Housekeeping notes

- First recording on Mediaspace: issue with screensharing after break, so the video is not available for the second hour. :(My apologies.
- Signup for group assignment closes tomorrow morning, Feb 28, 10 am. Make sure to sign up!
- Inspiration for grant writing:
  - https://data.snf.ch/grants
  - https://www.nature.com/search/advanced
  - https://www.feedly.com
  - Start a notebook for "Research Ideas" in digital or paper form
    - I note down interesting approaches, complementary strategies or actual ideas for experiments when I attend seminars locally or at international conferences, when I read/review papers, when I read books, etc. Usually, an idea comes **when I read or hear about someone else's work**.
    - **Start reading and going to research seminars! (www.memento.epfl.ch)**

Interactive exercise week 01

Question 1: What is Nanobiotechnology for you?

• **Blue (OTHER):** Nanoscale solutions to biological problems, CRISPR, drug delivery, biosensors, microfluidics. Combines **engineering fields for medical applications**.

• **Yellow (SV):** Engineering **nanodevices for biology & medicine** (vaccines, organoids, sensors, robotics). Drug delivery systems, precision medicine.

• **Pink (CGC_CHIM):** Engineering at the nanoscale (**proteins, DNA, biochemical interactions**). Study, control, and manipulate biological processes.

• **White (CGC_ING):** Nanotechnology in medicine (RNA, viruses, bacteria). **Engineering biomolecules** at nm-scale for diagnostics and treatments.

Interactive exercise week 01

Question 2: What do you hope to learn?

- **Blue (OTHER):** Prolonging life, nanoparticle interactions, targeted drug delivery, biosensors, nanoremediation, protein tracking.

- **Yellow (SV):** Applications in biology, gene delivery, lab techniques (microfluidics, aptamers, organoids), real-life technology use cases.

- **Pink (CGC_CHIM):** Cutting-edge methods, drug delivery, molecular biology, experimental approaches, AI, new materials, bio-based medicine.

- **White (CGC_ING):** Broad overview of nanobiotech, pharmaceutical applications, future trends, biotechnology career exploration.

CH-413 Nanobiotechnology

Single Molecule Fluorescence Detection

Angela Steinauer

February 27, 2025

William Moerner about single-molecule spectroscopy

- https://www.youtube.com/watch?v=q6nTXoErZzE&ab_channel=ChalmersUniversityofTechnology

- 12:35: single-molecule intro at RT
- 16:15: YFP (to 18:00)

"In normal microscopes the wavelength of light sets a limit to the level of detail possible. However this limitation can be circumvented by methods that make use of fluorescence, a phenomenon in which certain substances become luminous after having been exposed to light**. Around 2000, Eric Betzig and William E. Moerner helped create a method in which fluorescence in individual molecules is steered by light.** An image of very high resolution is achieved by combining images in which different molecules are activated. This makes it possible to track processes occurring inside living cells." From Nobel website: https://www.nobelprize.org/prizes/chemistry/2014/moerner/facts/

Let’s play connections

https://connections.swellgarfo.com/game/-NrCaTHe5L5xxRF7bgbG

How to Play

Find groups of four items that share something in common.

- Select four items and tap '**Submit**' to check if your guess is correct.
- Find the groups without making 4 mistakes!

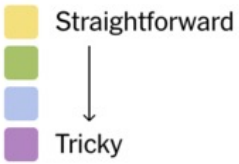
Category Examples

- FISH: Bass, Flounder, Salmon, Trout
- FIRE ___: Ant, Drill, Island, Opal

Categories will always be more specific than "5-LETTER-WORDS," "NAMES" or "VERBS."

Each puzzle has exactly one solution. Watch out for words that seem to belong to multiple categories!

Each group is assigned a color, which will be revealed as you solve:

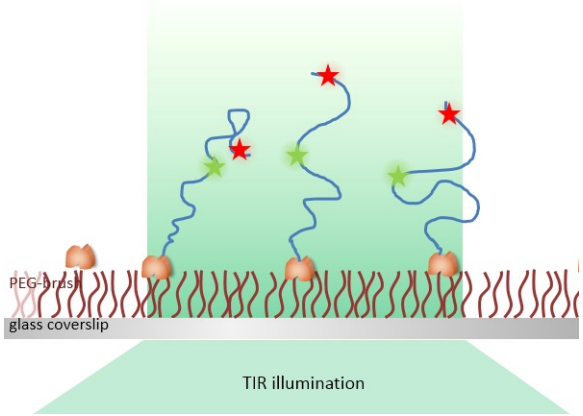


Single molecule fluorescence techniques

Immobilized molecules:

molecules are immobilized on a surface (coverslip) and can be observed for a long time, e.g. by total internal reflection fluorescence microscopy

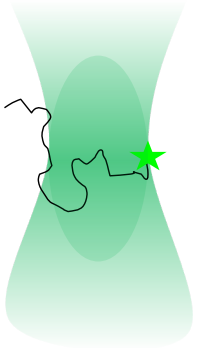
- Imaging and counting single molecules
- internal dynamics
- interaction dynamics
- Colocalization analysis



Freely diffusing molecules:

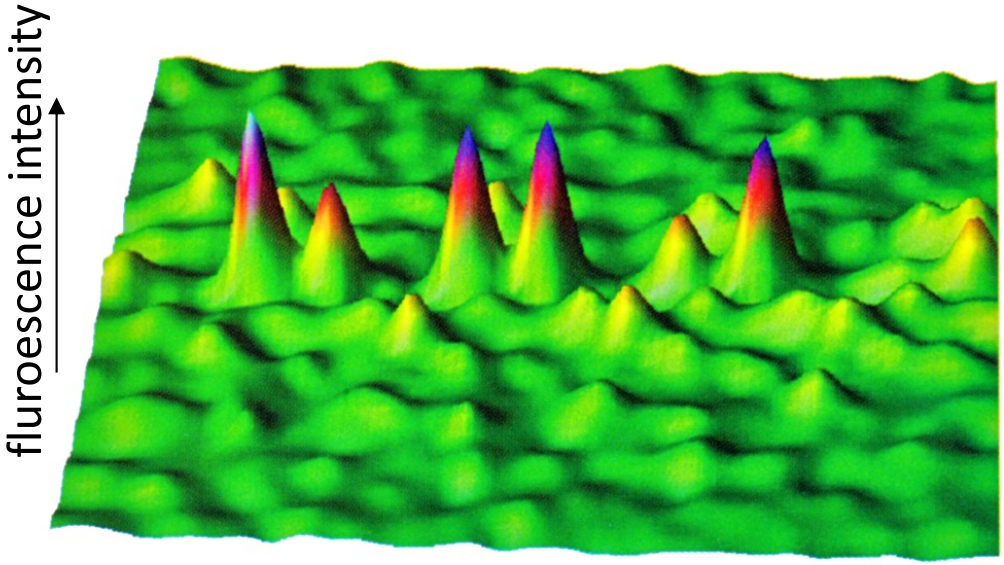
fluorescently labeled molecules are observed at very high dilution free in solution, using a confocal microscope:

- FRET statistics
- Burst analysis
- Photon counting histograms
- only fast dynamics are monitored (ms)



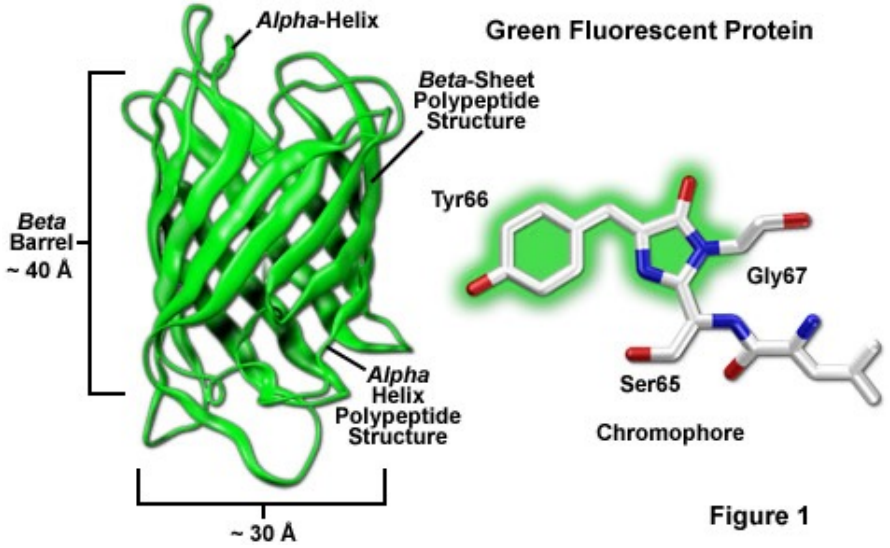
Imaging of single YFP molecules

YFP T203Y in polyacrylamide gel, imaged by TIRFM



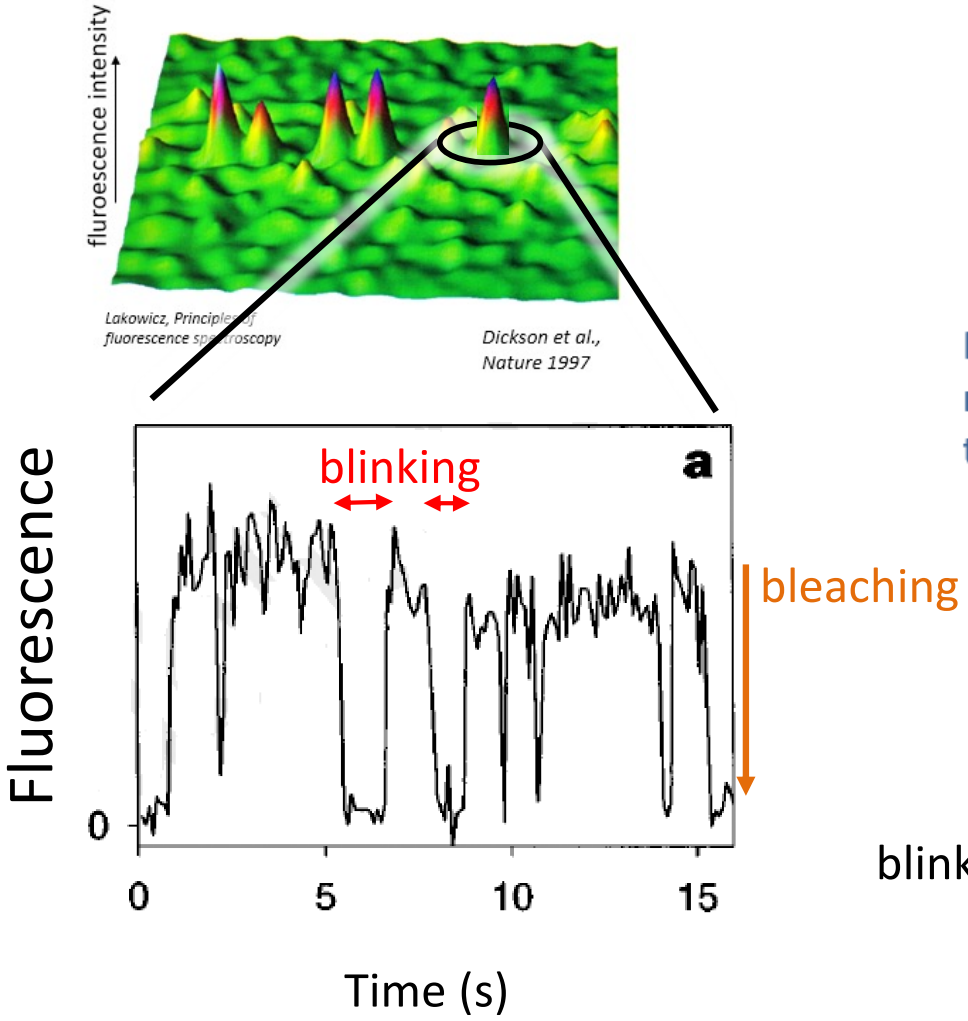
Lakowicz, Principles of fluorescence spectroscopy

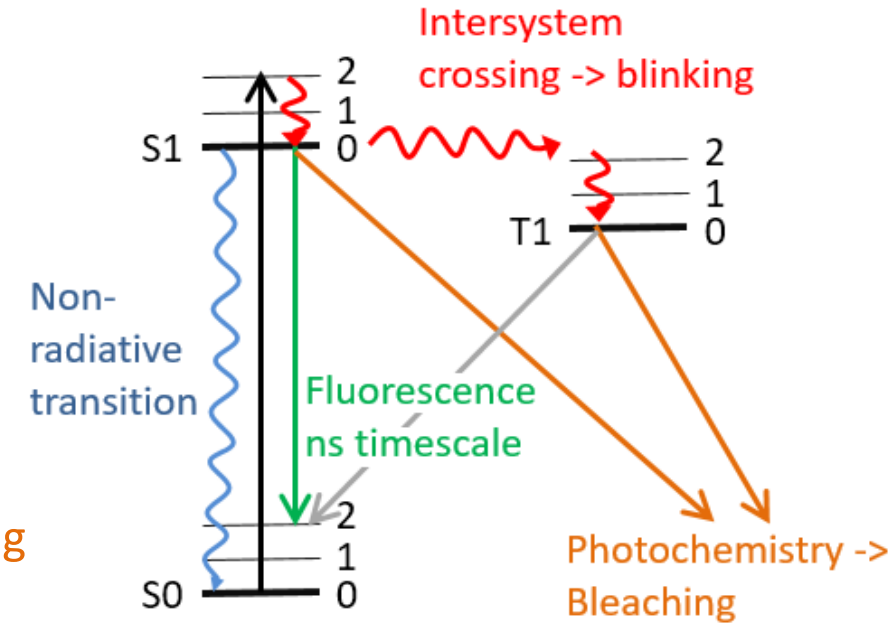
Dickson et al., Nature 1997



zeiss-campus.magnet.fsu.edu

How do we know these are single molecules?

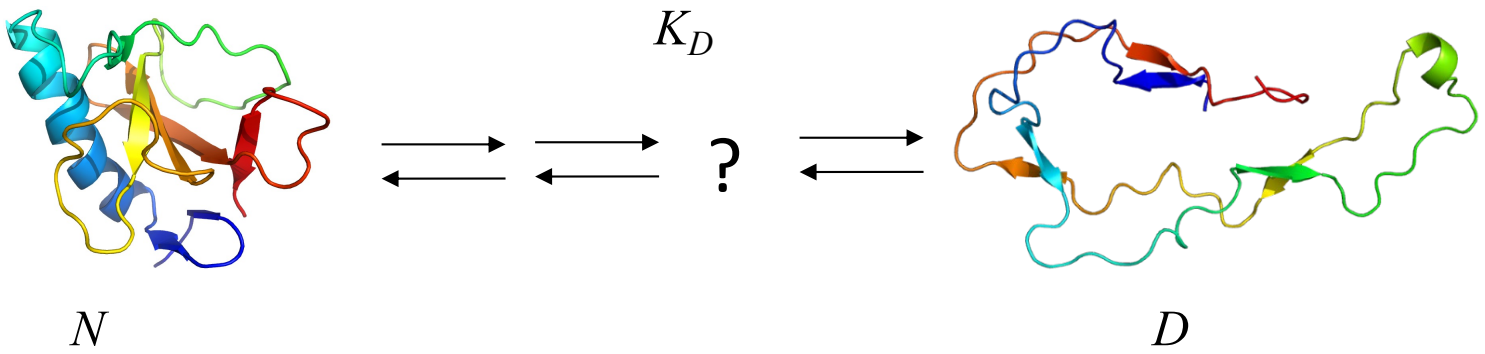




blinking

- Defined emission properties
- Bleach in one single step
- Blink in one single step

Design your own single-molecule experiment



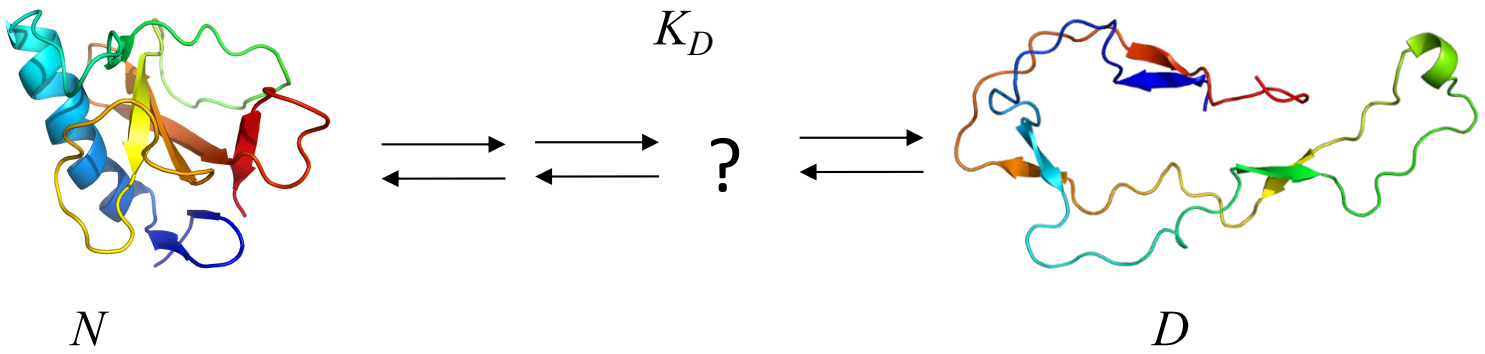
The questions you may ask yourself:

- What protein should I use?
- How do I make it unfold?
- How do I track single molecules? How do I track unfolding?
- Where would I attach a fluorophore? Should I use one fluorophore or more?
- Does it make sense to use FRET as a readout?
- Should I immobilize the molecule or look at it in solution?
- What microscopy setup is suitable for this experiment?
- Am I interested in an ensemble measurement or in tracking individual molecules?
- Etc.

Learning objectives

- **Understanding single-molecule experiments**: Gain a basic understanding of the purpose and methodology of single molecule experiments.
- **Molecular Labeling Techniques**: Grasp the most widely used method for labeling molecules for single molecule experiments.
- **Free vs. immobilized measurements**: Learn the differences between ensemble measurements and immobilized single molecule measurements, and their respective applications.
- **TIRF vs. confocal microscopy**: Understand the fundamental differences between TIRF (Total Internal Reflection Fluorescence) and confocal microscopy, and their relevance in single molecule experiments.
- **Conceptual application**: Begin to conceptualize how these fundamental aspects (measurement types, microscopy techniques, molecular labeling) are integrated and applied in specific experimental setups.

Design your own single-molecule experiment



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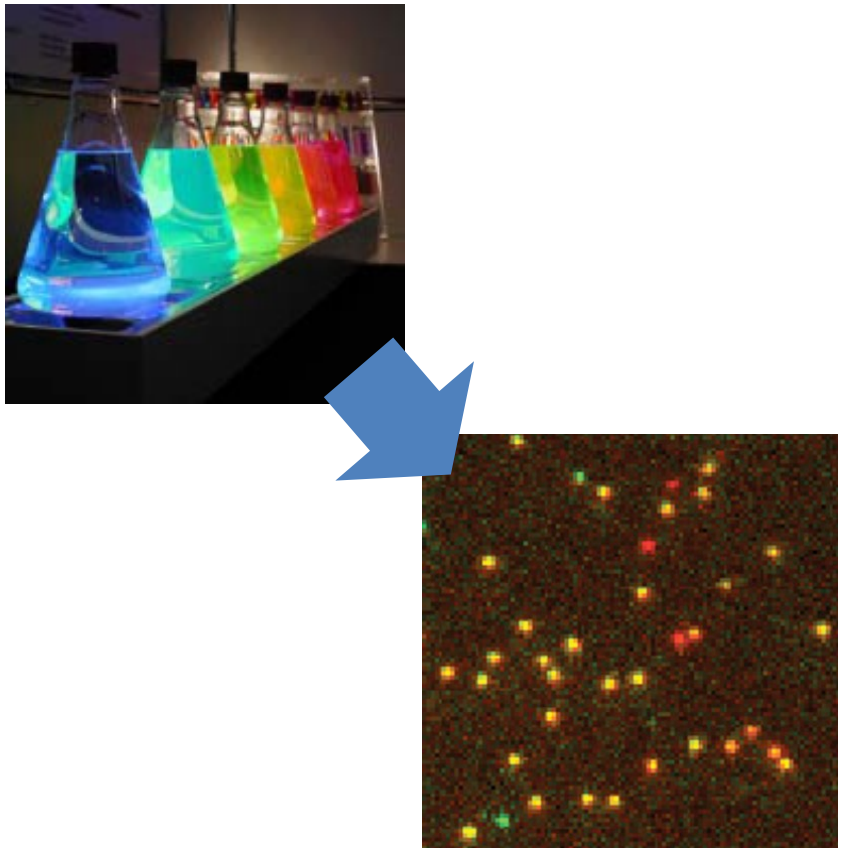
- Etc.

