Fluorescence applications

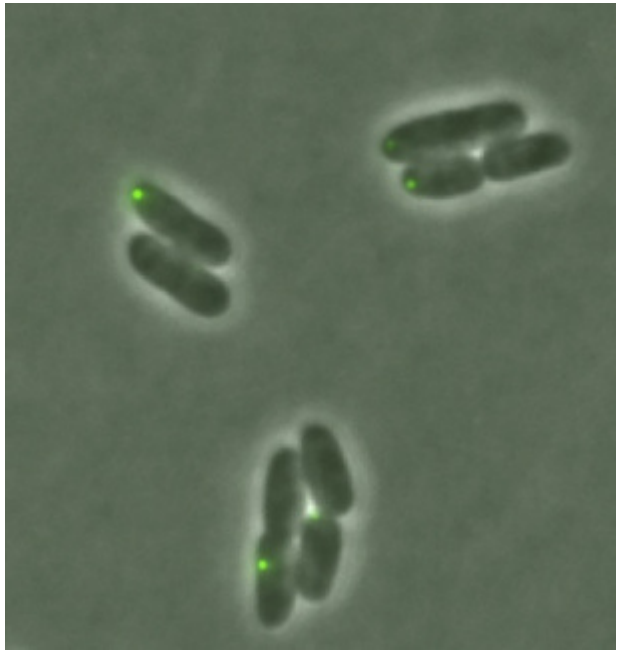
- Last class
  - Fluorescence toolbox
  - Some limitations of fluorescence
- This class
  - Quantifying fluorescence
  - FRAP/FLIP
  - Co-localization

