Lecture 21: Single-molecule (labeling) imaging of protein dynamic structural change and virus diffusion within living cells

- 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.

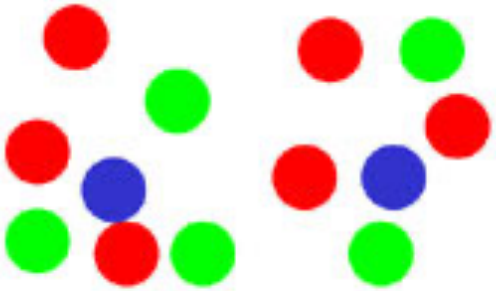
Part 1:
Imaging Protein dynamic structural change *via* ***evironment sensitive fluorophores, or fluorescence resonance energy transfer (FRET)***

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

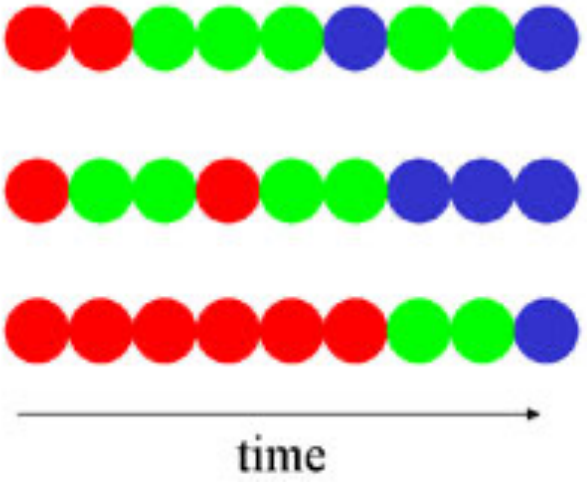
Distribution

-useful if population is heterogeneous.



Time trajectory

-useful if the dynamics is not synchronizable



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.

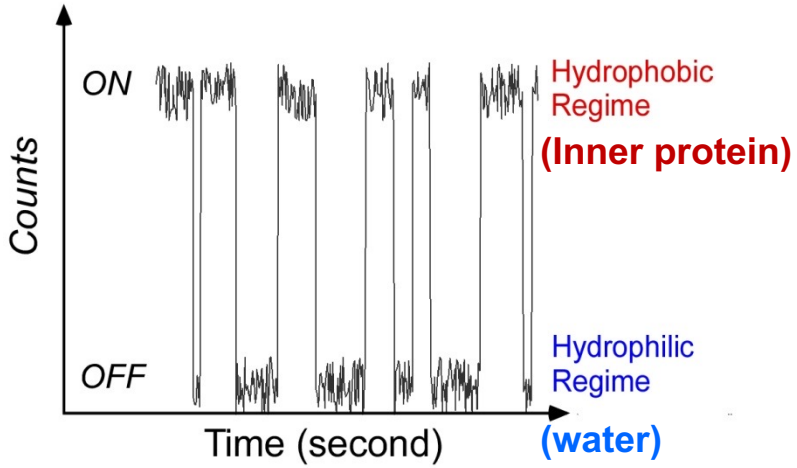
  Single molecule imaging/spectroscopy measures the entire time trajectories, which include multiple pathways and intermediates involved. See the last slide.

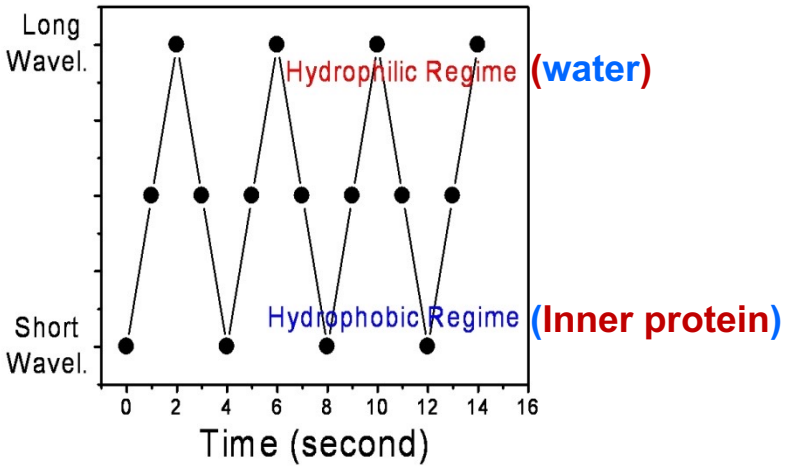
- Though elegant biochemical and structural methods are available, they don't provide with the real time measurements of conformational changes, and at single protein level.

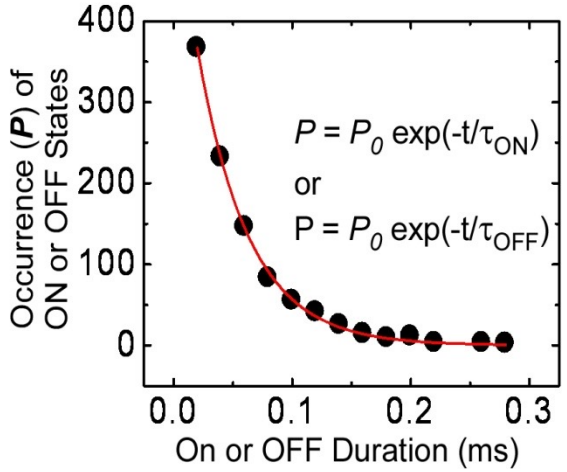
Typical fluorescence properties used for single molecule probing and imaging

- Fluorescence depends on local polarity --- for both wavelength and quantum yield (intensity). Such environment effect helps reveal different locations,e.g., inner protein vs. water. 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.

Single molecule measurement based on intensity and wavelength change (can also be used in monitoring of Virus moving and locations, see later slides)







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

(*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.

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Single-molecule rotation imaged as movie clips in various time intervals

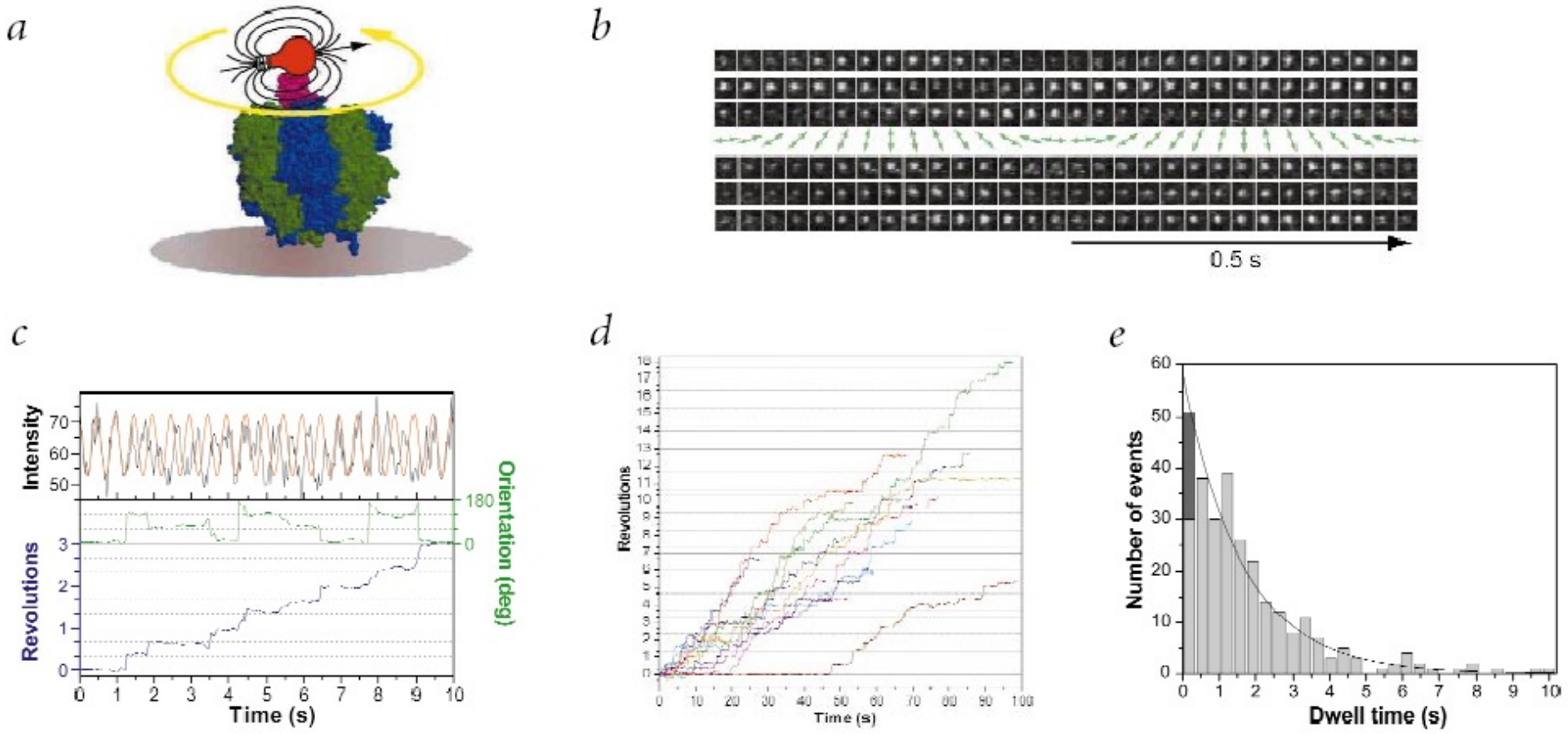

Fig. 4 Single molecule dipole rotation. ***a***, A cartoon of the F_1-ATPase motor with a single fluorescent probe attached to its rotor. ***b***, Two sequential fluorescence images (33 ms intervals) of single Cy3-F_1-ATPase molecules. The direction of the modulated excitation polarization is shown by 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).