Requirements for dyes used in sm-fluorescence experiments

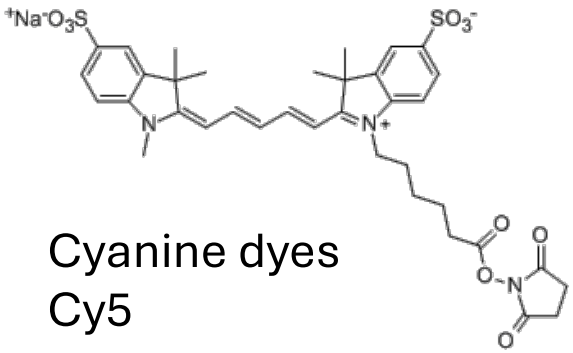
Pushing the limits to the single molecule scale!



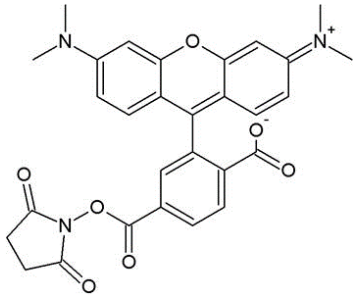
High sensitivity required to **detect $1/N_A$** ($1.6 \cdot 10^{-24}$ moles) of a molecule:

- Strong absorbance, high extinction coefficient ε
- For *in vivo* applications red-shifted absorbance is preferred
- Maximal fluorescence quantum yield
- Short fluorescence lifetime
- Low blinking probability (low probability of undergoing inter-system crossing)
- Photostability
- Small size

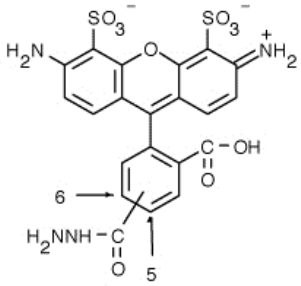
Fluorescent labels are required: dyes used for labeling



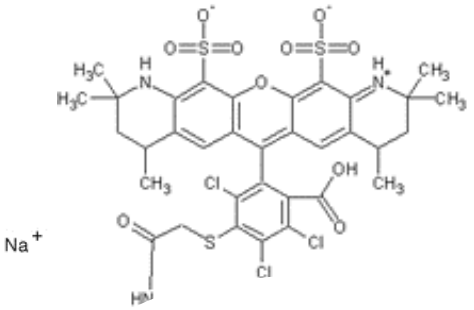
Cyanine dyes
Cy5



Tetramethyl rhodamine

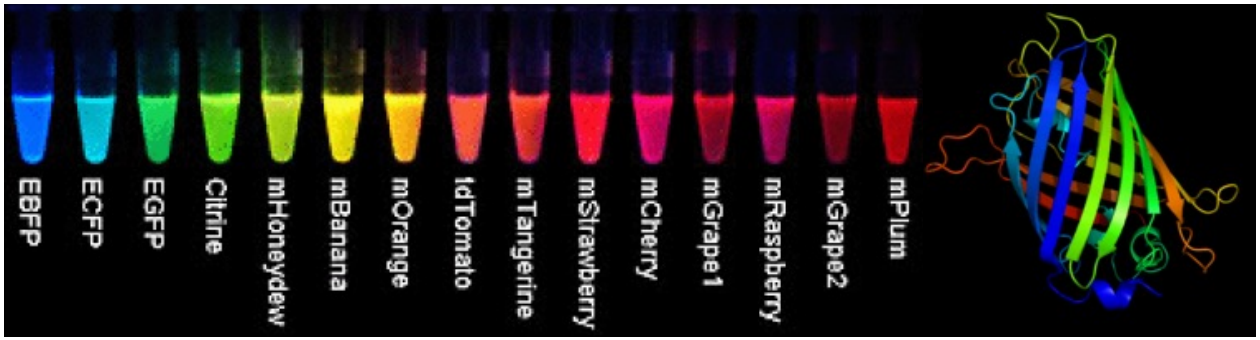


Alexa dyes
A488

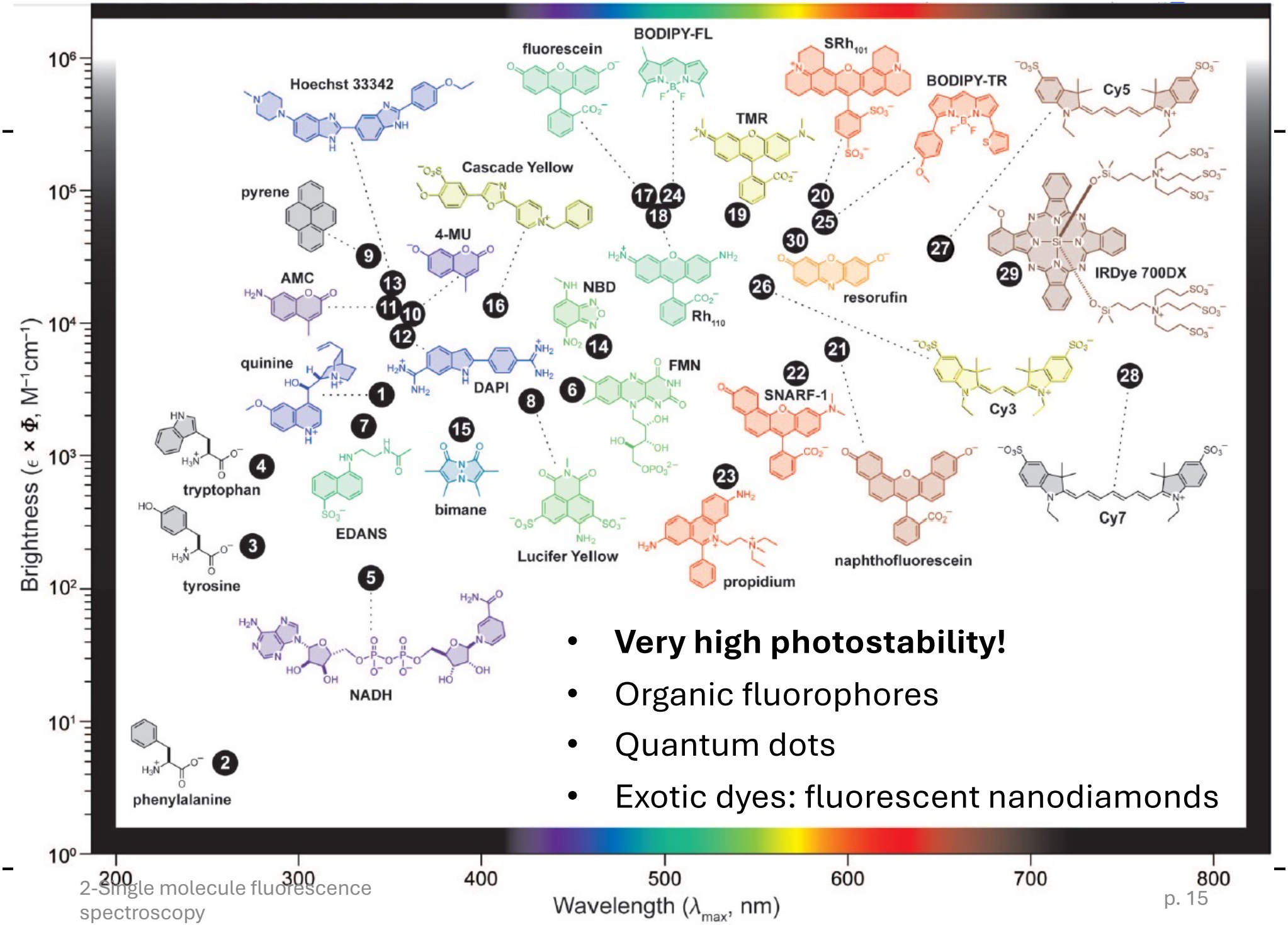


Alexa dyes
A546

Fluorescent proteins



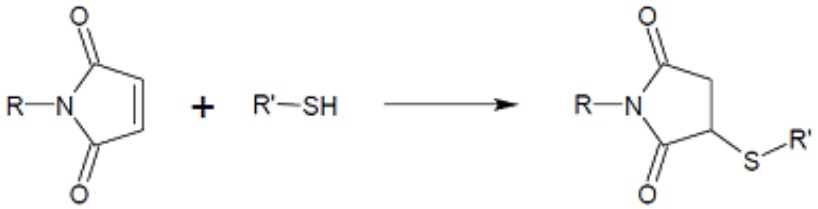
Tsien lab


Brightness (ε × Φ, M⁻¹cm⁻¹)
Wavelength (λmax, nm)
Hoechst 33342
fluorescein
BODIPY-FL
SRh101
BODIPY-TR
Cy5
TMR
Cascade Yellow
pyrene
4-MU
AMC
NBD
Rh110
resorufin
IRDye 700DX
quinine
DAPI
FMN
SNARF-1
Cy3
tryptophan
EDANS
bimane
Lucifer Yellow
propidium
naphthofluorescein
Cy7
tyrosine
NADH
phenylalanine
• Very high photostability!
• Organic fluorophores
• Quantum dots
• Exotic dyes: fluorescent nanodiamonds

Labeling methods

Thiol reactive probes

Maleimides



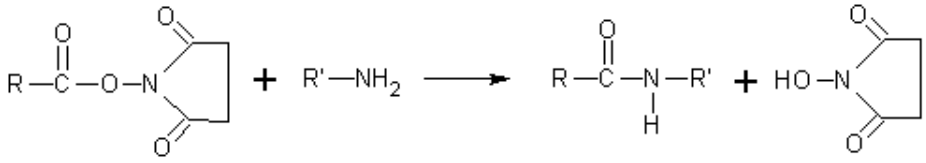
Iodoacetamide

Proteins:
Cysteine are rare and have unique chemical reactivity

Oligonucleotides:
Thiols are reactive and can be introduced at termini

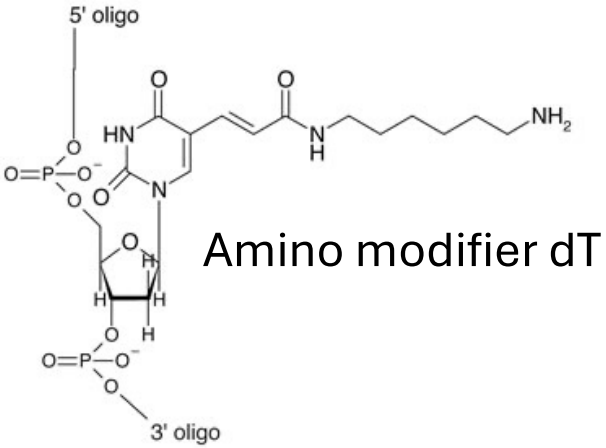
Amine reactive probes

NHS (N-hydroxysuccinimide) ester



Proteins:
non-specific labeling at lysine side chains, N-terminus

Oligonucleotides:
Specific introduction of amines at termini or in the interior of an oligo

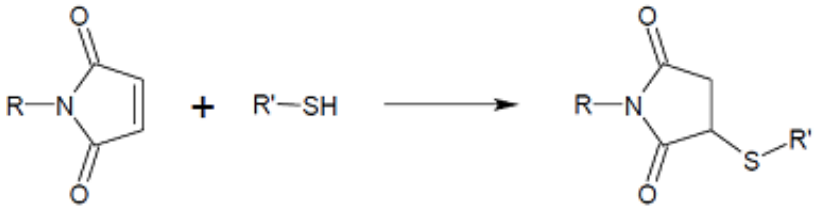


Amino modifier dT

Labeling methods - cysteines

Thiol reactive probes

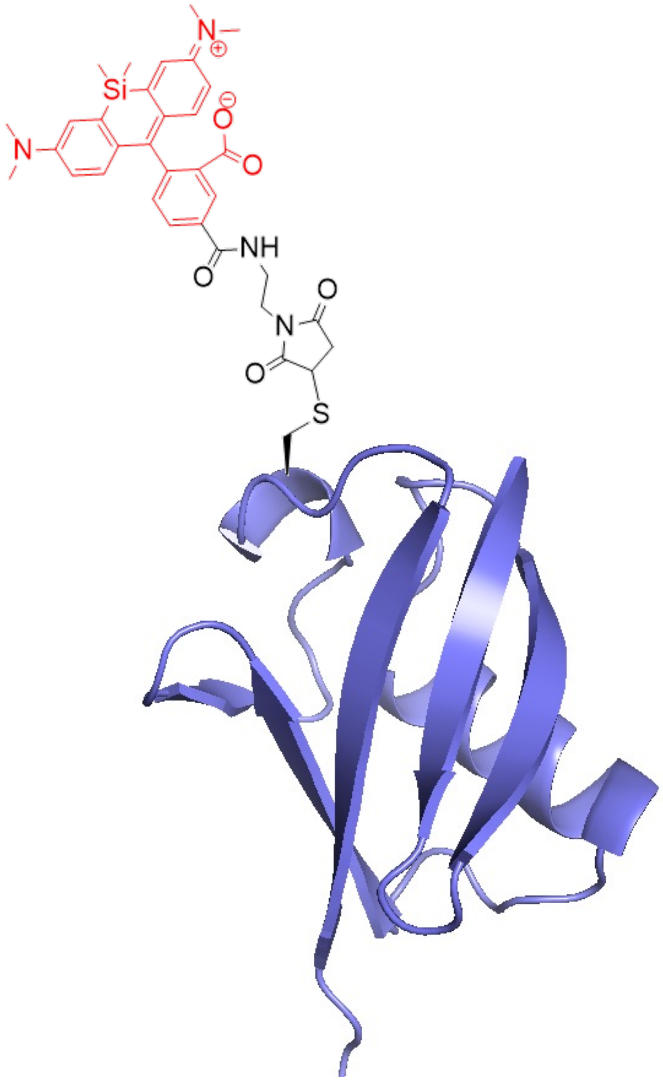
maleimides



iodoacetamide

Proteins:
Cysteine are rare and have unique chemical reactivity

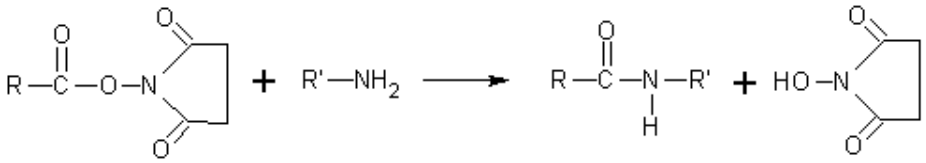
Oligonucleotides:
Thiols can be introduced at termini or internal positions



Labeling methods - amines

Amine reactive probes

NHS ester



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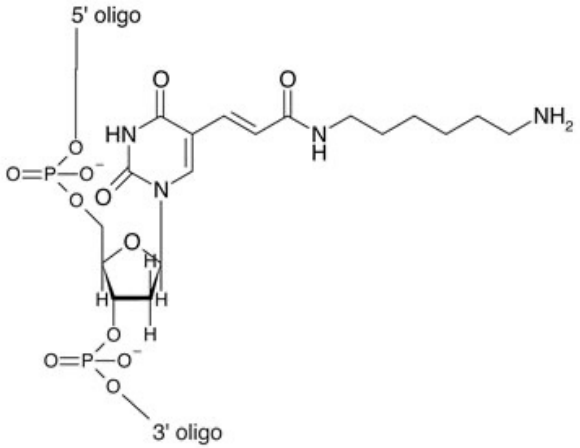
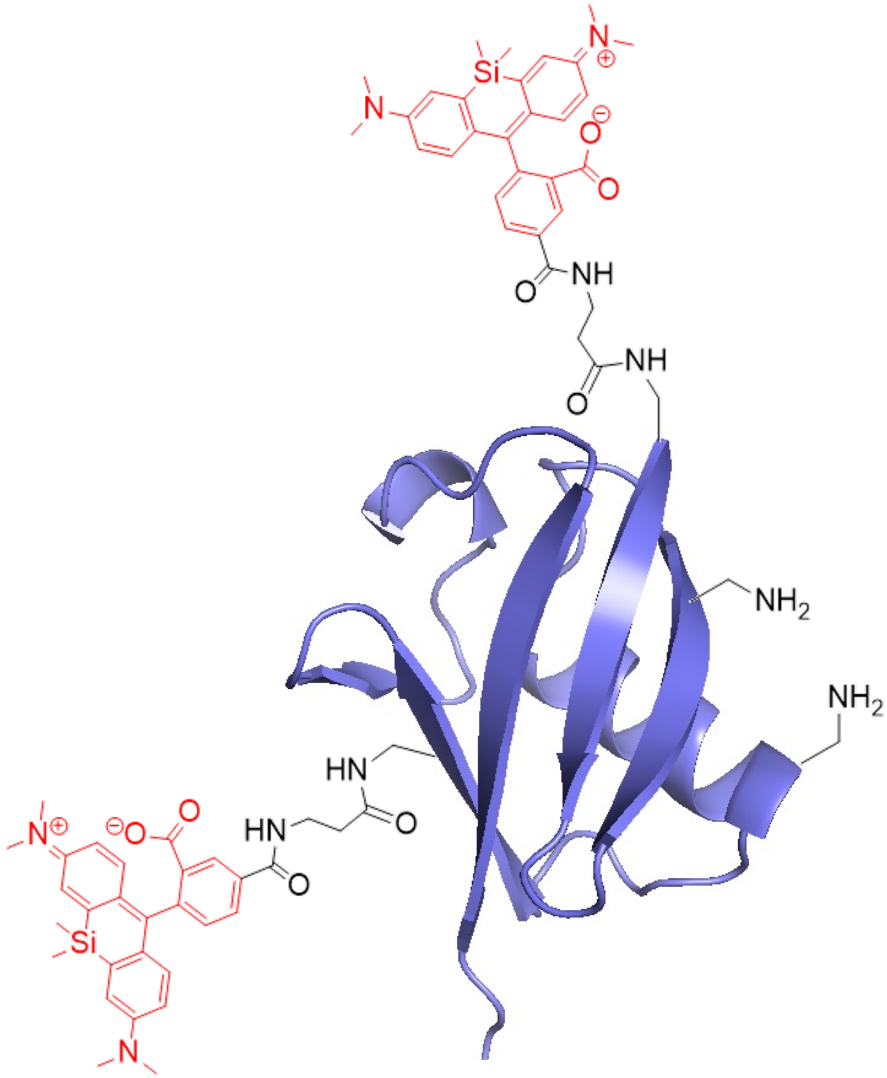


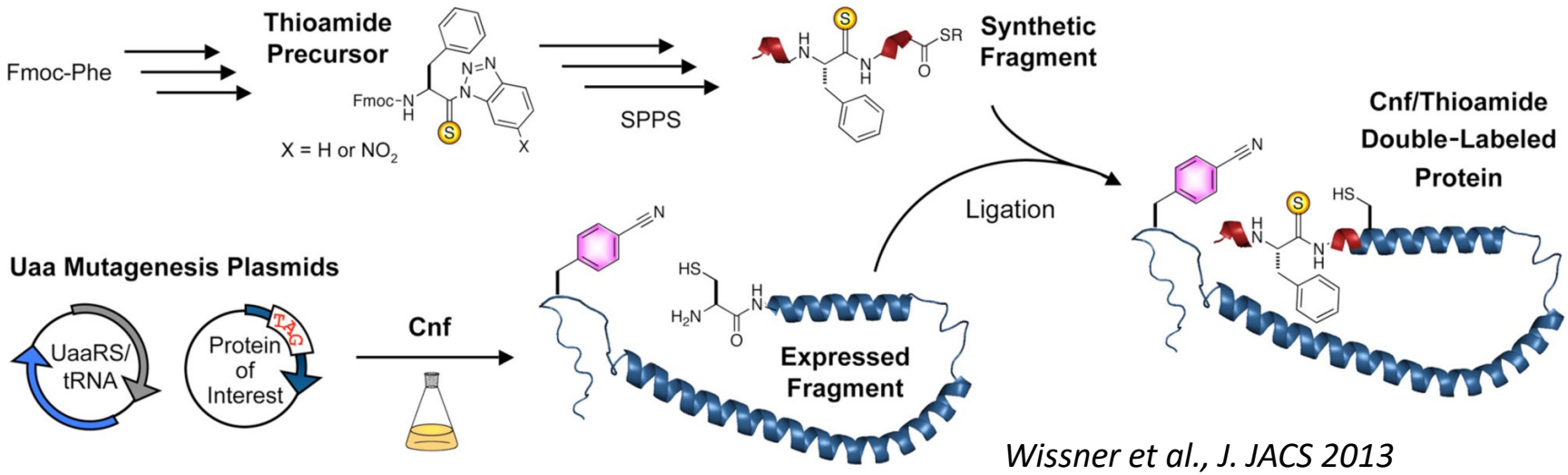
Amino modifier dT



Other ways to install fluorophores

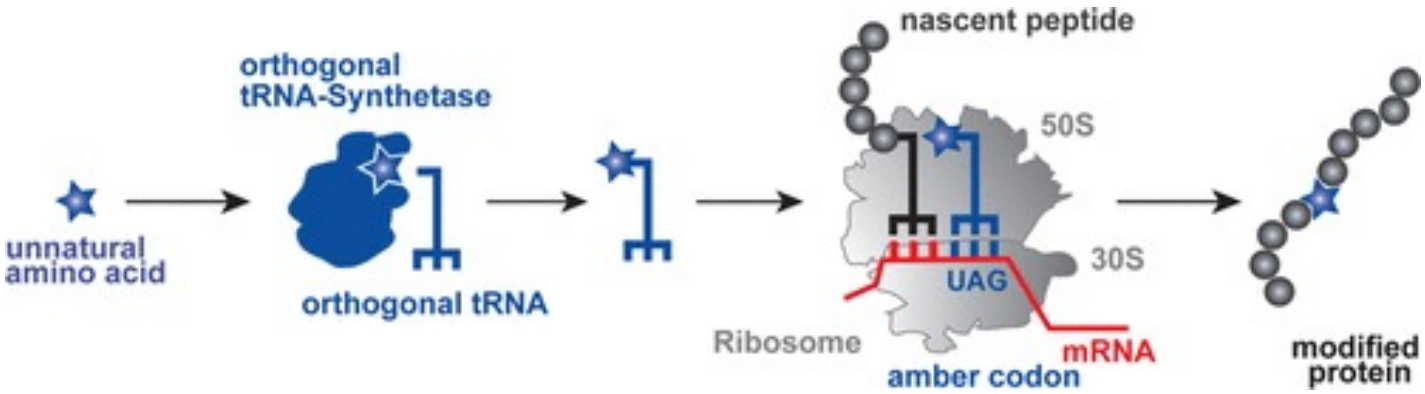
- Labeling of **protein termini** (particularly N-terminus)
- Using **noncanonical amino acid incorporation** through amber suppression combined with **click chemistry**
- **Protein tags:** SNAP-Tag, HaloTag (these are big!)