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

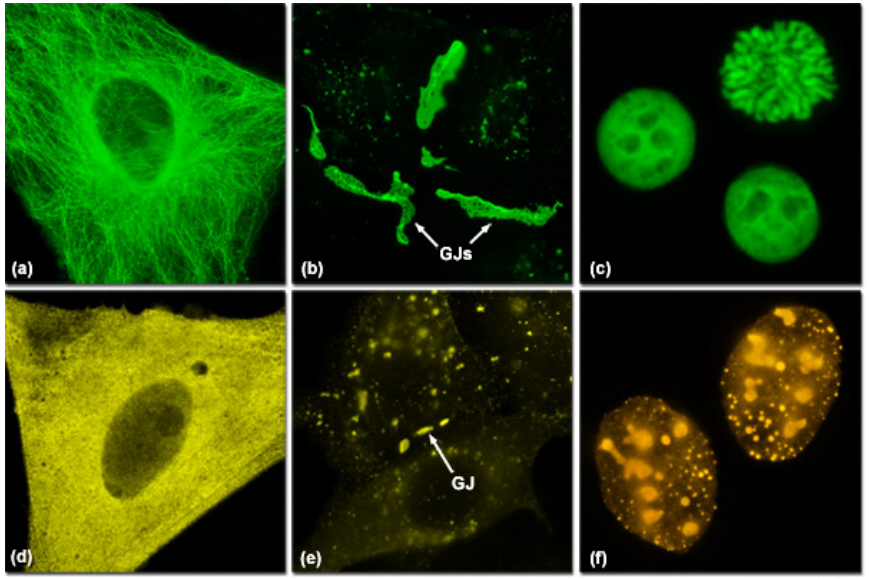


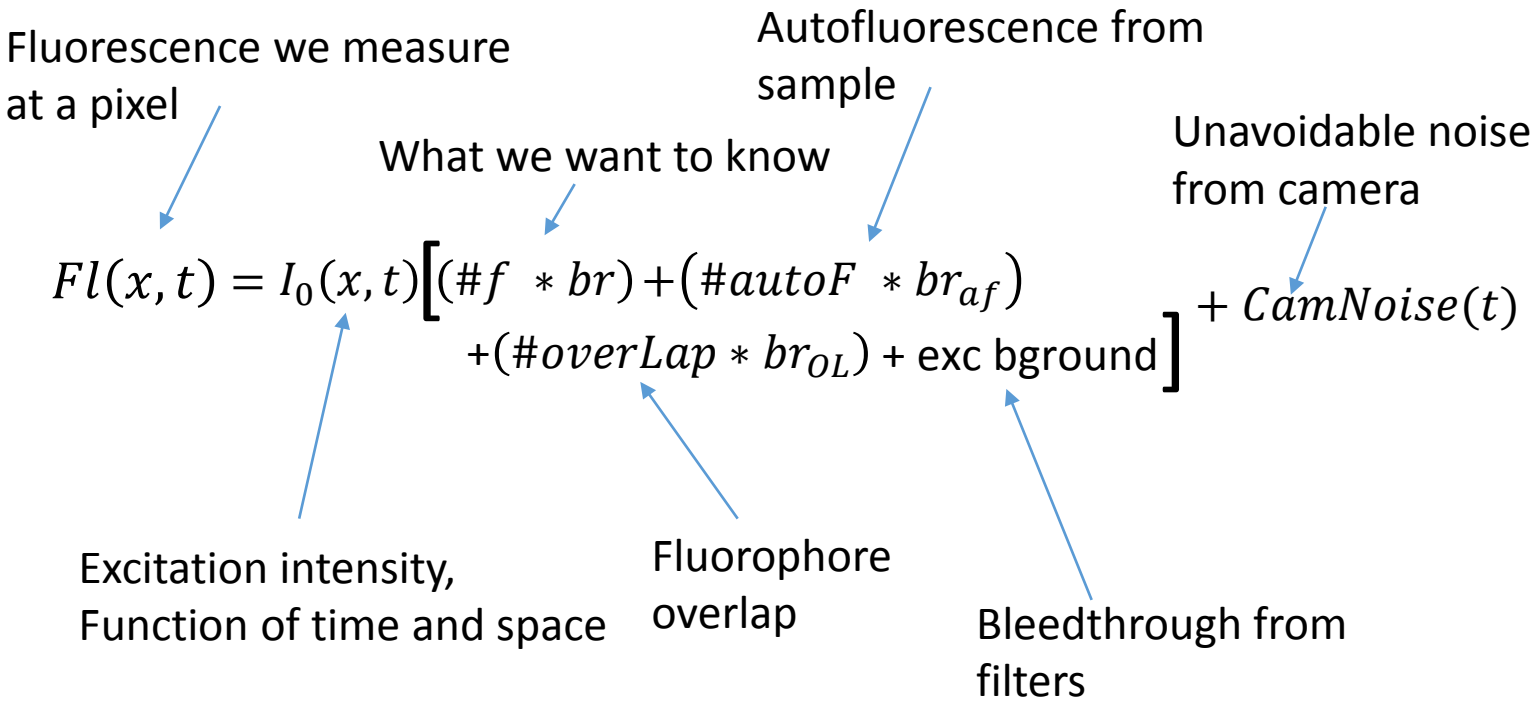
Figure 2

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

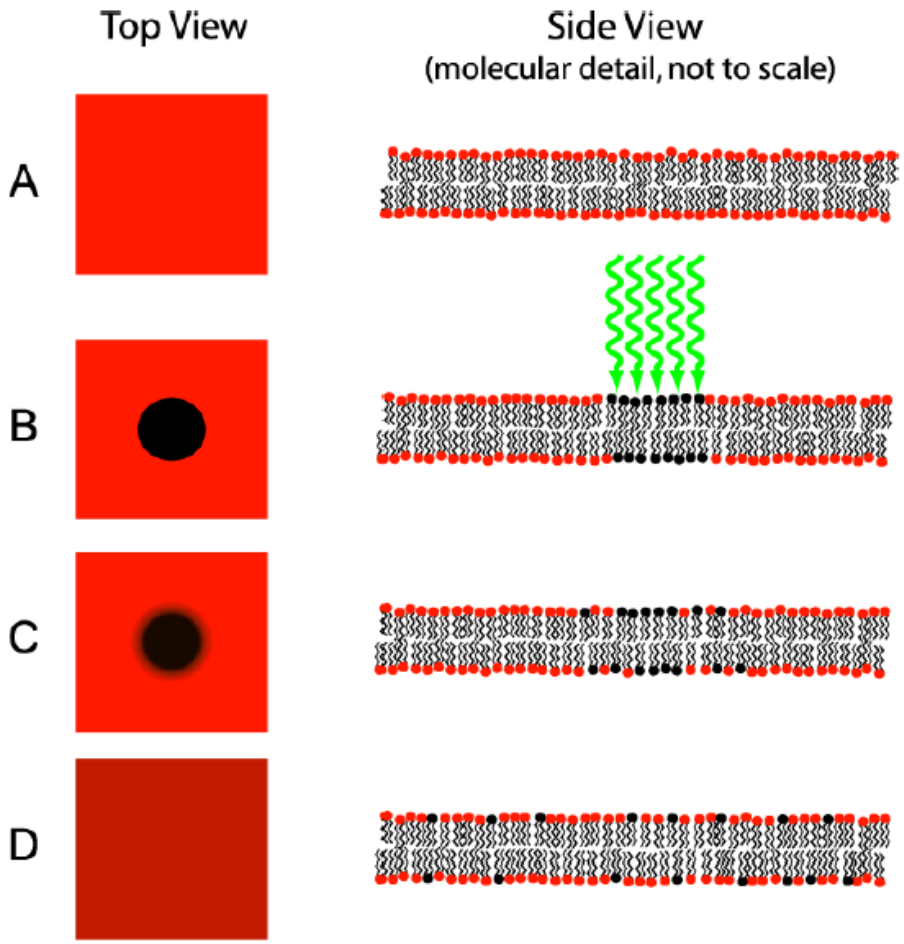


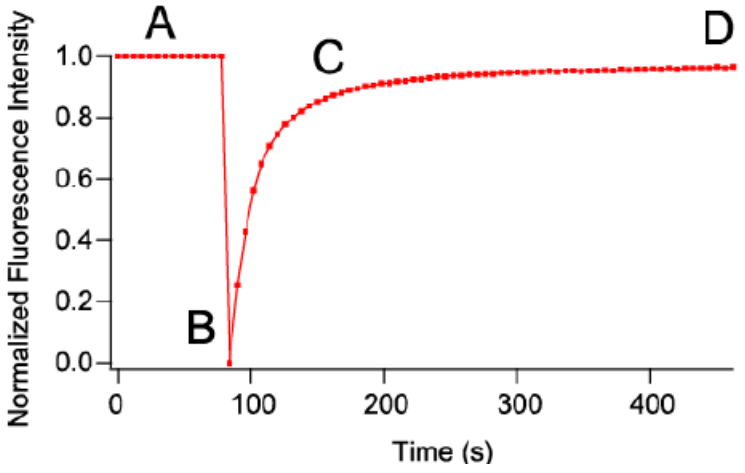
Dynamic applications of fluorescence microscopy

- Cavalcade of acronyms…

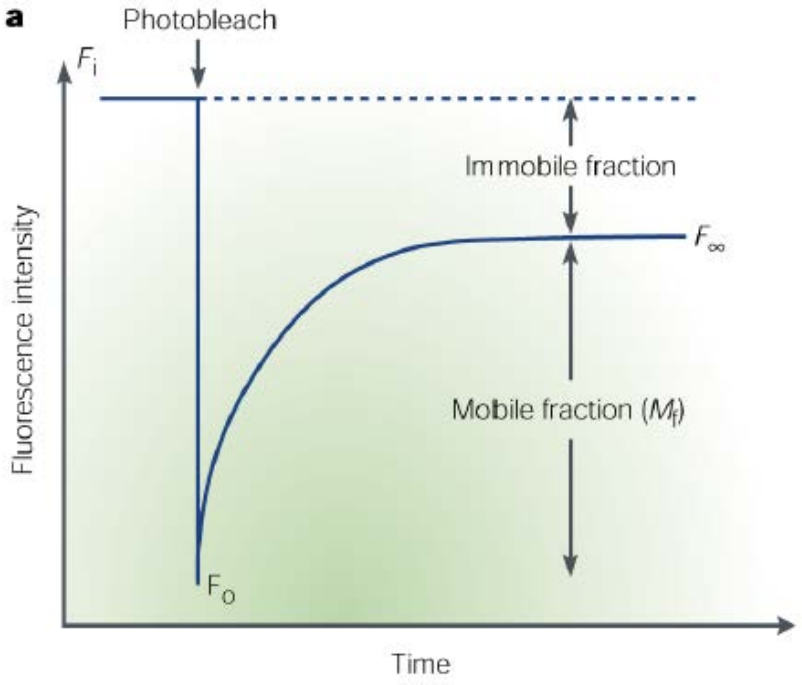
Fluorescence Recovery after Photobleaching (FRAP)

