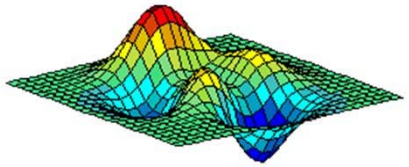

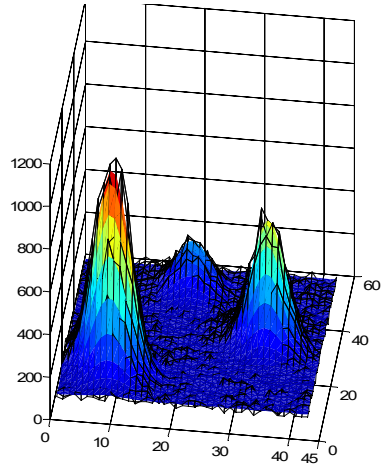

Diffusion in the cell membrane and its investigation by FRAP

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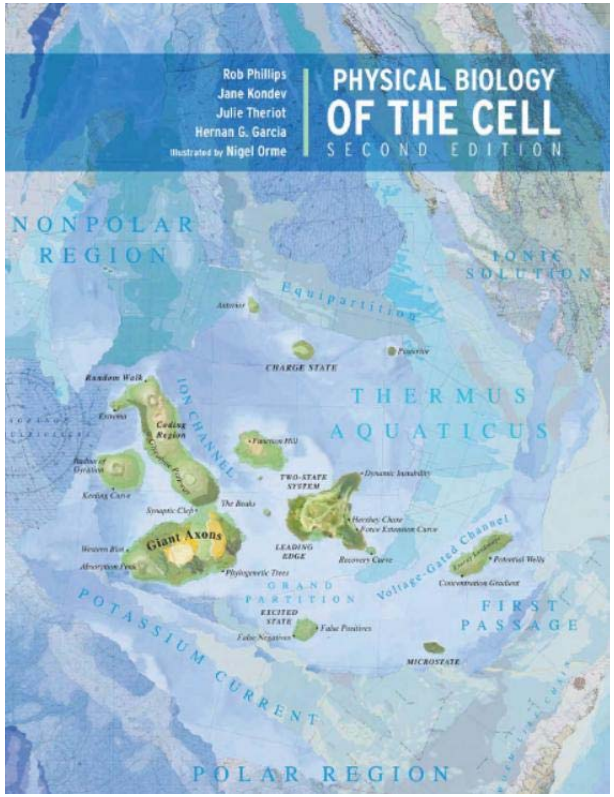
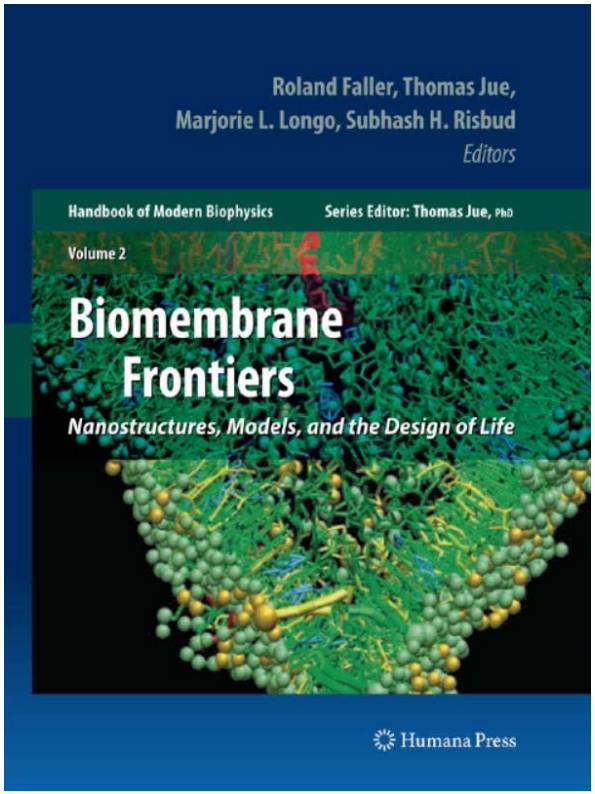
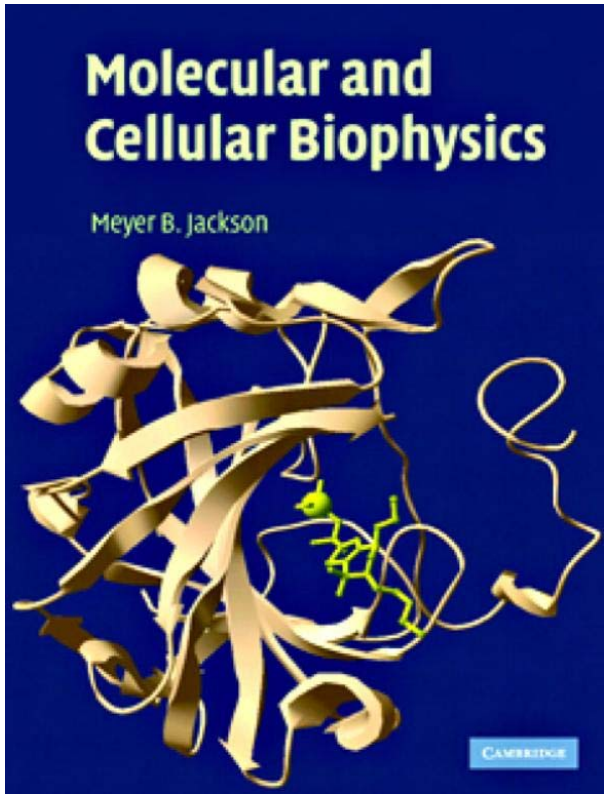


Physical Biology of the Cell
Rob Phillips, Jane Kondev, Julie Theriot, Hernan G. Garcia
Garland Science
978-0-8153-4450-6



Handbook of Modern Biophysics
Vol. 2, Biomembrane Frontiers
Roland Faller, Thomas Jue, Marjorie L. Longo, Subhash H. Risbud
Humana Press
978-1-60761-313-8



Molecular and Cellular Biophysics
Meyer B. Jackson
Cambridge University Press
978-0-521-62441-1

Aim

- How can such a simple process as diffusion play a role in a complex system like a eukaryotic cell (membrane) ?
- Isn’t diffusion too trivial to study nowadays?
- How to measure diffusion in a simple way?

Outline

- Diffusion in living systems
- Diffusion in membranes
- Clusters and crowding in membranes

} biophysics of living systems

- Basic principles of FRAP (fluorescence recovery after photobleaching)

} the method

Diffusion in the intra- and extracellular space

Diffusion is an important means of transportation in biological systems, but it has limitations

$$\left\langle \Delta r^2 \right\rangle = \left\langle \Delta x^2 \right\rangle + \left\langle \Delta y^2 \right\rangle + \left\langle \Delta z^2 \right\rangle = 6Dt \quad \Rightarrow \quad \sqrt{\left\langle \Delta r^2 \right\rangle} \sim \sqrt{t}$$

physical limitation 1

$$D = \frac{kT}{f} \Rightarrow D \sim \frac{1}{f} \sim \frac{1}{\sqrt[3]{MW}}$$

k – Boltzmann constant
T – absolute temperature
f – form factor
MW – molecular weight

physical limitation 2 **(weak)**

	glycine (MW=75)	glucose (MW=180)	ordinary (40 kDa) protein
D (m^2/s)	10^{-9}	$5 \cdot 10^{-10}$	10^{-10}
D (cm^2/s)	10^{-5}	$5 \cdot 10^{-6}$	10^{-6}
D ($\mu m^2/s$)	10^{3}	$5 \cdot 10^{2}$	10^{2}

approx. 1000x difference in MW

approx. $\sqrt[3]{1000} = 10$x difference in D

due to physical limitation 2

distance travelled	time of diffusion (sec)		
	protein	glucose	glycine
1 μm	0.0017	0.00033	0.00017
10 μm (size of a eukaryotic cell)	0.17	0.033	0.017
100 μm (max. distance of cells from capillaries)	16.7	3.3	1.7
1 mm	1667	333	166.7
1 cm	166667	33333	16667

Based on the above

- diffusion is an efficient way to transport molecules (up to ~100 μm).
- it is not efficient for transport to larger distances.

BUT: there are other, biological limitations (see next slide) → the diffusion of **proteins** is significantly **hindered or confined** in the cell

Diffusion in the intra- and extracellular space: filtering effect of the cytoplasm

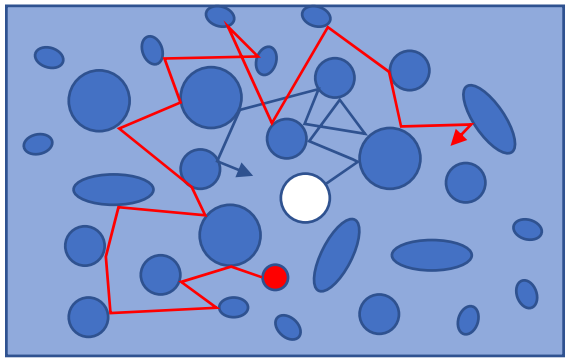
Finding: the diffusion of proteins declines steeply with their molecular mass → large proteins (300-500 kDa) basically stop diffusing.

The rate of diffusion is further decreased by:

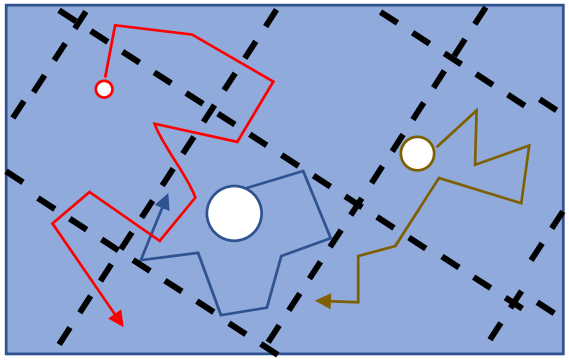
biological limitations

- molecular weight dependent (**static**) filtering effect of the cytosolic matrix with a pore size of ~50 nm
- **dynamic** filtering effect of macromolecules (see below)
- specific interaction of proteins with the cytoskeleton (or DNA in the nucleus)

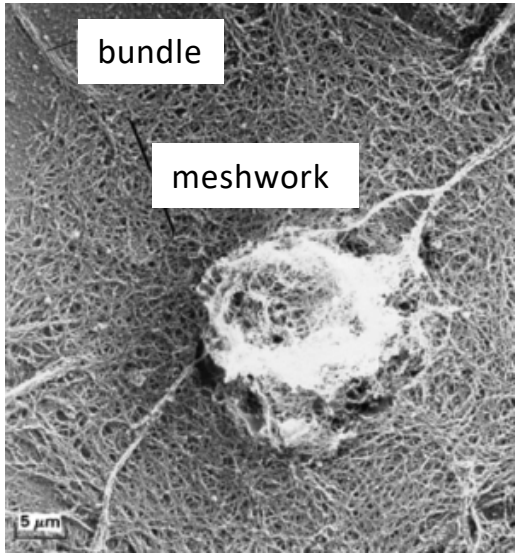
High concentration of dissolved macromolecules can also create a **dynamic** barrier.



effect of the cytoskeleton: besides the above dynamic barrier a **static** fence is also created by the cytoskeleton.