Often the most challenging part of a sm-experiment is to find a viable strategy for the synthesis of a doubly labeled protein, e.g.



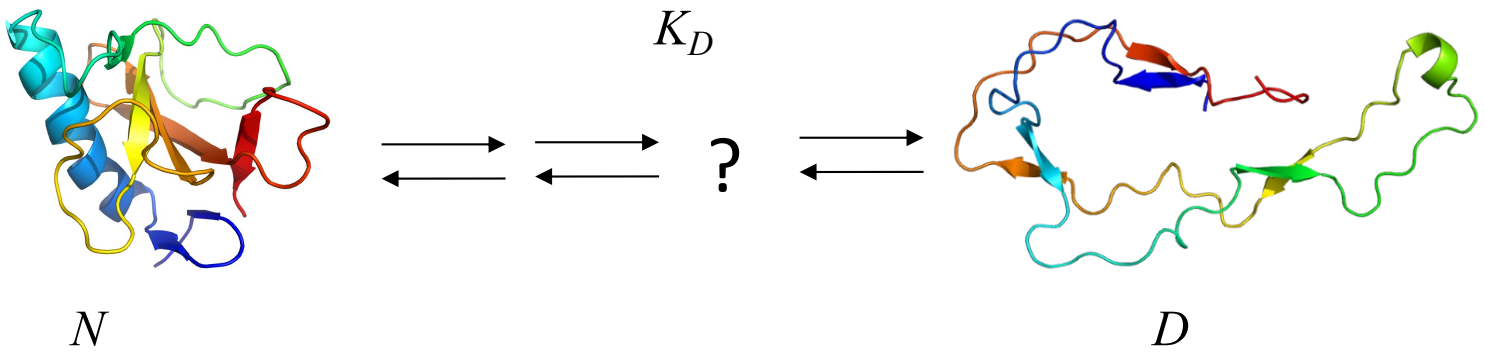
Wissner et al., J. JACS 2013
https://pubs.acs.org/doi/10.1021/ja4005943

Genetic code expansion to incorporate unnatural amino acids



Great review on the topic: https://pubs.acs.org/doi/10.1021/cr400355w

Design your own single-molecule experiment



The questions you may ask yourself:

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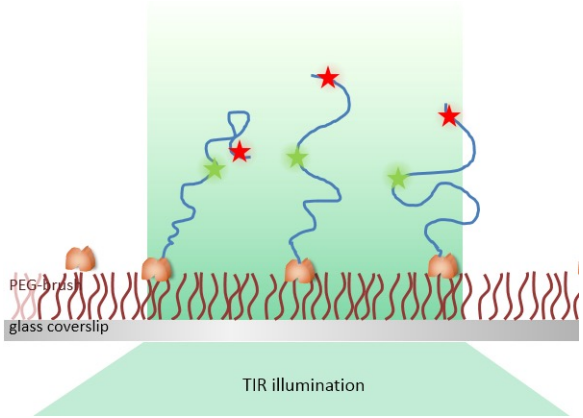
- **What microscopy setup is suitable for this experiment?**
- Am I interested in an ensemble measurement or in tracking individual molecules?
- Etc.

Following fixed or free molecules

Immobilized molecules:

molecules are immobilized on a surface (coverslip) and can be observed for a long time, e.g. **by total internal reflection fluorescence (TIRF) microscopy**

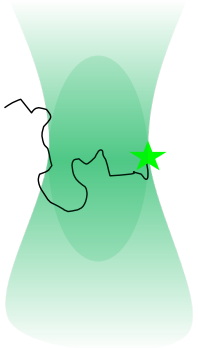
- Imaging and counting single molecules
- internal dynamics
- interaction dynamics
- Colocalization analysis



Freely diffusing molecules:

fluorescently labeled molecules are observed at very high dilution free in solution, using a **confocal microscope:**

- FRET statistics
- Burst analysis
- Photon counting histograms
- only fast dynamics are monitored (μs)



Total Internal Reflection Fluorescence (TIRF) Microscopy

Restriction of the sample volume at an interface:

Total internal reflection

at a boundary of changing refractive index, light arriving at a critical angle will be reflected (swimming pool!)

Critical (TIR) angle: (complete reflection)

$$\Theta_C = sin^{-1}\left(\frac{n_1}{n_2}\right)$$

n_1 and n_2: refractive indices of media

However, an **evanescent field** penetrates into solution (100-200 nm):

$$I(z) = \mathrm{I}_0 \mathrm{e}^{-\frac{z}{d}}$$

z: distance from the surface
d: penetration depth

$$d = \frac{\lambda}{4\pi}(n_2^2 sin\Theta_2 - n_1^2)^{-1/2}$$

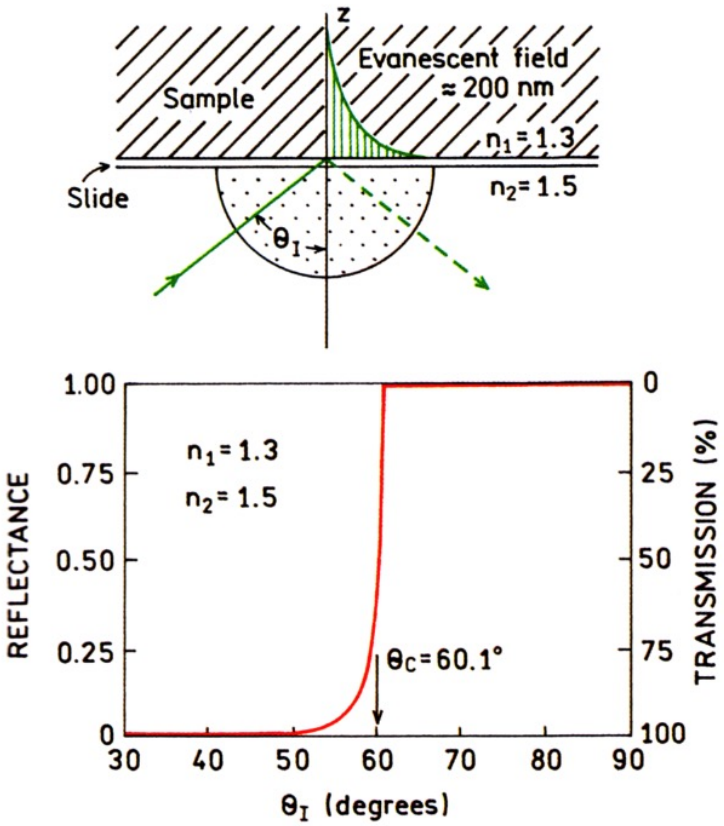


Figure 23.5. Top: Optical geometry for total internal reflection (TIR). **Bottom**: Calculated reflectance and transmittance for n_2 = 1.5 and n_1 = 1.3.

- **n_2 must be greater than n_1**
- **Θ must be greater than Θ_c**

Why is there an evanescent field?

1. Electromagnetic Wave Nature of Light

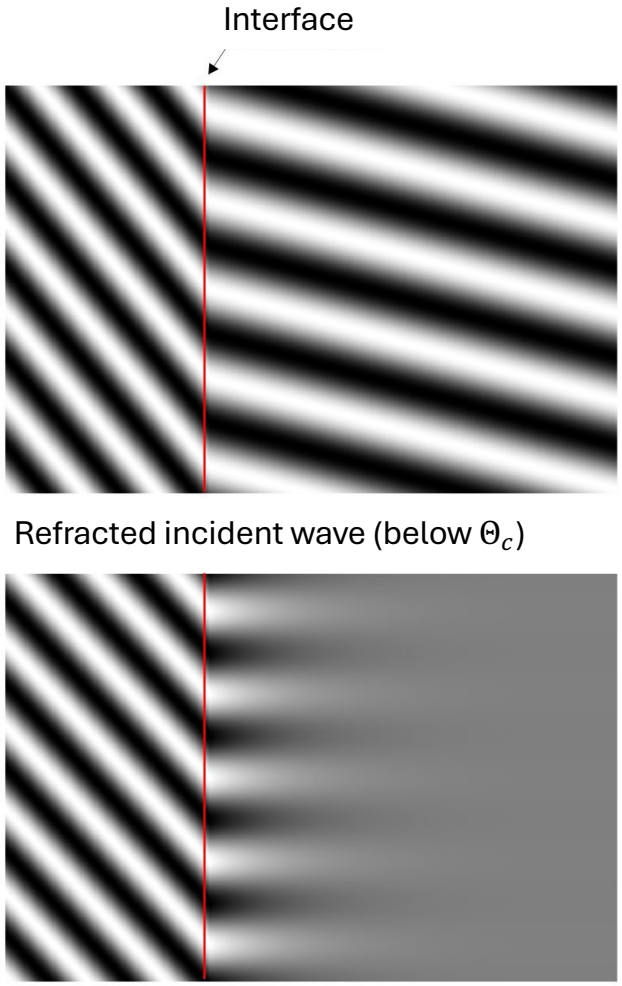
Light behaves as an electromagnetic wave, meaning it has both electric and magnetic field components that oscillate as it propagates.

At an interface, the **wave components must satisfy boundary conditions**, which require the electric field in the second medium (lower refractive index) to be nonzero, even though the light does not propagate into it.

2. Continuity of the Electric Field

When light undergoes total internal reflection (TIR) at the interface, **Maxwell's equations require that the electric field must be continuous across the boundary**.

This continuity condition forces the formation of an **exponentially decaying field**—the evanescent wave—**that extends into the second medium.**

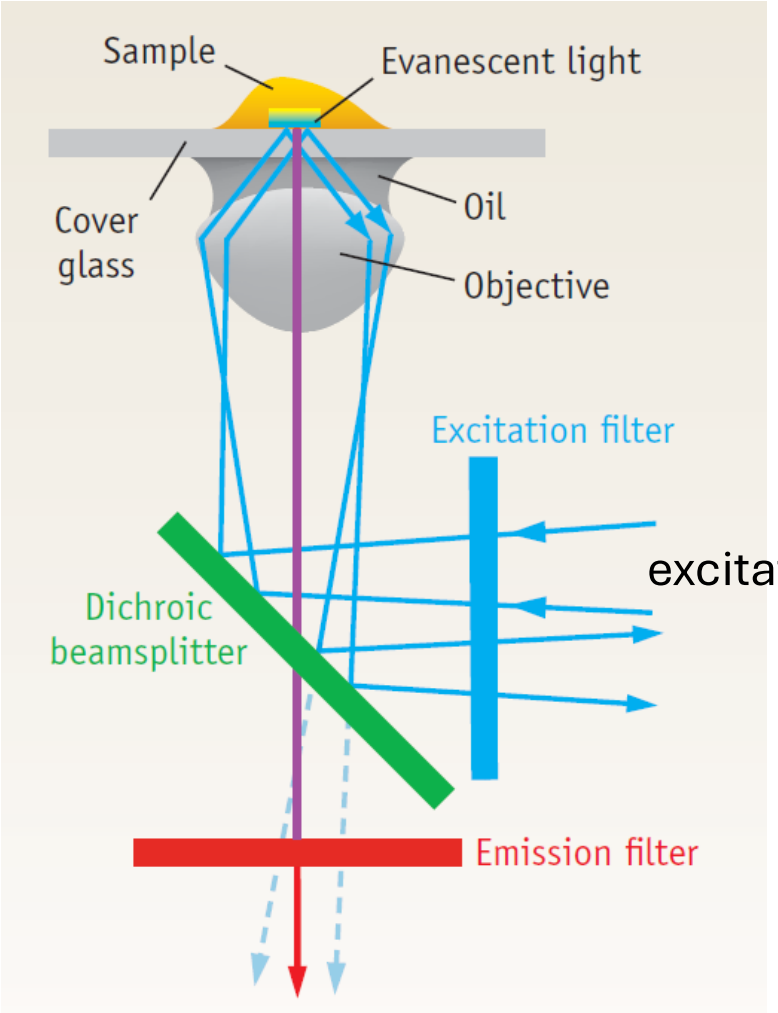


Refracted incident wave (below Θ_c)

Evanescent wave
(reflected waves not shown)

Image source: Wikipedia "Evanescent Field"
https://en.wikipedia.org/wiki/Evanescent_field

Microscope Setup



Normal microscope setup (widefield -> imaging of a large area)

Objective with very **high numerical aperture** required (NA > 1.38, ideally NA = 1.45)

Excitation with **lasers** for even illumination, high intensity

Detection using **EMCCD cameras** -> this allows the imaging of many single molecules at the same time, however at lower time resolution compared to confocal microscopy (ms)

Activity:
explain TIRF microscopy to each other

What questions do you have?

What is not clear?

This is a great video explaining TIRFM:
https://www.youtube.com/watch?v=lUrV6NrMRVg

Numerical aperture

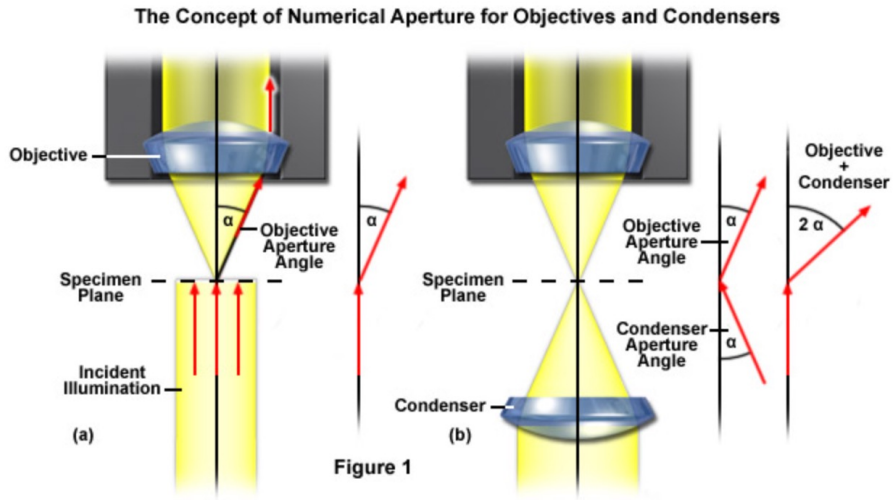


Figure 1

In order to enable two objectives to be compared and to obtain a quantitative handle on resolution, the numerical aperture, or the measure of the solid angle covered by an objective is defined as:

$$\text{Numerical Aperture (NA)} = \eta \cdot \sin(\alpha) \quad (1)$$

where **α** equals one-half of the objective's opening angle and **η** is the refractive index of the immersion medium used between the objective and the cover slip protecting the specimen (η = 1 for air; η = 1.51 for oil or glass). By examining **Equation (1)**, it is apparent that the refractive index is the limiting factor in achieving numerical apertures greater than 1.0. Therefore, in order to obtain higher working numerical apertures, the refractive index of the medium between the front lens of the objective and the specimen cover slip must be increased. The highest angular aperture obtainable with a standard microscope objective would theoretically be 180 degrees, resulting in a value of 90 degrees for the half-angle used in the numerical aperture equation. The sine of 90 degrees is equal to one, which suggests that numerical aperture is limited not only by the angular aperture, but also by the imaging medium refractive index. Practically, aperture angles exceeding 70 to 80 degrees are found only in the highest-performance objectives that typically cost thousands of dollars.

https://zeiss-campus.magnet.fsu.edu/articles/basics/resolution.html#:~:text=The%20numerical%20aperture%20of%20a,in%20Figure%201(a).

Why is TIRF microscopy required?

50 nM of a labeled protein

Moving in and out of the TIRF angle (Fierz lab)

Movement in and out of the TIRF angle: Under non-TIRF conditions, the background fluorescence is overwhelming the signal completely.