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



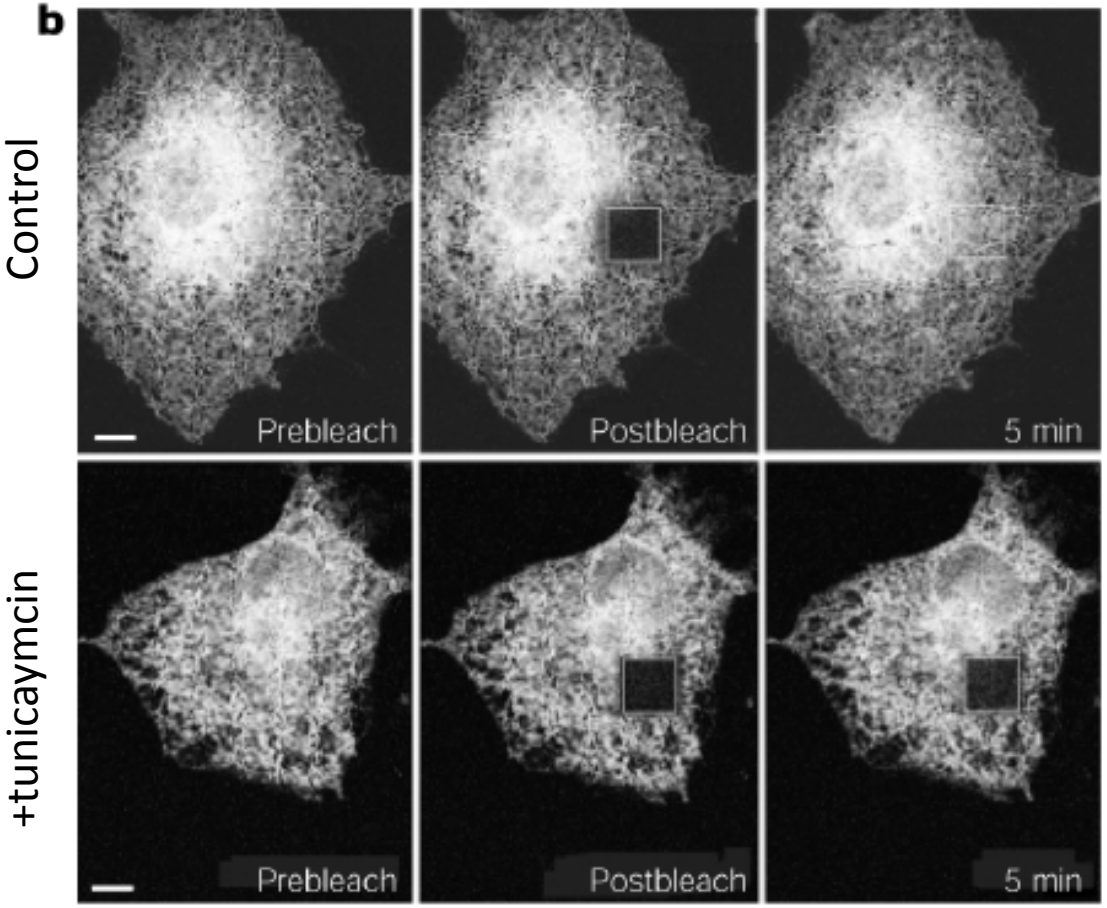


Quantifying FRAP



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

VsVg-GFP in ER



Quantifying FRAP

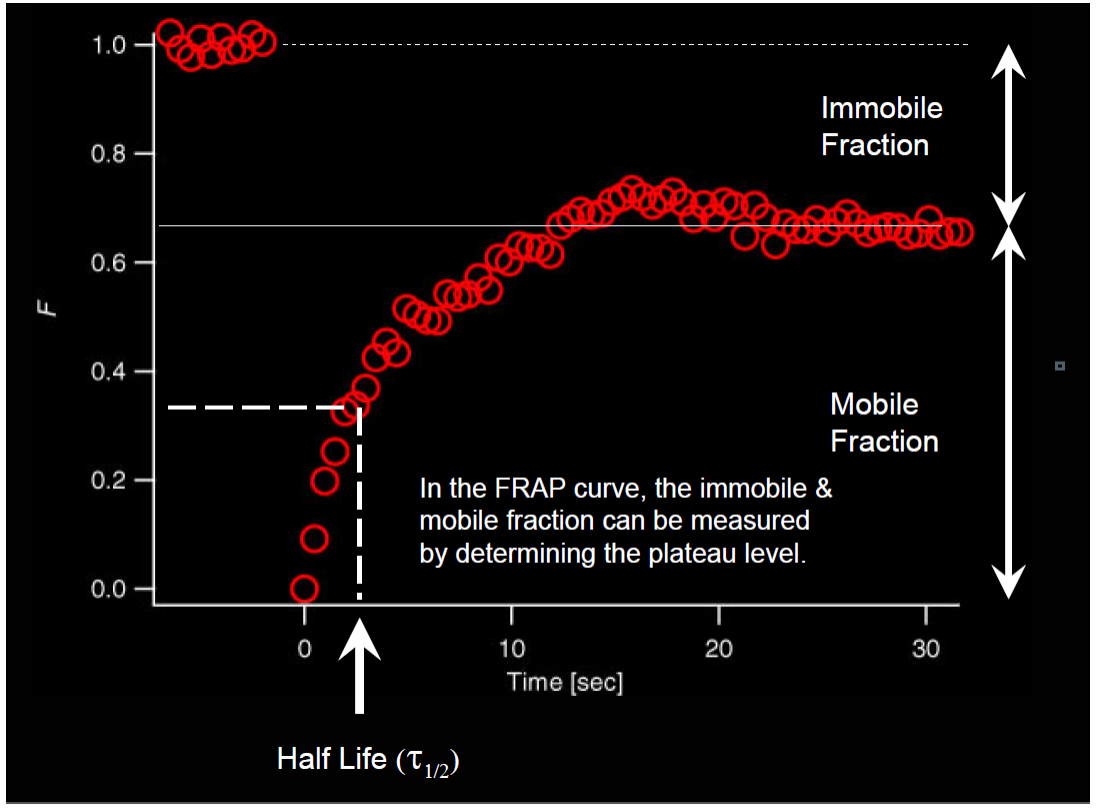
- We want to find the time constant of recovery

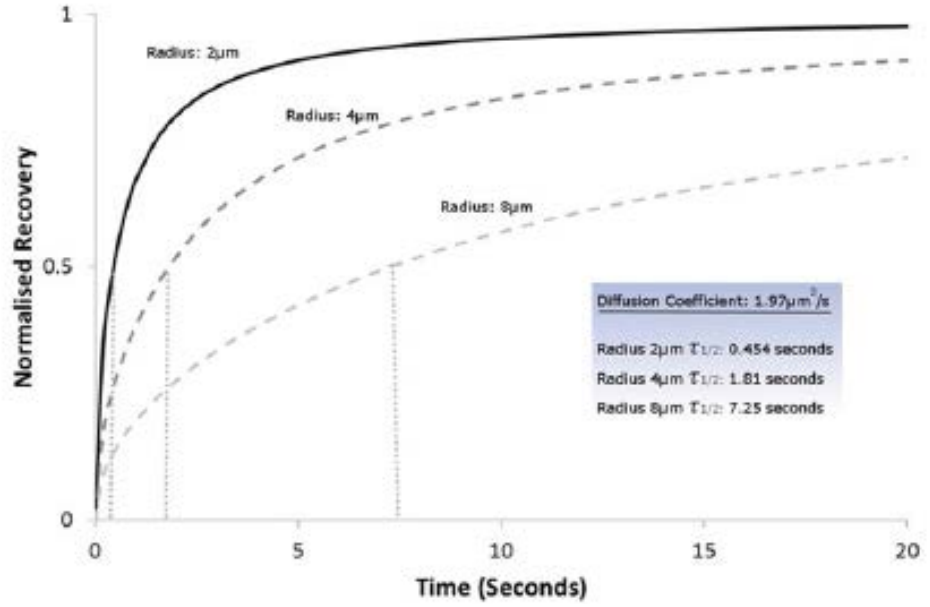
$$\mathrm{F}(t) = A\left(1 - e^{-t/\tau}\right)$$