completely locked | sometimes locked | completely free



Structure behind static filtering

Intracellular transport of	
proteins	**small molecules (e.g. glucose)**
diffusion is only efficient for short-range transport (up to ~1 µm)	diffusion is efficient for transport throughout the cell (up to ~100 µm)
cytoskeleton-mediated directed transport for transport of proteins throughout the cell	

Factors limiting diffusion in 2D and 3D (physical limitation 2)

Diffusion in 3D

Stokes-Einstein-Smoluchowski equation (1905)

$$D = \frac{kT}{f} = \frac{kT}{6\pi\eta r}$$

r – radius of the protein „sphere”
η – viscosity of the medium

$$D \sim \frac{1}{r} \sim \frac{1}{\sqrt[3]{MW}}$$

- strong dependence on size (diameter)
- weak dependence on mass

Diffusion in the membrane (2D)

Saffman-Delbrück equation (1975)

$$D = \frac{kT}{4\pi\eta_m h}\left(\ln\frac{\eta_m h}{\eta_w a} - 0.5772\right)$$

h – membrane thickness
a – radius of the protein „cylinder”
η_m – viscosity of the membrane
η_w – viscosity of the aqueous medium

D is insensitive to a and η_w

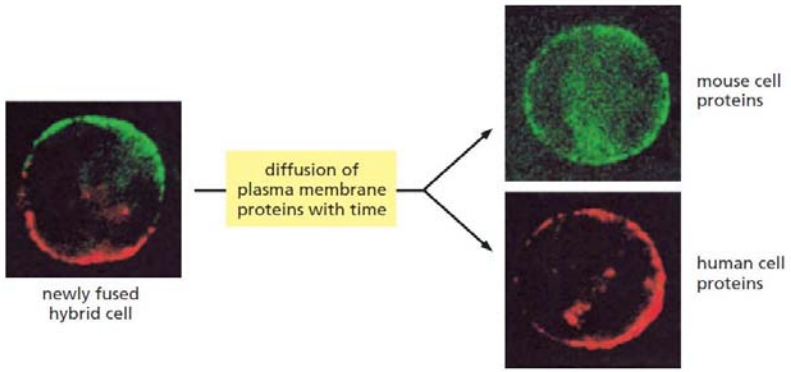
- 10× difference in diameter → 2× difference in D
- 12× difference in η_w → 2× difference in D

- weak dependence on size (diameter)
- strong dependence on membrane viscosity

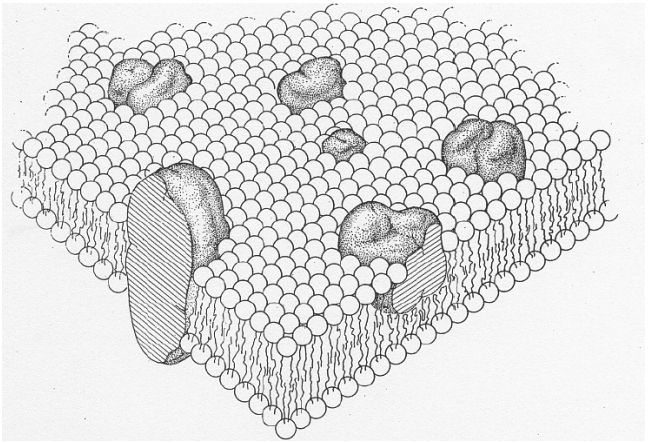
- Saffman, P. G. & Delbruck, M. Brownian motion in biological membranes. *Proc Natl Acad Sci U S A* **72**, 3111-3113 (1975).
- Einstein, Albert (1905). "Über die von der molekularkinetischen Theorie der Wärme geforderte Bewegung von in ruhenden Flüssigkeiten suspendierten Teilchen". Annalen der Physik. 17 (8): 549–560. doi:10.1002/andp.19053220806

Structure of the cell membrane: the fluid-mosaic model

Our view on the cell membrane was dominated for about three decades by the Frye-Edidin experiment (J Cell Sci. (1970) 7:319-35: The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons), and the Singer-Nicolson model based on it (Science (1972) 175:720-731)



Frye-Edidin experiment

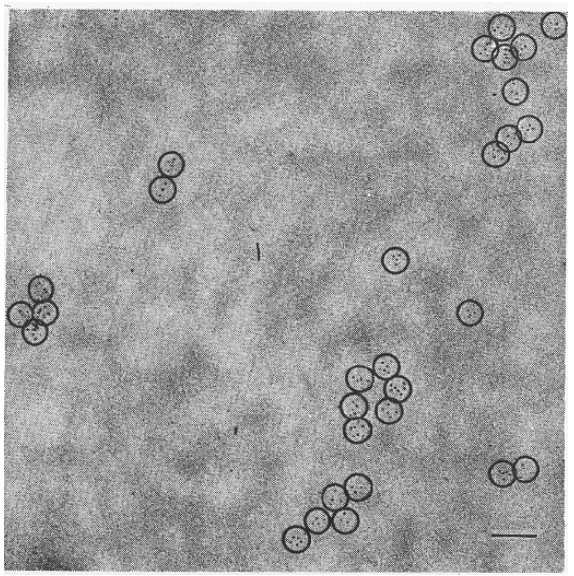


Singer-Nicolson model

Punch lines of the fluid-mosaic model (according to textbooks):

- structureless ocean of lipids
- in which proteins randomly diffuse around

… although Singer and Nicolson emphasized that some sort of order does exist in the lateral organization of the membrane (“ferritin appears bound to the membrane in discrete clusters 2-8 ferritin conjugates”)



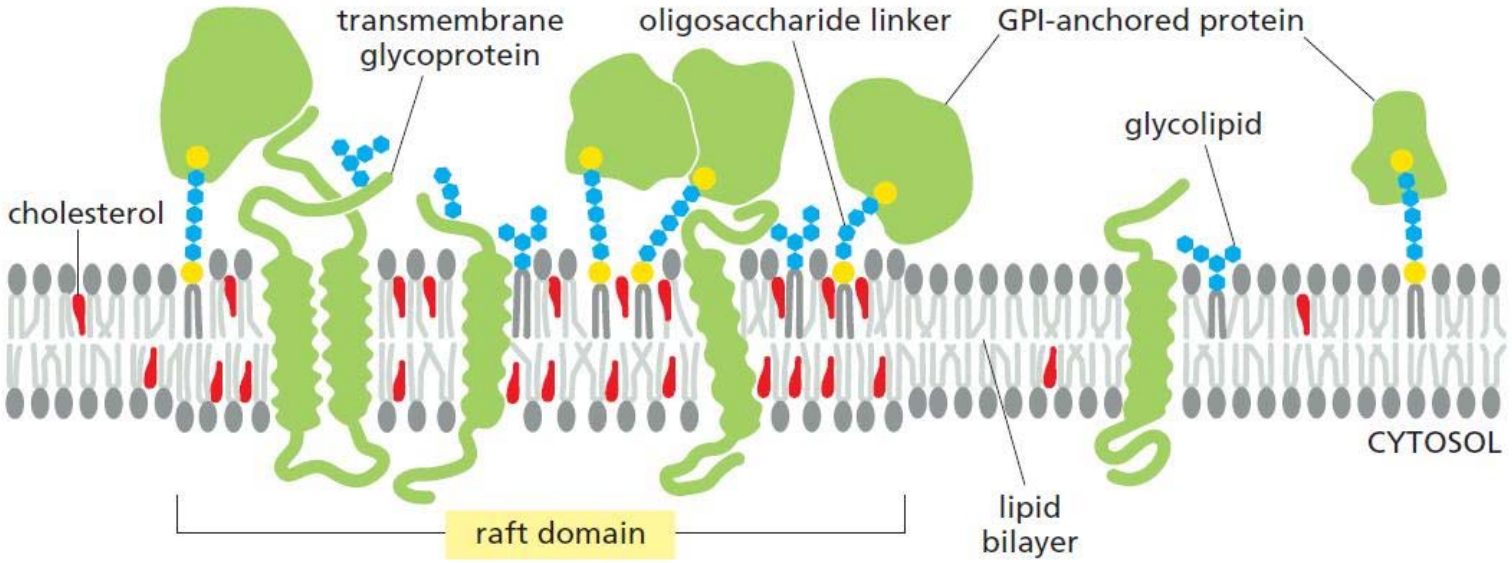
How is the membrane heterogeneous?

In space and time:

- lipid microdomains
- protein clusters

} Which comes first? Chicken-egg dilemma

The raft hypothesis: lipid domains exist in the absence of proteins
Simons, Ikonen (1997) Nature 387:569-572.
"A new aspect of cell membrane structure is presented, based on the **dynamic clustering of sphingolipids and cholesterol** to form rafts that move within the fluid bilayer. It is proposed that these rafts function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction."



Do rafts (lipid-based microdomains) indeed exist *in vivo*?

Sevcsik E & Schutz GJ (2016) With or without rafts? Alternative views on cell membranes. *Bioessays* 38(2):129-139.:
Most experimental evidence does not prove that sterol-dependent nanoscopic phases of different lipid chain order (rafts) compartmentalize proteins.

phase separation ⟷ homogeneous distribution

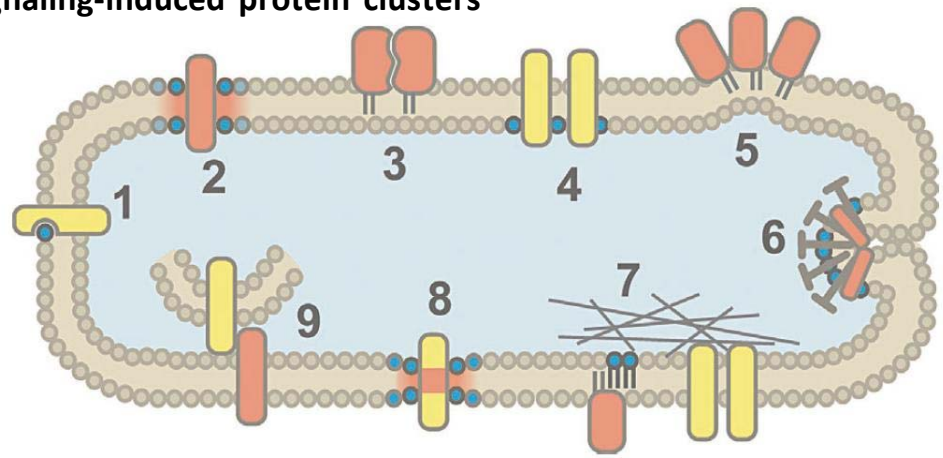
- phase separation tendency of lipids
- presence of proteins
- presence of the cytoskeleton and the ECM
- vesicular traffic

- large number of lipid species
- diffusion

- line tension
- entropy

Conclusion: raft-like domains only exist when the relatively weak phase separation tendency of lipids is precipitated by

- **protein clustering, signaling-induced protein clusters**
- **ceramide formation**
- **the cytoskeleton**



1. Lipid binding to proteins
2. Lipid shells around TM proteins
3. Protein clusters
4. Charge-mediated separation
5. Membrane bending
6. Vesicular traffic
7. Confinement
8. Hydrophobic mismatch
9. ER-plasma membrane interactions

Local and long-range diffusion in the plasma membrane

For almost every kind of molecule:
D_{local}≈0.3-0.7 μm²/s
weak dependence on size in accordance with the Saffman-Delbrück equation