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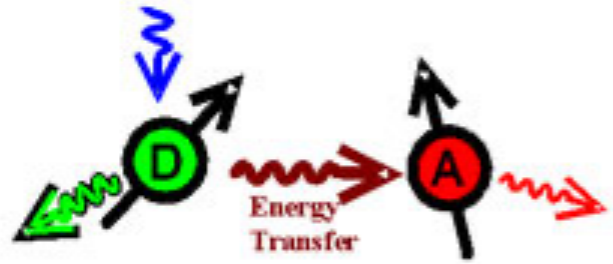
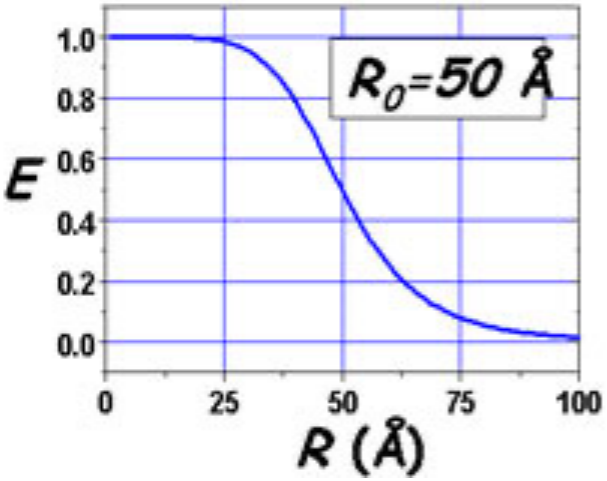
What is FRET



DONOR ACCEPTOR

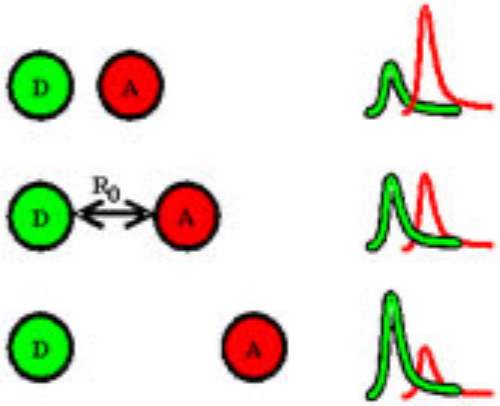


Energy Transfer Efficiency

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

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

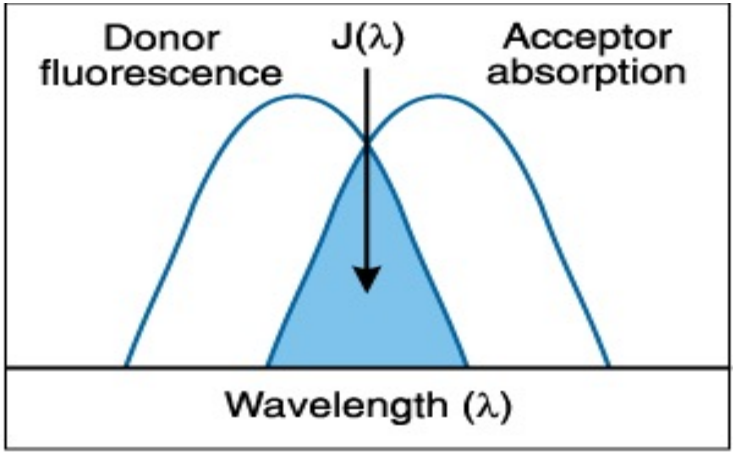
"Spectroscopic Ruler"



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.

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

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

Breakthroughs 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).

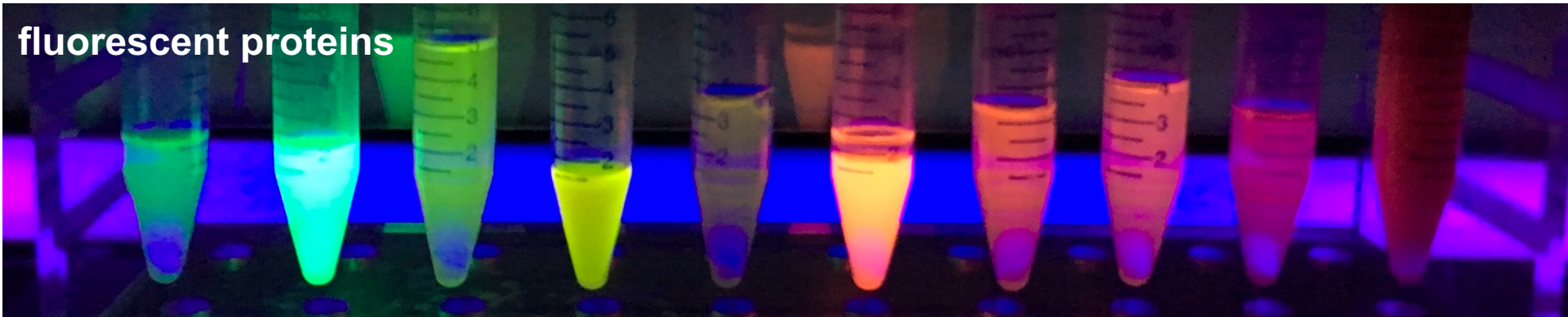
- Sako *et al* – extended it to invivo system and used it for studying epi'dermal growth factor dimerisation.

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

Fluorophores 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"

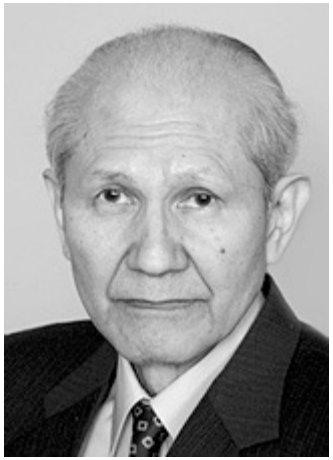


Photo: U. Montan

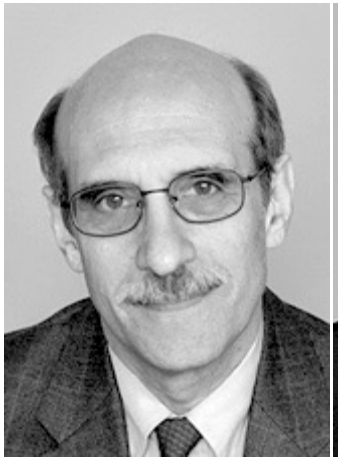


Photo: U. Montan

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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