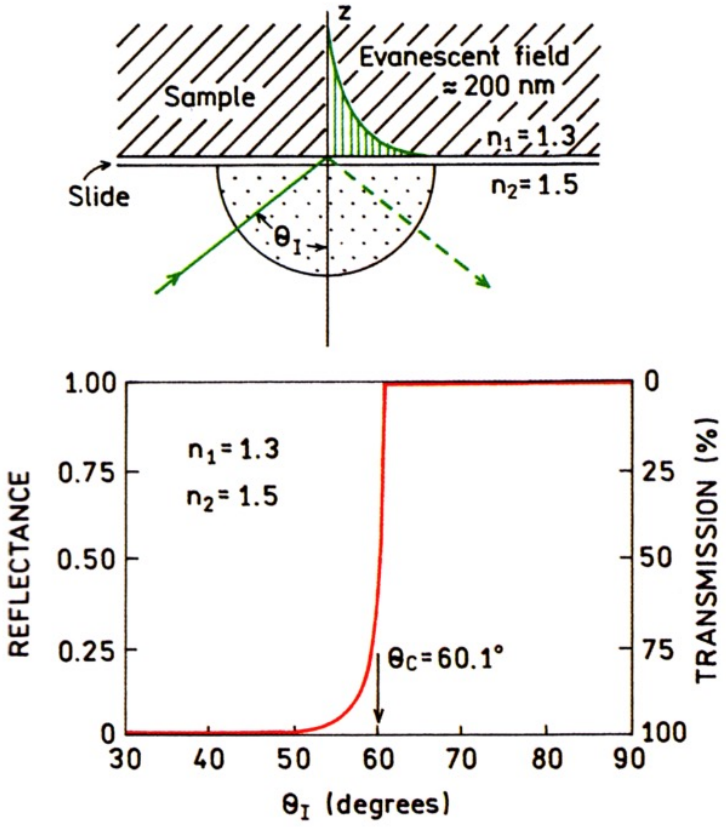
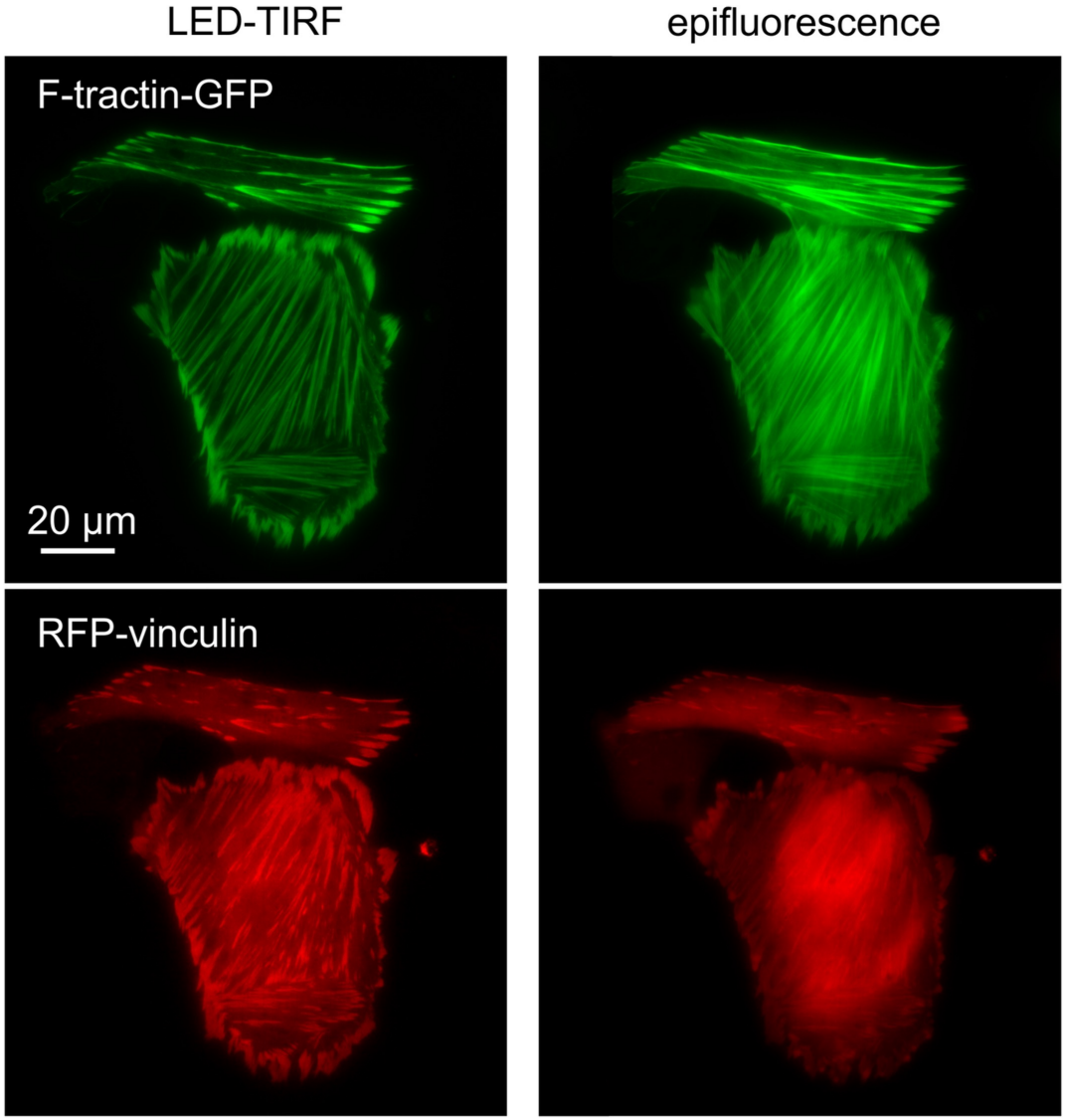


Figure 23.5. **Top**: Optical geometry for total internal reflection (TIR). **Bottom**: Calculated reflectance and transmittance for n_2 = 1.5 and n_1 = 1.3.

Epi- vs. total internal reflection fluorescence



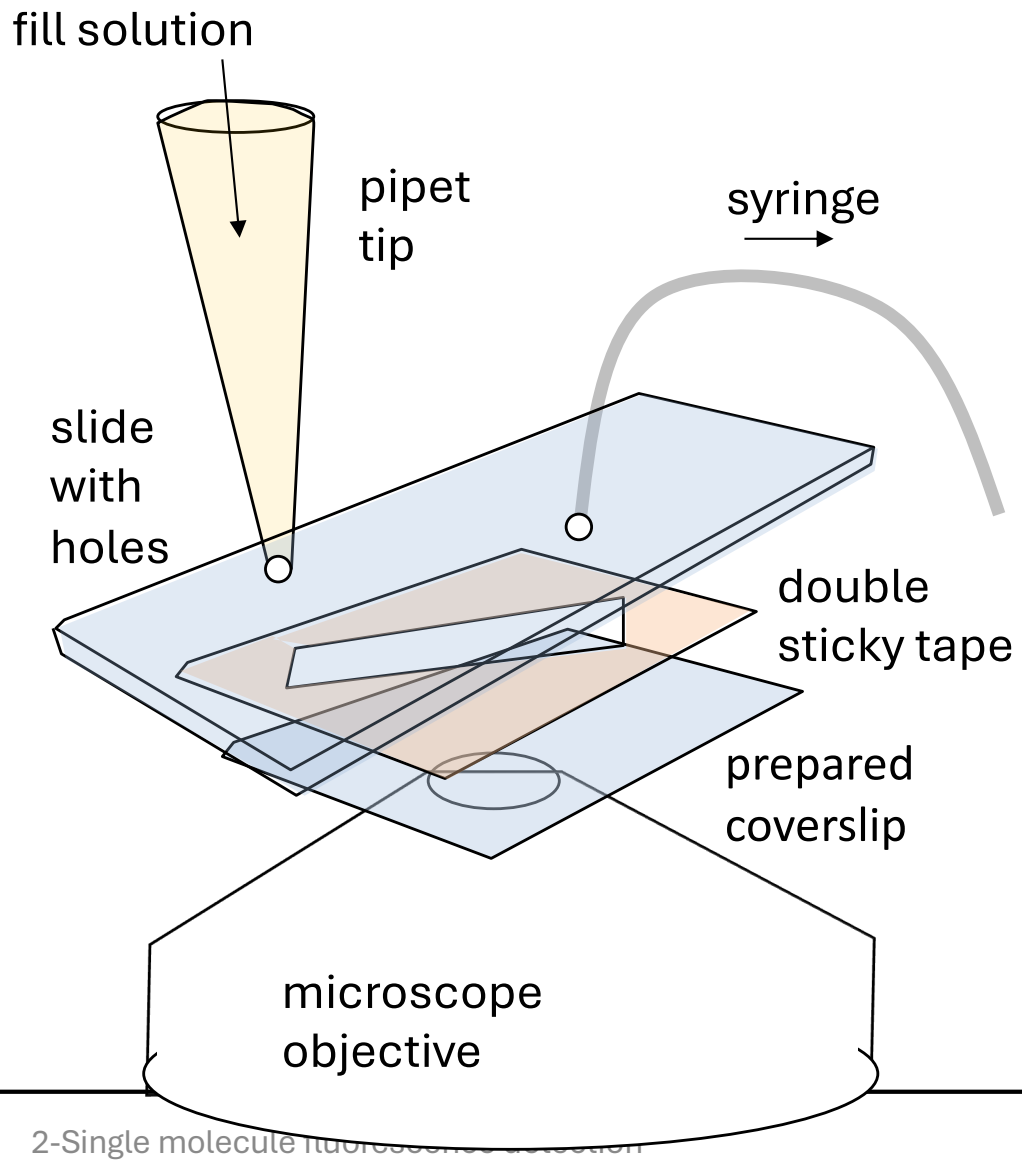
Good review of TIRF basics:

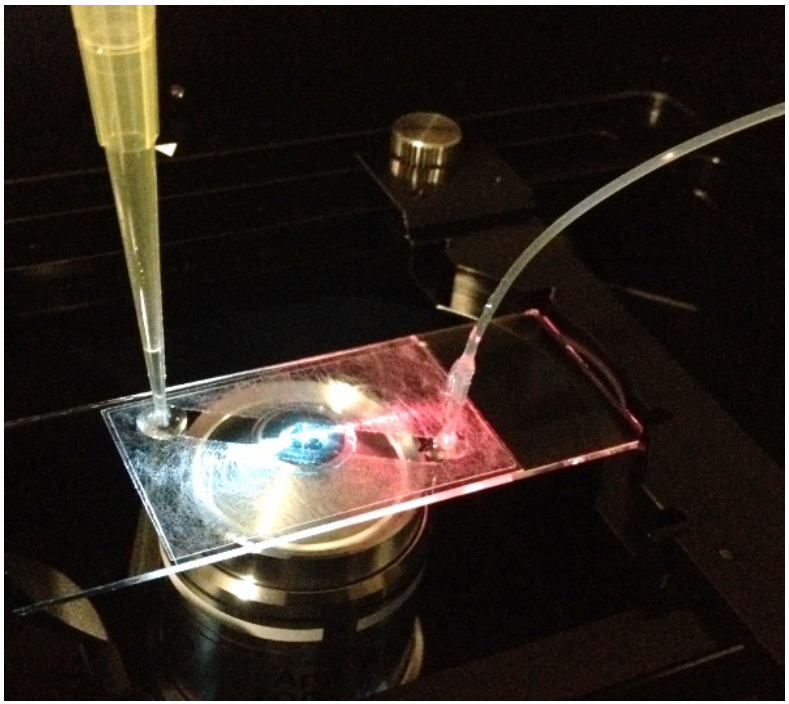
https://www.microscopyu.com/techniques/fluorescence/total-internal-reflection-fluorescence-tirf-microscopy

Kogel et al., J. Biophotonics 2019

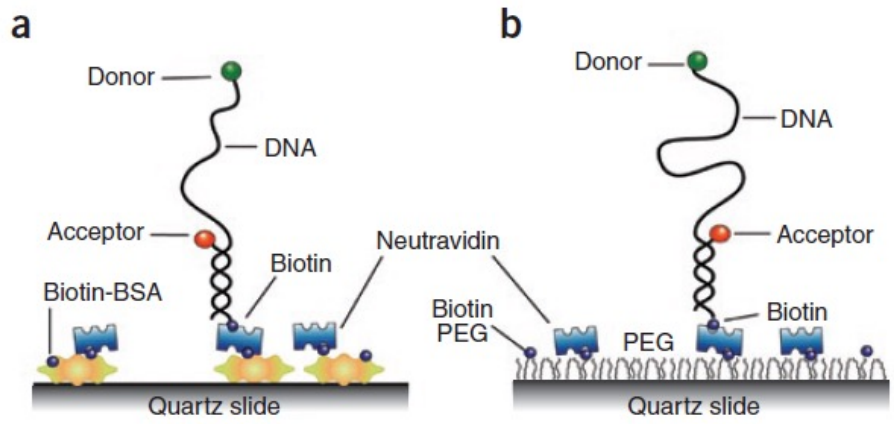
https://doi.org/10.1002/jbio.201900033

Flowcell construction





Immobilization Strategies



TIRF microscopy requires molecule immobilization on the surface:

Surface passivation:

Bovine serum albumin (BSA)

PEG layer

Attachment chemistry:

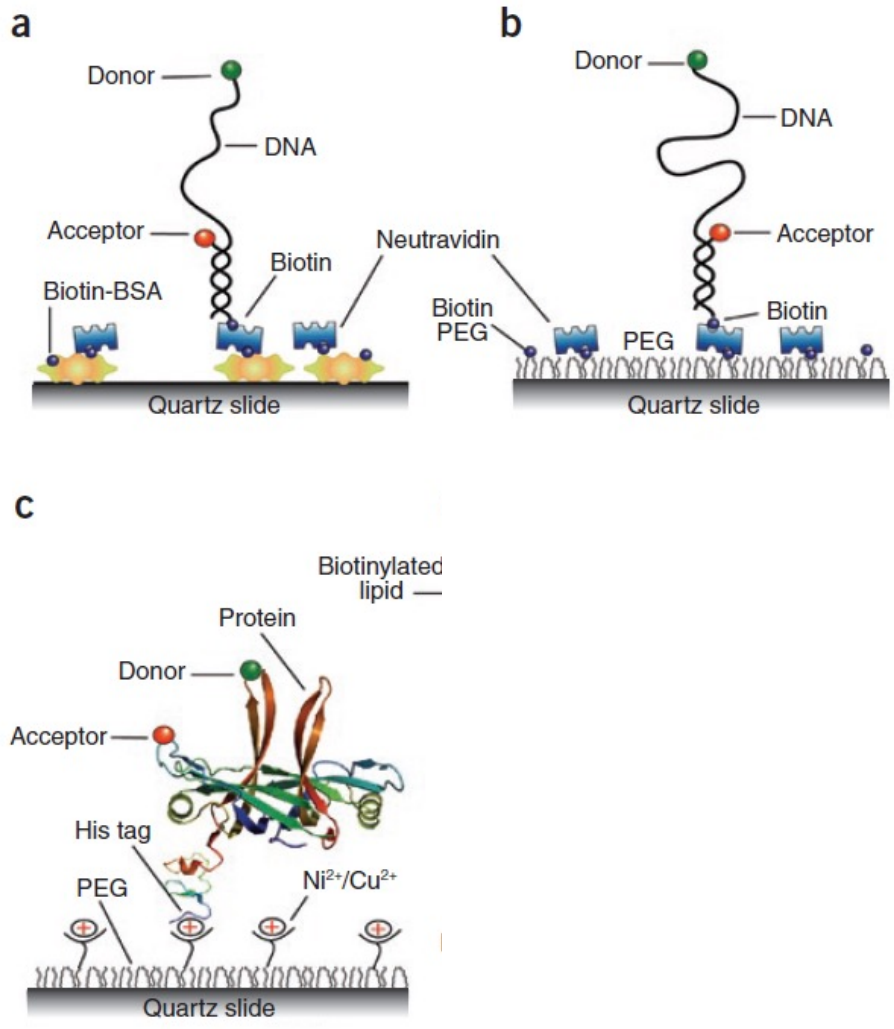
Biotin-avidin

Ni^{2+}:NTA – His_6 interaction

Encapsulation in lipid vesicles

Roy et al. Nat Methods 2008

Immobilization Strategies



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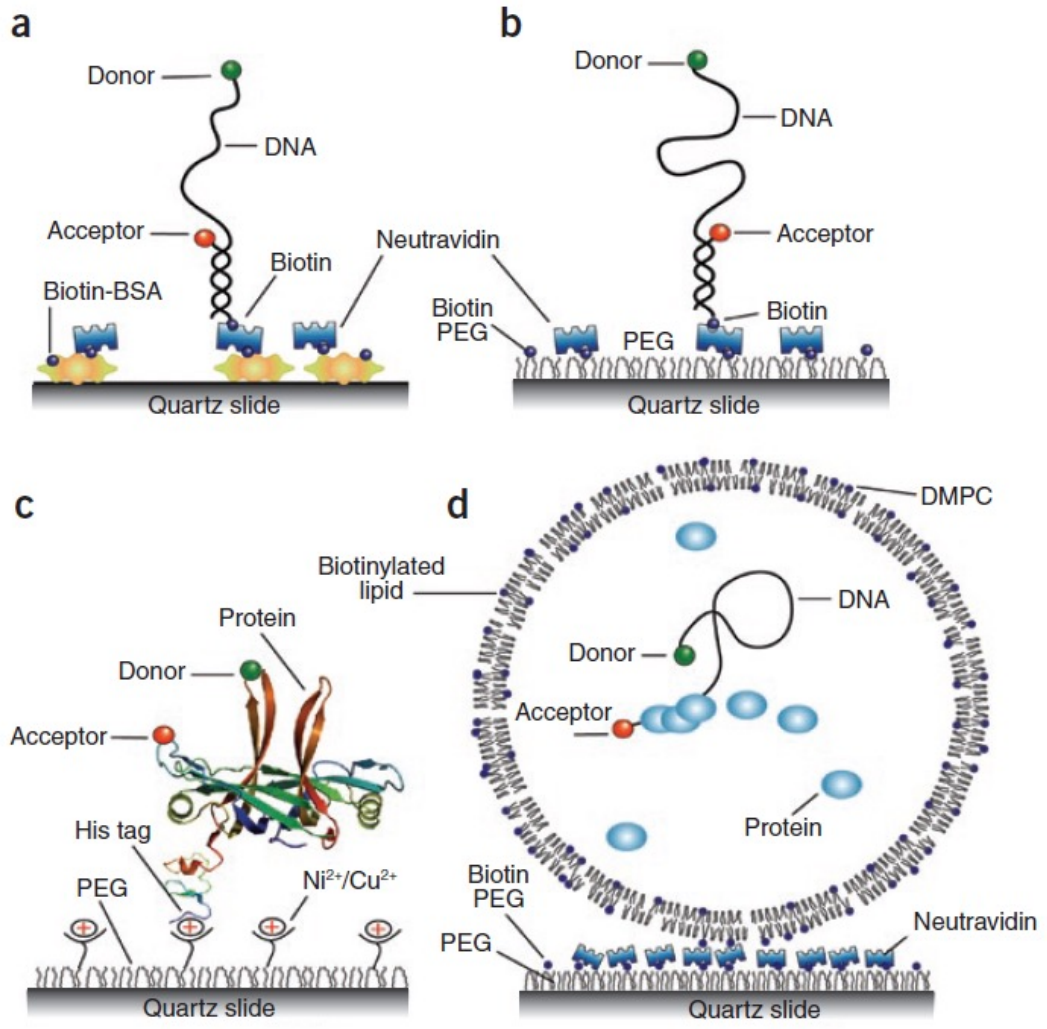
Immobilization Strategies



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PEG layer

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Biotin-avidin

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Encapsulation in lipid vesicles

Roy et al. Nat Methods 2008

Case study 1

Extreme Bendability of DNA Less than 100 Base Pairs Long Revealed by Single-Molecule Cyclization

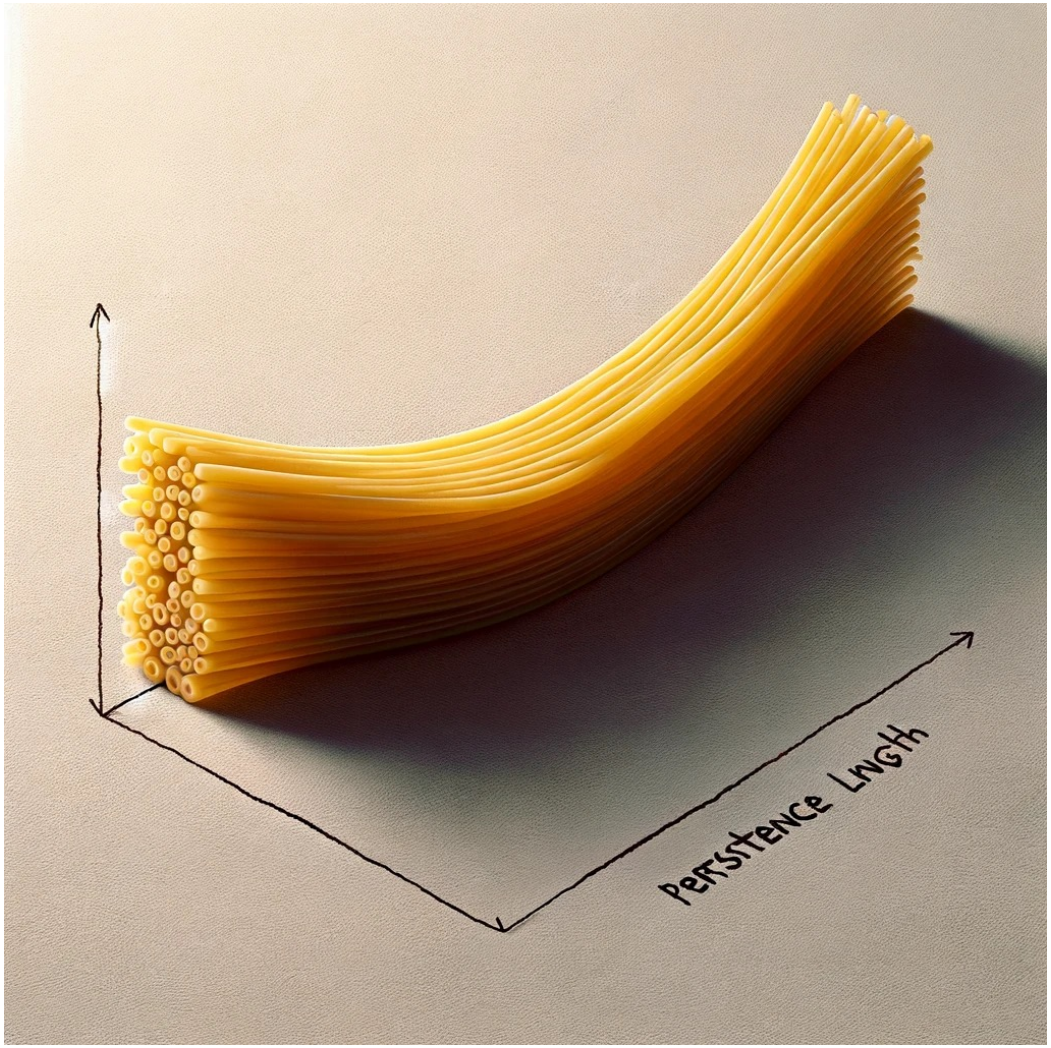
Reza Vafabakhsh[1] and Taekjip Ha[1,2]* Science 2012

Question:
DNA is stiff (persistence length ~150 bp). However, studies have indicated that it can bend below this length.