Long-range **free** diffusion is very rare in the plasma membrane due to
- trapping
- confinement

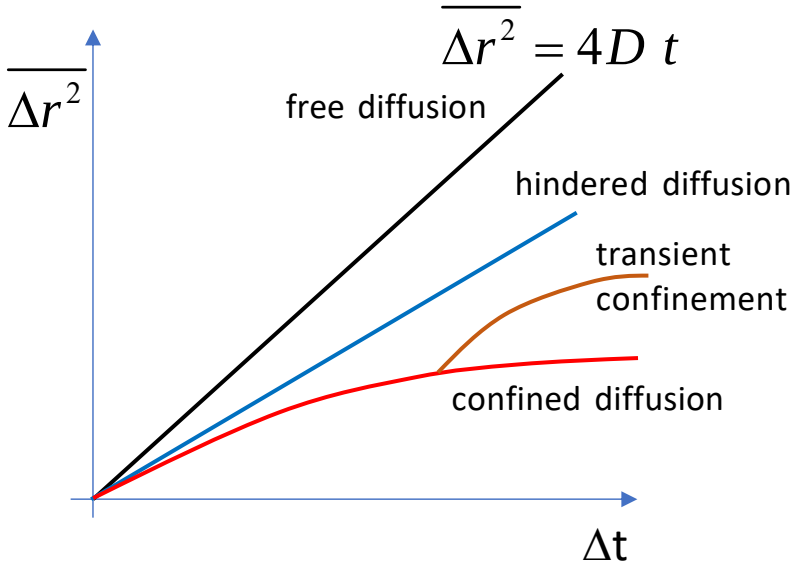
$$D_{long-range} \approx \frac{D_{local}}{5} - D_{local}$$

probability and duration of trapping

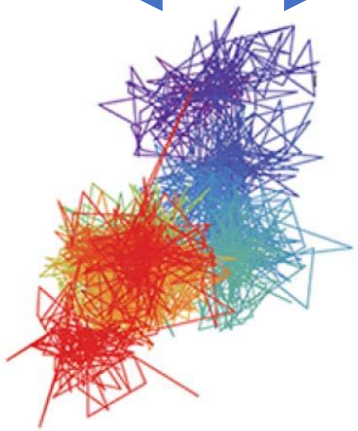
D_{local}

domain size

probability of domain hopping

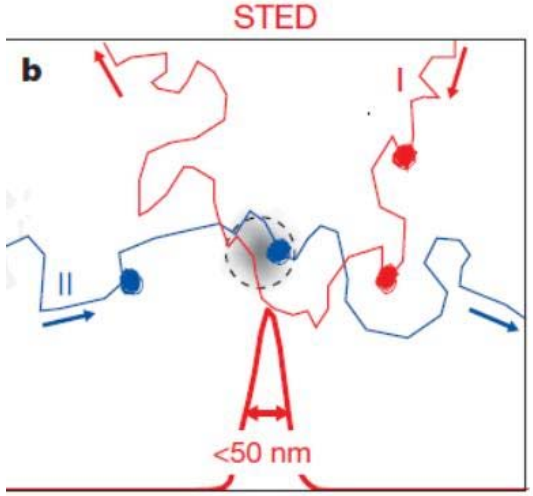


TCZ (transient confinement zone) 80-150 nm, 1-10 ms



confinement (practically everything, even all kinds of lipids)

trapping time≈5-10 ms



trapping (some proteins and sphingomyelin)

Nano Lett **13**, 2332-2337, doi:10.1021/nl303151f (2013); *Nature* **457**, 1159-1162, doi:10.1038/nature07596 (2009); *Nat Commun* **5**, 5412, doi:10.1038/ncomms6412 (2014); *Sci Rep* **5**, 11454, doi:10.1038/srep11454 (2015).

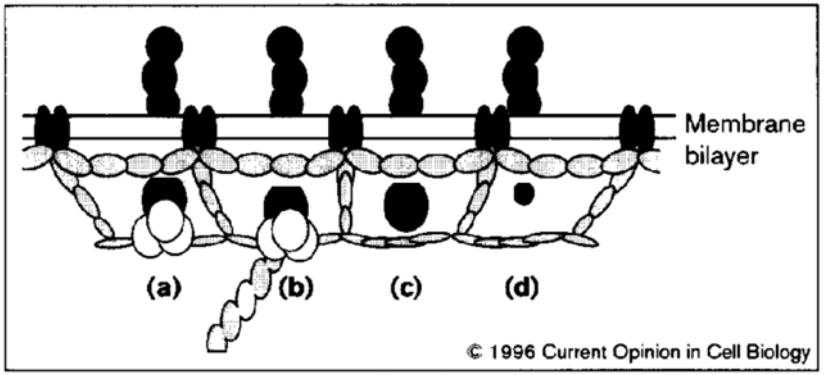
Biological background of trapping and confinement

Trapping:

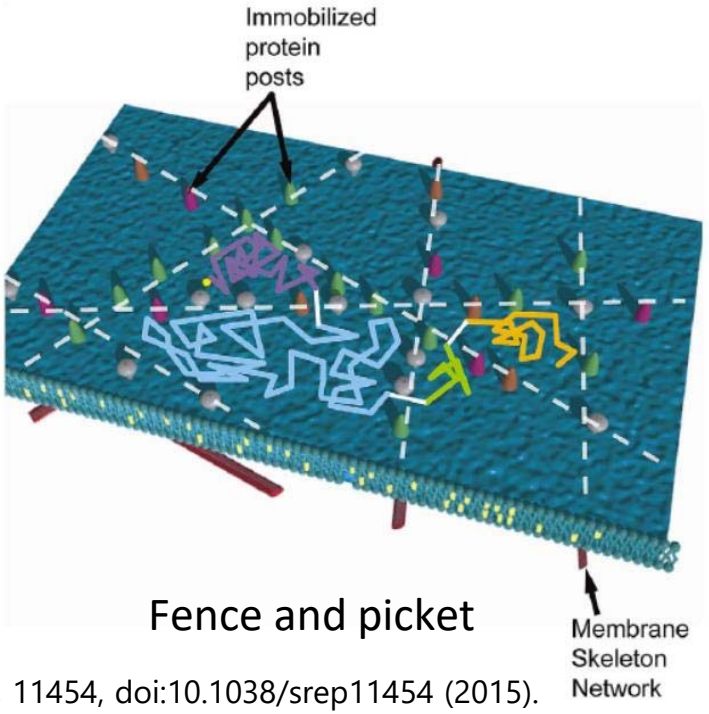
- binding to an immobile site
- proteins and sphingomyelin (but not cholesterol and other lipids)
- immobile site: **some believe it to be raft-like (Nature, 457), others deny it (Sci Rep, 5)**

Confinement:

- direct corralling by the actin cytoskeleton (“membrane skeleton”)
- indirect corralling by immobilized proteins (“fence and picket model’)
- **seem to be raft-related, but actin-dependent**



Membrane skeleton



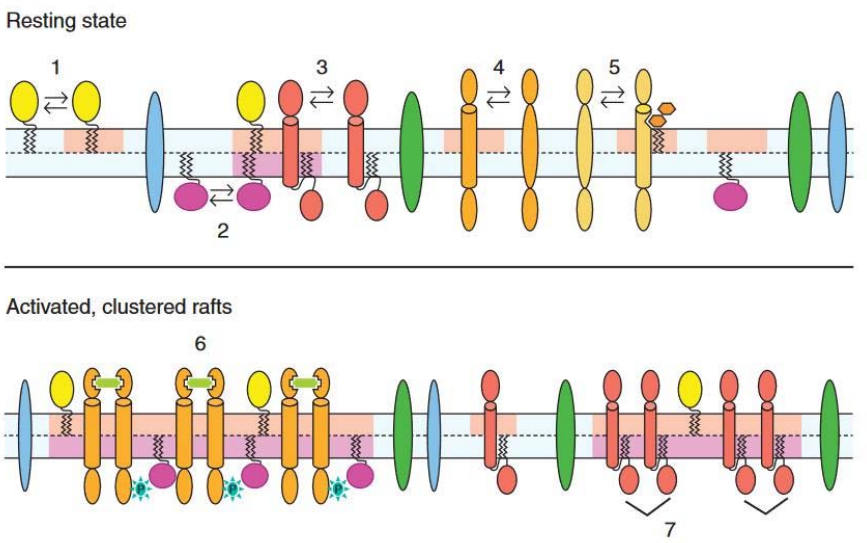
Fence and picket

Curr Opin Cell Biol **8**, 566-574 (1996); *Mol Membr Biol* **20**, 13-18 (2003); *Sci Rep* **5**, 11454, doi:10.1038/srep11454 (2015). *J Lipid Res* **57**, 159-175, doi:10.1194/jlr.R062885 (2016). *Nature* **457**, 1159-1162, doi:10.1038/nature07596 (2009); *Lipids* **39**, 1115-1119 (2004).

What is the advantage of having clusters in the membrane?

1. Regulation

- activation-induced clustering
- clustering-induced activation



2. Increased binding rate

Binding rate at the cell surface:

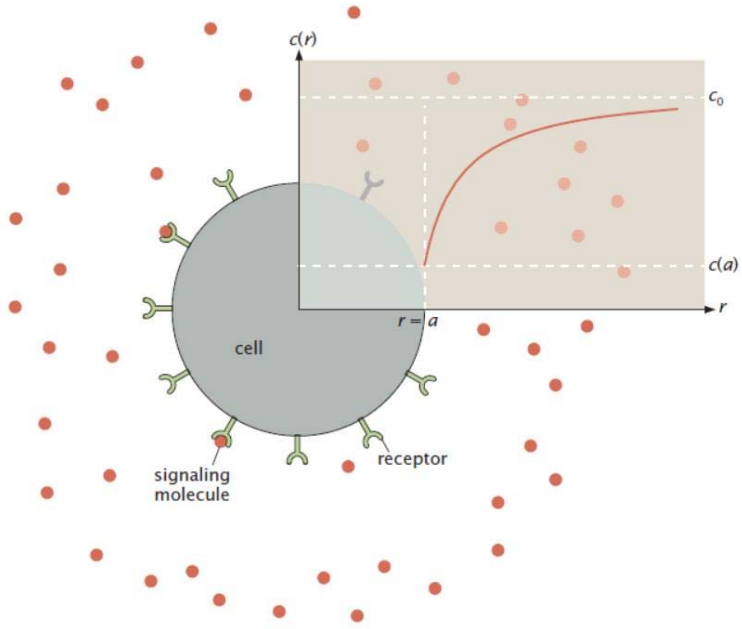
$$\frac{dn}{dt} = \frac{M\ k_{on}\ c_0}{1+\frac{M\ k_{on}}{4\pi D\ a}} \Rightarrow 4\pi D\ a\ c_0,\ if\ \ k_{on} \to \infty$$

To achieve half of the maximal binding permitted by diffusion ($2\pi Dac_0$):

$$\frac{M\ k_{on}\ c_0}{1+\frac{M\ k_{on}}{4\pi D\ a}} = 2\pi D\ a\ c_0 \Rightarrow M \approx 10^5 \text{ receptors}$$

Overexpression!

