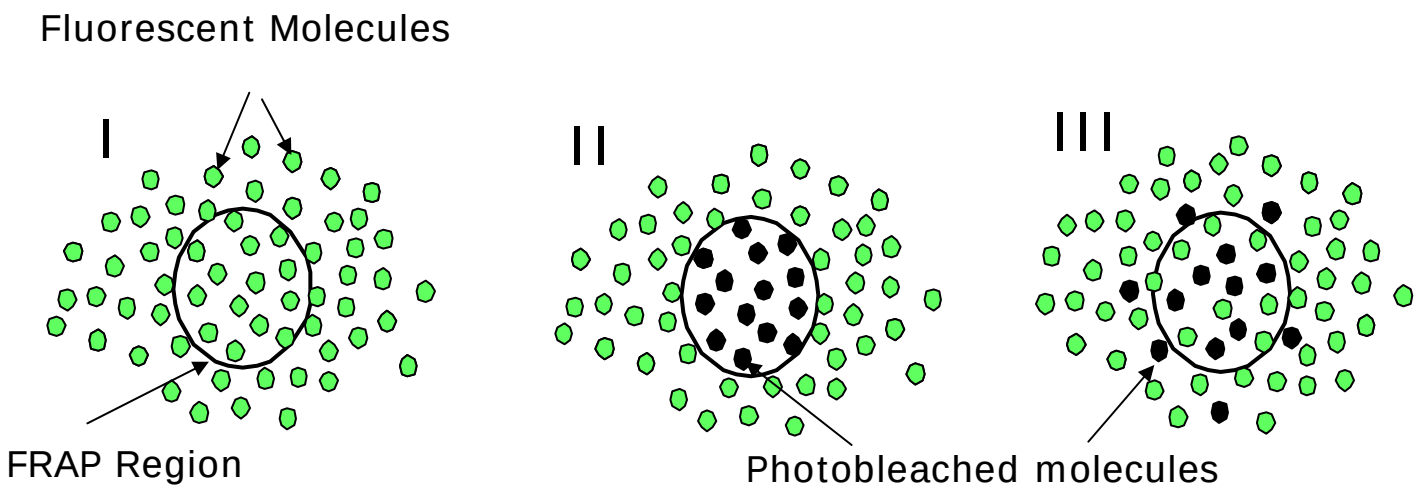
MAG Biosystems FRAP 3D™

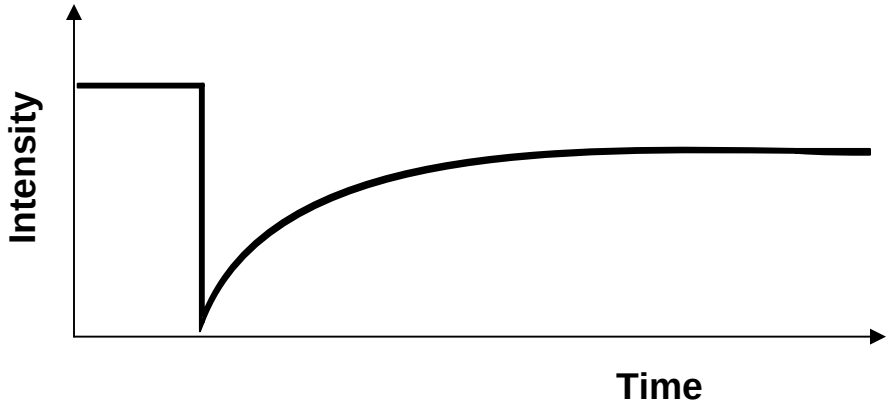
MAG BIOSYSTEMS™

FRAP and Photoactivation

An introduction

Fluorescence Recovery After Photobleaching

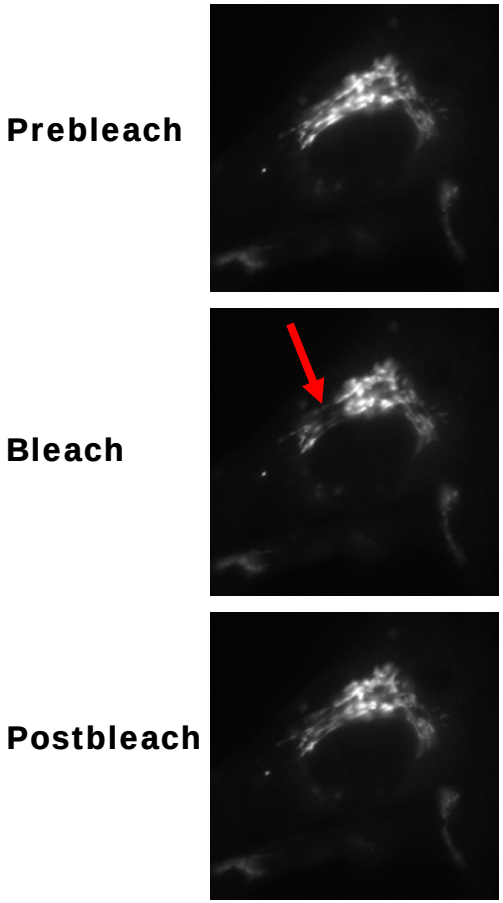


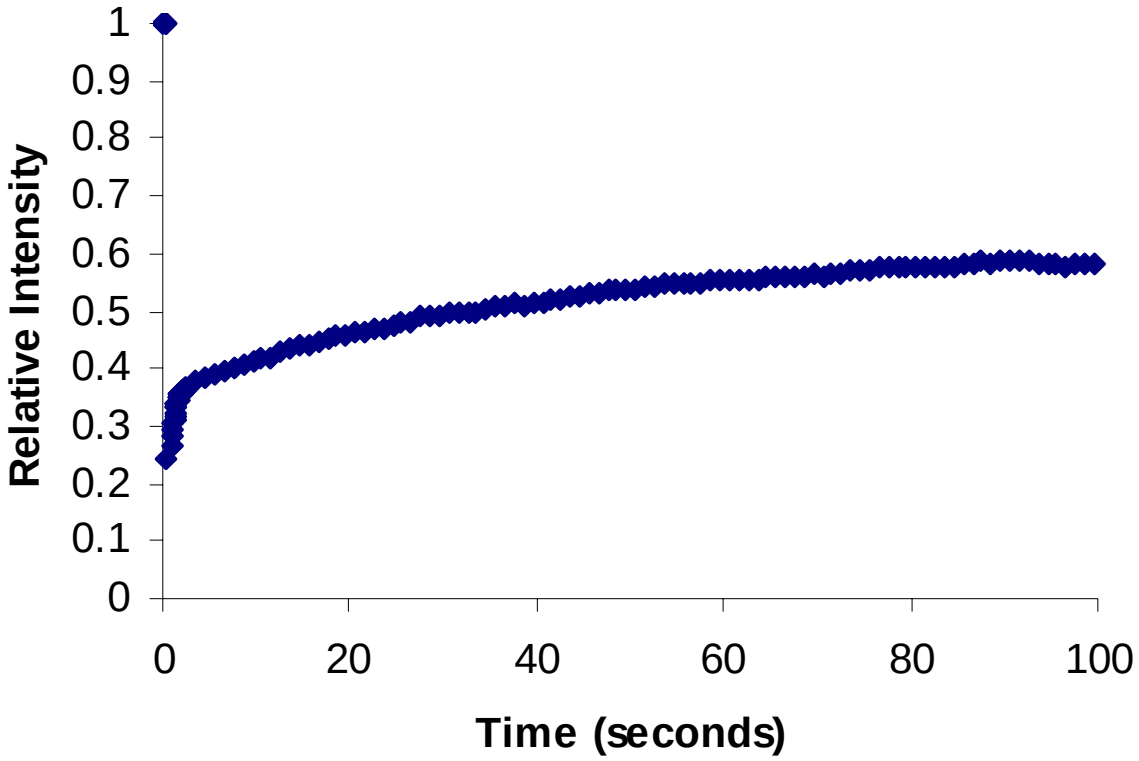


FRAP 3D

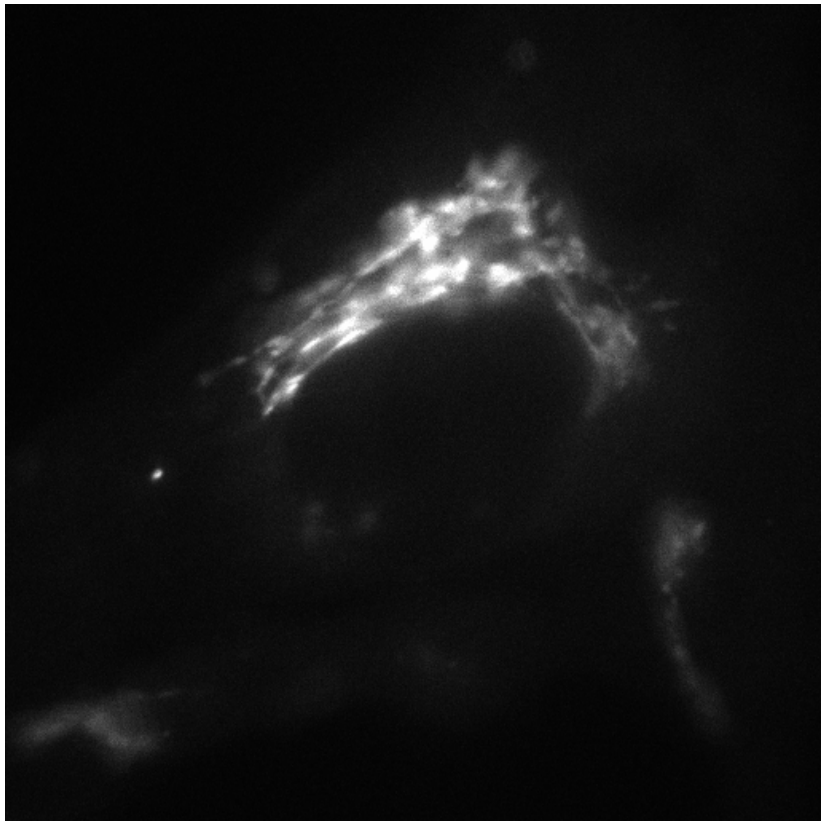
Fluorescence Recovery After Photobleaching