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All commercially avalable

FRET efficiency correlates with the Protein folding states

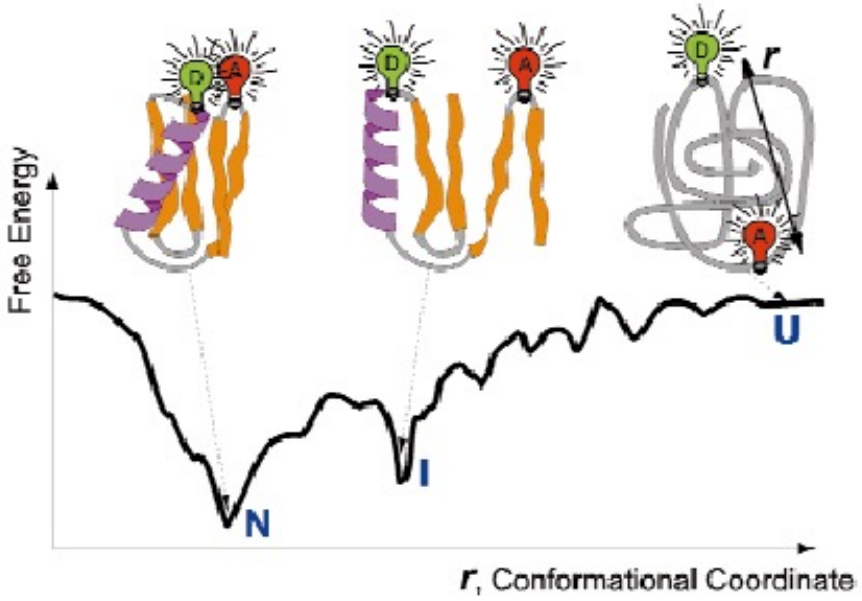
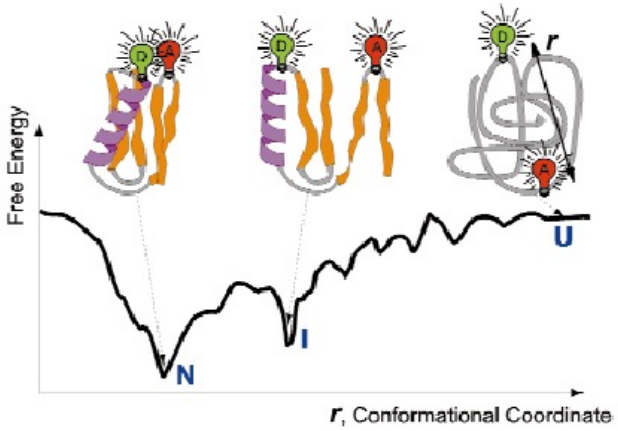


Fig. 1 Energy landscape for the folding reaction. A cartoon of free energy as a function of distance r between the fluorescence donor and acceptor. The rugged energy landscape has a funnel shape. U, the unfolded state; I, an intermediate state; N, the folded state.

A dynamic gauge for protein folding processes.

FRET efficiency correlates with the Protein folding states



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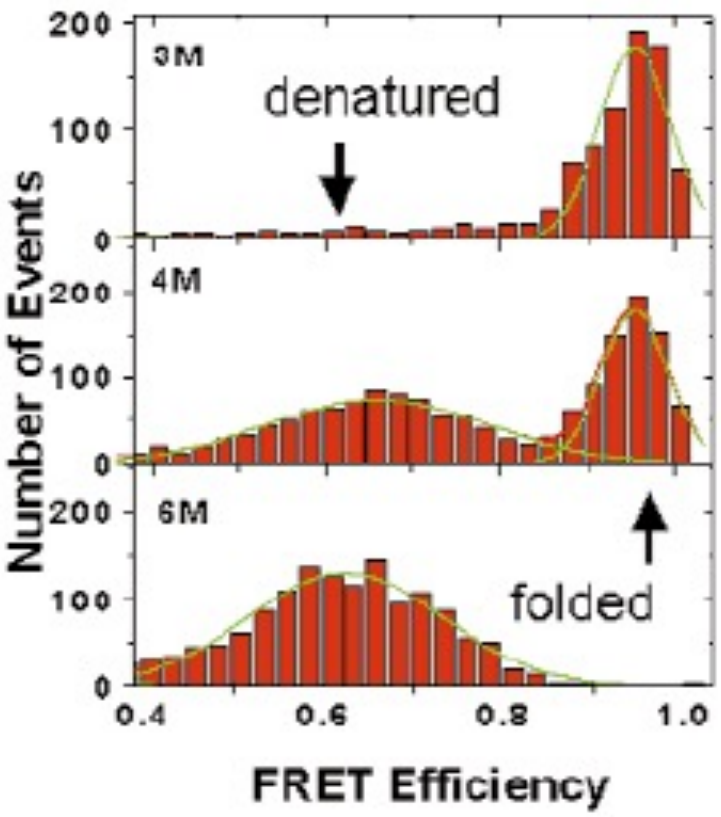
- Many methodologies have been developed and applied to protein folding, but most conducted on an **ensemble of proteins**.
- Since it is very difficult to synchronize the folding reactions over an ensemble of molecules, these measurements usually fail to resolve detailed dynamic information.
- **Single-molecule-FRET**, can provide **distance** information between a pair of points on a polypeptide chain as folding progresses (real-time) — 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.

FRET in living cells

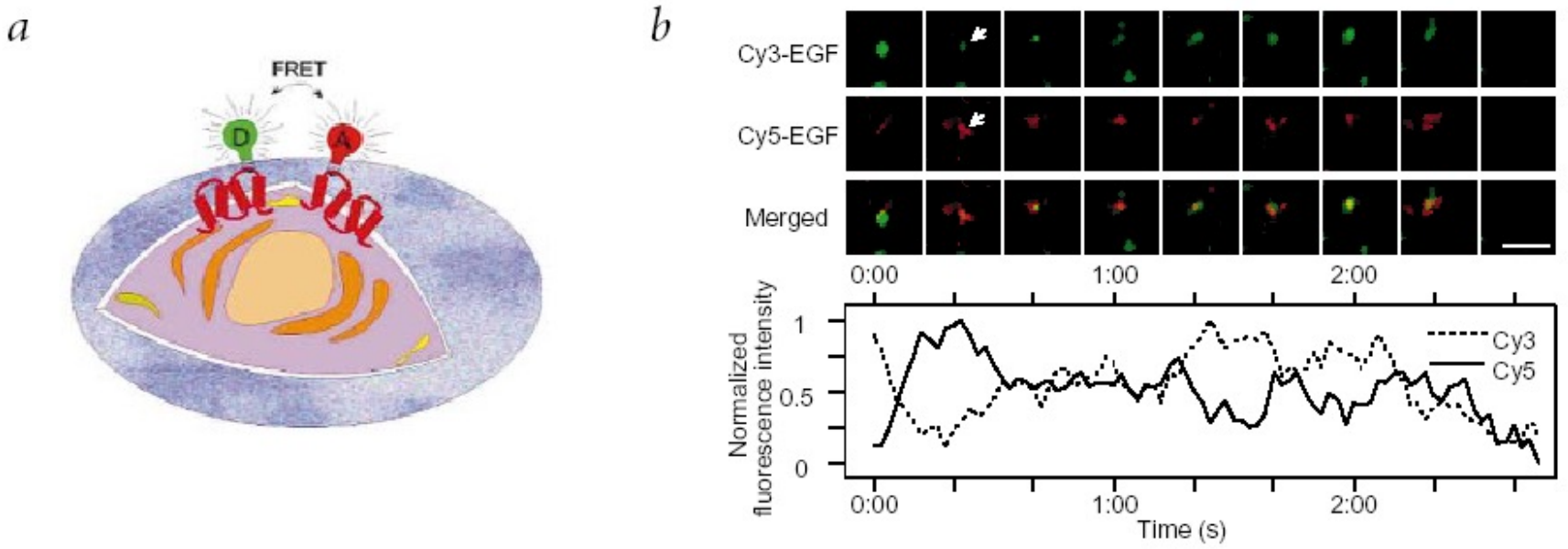


Fig. 5 Single pair FRET in a living cell. ***a***, A cartoon of spFRET between two epidermal growth factor receptors in the membrane of a live cell (a mixture of donor-labeled and acceptor-labeled extracellular ligands was used to label the receptors). ***b***, Top three rows: video frames showing the 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).

