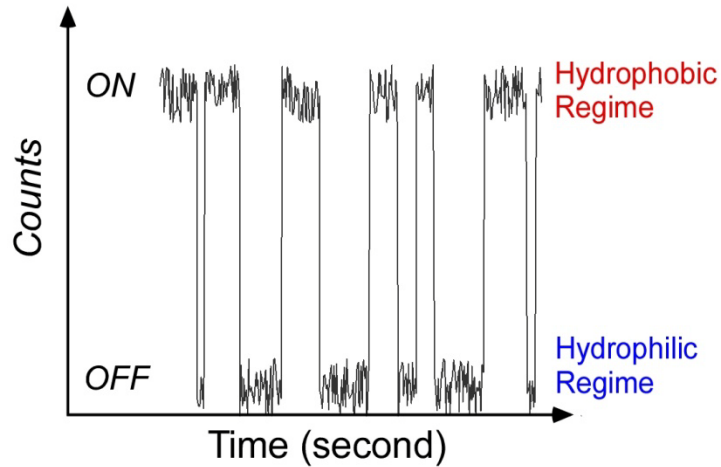
1. The shorter τ_{ON} or τ_{OFF}, the faster the conformation change, i.e. the higher the flexibility.

2. If τ_{ON} and τ_{OFF} are comparable, it means the labeled position has equal accessibility to hydrophilic (water) and hydrophobic (inside protein) phase. This is normally the case for an opened α-helix.

3. If $\tau_{ON} \gg \tau_{OFF}$, it implies that the labeled position is tightly wrapped inside the bundles.

$P = P_0 \exp(-t/\tau_{ON})$

or

$P = P_0 \exp(-t/\tau_{OFF})$

(*top panel*) Fluorescence fluctuation monitored with photon counting (left) and spectra recording (right). (*bottom panel*) A scheme showing the basic method to get the statistic mean value of ON and OFF times.

What is FRET

- FRET is a distance-dependent interaction between the excited state of the donor molecule and the ground state of the acceptor molecule, in which the excitation is transferred from the donor to an acceptor.
- The efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation,

- The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by FRET) is defined by the Förster radius (Ro).
- The magnitude of Ro is dependent on the spectral properties of the donor and acceptor dyes

- Donor and acceptor molecules must be in close proximity (typically 10–100 Å).
- The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor (see **Figure**).
- Donor and acceptor **transition dipole** orientations must be approximately parallel.
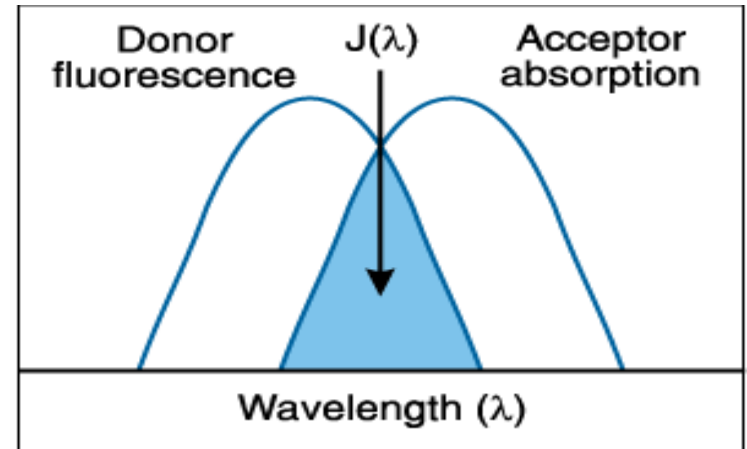
$$R_O = [8.8 \times 10^{23} \cdot \kappa^2 \cdot n^{-4} \cdot QY_D \cdot J(\lambda)]^{1/6} \text{ Å}$$

where κ^2 = dipole orientation factor (range 0 to 4; $\kappa^2 = 2/3$ for randomly oriented donors and acceptors)

QY_D = fluorescence quantum yield of the donor in the absence of the acceptor
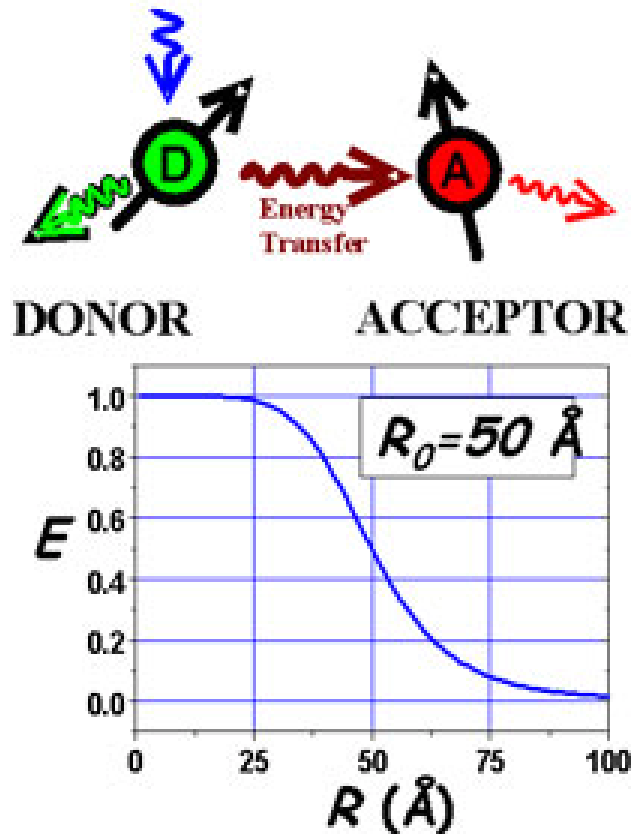
n = refractive index

$J(\lambda)$ = spectral overlap integral (see figure)

$$= \int \epsilon_A(\lambda) \cdot F_D(\lambda) \cdot \lambda^4 d\lambda \text{ cm}^3 \text{M}^{-1}$$

where ϵ_A = extinction coefficient of acceptor

F_D = fluorescence emission intensity of donor as a fraction of the total integrated intensity

What is FRET

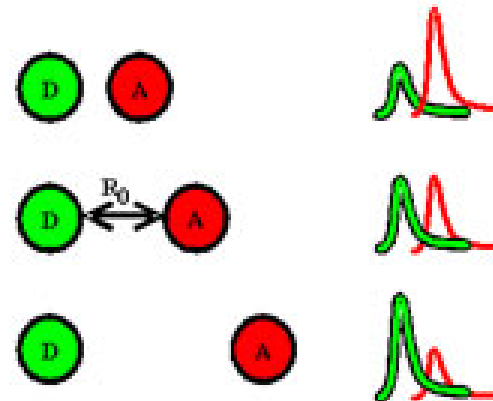Energy Transfer Efficiency

$$E = \frac{1}{1 + (R/R_0)^6}$$

R_0=50% transfer efficiency distance
3nm~7nm

"Spectroscopic Ruler"

R_0=50 Å

Determines changes in distance (conformation) rather than absolute distances as 'E' depends on orientation of the dyes. FRET is particularly useful for biological systems. most of single-molecule-FRET studies have been performed on bio-molecules.

T. Ha @ UIUC

Selected Applications of FRET bio-research

- Structure and conformation of proteins
- Spatial distribution and assembly of protein complexes
- Receptor/ligand interactions
- Immunoassays
- Probing interactions of single molecules
- Structure and conformation of nucleic acids
- Real-time PCR assays and SNP detection
- Detection of nucleic acid hybridization
- Primer-extension assays for detecting mutations
- Automated DNA sequencing
- Distribution and transport of lipids
- Membrane fusion assays
- Membrane potential sensing
- Fluorogenic protease substrates
- Indicators for cyclic AMP and zinc

Invitrogen, Molecular Probes

History of Single-molecule bio-imaging

- Ha *et al* - First FRET in single molecules between single donor and acceptor under non-physiologic conditions (dry glass surface).