MAG BIOSYSTEMS



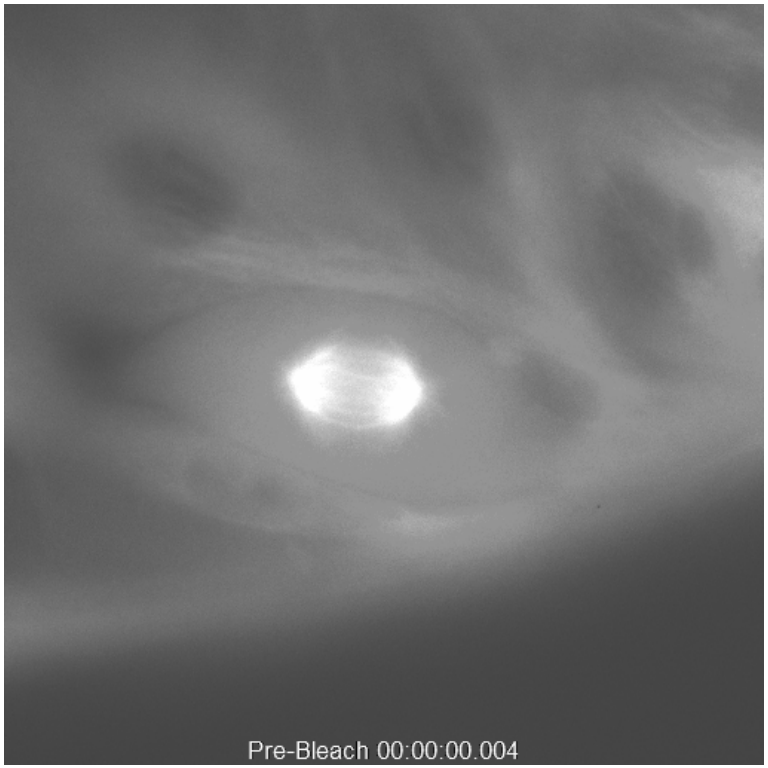


Fluorescence Recovery After Photobleaching



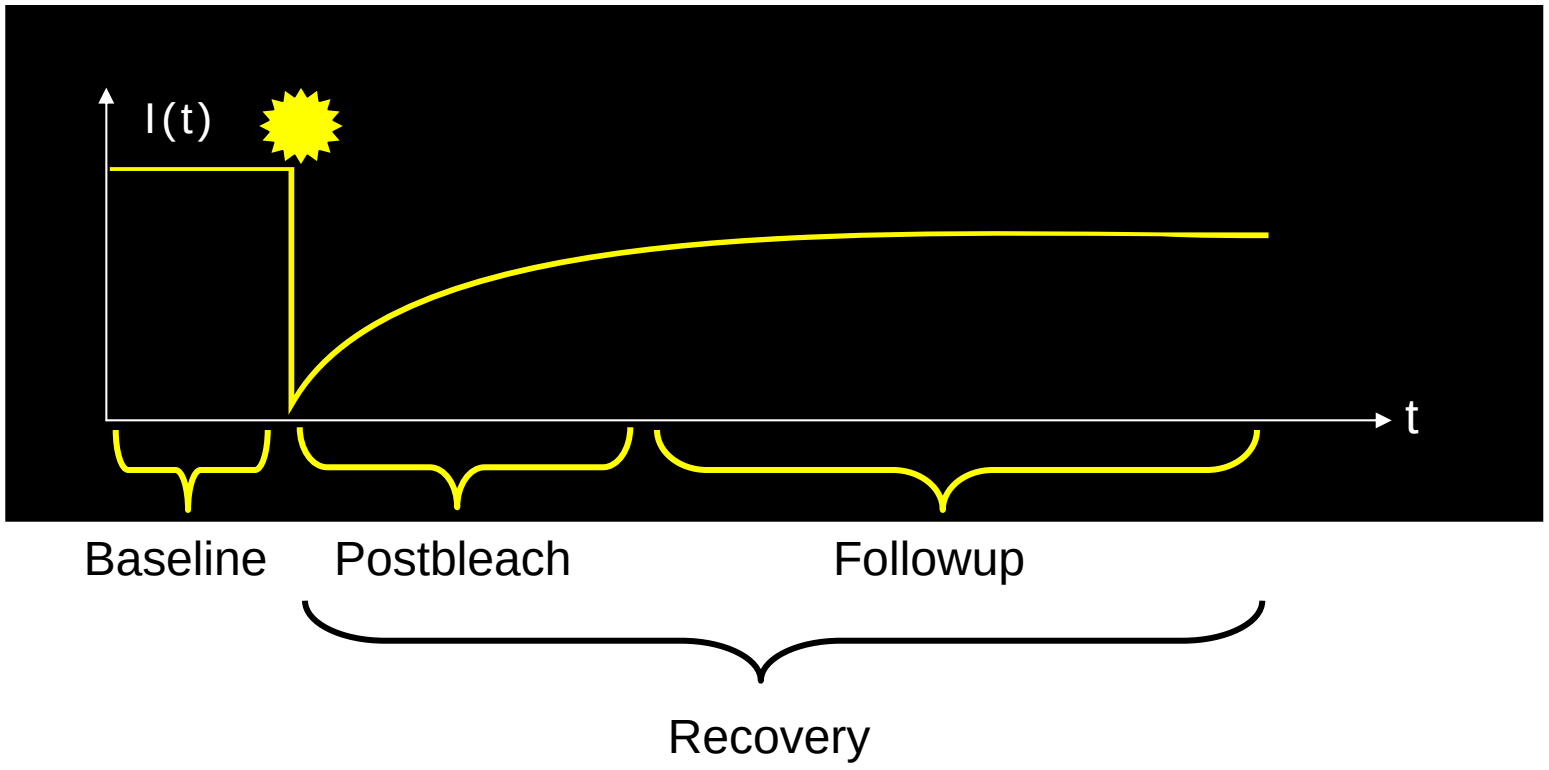
- EYFP targeted to Golgi

Fluorescence Recovery After Photobleaching

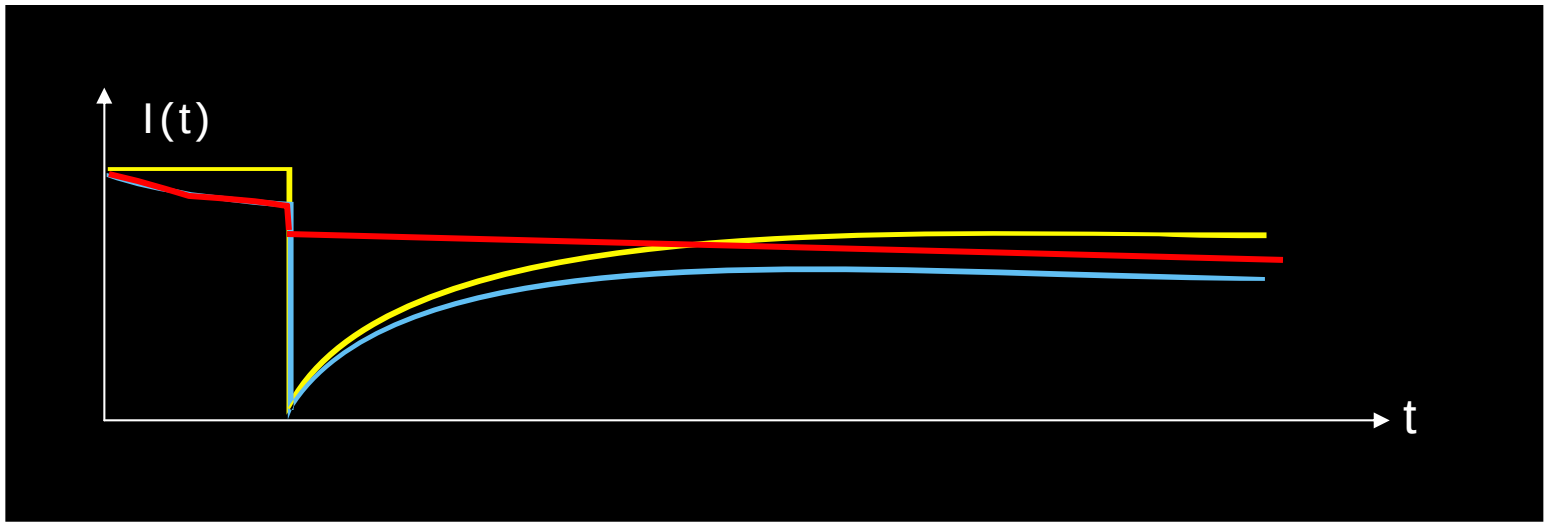


- EGFP-tubulin.
- FRAP region of mitotic spindle

Anatomy of a FRAP curve



Correcting a FRAP curve

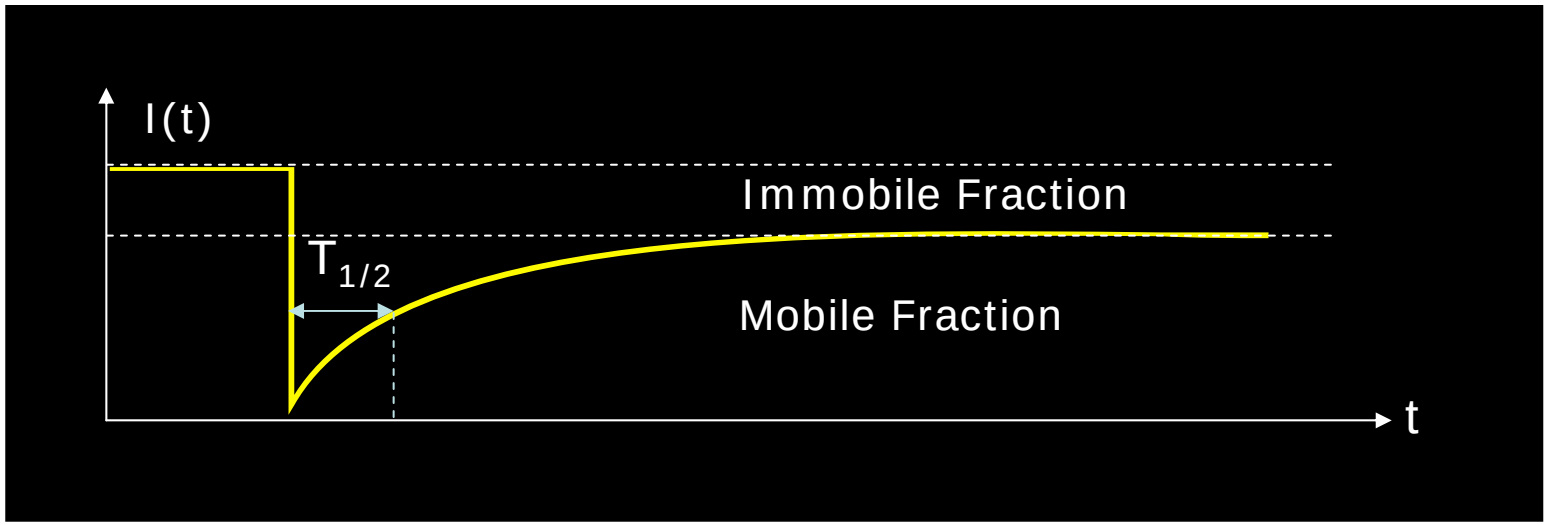


Start with raw FRAP data

Must correct FRAP data

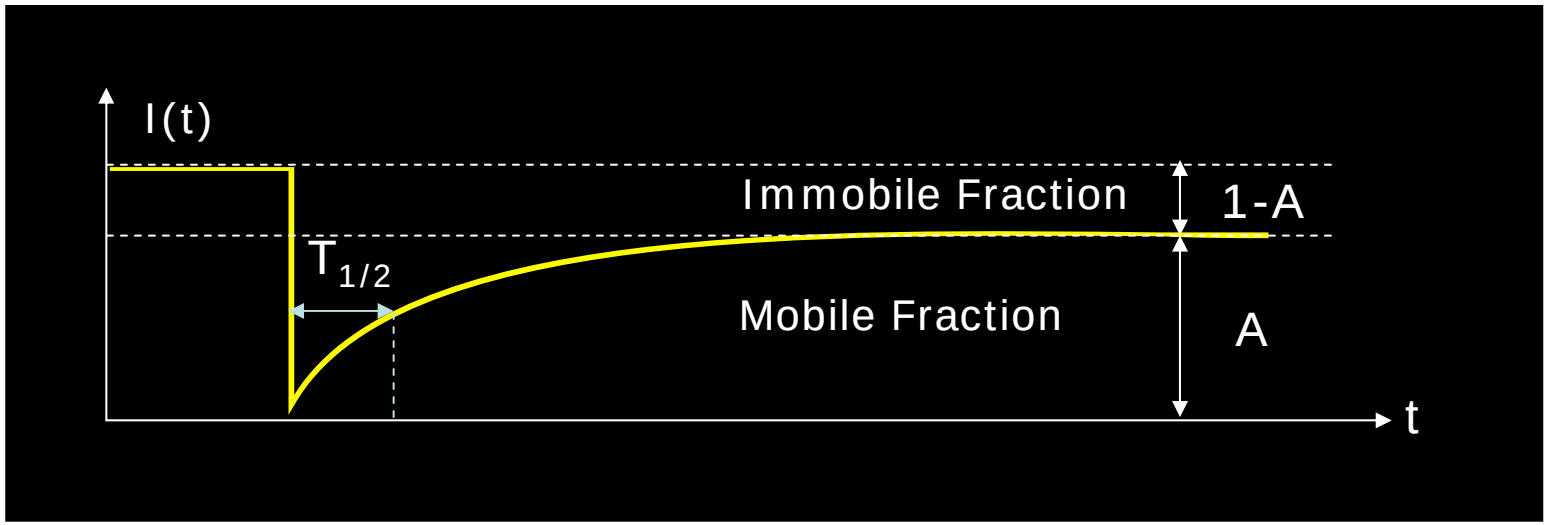
- Background
- Photobleaching due to imaging
- Decrease in total cellular fluorescence due to bleaching

What does a FRAP curve tell you?



Can determine these values by visual inspection or by curve fitting

What does a FRAP curve tell you?



$$f(t) = A\left(1 - e^{-\pi t}\right)$$

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

What is FRAP used for?

- Classically, FRAP was used to measure diffusion constants (D) of molecules in the membrane.
- $D=0.88*w^2/4t_{1/2}$ where w is the bleach radius
- Two major assumptions:
  - Bleach area is a circle
  - Diffusion only occurring in 2D

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What is FRAP used for?

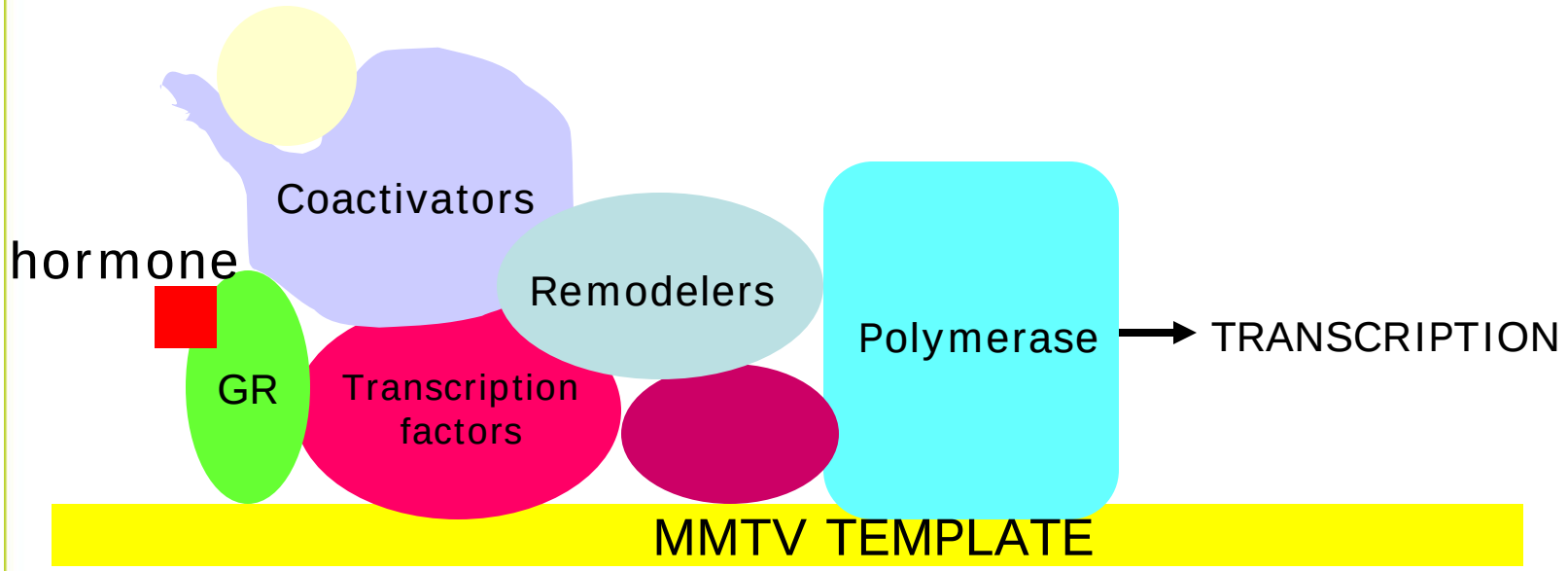
- More recently, FRAP has also been used to analyze protein “mobility” in the nucleus and cytoplasm.
  - This “mobility” is now being recognized as a combination of diffusion and binding.
  - FRAP can also be used to measure protein binding interactions in vivo.

What can FRAP tell us?