$$a = 10\,\mu m,\ D = 100\,\mu m^2/s,\ k_{on} = 10\ \mu m^{-1}s^{-1}$$

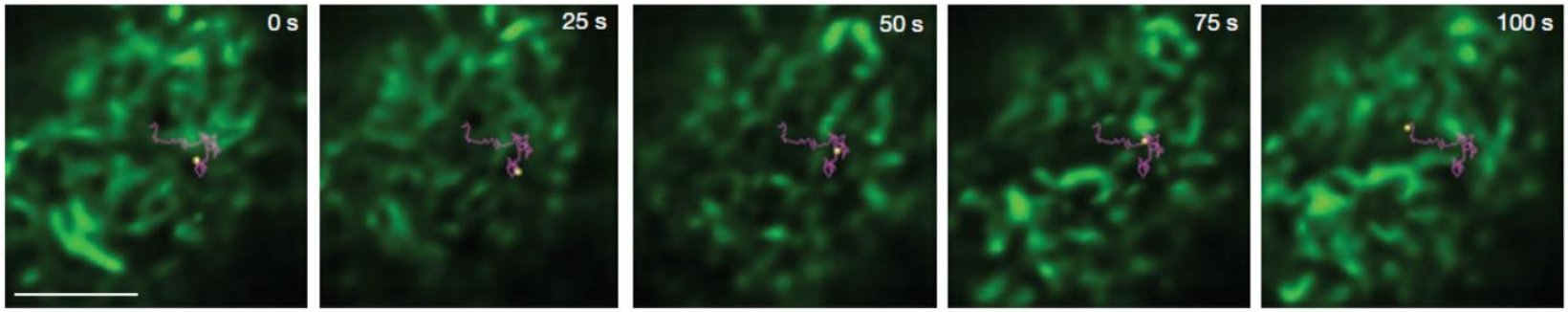


- Simons, K. & Sampaio, J. L.. *Cold Spring Harb Perspect Biol* **3**, a004697, doi:10.1101/cshperspect.a004697 (2011).
- Physical Biology of the Cell, Rob Phillips, Jane Kondev, Julie Therio, Garland Science

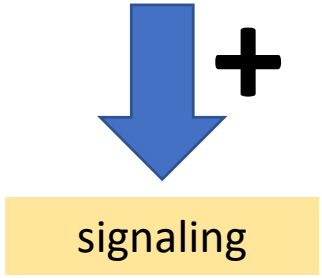
What is the advantage of having clusters in the membrane?

3. Shortened diffusion time in the membrane

- long-range diffusion in the membrane is very slow
- activated receptors diffuse even more slowly (dimerization, cytoskeletal anchoring)



- clustering reduces the diffusion distance between potential interacting partners
- clustering enhances the probability of rebinding

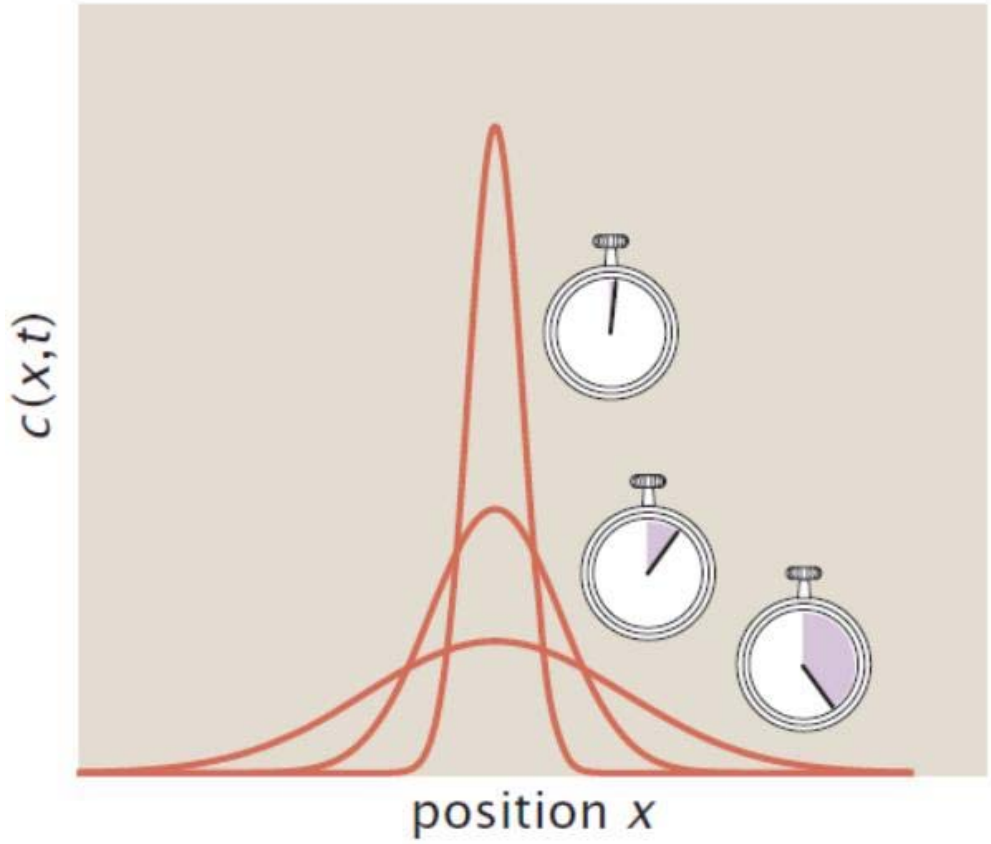


Andrews, N. L. *et al.* Actin restricts FceRI diffusion and facilitates antigen-induced receptor immobilization. *Nat Cell Biol* **10**, 955-963. 10.1038/ncb1755 (2008).
Mugler, A., Bailey, A. G., Takahashi, K. & ten Wolde, P. R. Membrane clustering and the role of rebinding in biochemical signaling. *Biophys J* **102**, 1069-1078, doi:10.1016/j.bpj.2012.02.005 (2012).

Summary of the biological / biophysical part

- The cell membrane is heterogeneous.
- Diffusion is free only locally.
- Long-range diffusion is limited by
  - confinement
  - trapping
- Advantage of clustering in signaling

How does diffusion spread molecules in the membrane?



$$c(x,t) = \frac{N}{\sqrt{4\pi Dt}} e^{-\frac{x^2}{4Dt}}$$

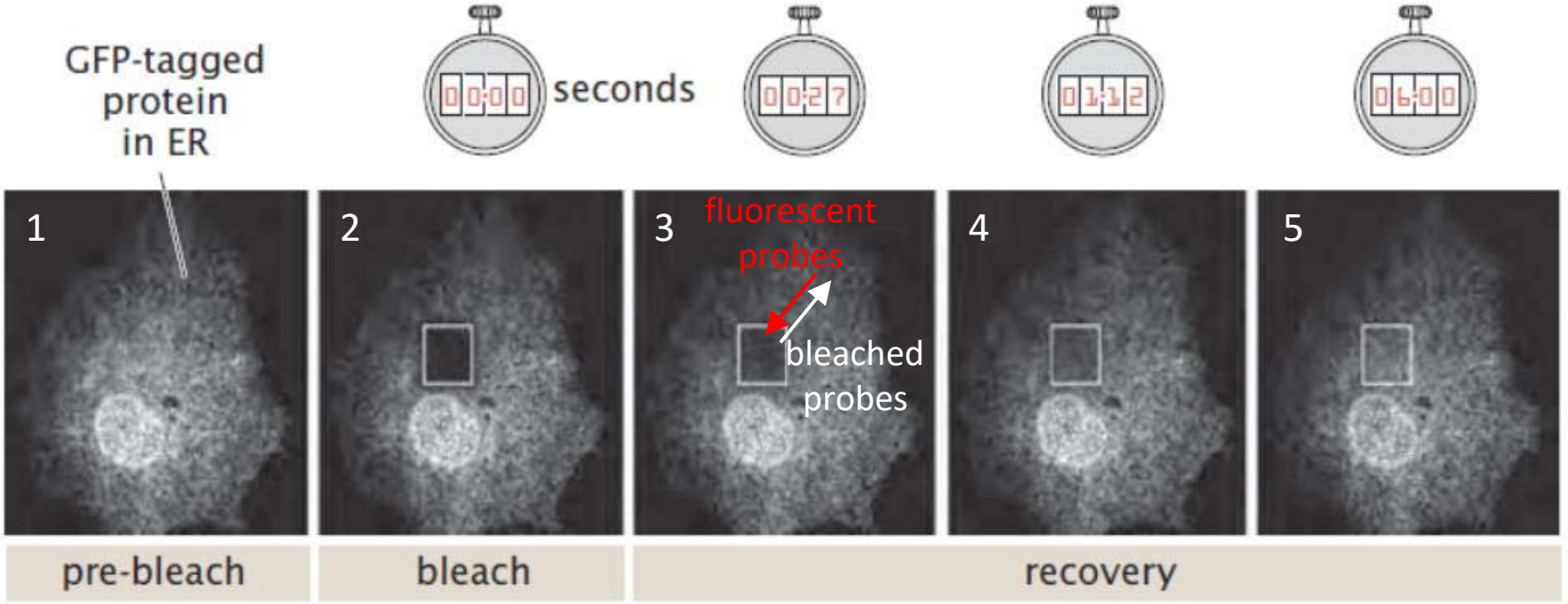
Normal distribution
mean – 0
SD - $\sqrt{2Dt}$

N – number of molecules at the origin at *t*=0

The basic principles of FRAP

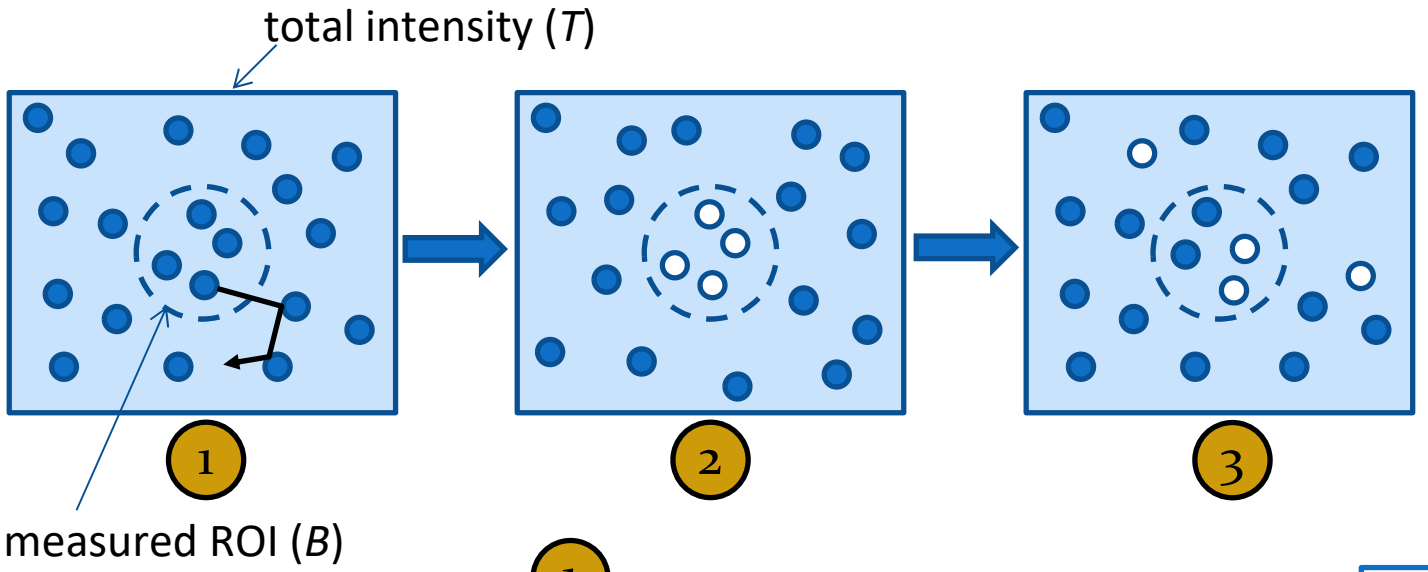
The most frequent questions addressed by FRAP

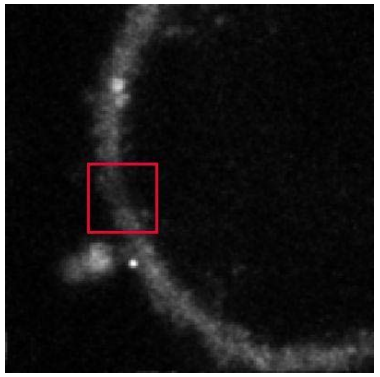
- how fast does a labeled molecule diffuse?
- how large is the immobile fraction?
- how are molecules exchanged between compartments (mainly by the FRAP-related techniques, see later) ?