The **persistence length** measures how far along a polymer remains directionally correlated before bending significantly.

→ The authors use a single-molecule assay to check in real time, if bending occur.

DNA: raw or cooked spaghetti?



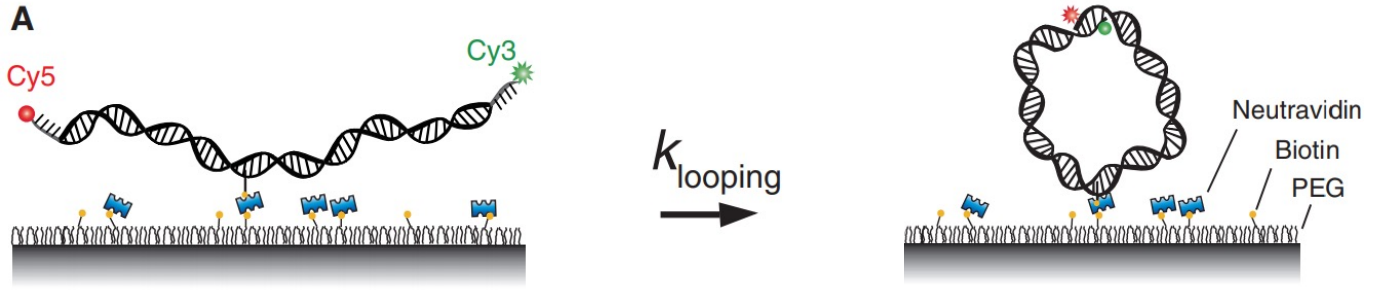
Raw spaghetti (rigid) =
high persistence length

Cooked spaghetti (flexible) =
low persistence length

Image source: ChatGPT

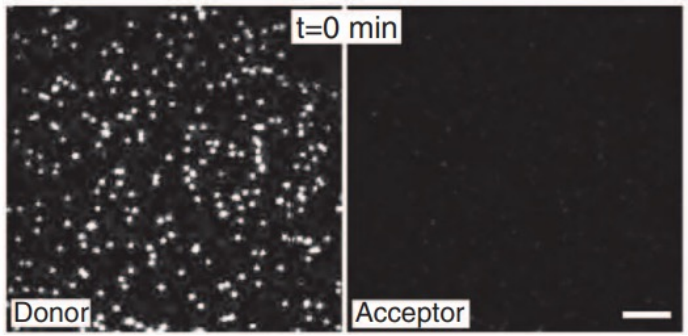
Observing DNA circle formation in real time

DNA: 70 bp



long sticky ends: permanent circle formation

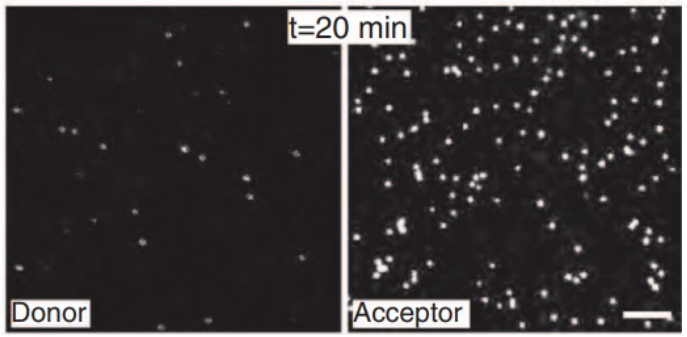
low FRET



Ex Cy3
Em Cy3

Ex Cy3
Em Cy5

high FRET

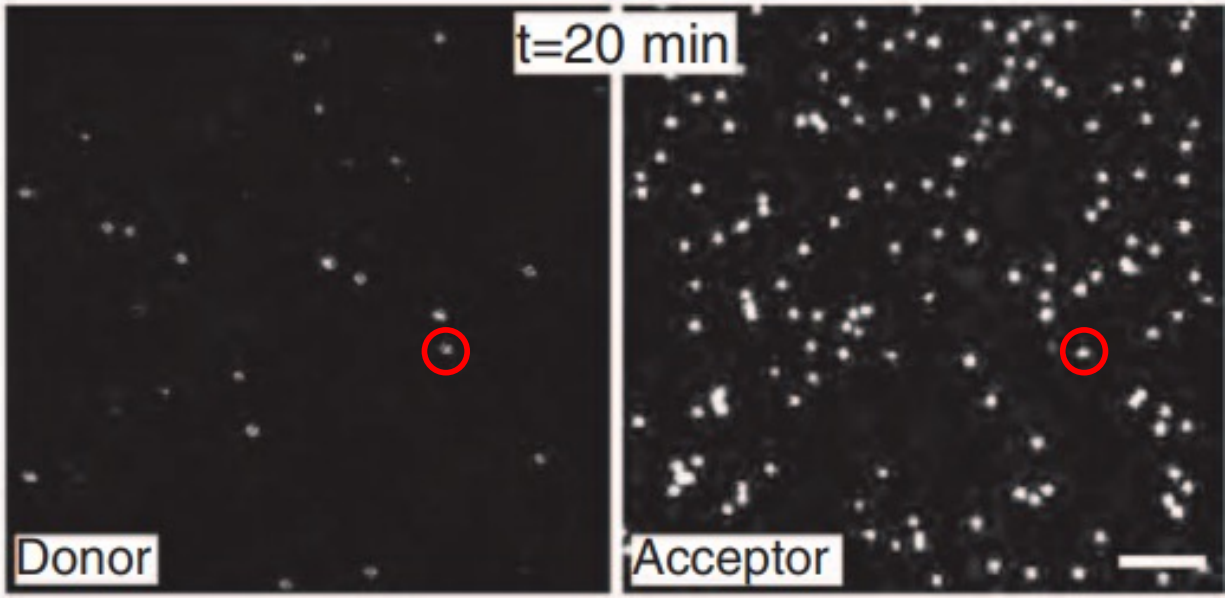


Ex Cy3
Em Cy3

Ex Cy3
Em Cy5

Vafabakhsh, Ha, Science 2012

Constructing FRET histograms



1. Select the same spot in both channels

2. Measure the emission intensities for all molecules

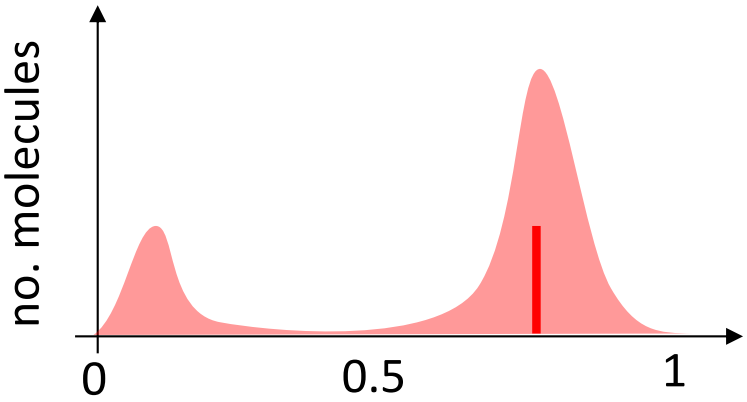
I_A , I_D

3. Calculate FRET

$E_{FRET} = I_A - \beta I_D / ([I_A - \beta I_D] + \gamma I_D)$

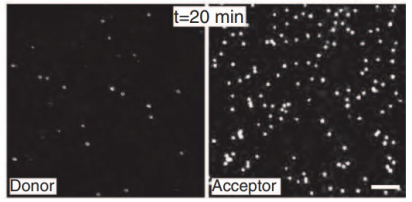
for all molecules

4. Construct FRET histogram for all molecules



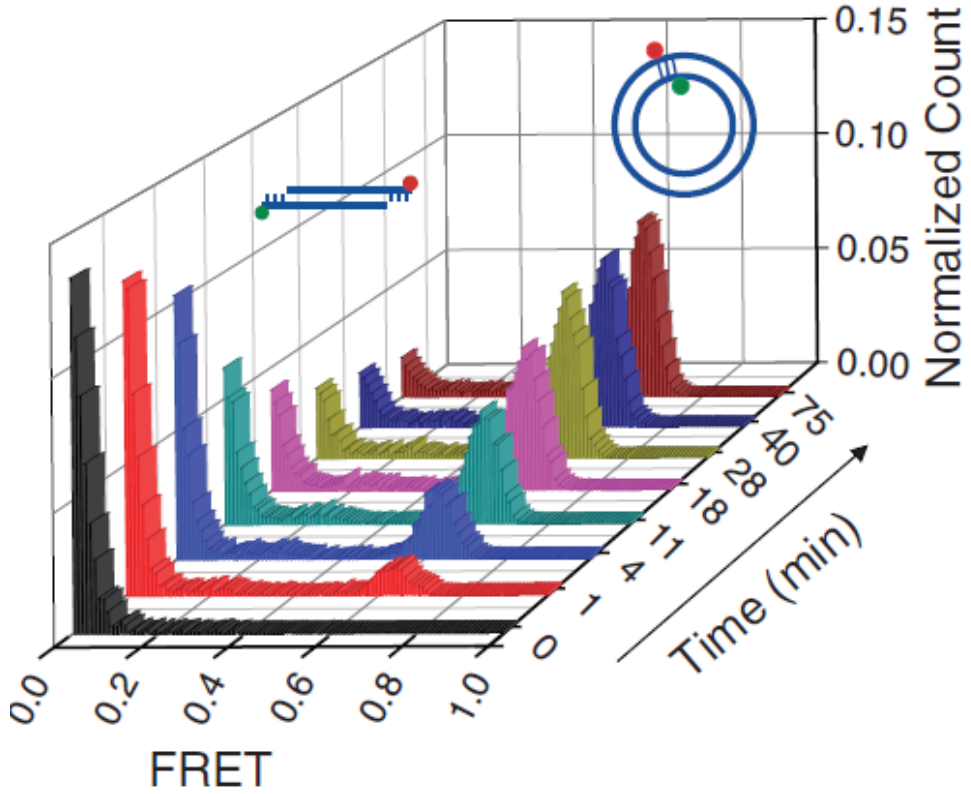
parameters:
β channel bleedthrough
γ detection efficiency for I_A and I_D

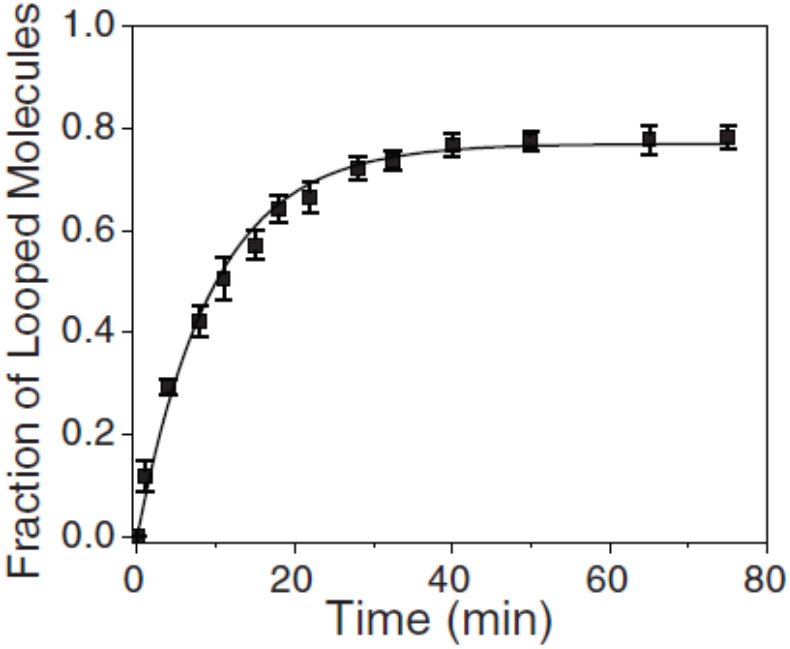
Observing DNA circle formation in real time



$$E_{FRET} = I_A - \beta I_D / ([I_A - \beta I_D] + \gamma I_D)$$

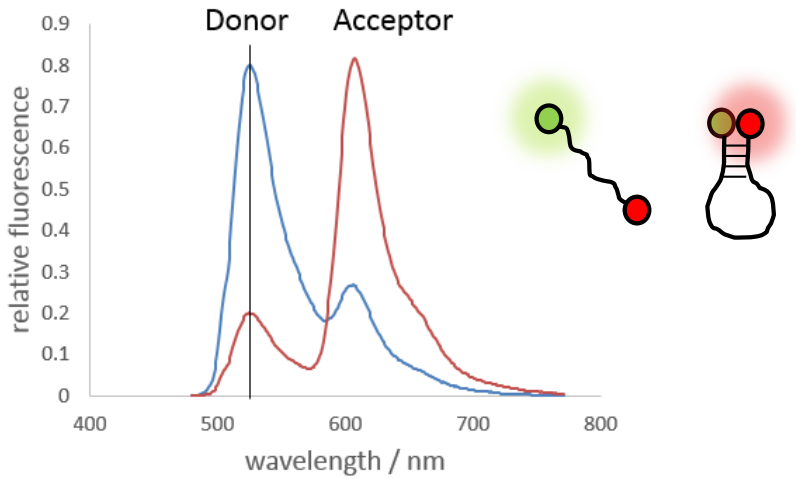
long sticky ends: permanent circle formation



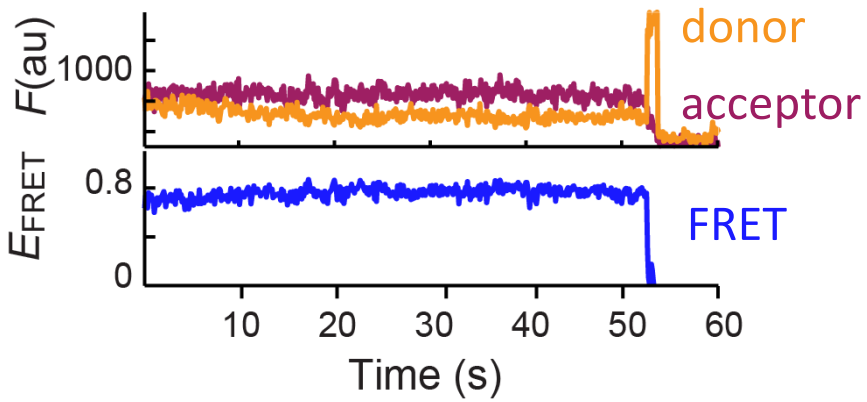


after ~20 min, **all DNA circles have formed**

Measuring the efficiency of energy transfer



Blue: open state
Red: closed state



FRET efficiency:

$$E_{FRET} = I_A / (I_A + I_D)$$

I_D : intensity of donor emission

I_A : intensity of acceptor emission

Both upon excitiation of donor only

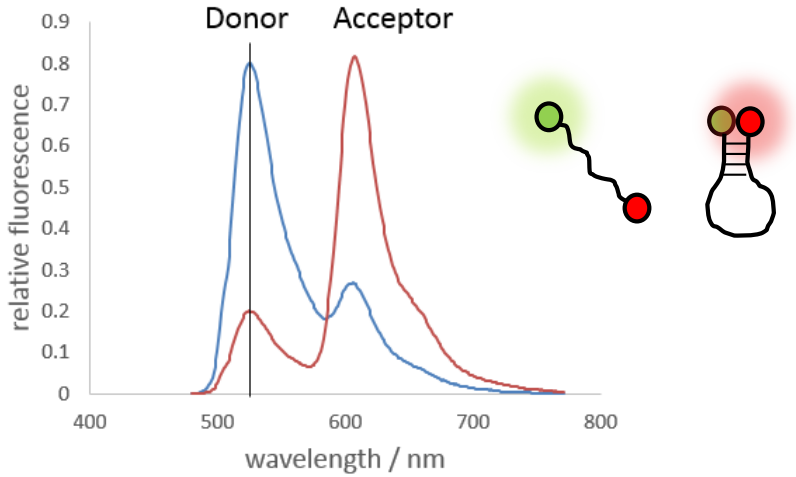
corrected:

$$E_{FRET} = I_A - \beta I_D / ([I_A - \beta I_D] + \gamma I_D)$$

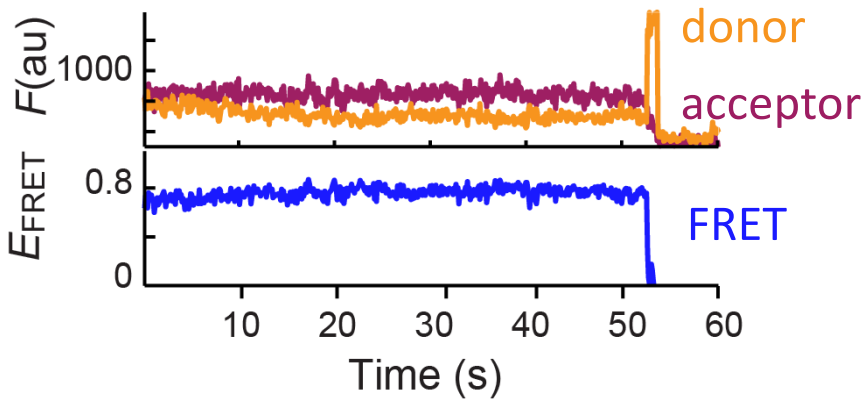
β : leakage of donor emission into acceptor channel ($\beta < 1$)

γ : detection efficiency of photons in donor and acceptor channels

Calculating FRET efficiency: option 1



Blue: open state
Red: closed state



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Both upon excitiation of donor only

corrected:

$$E_{FRET} = I_A - \beta I_D / ([I_A - \beta I_D] + \gamma I_D)$$

β : leakage of donor emission into acceptor channel ($\beta < 1$)

γ : detection efficiency of photons in donor and acceptor channels

For more information: Option 1

$$E_{FRET} = I_A / (I_A + I_D)$$

This formula calculates FRET efficiency based on the intensities of both the acceptor emission (I_A) and the donor emission (I_D). This method is used when both donor and acceptor fluorescence can be measured simultaneously.

I_A is the acceptor fluorescence intensity.

I_D is the donor fluorescence intensity.

This equation assumes that all of the acceptor fluorescence is due to FRET and that there is no direct excitation of the acceptor.

Corrected FRET Efficiency:

$$E_{FRET} = I_A - \beta I_D / ([I_A - \beta I_D] + \gamma I_D)$$

The corrected version of the formula **accounts for the bleed-through of donor fluorescence** into the acceptor channel (βI_D) and the direct excitation of the acceptor (γI_D).