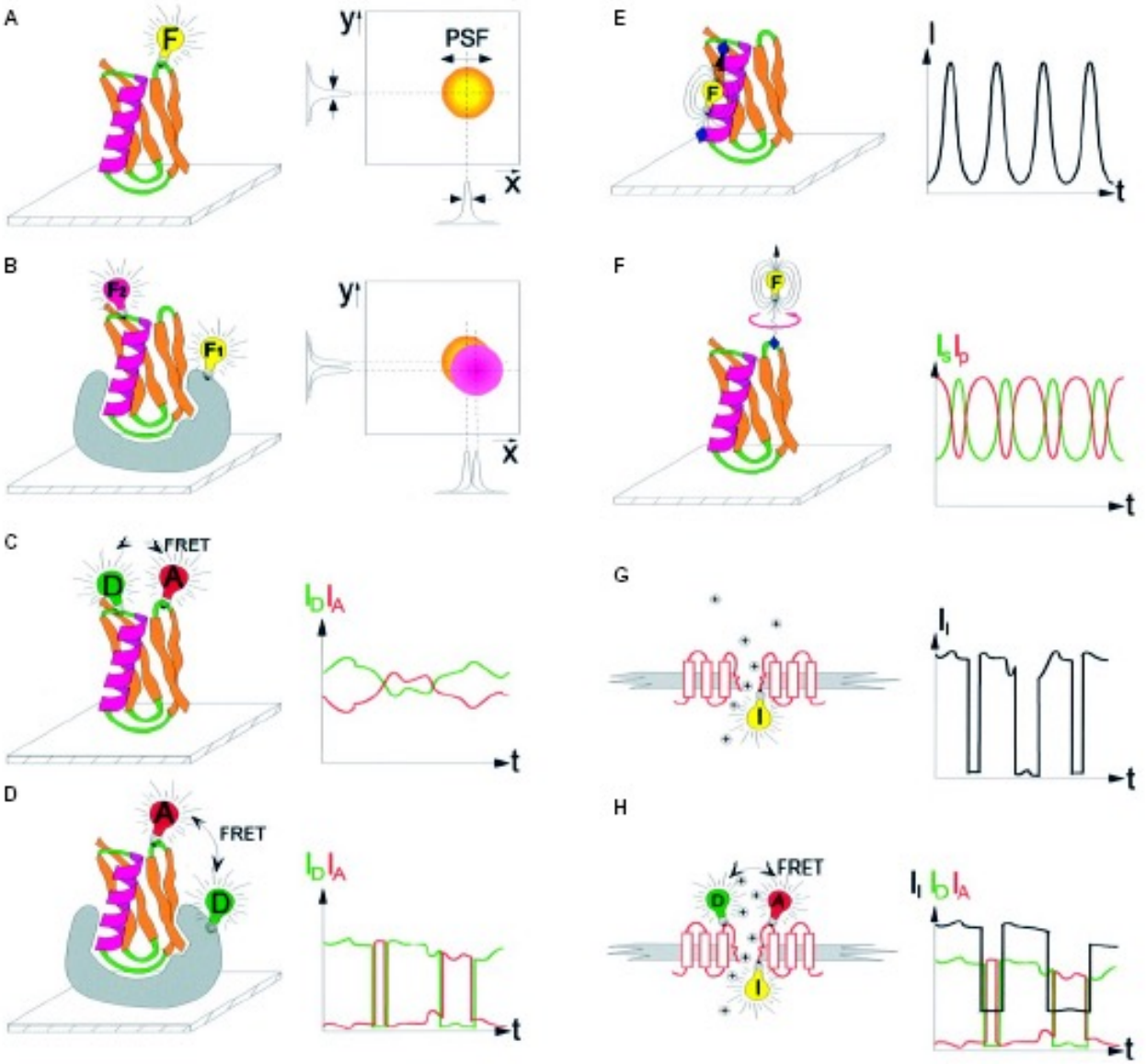


Fig. 1. Labeling schemes (left) and physical observables (right). (A) Localization of a macromolecule labeled with a single fluorophore F with nanometer accuracy. The point-spread-function (PSF) can be localized within a few tenths of a nanometer. (B) Colocalization of two macromolecules labeled with 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.

FRET for nuclease-DNA interaction

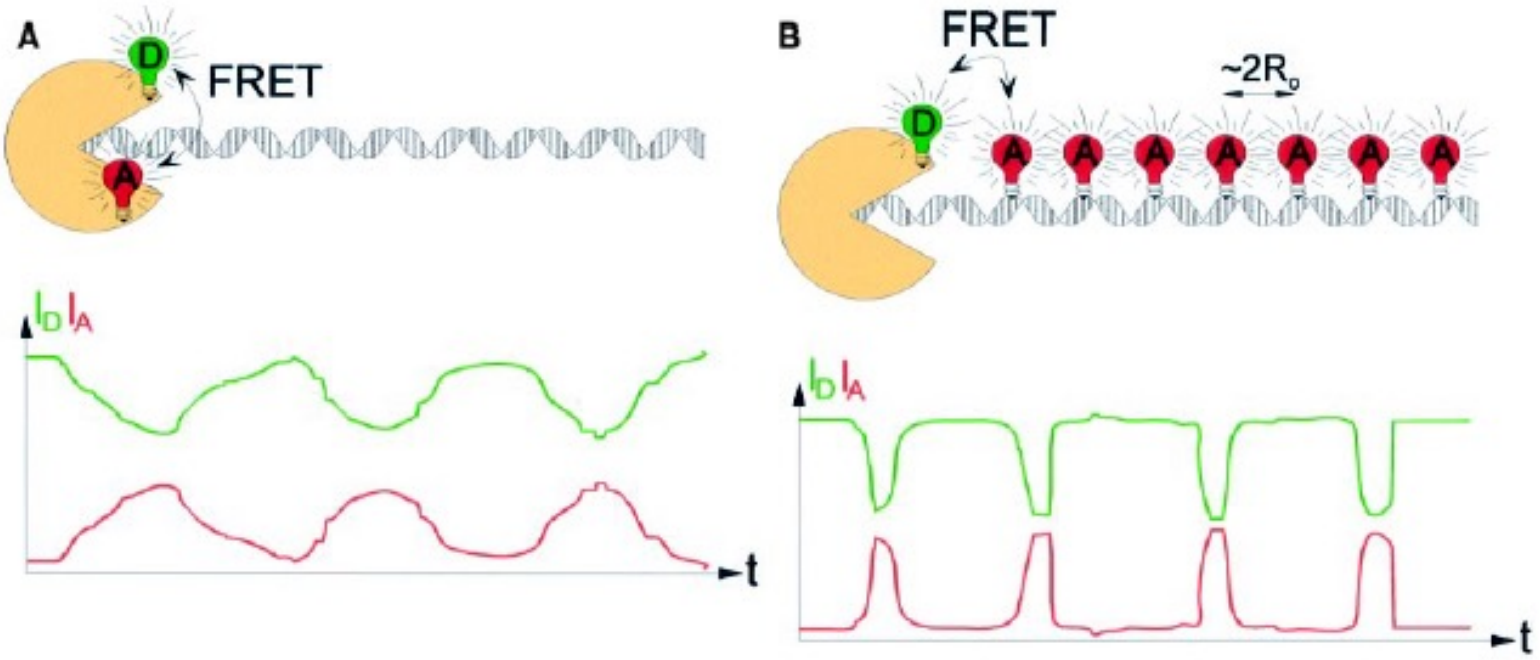


Fig. 5. A cartoon illustrating (**A**) intramolecular and (**B**) intermolecular spFRET nuclease-DNA interactions. Intramolecular spFRET measures conformational dynamics of the enzyme during catalysis. Intermolecular spFRET measures association, catalysis, and dissociation of substrate molecules. Multiple acceptors at equal distances on the DNA act as a "ruler." R_0 is the Förster radius (distance at which 50% of the energy is transferred). This scheme can be generalized to many other protein-DNA interactions.

Shimon Weiss, SCIENCE, VOL 283, page 1686.

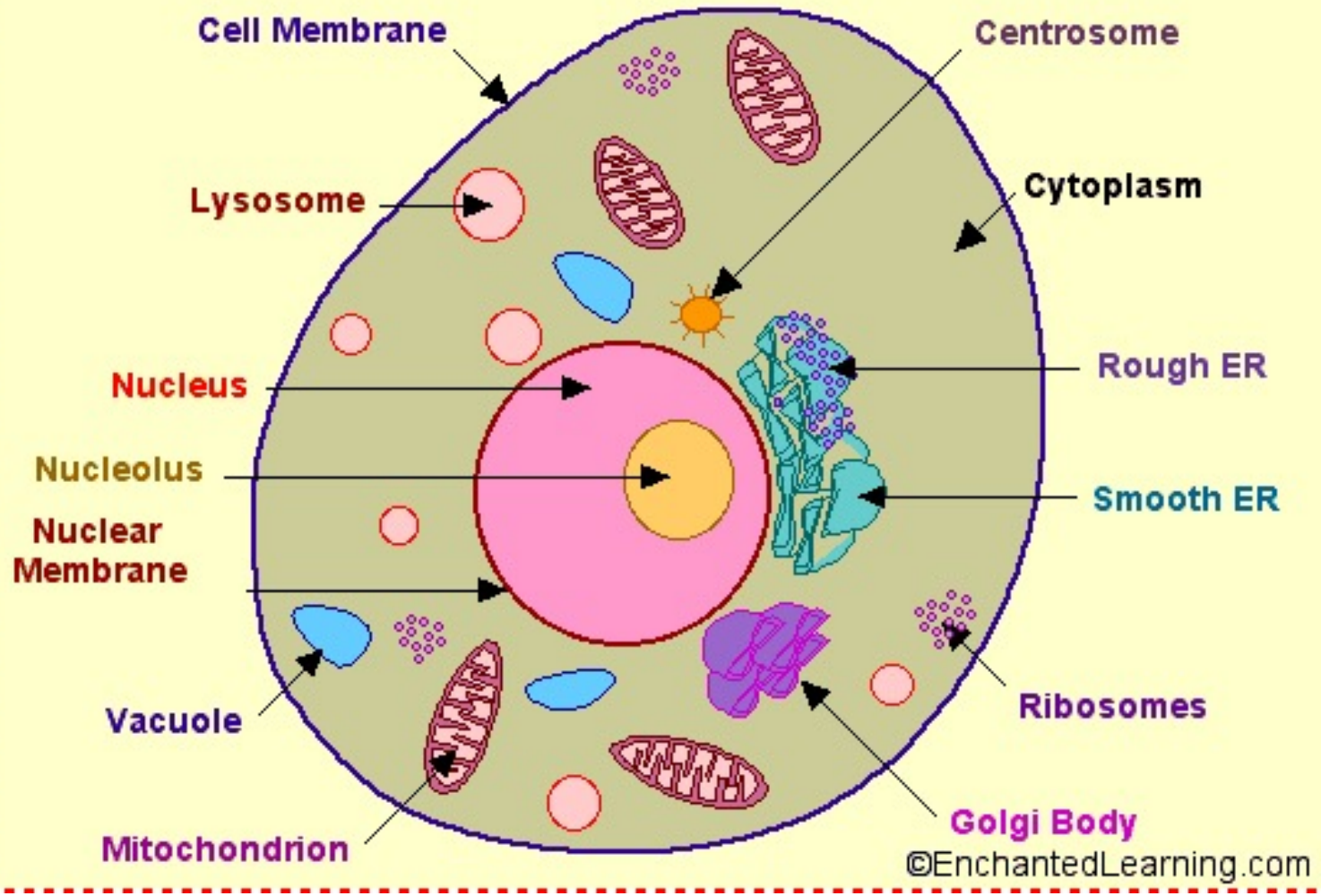
Part 2:
Imaging virus diffusion and attacking to living cell

