# Fluorescence microscopy: the beginning

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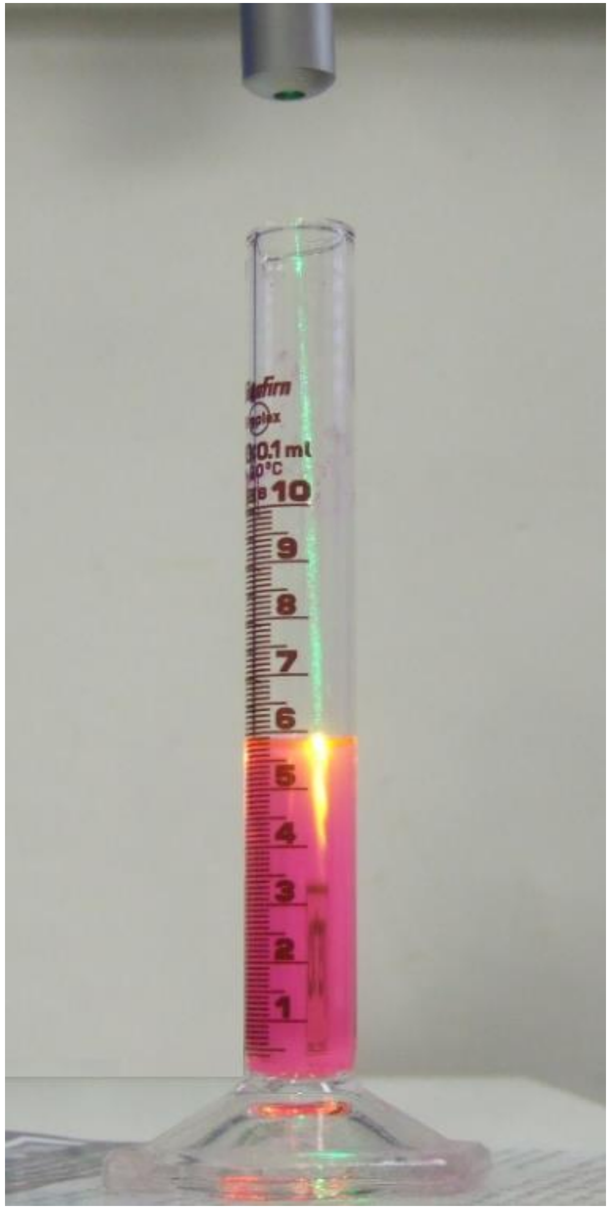
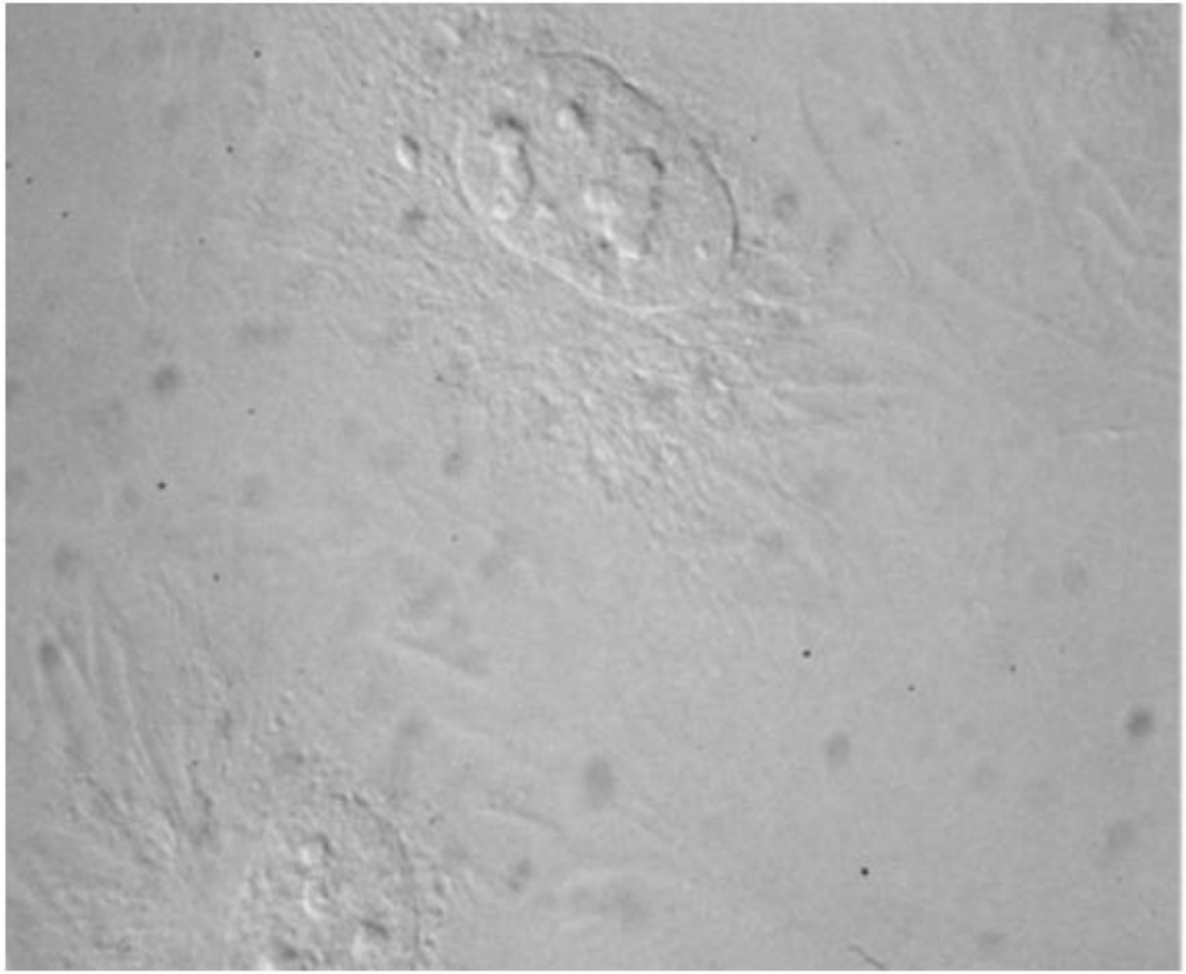
# Fluorescence: it’s better to see once, than to hear thousand times

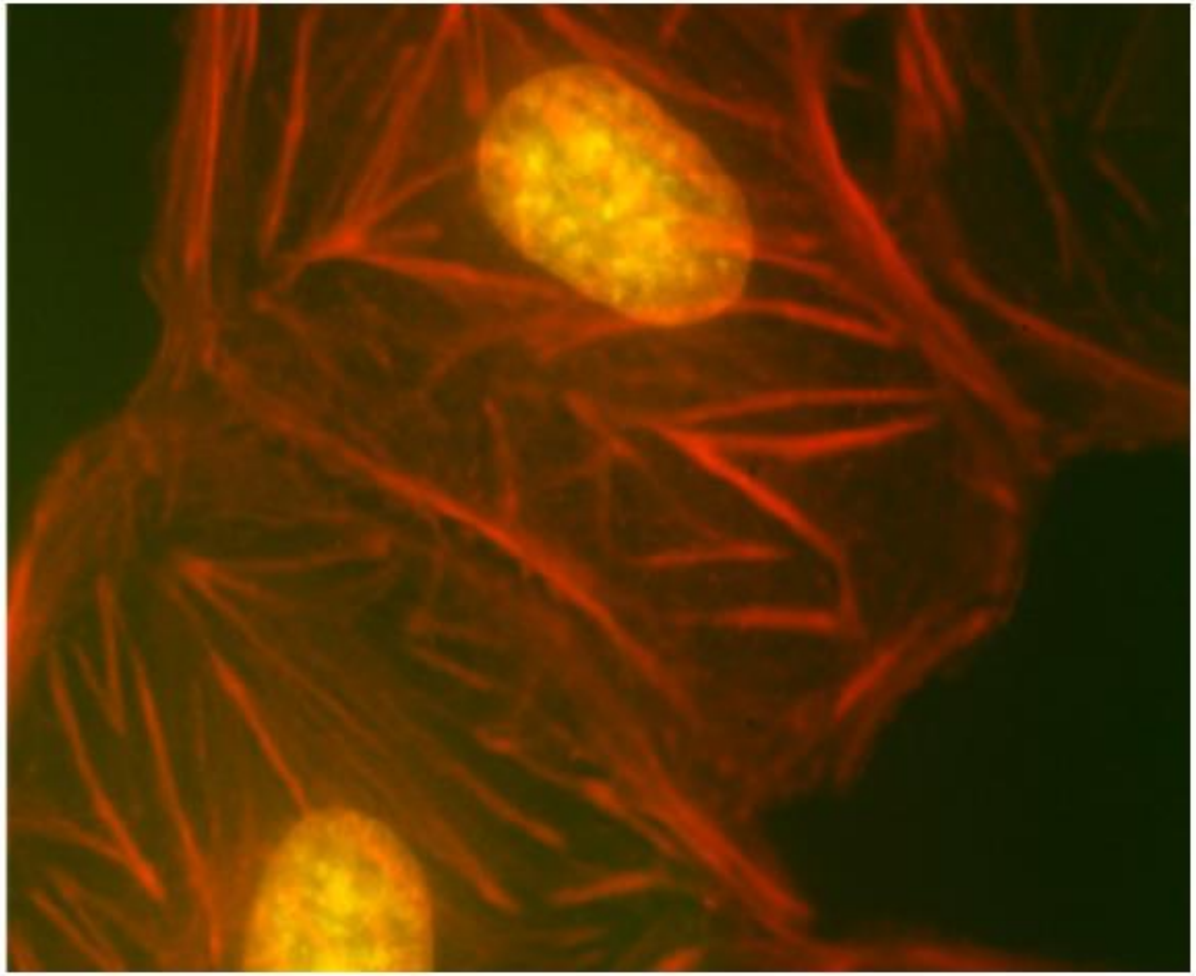



Fluorescence

and chemiluminescence

# DIC vs fluorescence





An image of the same field of BPAE cells captured using brightfield (left) and fluorescence (right) microscopy. Fluorescent labeling of the nucleus (yellow) and actin (red) makes it possible to see much more detailed cell structure.

High specificity, sensitivity and versatility

*In vivo* imaging/probing in real time

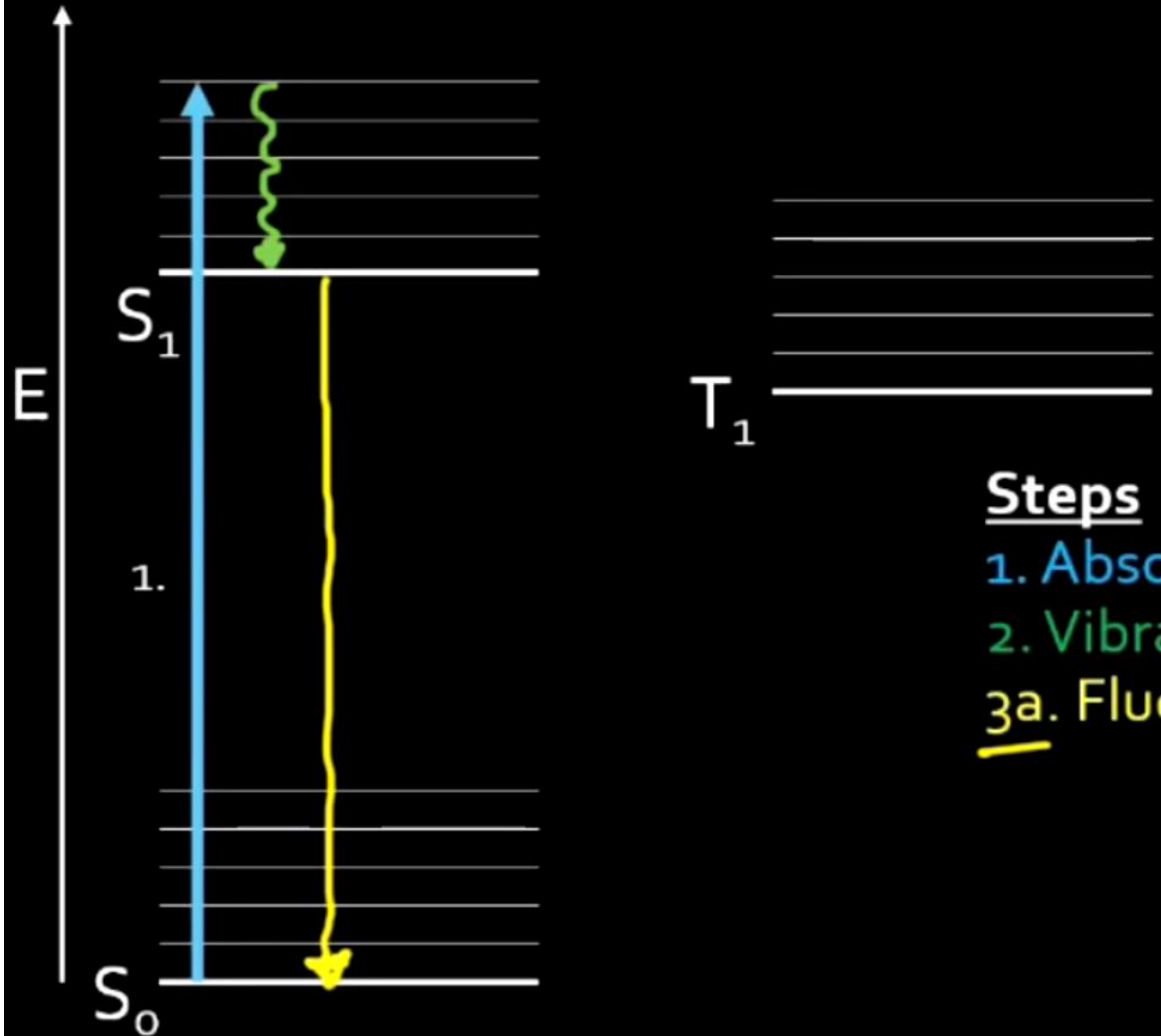
High and super-resolution imaging up to several nanometers scale

Not only imaging, but probing
(pH, $Ca^{2+}$, electric potential, mechanical force)

Indirect measurements

# Jablonski Diagrams

$$\Delta E = h\nu = hc/\lambda$$



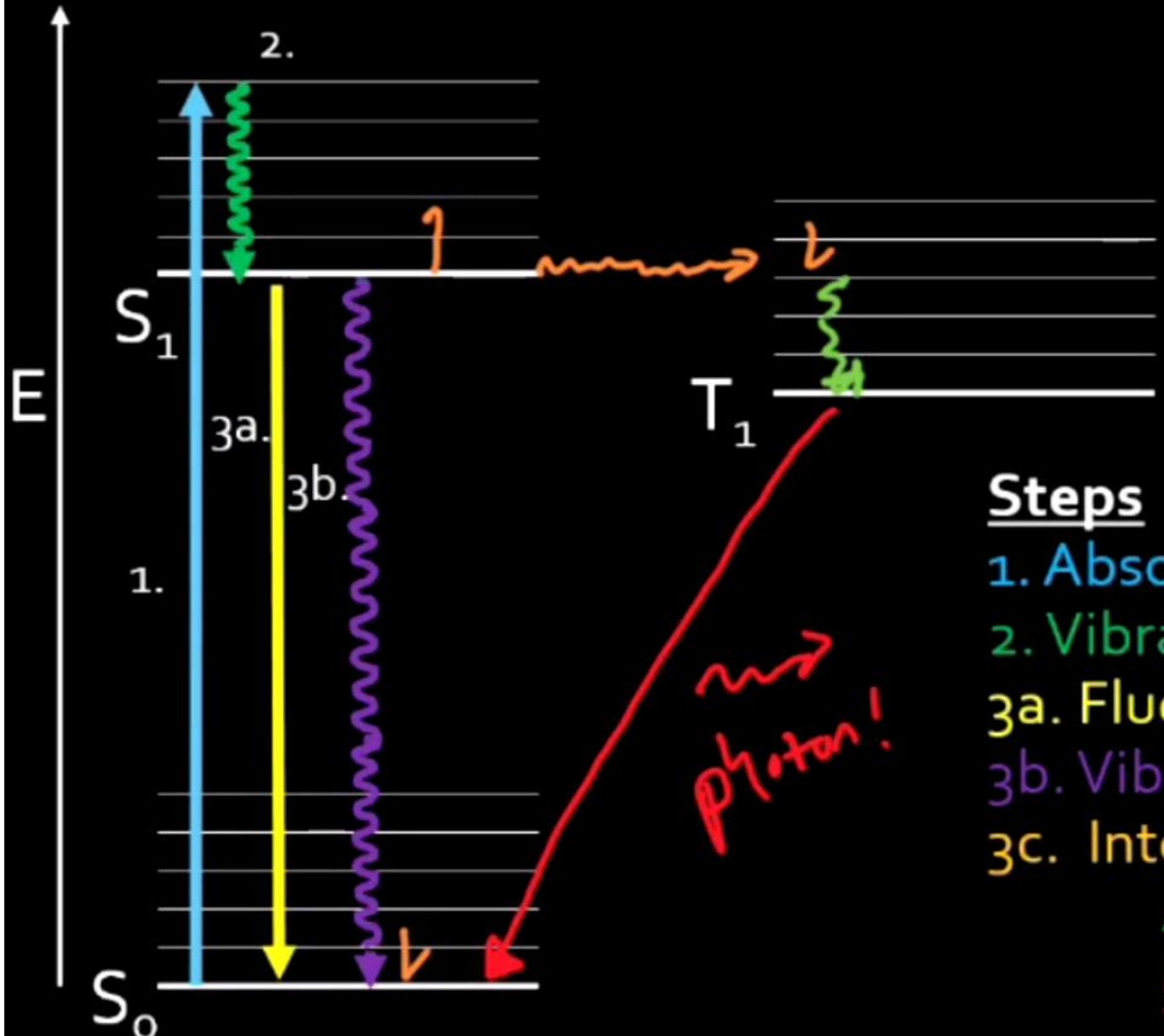

**Steps**

1. Absorption
2. Vibrational Relaxation

3a. Fluorescence

# Jablonski Diagrams

$$\Delta E = h\nu = hc/\lambda$$




**Steps**

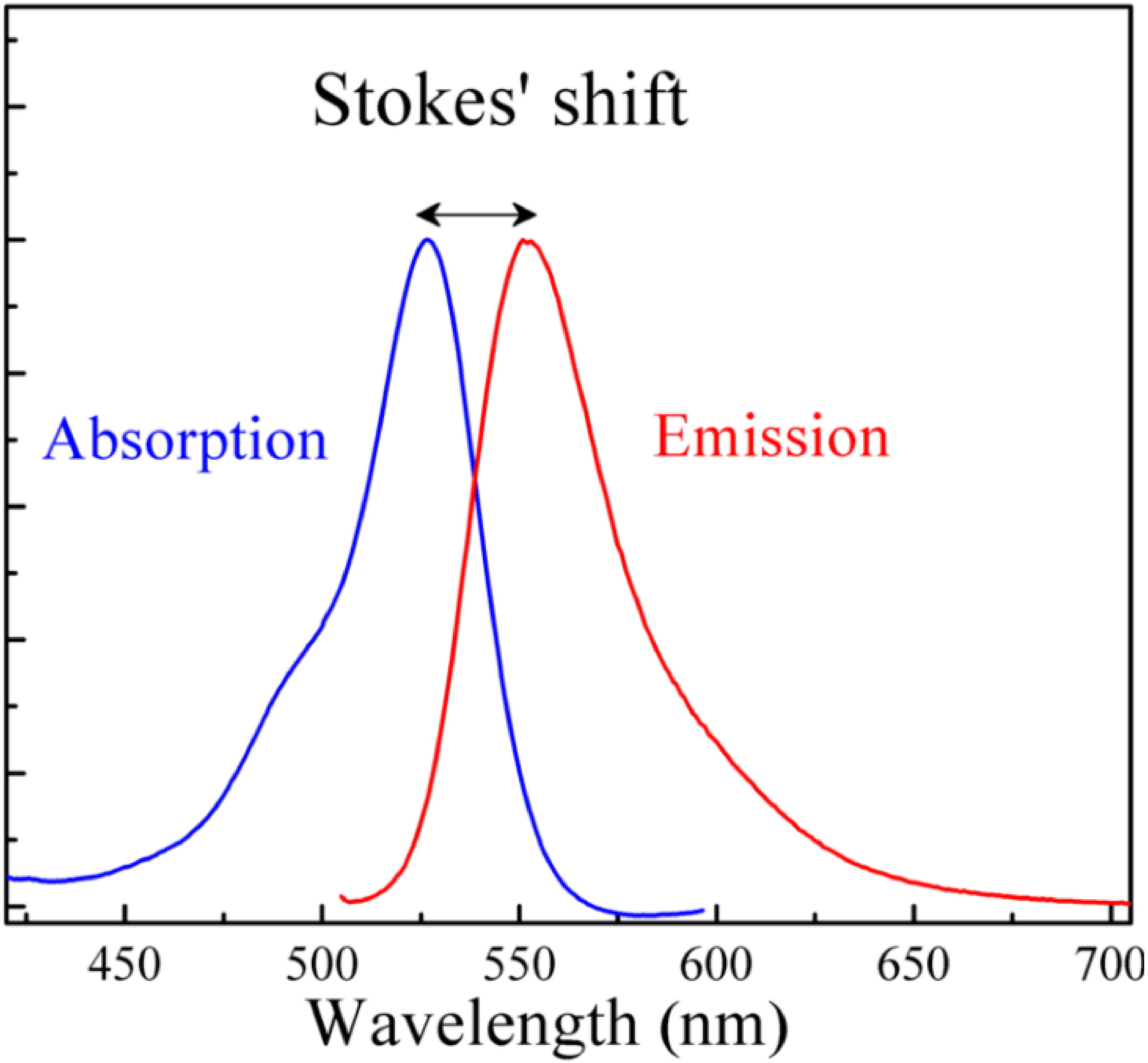
1. Absorption
2. Vibrational Relaxation

3a. Fluorescence

3b. Vibrational Relaxation + Internal Conversi

3c. Intersystem crossing

4. then Vibrational Relaxation
5. Phosphorescence!