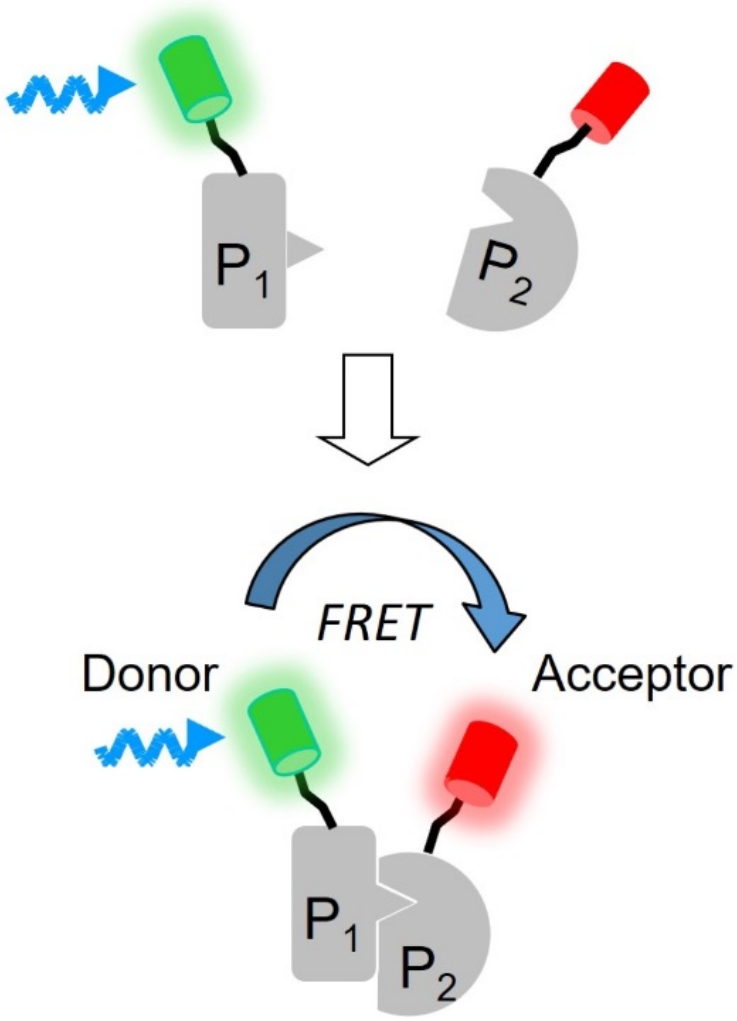
I_A is the acceptor fluorescence intensity.

βI_D is the contribution of donor fluorescence to the acceptor channel.

γI_D is the contribution from direct excitation of the acceptor.

This correction is important in cases where the spectral overlap of donor emission and acceptor excitation leads to significant bleed-through or when the acceptor can be excited directly by the excitation light source.

Calculating FRET efficiency: option 2



FRET efficiency:

$$E_{FRET} = 1 - I_{DA} / I_D$$

I_D : Donor emission in the absence of acceptor

I_{DA} : Donor emission in the presence of acceptor

Both methods are correct, but require **different observables** to be measured. When working with dually labeled molecules (as shown in the previous slide), option 1 is more convenient.

When working with a FRET acceptor that binds intermolecularly (as shown here), option 2 is more convenient.

For more information: Option 2

$$E_{FRET} = 1 - I_{DA} / I_D$$

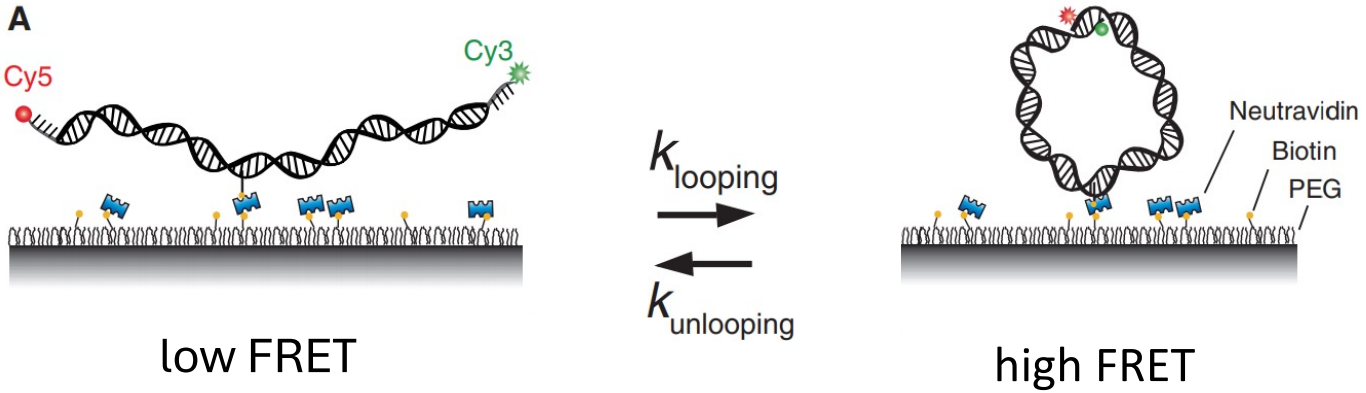
This formula is used when you **can directly measure the donor fluorescence both in the presence (I_{DA}) and absence (I_D) of the acceptor**. It is based on the quenching of donor fluorescence when FRET occurs. The rationale is that the donor fluorescence intensity decreases (is quenched) when energy is transferred to the acceptor.

I_{DA} is the donor fluorescence intensity in the presence of the acceptor.

I_D is the donor fluorescence intensity in the absence of the acceptor.

This method is straightforward but assumes that the only cause of donor quenching is energy transfer to the acceptor, which may not always be the case.

Shorter DNA overlap: Opening and closing dynamics

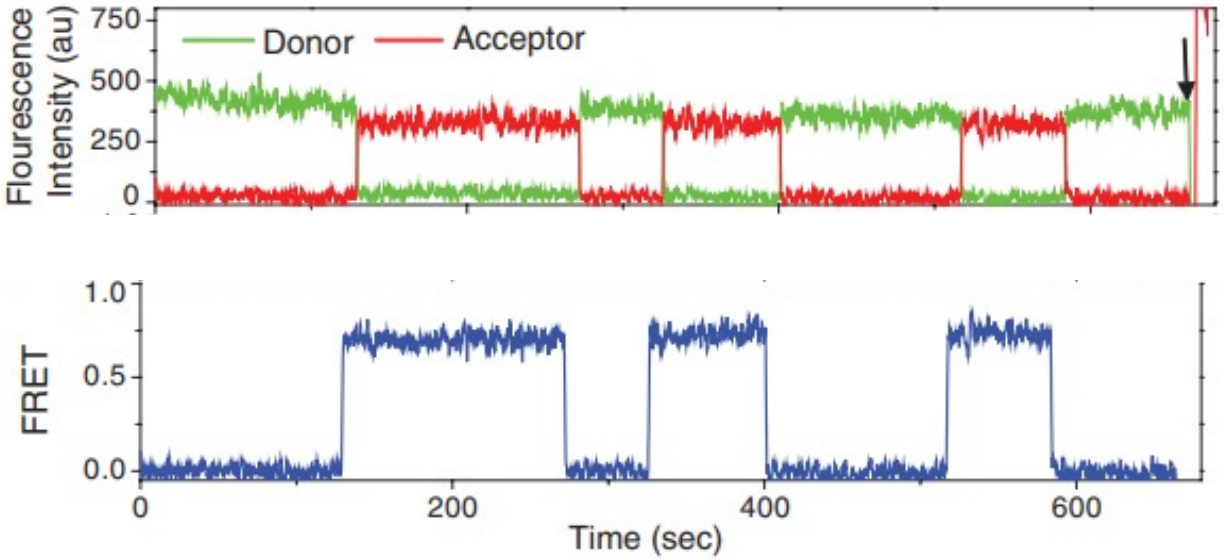


low FRET

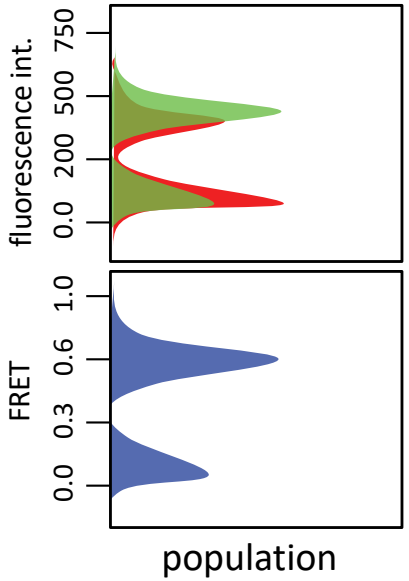
high FRET

short sticky ends: dynamic opening and closing

emission from a single molecule



sketch: populations

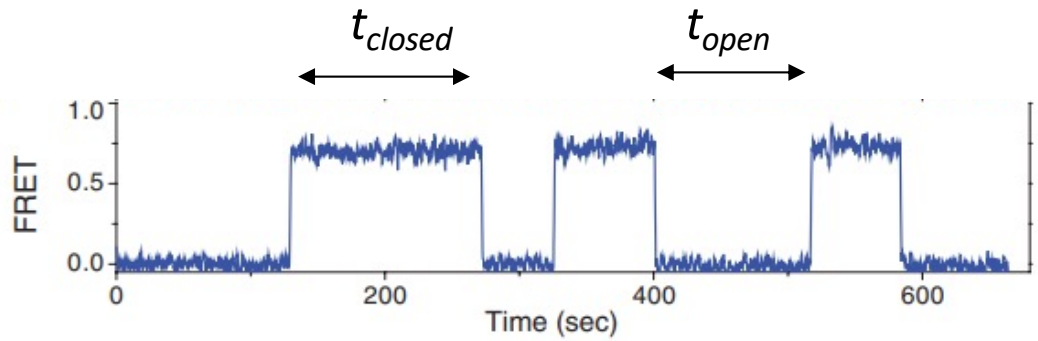


Vafabakhsh, Ha, Science 2012

Activity: explain the experiment on the previous slide to each other and answer questions below

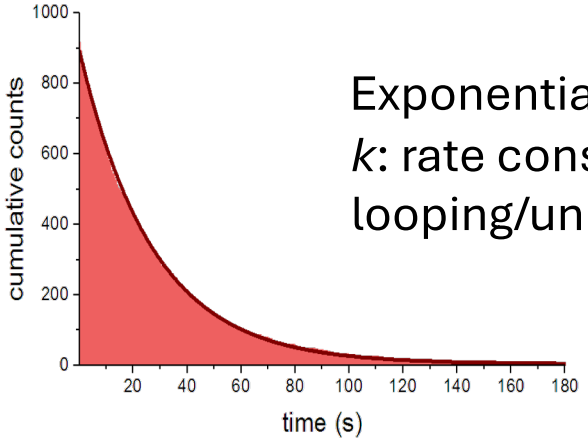
1. What's the difference of this experiment compared to the previous one?
2. How would you transform the fluorescence intensity (red/green plot) to the FRET efficiency (blue plot)? What formula would you use?
3. What questions do you have?/What is not clear?

Gathering kinetic information from dynamic traces



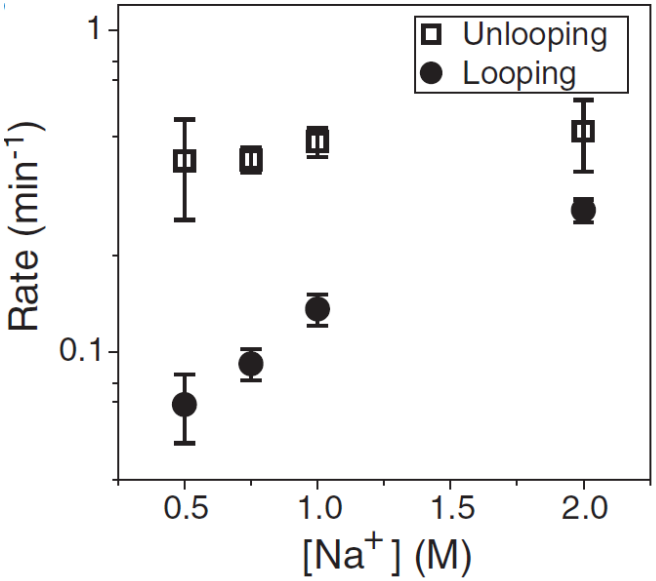
Histogram of closed times (t_{closed})
→ yield **opening kinetics**

Histogram of open times (t_{open})
→ yield **closing kinetics**



Exponential fit → $y = Ae^{-kt}$
k: rate constant of looping/unlooping

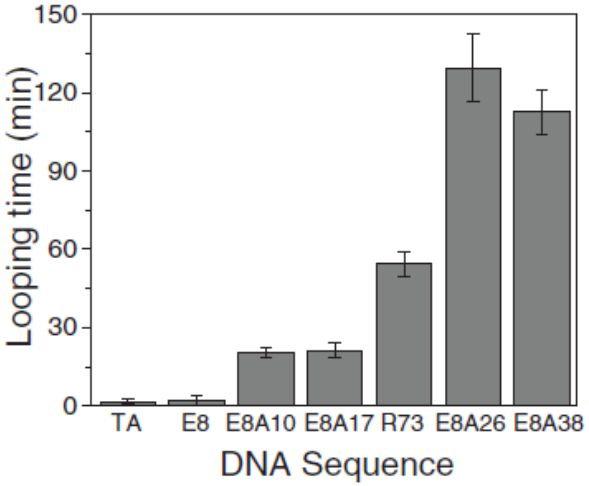
Rate constants



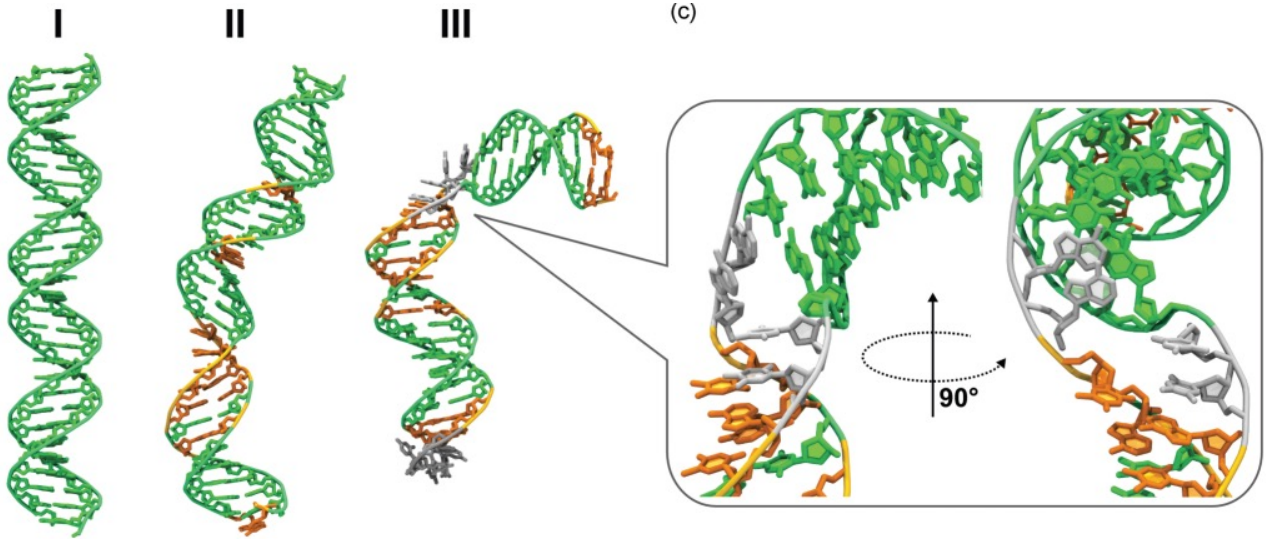
Opening dynamics are unaffected by salt, whereas closing is faster at higher salt

Conclusion from case study 1

- DNA is more flexible than thought previously
- Flexibility strongly depends on the sequence:



- Mechanisms might include distortion in the DNA structure:
  - transient kinks
  - bubbles
  - anharmonic elasticity of DNA



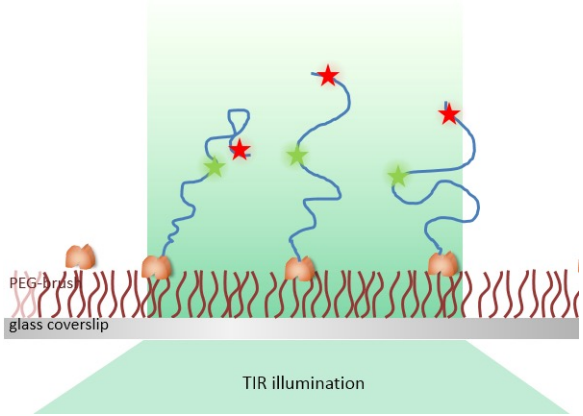
Zeida et al. Phys Rev A 2012

Single molecule fluorescence techniques

Immobilized molecules:

molecules are immobilized on a surface (coverslip) and can be observed for a long time, e.g. by total internal reflection fluorescence microscopy

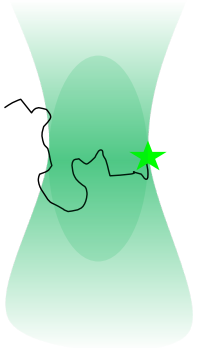
- Imaging and counting single molecules
- internal dynamics
- interaction dynamics
- Colocalization analysis



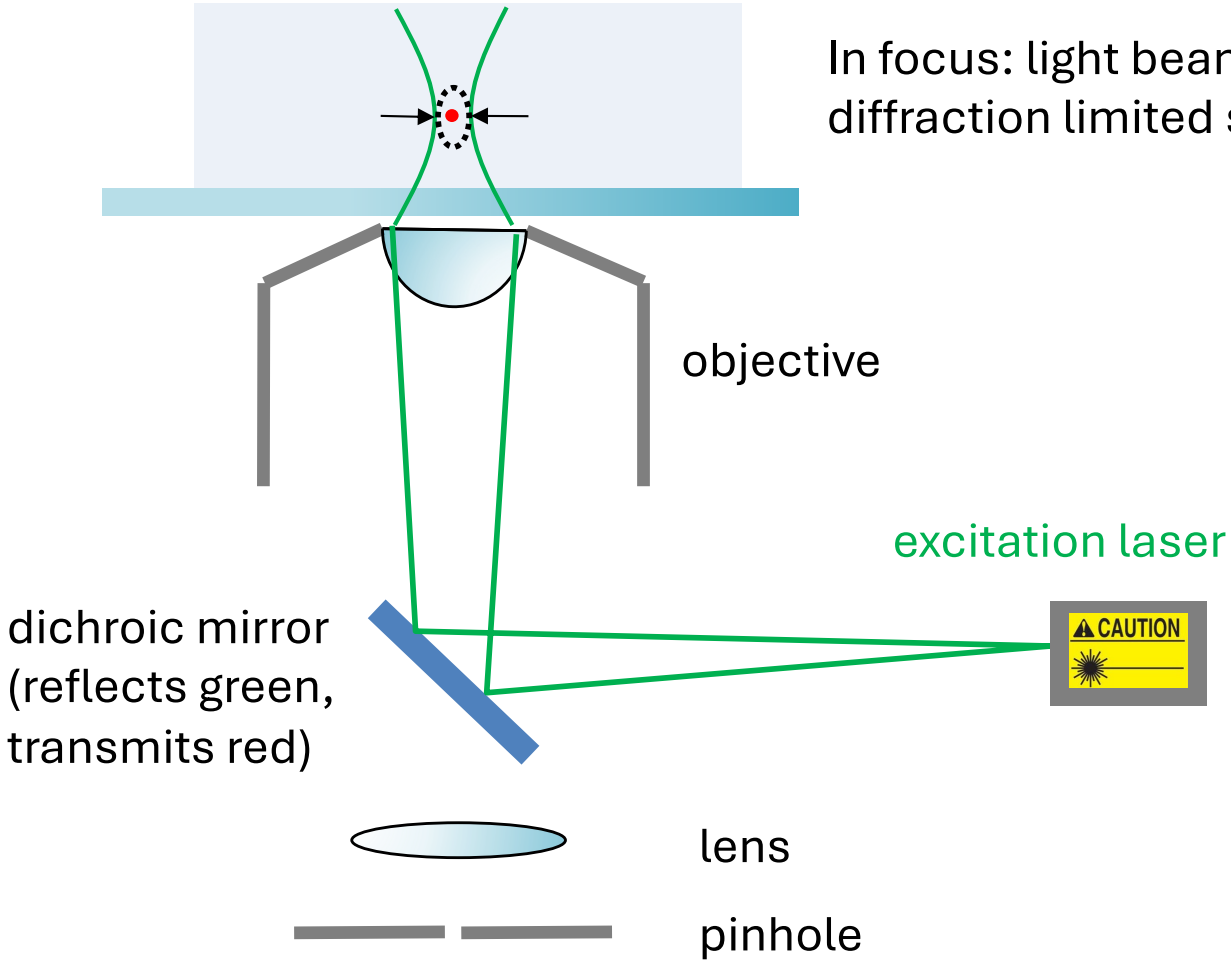
Freely diffusing molecules:

fluorescently labeled molecules are observed at very high dilution free in solution, using a confocal microscope:

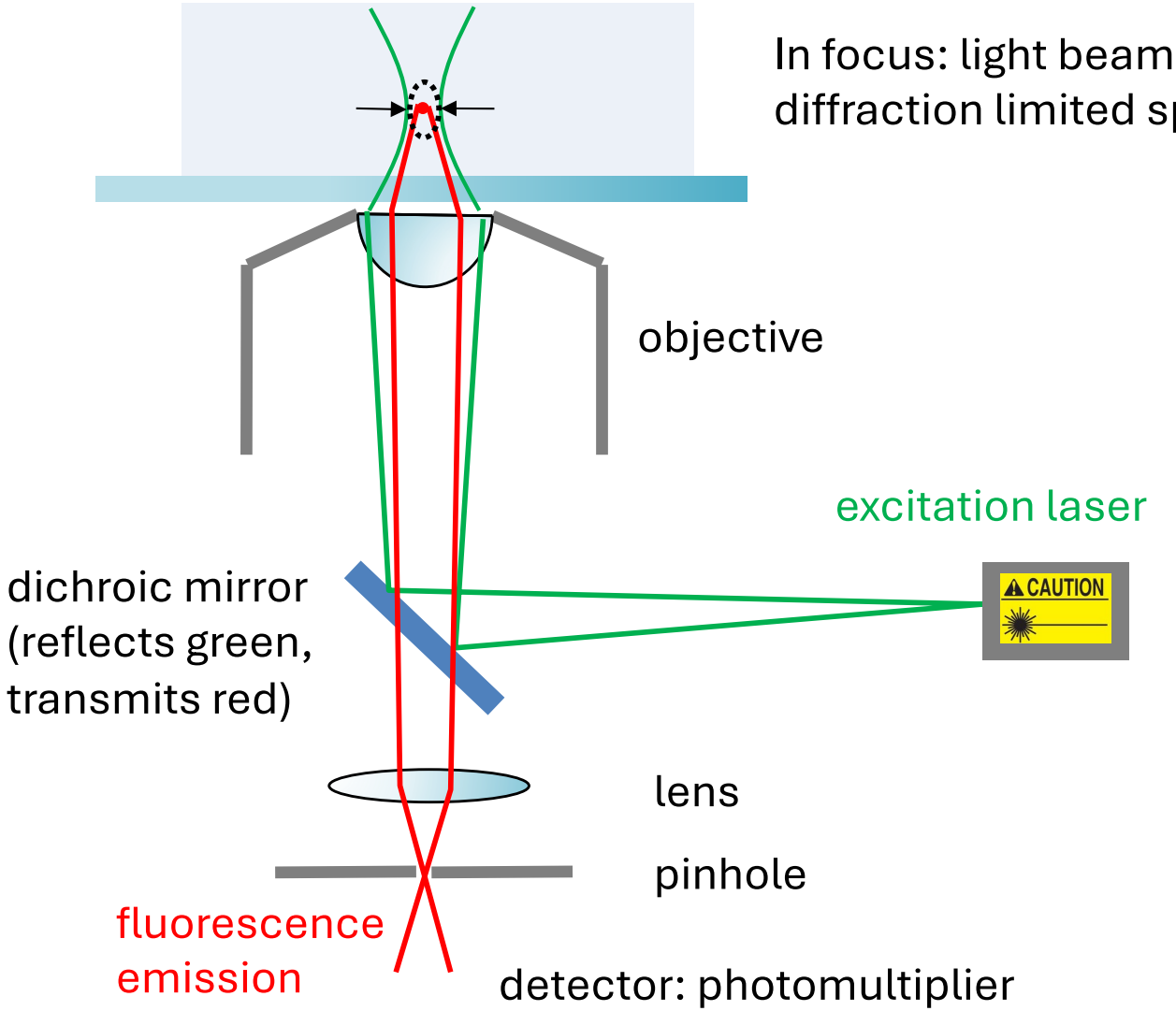
- FRET statistics
- Burst analysis (fluorescence correlation spectroscopy)
- Photon counting histograms
- Only fast dynamics are monitored (ms)



Confocal microscope



Confocal microscope

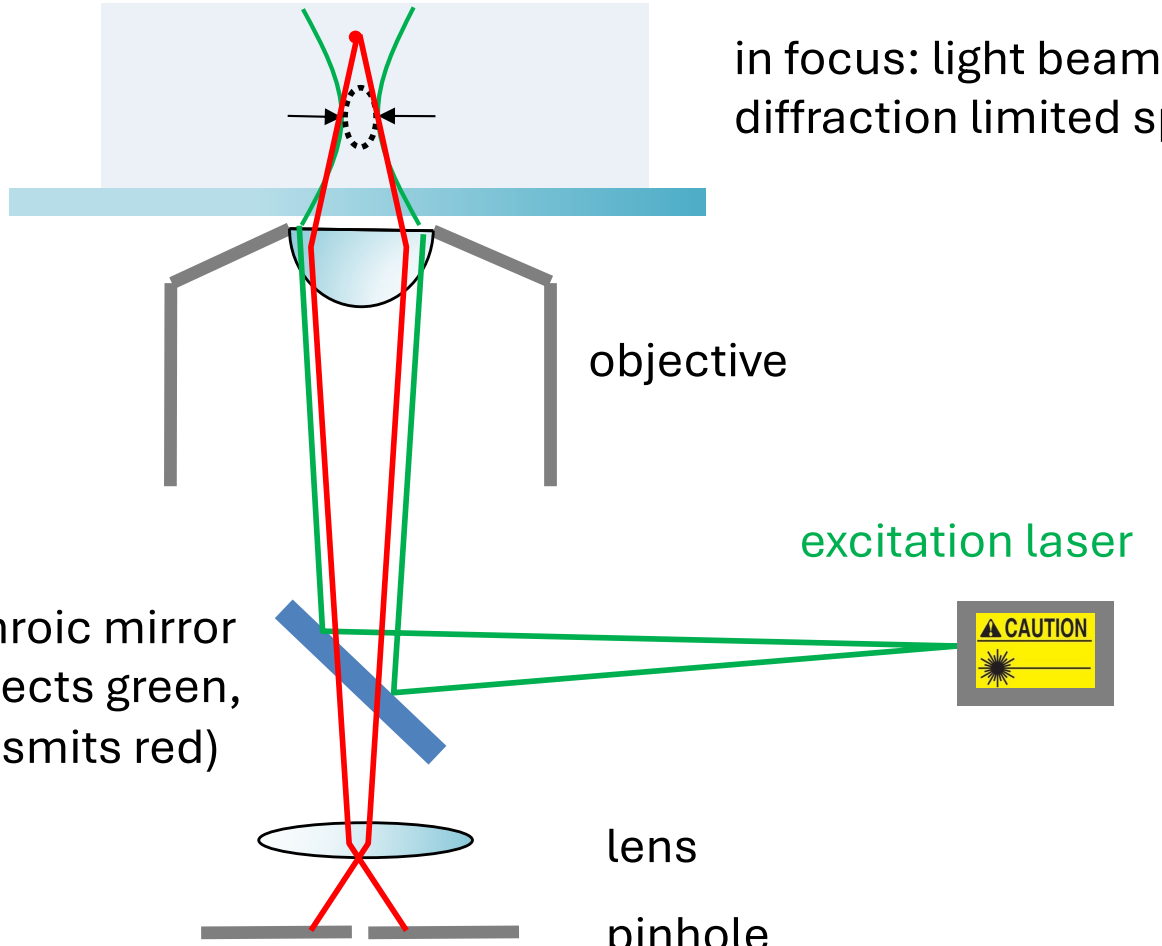


In focus: light beam is focused on a diffraction limited spot (d = 250 nm)

fluorescence emission from molecules **within** the confocal volume reach the detector through a pinhole

confocal volume: dashed circle, with a diameter of 250 nm and height 700 nm

Confocal microscope



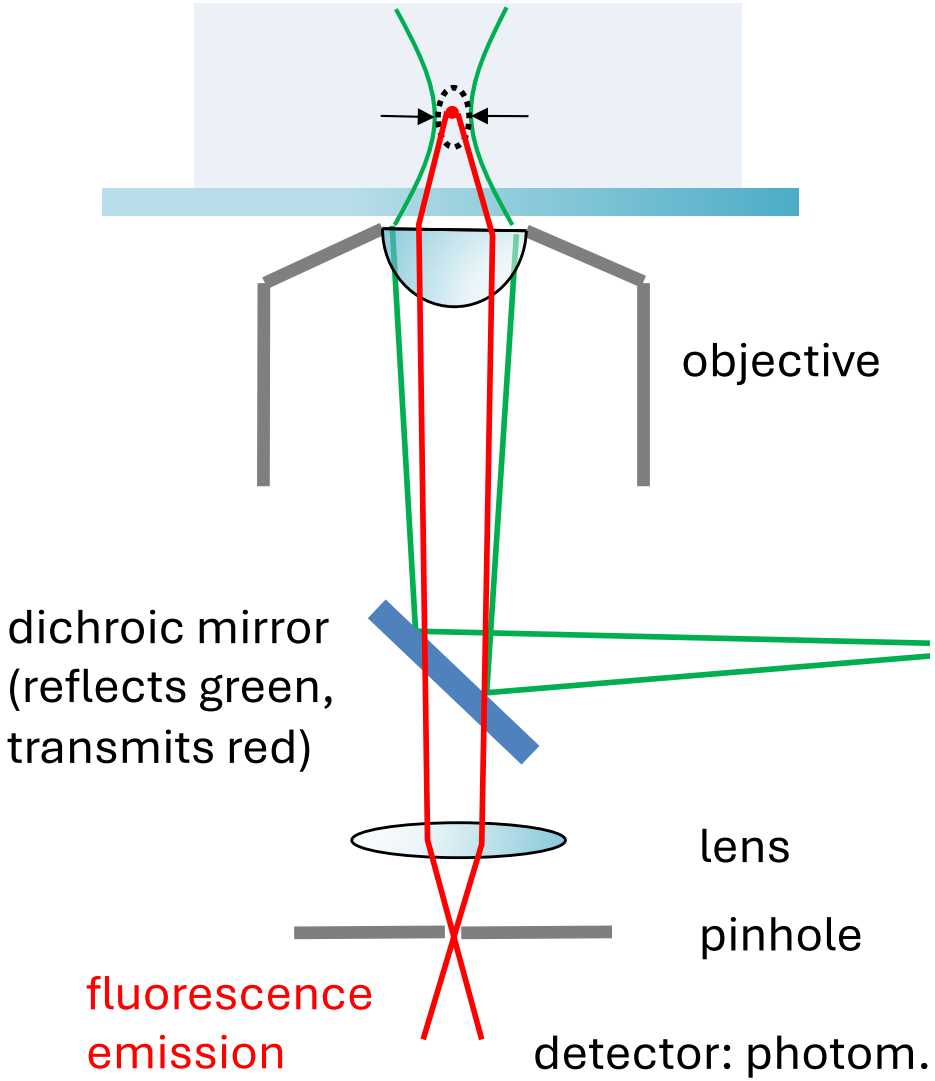
in focus: light beam is focused on a diffraction limited spot (d = 250 nm)

fluorescence emission from molecules **outside** the confocal volume are **blocked** as they do not pass the pinhole

Result: only molecules within the very small confocal volume are observed

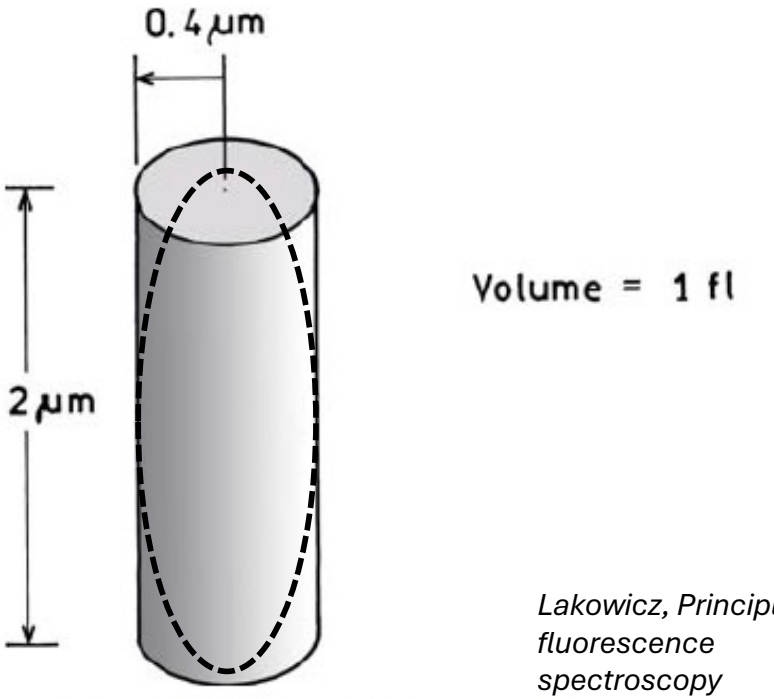
→ imaging by scanning in 3 dimensions

Confocal Imaging



Confocal imaging of molecules:

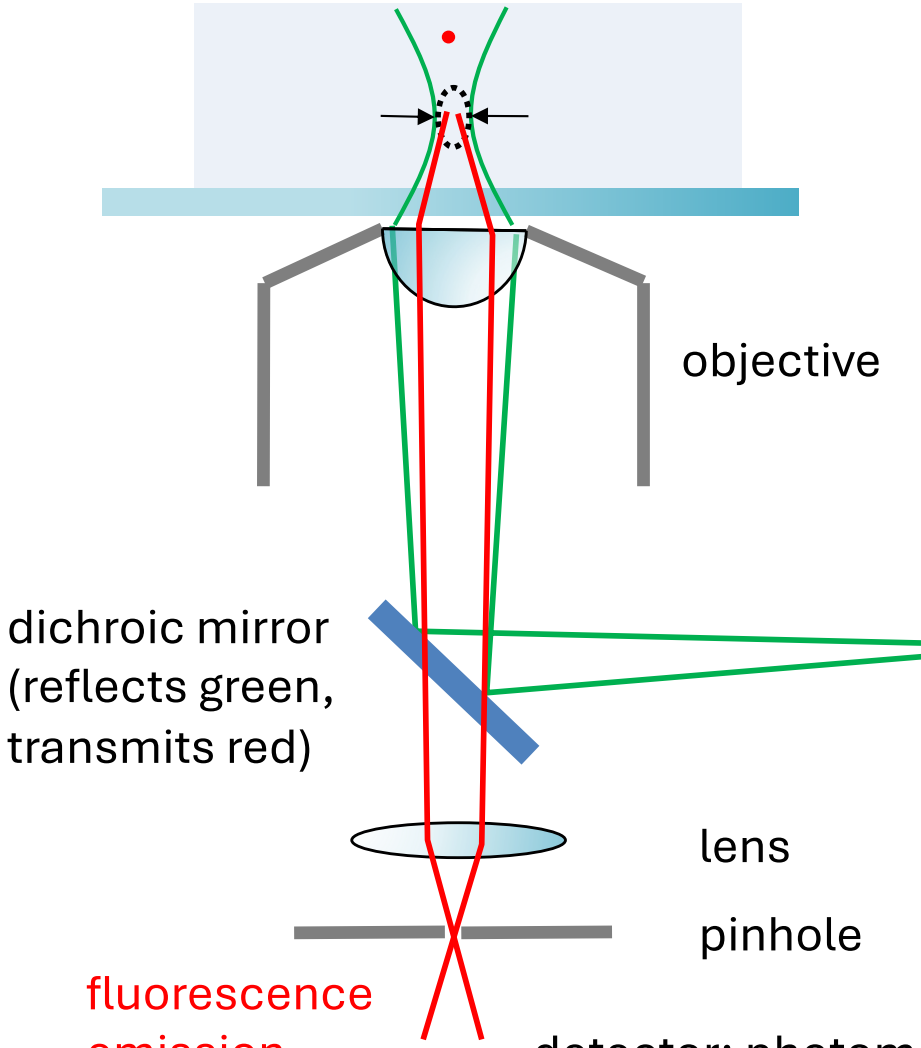
Only light from a small volume is recorded, all other light from the sample is blocked by a **pinhole**.

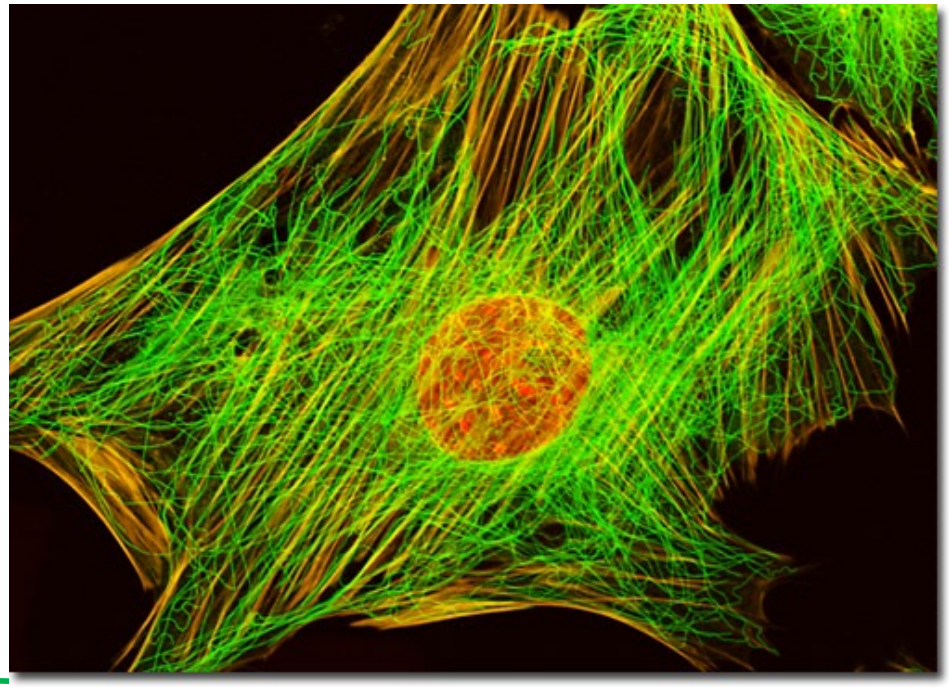


Lakowicz, Principles of fluorescence spectroscopy

Figure 24.1. Typical volume in an FCS experiment.