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

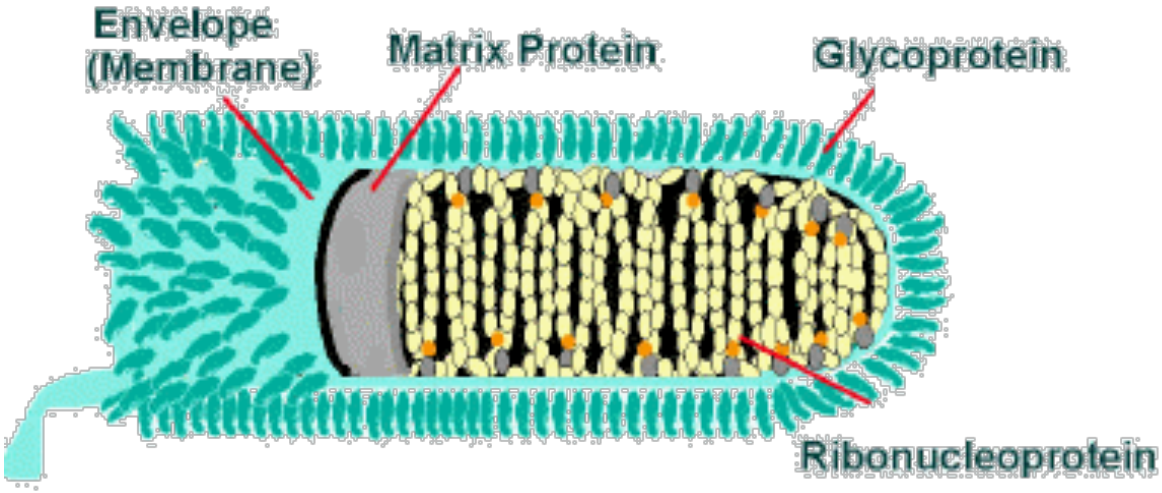
Basics of virus

- 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

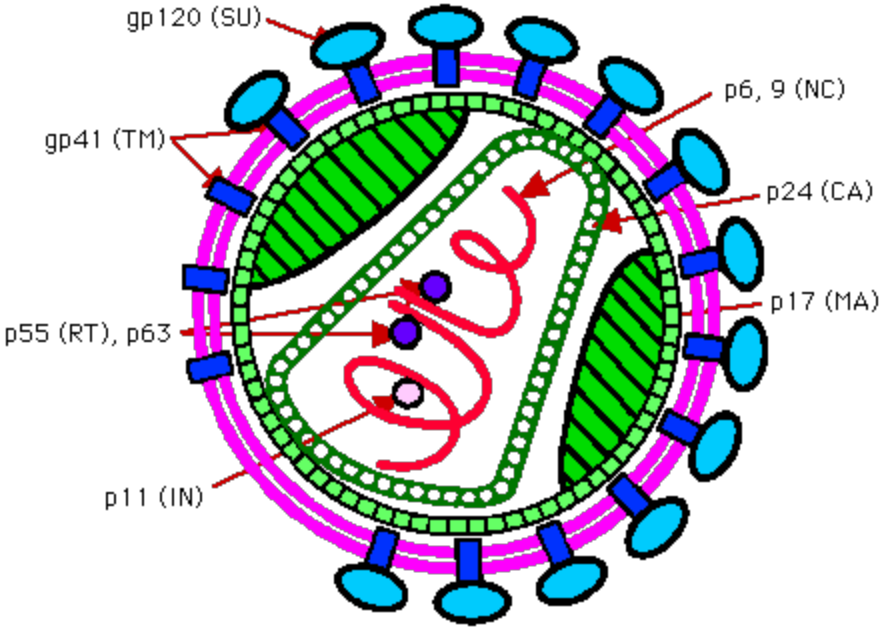


Helical virus



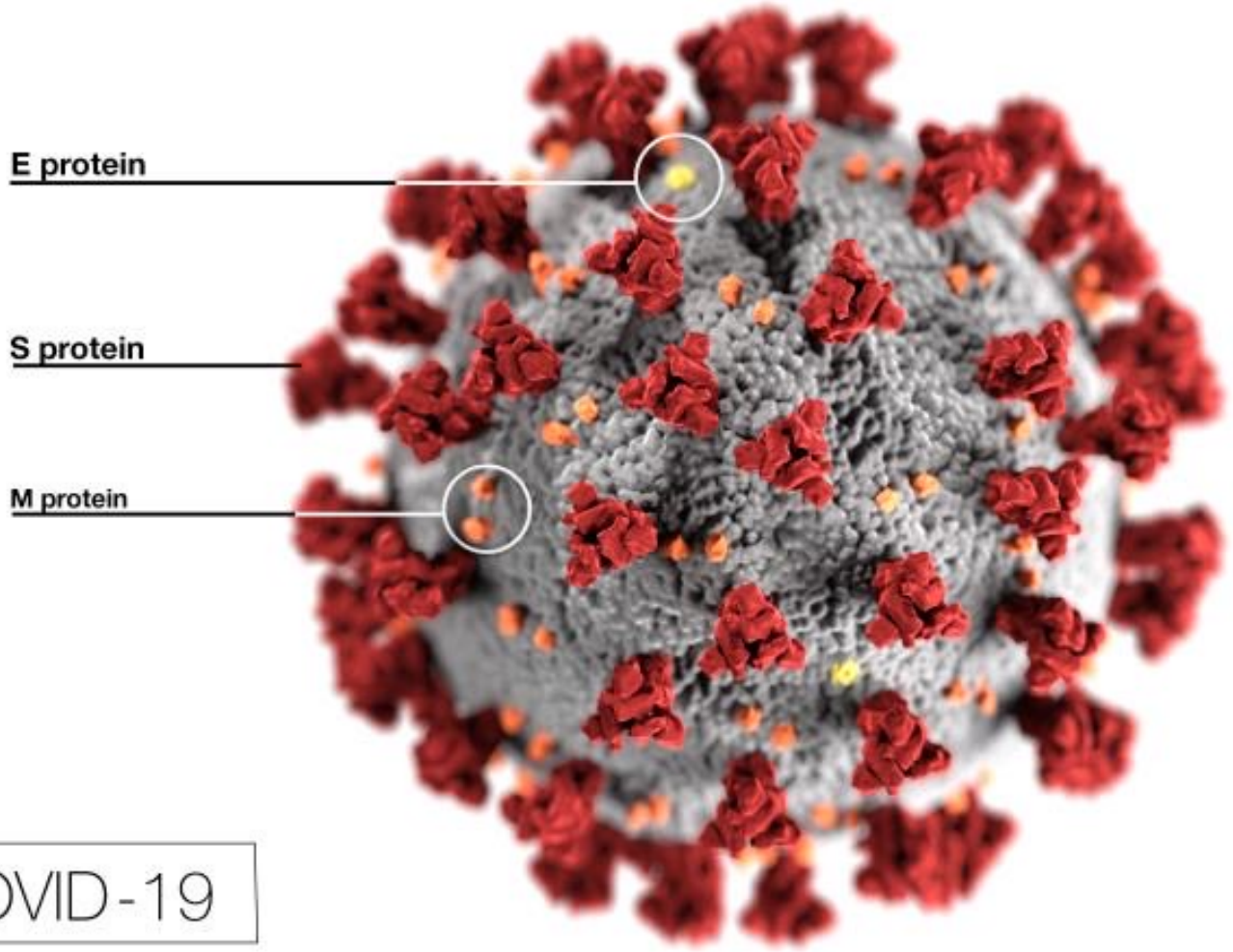
Rabies virions are bullet-shaped with 10-nm spike-like glycoprotein peplomers covering the surface. The ribonucleoprotein is composed of RNA encased in nucleoprotein, phosphorylated or phosphoprotein, and polymerase.