Rhodamine 6B absorption and emission (fluorescence) spectra

# What are the basic features of fluorescence?

1. Fluorescence intensity linearly increases with intensity of excitation light

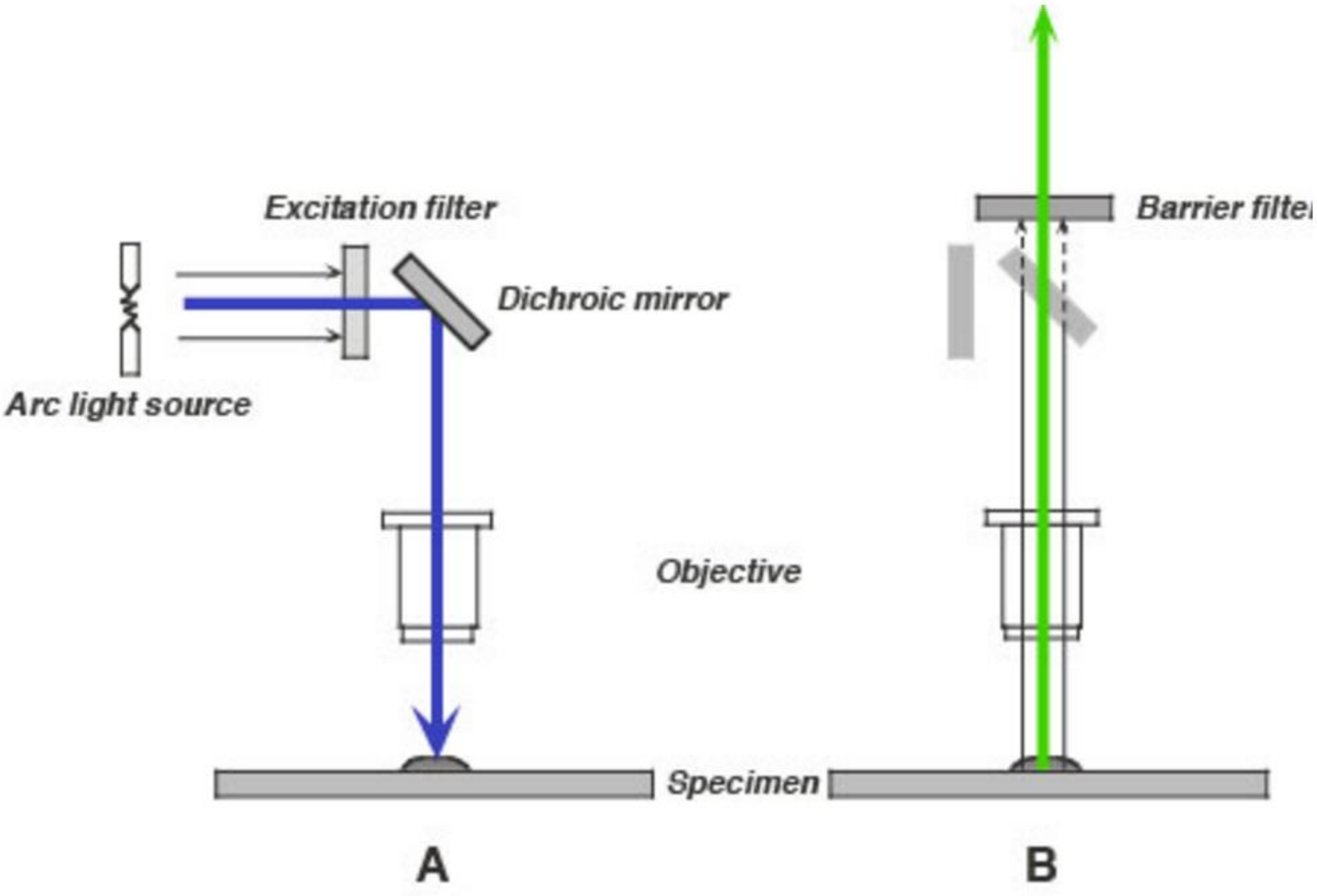
2. Occurs within the nanosecond - timescale after incoming photon absorption

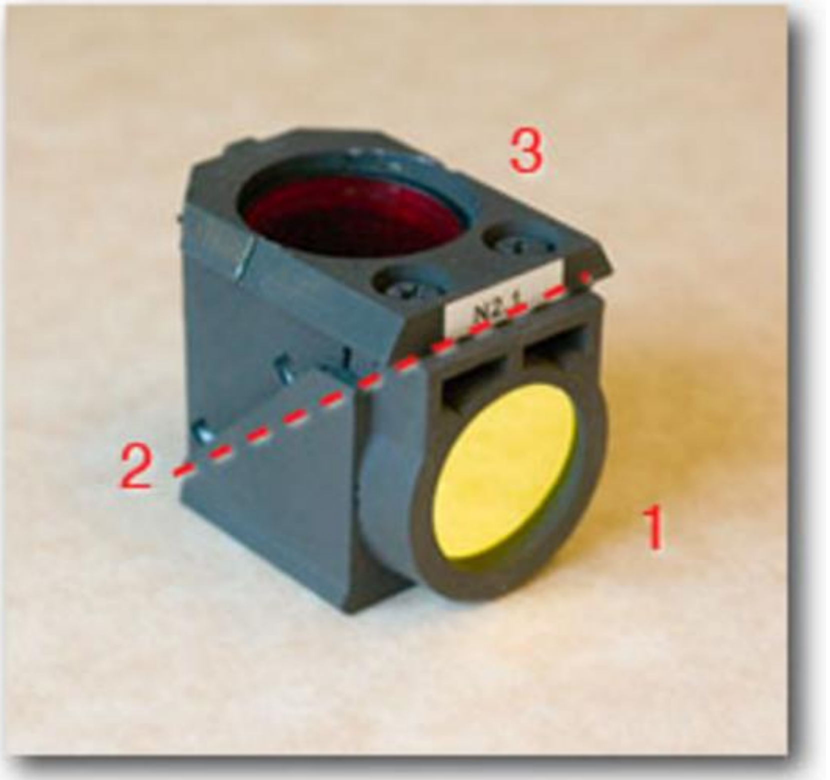
3. Normally emission occurs in all possible directions

4. Both efficieny of fluorescence and emission spectrum depend on molecular environment

5. Single fluorophore may be prone to blinking (reversible switching “on” and “off” ) and irreversible switching to the “dark” (“off” ) state – process called photobleaching

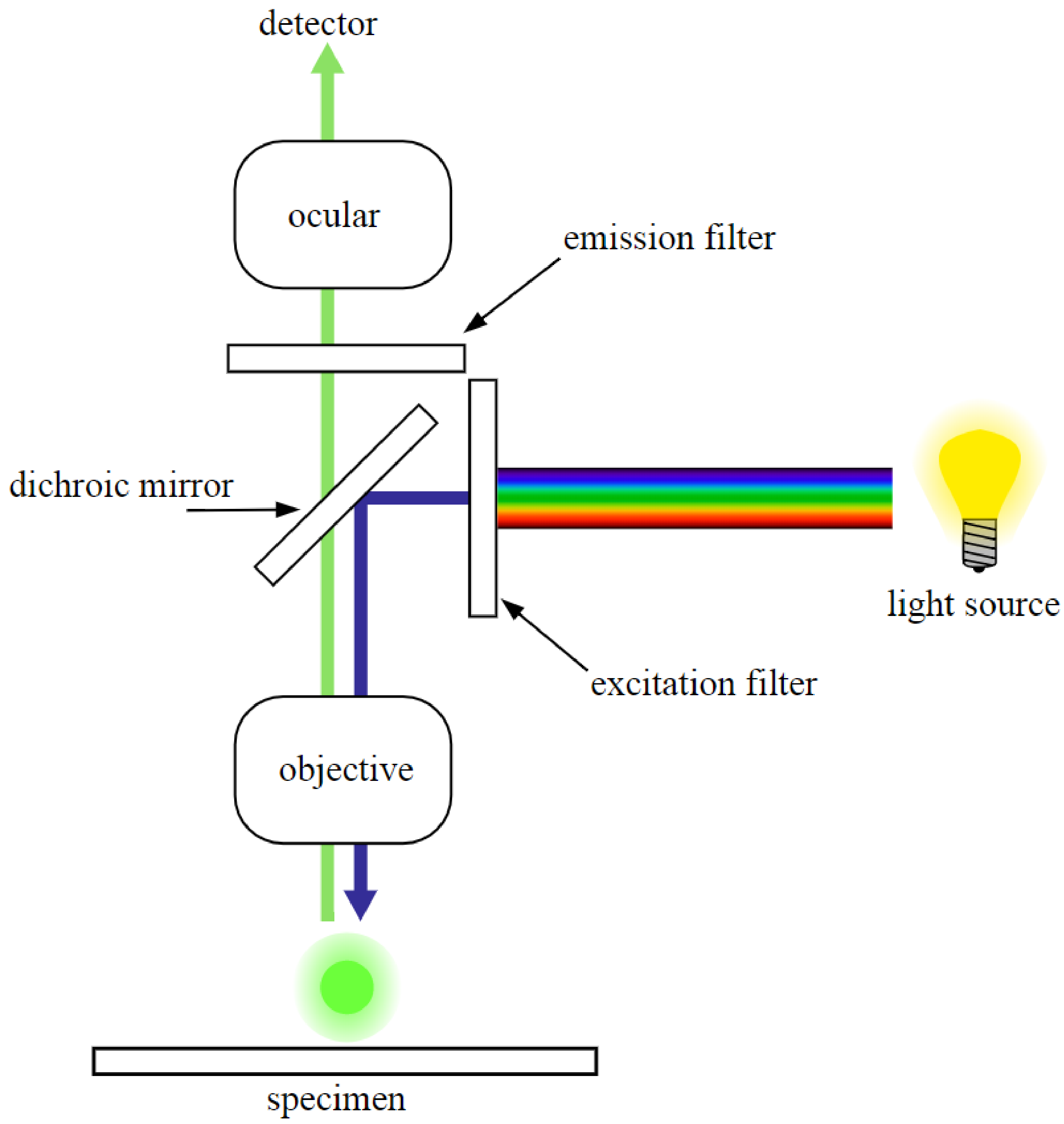
# Classics: epifluorescence microscopy



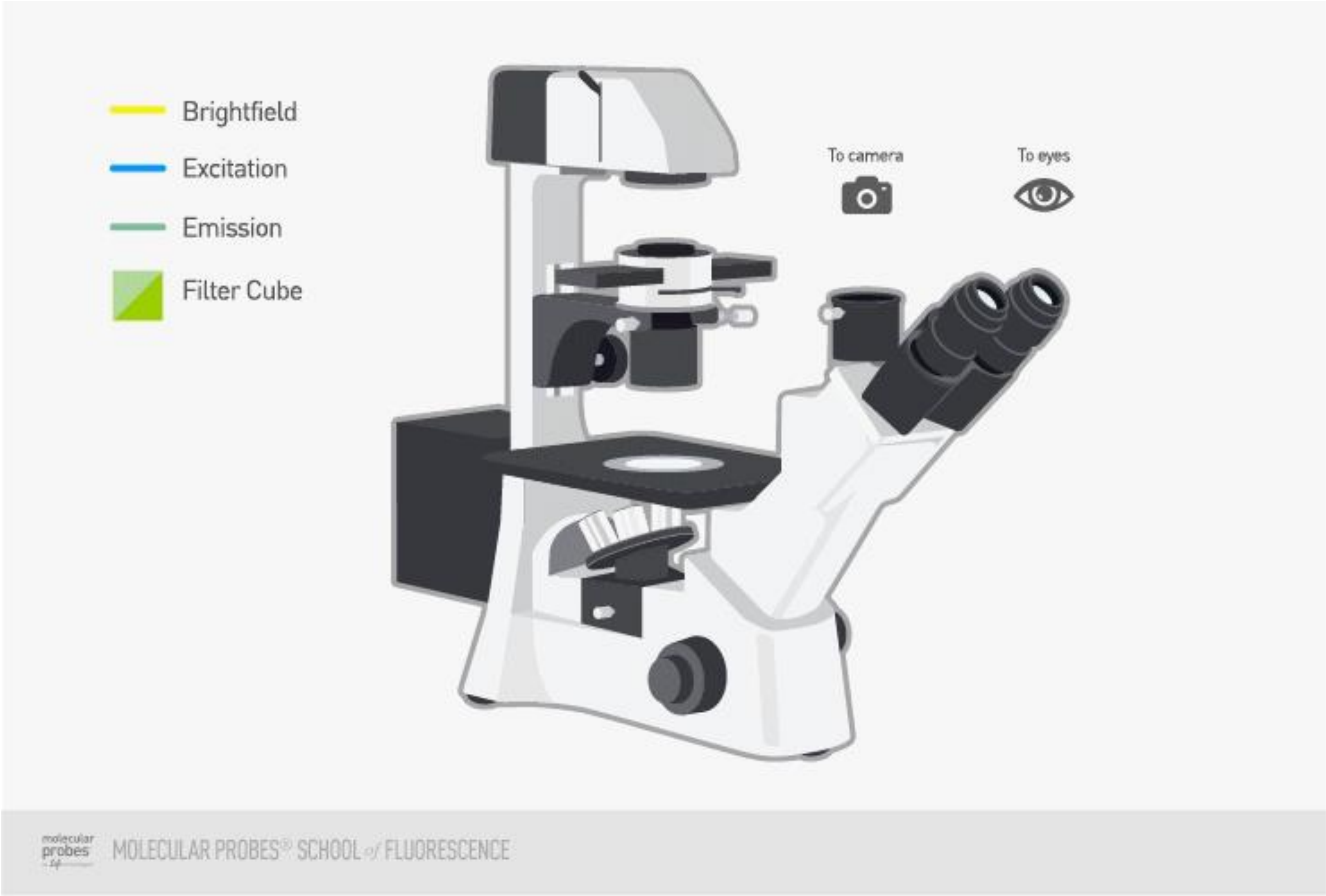



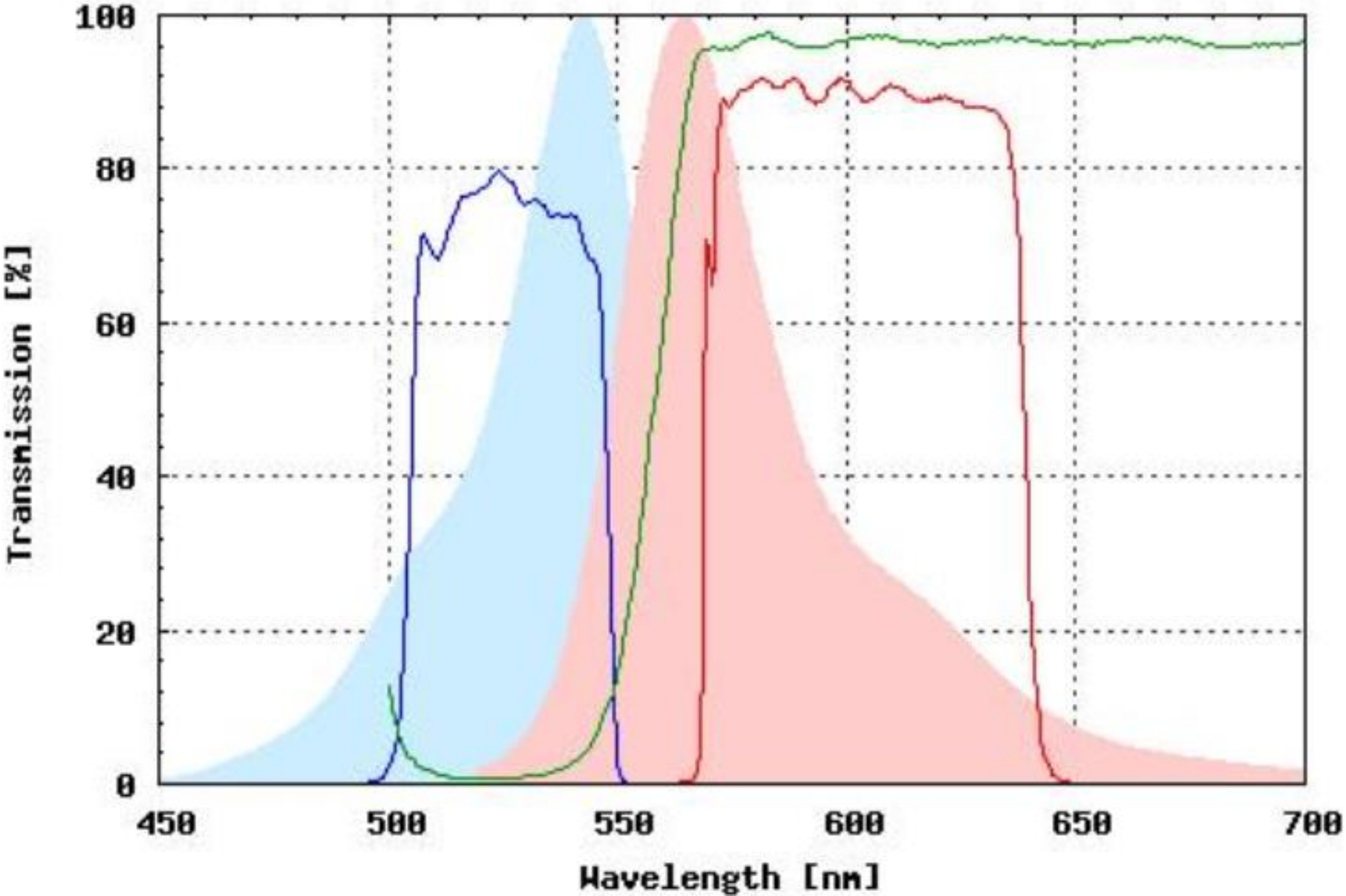

Fluorescent filter cube.

1: Ex filter
2: Dichroic mirror
3: Em (barrier) filter


detector
ocular
emission filter
dichroic mirror
light source
excitation filter
objective
specimen