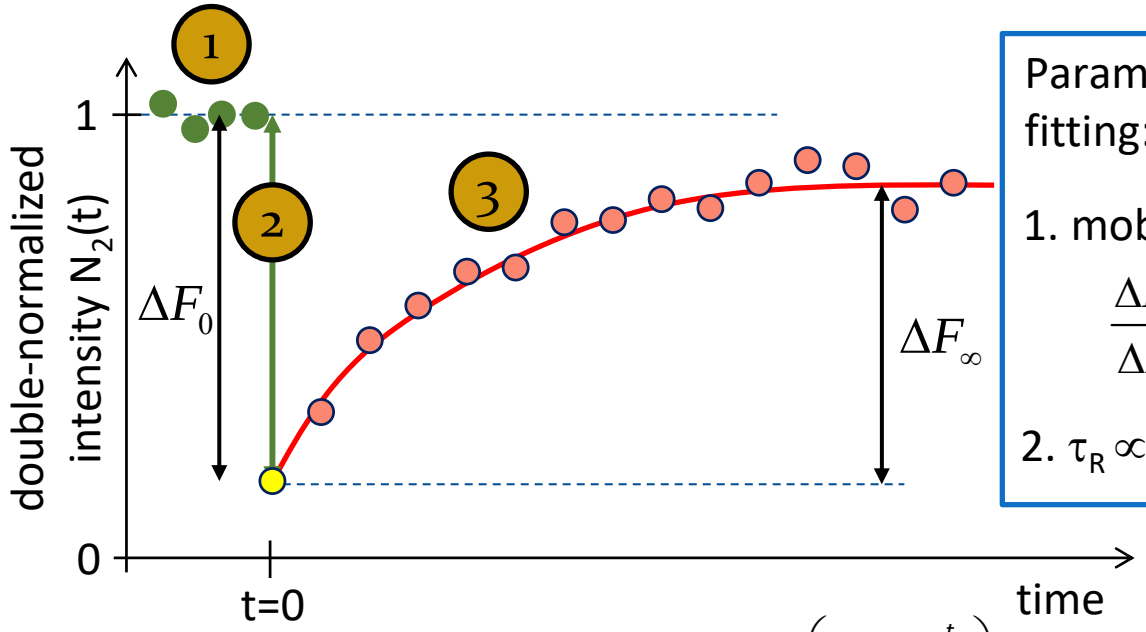
1. Label cells with a fluorescent probe
2. Bleach a region.
3-5. Follow how fluorescence returns (recovers) due to diffusion
    - fluorescent probes move from the unbleached area
    - bleached probes move out of the bleached area

Measurement of mobility of membrane components by FRAP





$$N_2(t) = \frac{\frac{B(t) - Bg(t)}{B(0) - Bg(0)}}{\frac{T(t) - Bg(t)}{T(0) - Bg(0)}}$$



Parameters obtained by fitting:

1. mobile fraction:

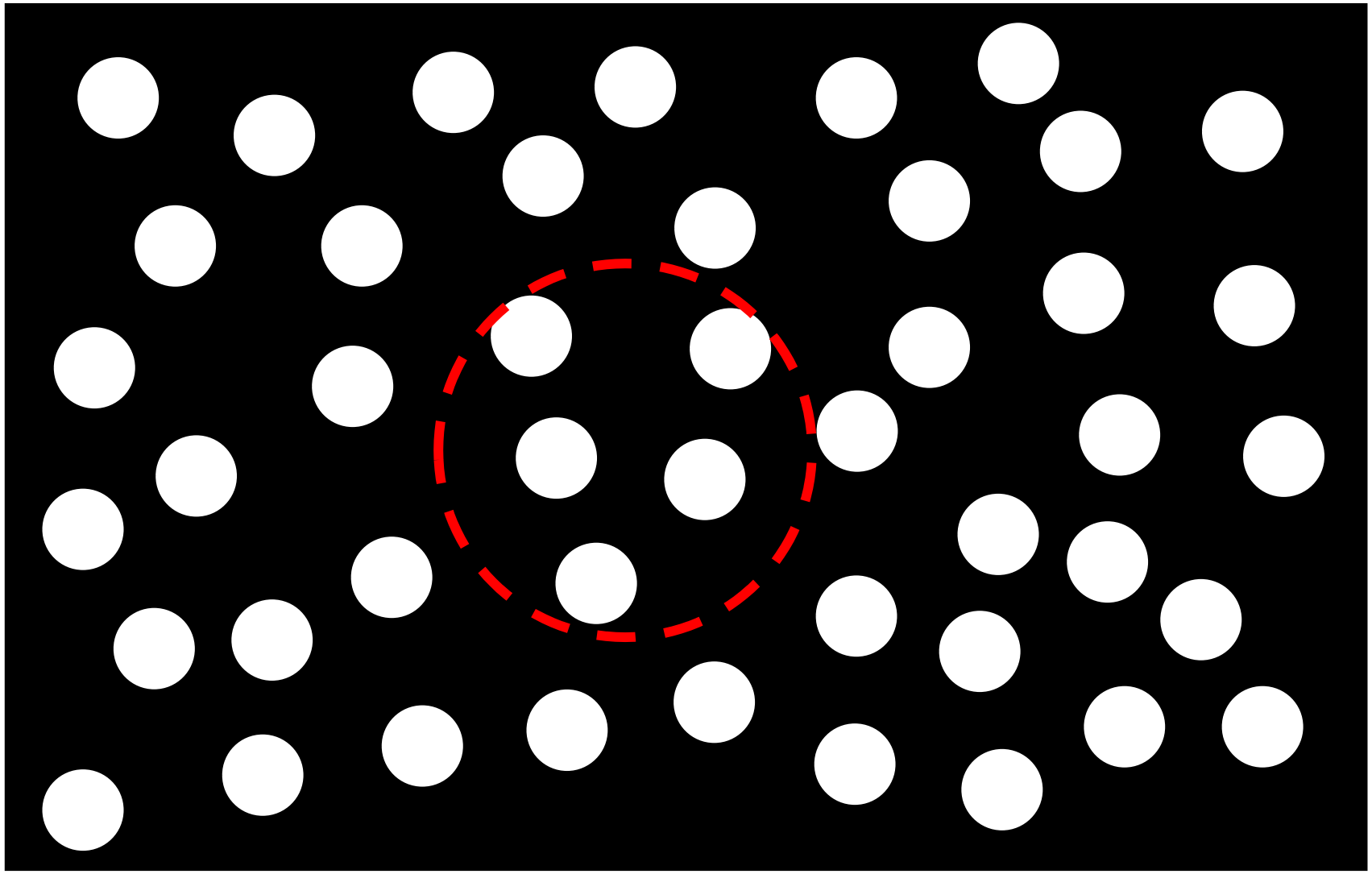
$$\frac{\Delta F_\infty}{\Delta F_0} = 1 - f_{immobile}$$

2. $\tau_R \propto$ 1/diffusion coeff.

Qualitative evaluation:

$$F(t) = F(0) - \Delta F_0 + \Delta F_0 (1 - f_{immobile}) \left(1 - e^{-\frac{t}{\tau_R}} \right)$$

FRAP in steps

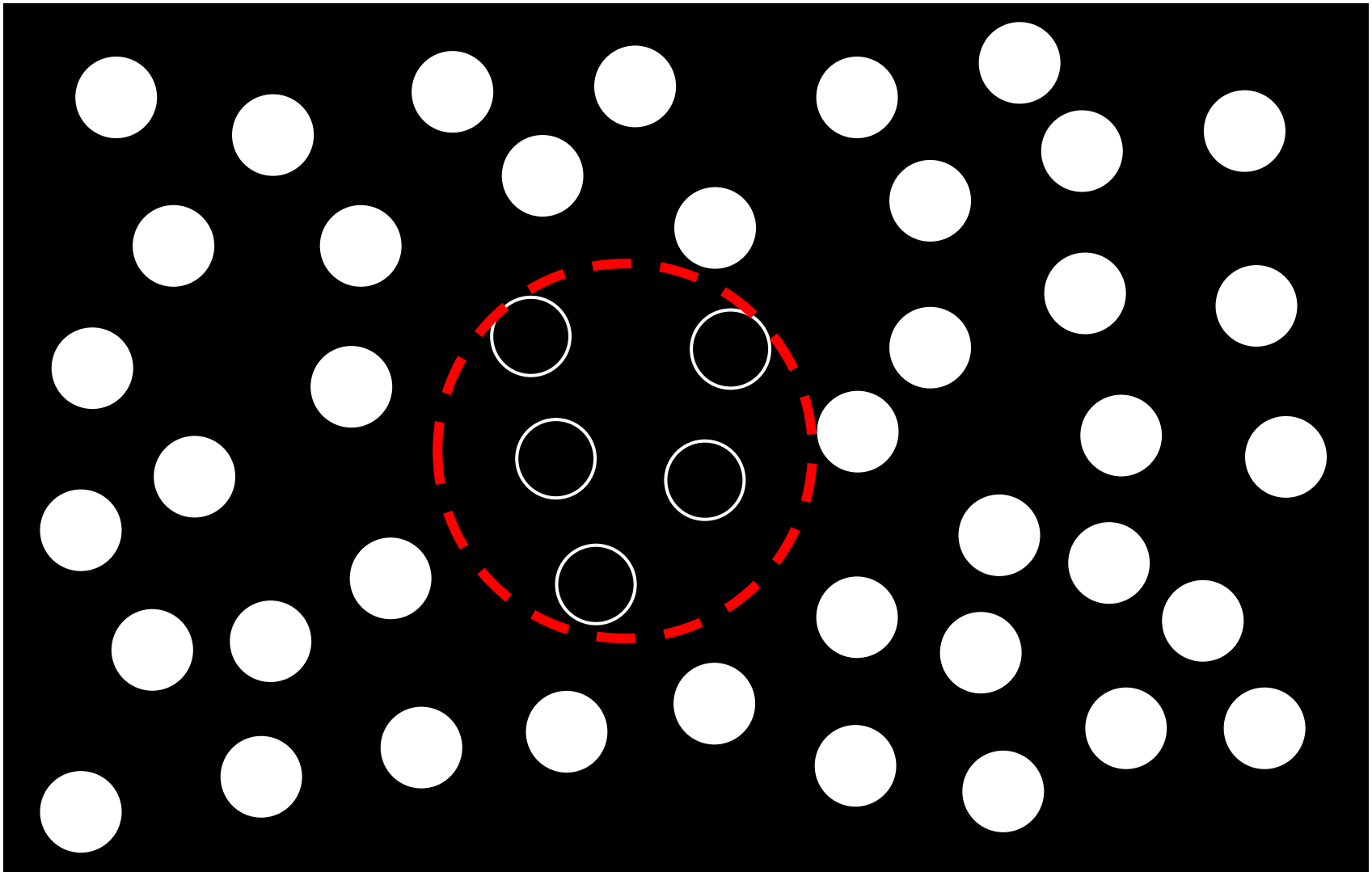


Fluorescent molecules

Idea by Kota Miura

A strong laser beam is focused at a spot.