Spherical virus

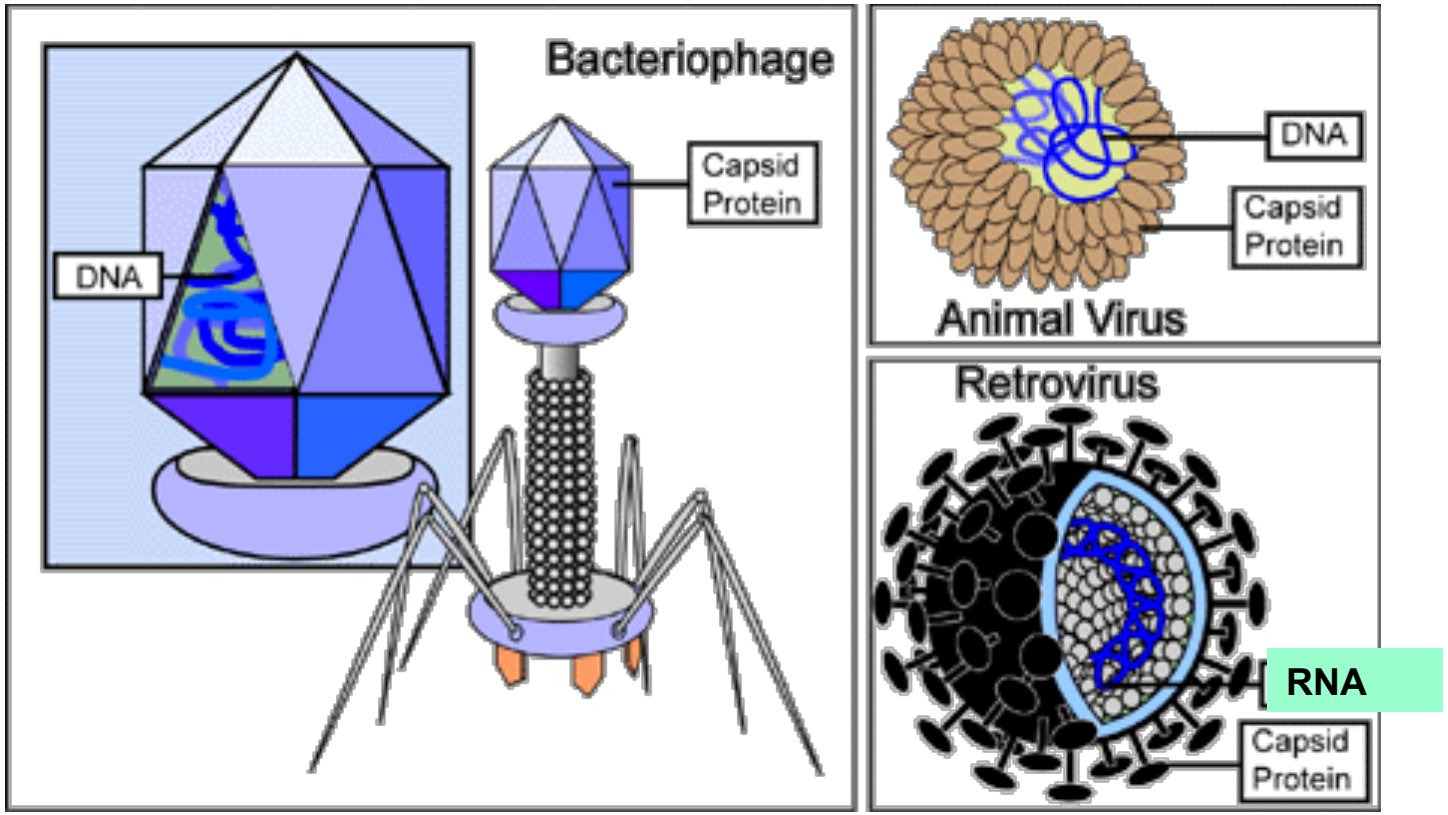


Schematic representation of the structure of HIV:

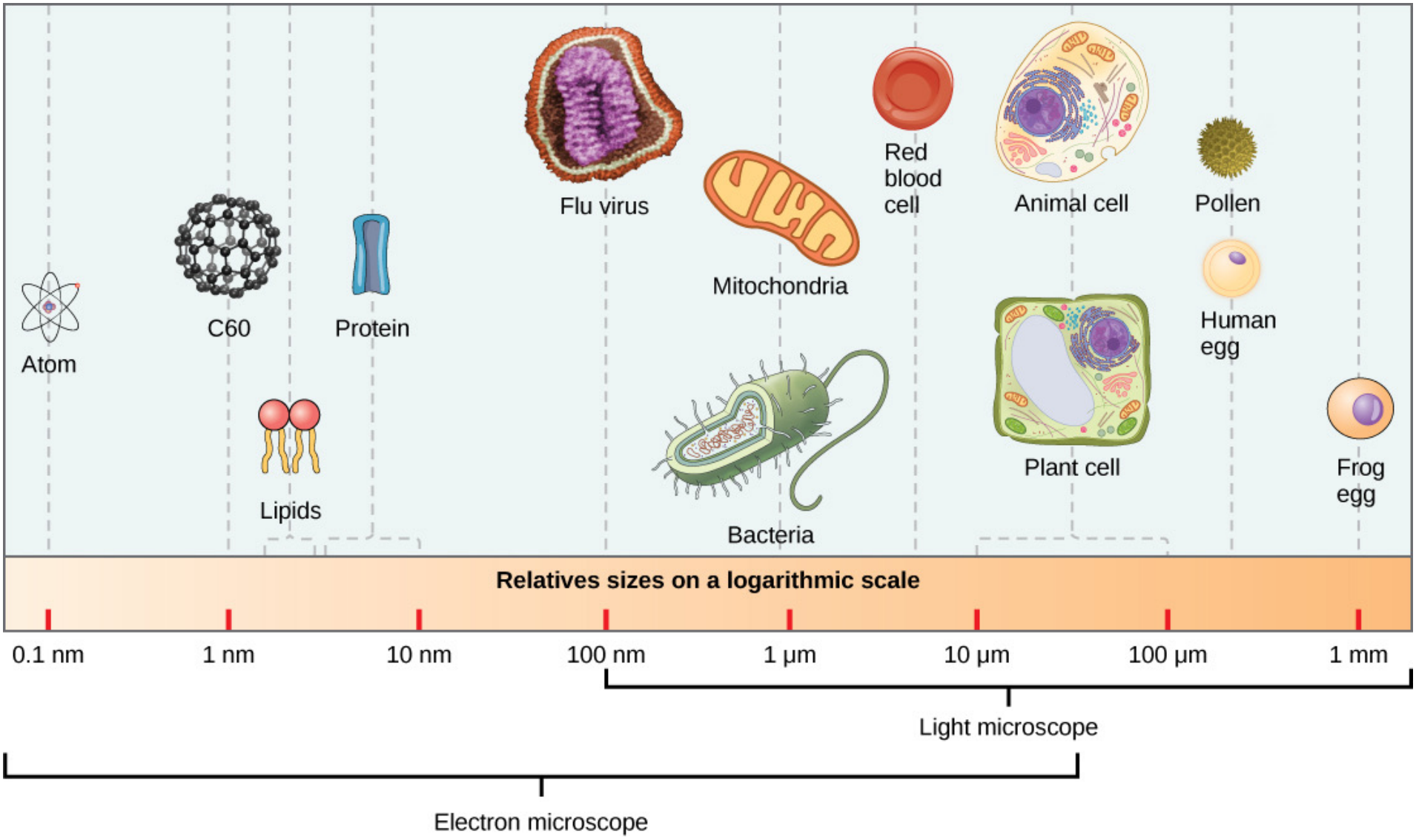
100 nm in diameter
Cases: 10.7M; Deaths: 243K



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.