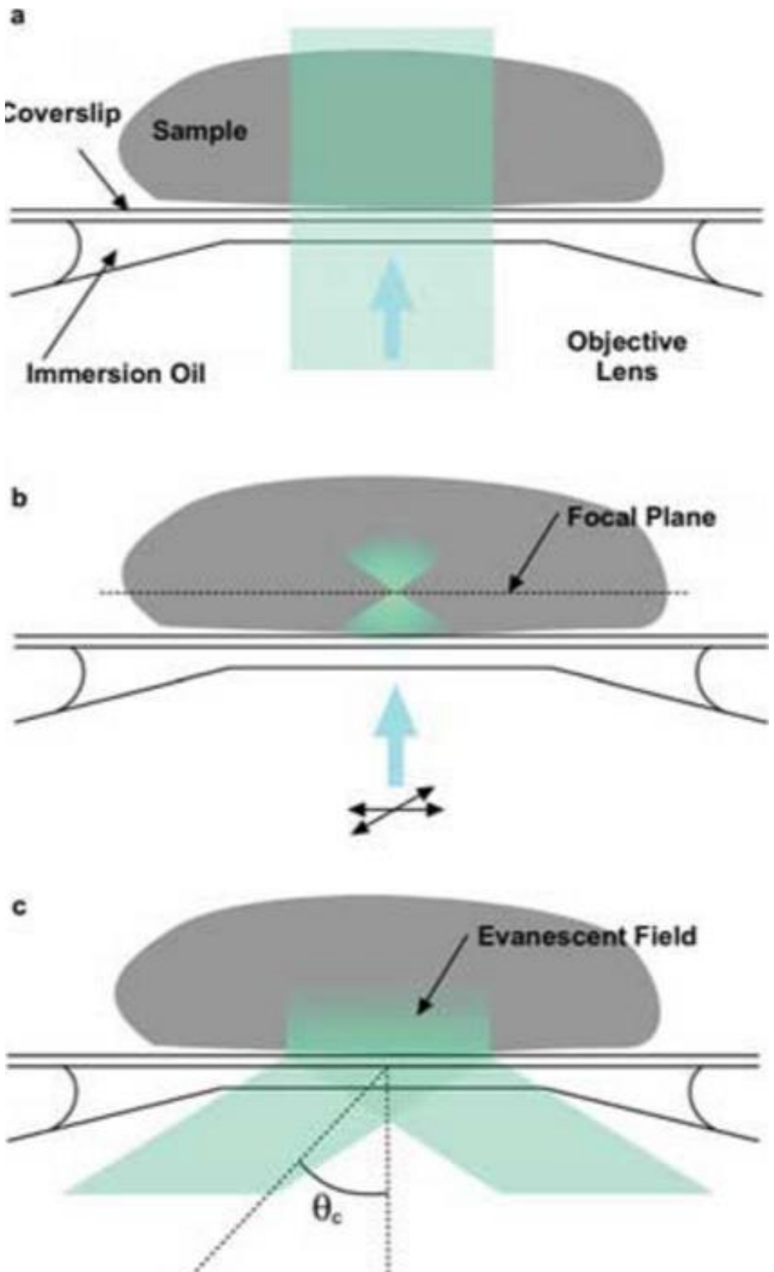
# Inverted microscope





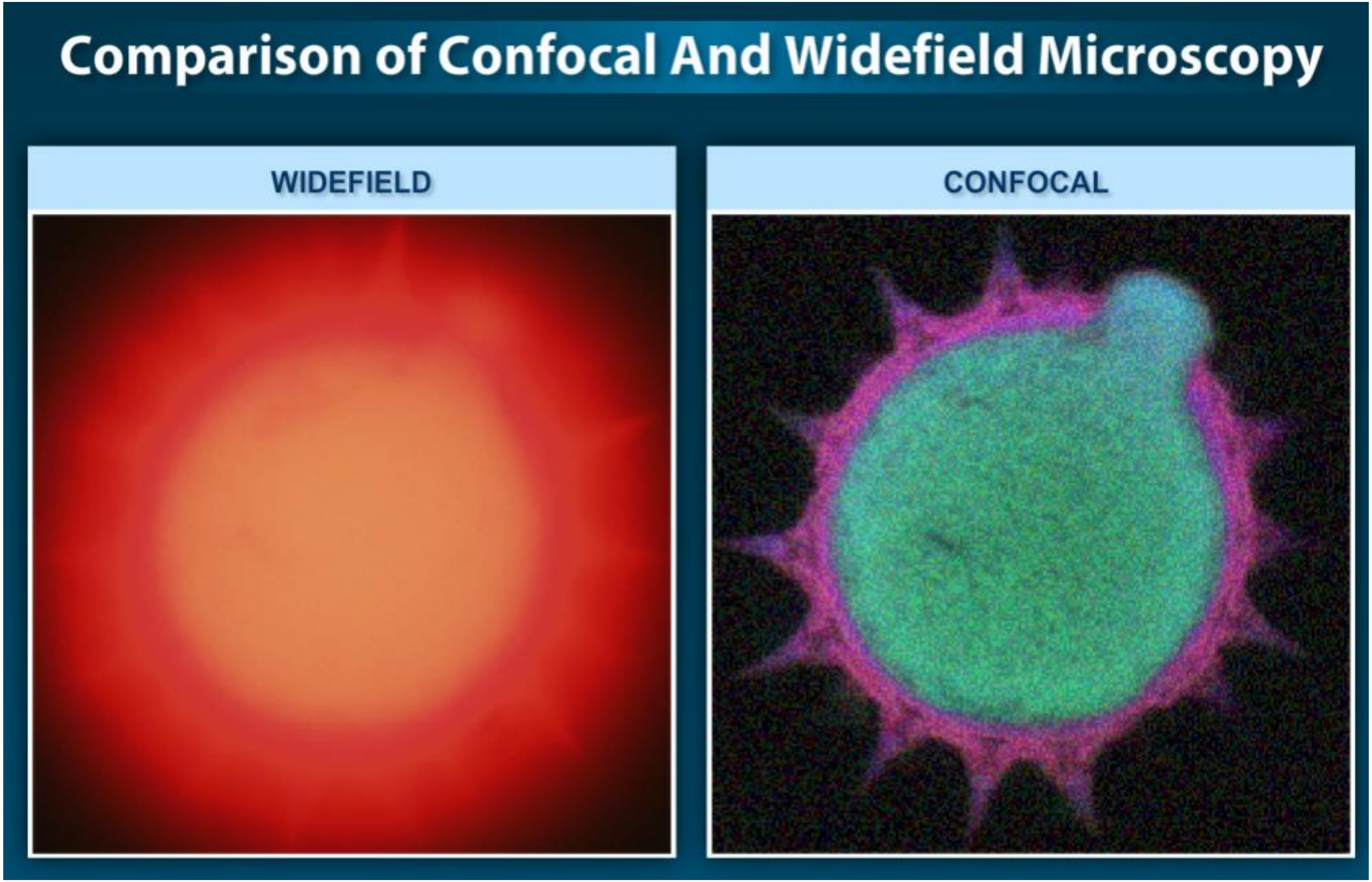
100
80
60
40
20
0
Transmission [%]
450
500
550
600
650
700
Wavelength [nm]

# Basic types of illumination

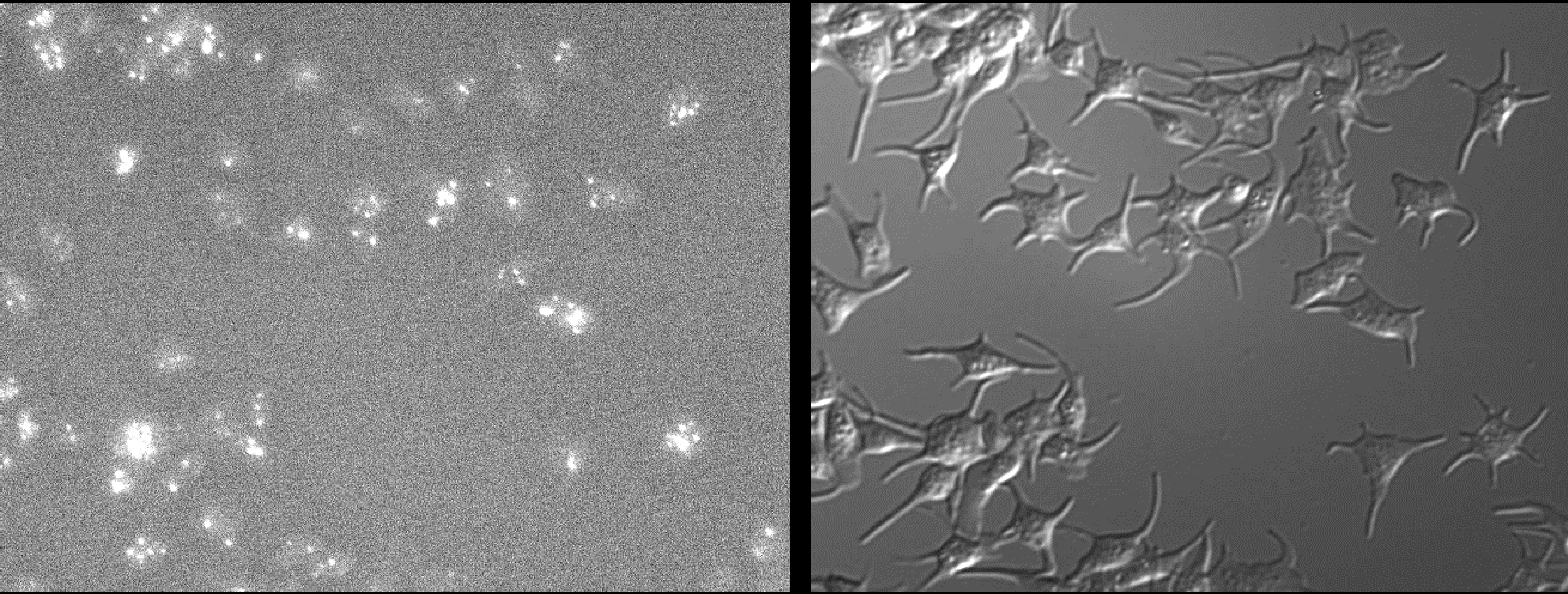



- Widefield (epifluorescence)
- Focused (confocal, multiphoton)
- Total internal reflection – based illumination (TIRF microscope)

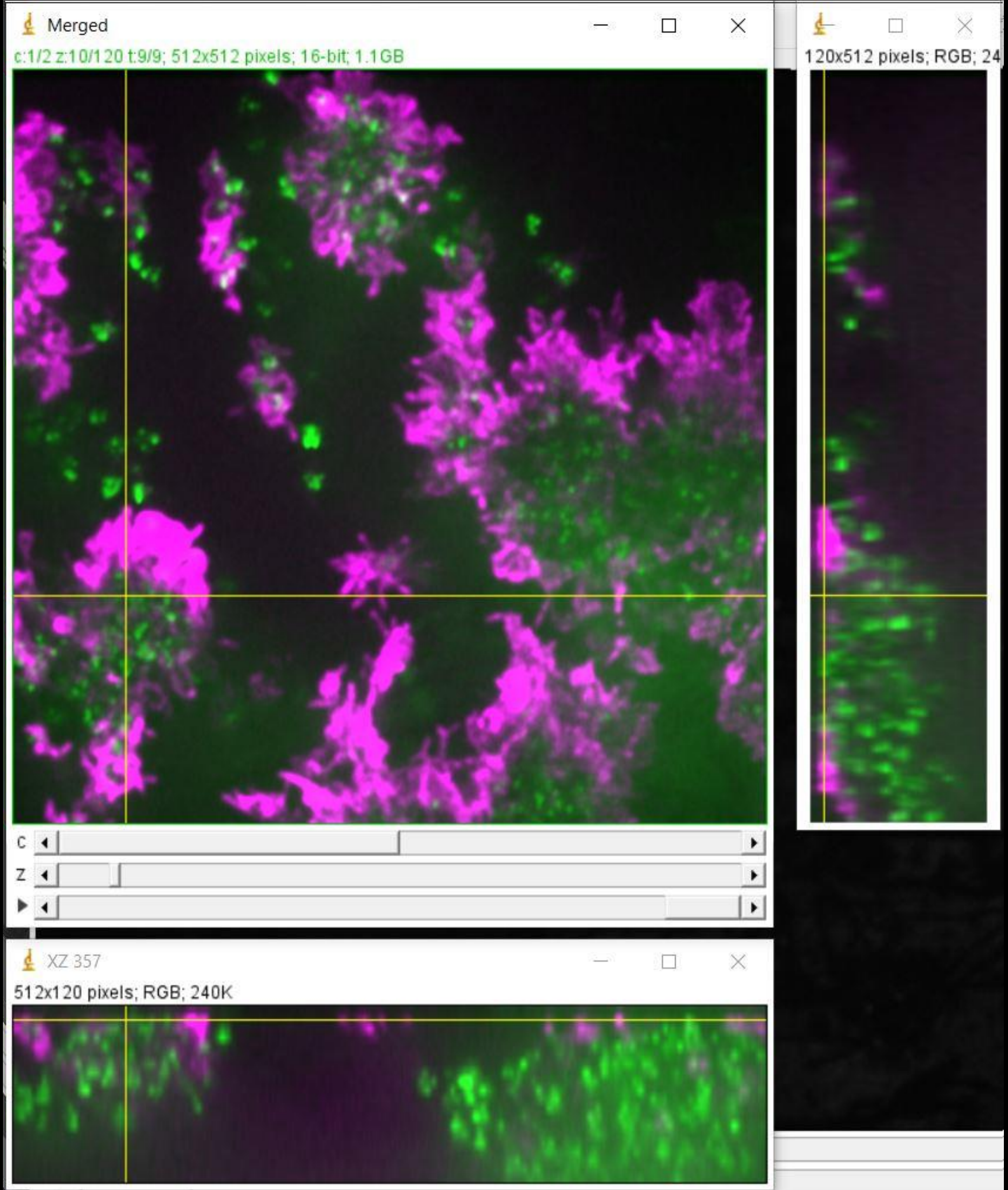
# Light Sheet Microscopy

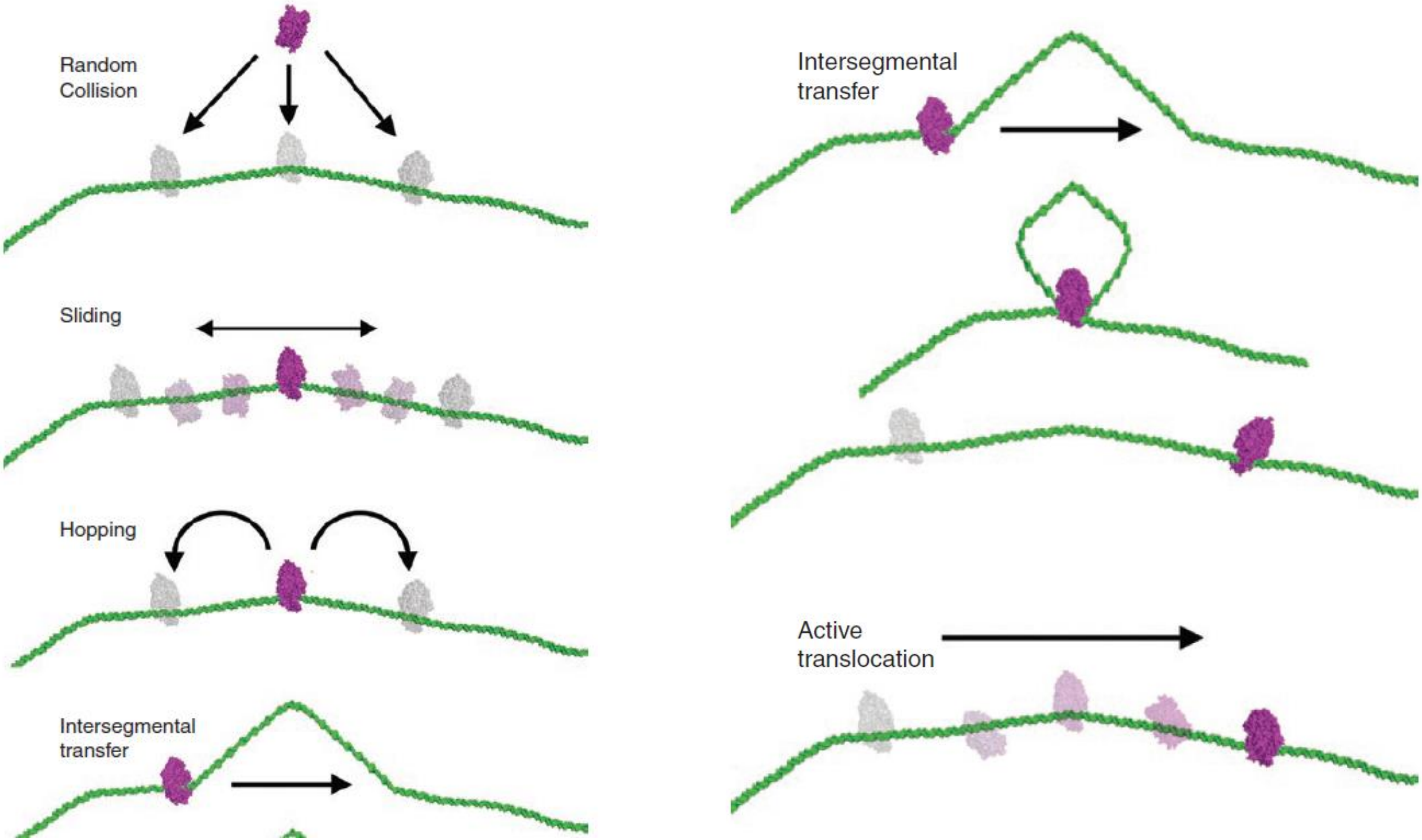

Comparison of Confocal And Widefield Microscopy
WIDEFIELD
CONFOCAL

# Mepacrine accumulation in dense granules

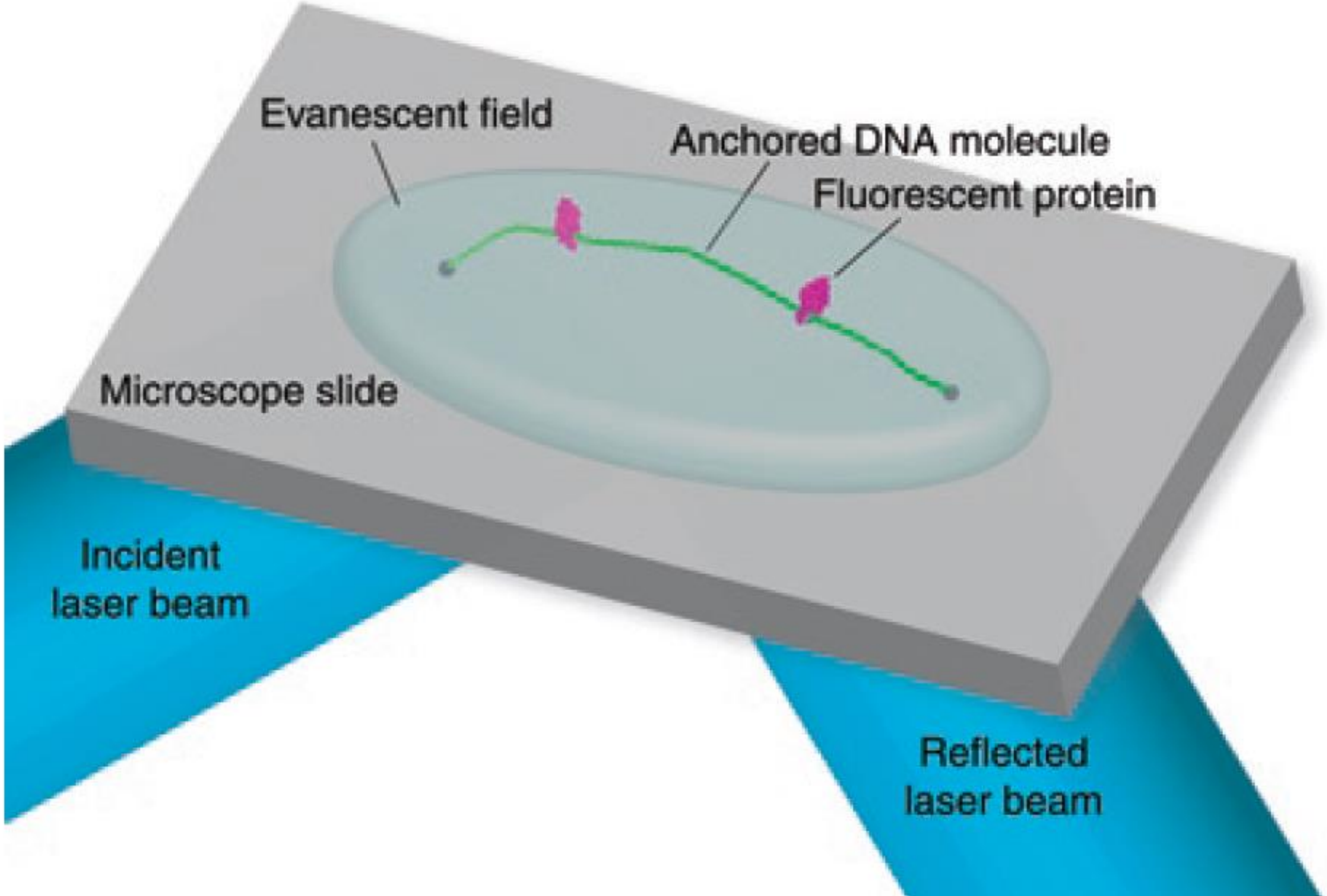


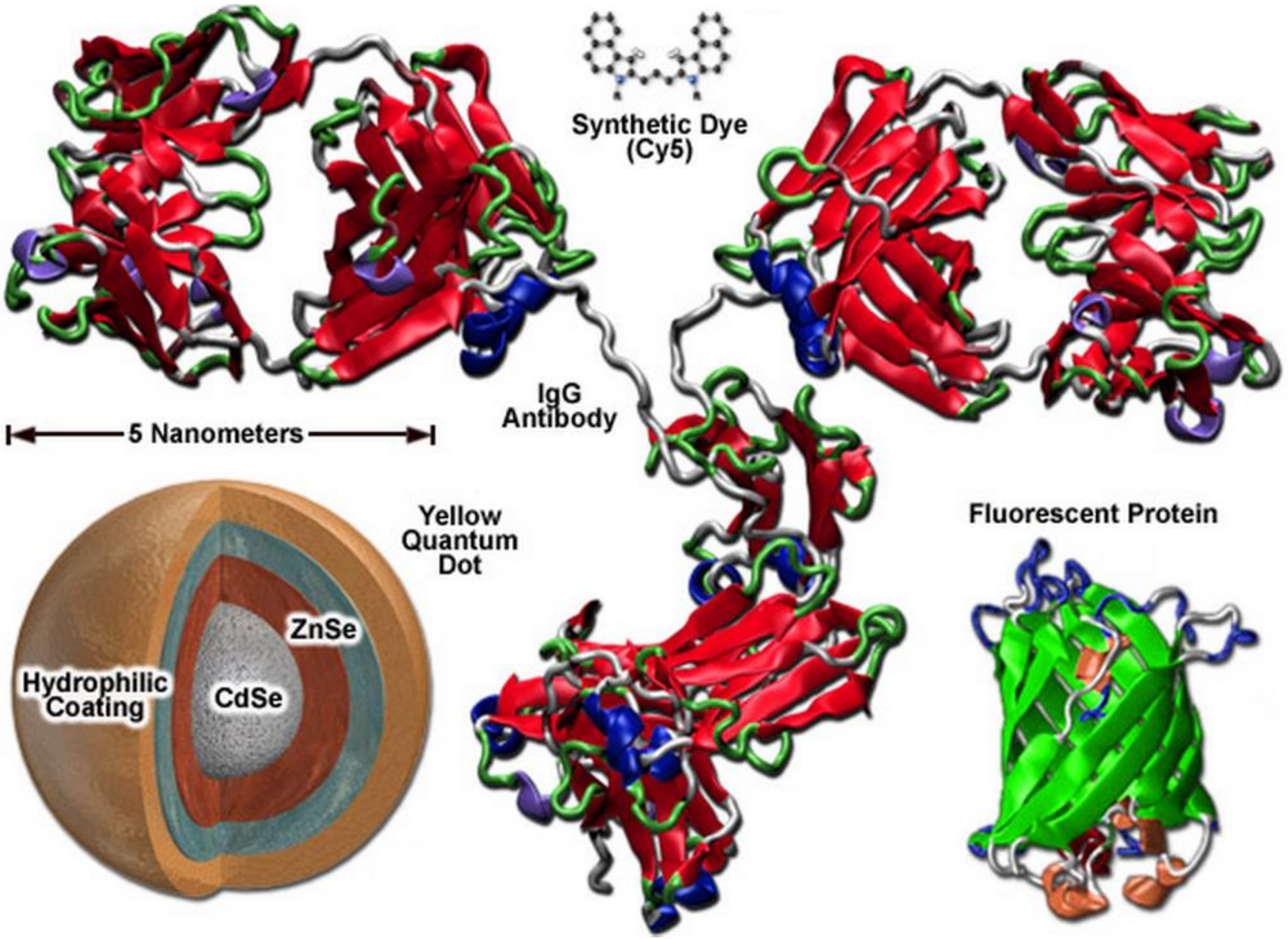
# Конфокальные срезы и сечения



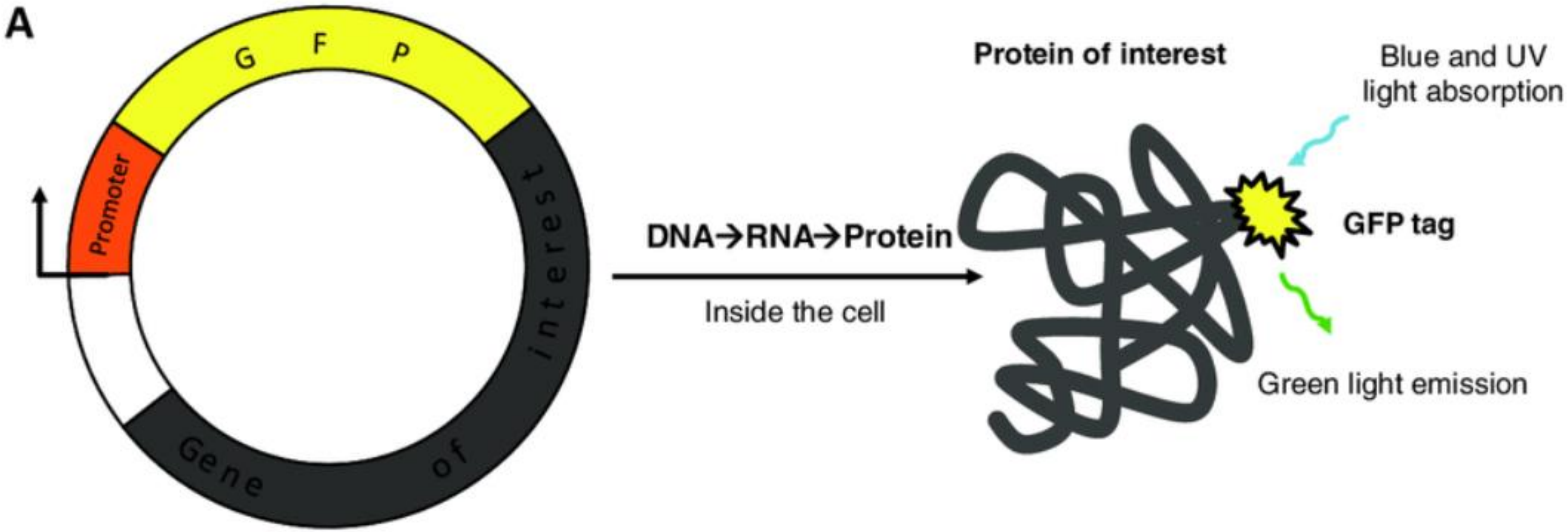

Random
Collision
Sliding
Hopping
Intersegmental
transfer
Intersegmental
transfer
Active
translocation