- Case study from work done by Jim McNally's group at the NCI
  - Sprague BL, Muller F, Pega RL, Bungay PM, Stavreva DA, Mcnally JG. 2006 Biophysical Journal 91(4) 1169-1191
  - Stavreva DA, Mcnally JG. 2006 Histochem Cell Biol 125(1-2) 83-89
  - Stavreva DA, Muller WG, Hager GL, Smith CL, McNally JG. 2004 Mol Cell Biol 24(7) 2682-2697
  - Becker M, Baumann CT, John S, Walker D, Vifneron M, McNally JG, Hager GL. 2002 EMBO 3(12) 1188-1194
- Studying the role of steroid hormone receptors in transcriptional regulation
- Thanks to Jim McNally for providing the following slides and data

FRAP as a tool to study transcription in vivo

Overview: transcription mediated by the glucocorticoid receptor (GR)



MMTV = mouse mammary tumor virus

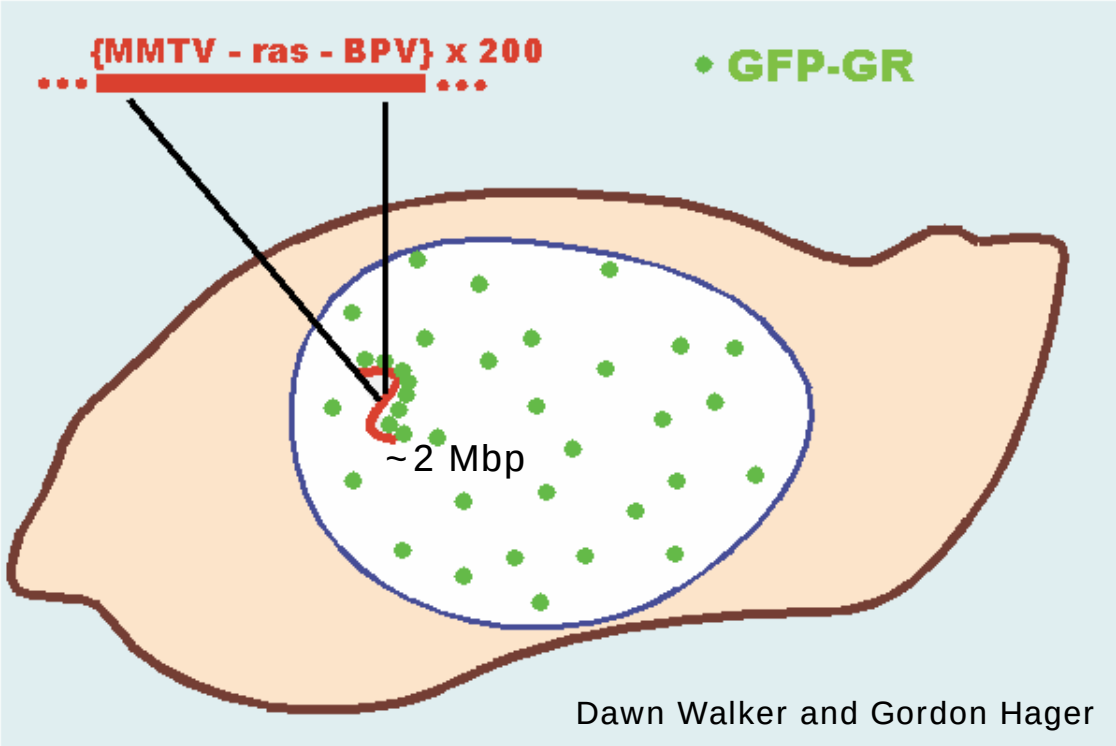
Tandem array of promoter sites

= promoter (MMTV)

= GFP-tagged transcription factor (GFP-GR)

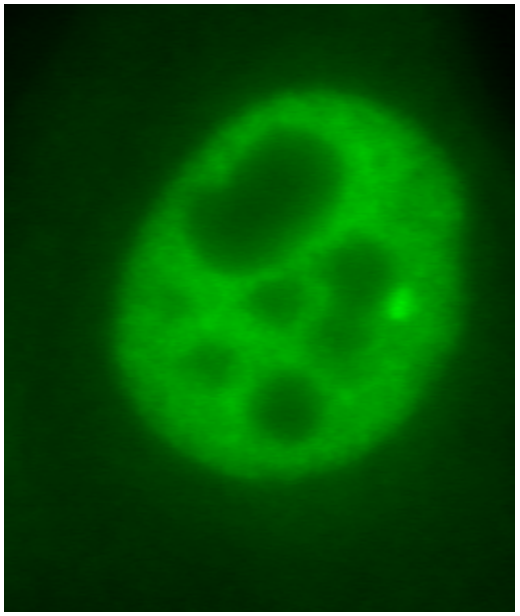
= downstream gene (ras)

A cell system for in vivo imaging of transcription

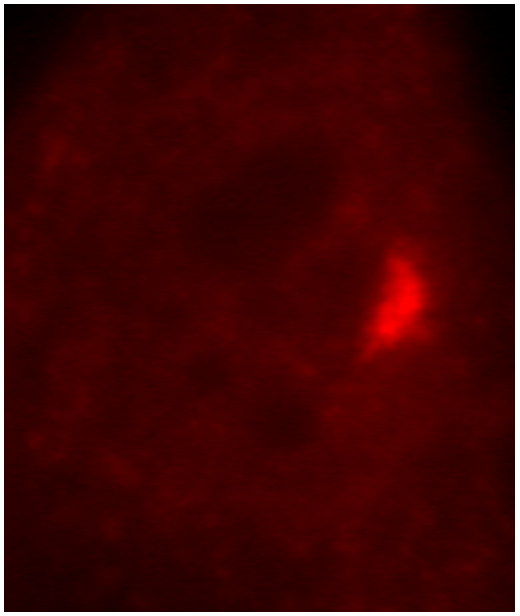


Cells are stably transfected with GFP-GR under tetracycline regulation.

MMTV arrays are visible and colocalize with transcript

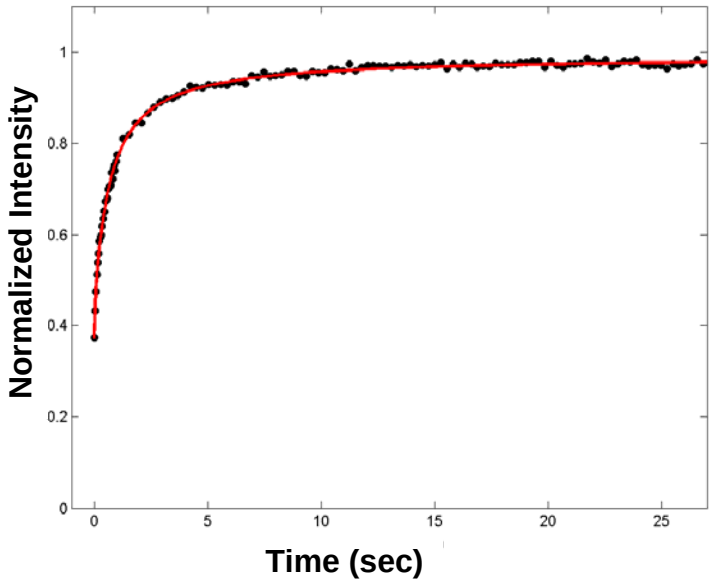


GFP-GR



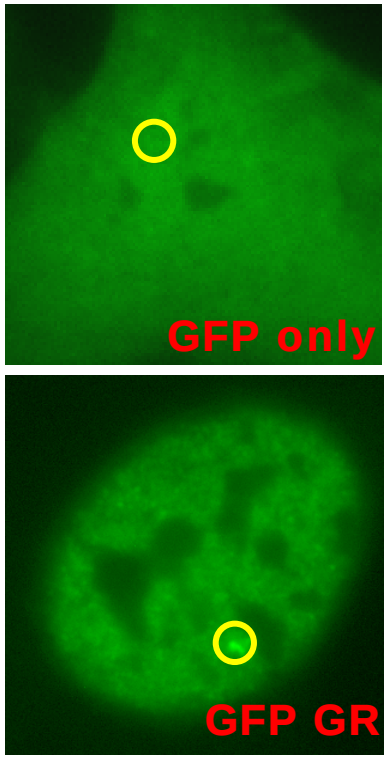
RNA FISH

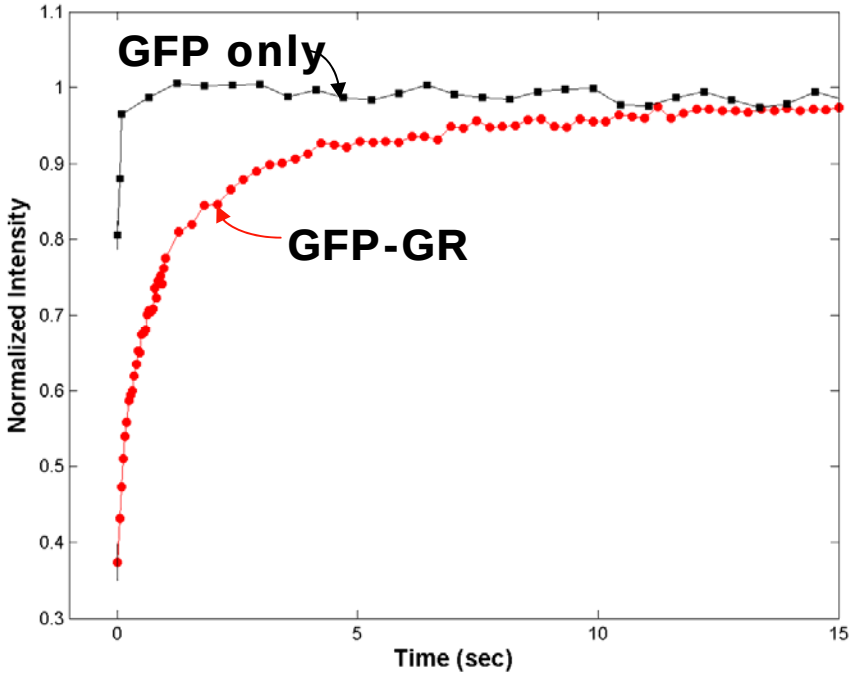
FRAP of GFP-GR bound to MMTV Promoter



GFP-GR recovers at the tandem array in seconds while transcription continues for hours

Analysis of GFP-GR binding



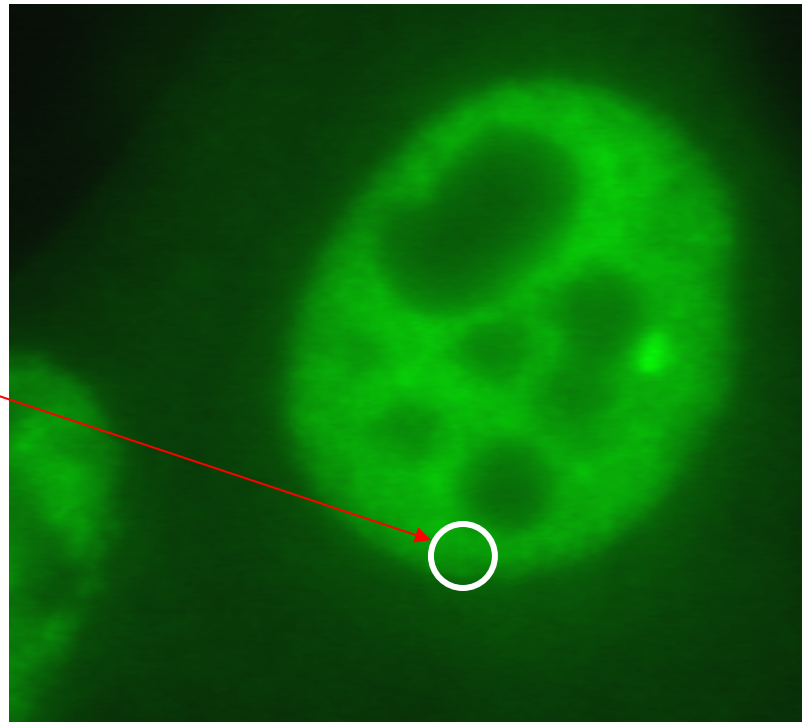


Slower recovery of GFP-GR not due to size of GR but to binding of GFP-GR

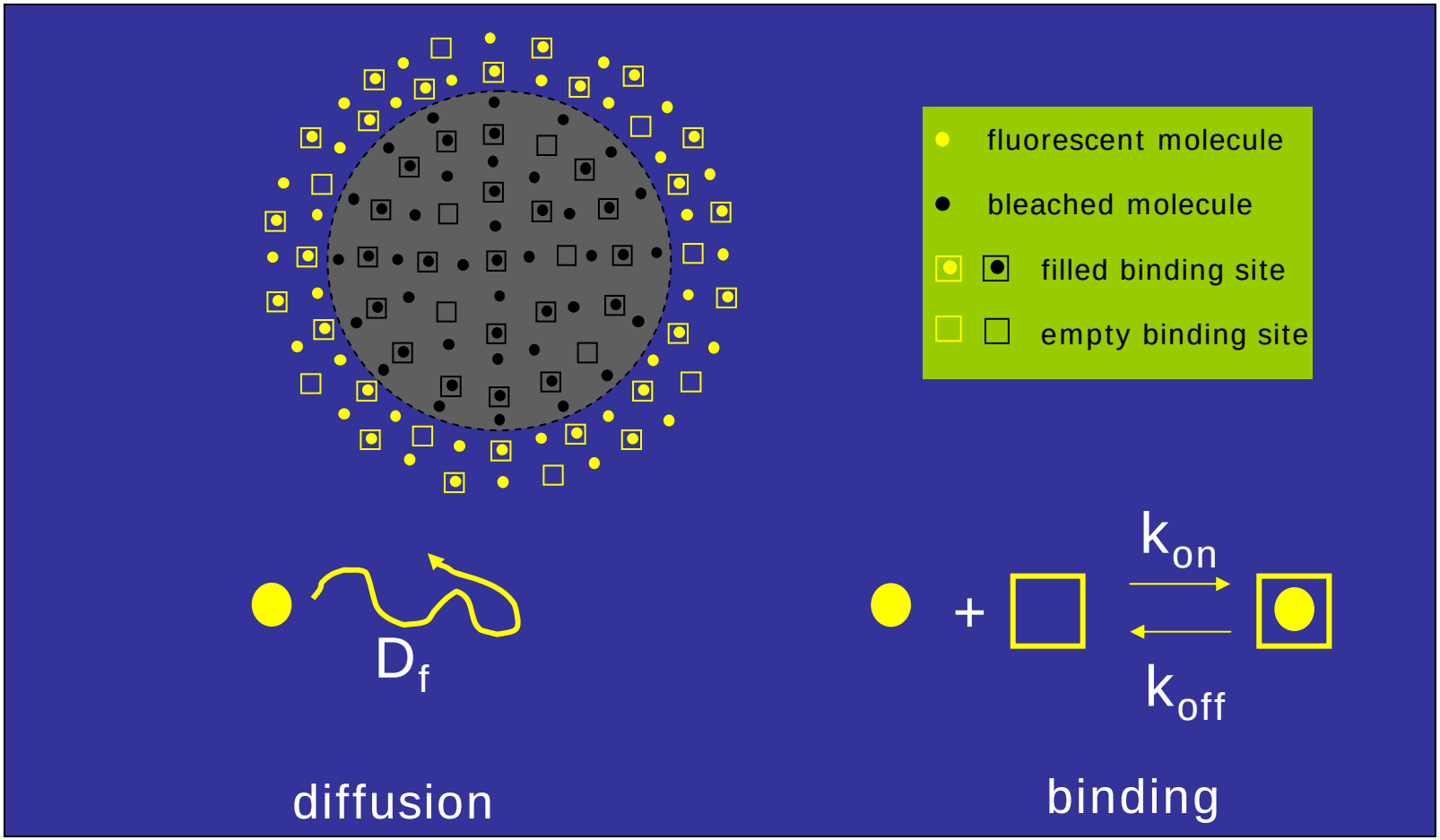
Analysis of GFP-GR binding

How does GFP-GR recover outside MMTV array?