Atom
C60
Protein
Lipids
Flu virus
Mitochondria
Bacteria
Red blood cell
Animal cell
Pollen
Human egg
Plant cell
Frog egg
Relatives sizes on a logarithmic scale
0.1 nm
1 nm
10 nm
100 nm
1 µm
10 µm
100 µm
1 mm
Light microscope
Electron microscope

Study and applications of viruses

Viruses as probes for exploring basic cellular processes

- Viruses are important to the study of molecular and cellular biology because they provide **simple** systems that can be used to manipulate and investigate the functions of cell types.
- Viral replication depends on the metabolism of the host ---- the study of viruses can provide fundamental information about aspects of cell biology and metabolism. With high sensitivity of fluorescence labeling, the metabolic dynamics can be revealed.
- Because of the complexity 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

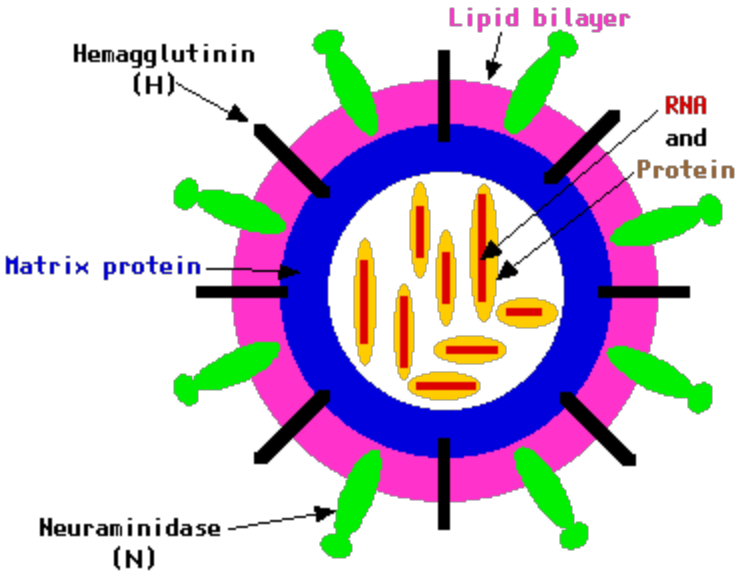
- Geneticists regularly use viruses as vectors to introduce DNA into cells that they are studying. Attempts to treat human diseases through genetic engineering have also made use of viruses in similar ways. Deaths have occurred through virus infections caused by virus vectors used in gene therapy, so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

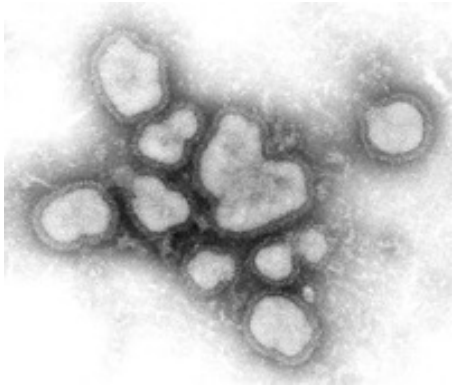
Both the two applications relate to gene delivery

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





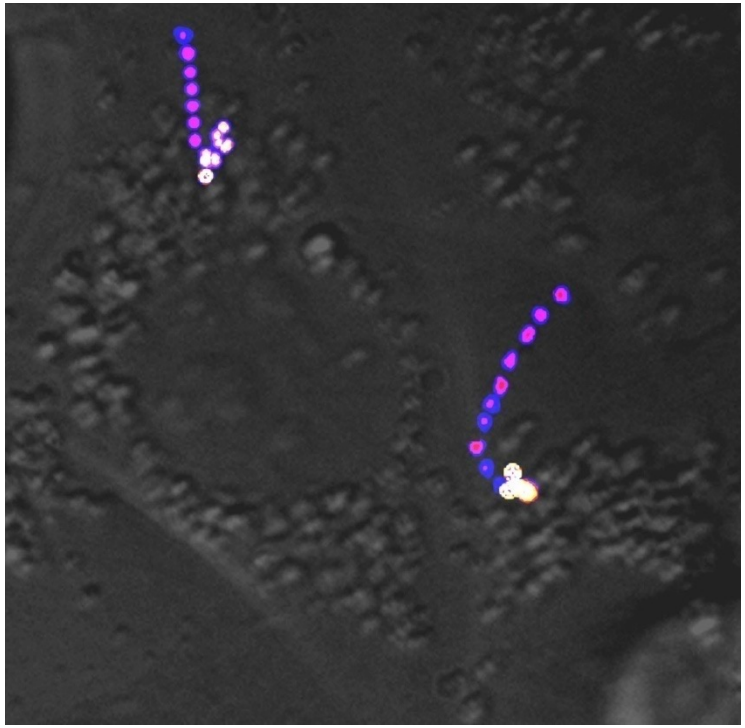
Electron microscope image of stained viurs

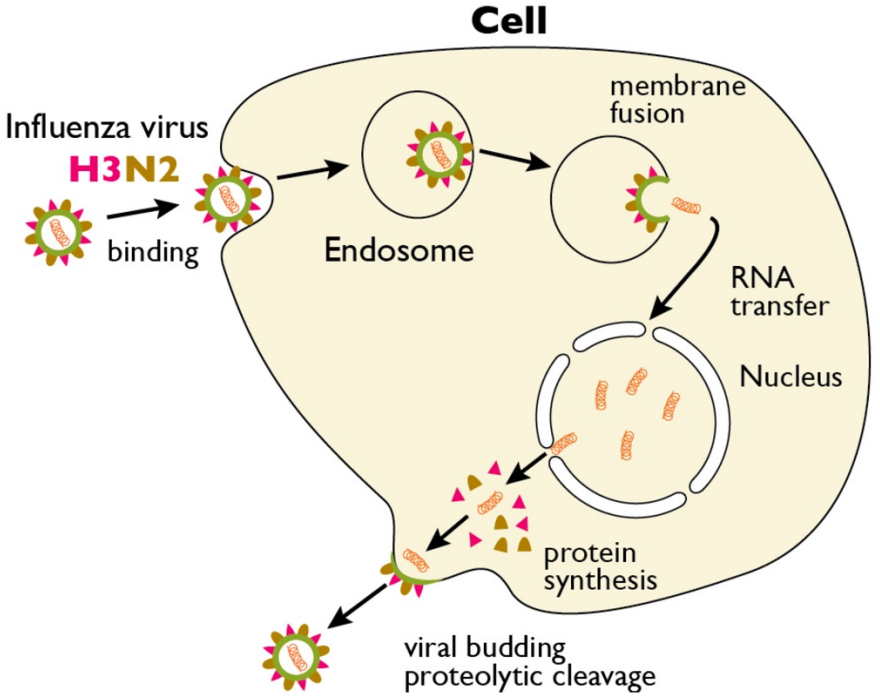
- A RNA virus: 8 single strand RNA.
- a globular particle (about **100 nm** in diameter).
- Each year, 10-20% US residents get infected by flu, and ~36,000 people die of flu-related comlications.
- 1918 Spanish flu, 21 M people died over the world --- but still a mystery why it was so deadly in terms 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.



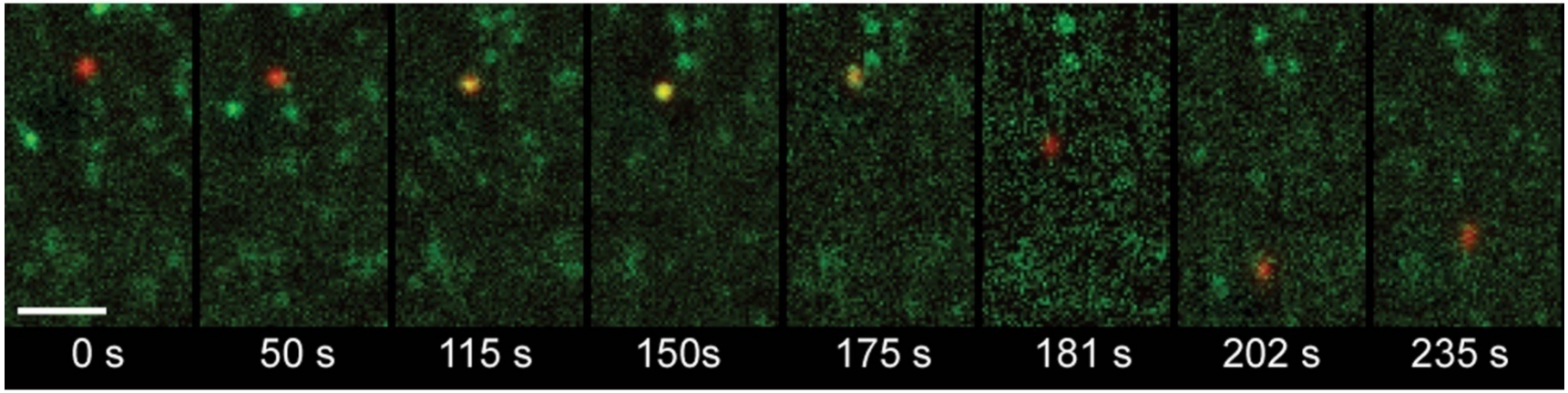


Stacked, time-lapsed images of two viruses in living cells. The sudden color change from blue/pink to white indicates a dramatic fluorescence dequenching, signaling the **fusion of the virus with an endosome**. The viruses are labeled with membrane dyes.