FRAP in steps



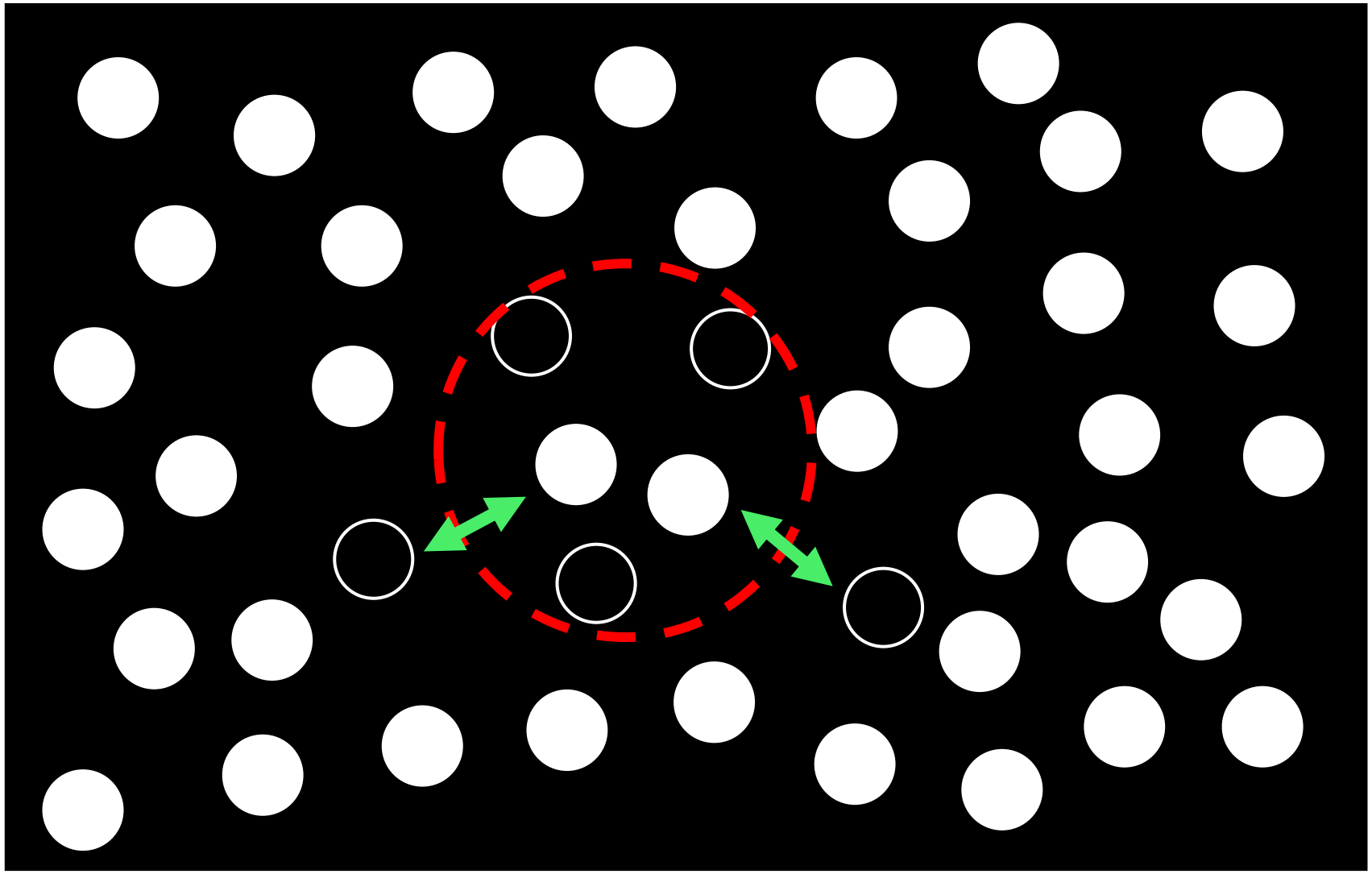
Fluorescent molecules

Bleached molecules

Idea by Kota Miura

The strong laser beam bleaches molecules in the spot.

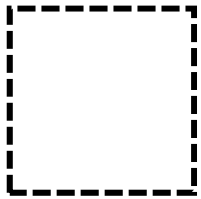
FRAP in steps



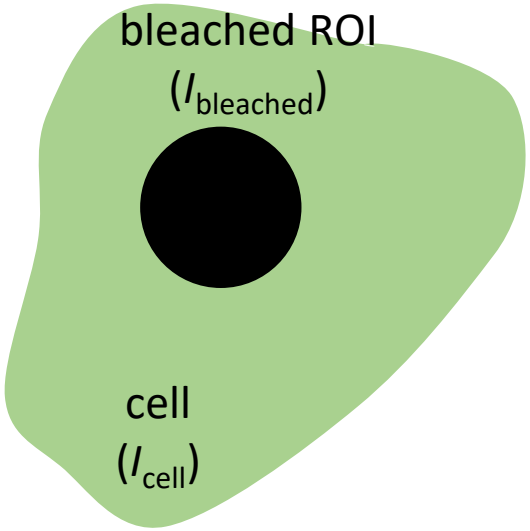
Fluorescent molecules Bleached molecules Idea by Kota Miura

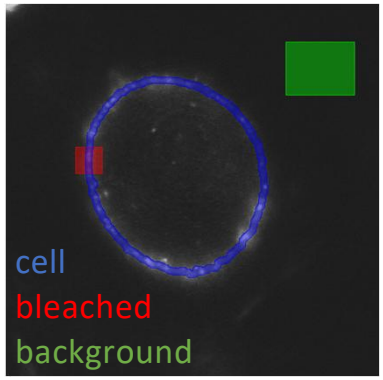
Fluorescence in the spot recovers due to exchange of bleached molecules for unbleached ones.

Normalization of fluorescence



background
(I_{bg})





Intensities are mean intensities.

1. No normalization

$$I_{bleached}(t)$$

2. Background subtraction

$$I_{bleached}(t) - I_{bg}(t)$$

3. Normalization to the pre-bleach intensity

$$FRAP3(t) = \frac{I_{bleached}(t) - I_{bg}(t)}{I_{bleached}(0) - I_{bg}(0)}$$

4. Double normalization

- normalize the intensity of the bleached ROI to the intensity of the cell
- corrects for laser instability and focal drift during imaging

$$FRAP4(t) = FRAP3(t)\frac{I_{cell}(0) - I_{bg}(0)}{I_{cell}(t) - I_{bg}(t)} = \frac{I_{bleached}(t) - I_{bg}(t)}{I_{bleached}(0) - I_{bg}(0)}\frac{I_{cell}(0) - I_{bg}(0)}{I_{cell}(t) - I_{bg}(t)}$$

5. Triple normalization: ensures that the FRAP curve drops to 0 after bleaching

- doesn't show the extent of bleaching

$$FRAP5(t) = 1 - \frac{1 - FRAP4(t)}{1 - FRAP4(0)}$$

Image registration

During a long recovery phase
- cells may move
- the stage may drift

You don't measure what you expect in the ROIs.

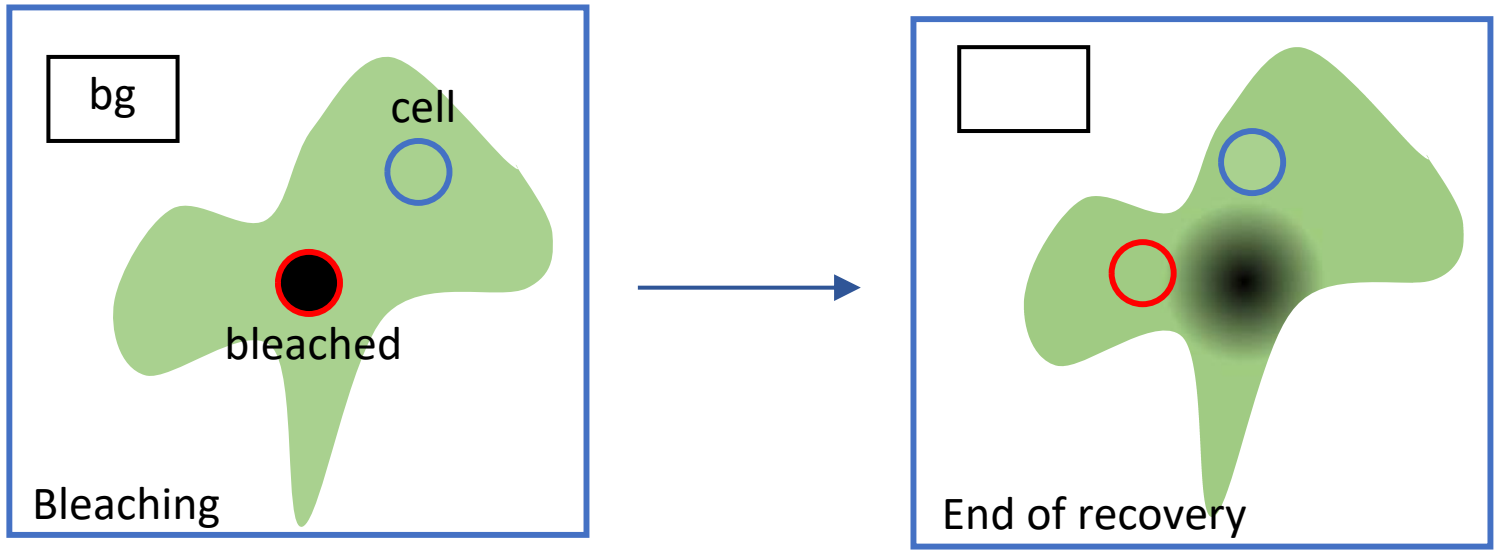
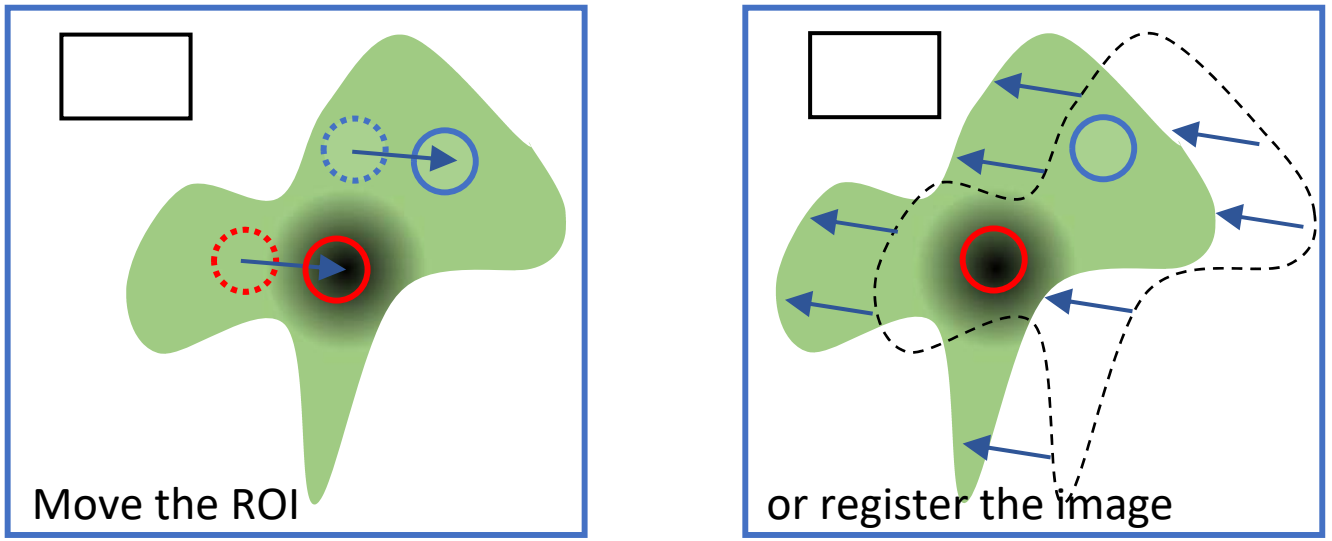


Image processing:



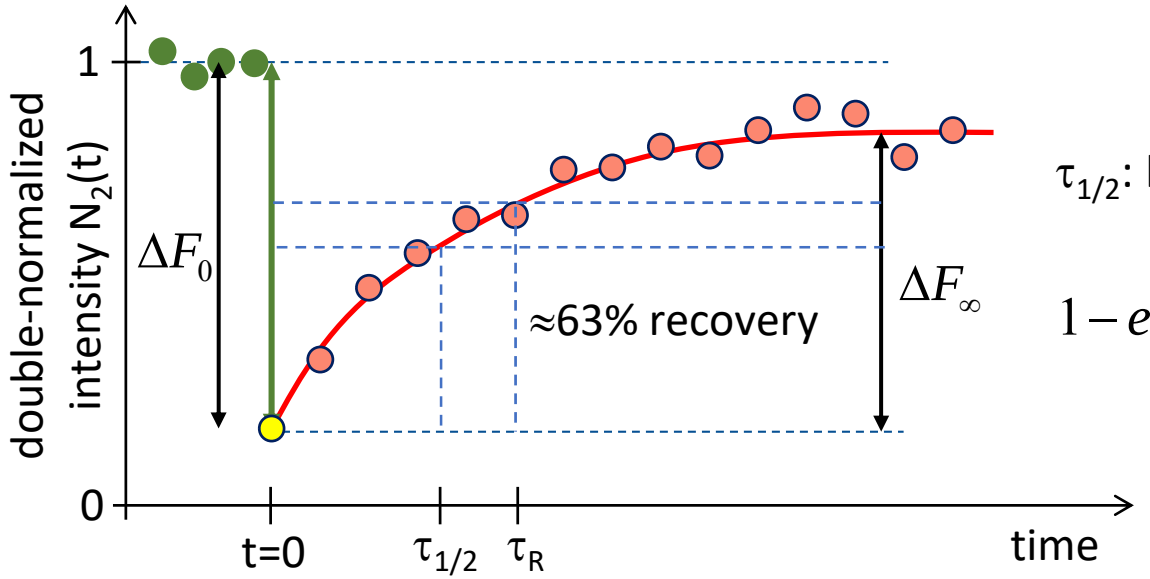
Evaluation of a FRAP experiment

1. Qualitative: fit an exponential function

e.g. $F(t) = F(0) - \Delta F_0 + \Delta F_0 (1 - f_{immobile}) \left(1 - e^{-\frac{t}{\tau_R}} \right)$ but it can be double-exponential, etc.

- recovery time constant ("speed of recovery", $\tau_R \propto$ 1 / diffusion coefficient)
- immobile fraction:

$$f_{immobile} = 1 - \frac{\Delta F_\infty}{\Delta F_0}$$



$$1 - e^{-1} \approx 0.63$$

$\tau_{1/2}$: half-time of recovery

$$1 - e^{-\frac{t}{\tau_R}} = 0.5 \Rightarrow \tau_{1/2} = \ln(2)\,\tau_R$$

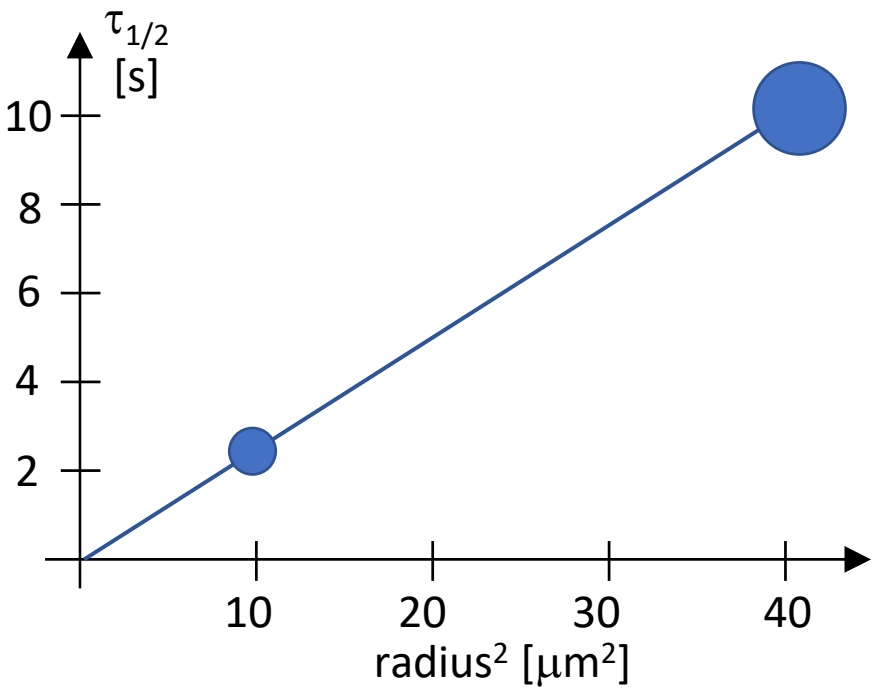
- Appropriate for comparing different samples measured under identical conditions.
- Not influenced by model-dependent assumptions.

Evaluation of a FRAP experiment

1. Qualitative (cont'd): Interpretation of the results

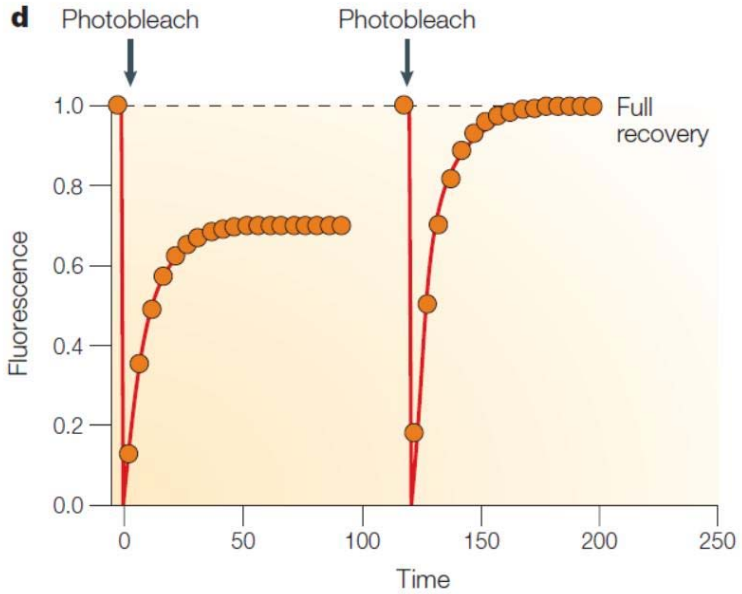
$\tau_{1/2}$

- qualitative measure of the recovery speed
- inversely related to the diffusion coefficient
- proportional to the area of the bleached spot if recovery is due to free diffusion



immobile fraction

- due to cytoskeletal anchoring and confinement
- depends on the area of the bleached spot (see later)
- can be artifactual created by photo-induced cross-linking by radicals. Repeat the FRAP experiment at the same spot
  - lower immobile fraction: "real" immobility
  - same immobile fraction: potential photo-induced cross-linking

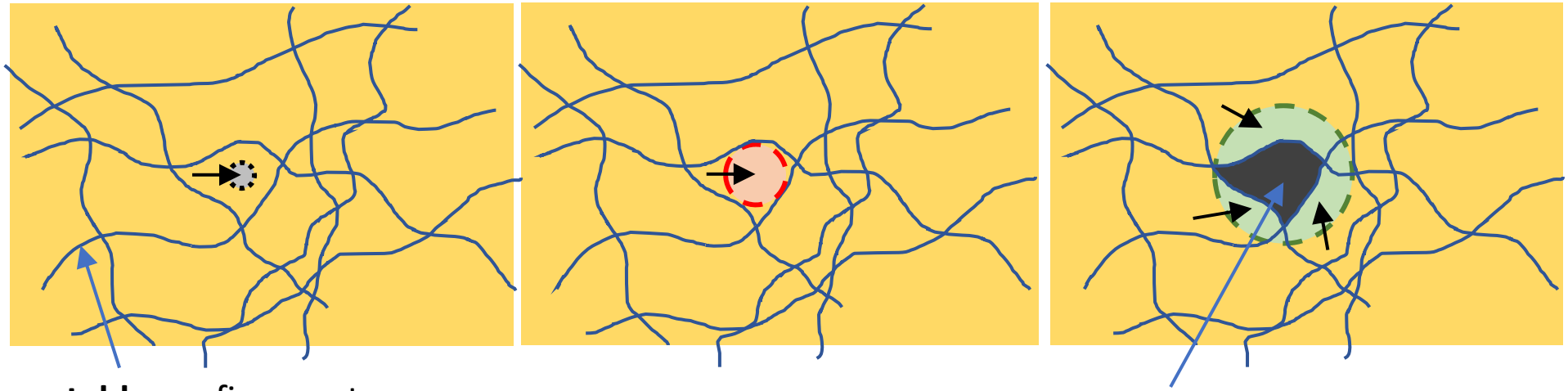


Lippincott-Schwartz, J., Altan-Bonnet, N. & Patterson, G. H. *Nat Cell Biol* **Suppl**, S7-14 (2003).