  Ha, T. *et al. Proc. Natl. Acad. Sci. USA* **93**, 6264–6268 (1996).

- Deniz *et al* – Single molecule FRET and predicted distance.

  Deniz, A.A. *et al. Proc. Natl. Acad. Sci. USA.* **97**, 5179–5184 (2000).

- Brasselet *et al* – GFP's in single-molecule-FRET for studying calmodulin.

  Brasselet, S., Peterman, E.J.G., Miyawaki, A. & Moerner, W.E. *J. Phys. Chem. B* **104**, 3676–3682 (2000).

- Sako *et al* – extended it to invivo system and used it for studying epi'dermal growth factor dimerisation.

  Sako, Y., Minoguchi, S. & Yanagida, T. *Nature Cell Biol.* **2**, 168–172 (2000).

Dyes Used

- **Green fluorescent protein (GFP) and siblings** (RFP, CFP, YFP, BFP) – unique and amenable for mutation within the host proteins without disturb the protein structure or functions. But suffers from limited sensitivity, low stability against photobleaching, and time consuming for sample assembly.

- Cysteine reactive dyes --- selectively reactive to cysteine moiety, which can be selectively mutated inside the proteins.

- Amine reactive dyes --- reactive to amine groups at some amino acids, but the selectivity is not as good as the Cysteine reactive dyes, since there several amino acids have amine groups.

- Membrane permeant fluorescein and rhodamine

- Cyanine Dyes (Cy3, Cy5, Cy7) – well separated emission maxima Cy3-Cy5 pair widely used for cellular imaging.

- Semiconductor nanocrystals (Q-dots) --- CdSe, CdTe or the core-shell nanocomposites. Good photostability, narrow emission band, tunable emission wavelength. But suffering from surface modification to get water soluble, and the potential poisonous.

The Nobel Prize in Chemistry 2008

"for the discovery and development of the green fluorescent protein, GFP"

Photo: U. Montan

Photo: U. Montan

Photo: U. Montan

Osamu Shimomura

1/3 of the prize

USA

Marine Biological Laboratory (MBL)
Woods Hole, MA, USA; Boston
University Medical School
Massachusetts, MA, USA

b. 1928
(in Kyoto, Japan)

Martin Chalfie

1/3 of the prize

USA

Columbia University
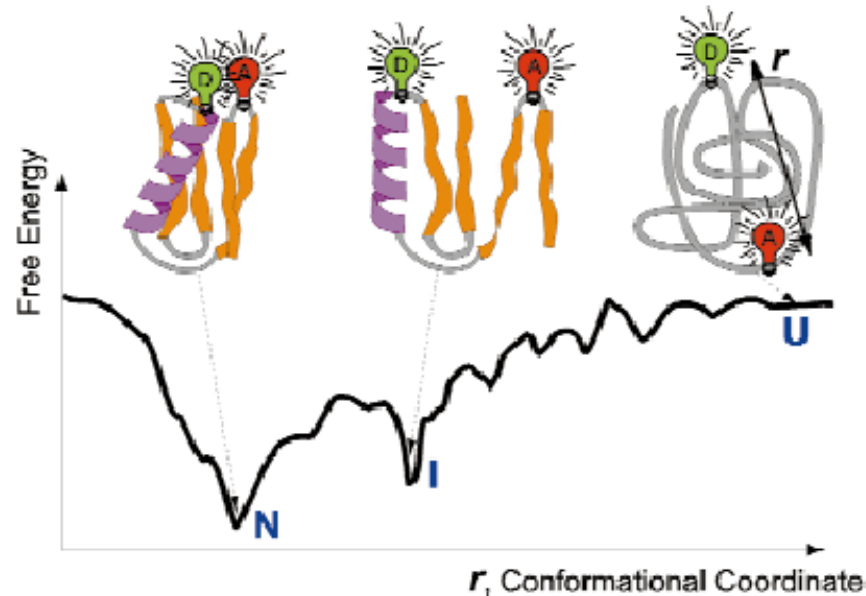New York, NY, USA

b. 1947

Roger Y. Tsien

1/3 of the prize

USA

University of California
San Diego, CA, USA; Howard Hughes
Medical Institute

b. 1952

FRET efficiency correlates with the Protein folding states

Fig. 1 Energy landscape for the folding reaction. A cartoon of free energy as a function of distance r between the fluorescence donor and acceptor. The rugged energy landscape has a funnel shape. U, the unfolded state; I, an intermediate state; N, the folded state.

A dynamic gauge for protein folding processes.

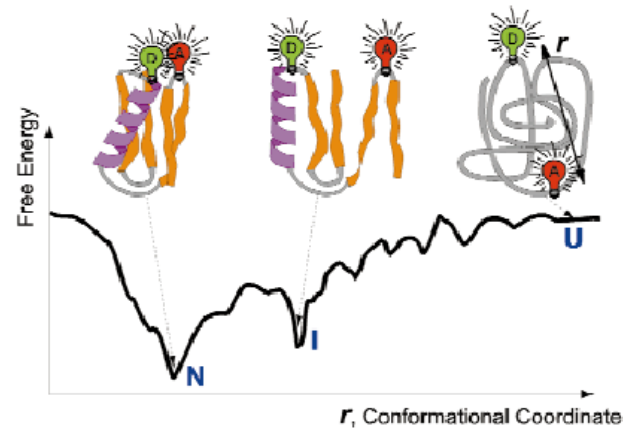# FRET efficiency correlates with the Protein folding states

Fig. 1 Energy landscape for the folding reaction. A cartoon of free energy as a function of distance r between the fluorescence donor and acceptor. The rugged energy landscape has a funnel shape. U, the unfolded state; I, an intermediate state; N, the folded state.

- Many methodologies have been developed and applied to protein folding, but most conducted on an ensemble of proteins.
- Since it is very difficult to synchronize the folding reactions over an ensemble of molecules, these measurements usually fail to resolve detailed dynamic information.
- SMS, and in particular single-molecule-FRET, can provide **distance** information between a pair of points on a polypeptide chain as folding progresses — providing a reaction coordinate that affords a global view of the conformational distributions, intermediates and dynamics as the protein tumbles down the free energy folding funnel.

Single-molecule-FRET histograms of wild type CI2 at 3, 4 and 6 M denaturant concentrations

Shimon Weiss lab
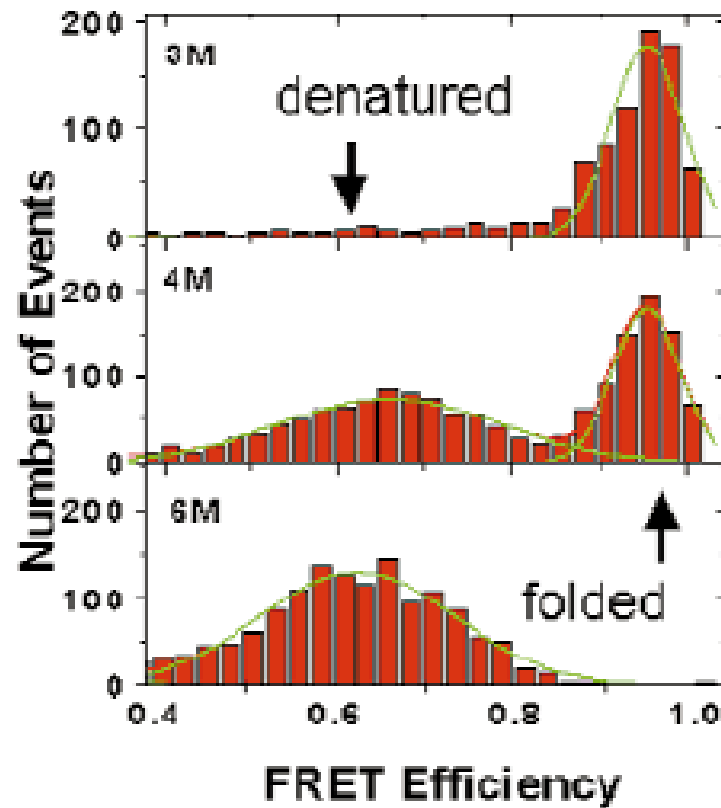
Immobilization of Bio-Systems

- Why immobilization? – Observing conformational changes can be impeded by molecule diffusion in solution (see last lecture). To quantitatively measure the changes in the conformation, immobilization is required.