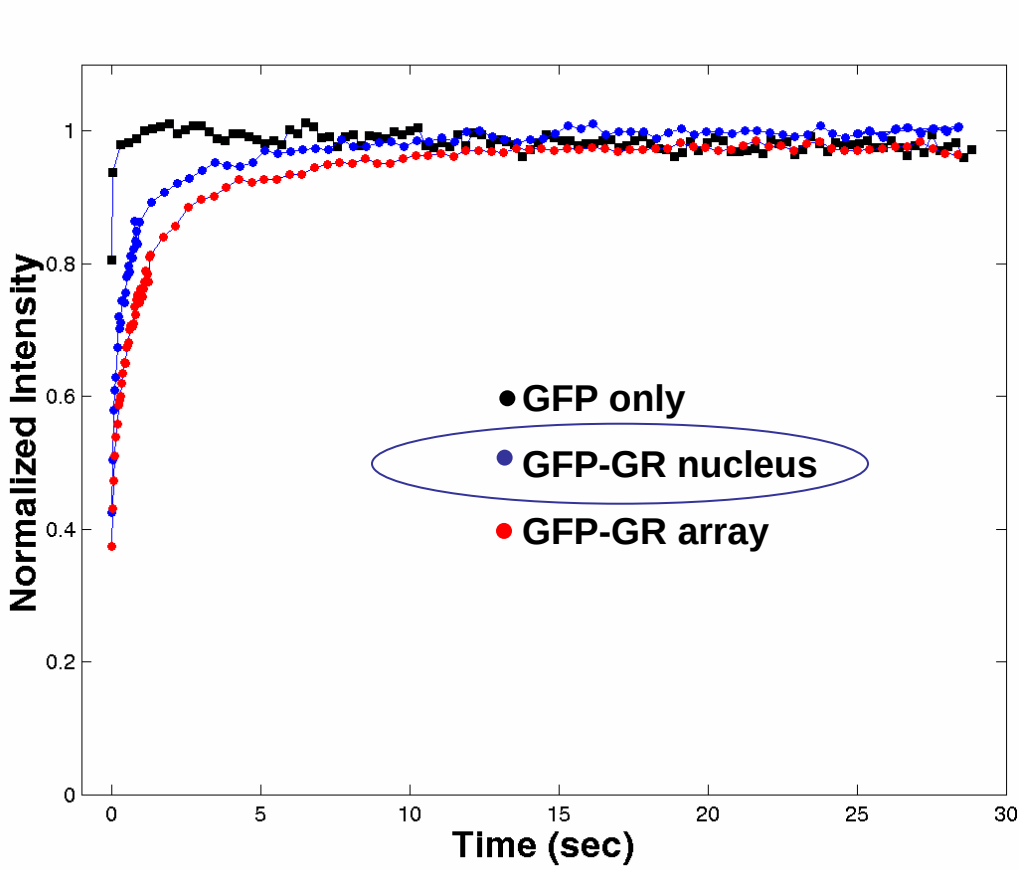
FRAP here



GFP-GR binding model

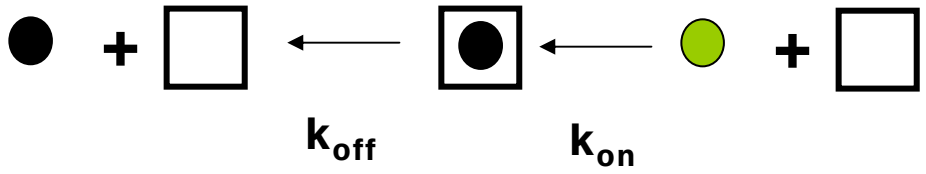


GFP-GR binding model

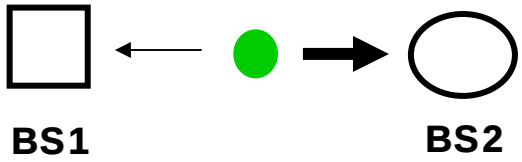


GFP-GR binding model

- Do slower recoveries imply longer residence times and/or slower on rates?



- Does the FRAP curve reflect diffusion plus one binding state, or two different binding states?



Need a comprehensive model for FRAP incorporating diffusion and multiple binding states.

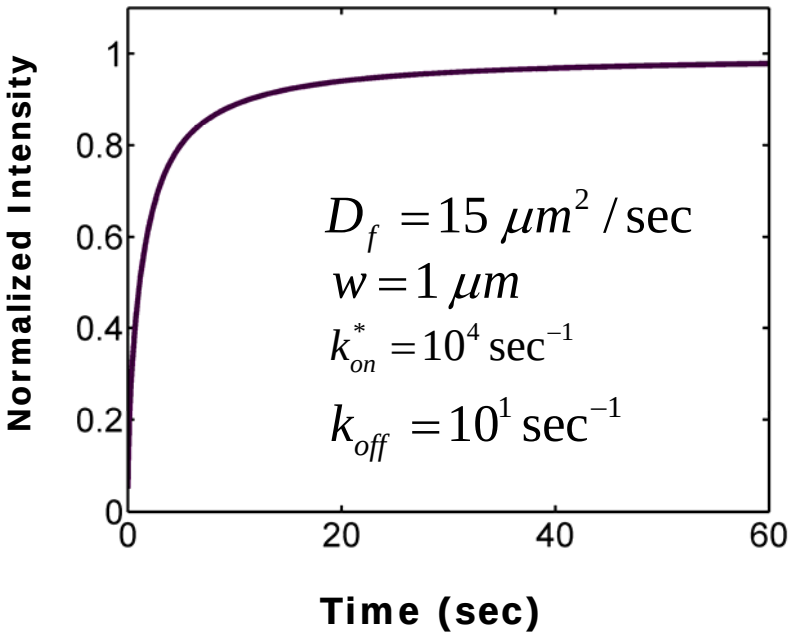
GFP-GR binding model

$$\frac{\partial f}{\partial t} = D_f \nabla^2 f - k_{on}^* f + k_{off} c$$

$$\frac{\partial c}{\partial t} = k_{on}^* f - k_{off} c$$

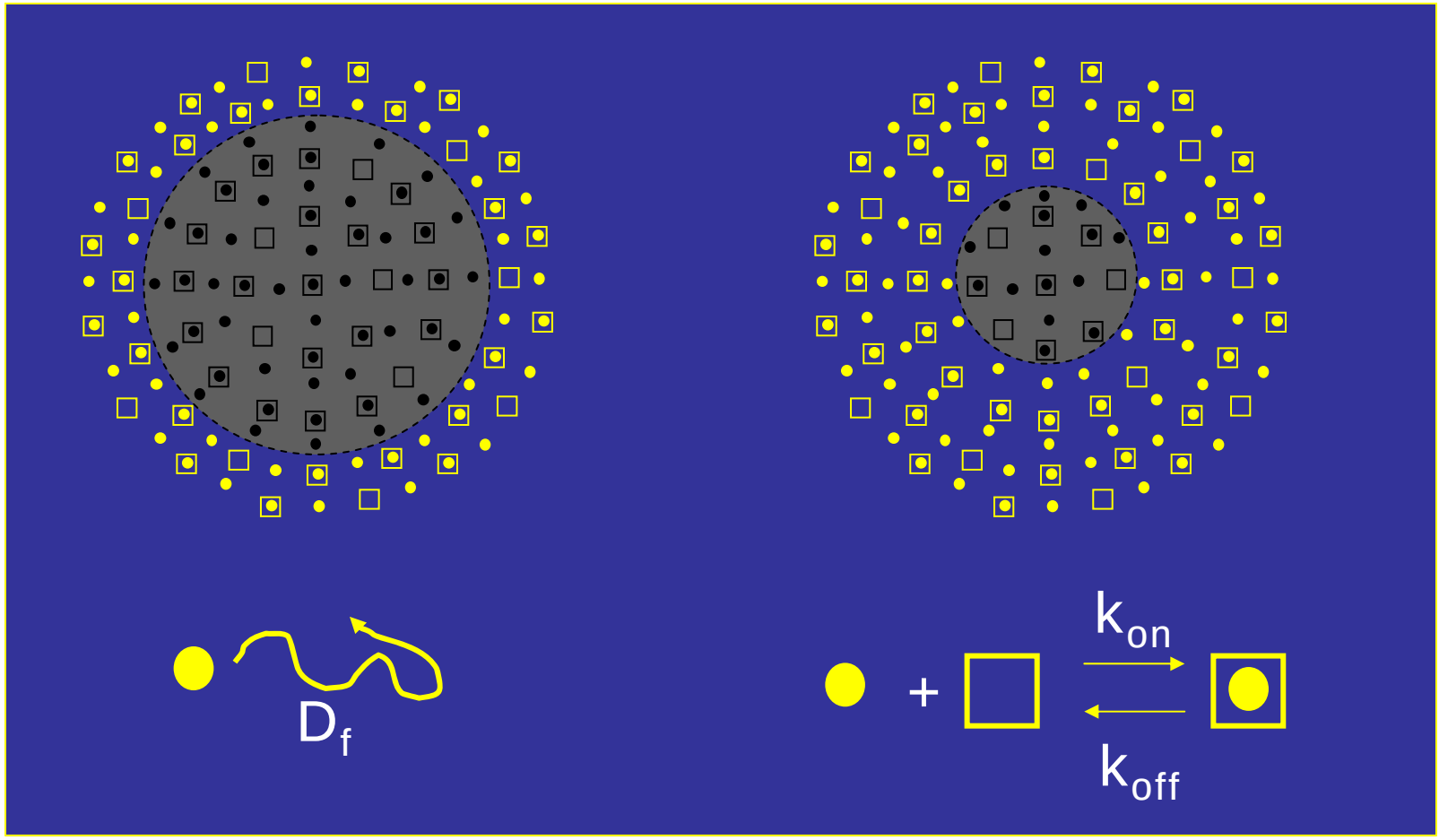
f = [free molecules]

c = [bound molecules]

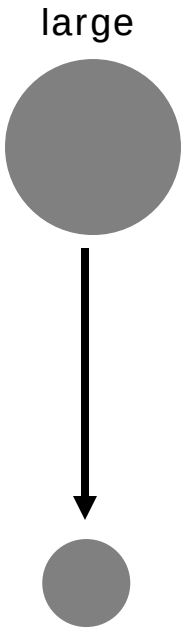


- Assuming that binding sites are immobile and that the system is at equilibrium
- The solution generates a FRAP recovery curve that depends on diffusion, binding and the bleach spot size

GFP-GR binding model



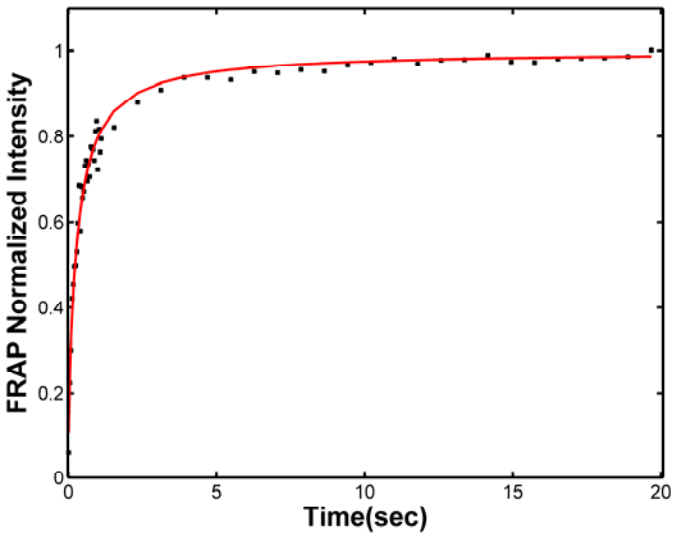
The full model solution reduces to other simplified equations depending on the bleach spot size



1. Effective diffusion (time to bind is short compared to time to diffuse across the spot)
2. Full model solution
3. Reaction dominant (time to bind is long compared to time to diffuse across the spot)