Zhuang, PNAS, 2003, vol. 100, 9280–9285

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.



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

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

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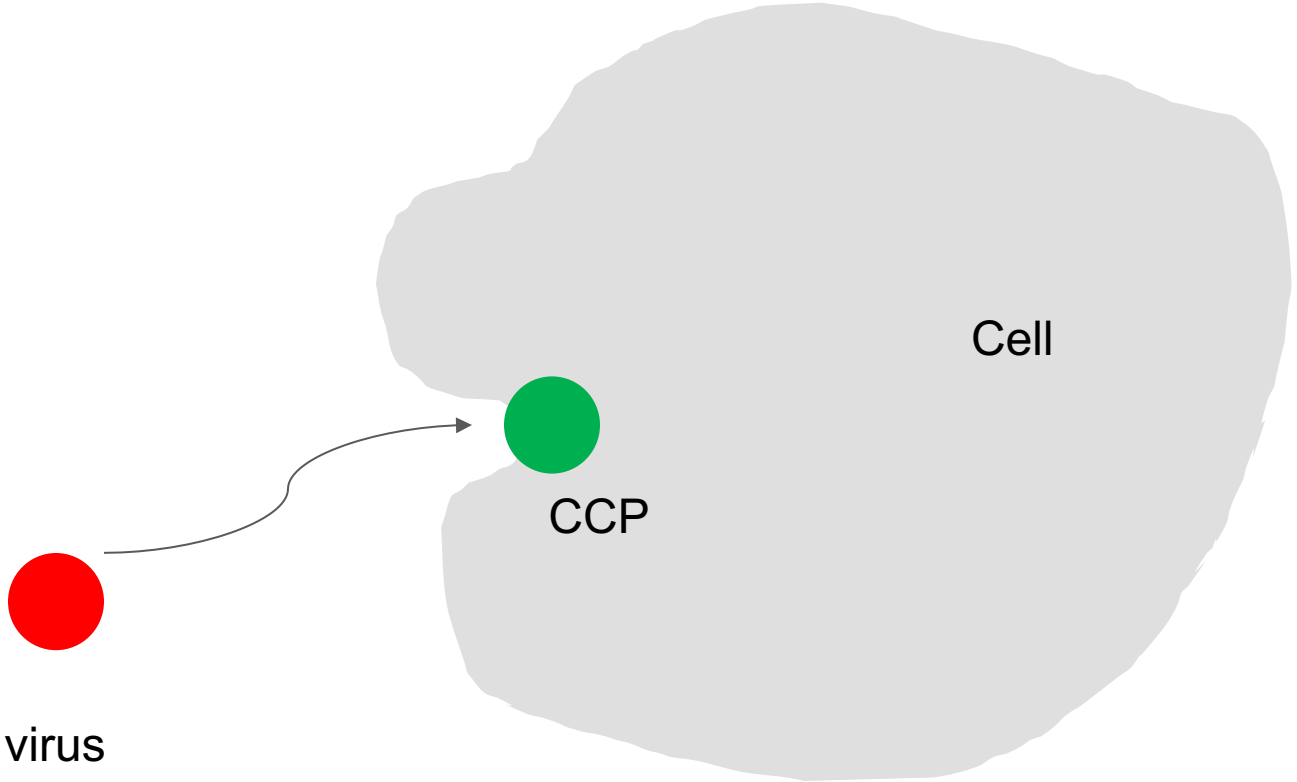

CCP: clathrin-coated pit

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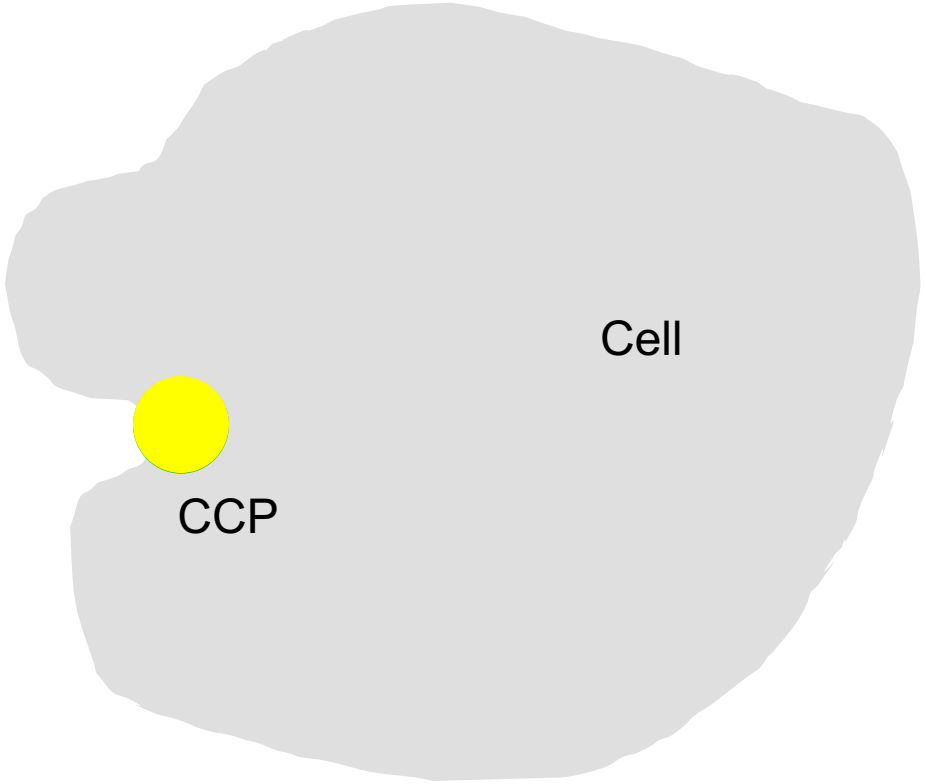
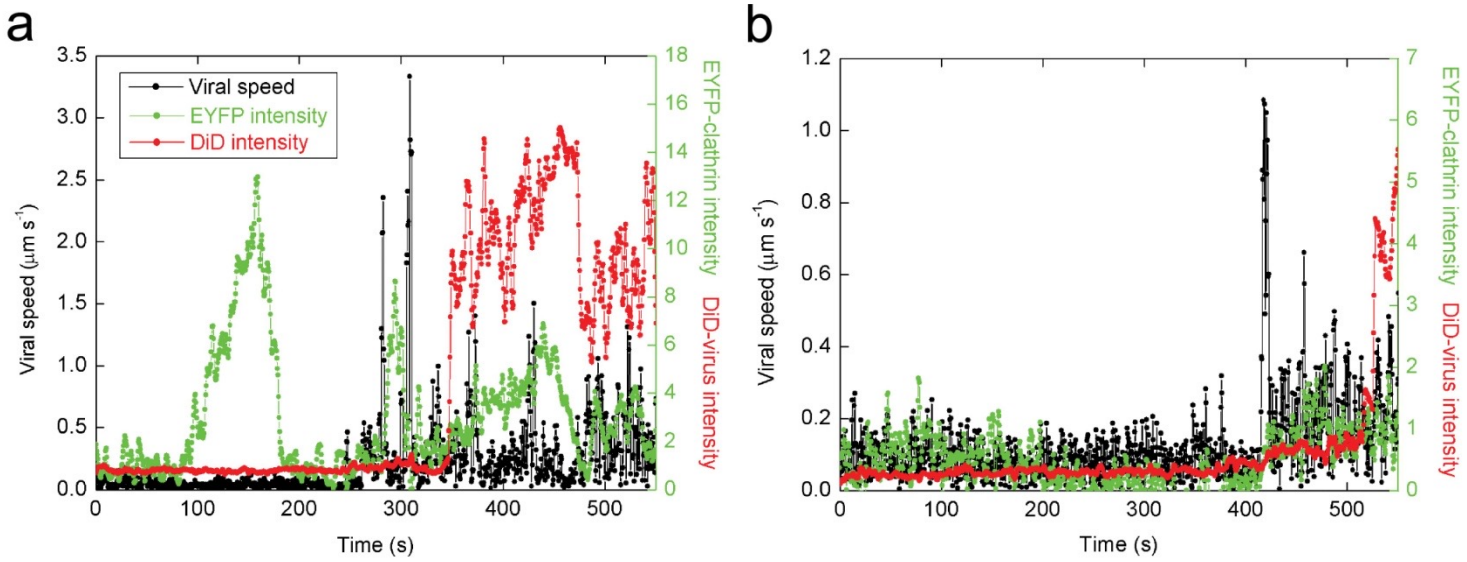


Zhuang, ***Nature Struct. Mol. Biol.*** **11** , 567-573 (2004)

Entry mechanisms of influenza viruses: without CCP



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.

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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.