# DNA-protein interaction: TIRF insight



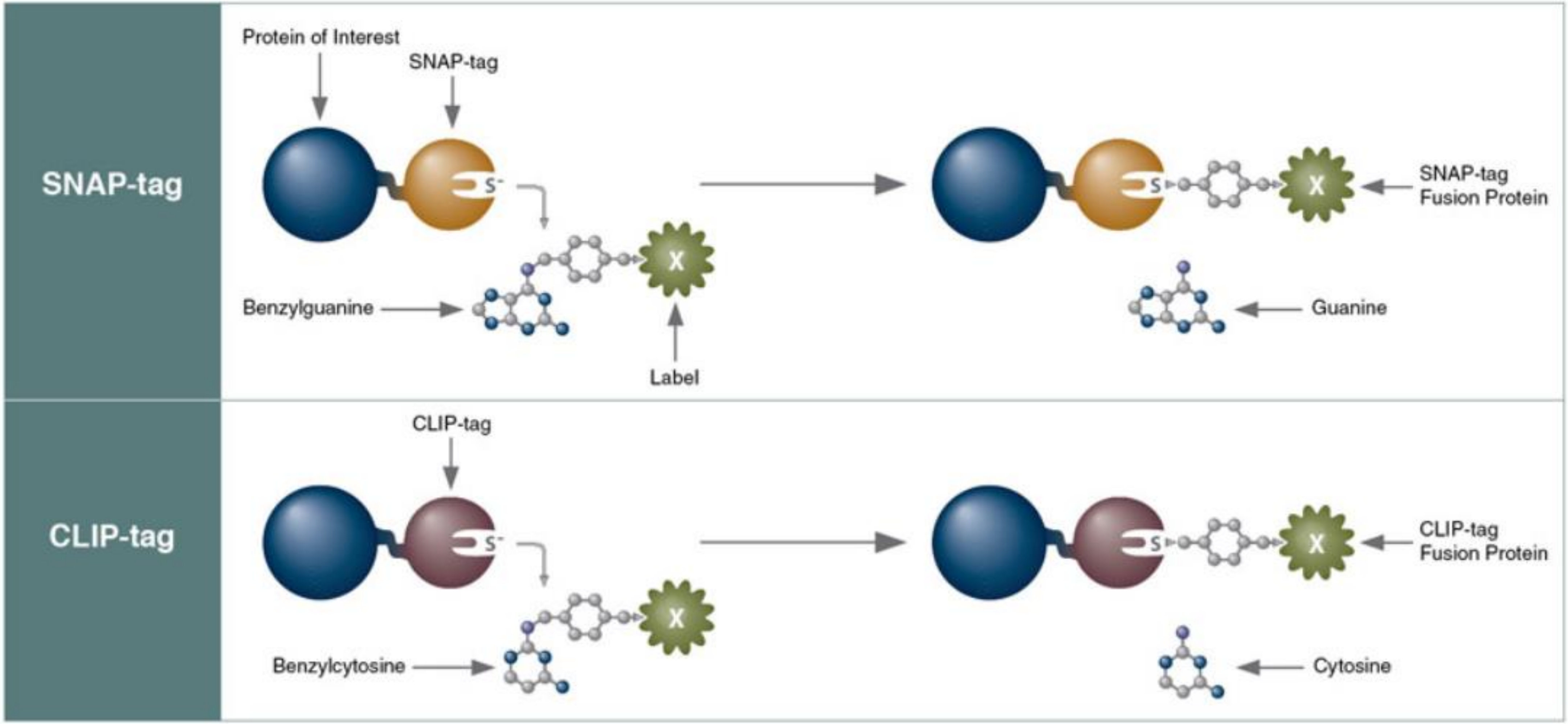

Synthetic Dye
(Cy5)
IgG
Antibody
5 Nanometers
Yellow
Quantum
Dot
ZnSe
Hydrophilic
Coating
CdSe
Fluorescent Protein

# GFP-tag




- https://www.researchgate.net/figure/Fluorescence-labeling-using-the-GFP-tags-the-most-common-genetic-method-increasing-the_fig3_286220224

# SNAP & CLIP-tags




- https://international.neb.com/tools-and-resources/feature-articles/snap-tag-technologies-novel-tools-to-study-protein-function

# The most important characteristics of fluorophores

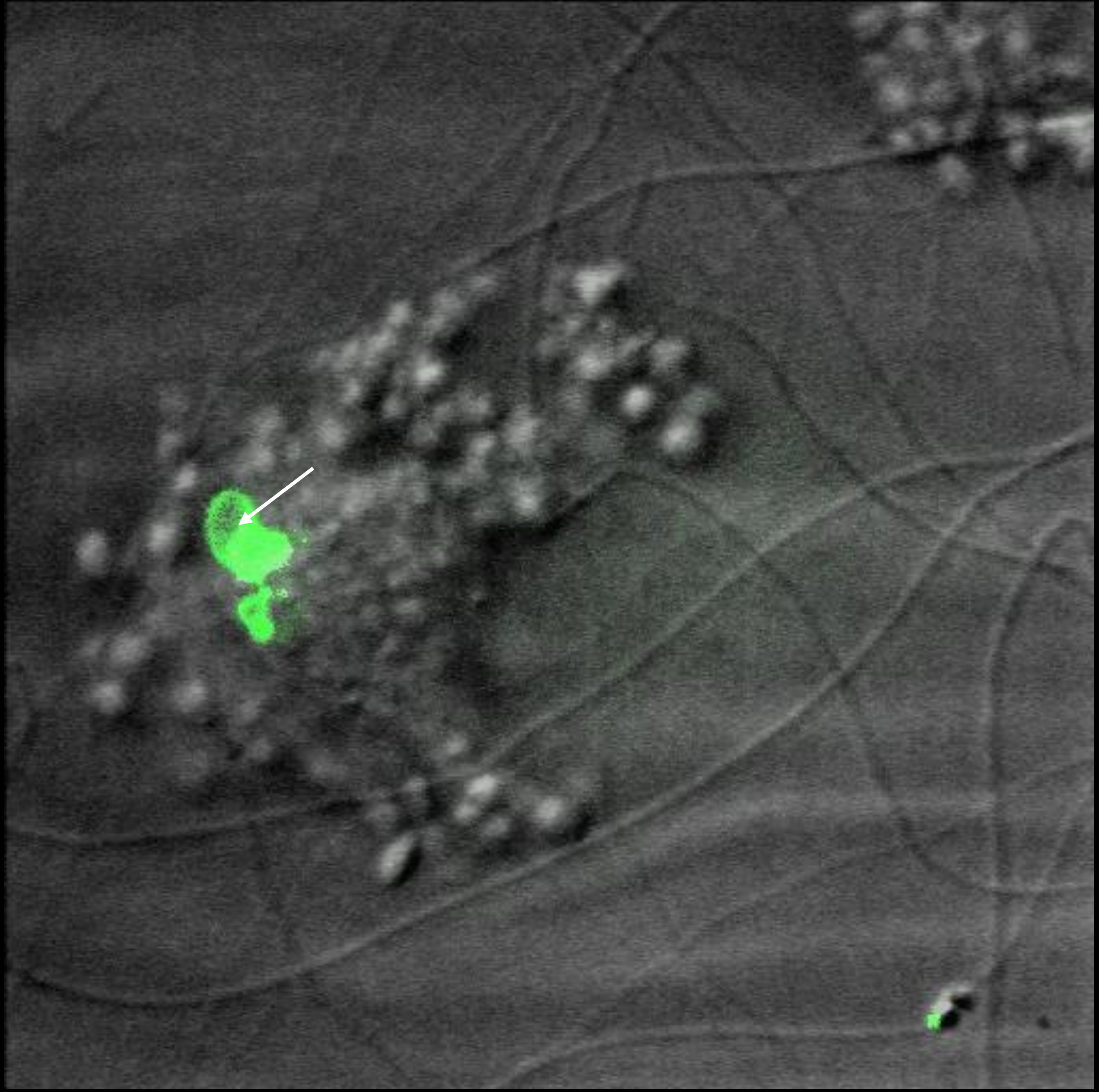
- Spectral ( absorption max, emission max )
- Brightness = efficiency of absorption * quantum yield
- Photostability (mean number of excitation cycles before photobleaching)
- Fluorescence lifetime

# Stability: a number of cycles before photobleaching

| | | |
|---|---|---|
| Green fluorescent protein: | $10^4$-$10^5$; | 0.1-1 s |
| Typical organic dye: | $10^5$-$10^6$; | 1-10 s |
| CdSe/ZnS Quantum dot: | $10^8$; | > 1000 s |