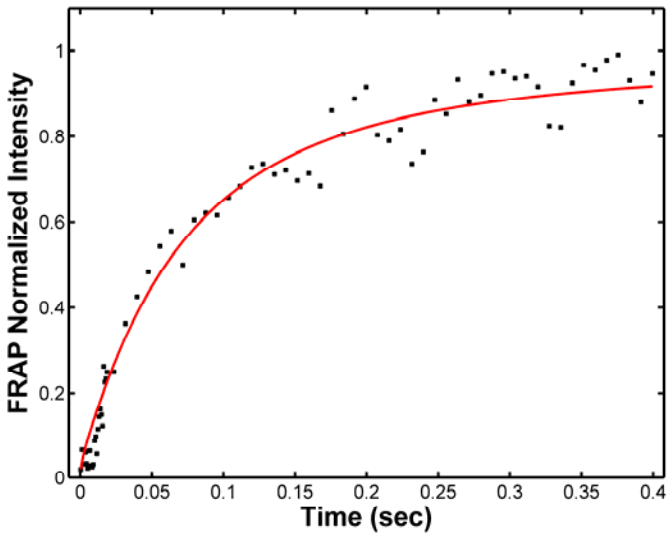
For larger bleach spots, GFP-GR FRAPs also are fit by effective diffusion

W = 1.0 mm



Effective diffusion fit

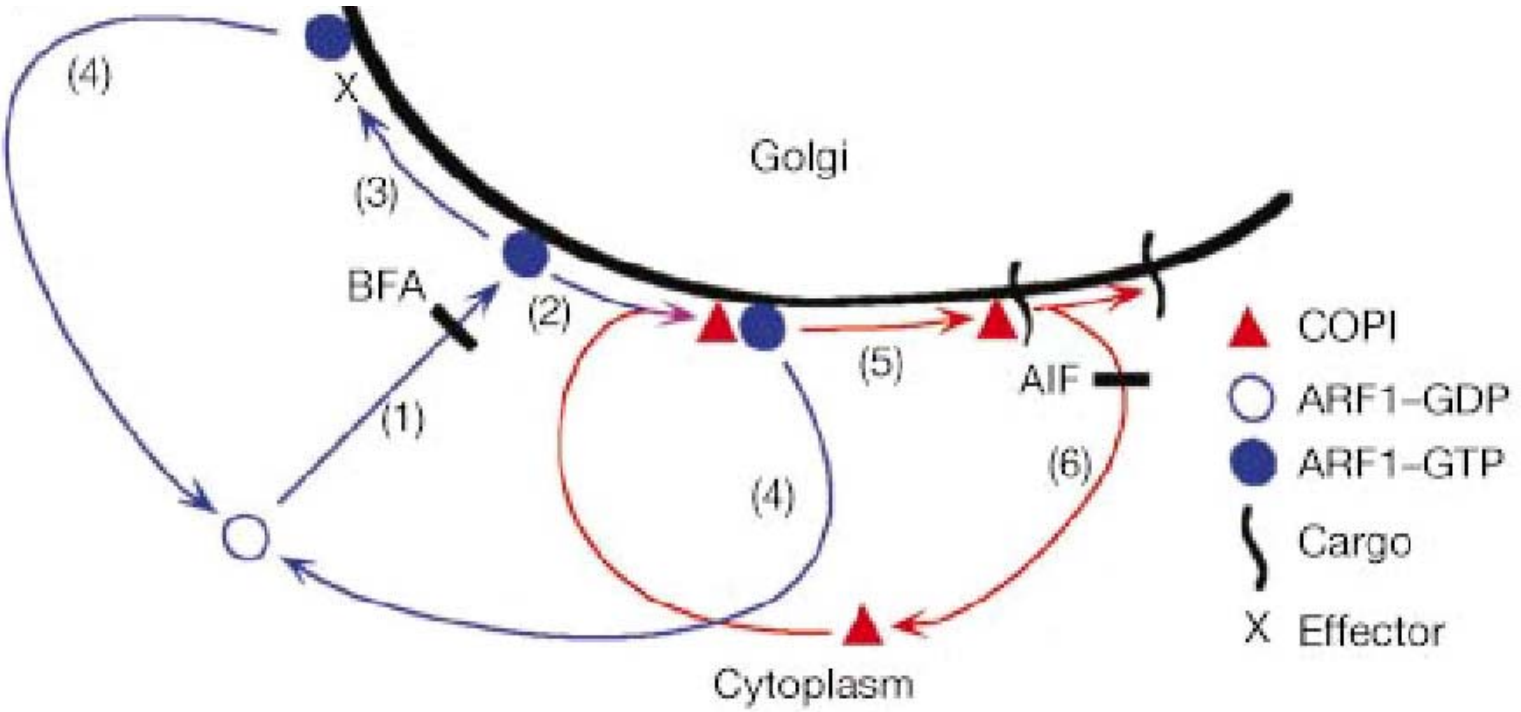
w = 0.4 mm



Full model fit

For smaller bleach spots, the full model yields a better fit, and therefore independent estimates of on and off rates

FRAP analysis can get very advanced



Presley JF *et al.* (2002) Nature.9;417(6885):187-93.

FRAP analysis can get very advanced

$$\frac{dfARF_g}{dt} = k_{gef}\ fARF_{cyto} - k_{ae}\ fARF \cdot effector - k_{ac}\ fARF_g \cdot COP_{cyto} - k_{bleachARF} \cdot fARF_g$$

$$\frac{dfARF_{effector}}{dt} = k_{ae}\ fARF_g \cdot effector - k_{gtpase1}\ fARF_{effector} - k_{bleachARF}\ fARF_{effector}$$

$$\frac{dfARF_{cop}}{dt} = k_{ac}\ fARF_g \cdot COP_{cyto} - k_{gtpase2}\ fARF_{cop} - k_{bleachARF}\ fARF_{cop}$$

$$\frac{dfARF_{cyto}}{dt} = k_{gtpase1}\ fARF_{effector} + k_{gtpase2}\ fARF_{cop} - k_{gef}\ fARF_{cyto}$$

$$\frac{dfARF_{exch}}{dt} = k_{fxarf}\ fARF_g + k_{rxarf}\ fARF_{exch} - k_{bleachARF}\ fARF_{exch}$$

$$\frac{dfARF_{cyto}}{dt} = k_{uncoat}\ fARF_{Xg} + k_{ac}\ fARF_g \cdot fCOP_{cyto}$$

$$\frac{dfCOP_{Xg}}{dt} = k_{gtpase2}\ ARF_{fCOP} - k_{uncoat}\ fCOP_{Xg} - k_{bleachCOP}\ fCOP_{Xg} - k_{fexch}\ fCOP_{Xg} + k_{rexch}\ fCOP_{exch}$$

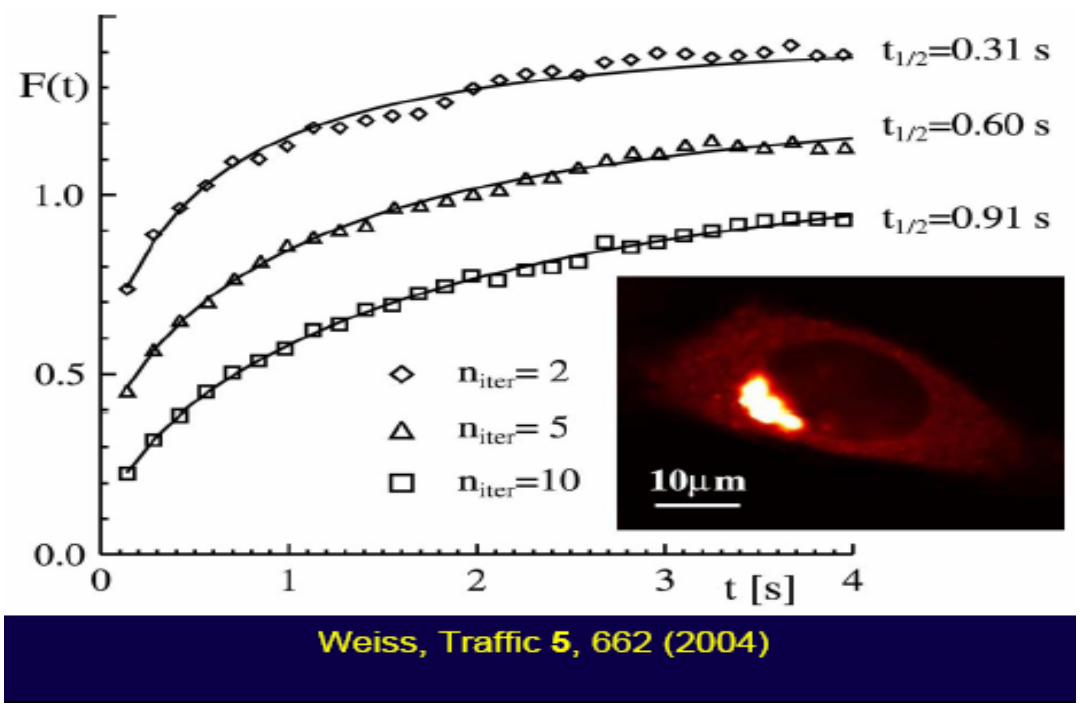
$$\frac{dfARF_{fCOP}}{dt} = k_{ac}\ ARF_g \cdot fCOP_{cyto} - k_{gtpase2}\ ARF_{fCOP} - k_{bleachCOP}\ ARF_{fCOP}$$

$$\frac{dfCOP_{exch}}{dt} = k_{fexch}\ fCOP_{Xg} - k_{rexch}\ fCOP_{exch} - k_{bleachCOP}\ fCOP_{exch}$$

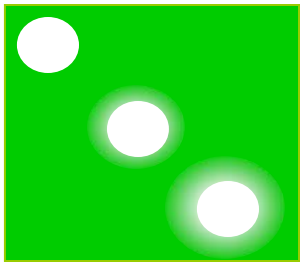
Presley JF *et al.* (2002) Nature.9;417(6885):187-93.

What can complicate a FRAP experiment?

- Bleach protocol
  - Time of bleach pulse and intensity of bleach pulse

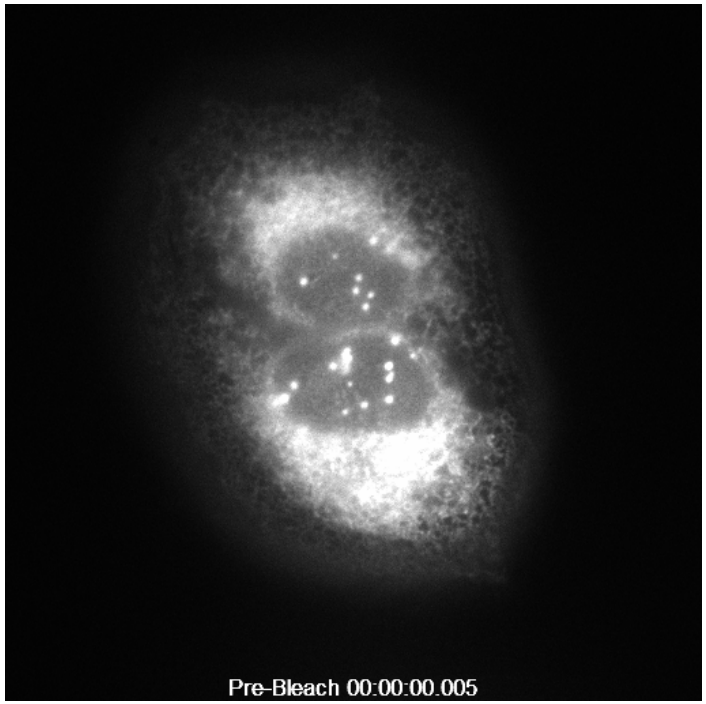


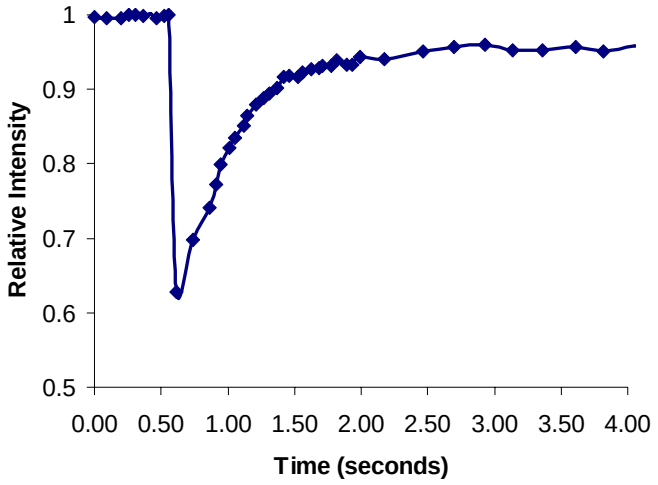
Weiss, Traffic **5**, 662 (2004)



What can complicate a FRAP experiment?

- Time of first recovery image



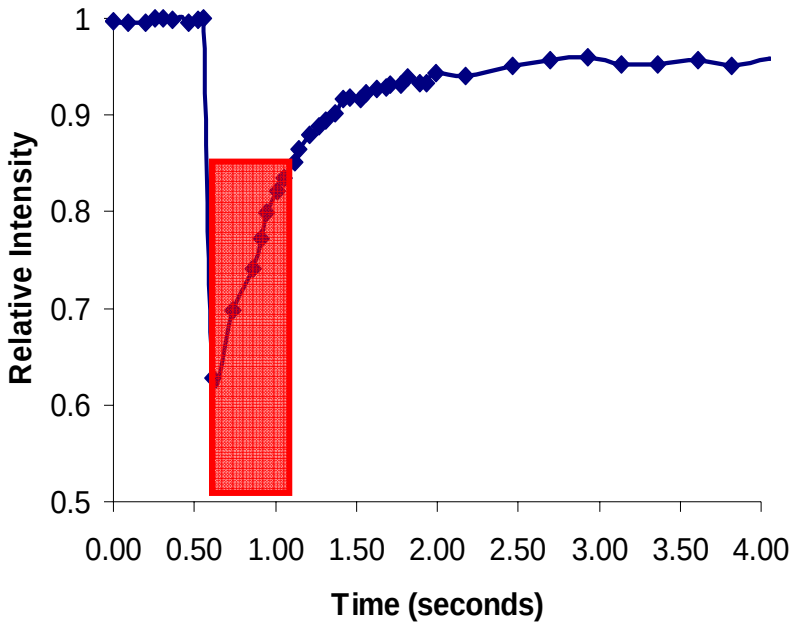


- mEmerald targeted to the endoplasmic reticulum
- Recovery complete within ~ 1 second of bleach

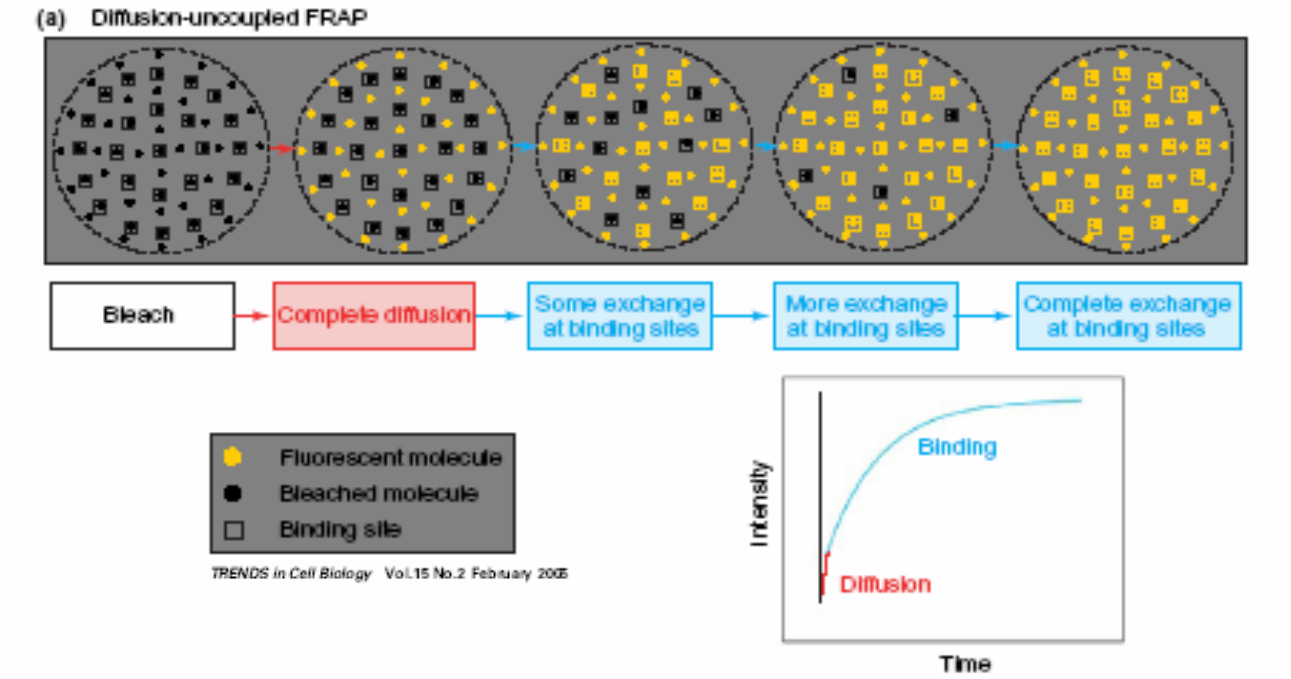
What can complicate a FRAP experiment?

Why does speed matter?
(What am I missing?)

- Image and quantify the rapid diffusion events
  - If first post bleach image took ~ 500 msec to happen, you would miss most of the recovery

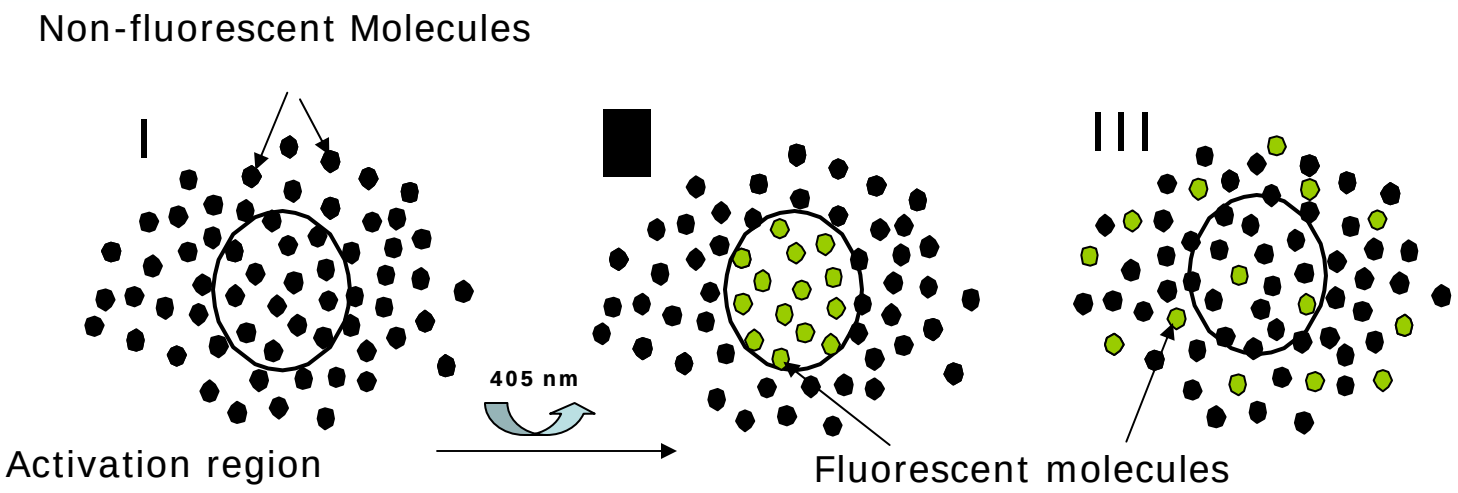


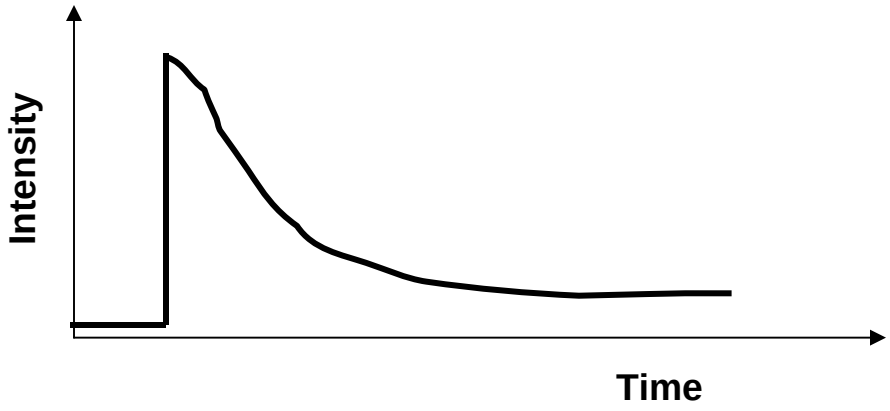
What can complicate a FRAP experiment?



- The recovery curve of many proteins have two components… a rapid diffusion component and a slower binding component.
  - Being able to quantify the initial diffusion component is critical for proper analysis

Photoactivation





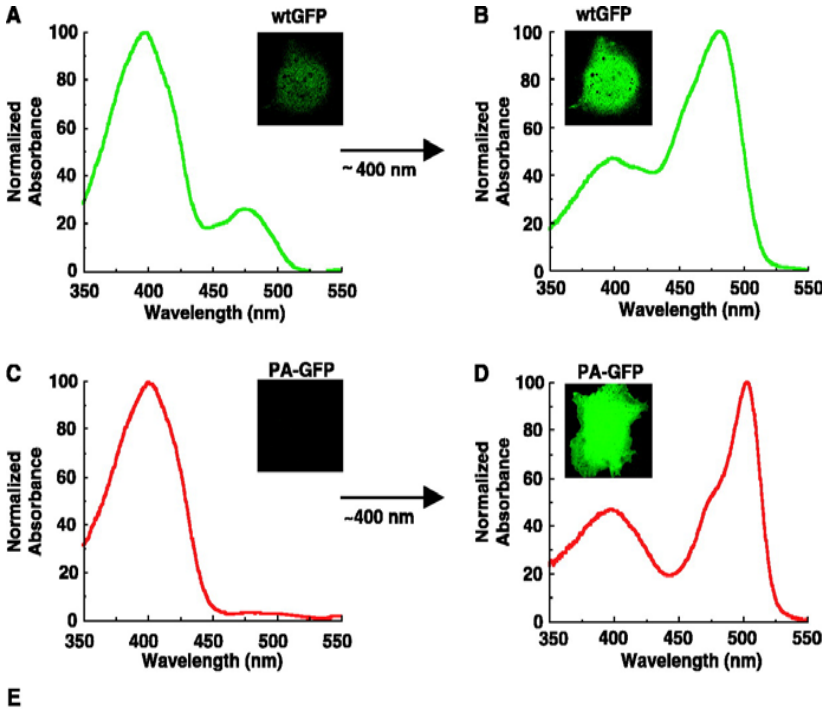
PA-GFP

Dronpa

Kindling Fluorescent Protein

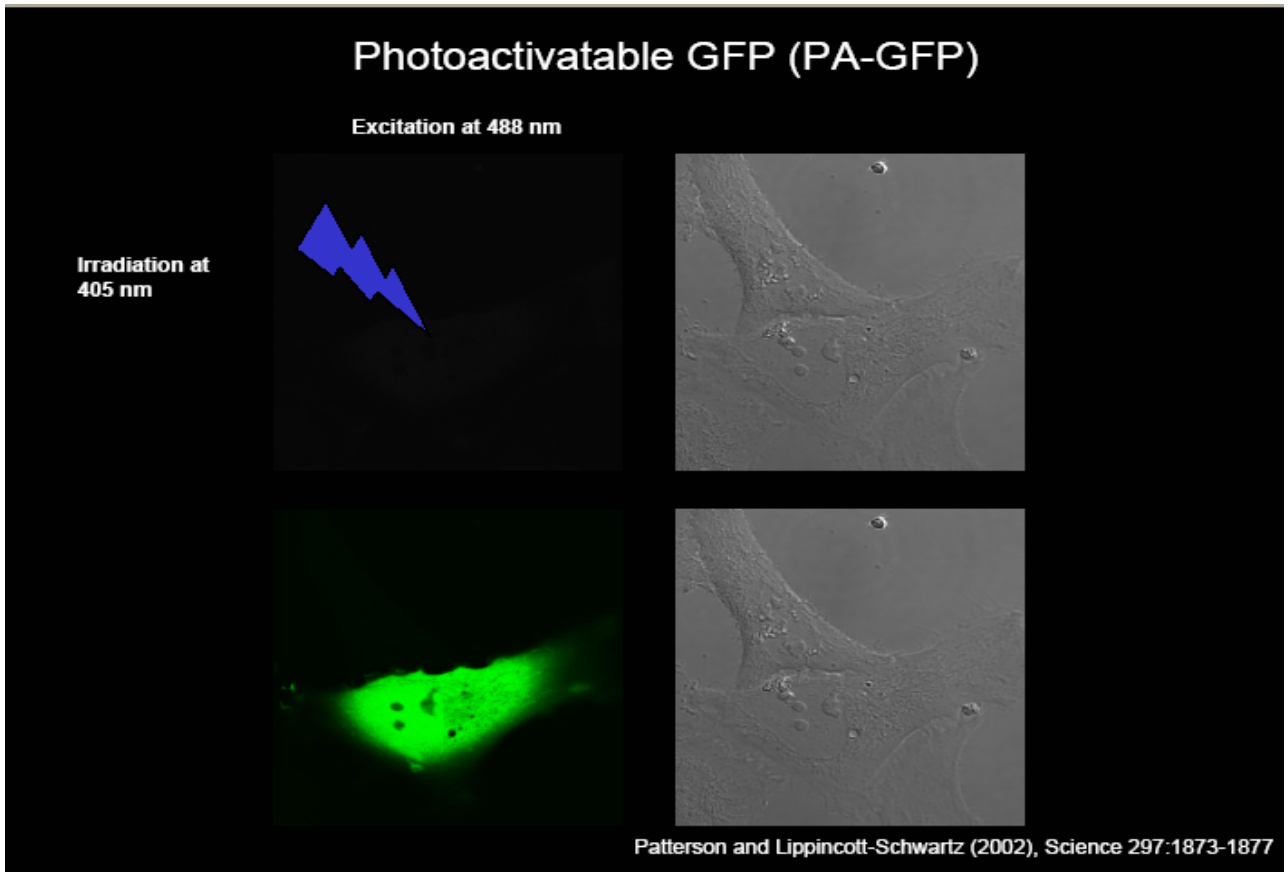
PA-GFP

- Photoactivatable GFP
  - Derivative of wtGFP
  - Essentially nonfluorescent before activation when illuminated at 488
  - After activation with violet light, strong fluorescence is seen when illuminated with 488