- Nonspecific immobilization:
  - DNA or proteins attached to charged surface like aminopropylsilane coated surface via electrostatic interaction. This is a easy and universal technique.
  - Trapping molecules in pores of agarose gels. Agarose is a widely used support material in molecular biology. Agarose gels have large "pore" size, which would be just appropriate for hosting and immobilizing proteins (MW 50 – 150 kDa), and on the other hand, there is still enough free room for the proteins to keep the dynamic activity.

- Specific immobilization
  - Using a biotin – streptavidin tether to the surface.
  - Histidine tags for recombinant proteins immobilized on Ni – NTA coated surface.

Control of molecular rotation for FRET measurement

- Since the diffusion is quite limited due to the binding to the host, most control is for molecular rotation, which determines the fluorescence polarization. Also the FRET efficiency depends on the relative orientation between donor and acceptor --- parallel gives the highest efficiency.

- Check for anisotropy in solution at ensemble levels. If the anisotropy is low, it means that the dye molecule interacts weakly with the host molecule (large flexibility for rotation) --- this assumes the host motion (rotation) is slower than the lifetime of fluorescence.

- In case the molecular rotation is significant, another polarization control should be done at single molecule level with immobilization of proteins on surface. This measures the rotation rate --- if the rotation is far slower or faster than the FRET process, the signal can be resolved for FRET. But if the two rates are close, it would be a problem for FRET measurement.

FRET in living cells
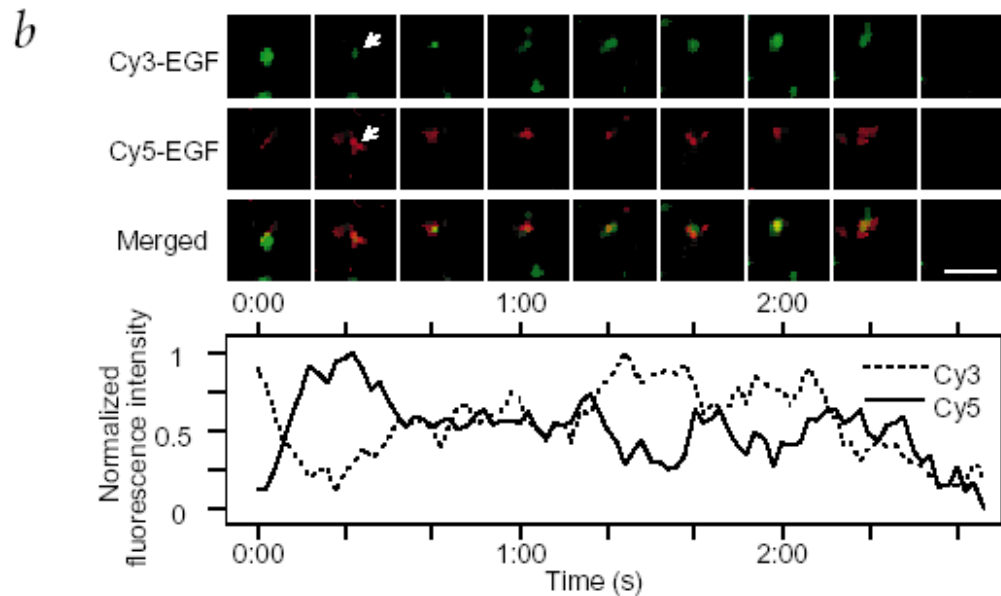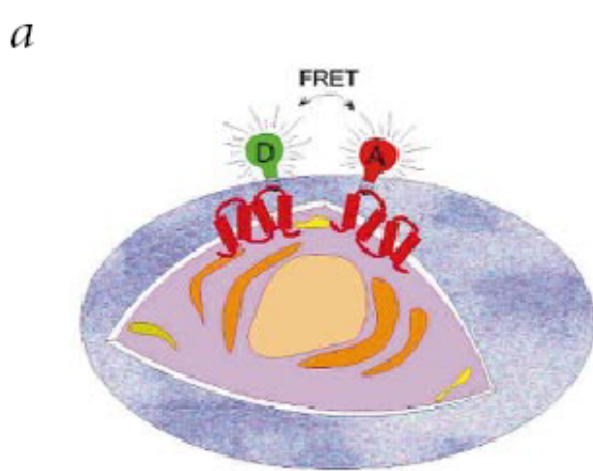
Fig. 5 Single pair FRET in a living cell. *a*, A cartoon of spFRET between two epidermal growth factor receptors in the membrane of a live cell (a mixture of donor-labeled and acceptor-labeled extracellular ligands was used to label the receptors). *b*, Top three rows: video frames showing the change in fluorescence intensity over time of a donor-labeled receptor (green) interacting with an acceptor-labeled receptor (red) and an overlay of these two signals (third row). Bottom panel: donor (dashed line) and acceptor (solid line) time trajectories extracted from the video data. The two trajectories are anticorrelated as expected for spFRET. (Figure prepared modified from ref. 51 with permission.)