Confocal Imaging of Biological Samples





Confocal imaging allows 3D sectioning of samples by **scanning** the confocal volume through the sample and recording with different excitation / emission wavelengths (3T3 cell stained for: **orange: actin**, **green: tubulin**, **red: DNA**)

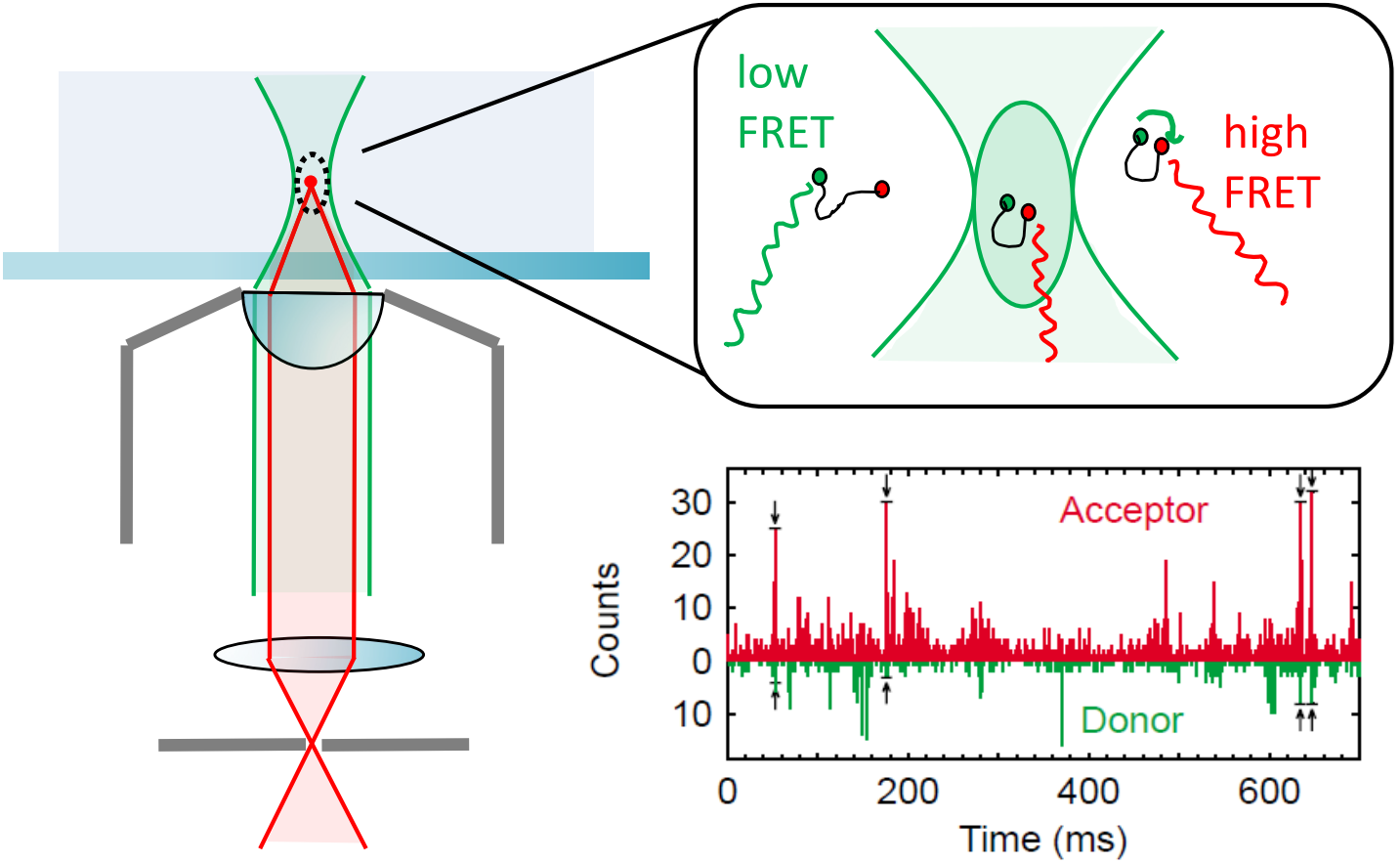
http://www.olympusfluoview.com/gallery/cells/3t3/3t3large.html

Xiaowei Zhuang about the diffraction limit

- https://www.youtube.com/watch?v=w2Qo__sppcI&ab_channel=ScienceCommunicationLab

- Start at 7:00

Single Molecule FRET: Freely Diffusing Molecules



Diffusing doubly-labeled molecules:

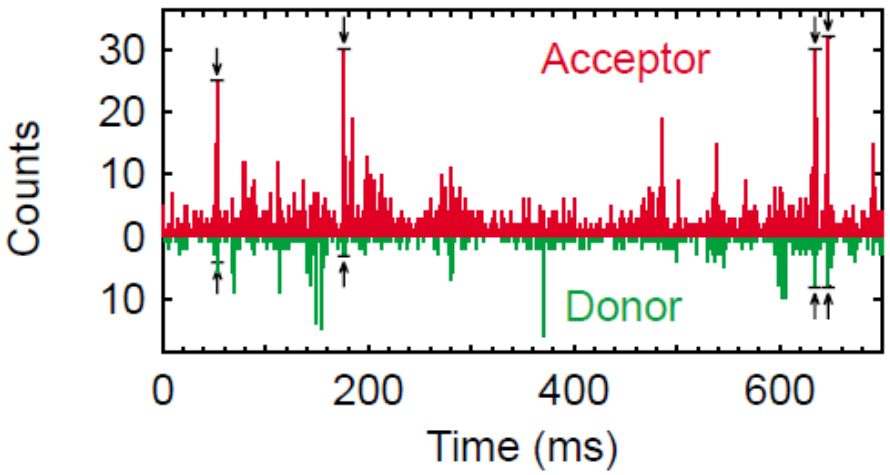
Whenever a molecule diffuses through the confocal volume it is excited by the laser beam and emits fluroescence in a **short burst (μs)**. The concentration is so low that only one molecule enters the volume at once.

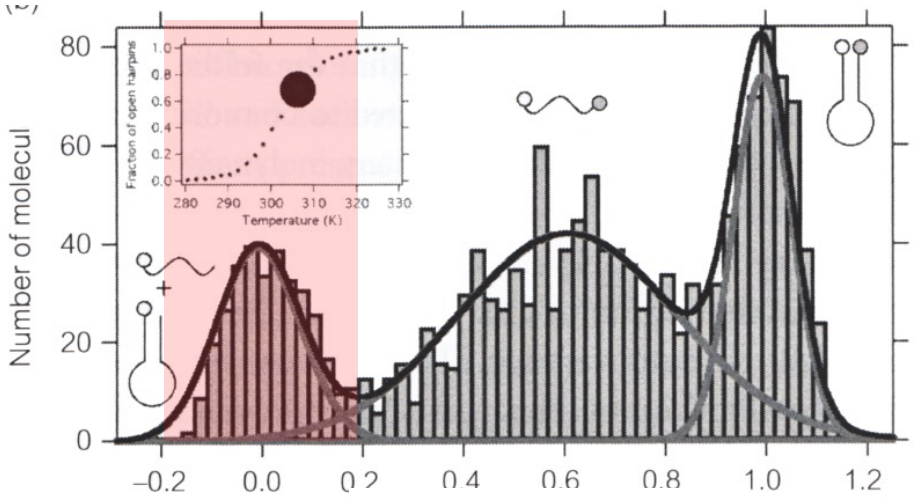
Simultaneous detection from **donor** and **acceptor** dye at two wavelengths

If it is in a closed conformation → **FRET, and red-shifted fluorescence is detected**

in open conformation → no FRET, **fluorescence at lower wavelength**

FRET Efficiency Histograms





Analysis:

Find significant bursts using a threshold value

Calculate **FRET efficiency:**

$E_{FRET} = I_A - \beta I_D / ([I_A - \beta I_D] + \gamma I_D)$
for each burst

Create histogram of all recorded events

Zero FRET molecules that **lack an acceptor**

Medium FRET molecules in an **open conformation**

High FRET molecules in a **closed conf.**

Case study 2

Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy

nature

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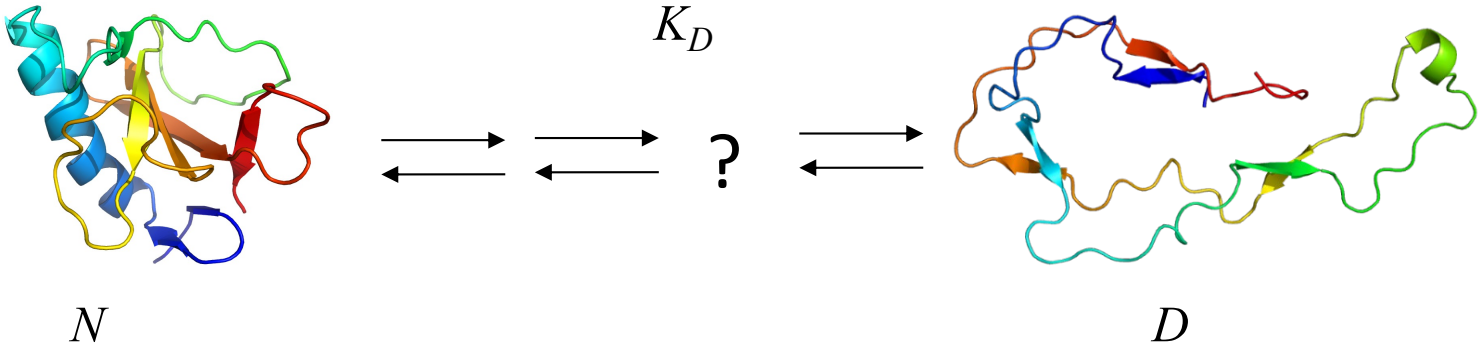
† These authors contributed equally to this work

Protein folding

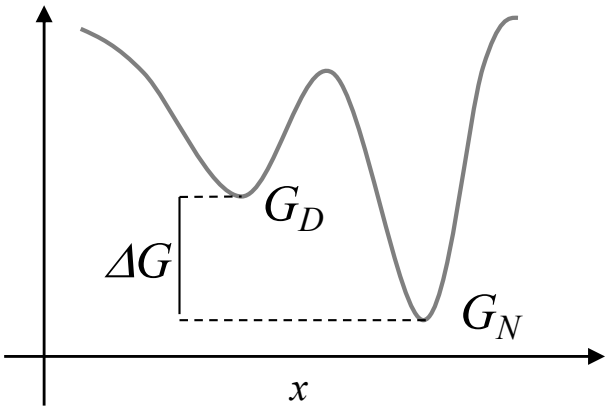


Every protein "flickers" in and out of its native conformation constantly.

The stabilities of the native and unfolded state determine the ratio of dwell times.

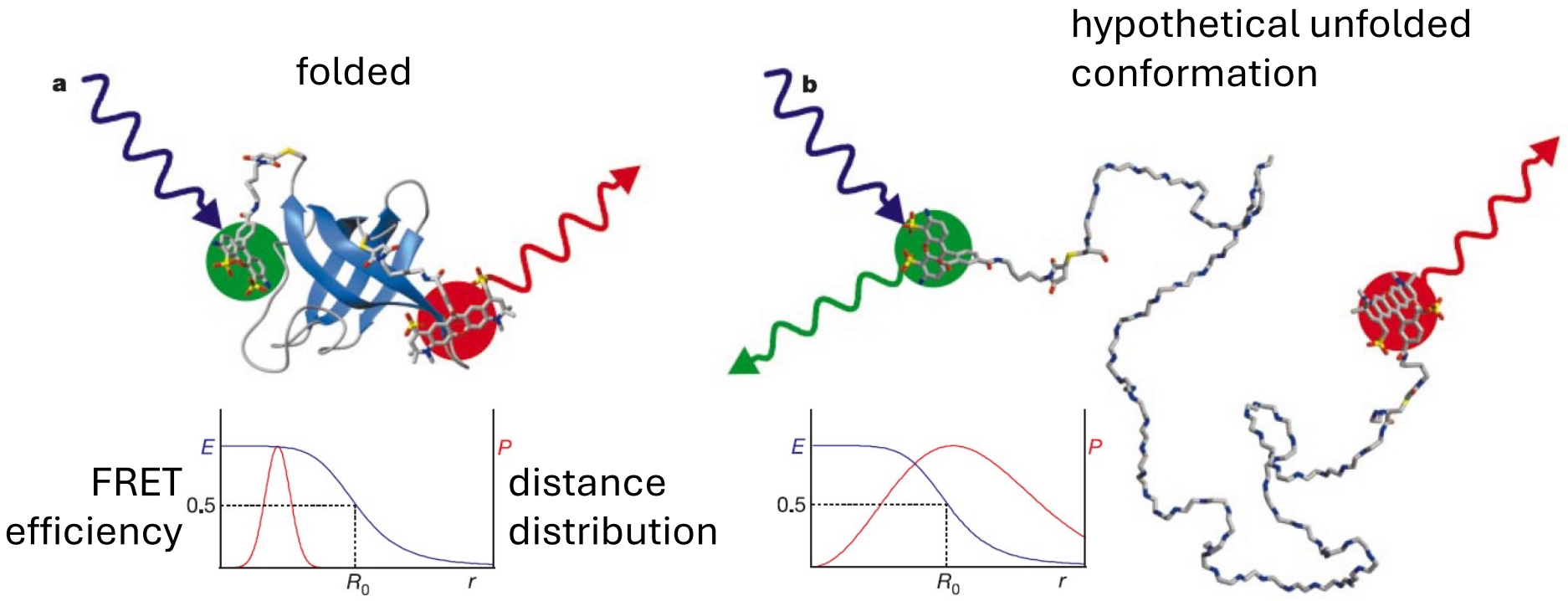
The kinetics of the transition are given by the energy barriers separating the folded from the unfolded state

The molecular process of folding is still not completely understood.



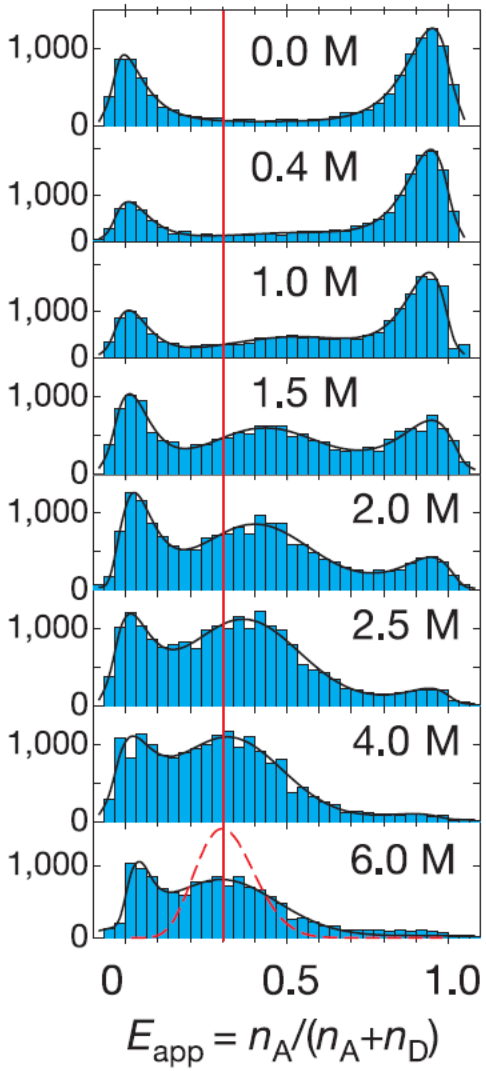
Watching a Single Protein (Un)fold

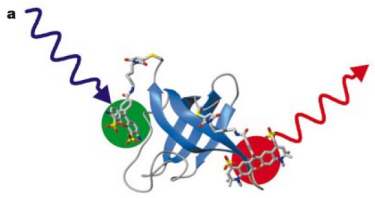
Cold shock protein from
*Thermotoga maritima (*Csp*Tm)*



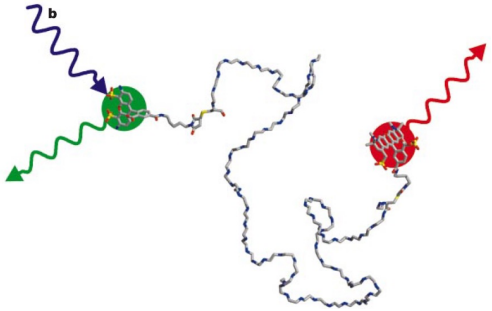
Schuler, Lipman & Eaton,
Nature 2002

Watching a Single Protein (Un)fold





Chemical denaturant: guanidinium hydrochloride

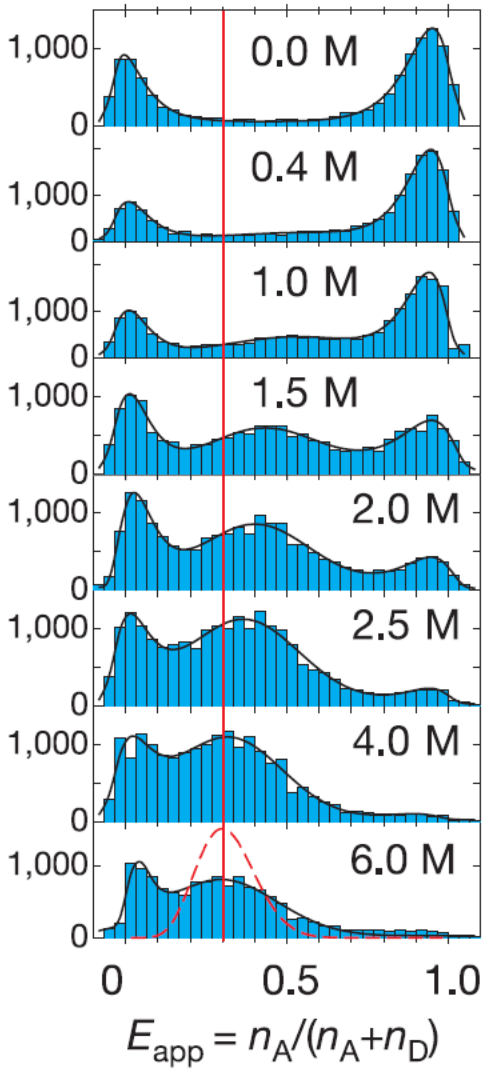


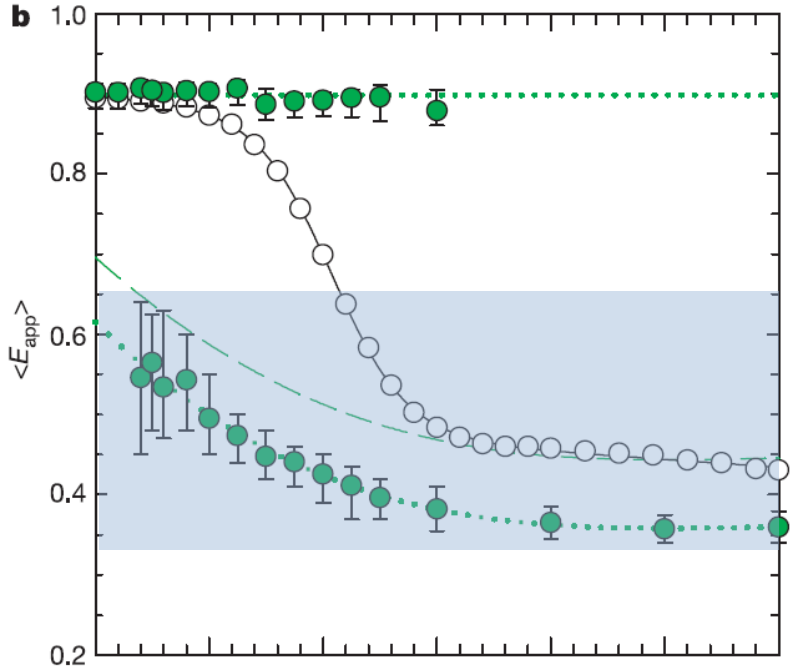
Single molecule analysis does **not reveal any** intermediates

Two states populated under all conditions

Schuler, Lipman & Eaton, Nature 2002

Analysis of the protein conformation during unfolding





Filled green circles: Single-molecule mean values (high & low)

Open circles: Ensemble FRET efficiencies

FRET efficiency of unfolded state shifts to lower values at high denaturant.

→ Distance is increased, protein chain more loosely arranged

Conclusions of case study 2

- Single-molecule FRET allows to observe dynamic changes in proteins
- States and processes hidden in the ensemble can be revealed
- Unfolding measurements on a small heatshock protein did not reveal further intermediates (→ two-state folding, all-or-none transition)
- Analysis of peak-shape and position revealed collapse of the protein chain under native conditions

Next week: Readings

- **Optical tweezers**
  - Direct observation of base-pair stepping by RNA polymerase. Abbondanzieri EA, Greenleaf WJ, Shaevitz JW, Landick R, Block SM. Nature. 2005 Nov 24;438(7067):460-5. Epub 2005 Nov 13.

- **Protein dynamics using AFM force spectroscopy**
  - Science. 2009 Jan 30;323(5914):633-7. Ligand-dependent equilibrium fluctuations of single calmodulin molecules. Junker JP1, Ziegler F, Rief M.

Let’s play connections

https://connections.swellgarfo.com/game/-NrjFeoEXc7tZYtSDmpG

How to Play

Find groups of four items that share something in common.

- Select four items and tap '**Submit**' to check if your guess is correct.
- Find the groups without making 4 mistakes!

Category Examples

- FISH: Bass, Flounder, Salmon, Trout
- FIRE ___: Ant, Drill, Island, Opal

Categories will always be more specific than "5-LETTER-WORDS," "NAMES" or "VERBS."

Each puzzle has exactly one solution. Watch out for words that seem to belong to multiple categories!

Each group is assigned a color, which will be revealed as you solve: