

Fluorescence applications

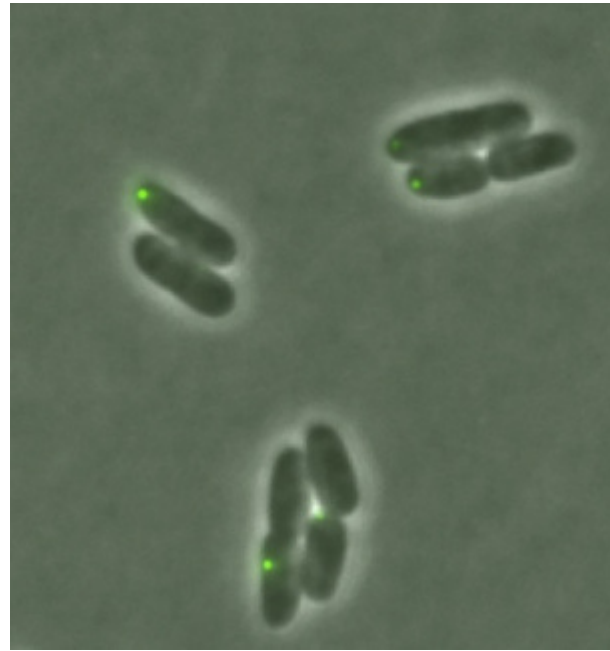
- Last class
 - Fluorescence toolbox
 - Some limitations of fluorescence
- This class
 - Quantifying fluorescence
 - FRAP/FLIP
 - Co-localization

A few more limitations

- Resolution
- Autofluorescence
- Bleedthrough
- Blinking
- Photostability
- Phototoxicity

Labeling

- Does the presence of the fluorescent molecule affect the function you're trying to study?
- Attaching FP sequence will change transcription factor concentration, ribosome occupancy, already crowded cytoplasm, trafficking of the protein of interest?
- Adding dye can also affect cellular properties, they're just smaller



Proteolysis can be very different if you have a FP Tag

Same types of cells labeled with GFP and tdTomato

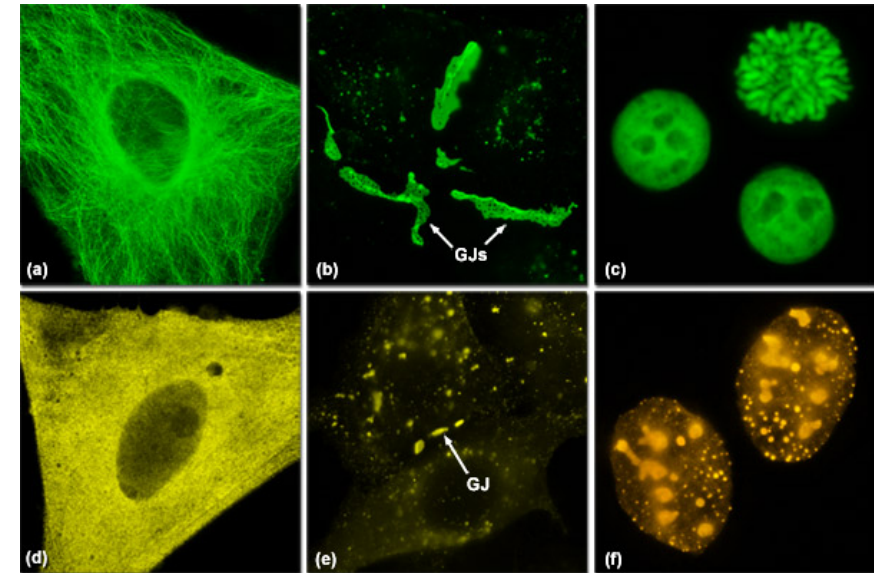


Figure 2

The Helical MreB Cytoskeleton in Escherichia coli MC1000/pLE7 Is an Artifact of the N-Terminal Yellow Fluorescent Protein Tag

1. [Matthew T. Swulius^a](#) and
2. [Grant J. Jensen^{a, b}](#)

Signal Quantitation

- Limited by the above factors
- Have to consider photobleaching and background
- We want to know #fl at a given pixel
- **To quantitate fluorescence, we try to model other parameters**

Fluorescence we measure at a pixel

What we want to know

Autofluorescence from sample

Unavoidable noise from camera

$$Fl(x, t) = I_0(x, t) \left[(\#f * br) + (\#autoF * br_{af}) + (\#overLap * br_{OL}) + exc \text{ bground} \right] + CamNoise(t)$$

Excitation intensity, Function of time and space

Fluorophore overlap

Bleedthrough from filters

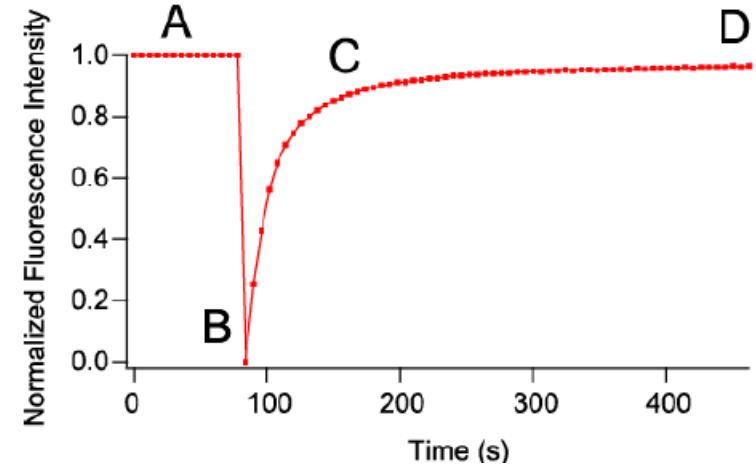
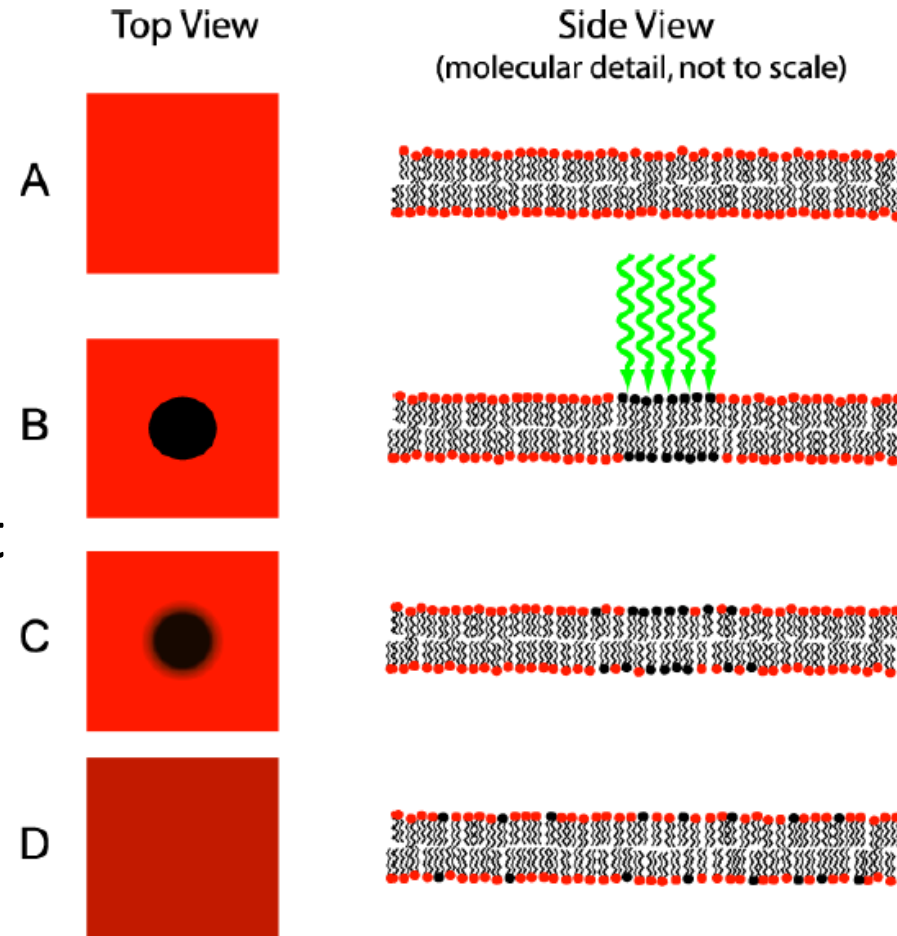
Detailed description: The diagram illustrates the components of the fluorescence signal equation. The equation is $Fl(x, t) = I_0(x, t) [(\#f * br) + (\#autoF * br_{af}) + (\#overLap * br_{OL}) + exc \text{ bground}] + CamNoise(t)$. Blue arrows point from descriptive text to each part of the equation: 'Fluorescence we measure at a pixel' points to the left side; 'What we want to know' points to the entire equation; 'Excitation intensity, Function of time and space' points to $I_0(x, t)$; 'Autofluorescence from sample' points to the $(\#autoF * br_{af})$ term; 'Fluorophore overlap' points to the $(\#overLap * br_{OL})$ term; 'Bleedthrough from filters' points to the 'exc bground' term; and 'Unavoidable noise from camera' points to the $+ CamNoise(t)$ term.

Dynamic applications of fluorescence microscopy

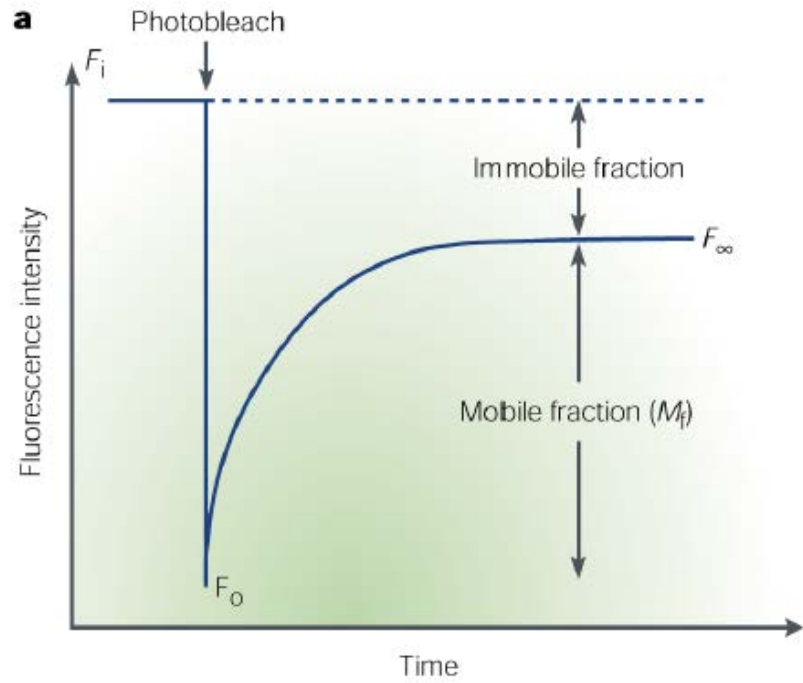
- Cavalcade of acronyms...

Fluorescence Recovery after Photobleaching (FRAP)

- Selectively bleach some portion of the sample
- Image fluorescence return to that spot
- Originally developed to monitor lipid movement in membranes

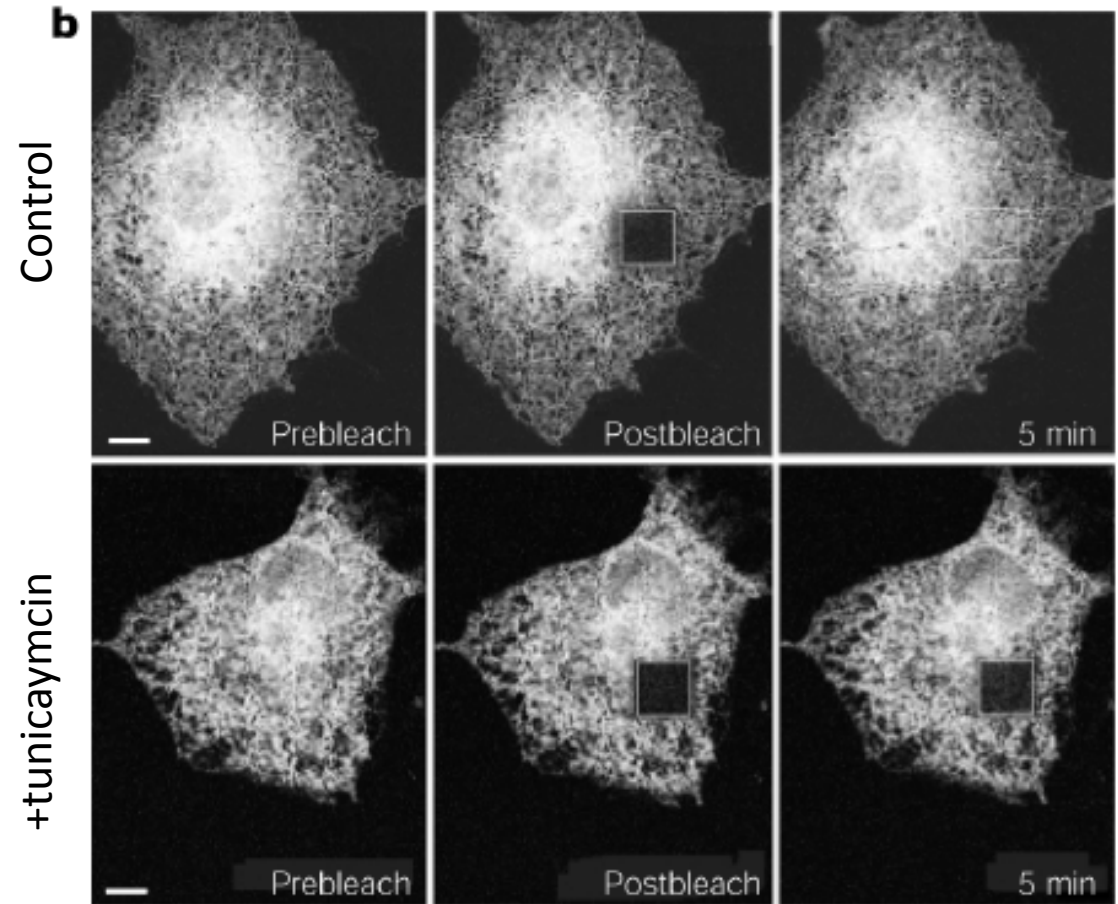


Quantifying FRAP



$$\%Recovery = \frac{F_\infty - F_0}{F_\infty - F_1}$$

VsVg-GFP in ER



Quantifying FRAP

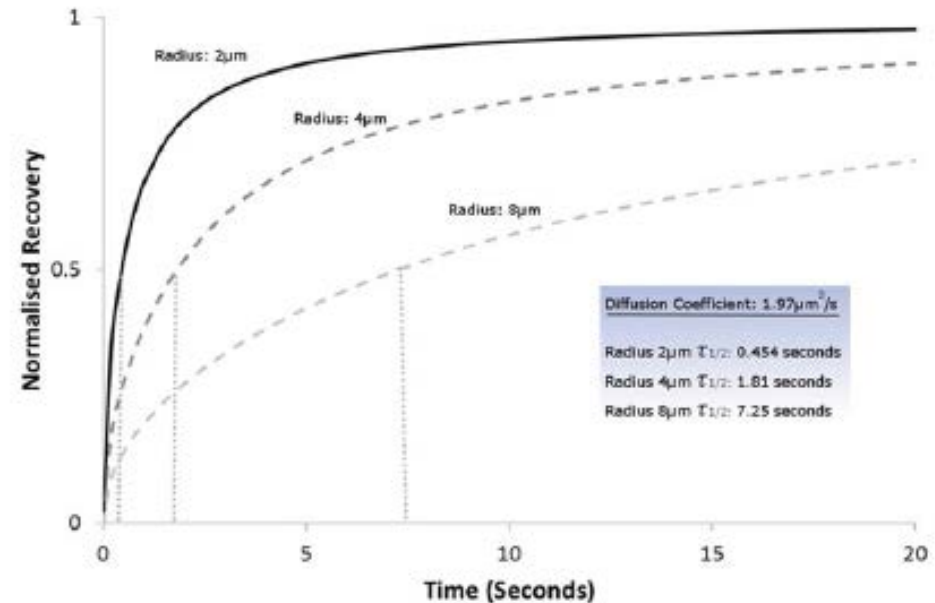
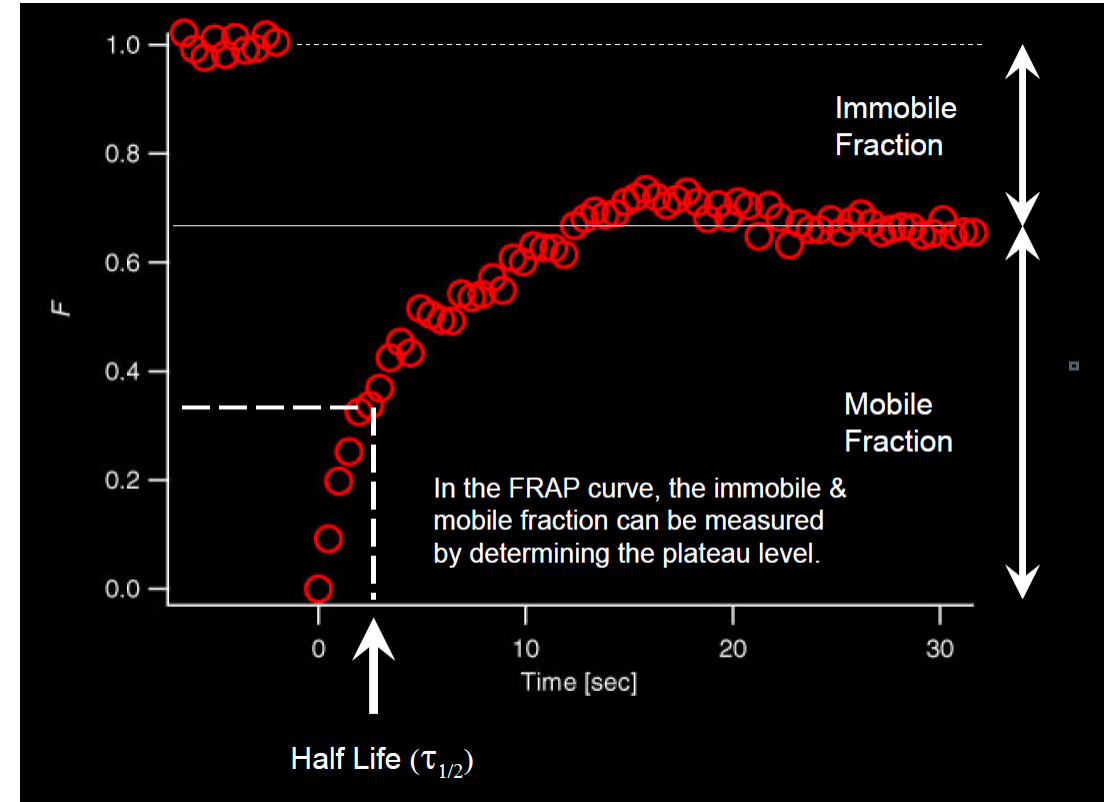
- We want to find the time constant of recovery

$$F(t) = A(1 - e^{-t/\tau})$$

$$\tau_{1/2} = \frac{\ln 0.5}{-\tau}$$

Assuming disc bleach and diffusion is in 2D

$$D = \frac{0.88 w^2}{4t_{1/2}} \quad t \approx \frac{x^2}{2D}$$



Diffusion

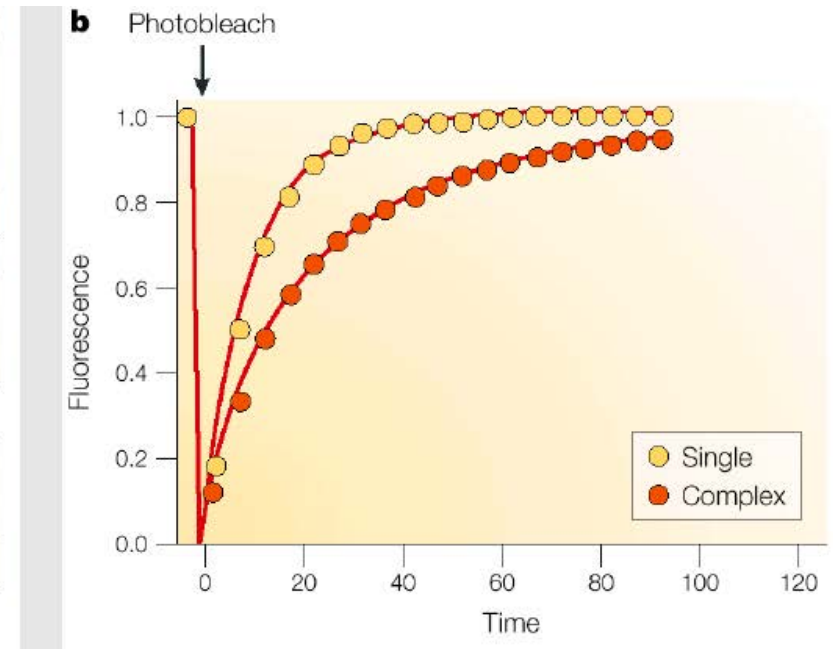
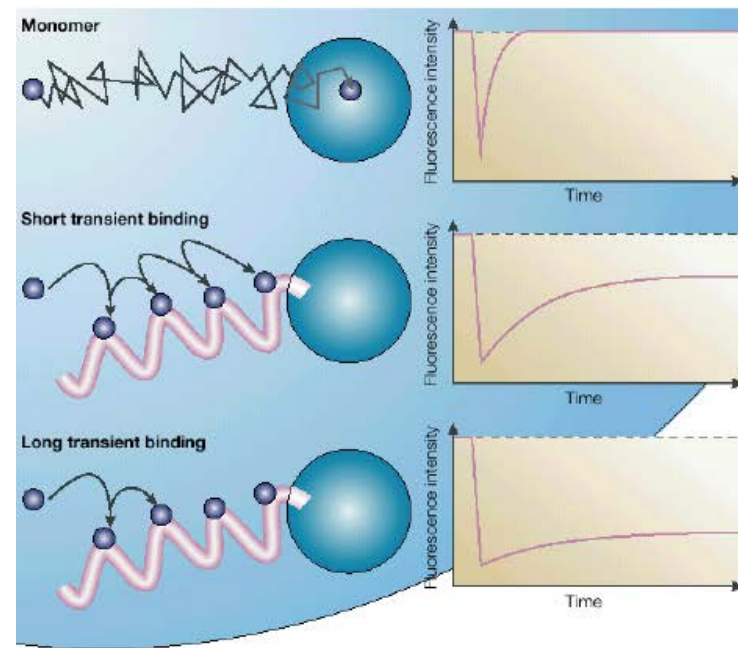
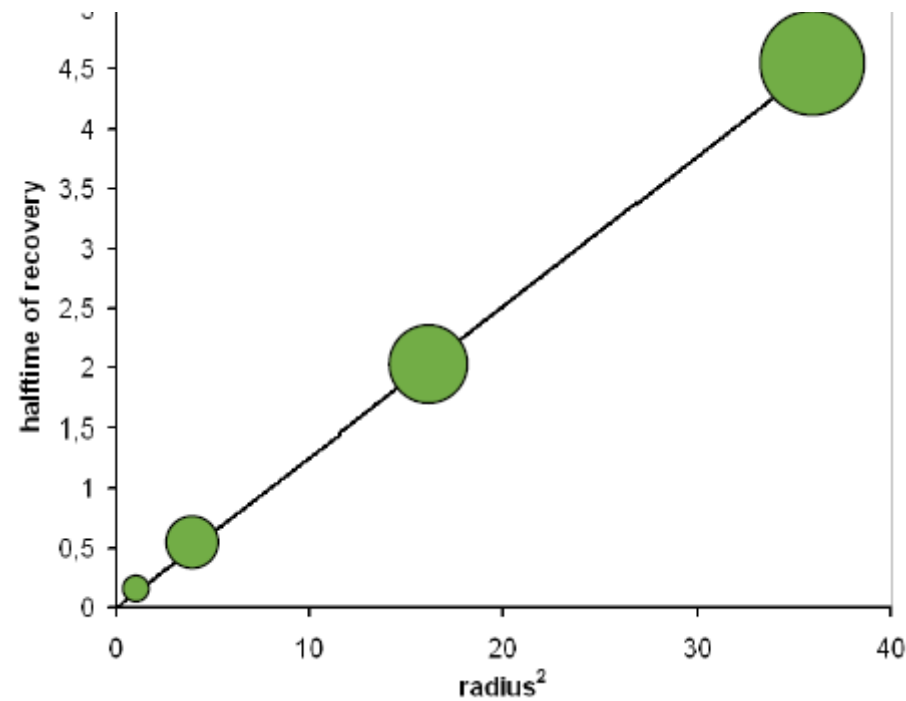
$$D = \frac{0.88 w^2}{4t_{1/2}}$$

$$t \approx \frac{x^2}{2D}$$

$$D = \frac{k_B T}{6\pi\eta r}$$

molecule	measured context	diffusion coefficient ($\mu\text{m}^2/\text{s}$)	BNID
H ₂ O	water	2000	104087, 106703
H ₂ O	nucleus of chicken erythrocyte	200	104645
H ⁺ (from H ₃ O ⁺ to H ₂ O)	water	7000	106702
O ₂	water	2000	104440
CO ₂	water	2000	102625
tRNA (≈ 20 kDa)	water	100	107933, 107935
protein (≈ 30 kDa GFP)	water	100	100301
protein (≈ 30 kDa GFP)	eukaryotic cell (CHO) cytoplasm	30	101997
protein (≈ 30 kDa GFP)	rat liver mitochondria	30	100300
protein (NLS-EGFP)	cytoplasm of <i>D. melanogaster</i> embryo	20	109209
protein (≈ 30 kDa)	<i>E. coli</i> cytoplasm	7-8	100193, 107985
protein (≈ 40 kDa)	<i>E. coli</i> cytoplasm	2-4	107985
protein (≈ 70 -250 kDa)	<i>E. coli</i> cytoplasm	0.4-2	107985
protein (≈ 140 kDa Tar-YFP)	<i>E. coli</i> membrane	0.2	107985
protein (≈ 70 kDa LacY-YFP)	<i>E. coli</i> membrane	0.03	107985
fluorescent dye (carboxy-fluorescein)	<i>A. thaliana</i> cell wall	30	105033
fluorescent dye (carboxy-fluorescein)	<i>A. thaliana</i> mature root epidermis	3	105034
transcription factor (LacI)	movement along DNA (1D, <i>in vitro</i>)	0.04 ($4 \times 10^5 \text{ bp}^2 \text{ s}^{-1}$)	102036
morphogen (bicoid-GFP)	cytoplasm of <i>D. melanogaster</i> embryo	7	109199
morphogen (wingless)	wing imaginal disk of <i>D. melanogaster</i>	0.05	101072
mRNA	HeLa nucleus	0.03-0.10	107613
mRNA	various localizations and sizes	0.005-1	110667
ribosome	<i>E. coli</i>	0.04	108596

FRAP and diffusion



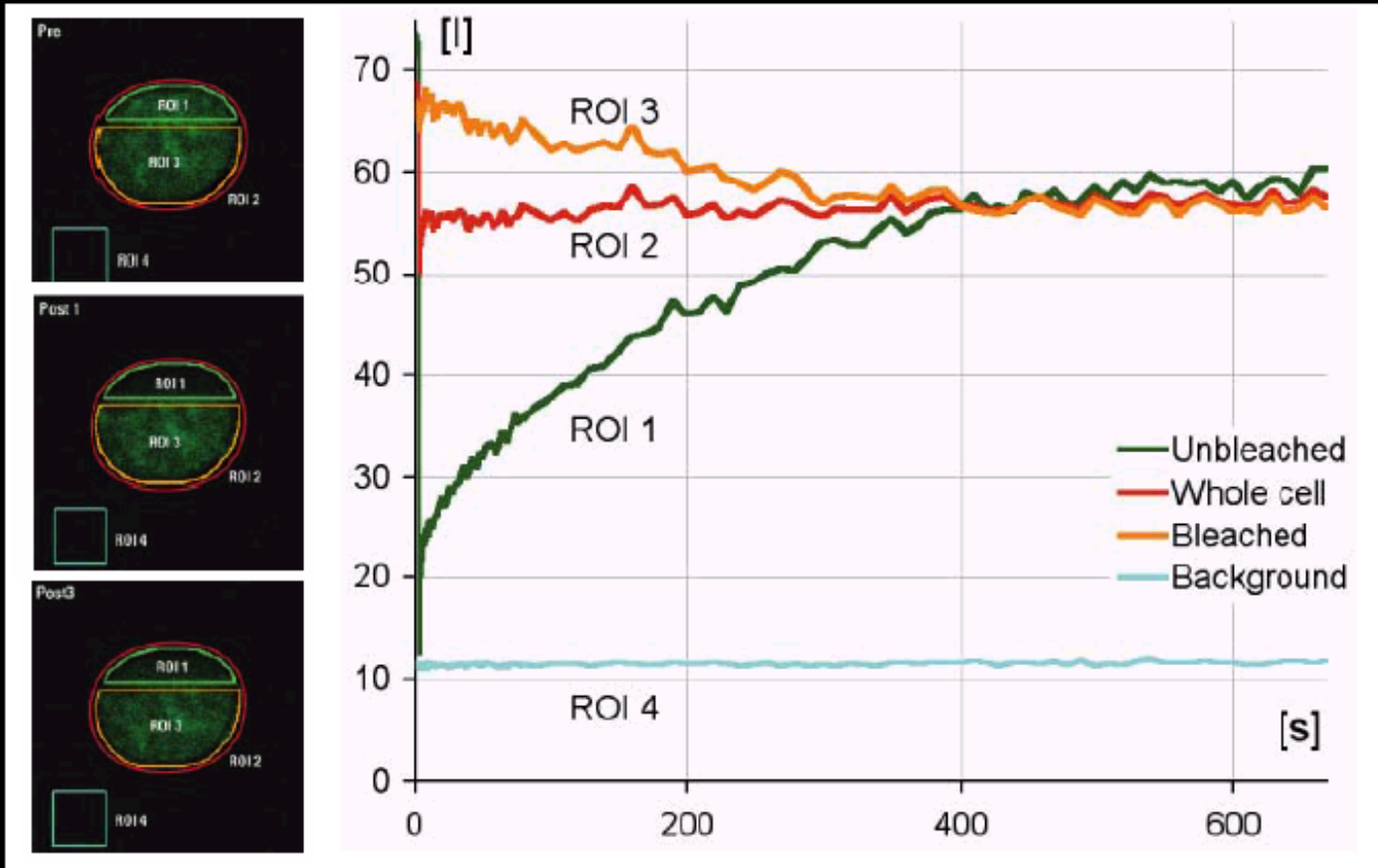
$$F(t) = A(1 - e^{-t/\tau})$$

$$D = \frac{0.88 w^2}{4t_{1/2}}$$

More on... Quantifying FRAP

- Corrections
 - Background
 - Photobleaching

FRAP experimental data



Kappel and Eils, Leica App.Letter 2004

$$F_{ROI1}(t)$$

1. Subtract background

$$F_{ROI1,BG}(t) = F_{ROI1}(t) - F_{ROI4}(t)$$

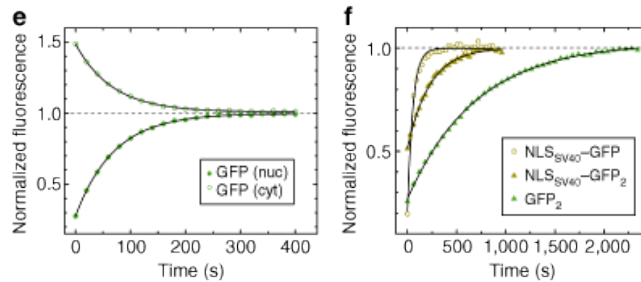
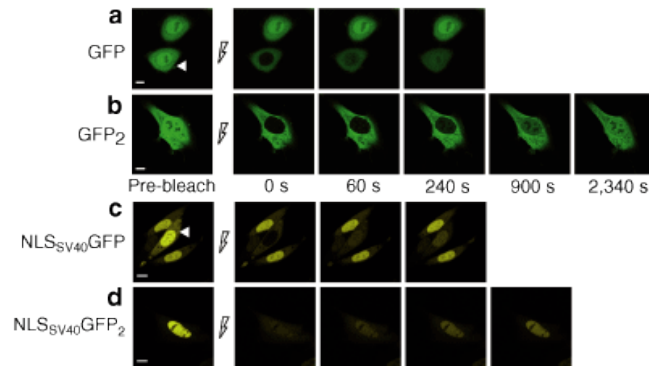
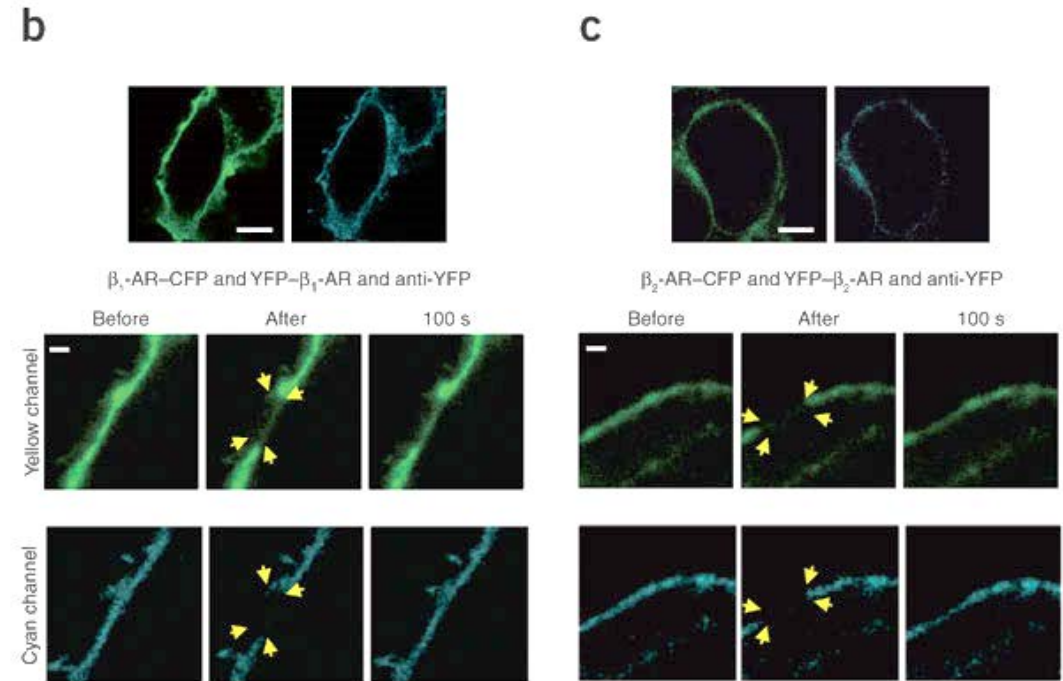
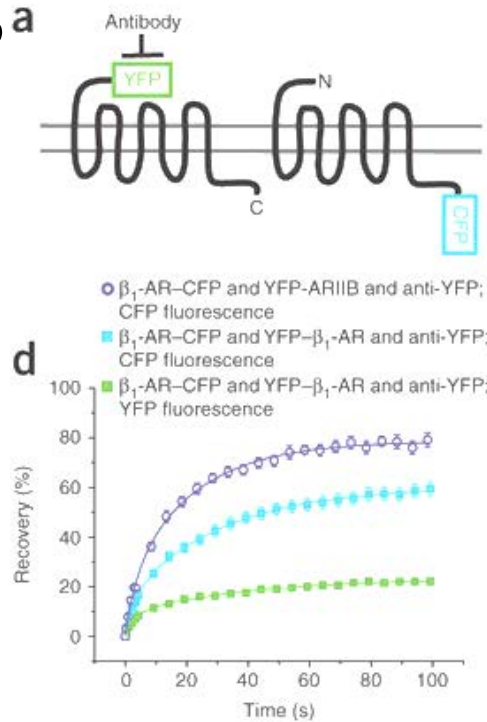
2. Correct photobleaching

$$F_{ROI1,BG,PB}(t) = \frac{F_{ROI1,BG}(t)}{F_{ROI2}(t) - F_{ROI4}(t)}$$

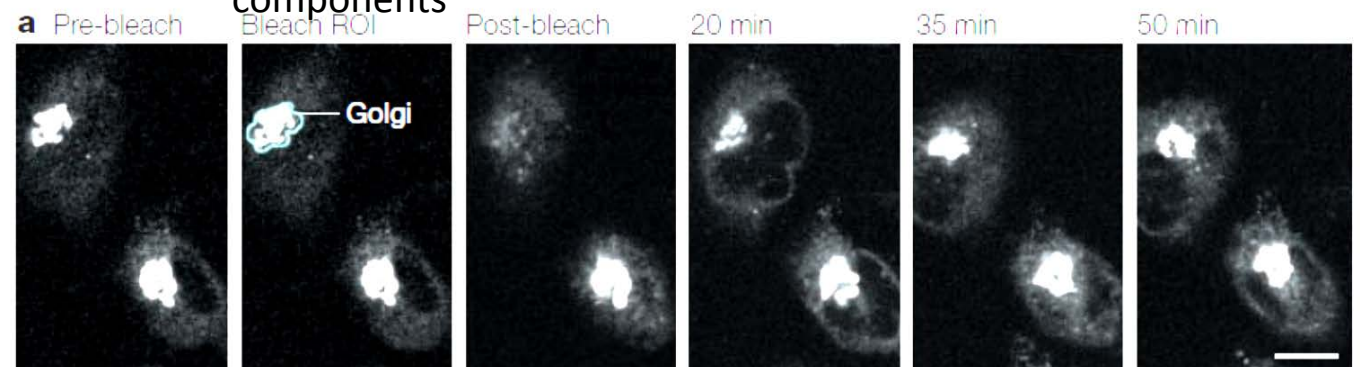
3. Extract time constants

FRAP applications

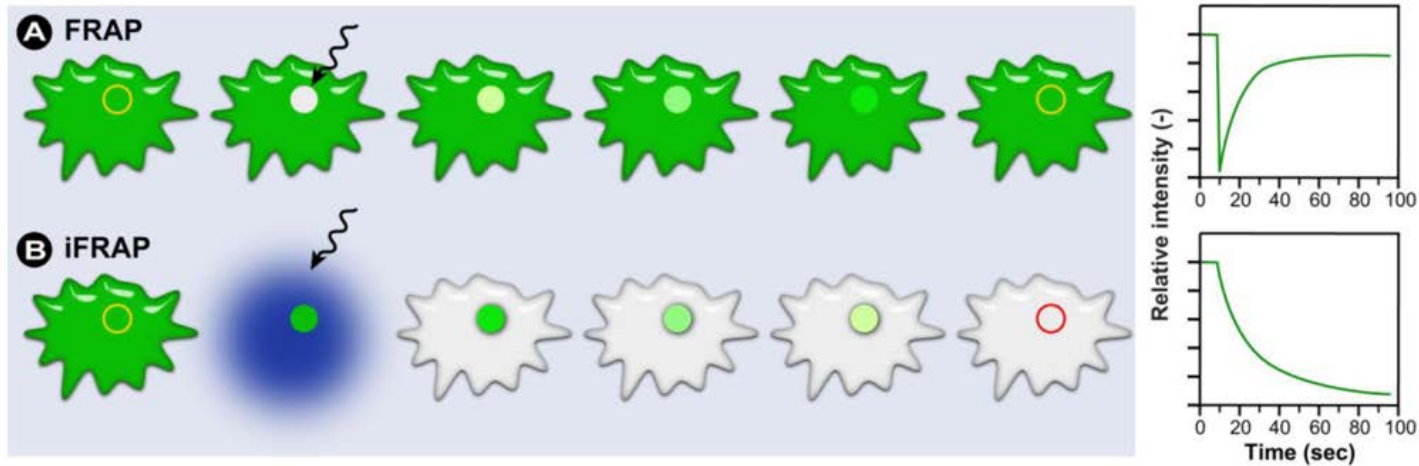
- Lipid diffusion
- Receptor binding
- Organelle diffusion
- Protein dynamics



Galtase-GFP in the Golgi. A FRAP experiment showed Galtase is exchangeable with other components

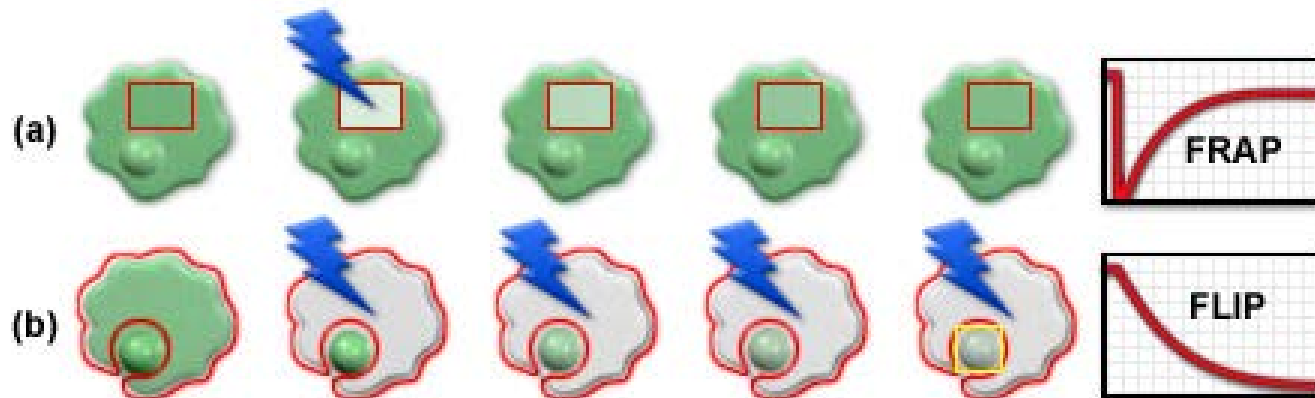


FLIP – fluorescence loss in photobleaching/iFRAP



$$F(t) = Ae^{-\tau t}$$

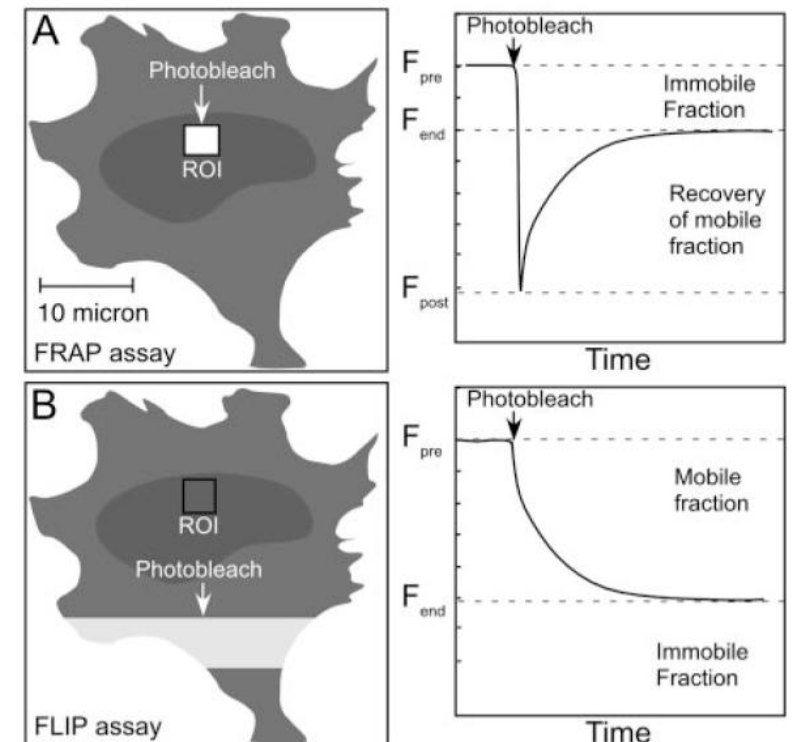
Photobleaching and Photoactivation Techniques



Measurements are not recorded from photobleached area.

Watch diffusion back into the rest of the cell.

FLIP uses continuous inactivation. iFRAP does it once

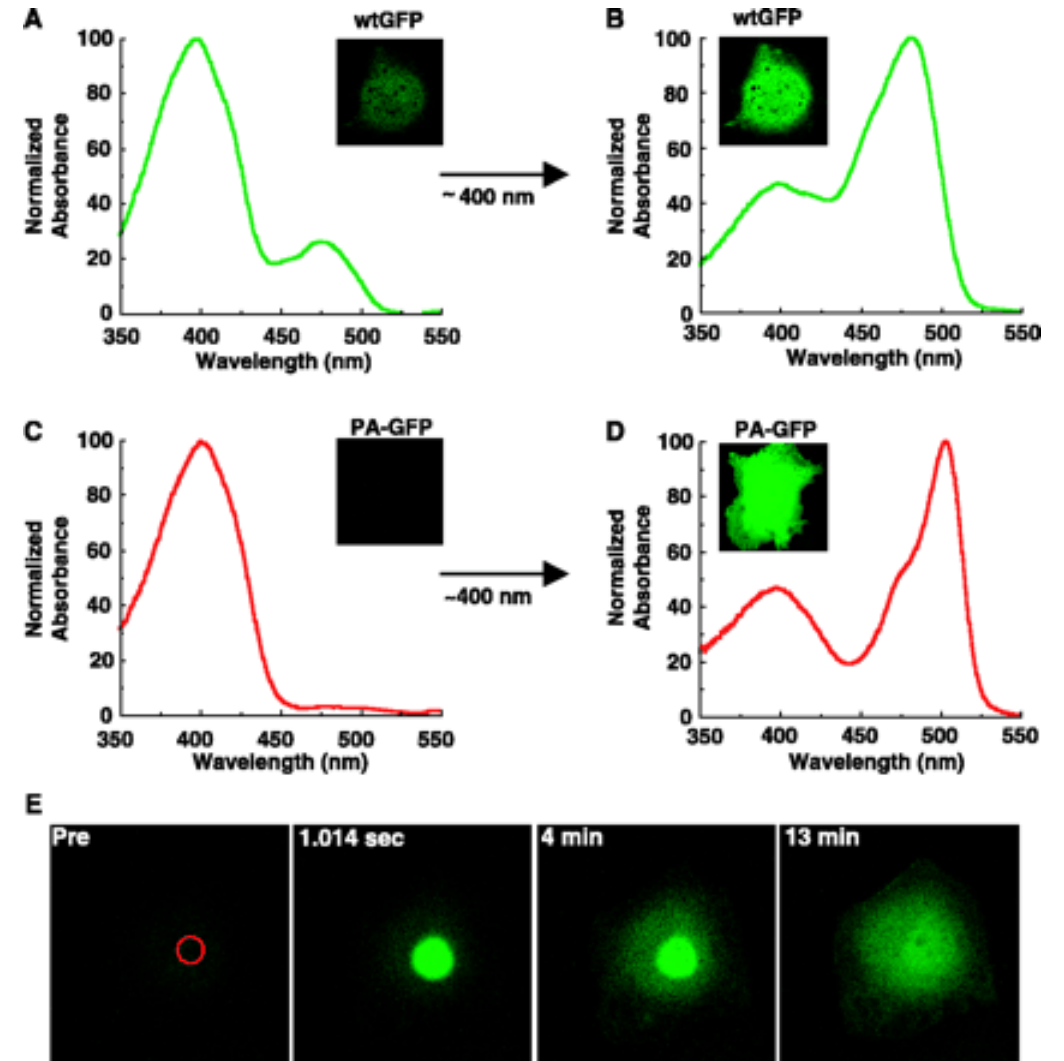
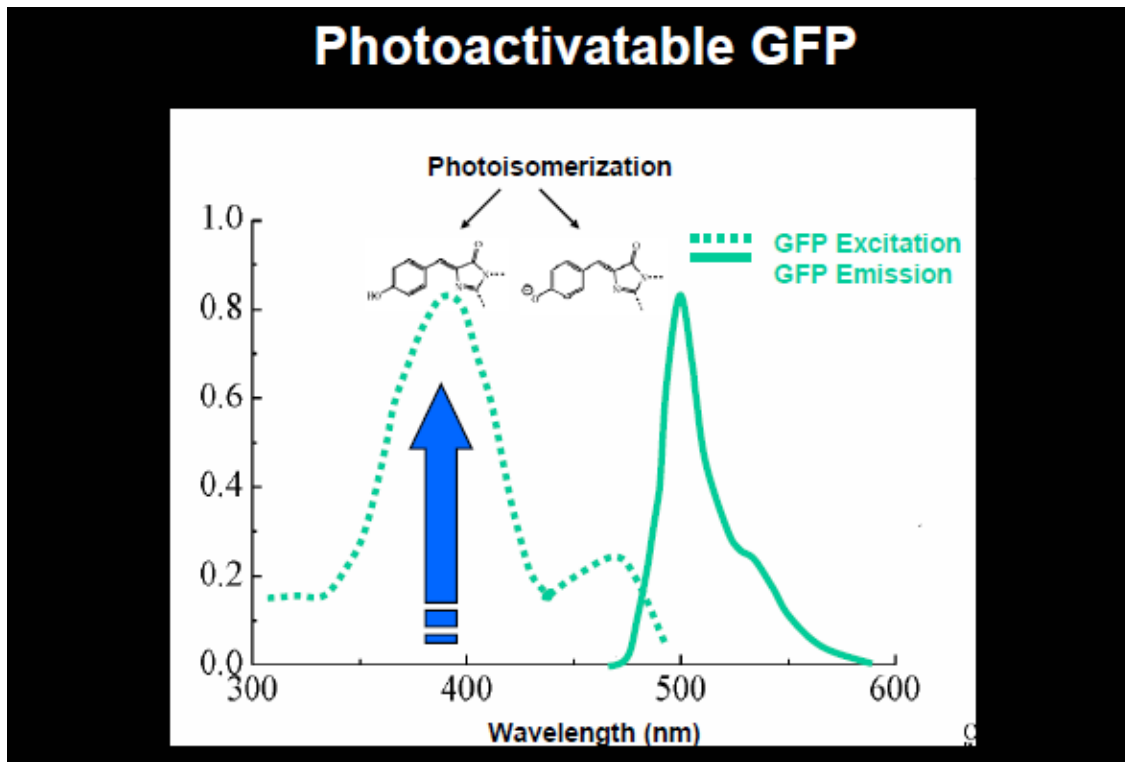


FRAP/FLIP limitations

- Photobleaching must be very quick (assumed to be instantaneous)
- High intensities of light must be used
- Potential photodamage
- Photobleaching, or triplet state?
- Incorporating models of 3D diffusion is challenging

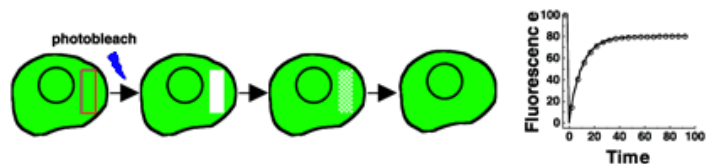
Photoactivatable proteins to the rescue

- Photoactivatable GFPs are dark until illumination with 405 nm light

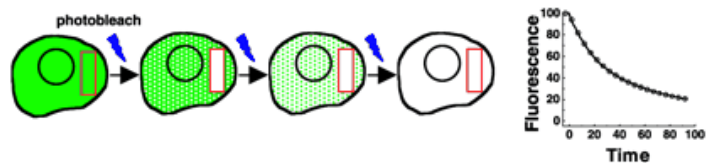


Photoactivation

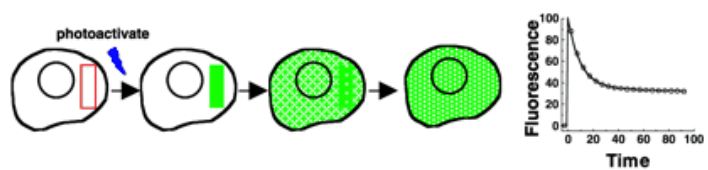
A Fluorescence Recovery After Photobleaching (FRAP)



B Fluorescence Loss in Photobleaching (FLIP)

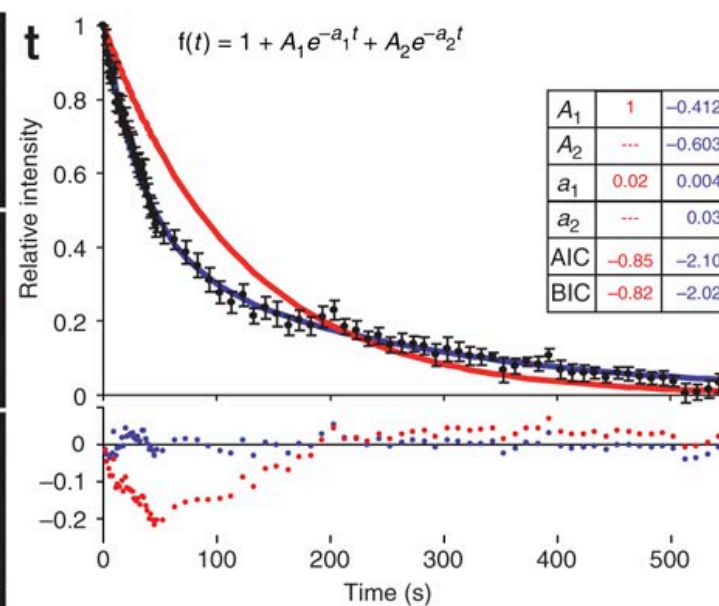
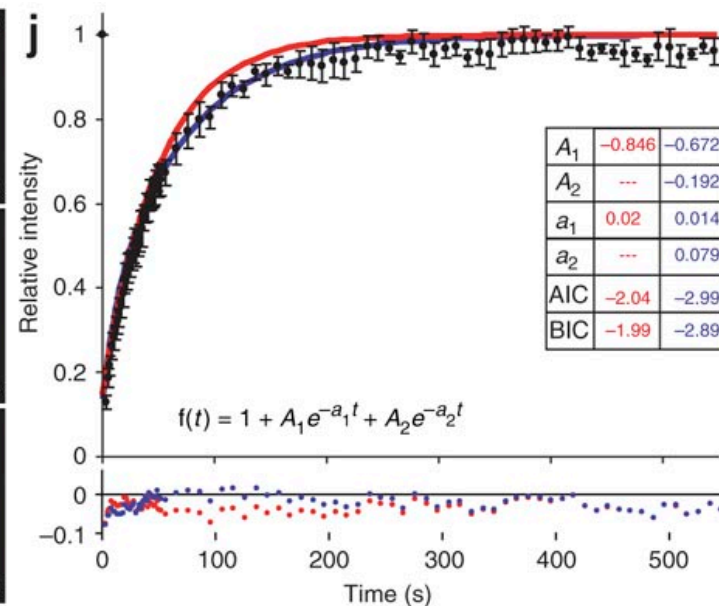
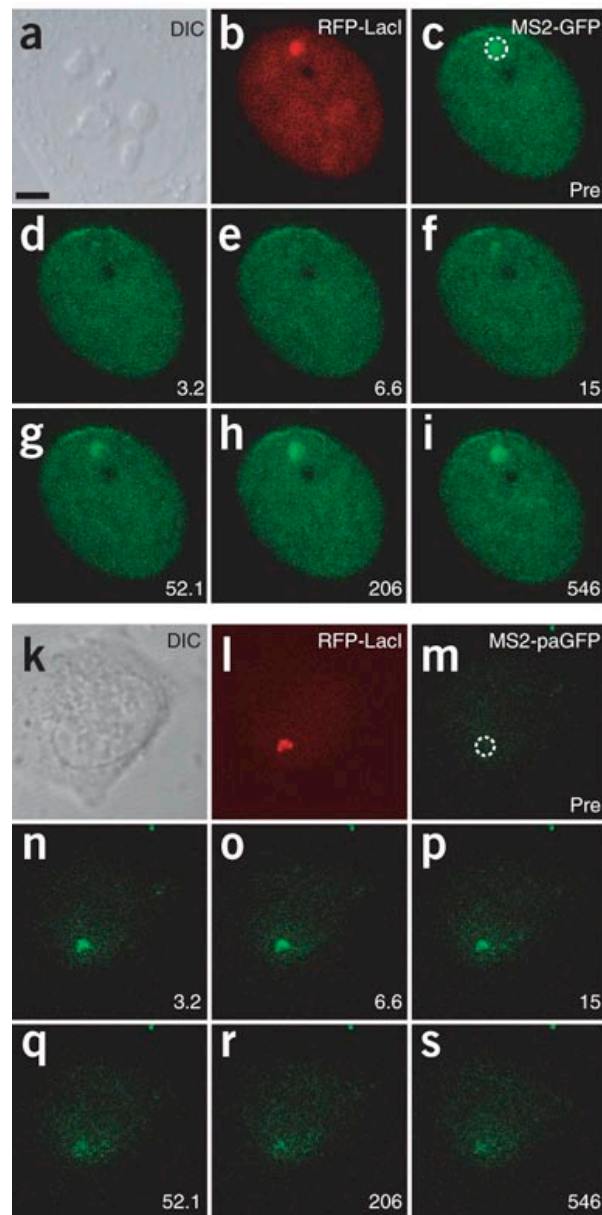


C Photoactivation



$$F(t) = Ae^{-\tau t}$$

mRNA synthesis in vivo



Advanced techniques

Photoconversion Reactions in Optical Highlighters

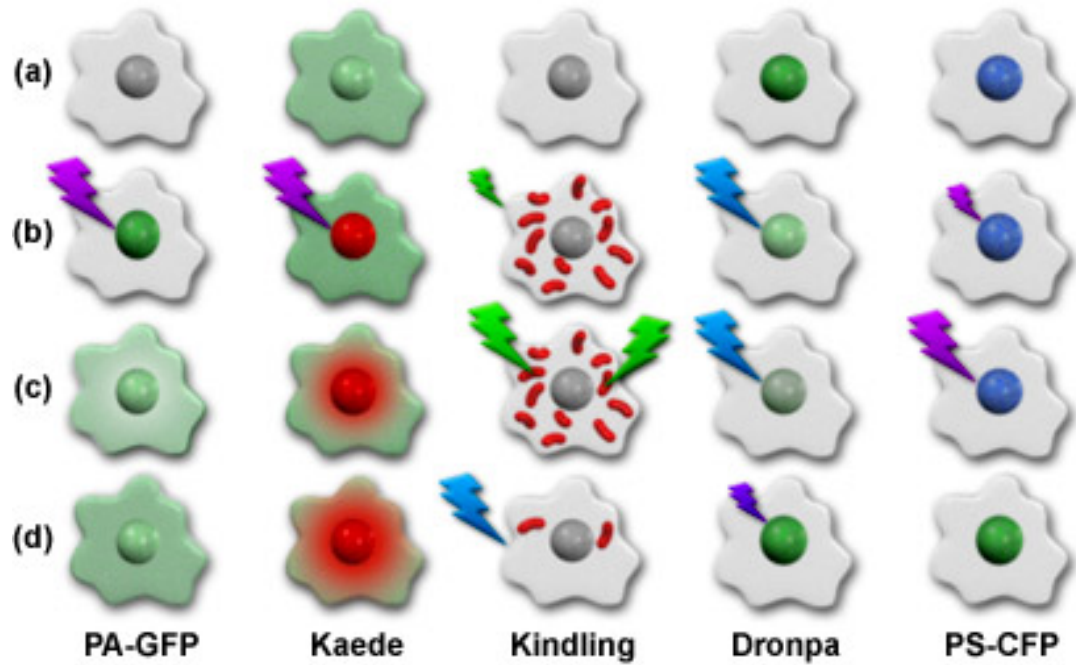
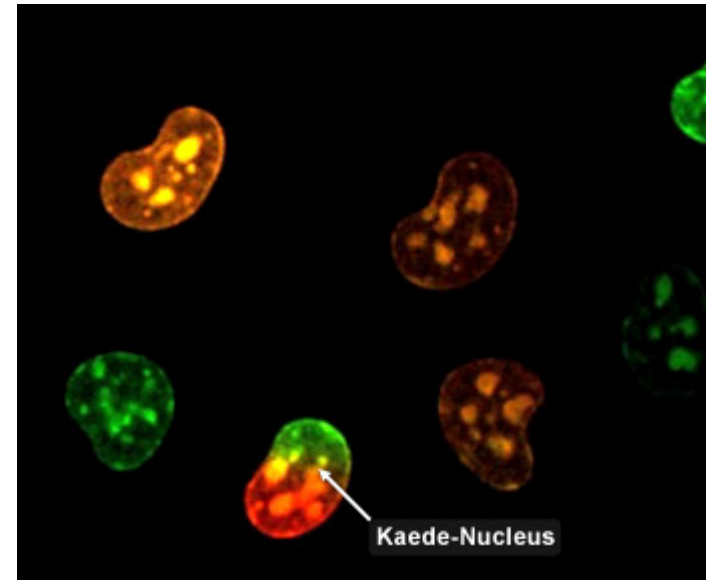
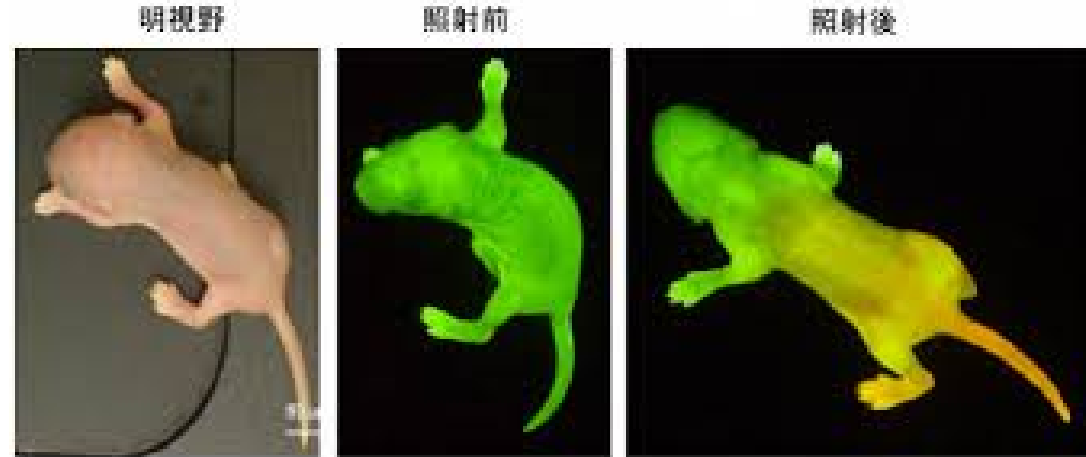


Figure 1



And on to Matlab...