Sako, Y., Minoguchi, S. & Yanagida, T. *Nature Cell Biol.* **2**, 168–172 (2000).
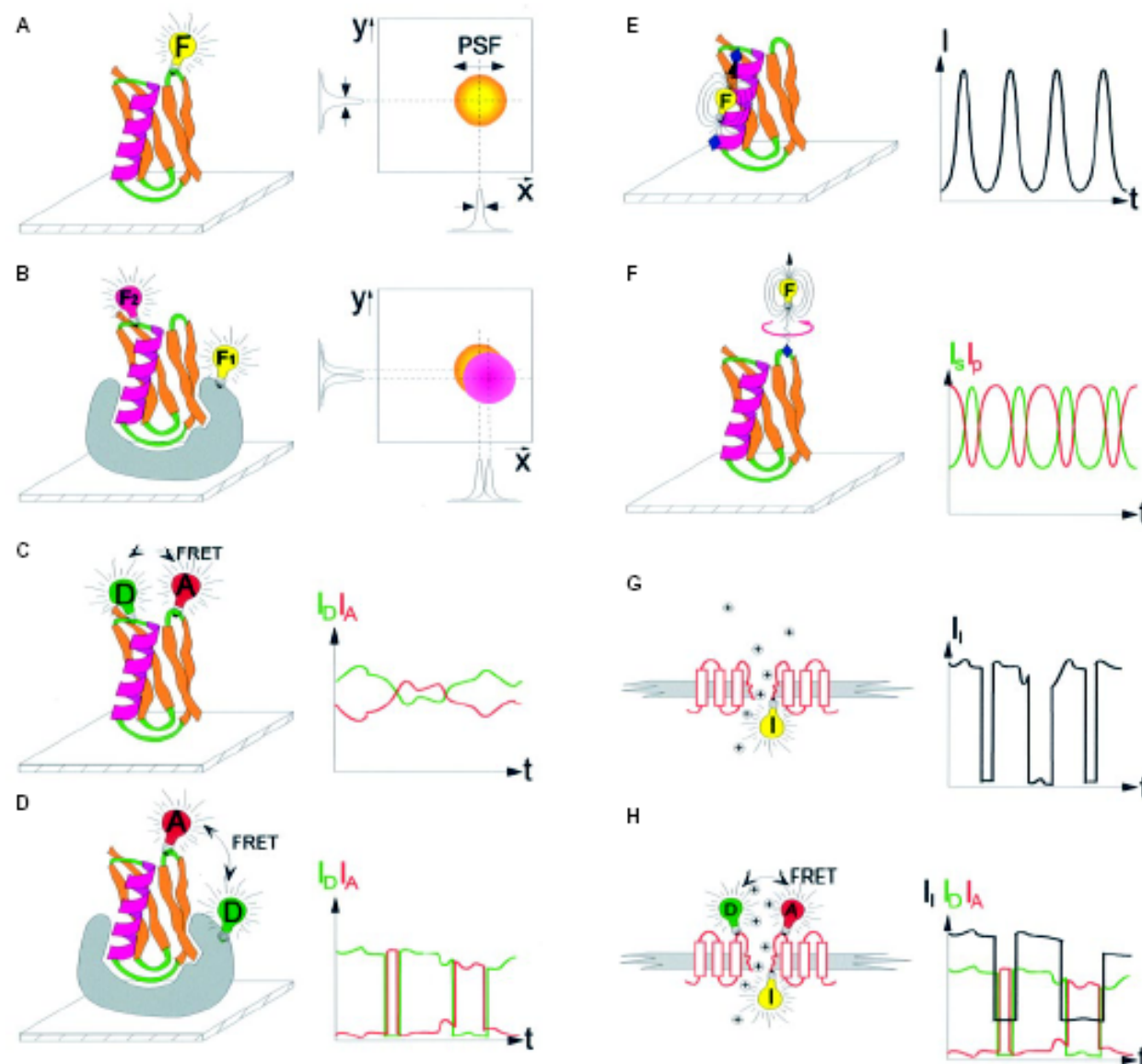
Fig. 1. Labeling schemes (left) and physical observables (right). (A) Localization of a macromolecule labeled with a single fluorophore F with nanometer accuracy. The point-spread-function (PSF) can be localized within a few tenths of a nanometer. (B) Colocalization of two macromolecules labeled with two noninteracting fluorophores, F_1 and F_2. Their distance can be measured by subtracting the center positions of the two PSFs. (C) Intramolecular detection of conformational changes by spFRET. D and A are donor and acceptor; I_D and I_A are donor and acceptor emission intensities; t is time. (D) Dynamic colocalization and detection of association or dissociation by intermolecular spFRET. Donor and acceptor intensities are anticorrelated both in (C) and (D). (E) The orientation of a single immobilized dipole can be determined by modulating the excitation polarization. The fluorescence emission follows the angle modulation. (F) The orientational freedom of motion of a tethered fluorophore can be measured by modulating the excitation polarization and analyzing the emission at orthogonal s and p polarization detectors. I_S and I_P are emission intensities of s and p detectors. (G) Ion channel labeled with a fluorescence indicator I. Fluctuations in its intensity I_I report on local ion concentration changes. (H) Combination of (C) and (G). D and A report on conformational changes whereas I reports on ion flux.

Fig. 5. A cartoon illustrating (A) intramolecular and (B) intermolecular spFRET nuclease-DNA interactions. Intramolecular spFRET measures conformational dynamics of the enzyme during catalysis. Intermolecular spFRET measures association, catalysis, and dissociation of substrate molecules. Multiple acceptors at equal distances on the DNA act as a "ruler." R_o is the Förster radius (distance at which 50% of the energy is transferred). This scheme can be generalized to many other protein-DNA interactions.
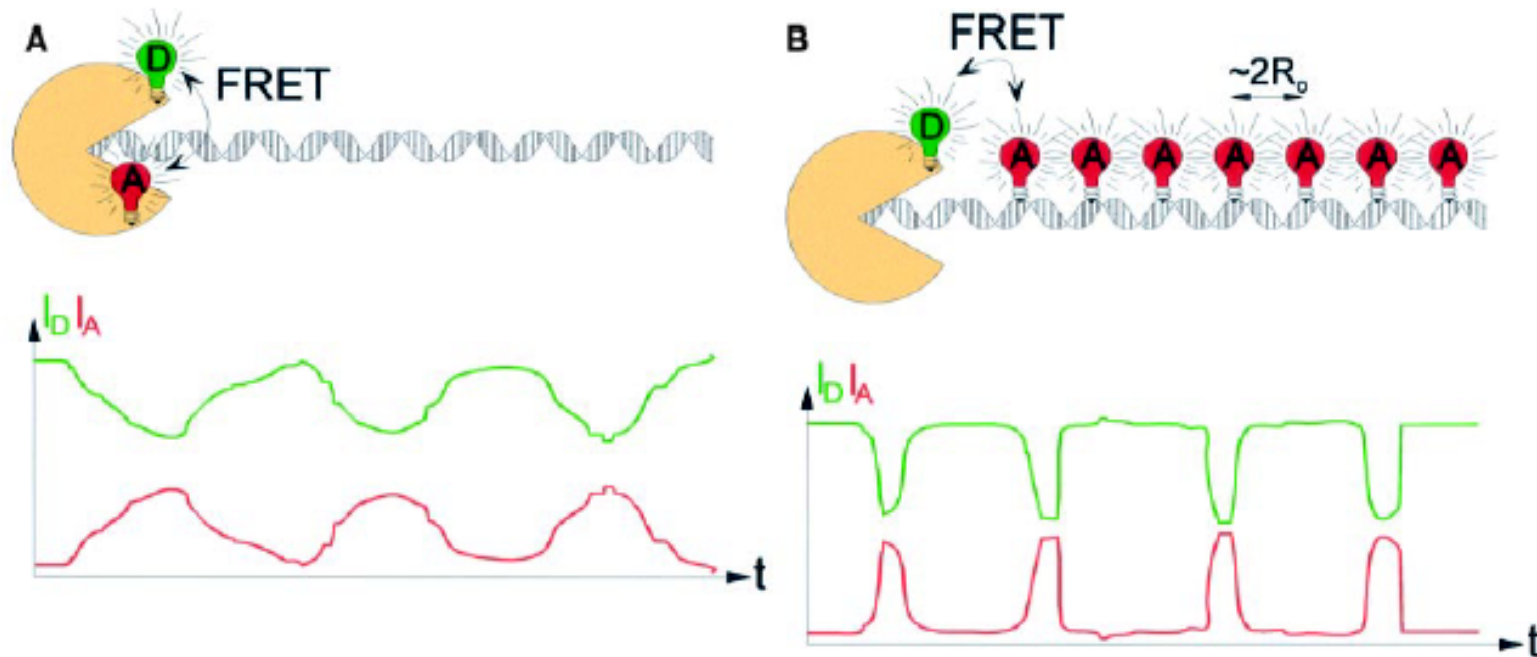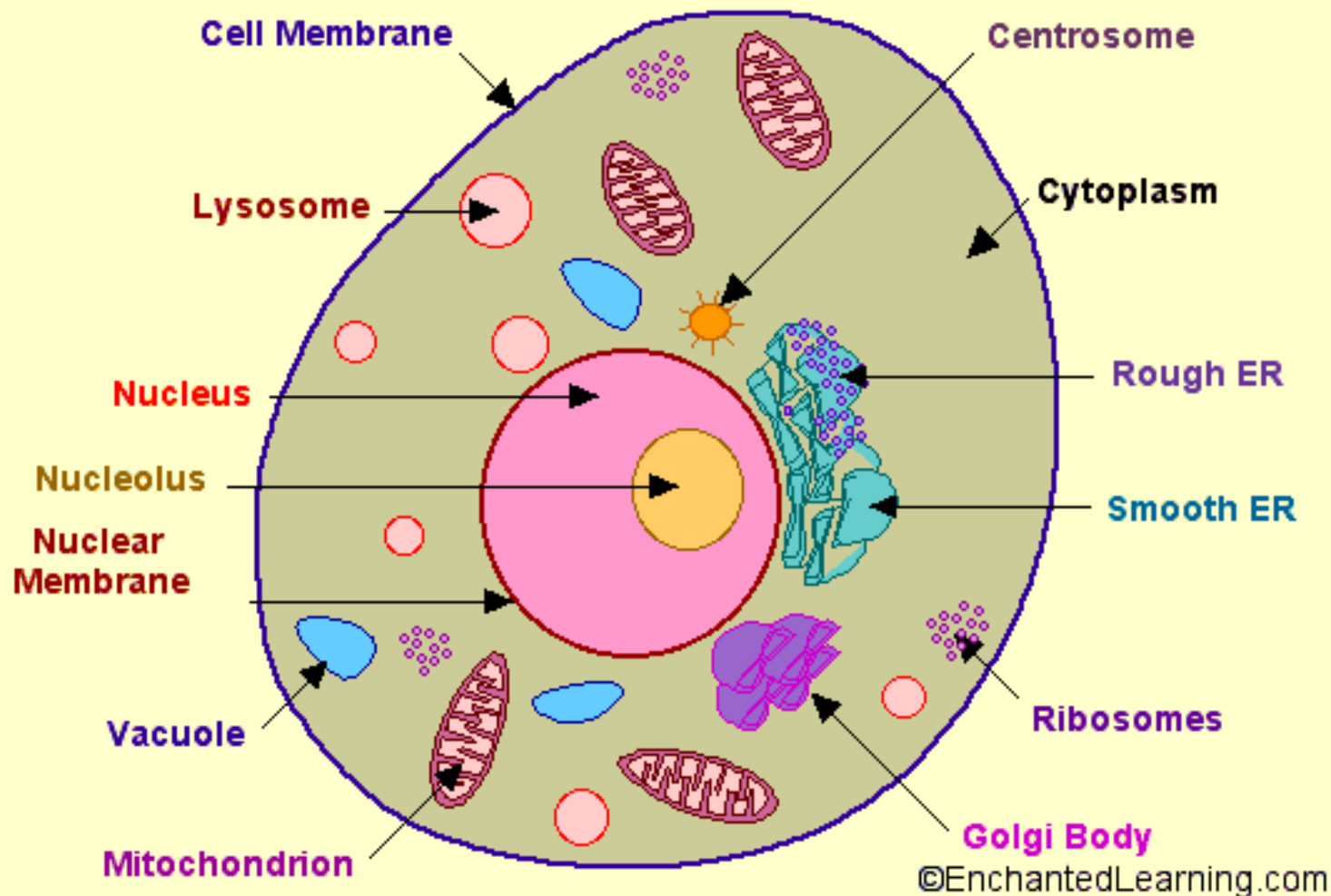
Part 3:
Imaging virus diffusion and attacking to living cell