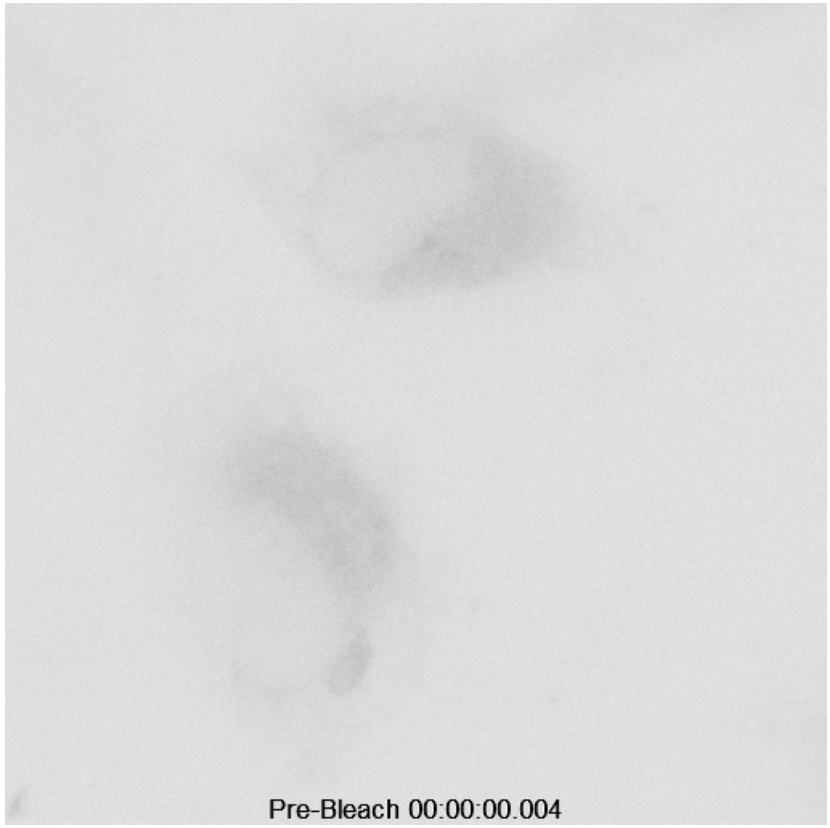


J. Lippincott-Schwartz et al., Science 300, 87 -91 (2003)

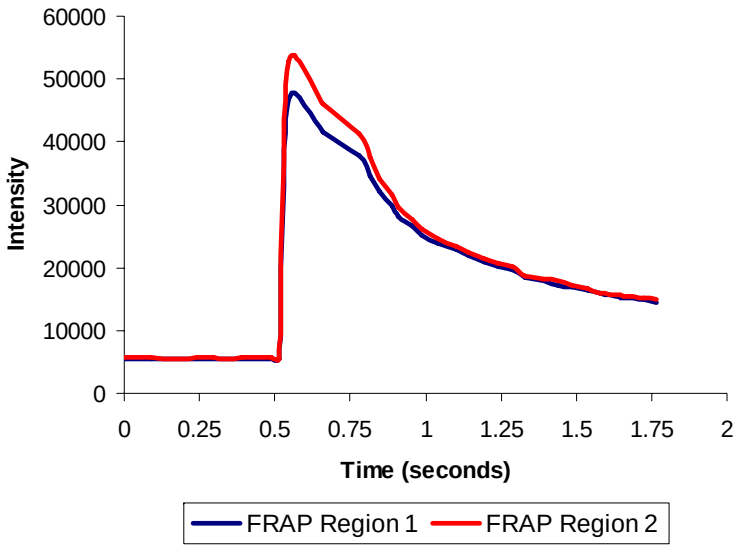
PA-GFP


Photoactivatable GFP (PA-GFP)
Excitation at 488 nm
Irradiation at 405 nm
Patterson and Lippincott-Schwartz (2002), Science 297:1873-1877

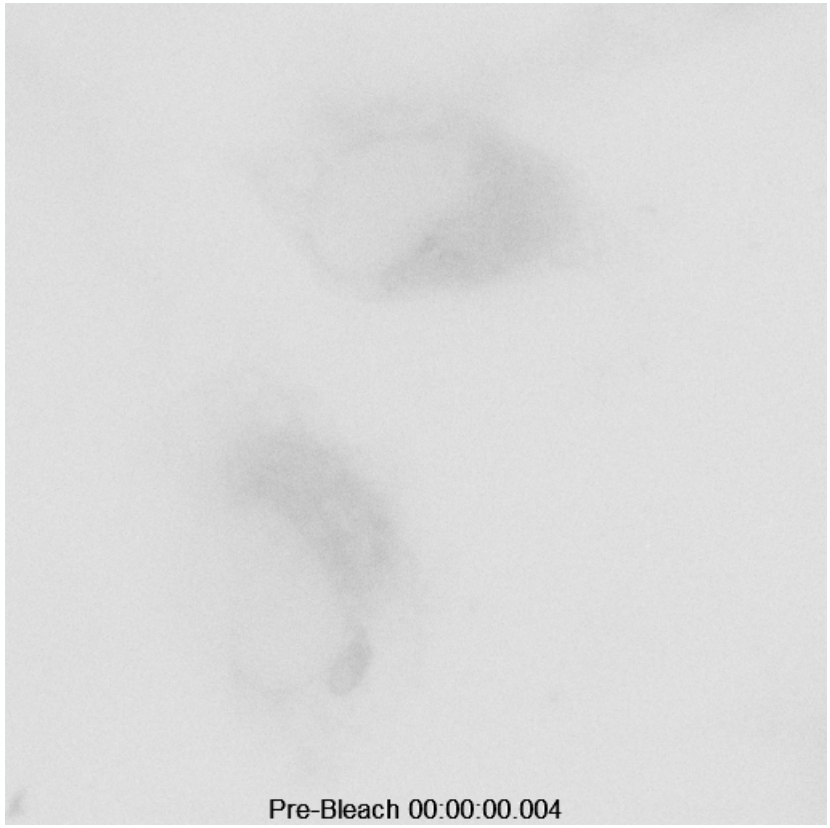
PA-GFP



- PA-GFP labelled targeted to endoplasmic reticulum
- Data shown with an inverted LUT for better contrast
- Activation with 405 nm laser



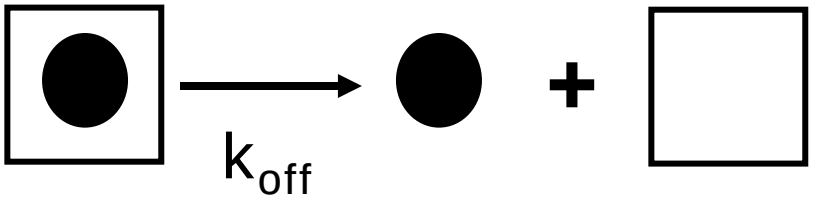
PA-GFP



The loss of signal at the activated region is only due to the k_{off} of the activated protein and diffusion

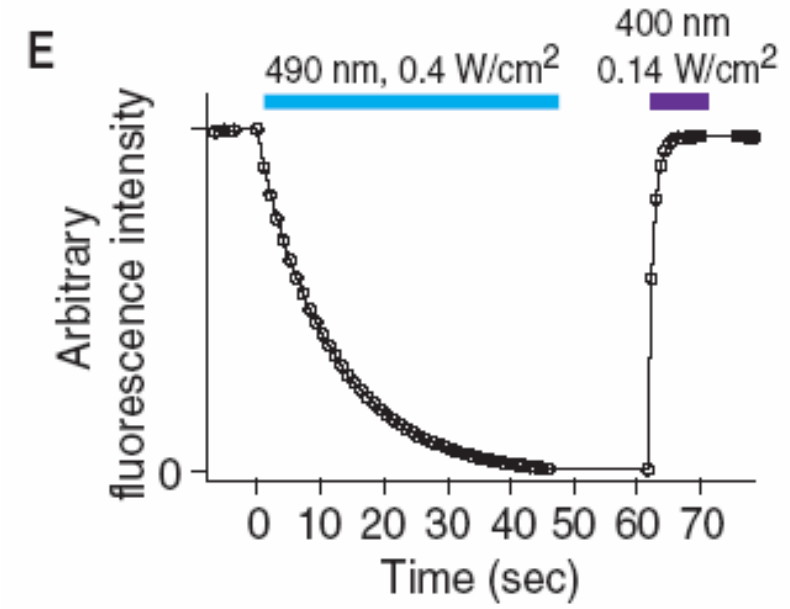
Similar results as iFRAP or inverse FRAP where you bleach the entire cell except for a specific ROI

Excellent complement to FRAP experiments



Dronpa

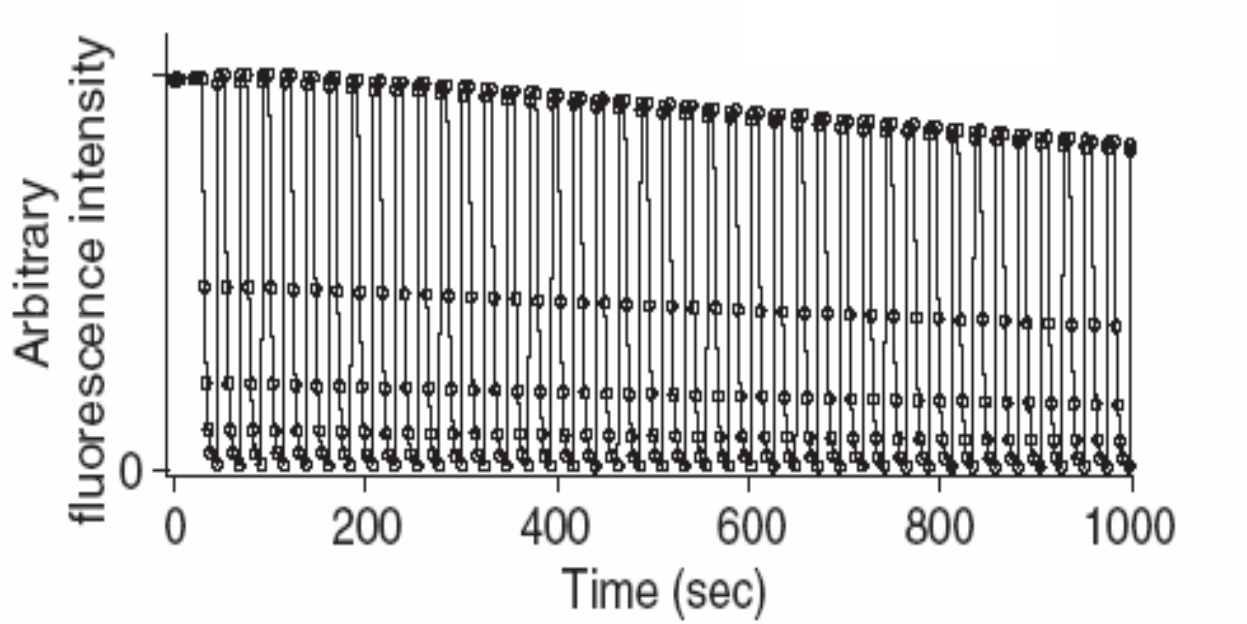
- Fluorescence switch
- Activated by 405 nm
- Turned off (erased) after bright exposure of 488



Ando et al., 2004 Science Vol 306

Dronpa

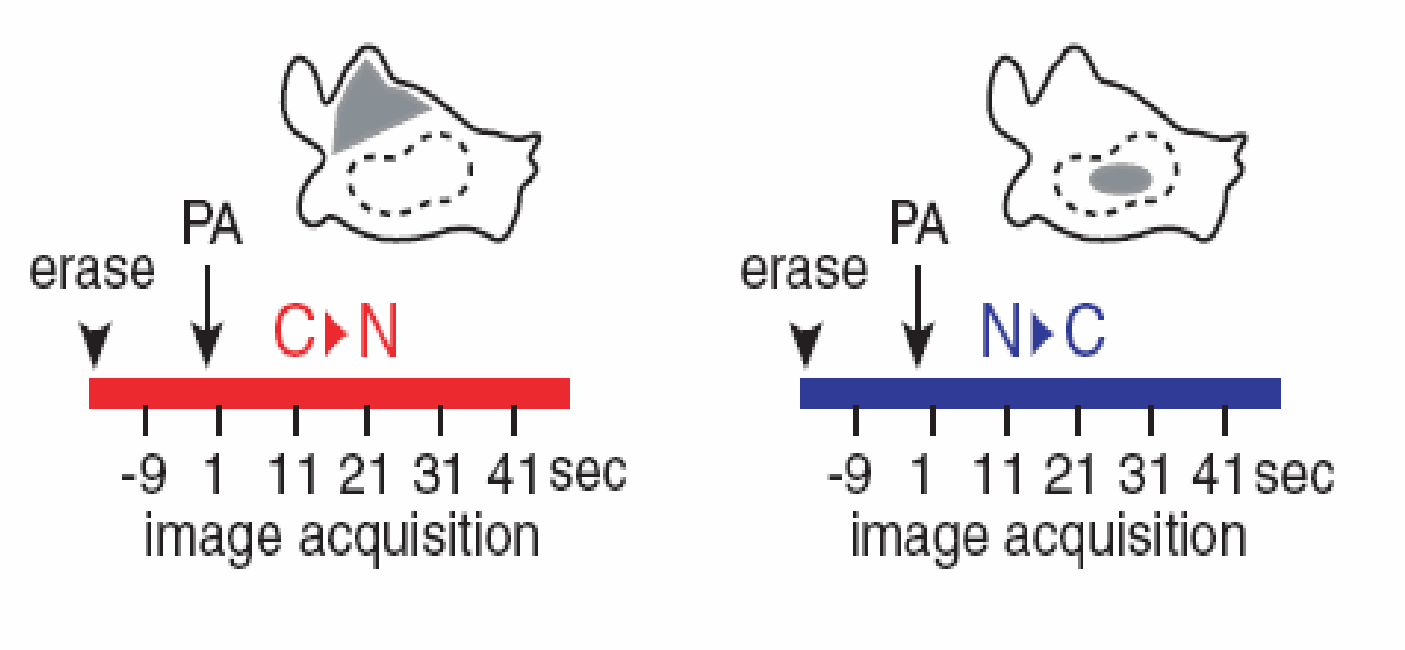
- Can be turned on and off 100's of times



Ando et al., 2004 Science Vol 306

Dronpa in the study of nuclear/cytoplasmic shuttling

- Ando et al., 2004 Science Vol 306
  - Turned Dronpa on to visualize cells and identify regions of interest
  - “Erase” Dronpa with strong blue light
  - Activate small region of either cell or nucleus with 405 nm light
  - Watch change in intracellular distribution over time



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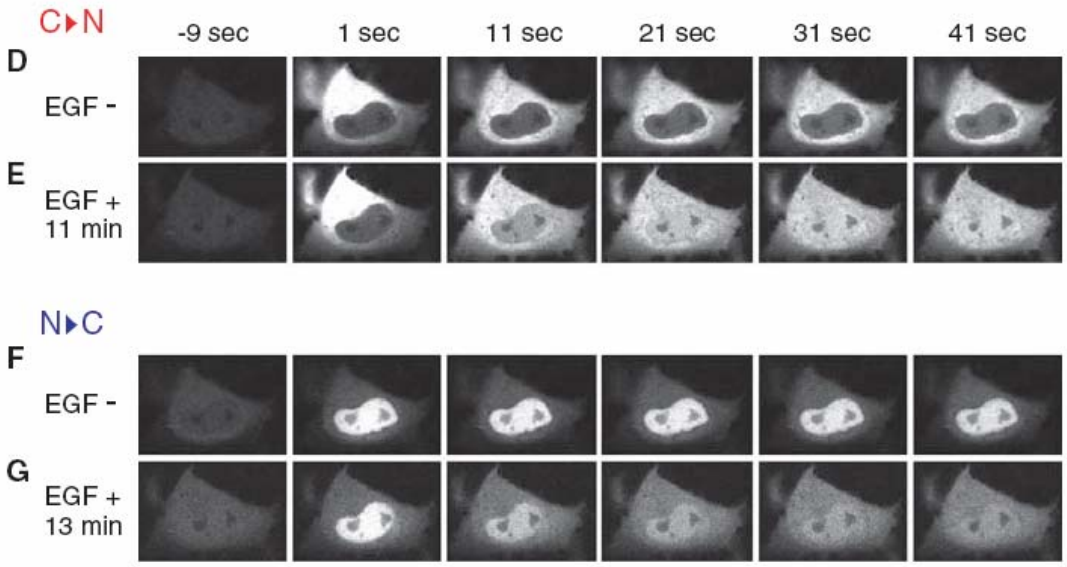
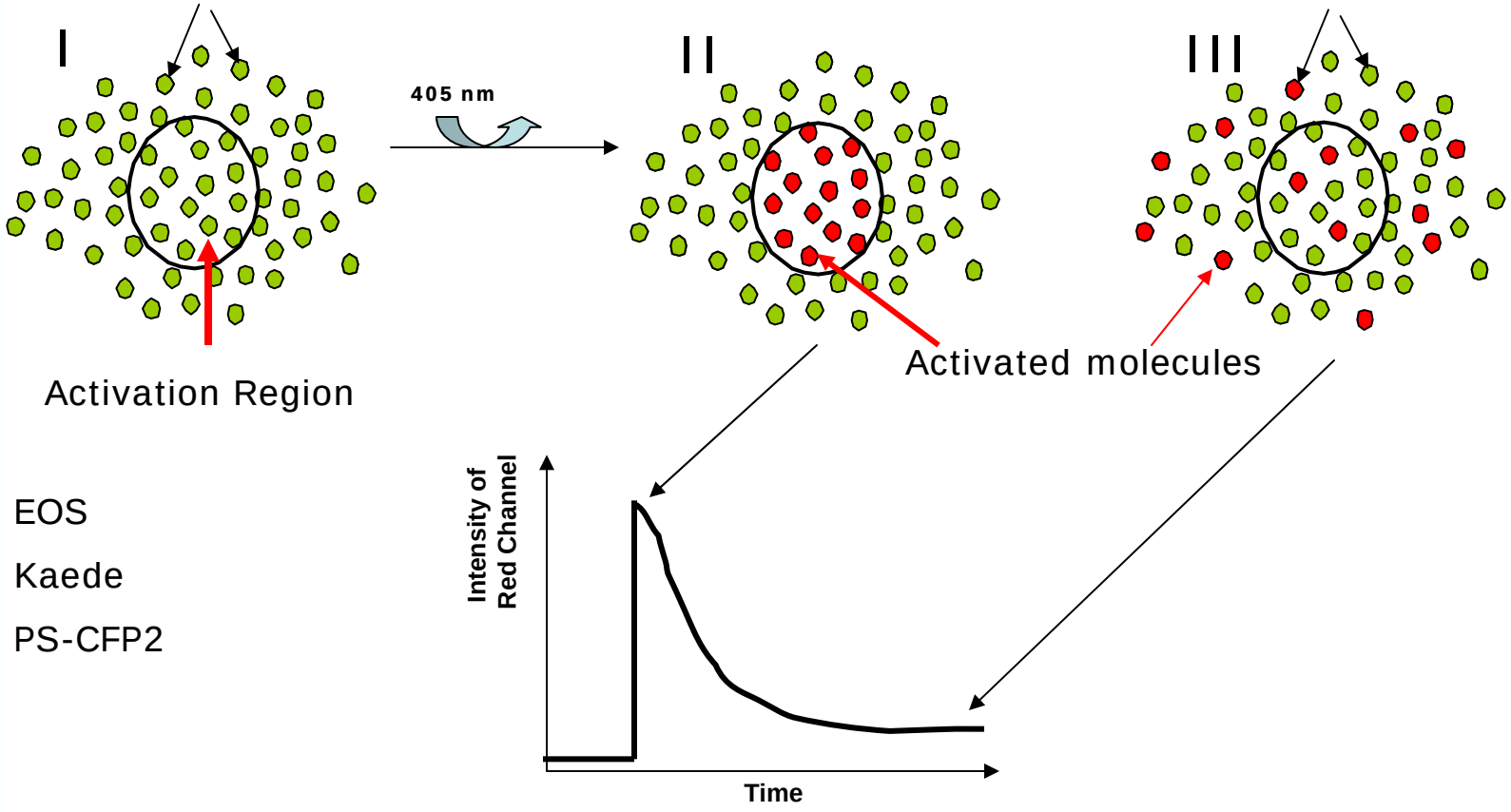


Photoconversion

Unconverted Molecules



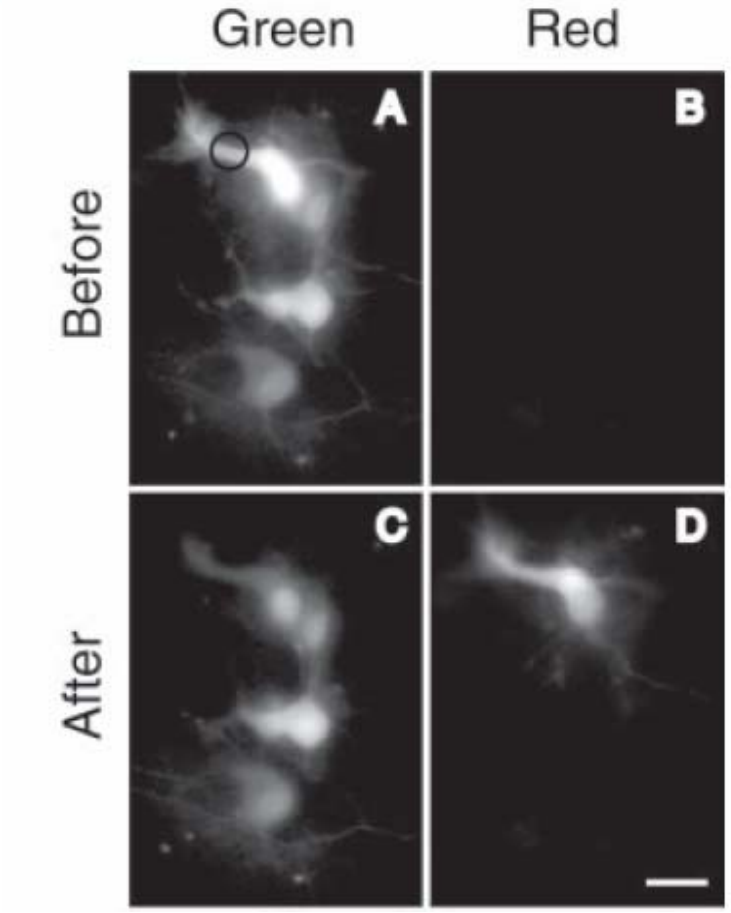
EOS

Kaede

PS-CFP2

Kaede

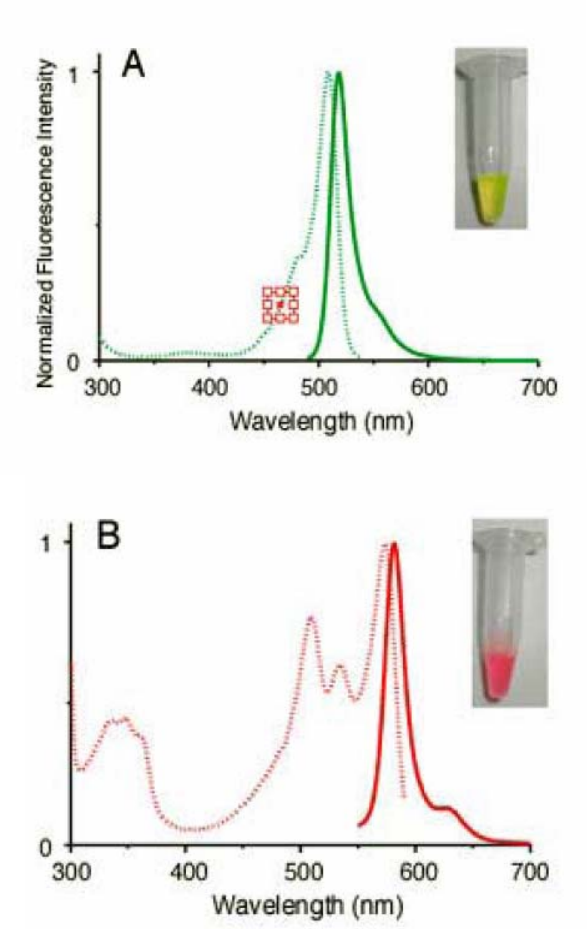
- Isolated by the Miyawaki lab from a coral purchased in a petshop (**Trachyphyllia geoffroyi**)
- Strong green fluorescence prior to conversion
- After illumination with violet light, the green fluorescence decreases and a red fluorescence is deveveloped



Ando et al., PNAS 2002

Kaede

- The photoconversion properties of Kaede were discovered by accident.
- When originally isolated, Kaede was thought to have only green fluorescecne
- A tube of the purified protein was left on the lab bench over night and the next day the protein was red.
- Determined that the conversion was a result of the UV irradiation from the sunlight coming through the windows



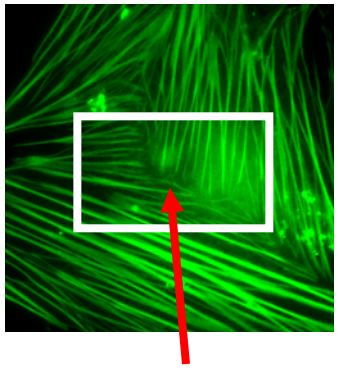
FRAP 3D

EOS

MAG BIOSYSTEMS

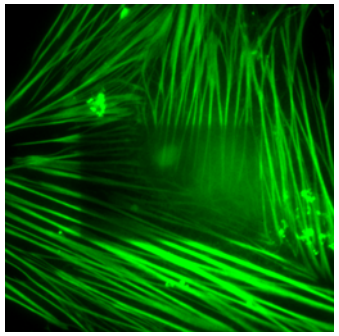
tdEOS-actin in fixed cells

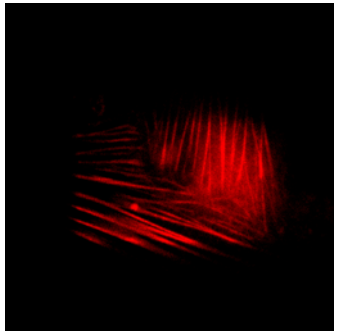
Pre-Activation



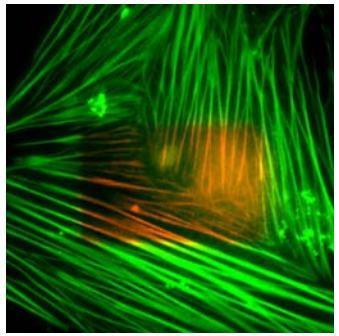
Area illuminated With 405 nm laser

Post-Activation





Overlay



Application with photoactivatable and photoconvertable proteins

Protein tracking

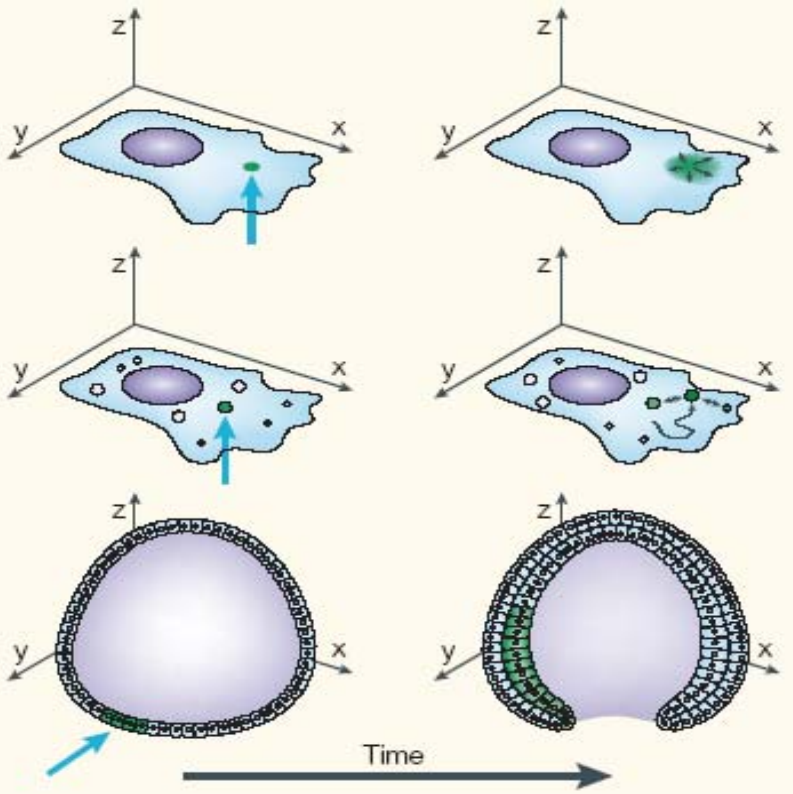
Parameters determined:
- Movement rate and direction
- Diffusion coefficient
- Mobile and immobile fractions
- Time parameters of compartmental residency and exchange between compartments
- Rate of turnover

Organelle tracking

Parameters determined:
- Movement rate and direction
- Rate of content interchange
- Fission and fusion events

Cell tracking

Parameters determined:
- Movement rate and direction
- Cell localization
- Rate of cell division
- Shape and volume of cells



Lukyanov et al., Nature Reviews, MCB 6, Nov 2005