Annexin V – AlexaFluor647

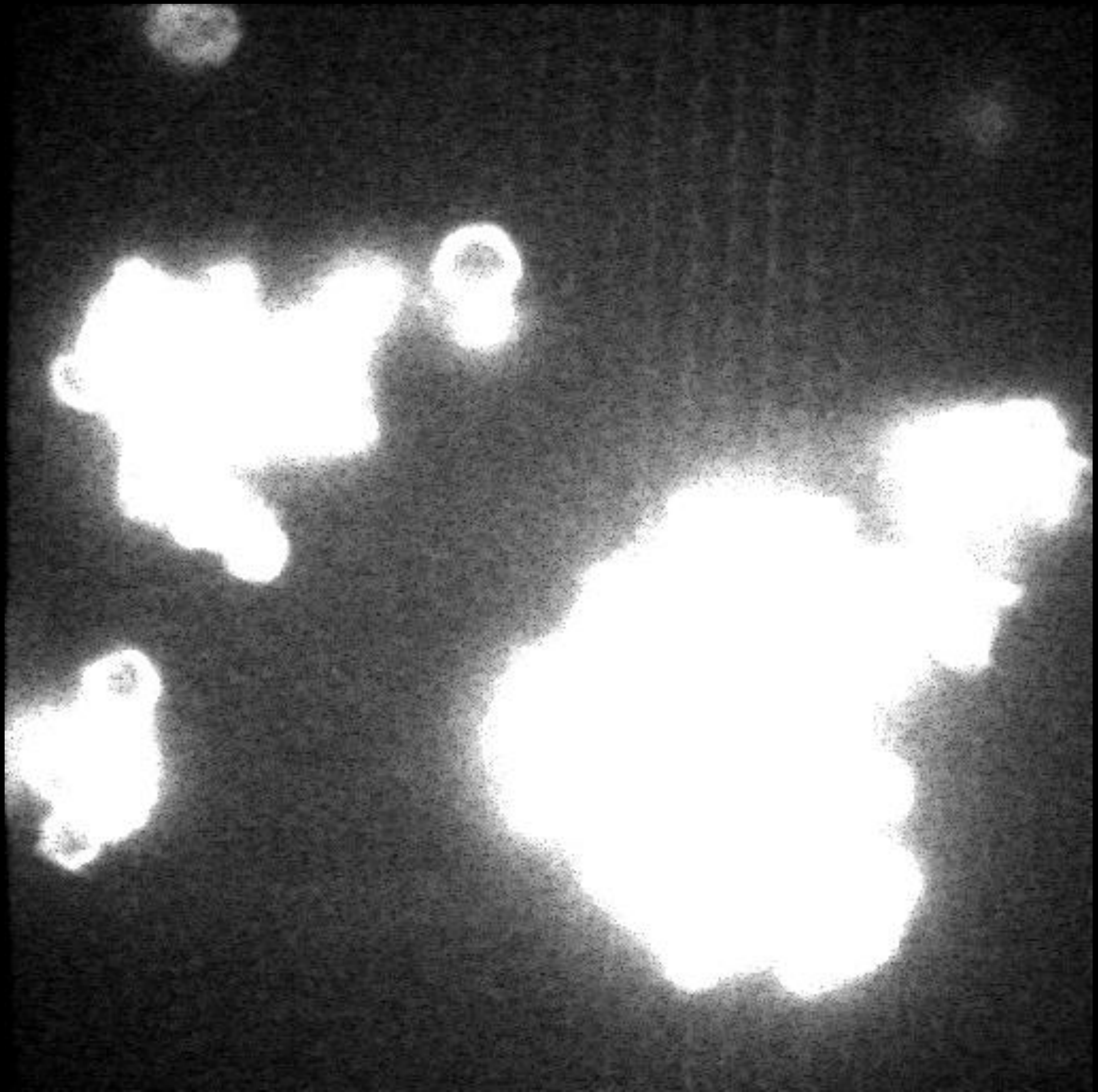
Flow



10 μ

**T ~ 2 min**

CD42b – FITC

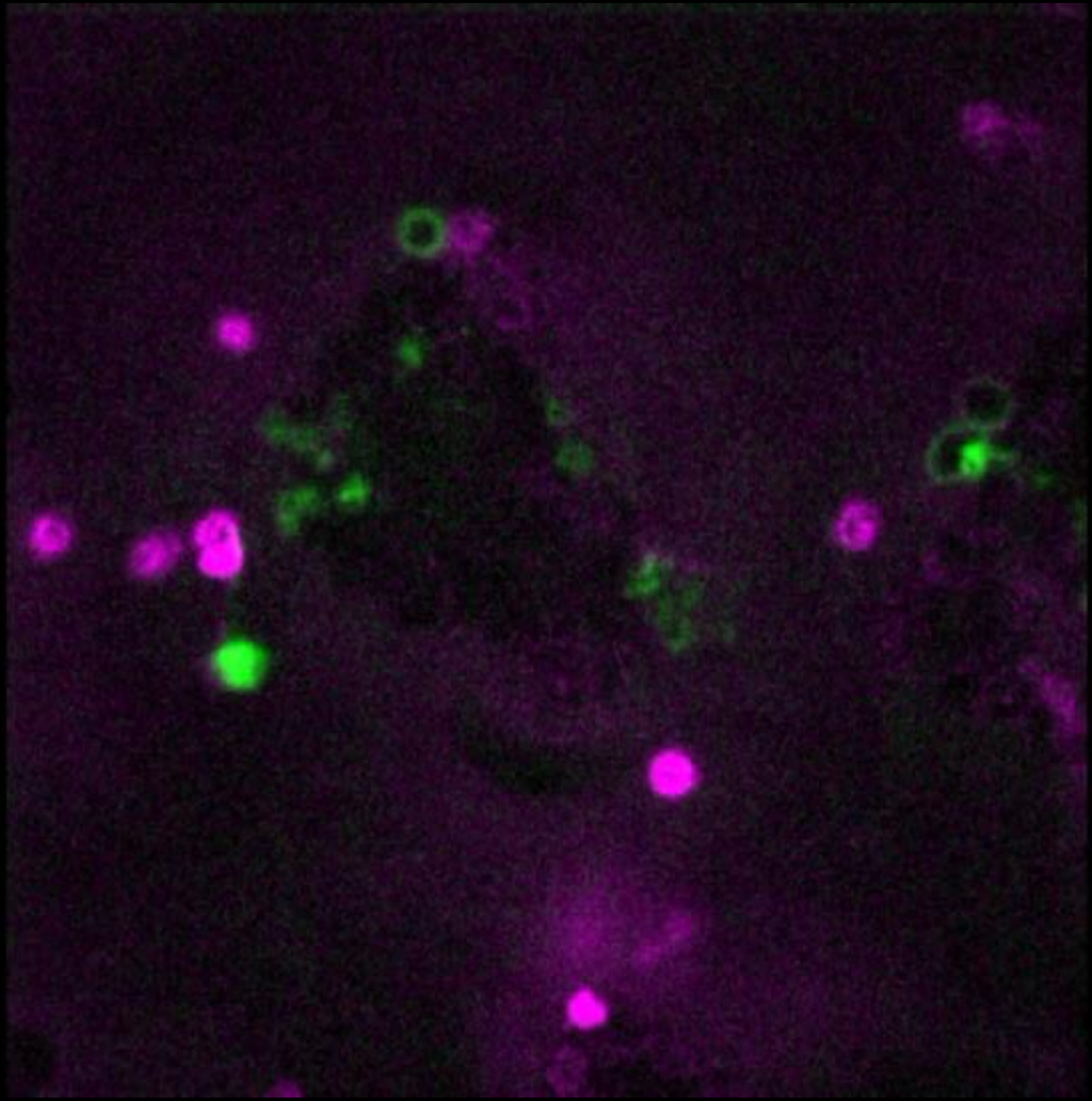


Annexin V – AlexaFluor647

CD42b – FITC

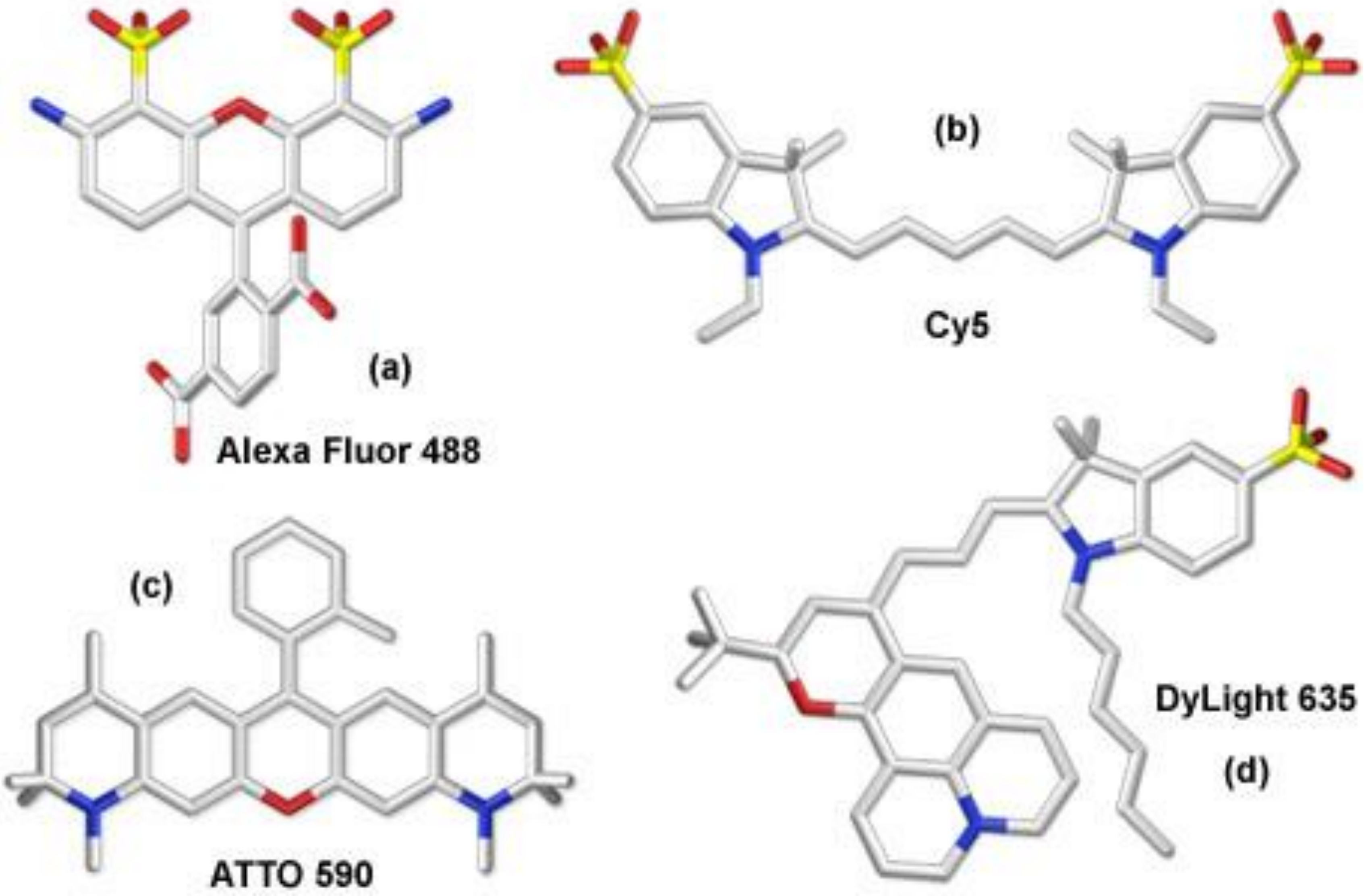
Flow

**T ~ 2 minutes**

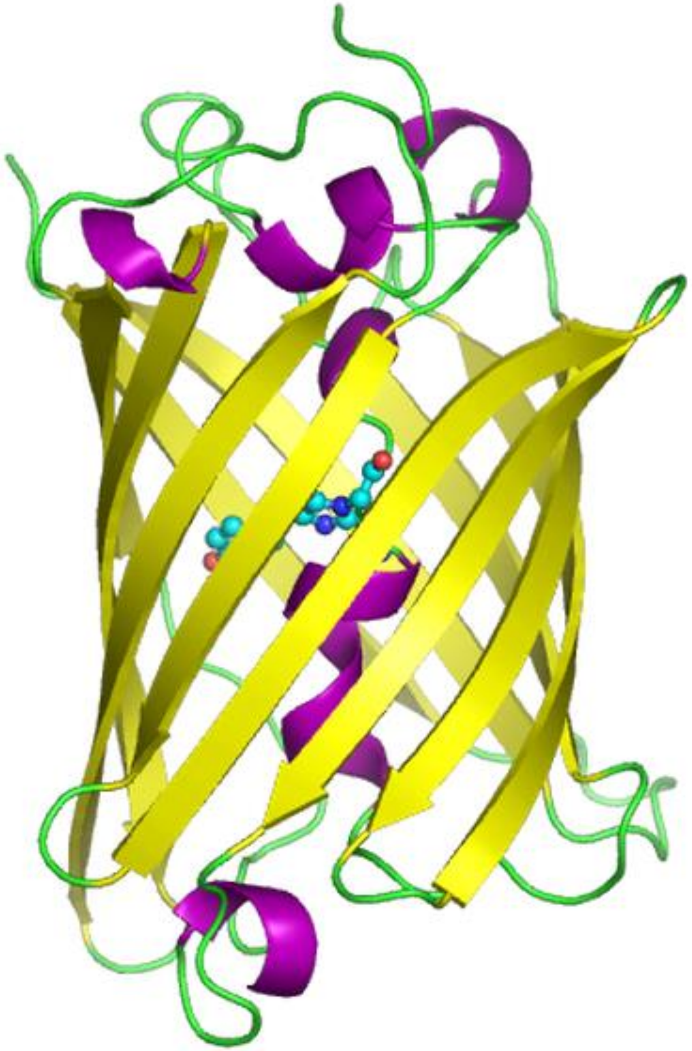


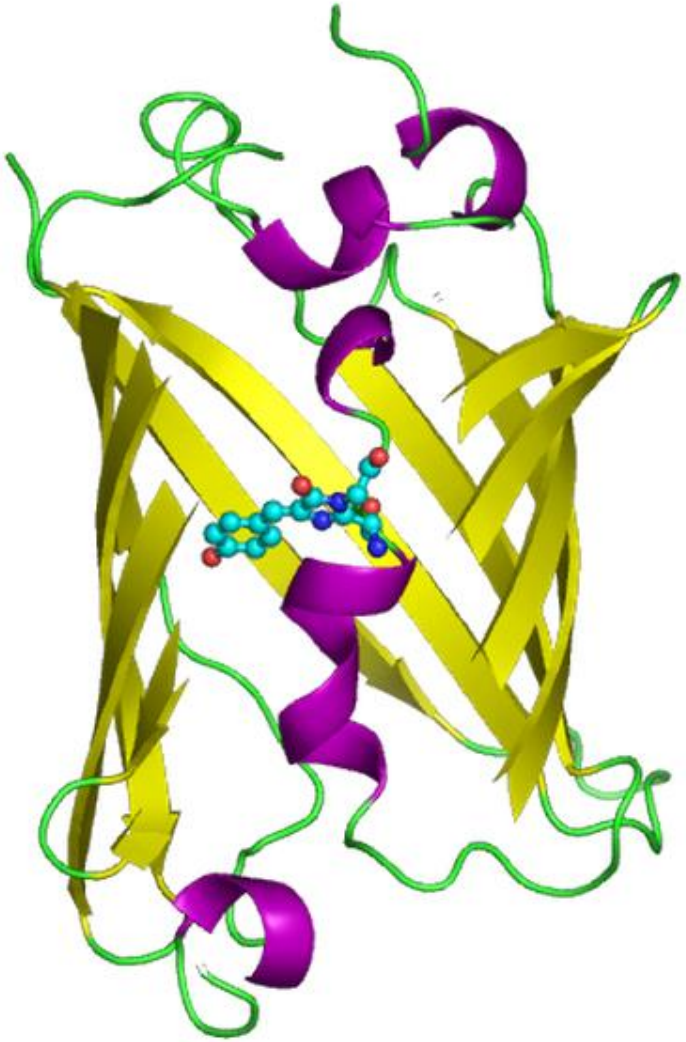
10 µ

# Synthetic fluorophores

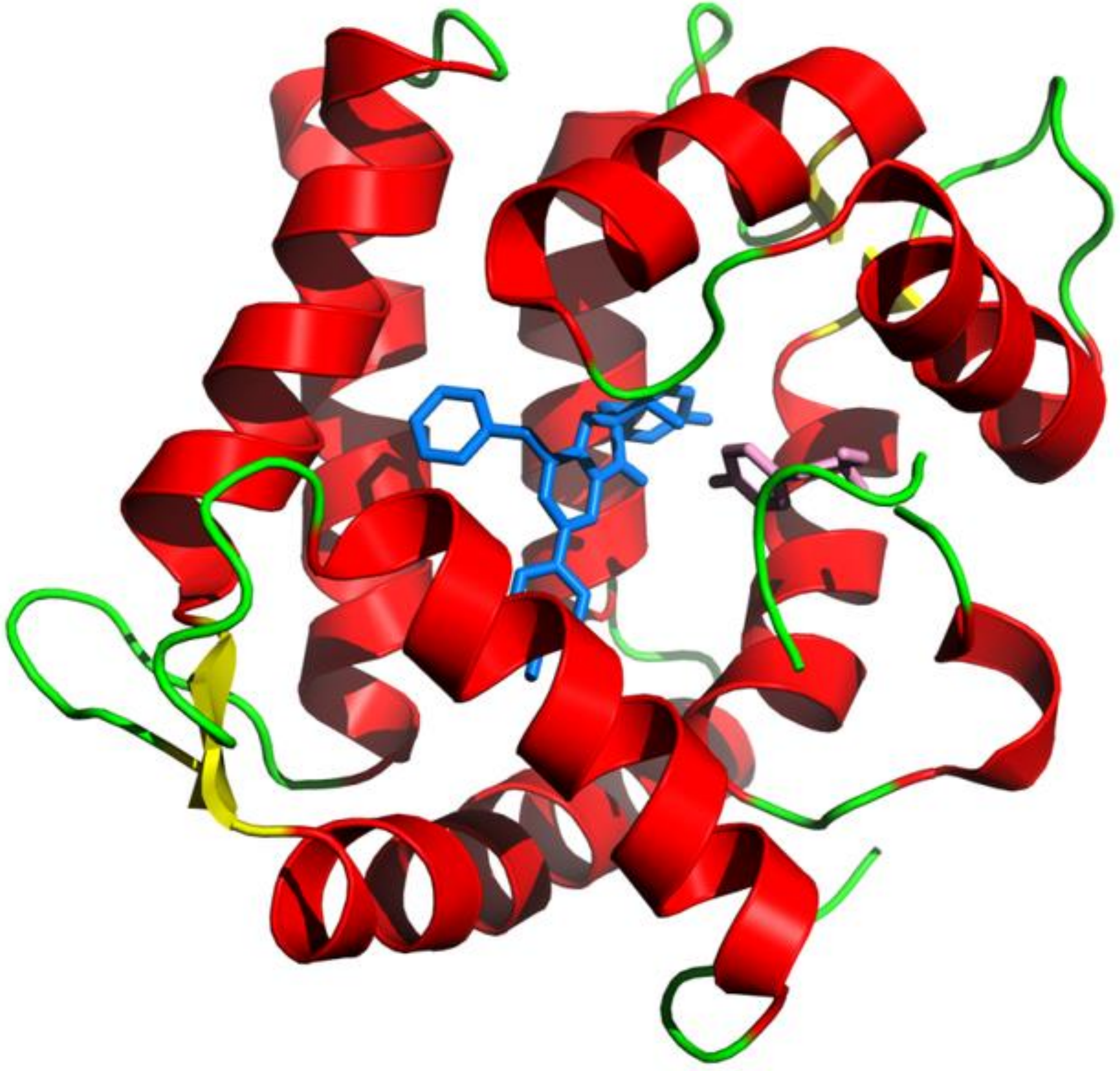


# GFP: CFP, YFP etc



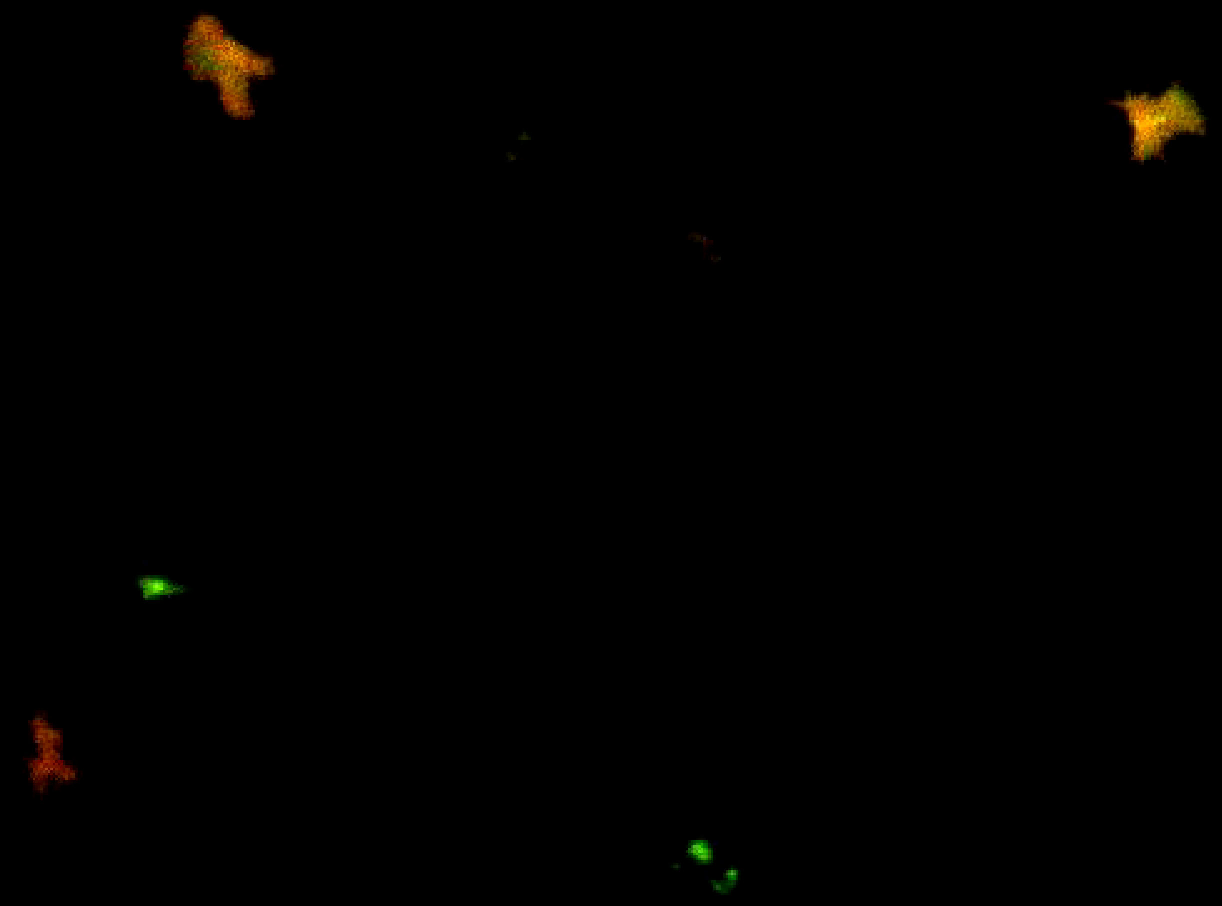


# Aequorin

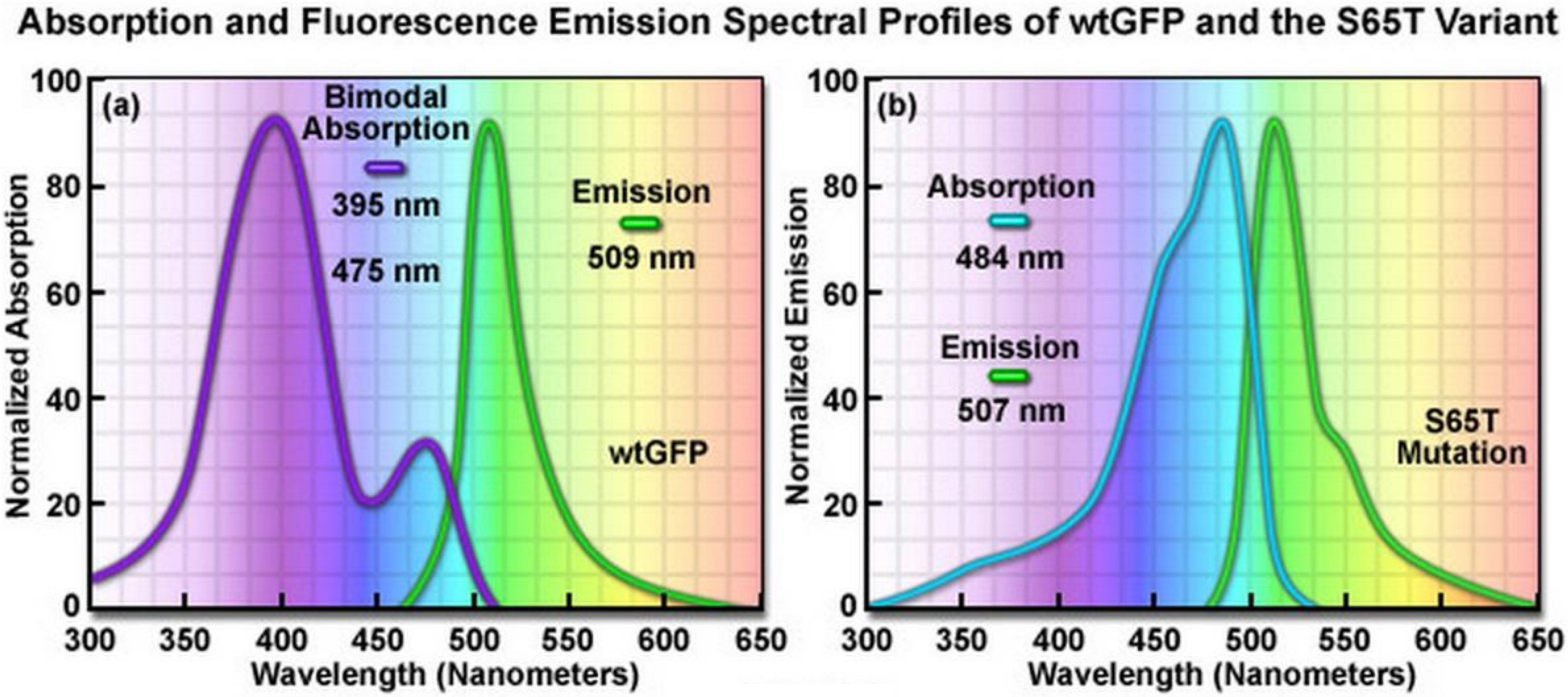


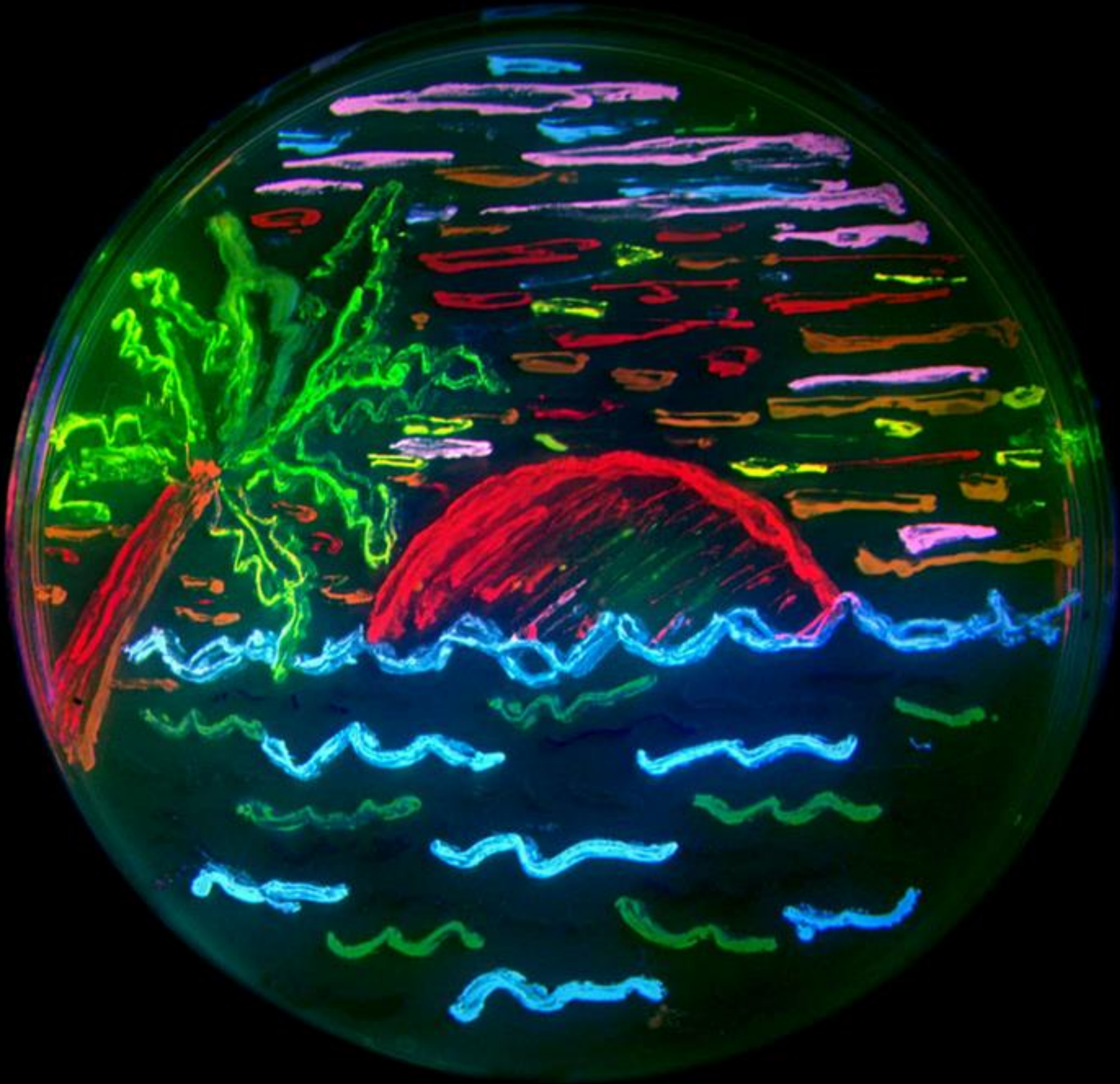
# Measuring $Ca^{2+}$ in single cells in vitro

Fura RED



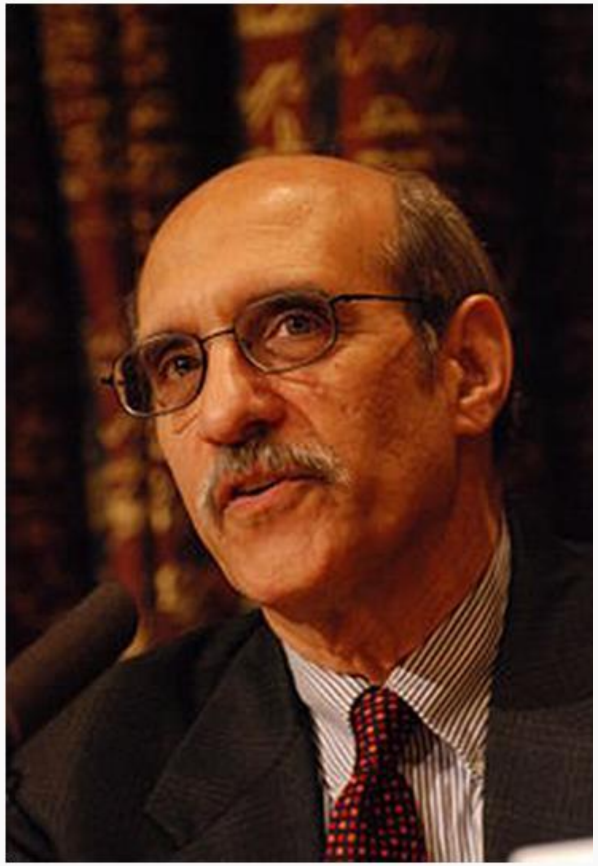
# Spectra of of wtGFP and S65T mutant





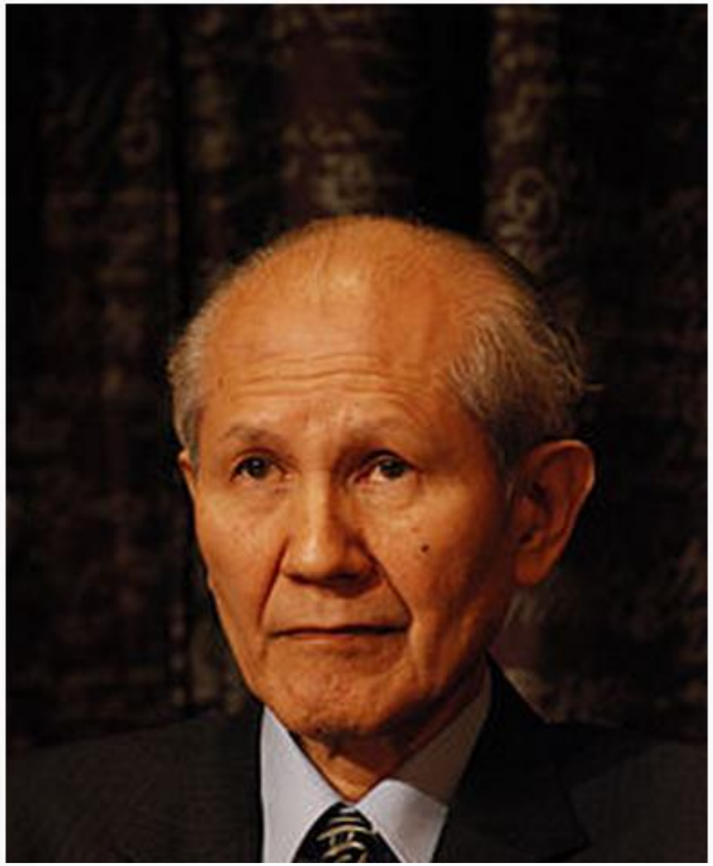
# GFP: nobel prize, 2008

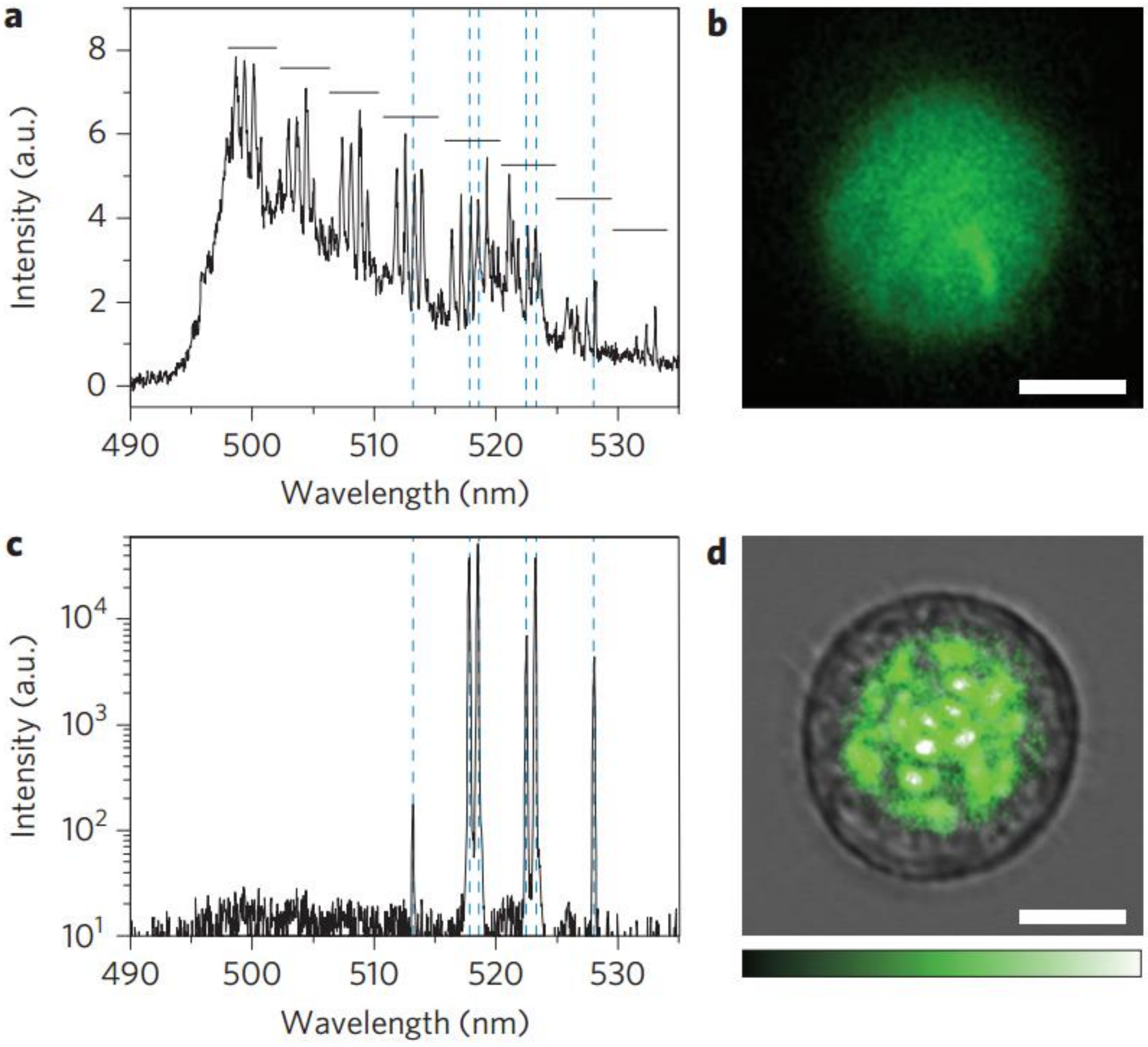
**Martin Chalfie**



**Roger Y. Tsien**
錢永健

下村 脩
**Shimomura Osamu**






- GFP laser within the single cell

Gather, M.C. and Yun, S.H., 2011. Single-cell biological lasers. *Nature Photonics*, *5*(7), p.406.

# FRAP, FLIM, FRET

***Fluorescence Recovery After Photobleaching**

**= FRAP**

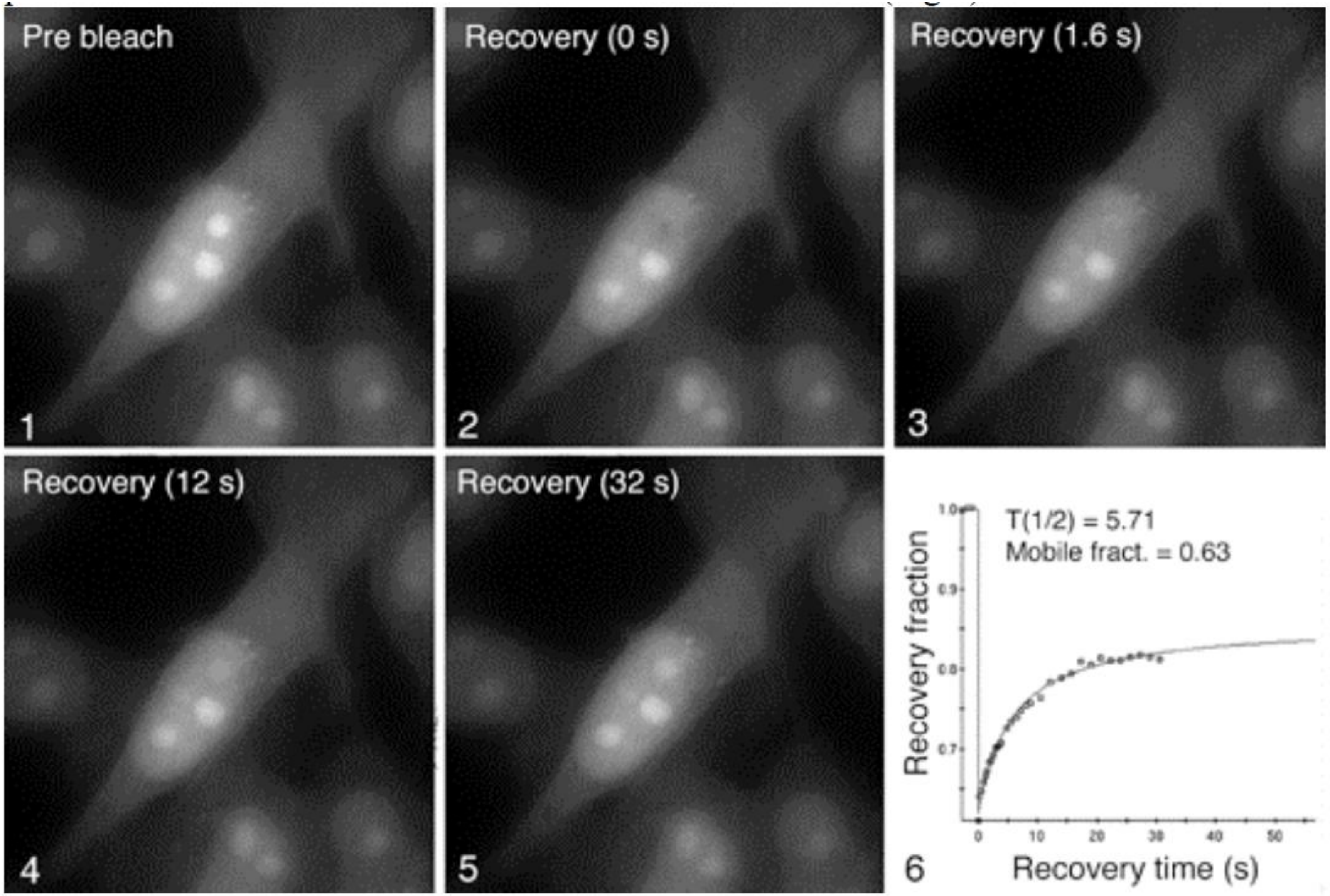
***Fluorescence Lifetime Imaging Microscopy**

**= FLIM**

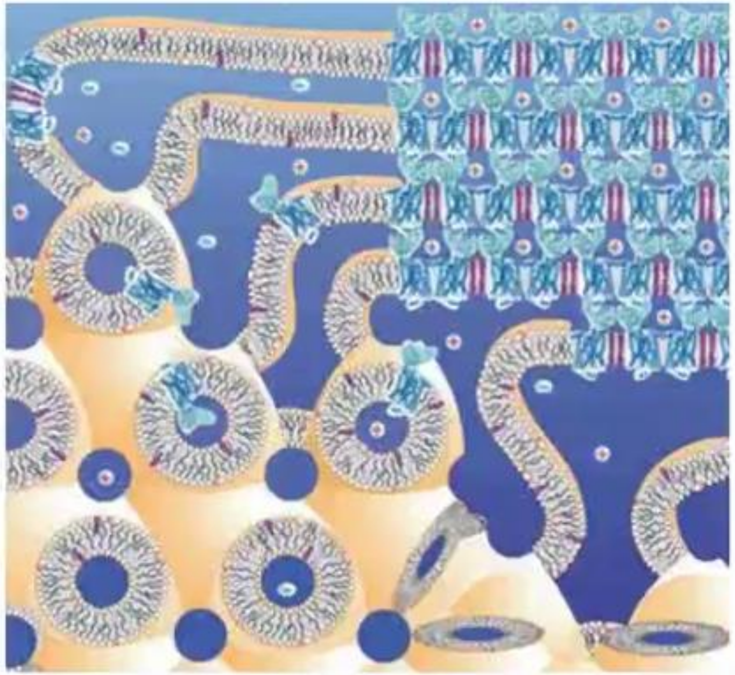
***Förster Resonance Energy Transfer**

**= FRET**

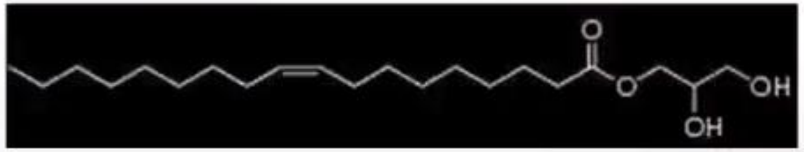
# FRAP: bleaching and measuring recovery rate



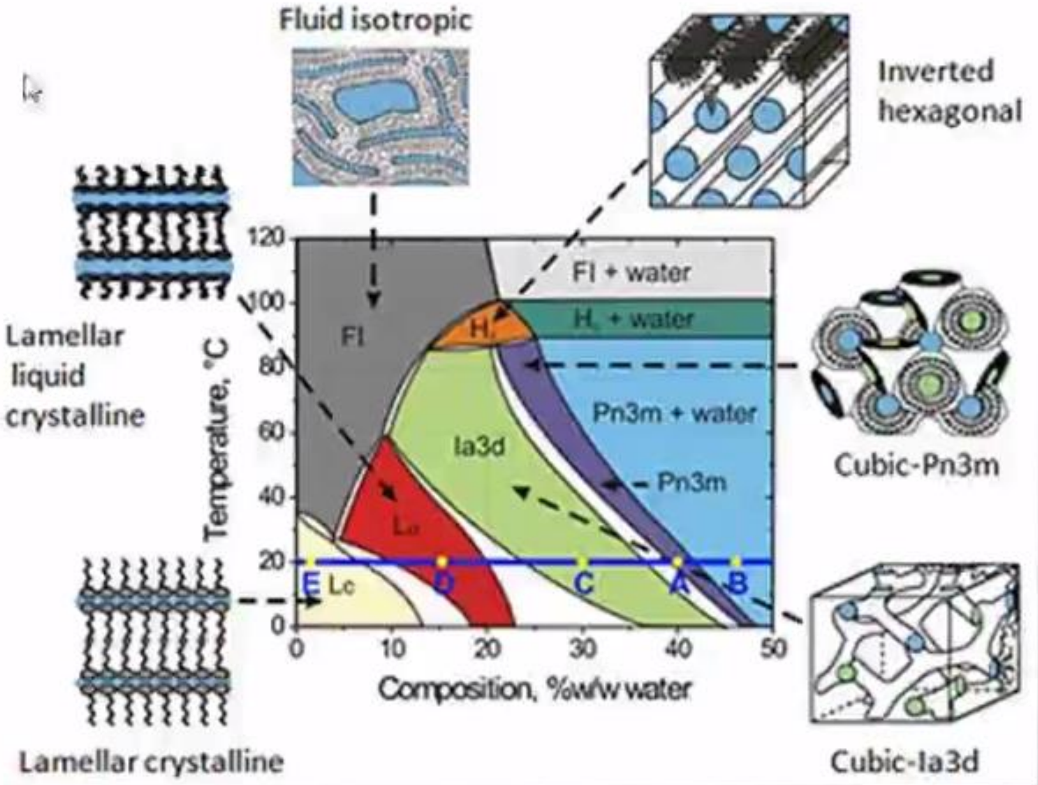
# Lipidic Cubic Phase (LCP)



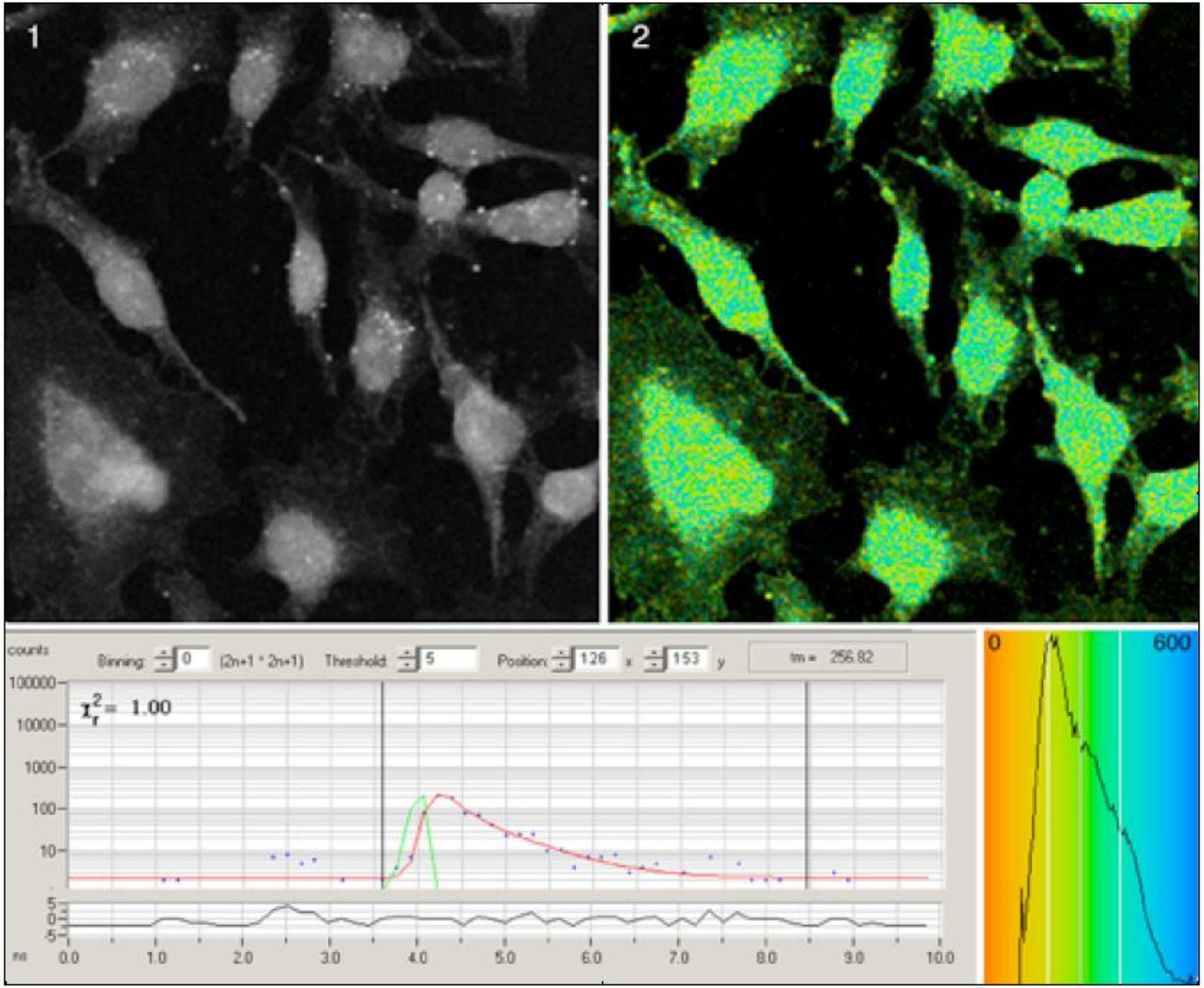
Caffrey. An Rev Biophys. 2009



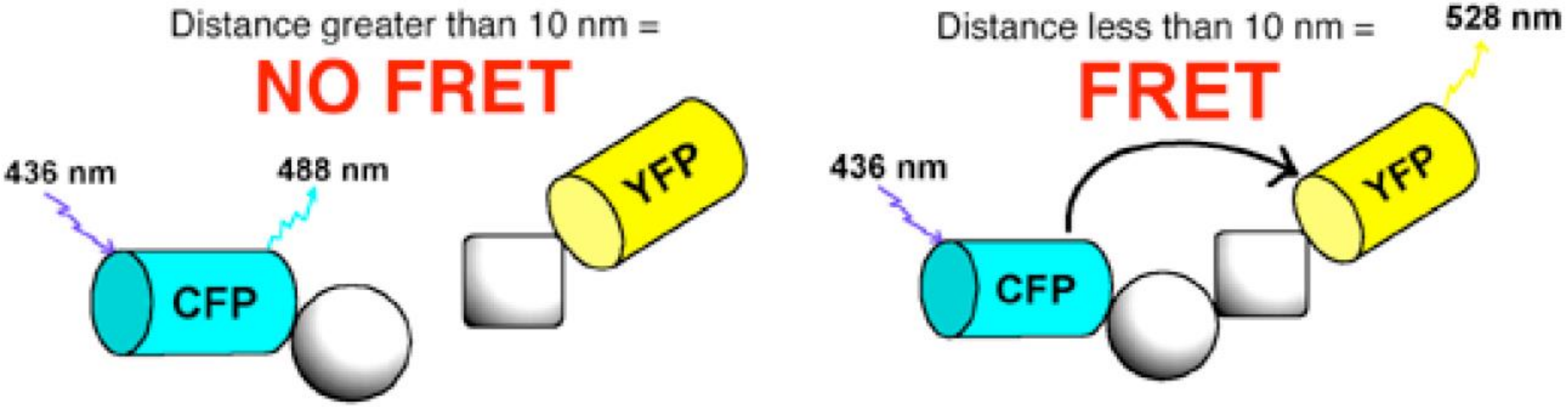

**Monoolein**

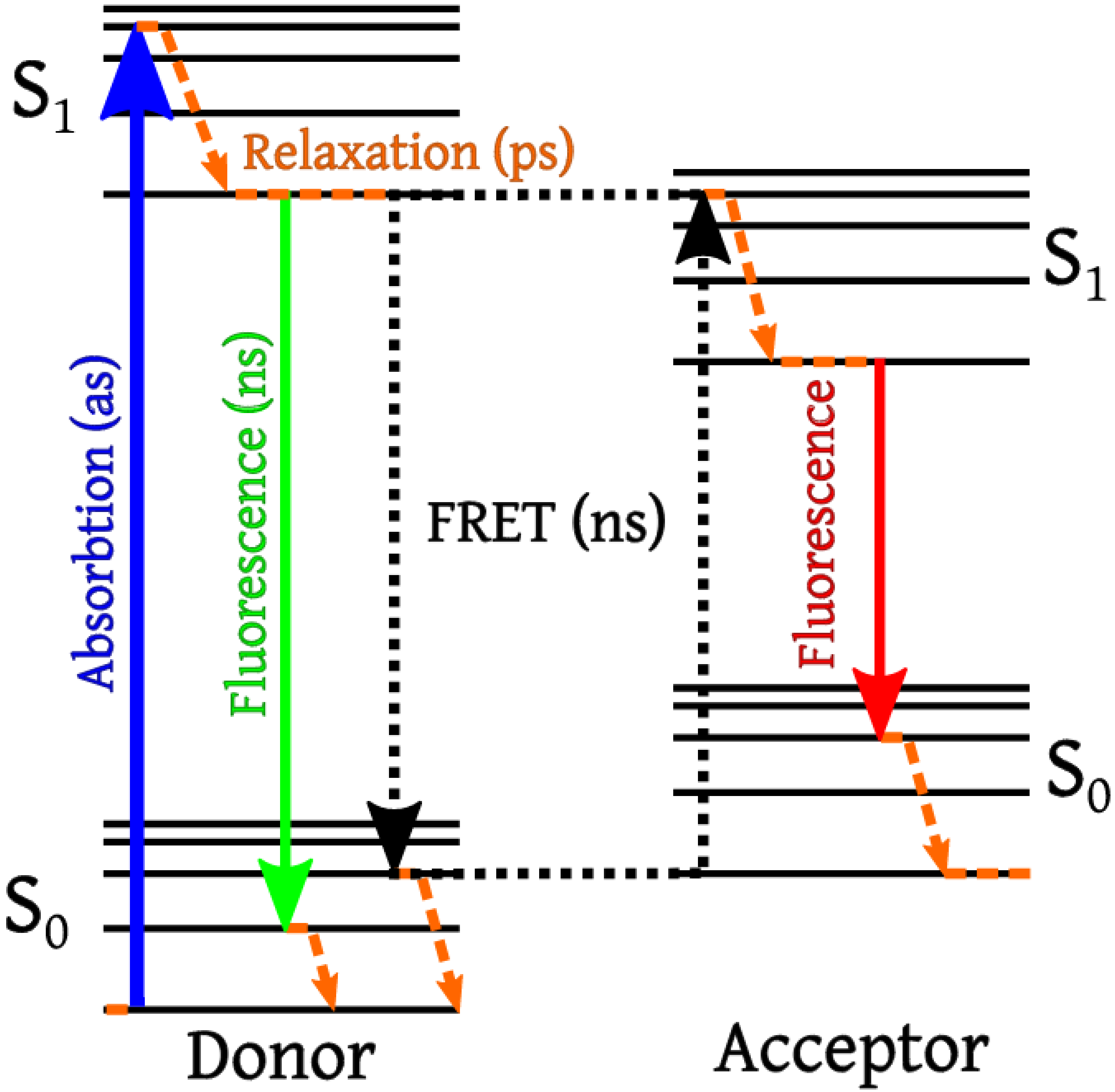


# FLIM


1
2
counts
Binning: 0 (2n+1 * 2n+1)
Threshold: 5
Position: 126 x 153 y
tm = 256.82
100000
10000
1000
100
10
$\chi_r^2$ = 1.00
5
0
-5
ns
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
0
600

# FRET


Distance greater than 10 nm =
NO FRET
436 nm
488 nm
CFP
YFP
Distance less than 10 nm =
FRET
528 nm
436 nm
CFP
YFP




# FRET features:

- Strong dependence on the distance between the molecules ( eff ~ 1/ $R^6$ )
- Depends on the spectral overlap for donor emission and acceptor absorption spectra
- Depends on relative oriention of the pair
- Non-radiative transfer!

# Main registration types

- ***FLIM-FRET***
- ***Acceptor photobleaching***
- ***Direct measurement of FRET using acceptor fluorescence***

# Acceptor photobleaching

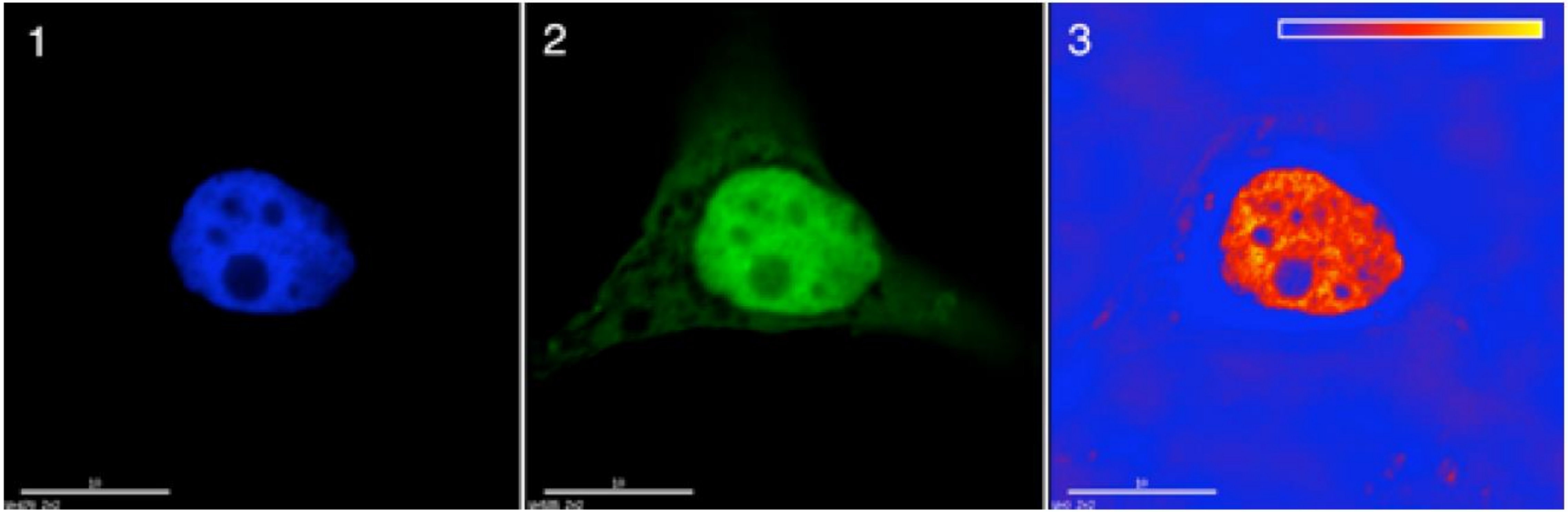
- Measuring fluorescence in the donor channel

- Acceptor photobleaching using the appropriate pulse of light

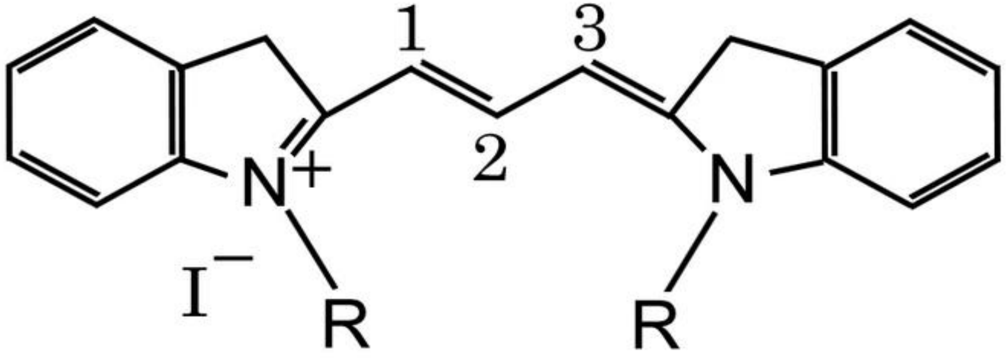
- Repeat fluorescence measurement in the donor channel

Drawbacks: photodamage, presence of FRAP, presense of acceptor signal

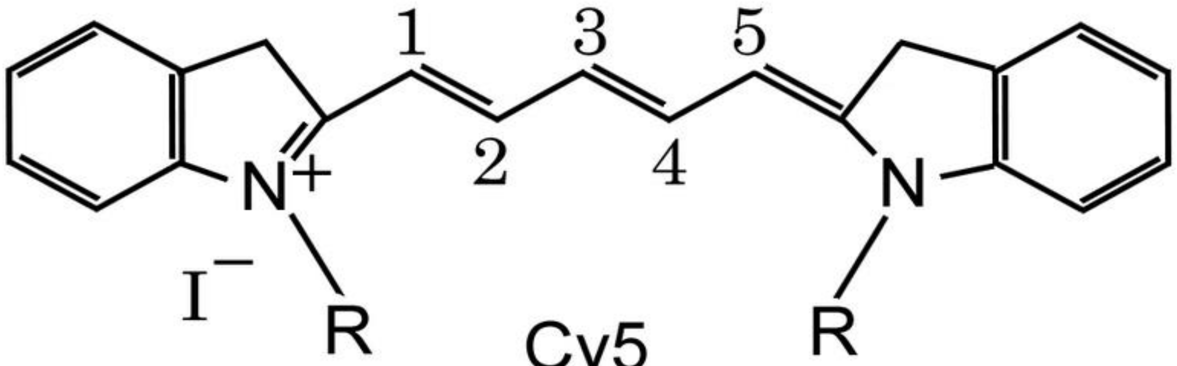
# *Measuring FRET using the acceptor channel*



# Popular FRET pair



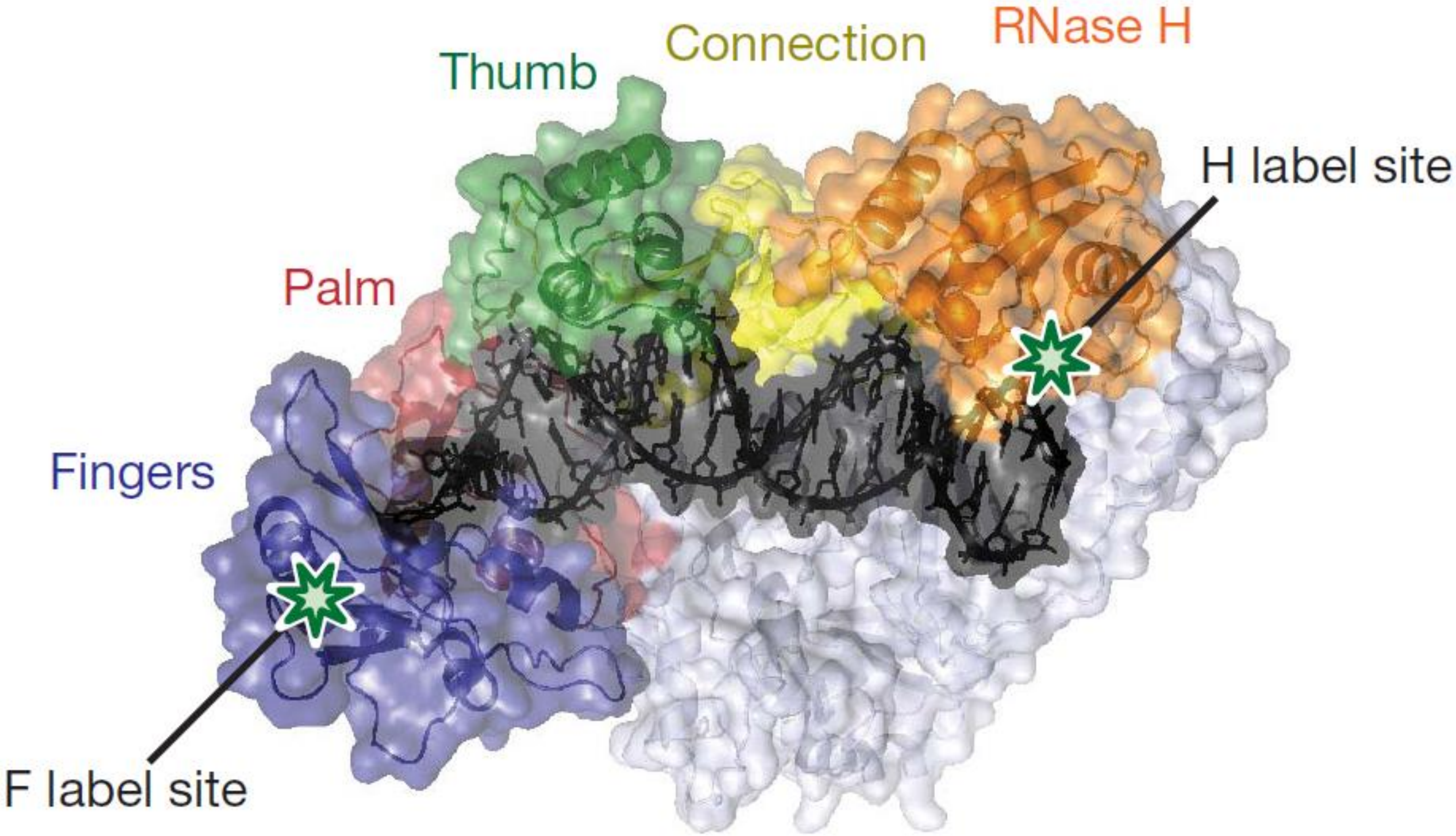

Cy3

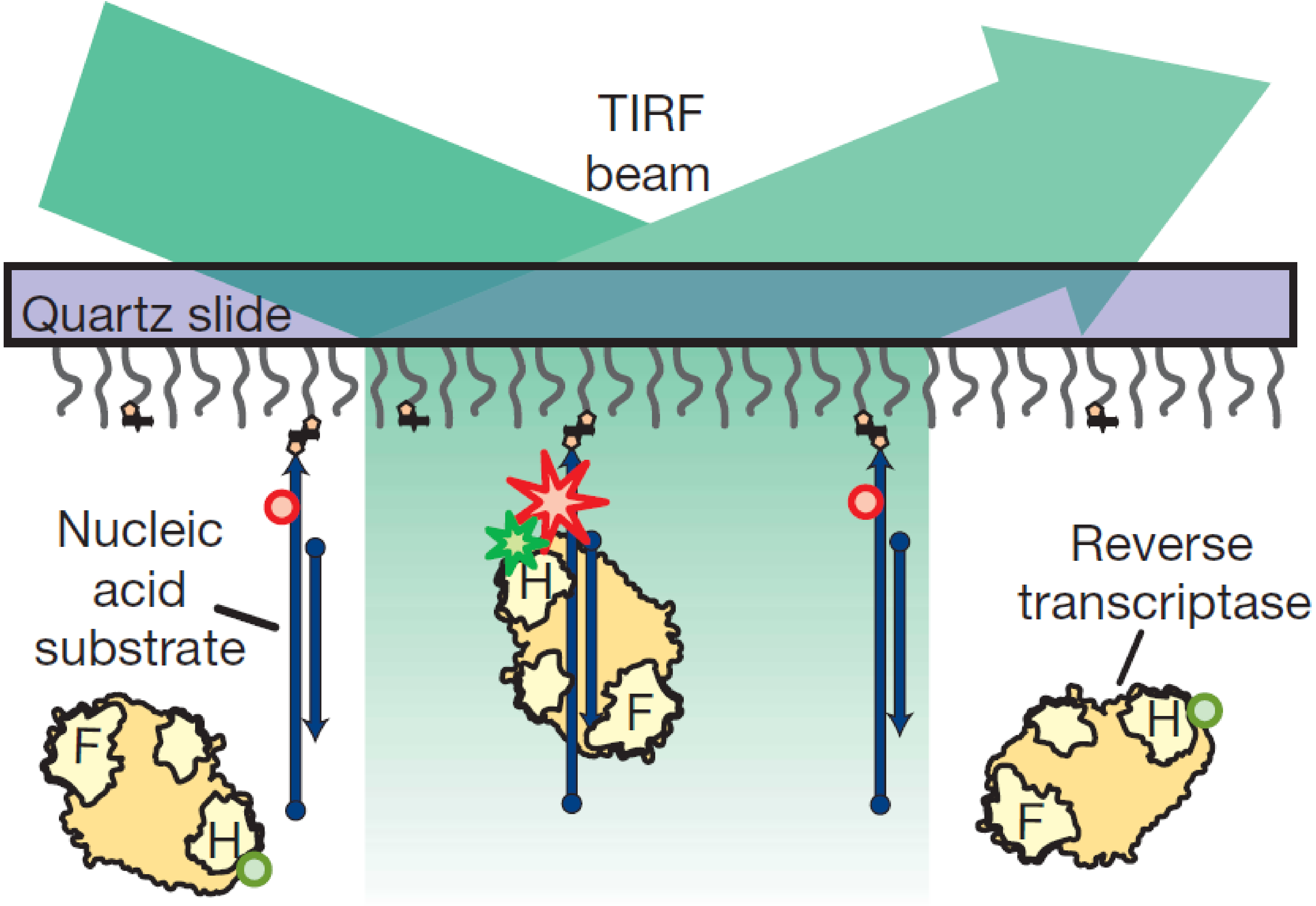



Cy5

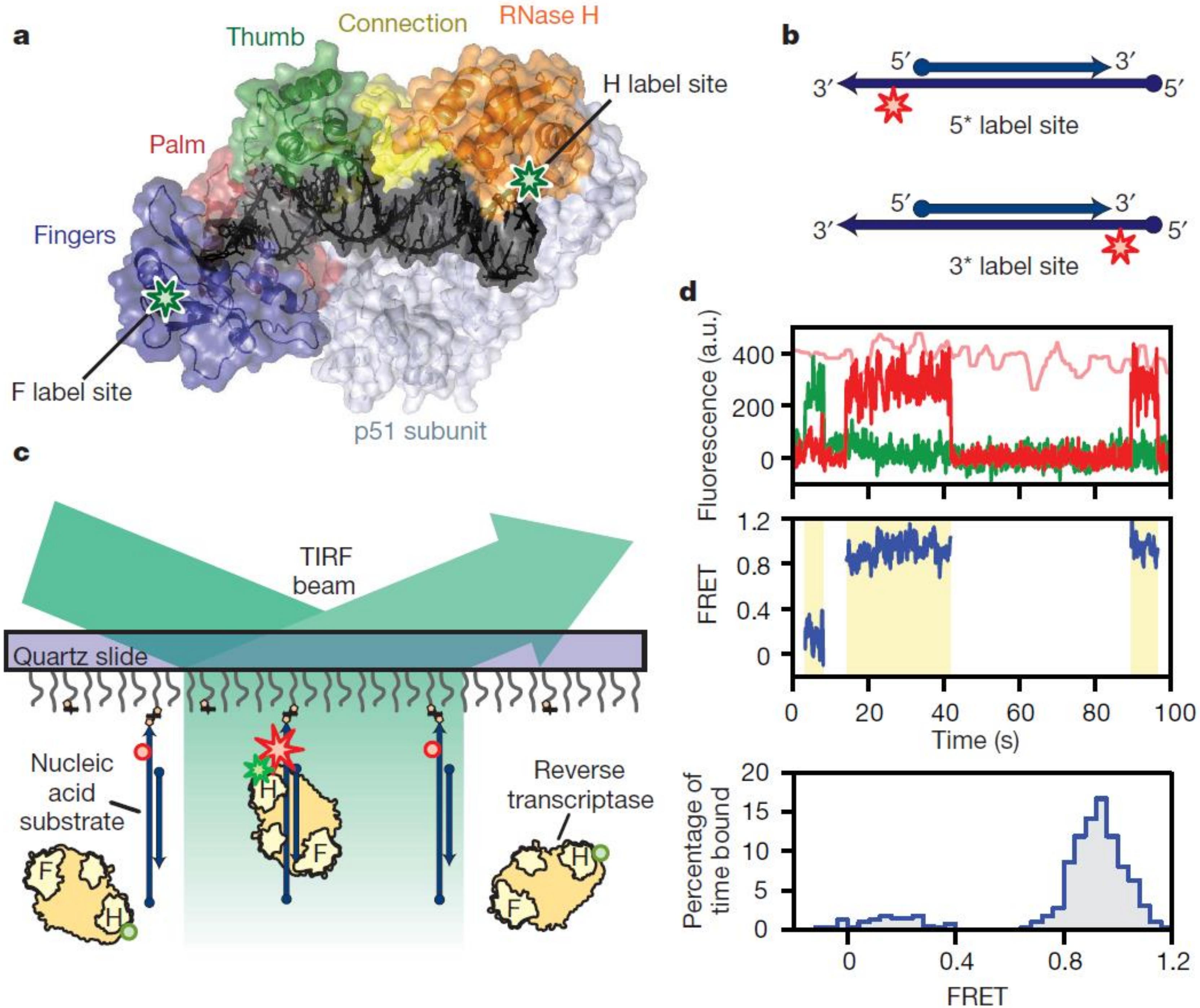
| Dye | Absorbance Max | Emission Max | Quantum yield in PBS buffer | Molecular weight (Da) |
|---|---|---|---|---|
| **Cy3** | 550 nm | 570 nm | 0.04[5] | 766 |
| **Cy5** | 649 nm | 670 nm | 0.28 | 792 |

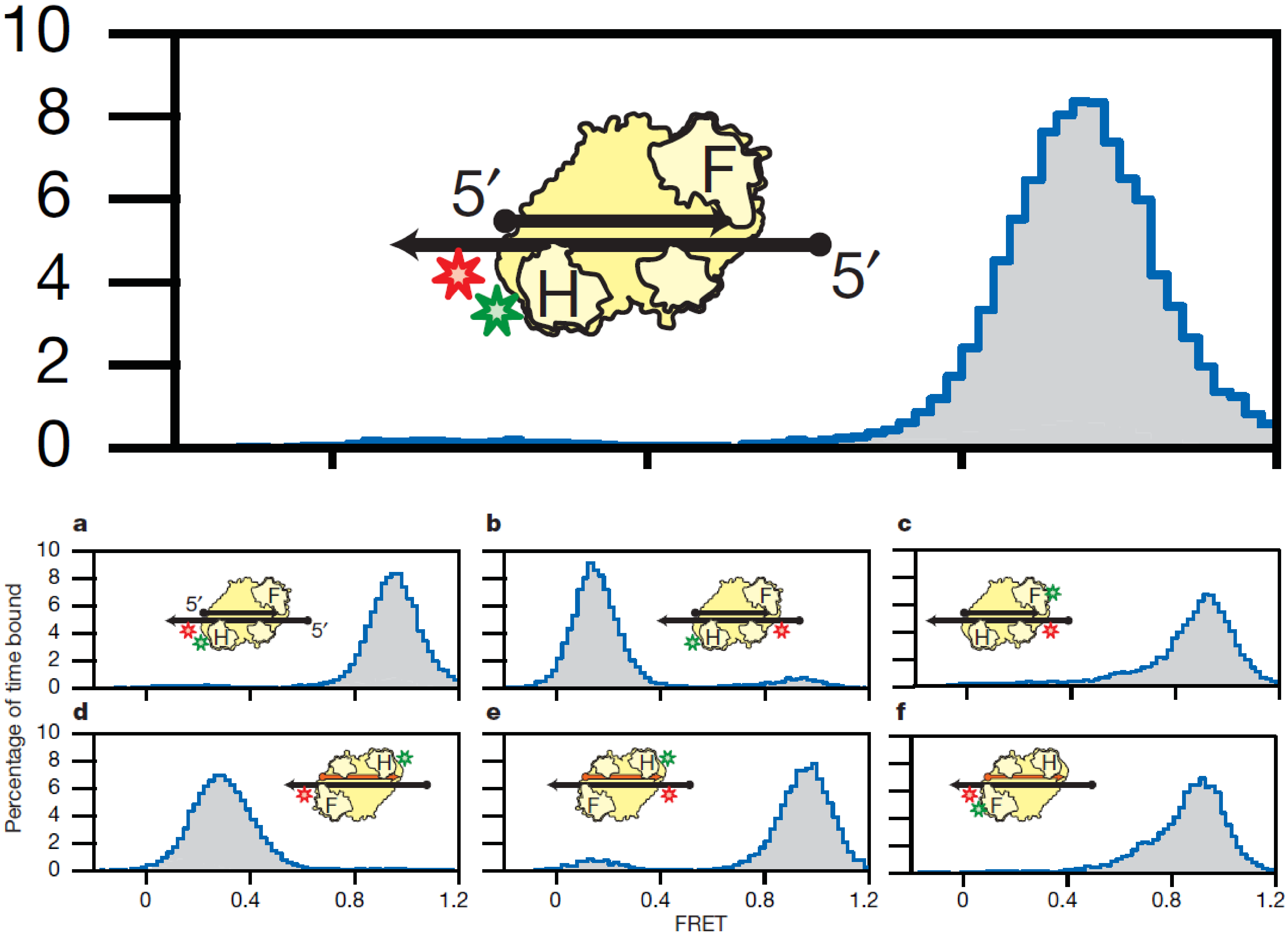
# TIRF + FRET: HIV reverse transcriptase dynamics during interaction with template






Abbondanzieri, E.A., Bokinsky, G., Rausch, J.W., Zhang, J.X., Le Grice, S.F. and Zhuang, X., 2008. Dynamic binding orientations direct activity of HIV reverse transcriptase. *Nature*, *453*(7192), p.184.


a
Thumb
Connection
RNase H
H label site
Palm
Fingers
F label site
p51 subunit
b
5′
3′
3′
5′
5* label site
5′
3′
3′
5′
3* label site
c
TIRF
beam
Quartz slide
Nucleic
acid
substrate
Reverse
transcriptase
H
F
d
Fluorescence (a.u.)
400
200
0
FRET
1.2
0.8
0.4
0
0
20
40
60
80
100
Time (s)
Percentage of
time bound
20
15
10
5
0
0
0.4
0.8
1.2
FRET

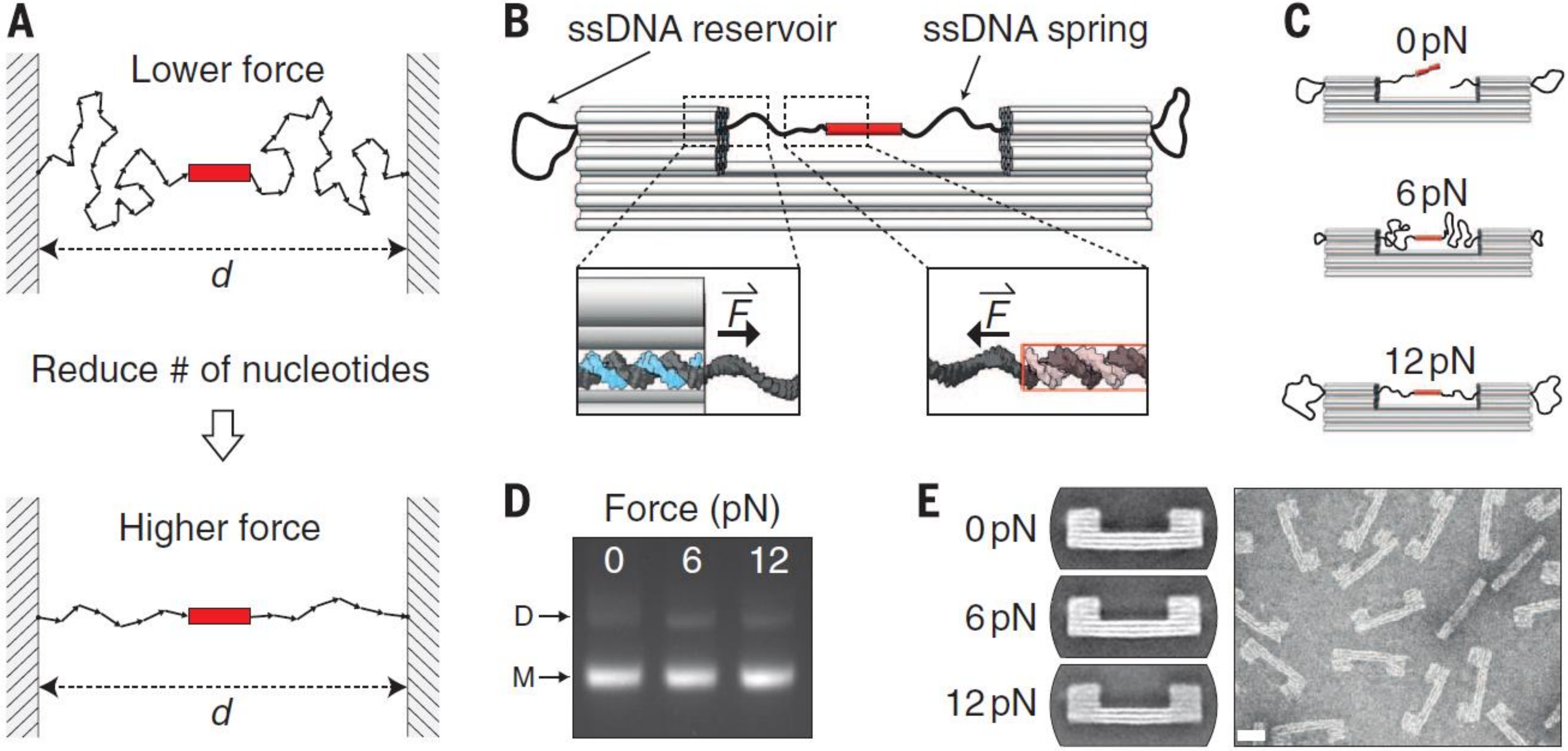

10
8
6
4
2
0
5′
5′
F
H
a
b
c
d
e
f
Percentage of time bound
FRET
0
0.4
0.8
1.2

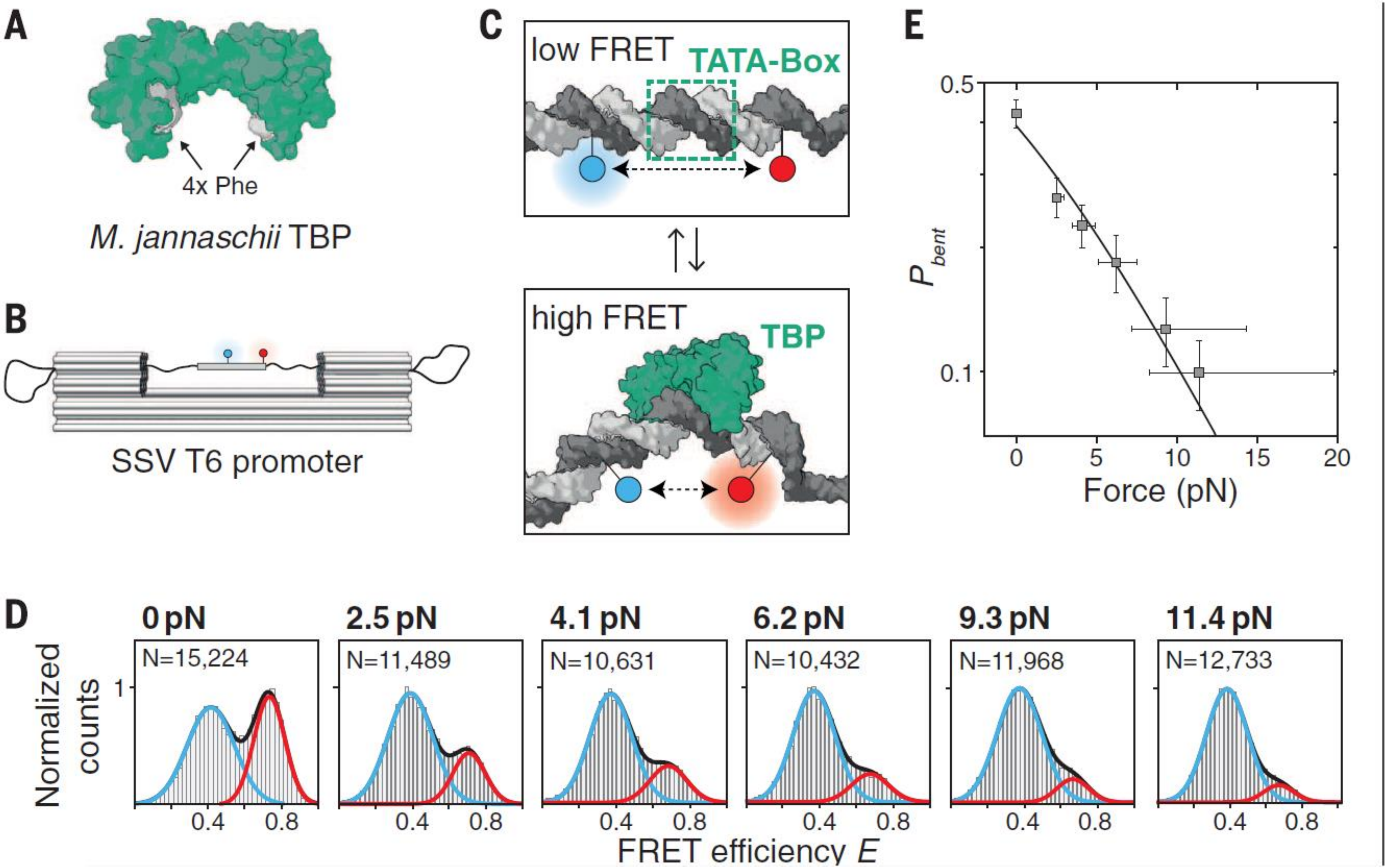
FORCE SPECTROSCOPY

# Molecular force spectroscopy with a DNA origami–based nanoscopic force clamp

Philipp C. Nickels,[1] Bettina Wünsch,[2] Phil Holzmeister,[2*] Wooli Bae,[1] Luisa M. Kneer,[1] Dina Grohmann,[2†] Philip Tinnefeld,[2‡] Tim Liedl[1‡]

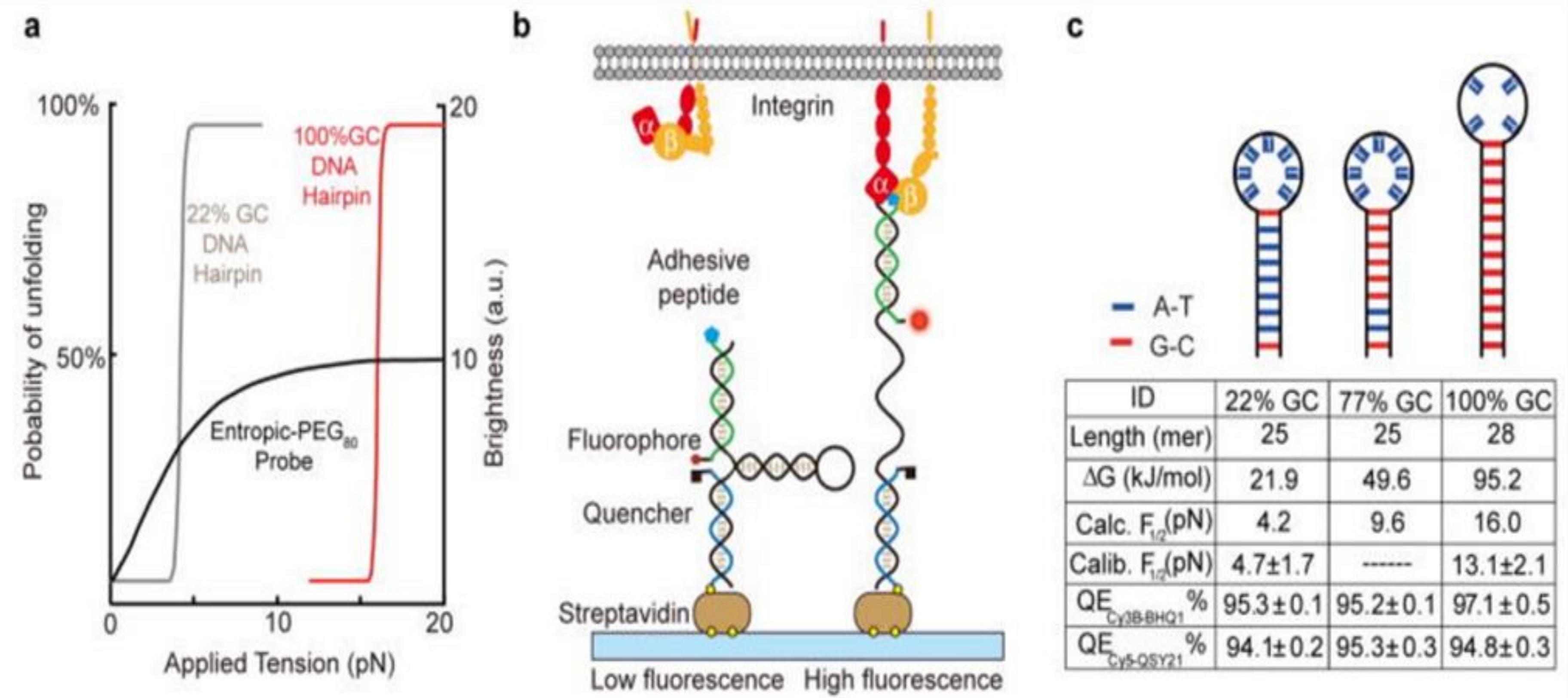
# When FRET and bioengineering are combined



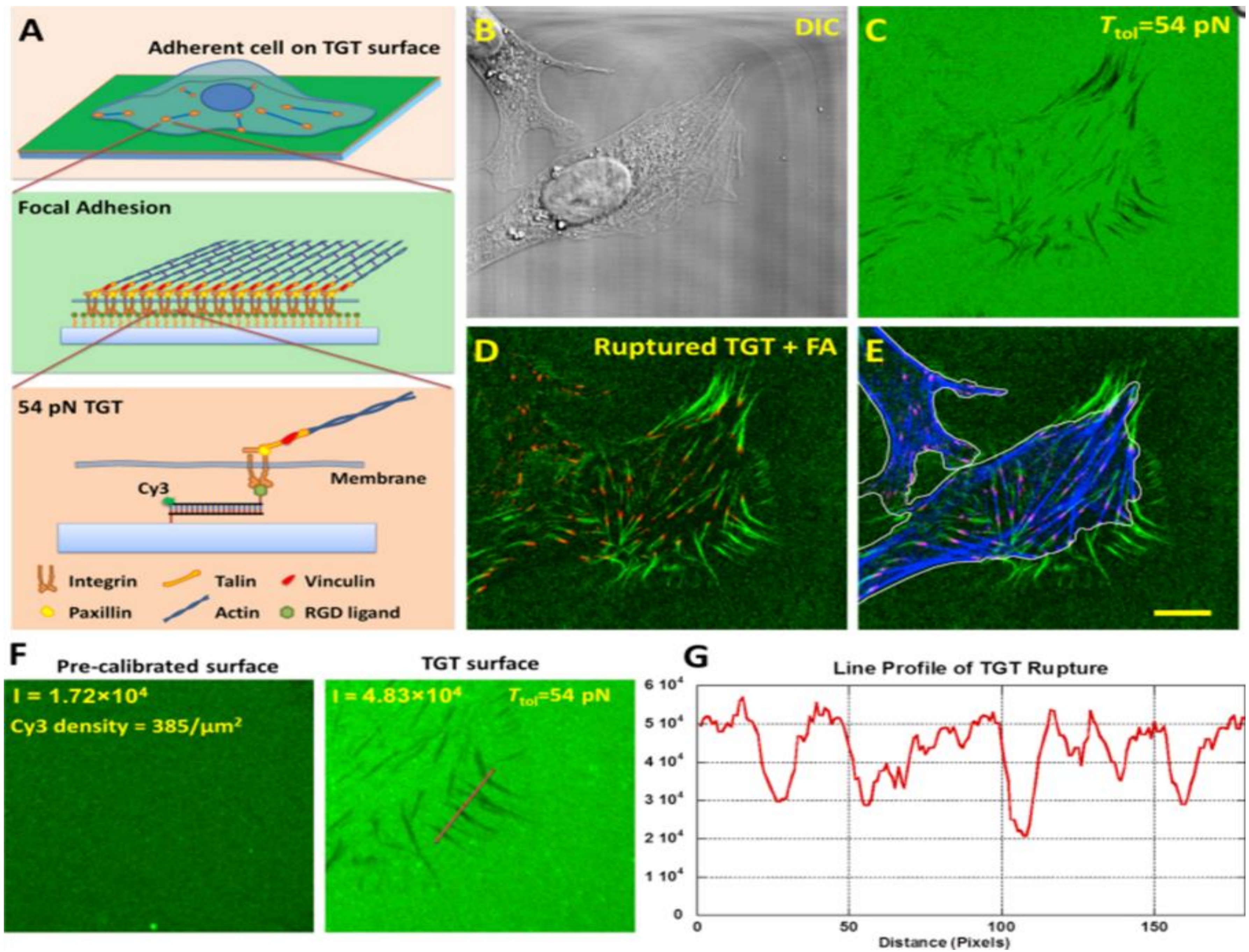


A
4x Phe
M. jannaschii TBP
B
SSV T6 promoter
C
low FRET
TATA-Box
high FRET
TBP
E
P_bent
Force (pN)
D
0 pN
2.5 pN
4.1 pN
6.2 pN
9.3 pN
11.4 pN
N=15,224
N=11,489
N=10,631
N=10,432
N=11,968
N=12,733
Normalized counts
FRET efficiency E

# FRET-based force sensor




| ID | 22% GC | 77% GC | 100% GC |
|---|---|---|---|
| Length (mer) | 25 | 25 | 28 |
| ΔG (kJ/mol) | 21.9 | 49.6 | 95.2 |
| Calc. $F_{1/2}$(pN) | 4.2 | 9.6 | 16.0 |
| Calib. $F_{1/2}$(pN) | 4.7±1.7 | ------- | 13.1±2.1 |
| $QE_{Cy3B\text{-}BHQ1}$% | 95.3±0.1 | 95.2±0.1 | 97.1±0.5 |
| $QE_{Cy5\text{-}QSY21}$% | 94.1±0.2 | 95.3±0.3 | 94.8±0.3 |


A
Adherent cell on TGT surface
Focal Adhesion
54 pN TGT
Membrane
Cy3
Integrin
Talin
Vinculin
Paxillin
Actin
RGD ligand
B
DIC
C
$T_{tol}$=54 pN
D
Ruptured TGT + FA
E
F
Pre-calibrated surface
I = 1.72×10⁴
Cy3 density = 385/µm²
TGT surface
I = 4.83×10⁴
$T_{tol}$=54 pN
G
Line Profile of TGT Rupture
6 10⁴
5 10⁴
4 10⁴
3 10⁴
2 10⁴
1 10⁴
0
0
50
100
150
Distance (Pixels)

Thank you!