Learn from the bad thing;
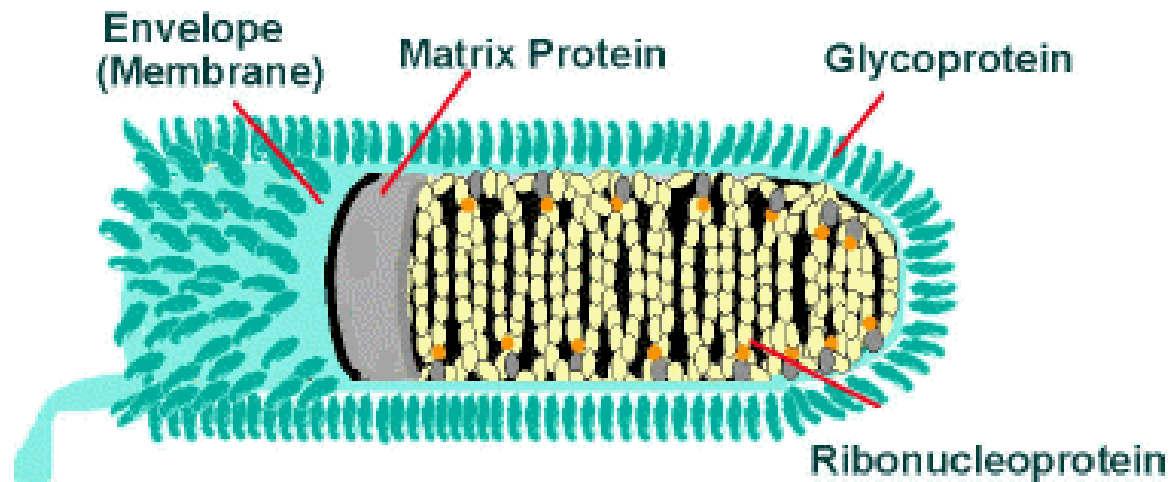*Turn it into something good.*

Basics of virus

- **Definition:** A **virus** is a small particle that infects cells in biological organisms. Viruses are obligate intracellular parasites; they can only reproduce by invading and taking over other cells as they lack the cellular machinery for self reproduction.

- **Structure:** virus carry a small amount of nucleic acid (either DNA or RNA) surrounded by some form of protective coat consisting of proteins, lipids, and glycoproteins. Importantly viral genomes code not only for the proteins needed to package its genetic material, but for proteins needed by the virus during lysogenic and lytic cycles, the reproductive cycles.

- **Non-living or alive:** A virus makes use of existing enzymes and other molecules of a host cell to create more virus particles. Viruses are neither unicellular nor multicellular organisms; they are somewhere between being living and non-living. Viruses have genes and show inheritance, but are reliant on host cells to produce new generations of viruses.

- **A supra-molecule?** Many viruses have similarities to complex molecules. Like DNA, viruses undergo molecular replication and they can often be crystallized.

- **Size:** The viral capsid may be either spherical or helical and is composed of proteins encoded by the viral genome. Helical virus: 1 μm to a few μm; spherical virus: 20 – 400 nm. Hard to see by optical microscope, but observable by confocal microscope based on fluorescence labeling.

Cross-Section of an Animal Cell

Cell Membrane

Centrosome

Lysosome

Cytoplasm

Rough ER

Nucleus

Nucleolus

Smooth ER

Nuclear
Membrane

Vacuole

Ribosomes

Mitochondrion
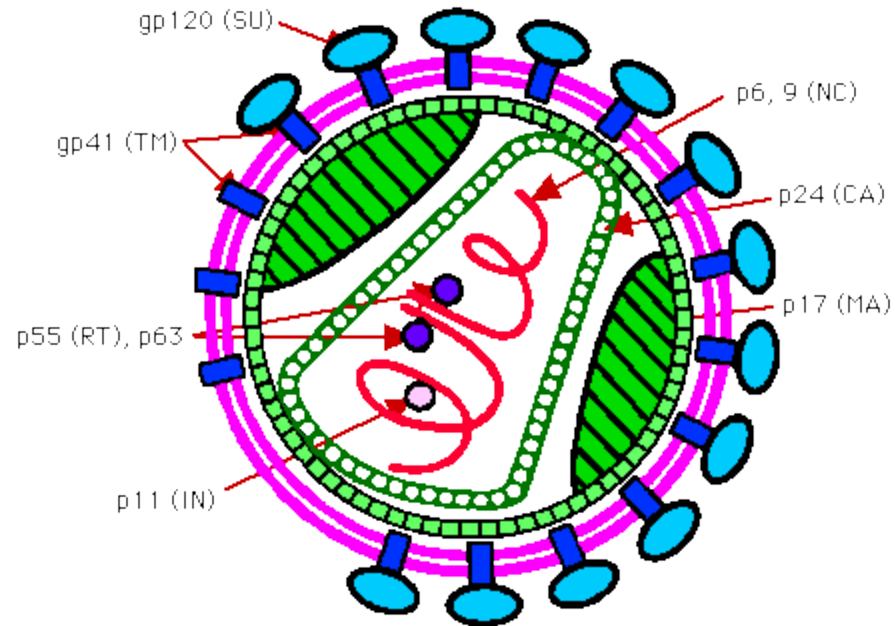
Golgi Body

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Helical virus

Rabies virions are bullet-shaped with 10-nm spike-like glycoprotein peplomers covering the surface. The ribonucleoprotein is composed of RNA encased in nucleoprotein, phosphorylated or phosphoprotein, and polymerase.
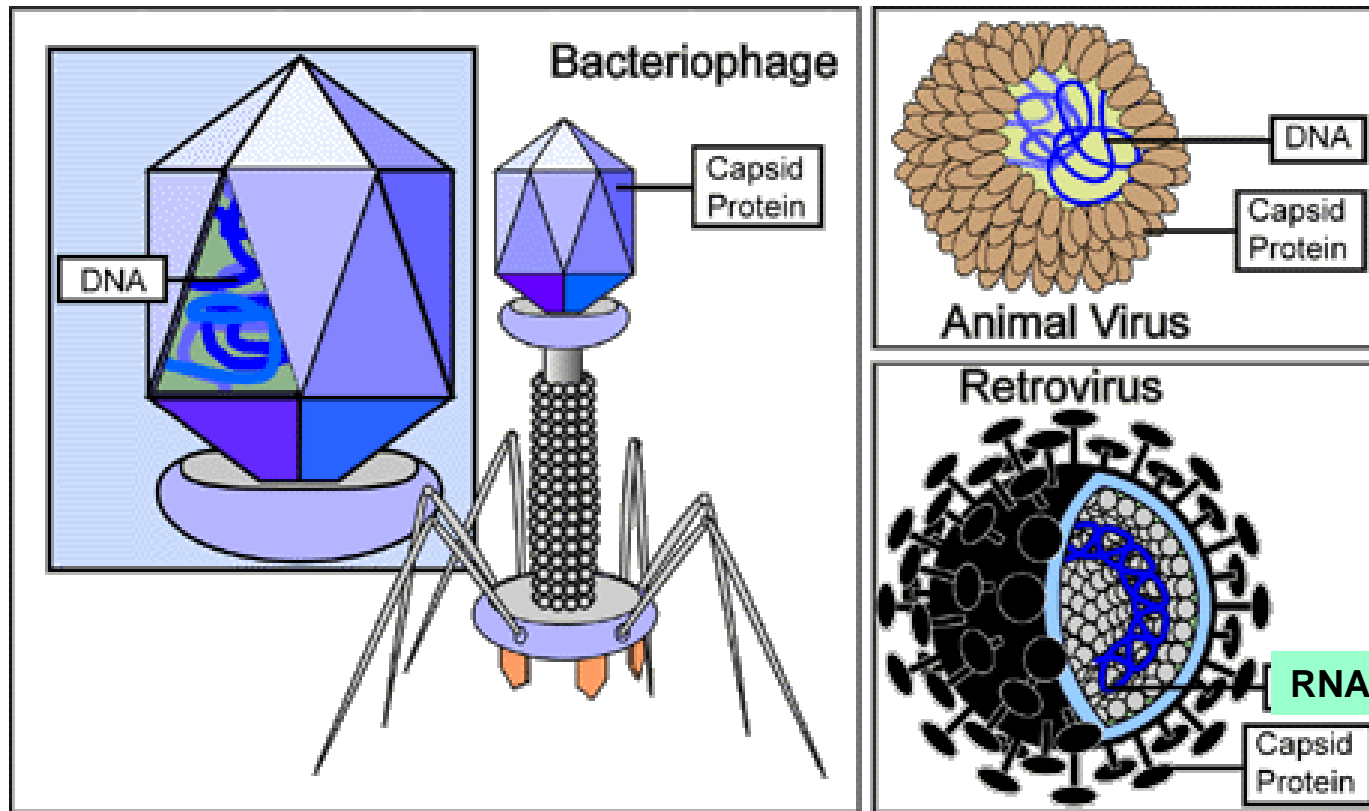
Spherical virus

Schematic representation of the structure of HIV:

Three types of virus

Three types of viruses: a bacterial virus, otherwise called a bacteriophage (left center); an animal virus (top right); and a retrovirus (bottom right). Viruses depend on the host cell that they infect to reproduce. When found outside of a host cell, viruses consist of genomic nucleic acid, either DNA or RNA (depicted as blue), surrounded by a protein coat, or capsid, with or without a glycoprotein envelope. Retroviruses contain RNA and reverse transciptase. it relates to some tumor formation.

Study and applications of viruses

Viruses as <u>probes</u> for exploring basic cellular processes

- Viruses are important to the study of <u>molecular</u> and <u>cellular biology</u> because they provide **simple** systems that can be used to <u>manipulate</u> and <u>investigate</u> the functions of cell types.

- Viral replication depends on the <u>metabolism</u> of the host ---- the study of viruses can provide fundamental information about aspects of cell <u>biology</u> and <u>metabolism</u>. With high sensitivity of fluorescence labeling, the metabolic dynamics can be revealed.

- Because of the <u>complexity</u> of an animal cell genome, viruses have been even more important in studies of animal cells than in studies of bacteria.

**Viruses as tools for genetic engineering**

- <u>Geneticists</u> regularly use viruses as <u>vectors</u> to introduce DNA into cells that they are studying. Attempts to treat human diseases through genetic engineering have also made use of viruses in similar ways. Deaths have occurred through virus infections caused by virus vectors used in <u>gene therapy</u>, so their application to human subjects is still nascent --- **how about artificial vector?**

Both the two applications relate to gene delivery

The steps of gene delivery and replication: draw scheme

- **Attachment**, sometimes called absorption: The virus attaches to receptors on the host cell wall.

- **Injection**: The nucleic acid of the virus moves through the plasma membrane and into the cytoplasm of the host cell. For bacterial virus, the capsid remains on the outside. For animal virus, they enter the host cell **intact**.

- **Replication:** The viral genome contains all the information necessary to produce new viruses. Once inside the host cell, the virus induces the host cell to synthesize the necessary components for its replication.

- **Assembly:** The newly synthesized viral components are assembled into new viruses --- self-assembly?

- **Lysis:** Assembled viruses are released from the cell and can now infect other cells, and the process begins again.

The influenza A virus
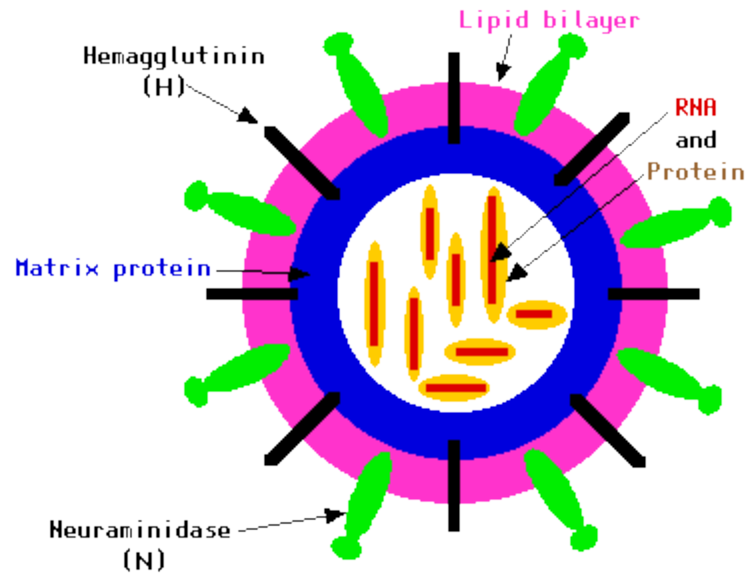
Electron microscope
image of stained viurs

- A RNA virus: 8 single strand RNA.
- a globular particle (about **100 nm** in diameter).
- Each year, 10-20% US residents get infected by flu, and ~36,000 people die of flu-related comlications.
- 1918 Spanish flu, 21 M people died over the world --- but still a mystery why it was so deadly in terms of genomes.
- Studying the cellular entry and the replication dynamics helps to understand the mystery of the special class of supra-molecules, and to avoid another time of pandemic attack of flu.

# Visualizing individual influenza virus particles in living cells

- Cellular replication of Influenza viruses: receptor-mediated endocytosis → endocytic trafficking of influenza to acidic endosomes → fusion of the viral membrane with endosomes → deliver the viral genome (ribonucleoprotein, vRNPs) into the cell → the vRNPs are then imported to the nucleus to initiate viral gene expression and replication.

- Despite intensive studies of influenza infection, many important aspects of cellular entry process of influenza still remain elusive.

- Labeling the lipid membrane of virus with fluorophores.

- Using fluorescence microscope equipped with oil immersion objective, NA1.4.

- 3 stages of virus transportation to be imaged (see above).

Three Stages of action: 1. moving at cell periphery, 2. moving from cell periphery to nuclear periphery, 3. moving at nuclear periphery.
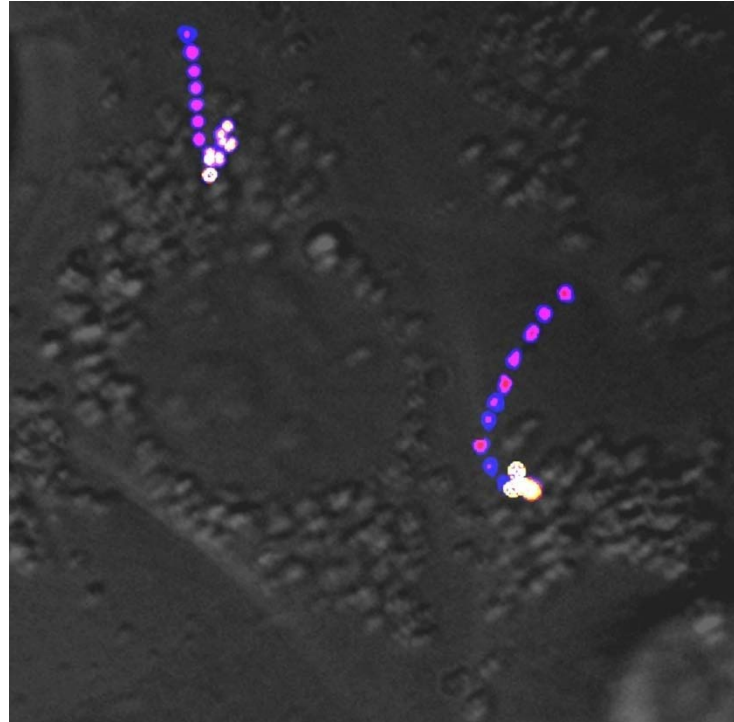
Stacked, time-lapsed images of two viruses in living cells. The sudden color change from blue/pink to white indicates a dramatic fluorescence dequenching, signaling the fusion of the virus with an endosome. The viruses are labeled with membrane dyes.

Three Stages of action: 1. moving at cell periphery, 2. moving from cell periphery to nuclear periphery, 3. moving at nuclear periphery.
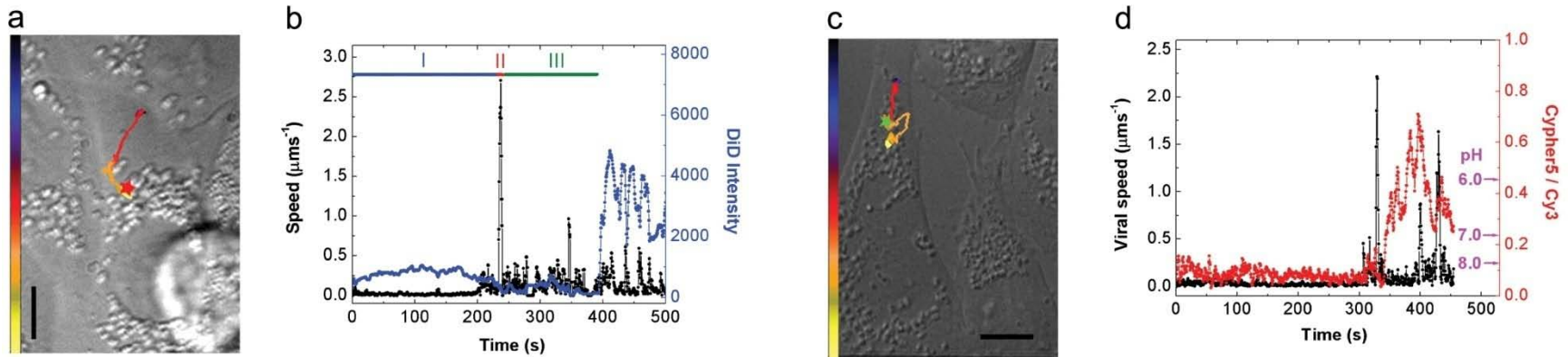

Endocytic trafficking and fusion of individual viruses. **(a)** The trajectory of a DiD-labeled virus inside a cell. The color of the trajectory codes time with the colored bar indicating a uniform time axis from 0 s (black) to 500 s (yellow). The red star indicates the fusion site. **(b)** Time trajectories of the velocity and the DiD fluorescence intensity of the virus. The labels I, II, and III indicate stage I, II, and III movement, respectively. The fluorescence dequenching signal of the lipophilic dye, DiD, near 400 s indicates viral fusion. Movie shown on the last slide.
(c) The trajectory of a Cy3/CypHer5-labeled virus inside a cell. The green star indicates the initial acidification site (to pH 6). **(d)** Time trajectories of the velocity and the fluorescent emission ratio of CypHer5 (a pH-dependent dye) and Cy3 (a pH-independent dye) of the virus. Scale bars: 10 μ m.

Entry mechanisms of influenza viruses: via CCP internalization

While many viruses are known to infect cells via receptor-mediated endocytosis, the exact endocytic mechanisms have remained unclear for most of them.
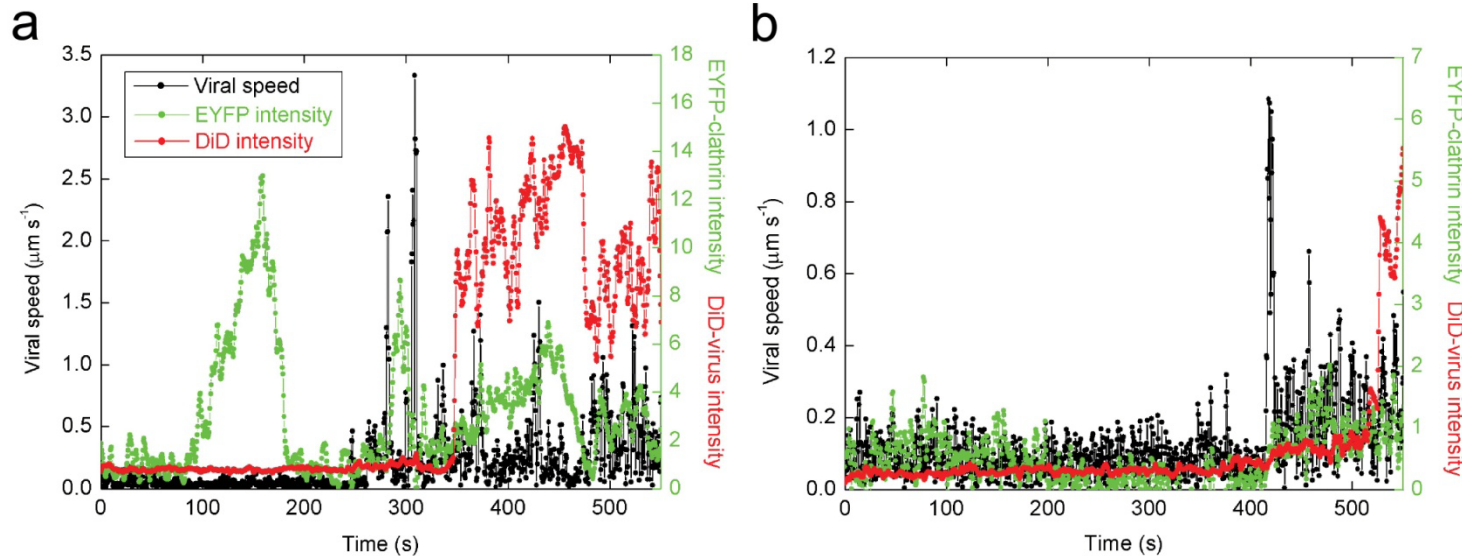

| 0 s | 50 s | 115 s | 150s | 175 s | 181 s | 202 s | 235 s |
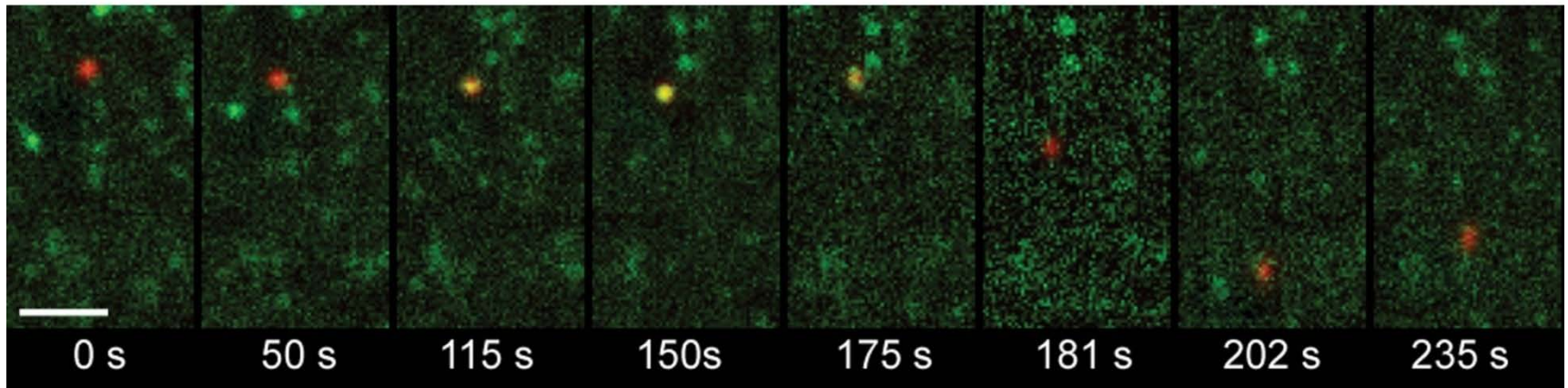
Snapshots of a virus (labled with DiD, red) internalized by a clathrin-coated pit (CCP, labeled with EYFP, green). Overlay of green and red signals appears yellow. T = 0 s: the virus (red) binds to the cell. t = 115 s: a CCP labeled with EYFP (green) begins to form at the virus site. T = 175 s: the clathrin coat rapidly disassembles. T = 181 s, 202 s, and 235 s: transport of the virus on microtubules.

Clathrin-coated pits are 150 nm invaginated structures on the plasma membrane that occupy about 2% of the plasma membrane surface
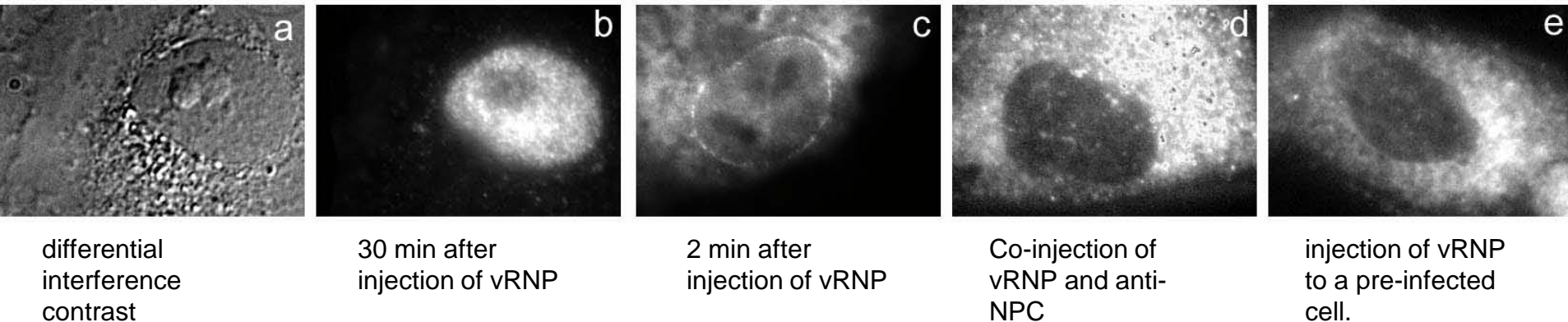
Time-trajectories of viruses fused with endosomes. (**a**) A virus internalized via a CCP. (**b**) A virus internalized without association with a CCP. Black symbols are the velocity time-trajectories of the viruses. Red symbols are the integrated DiD fluorescence intensities of the viruses. Viral fusion can be identified as a dramatic increase of the DiD signal. Green symbols are the integrated fluorescence intensities of EYFP-clathrin associated with the viruses.

Zhuang, ***Nature Struct. Mol. Biol.*** **11** , 567-573 (2004)

Nuclear import of influenza genome

| differential interference contrast | 30 min after injection of vRNP | 2 min after injection of vRNP | Co-injection of vRNP and anti-NPC | injection of vRNP to a pre-infected cell. |

Regulated nuclear import of influenza vRNPs. (**a**) A DIC image of an injected cell. (**b**) A fluorescence image of the same cell, taken 30 minutes after the microinjection of dye-labeled vRNPs, showing nuclear import of the labeled vRNPs. (c) A fluorescence image of a cell taken 2 minutes after injection of vRNP. The ring at the nuclear envelope indicates association of vRNPs to the nuclear envelope. (d) A fluorescence image of a cell that was co-injected with labeled vRNP and anti-NPC antibodies. (e) A fluorescence image of a pre-infected cell taken 10 min after injection with vRNPs. The cell was infected with influenza viruses 6 hours prior to injection. No fluorescent ring is observed at the nuclear envelope. DIC: differential interference contrast; NPC: nuclear pore complexes

Cellular entry and trafficking of polymeric gene-delivery vectors

- Gene delivery: emerging as a promising therapeutic method for a variety of diseases including cystic fibrosis, Parkinson's disease, and certain cancers.

- Polymer-mediated gene delivery replaces the conventional viral vector (potential infection) with a cationic polymer, which binds to the anionic DNA or siRNA, causing it to condense into a small complex with a net positive charge --- similar to the virus structure.

- At present, the best polymeric gene-delivery vectors is still a few orders of magnitude less efficient than viral vectors.

- A detailed understanding of the entry and trafficking mechanisms of polymer-DNA complexes is critical for the rational design of polymer vectors with much improved efficiency.

- A movie made for the polymer vectors.

Zhuang, at **Harvard**