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

Assuming disc bleach and diffusion is in 2D

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





Diffusion

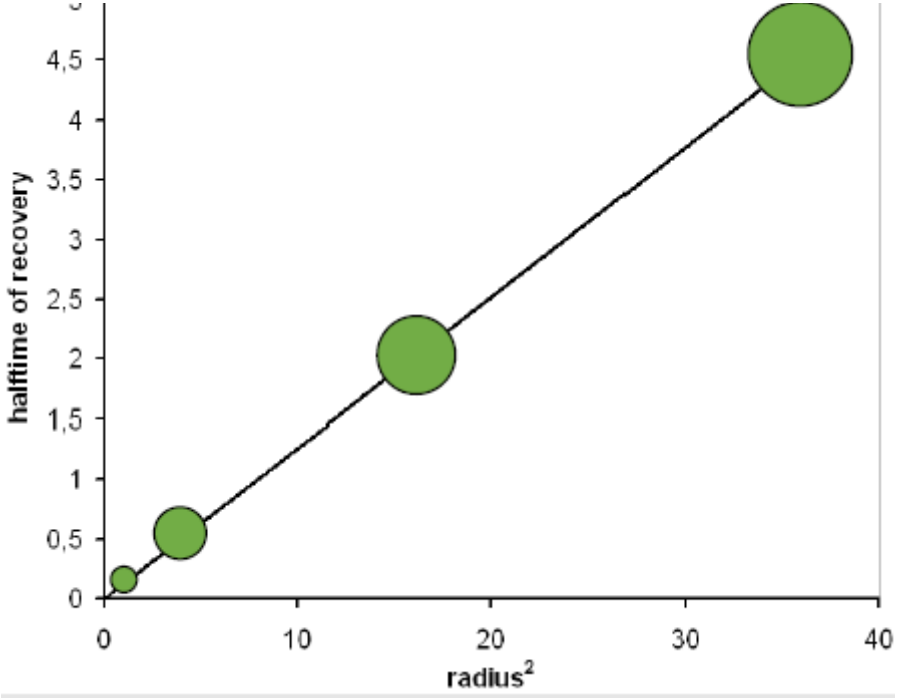
$$D = \frac{0.88\, w^2}{4t_{1/2}}$$

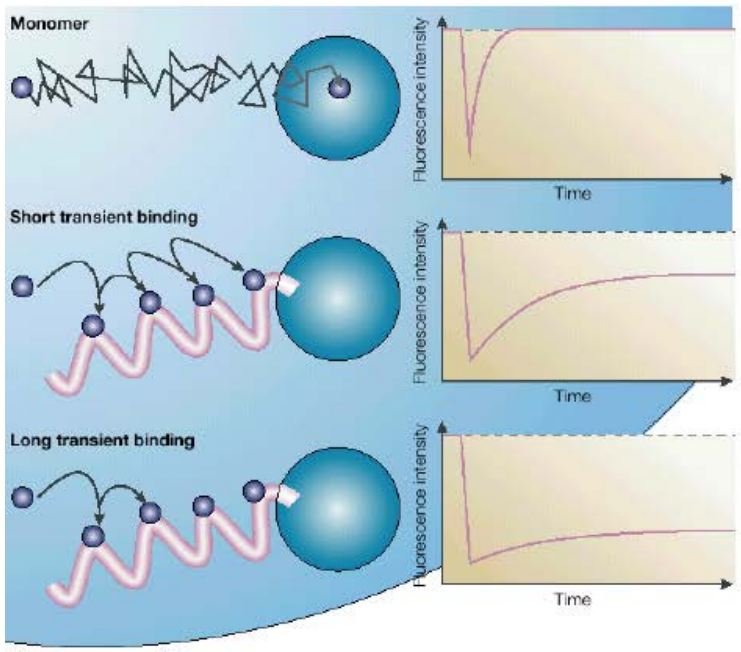
$$t \approx \frac{x^2}{2D}$$

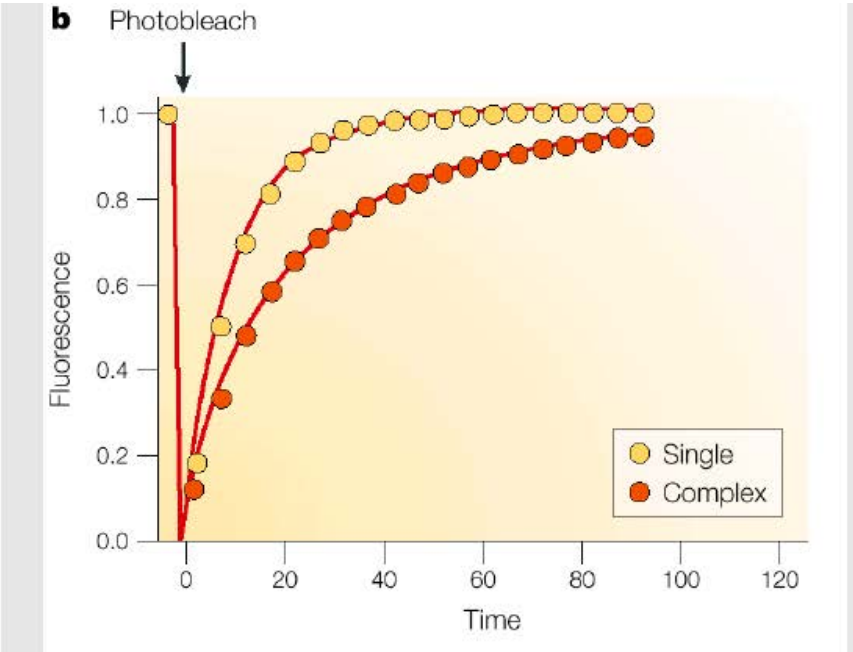
$$D = \frac{k_B T}{6\pi\eta r}$$

molecule	measured context	diffusion coefficient ($\mu m^2/s$)	BNID
H_2O	water	2000	104087, 106703
H_2O	nucleus of chicken erythrocyte	200	104645
H^+ (from H_3O^+ to H_2O)	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 *D. melanogaster* embryo	20	109209
protein (≈30 kDa)	*E. coli* cytoplasm	7-8	100193, 107985
protein (≈40 kDa)	*E. coli* cytoplasm	2-4	107985
protein (≈70-250 kDa)	*E. coli* cytoplasm	0.4-2	107985
protein (≈140 kDa Tar-YFP)	*E. coli* membrane	0.2	107985
protein (≈70 kDa LacY-YFP)	*E. coli* membrane	0.03	107985
fluorescent dye (carboxy-fluorescein)	*A. thaliana* cell wall	30	105033
fluorescent dye (carboxy-fluorescein)	*A. thaliana* mature root epidermis	3	105034
transcription factor (LacI)	movement along DNA (1D, *in vitro*)	0.04 (4×10^5 bp^2s^{-1})	102036
morphogen (bicoid-GFP)	cytoplasm of *D. melanogaster* embryo	7	109199
morphogen (wingless)	wing imaginal disk of *D. melanogaster*	0.05	101072
mRNA	HeLa nucleus	0.03-0.10	107613
mRNA	various localizations and sizes	0.005-1	110667
ribosome	*E. coli*	0.04	108596

FRAP and diffusion



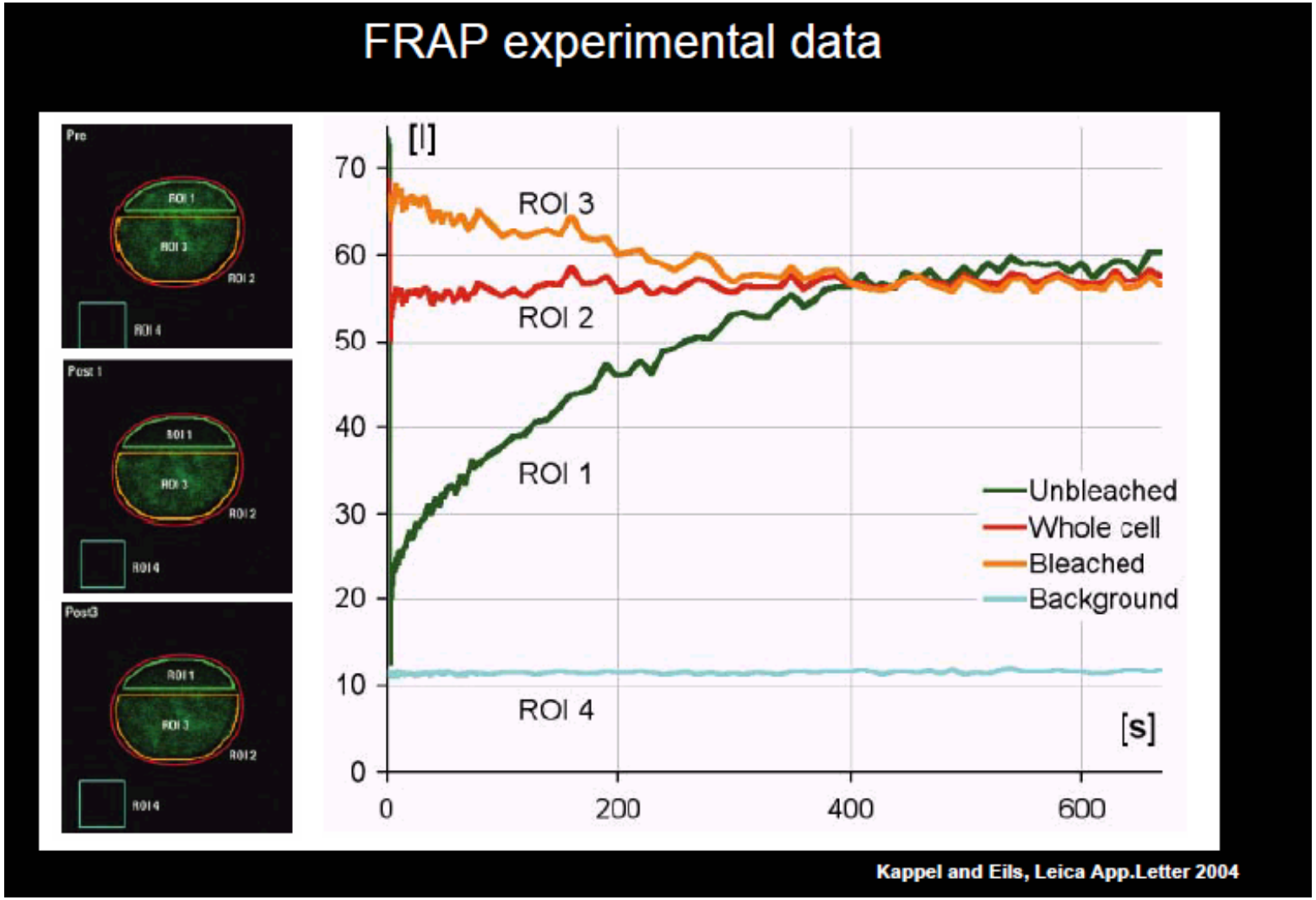




$$\mathrm{F}(t) = A\left(1 - e^{-t/\tau}\right)$$

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

More on… Quantifying FRAP



- Corrections
  - Background
  - Photobleaching

$$F_{ROI1}\,(t)$$

1. Subtract background

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

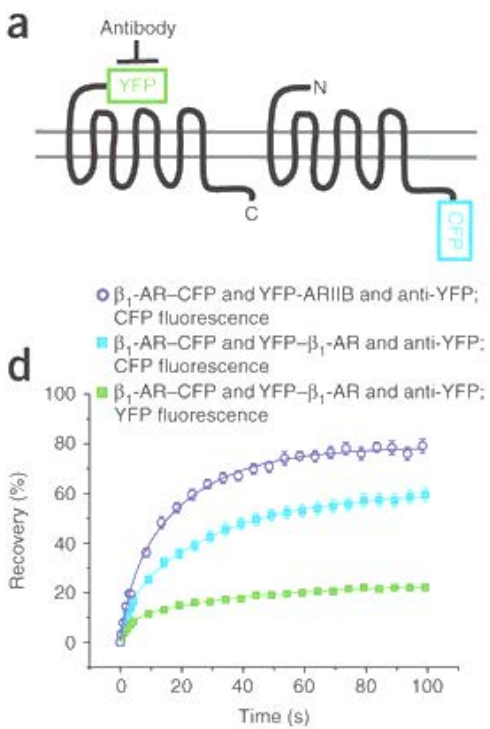
2. Correct photobleaching

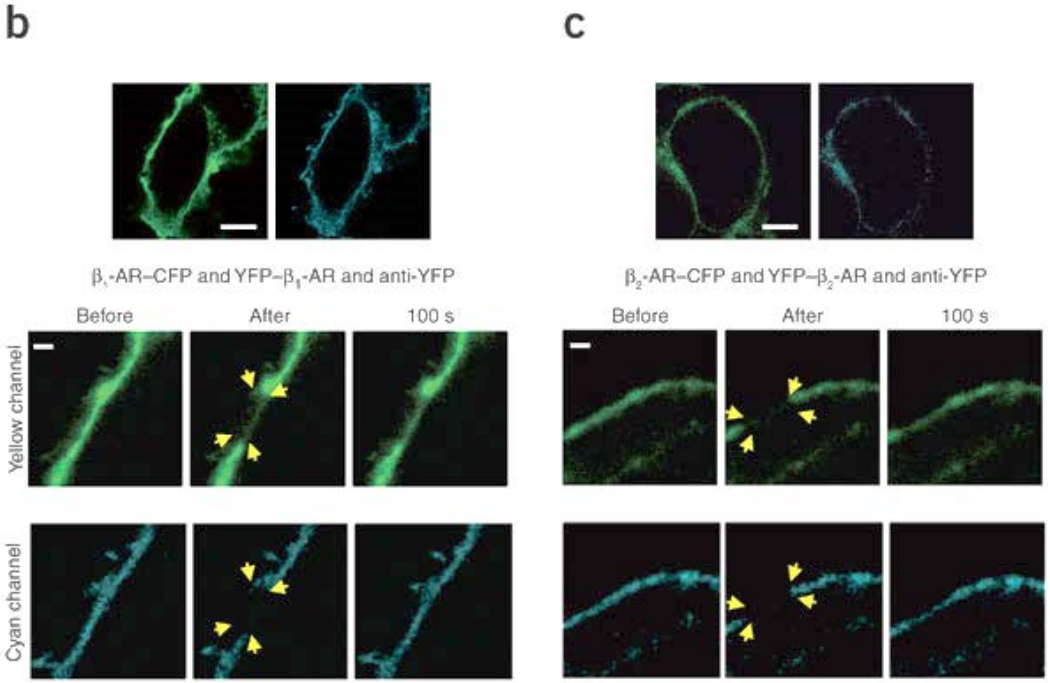
$$F_{ROI1,BG,PB}(t) = \frac{F_{ROI1,BG}\,(t)}{F_{ROI2}\,(t) - F_{ROI4}\,(t)}$$

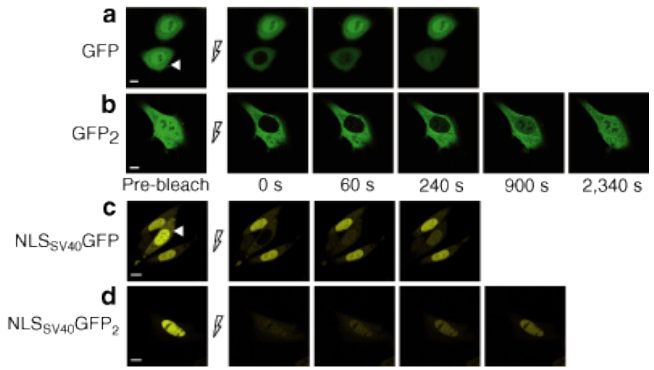
3. Extract time constants

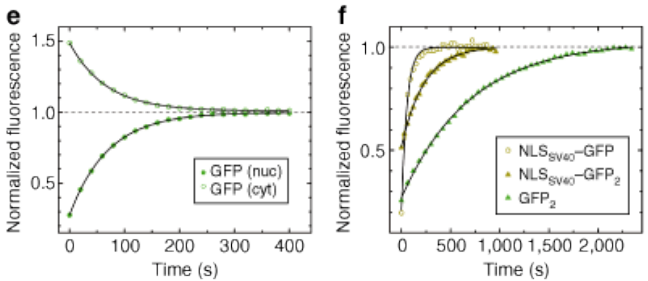
FRAP applications

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

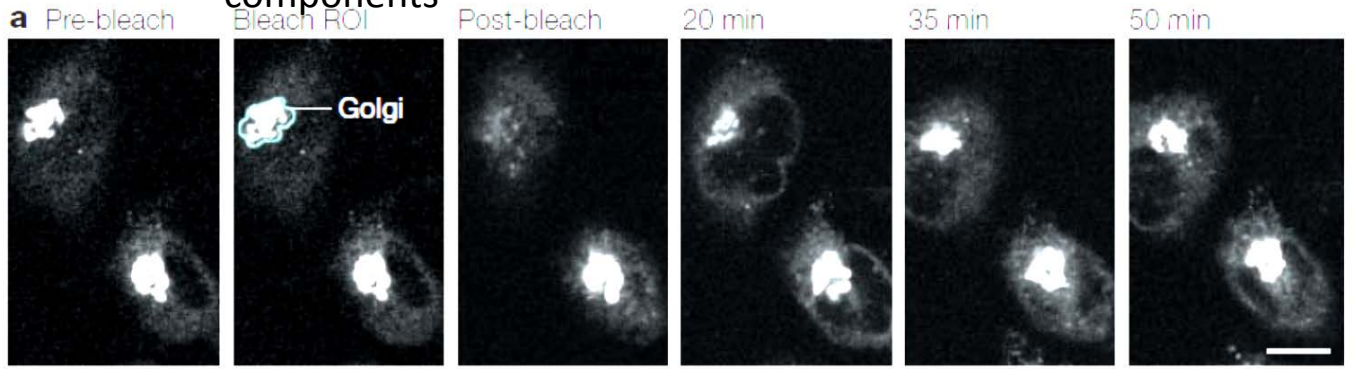




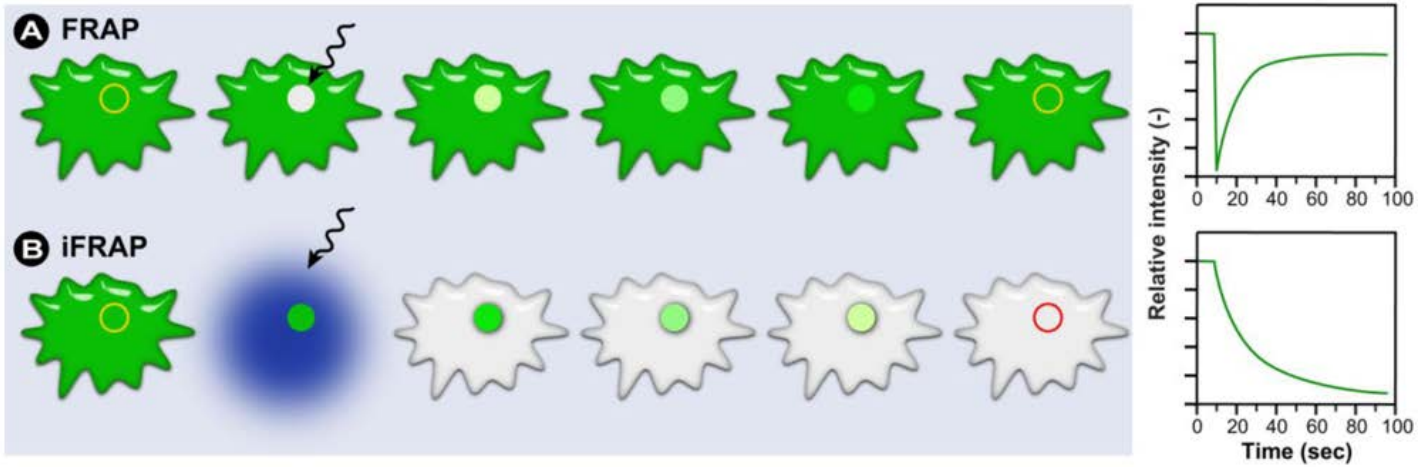




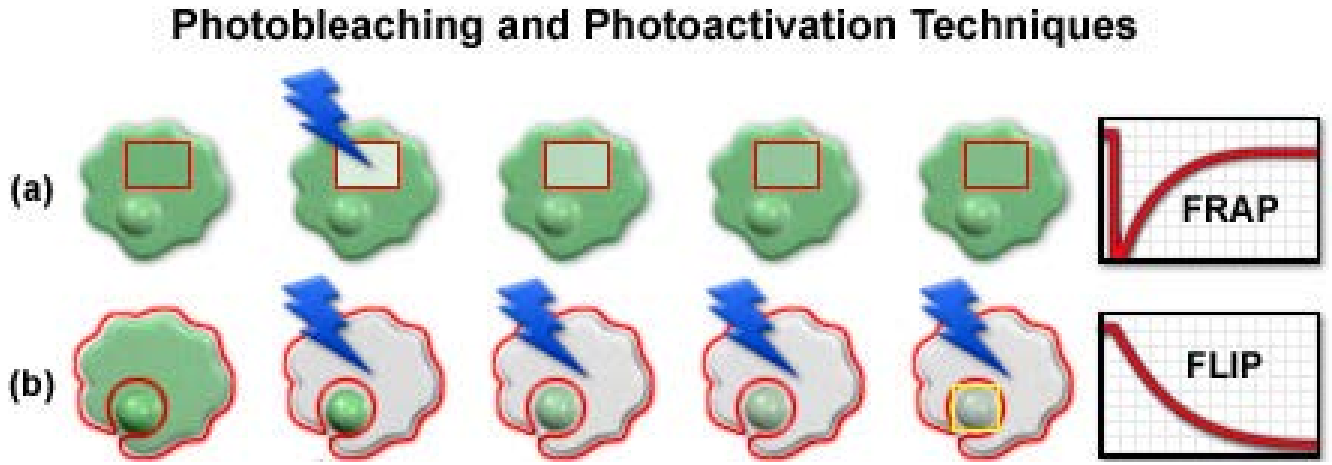
Galtase-GFP in the Golgi. A FRAP experiment showed Galtase is exchangeable with other components



FLIP – fluorescence loss in photobleaching/iFRAP



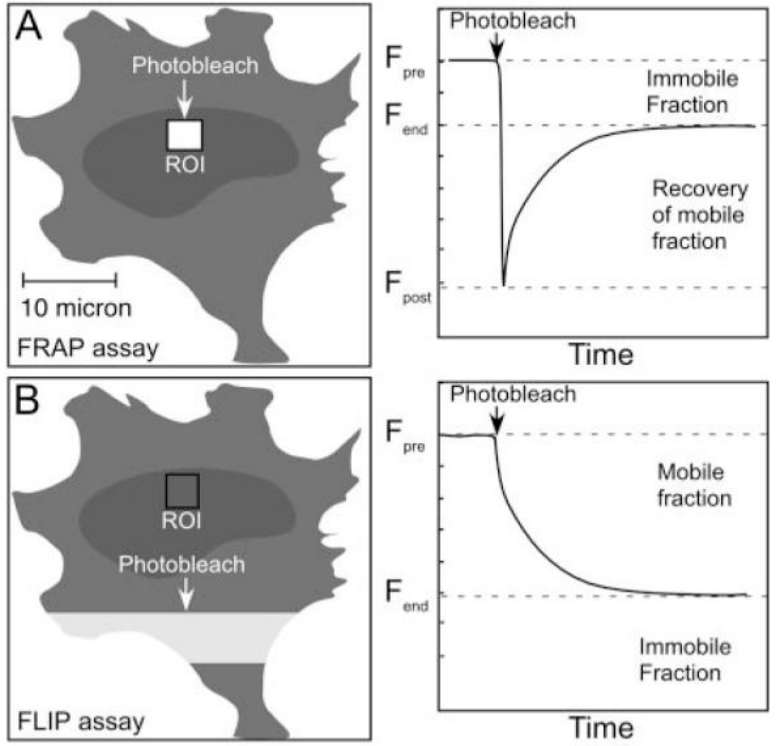
$$\mathrm{F}(t) = Ae^{-\tau t}$$



Measurements are not recorded from photobleached area.

Watch diffusion back into the rest of the cell.

FLIP uses continuous inactivation. iFRAP does it once

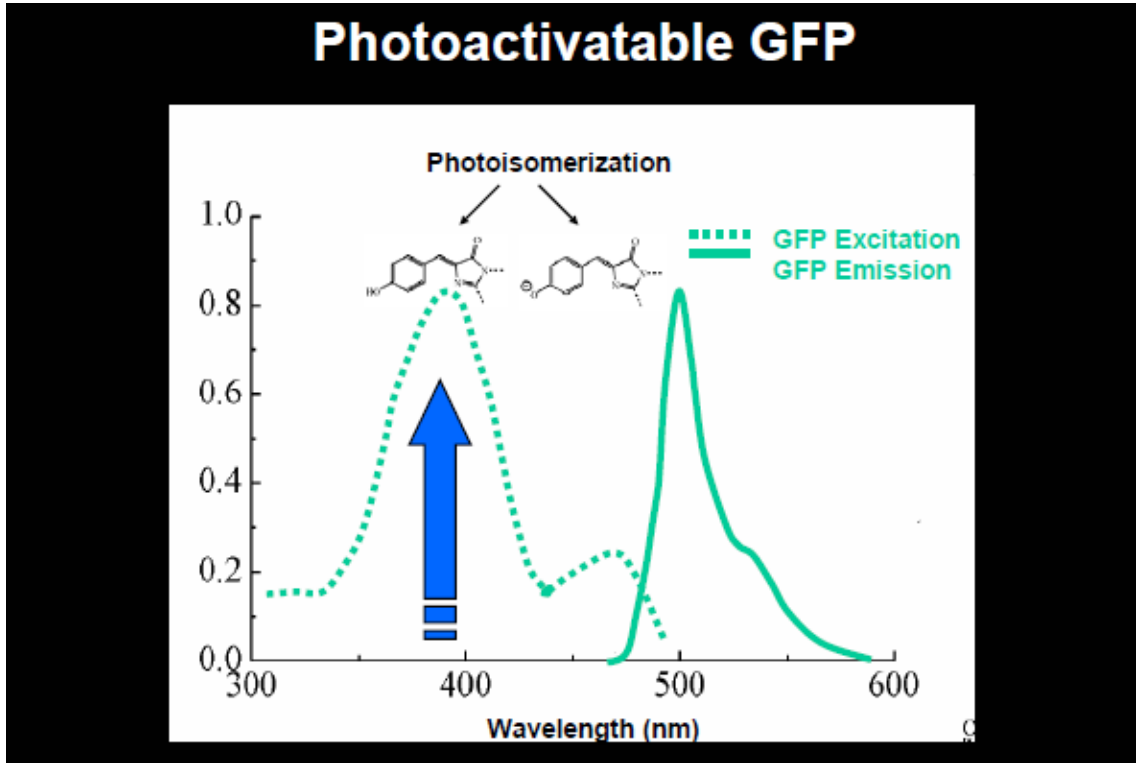


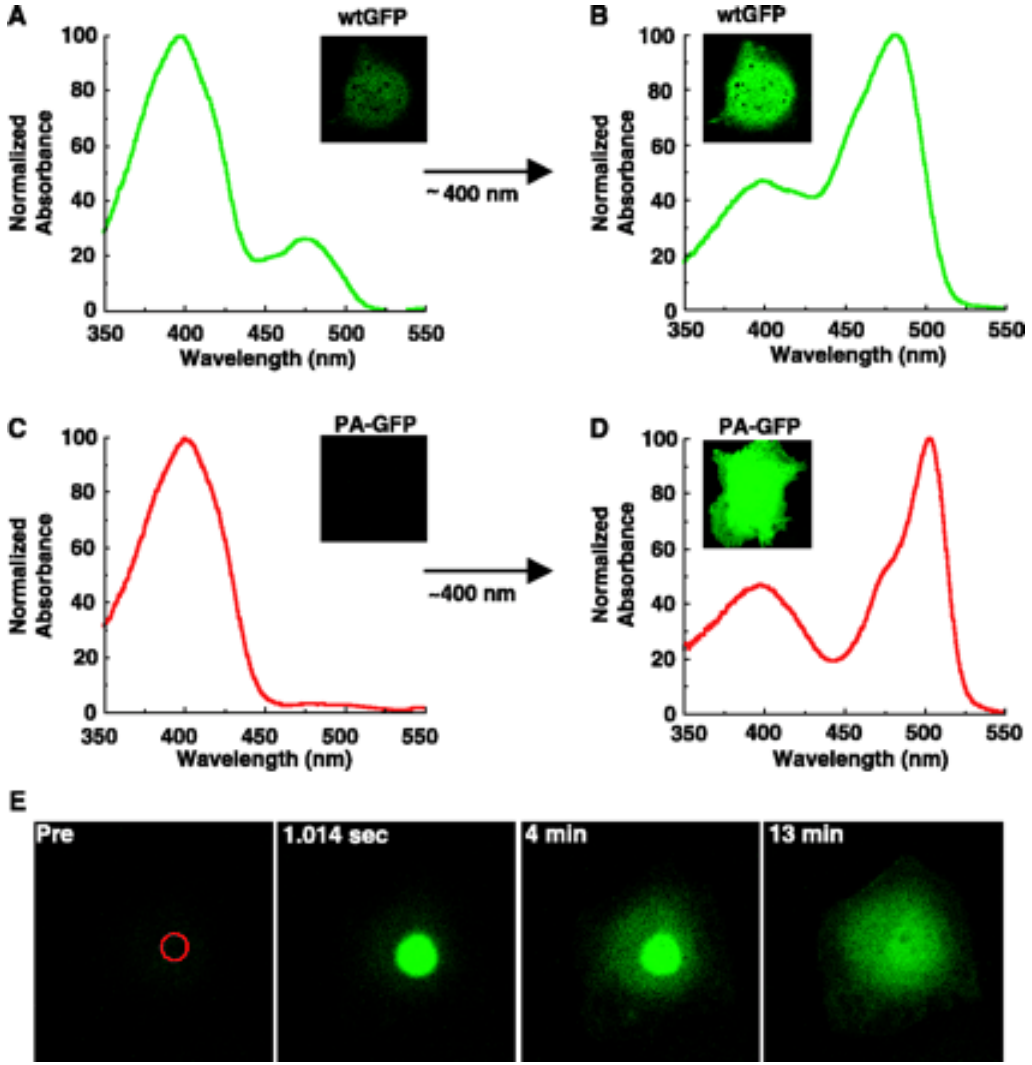
FRAP/FLIP limitations

- 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

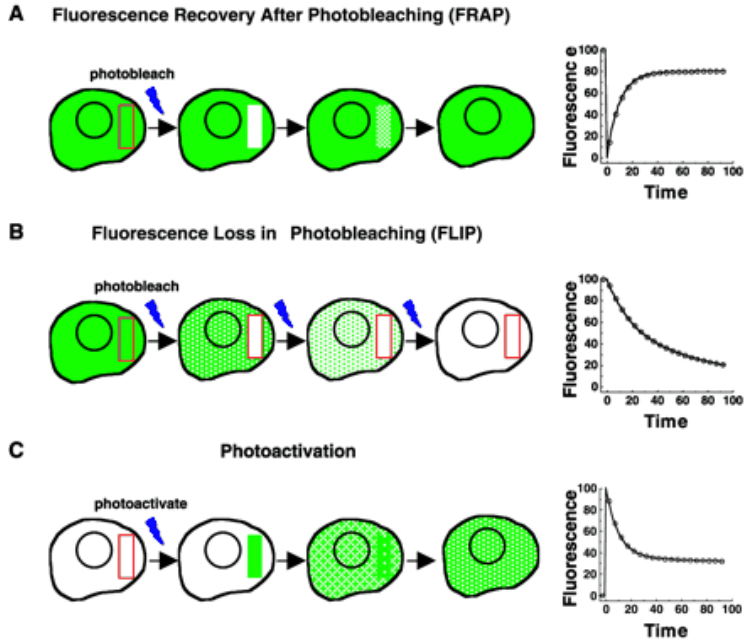
Photoactivatible proteins to the rescue

- Photoactivable GFPs are dark until illumination with 405 nm light



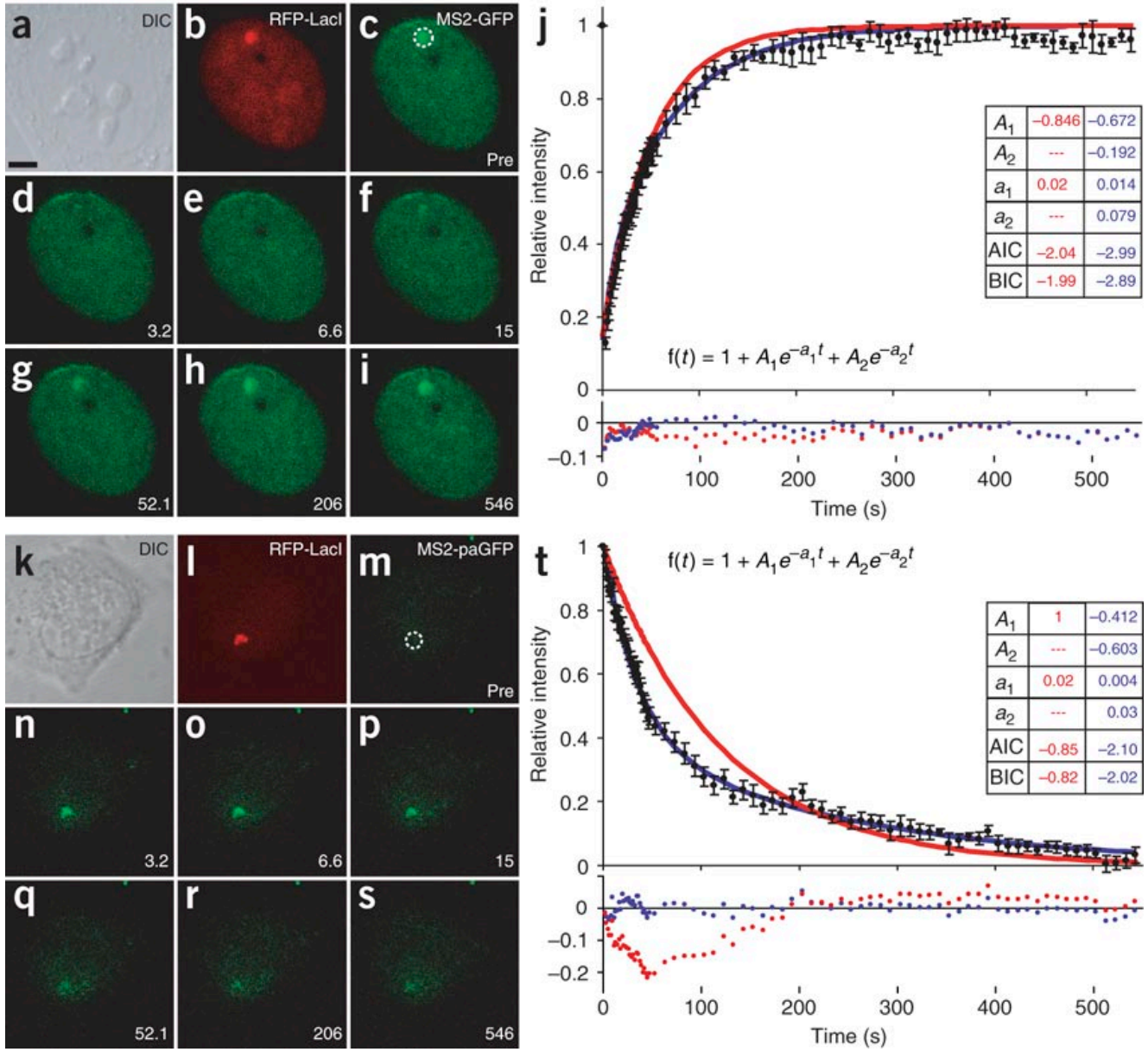


Photoactivation



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

mRNA synthesis in vivo



Advanced techniques

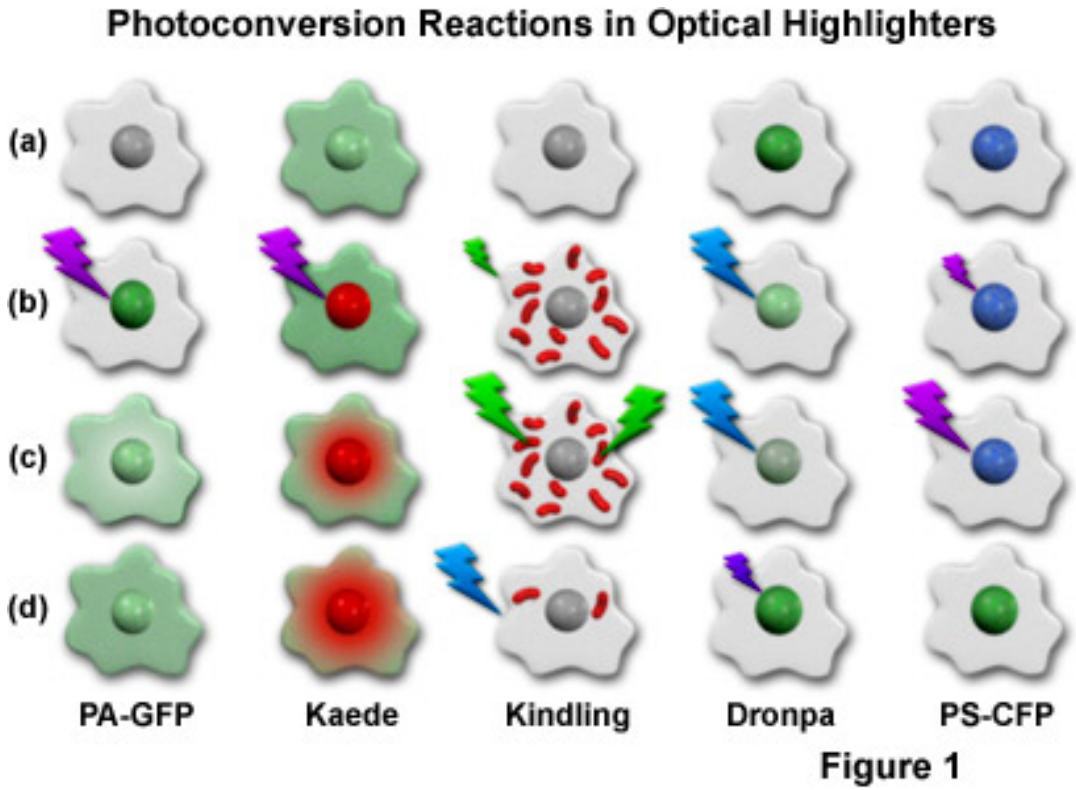
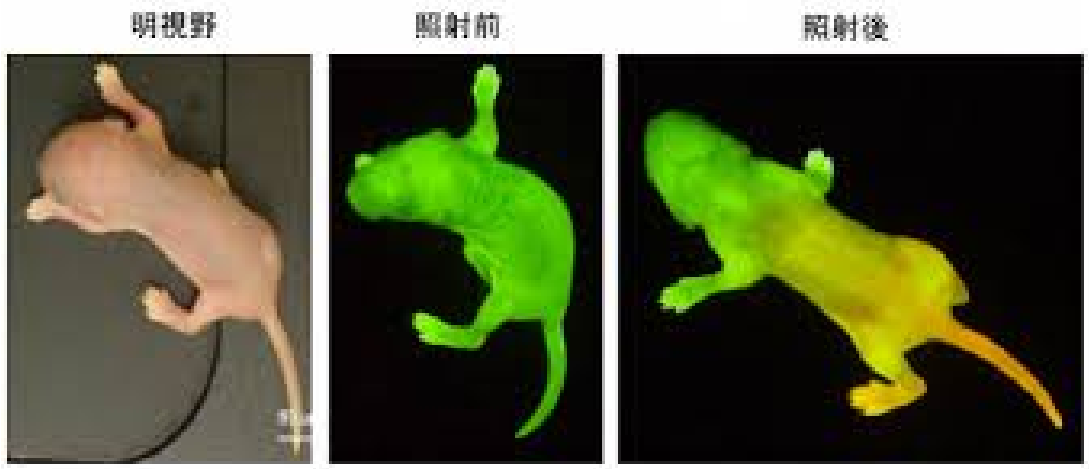
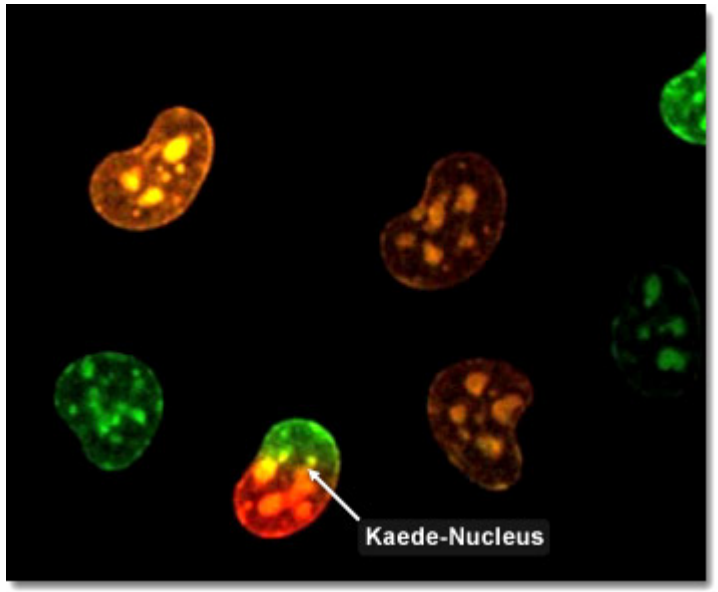


Figure 1





And on to Matlab…