Evaluation of a FRAP experiment

1. Qualitative (cont'd): dependence of immobile fraction on the area of the bleached spot

→ recovery



<u>stable</u> confinement zone boundaries (not the same as TCZ)

- bleached spot inside confinement zone
- bleached area much smaller than confinement zone

- low immobile fraction

- bleached spot inside confinement zone
- bleached area comparable to confinement zone

- high immobile fraction since ~50% of fluorophores are bleached in the confinement zone

won't recover at all

- bleached spot larger than confinement zone

- high immobile fraction since black area won't recover at all

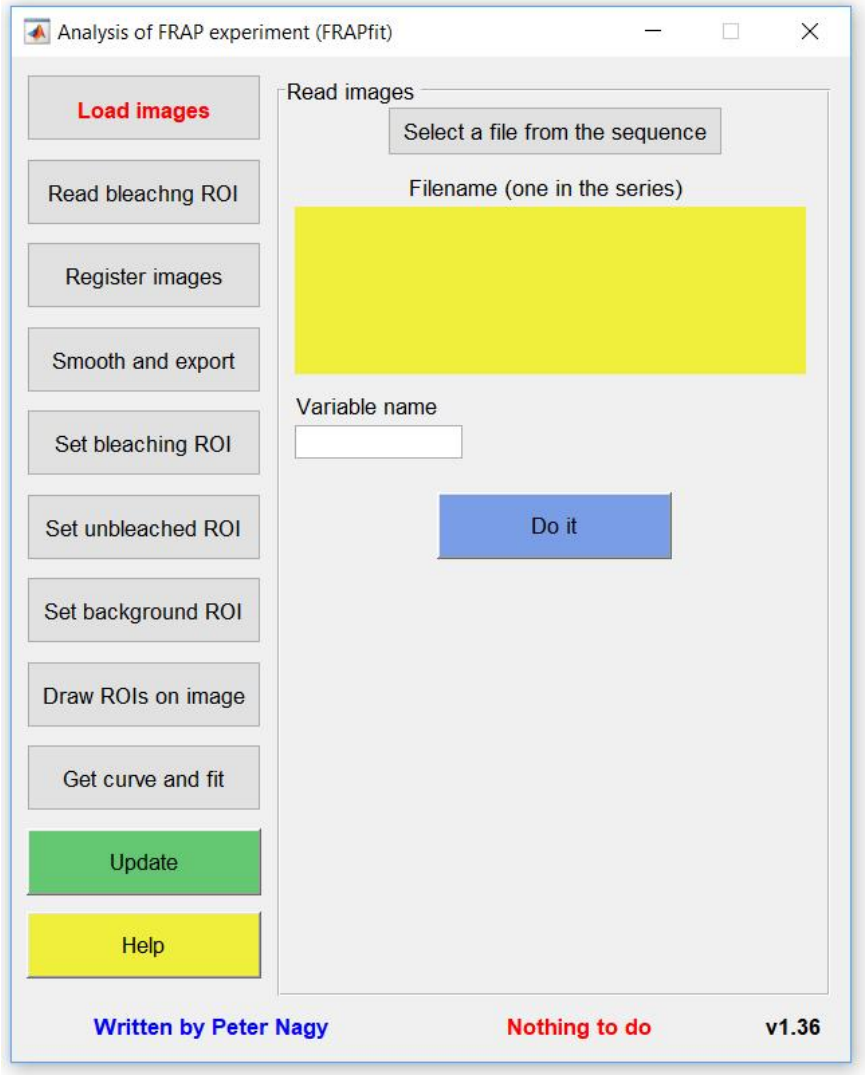
If the size of the bleached spot approaches the size of the confinement zone, the recovery fraction decreases.

Schram et al., *Eur Biophys J* **23**, 337-348 (1994)
Yechiel and Edidin, *J Cell Biol* **105**, 755-760 (1987)

Evaluation of a FRAP experiment

1. Qualitative (cont'd)

Program for evaluating a FRAP experiment in the practical part:



https://peternagyweb.hu/frap

Evaluation of a FRAP experiment

2. Quantitative: fitting of analytically solved equations

- the aim is to determine the diffusion coefficient
- an equation based on assumptions regarding the experiment is fitted to the data

- Influenced by the model-dependent assumptions.
- Very difficult to derive a model incorporating all relevant assumptions (confinement, trapping, hindered diffusion).

Original solution published by Axelrod (1976):

$$F(t) = C_0 \sum_n \left(\frac{(-K)^n}{n!} \right) \left(1 + n\left(1 + \frac{2t}{\tau_D} \right) \right)^{-1} \qquad D = \frac{\omega^2}{4\tau_D}$$

ω - half-width of beam at e^{-2} intensity
K – bleaching constant

- Axelrod D. et al, Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys J* **16**, 1055-1069.
- Soumpasis, D. M. Theoretical analysis of fluorescence photobleaching recovery experiments. *Biophys J* **41**, 95-97, doi:10.1016/S0006-3495(83)84410-5 (1983).
- Ellenberg, J. *et al.* Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J Cell Biol* **138**, 1193-1206 (1997).
- Yang, J., Kohler, K., Davis, D. M. & Burroughs, N. J. An improved strip FRAP method for estimating diffusion coefficients: correcting for the degree of photobleaching. *J Microsc* **238**, 240-253, doi:10.1111/j.1365-2818.2009.03347.x (2010).

Evaluation of a FRAP experiment

2. Quantitative: fitting of analytically solved equations (specific cases)

Circular ROI (Soumpasis, 1983)

$$FRAP(t) = a_0 + a_1 e^{=\frac{2\tau_D}{t}} \left(I_0 \frac{2\tau_D}{t} + I_1 \frac{2\tau_D}{t} \right)$$

$$\tau_D = \frac{w^2}{4D}$$

I_0, I_1 – modified Bessel functions
w – radius of the circular beam

Stripe ROI (Ellenberg, 1997)

$$FRAP(t) = a_0 + a_1 \left(1 - \sqrt{\frac{w^2}{w^2 + 4\pi D t}} \right)$$

w – width of the stripe

- Axelrod D. et al, Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys J* **16**, 1055-1069.
- Soumpasis, D. M. Theoretical analysis of fluorescence photobleaching recovery experiments. *Biophys J* **41**, 95-97, doi:10.1016/S0006-3495(83)84410-5 (1983).
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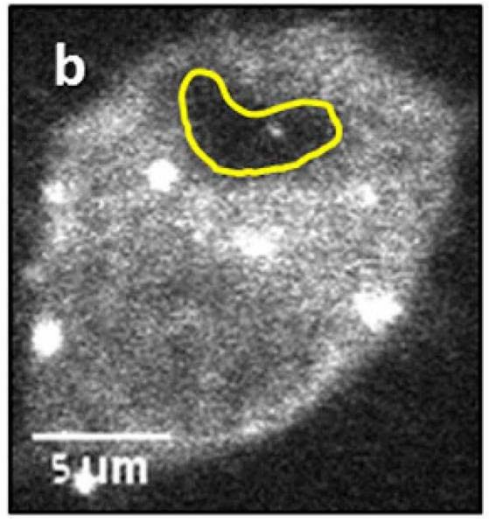
Evaluation of a FRAP experiment

3. Quantitative: simulation



arbitrary bleach pattern, measure recovery

include an (almost) arbitrary number of experimental parameters in the model

set up a differential equation set describing the system

solve the equation system numerically (simulation), or sometimes analytically

correspondence between simulation and measurement good enough?

no

yes

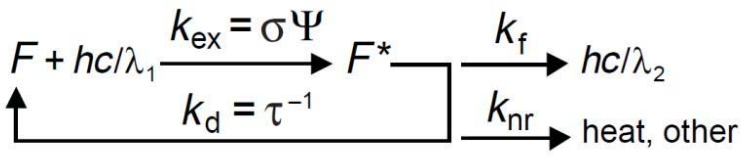
PUBLISH

- Blumenthal, D. et al, Universal Approach to FRAP Analysis of Arbitrary Bleaching Patterns. *Sci Rep* **5**, 11655.

Things to consider for bleaching

- Too much bleaching will cause phototoxicity, photo-induced cross-linking.
- Therefore, ~50% bleaching is sufficient.
- Avoid bleaching during the recovery phase
- Too much laser power is to be avoided because
  - it is not necessary due to fluorophore saturation
  - it can cause photodamage

- **Fluorophore saturation**
  - **biological analogue**: a fluorophore is an "enzyme" converting absorbed photons to emitted photons.



$$v = v_{max}\frac{[S]}{K_M + [S]}$$

[*S*] is the substrate concentration
K_M is the Michaelis-Menten constant
v is the reaction rate
v_{max} is the maximal reaction rate.

- **Photophysical counterpart**:

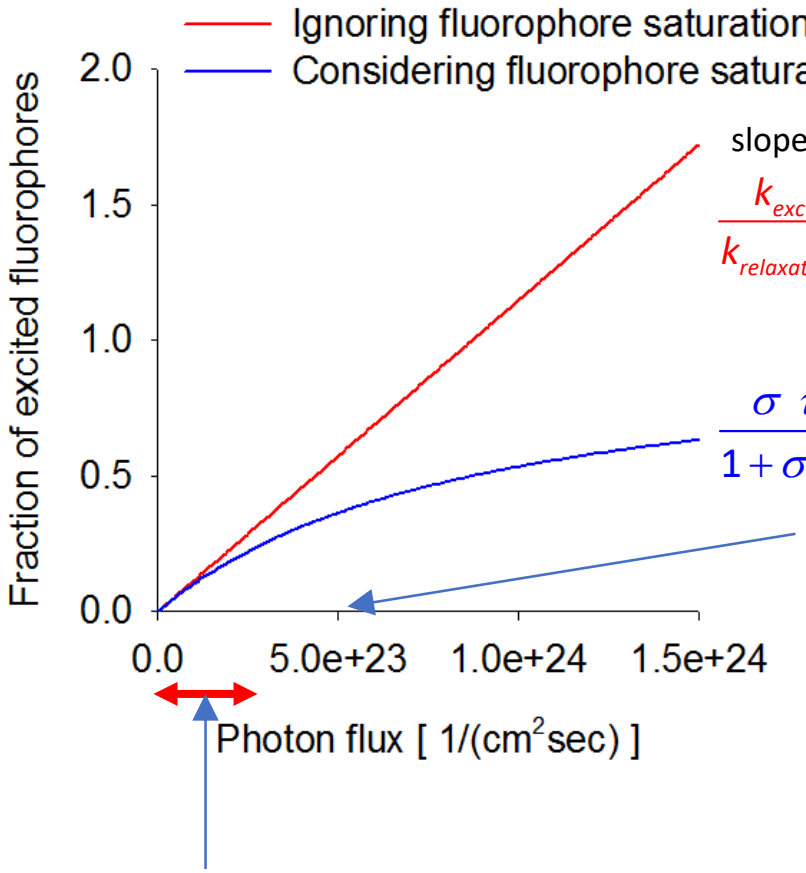
$$I = c\,k_f\frac{F_{all}\;\sigma\;\tau\;\Phi}{1+\sigma\;\tau\;\Phi} = c\,k_f\;F_{all}\frac{\Phi}{\frac{1}{\sigma\tau}+\Phi}$$

"K_M" of the fluorophore

F_{all} is the fluorophore concentration
σ is absorption cross-section of the fluorophore
τ is the fluorescence lifetime
Φ is the excitation photon flux
k_f is the rate constant of fluorescence
c is a proportionality constant

$$\sigma\left[cm^2/molecule\right] = \frac{1000\;\ln(10)}{6\cdot10^{23}}\varepsilon\left[M^{-1}cm^{-1}\right]$$

Things to consider for bleaching



slope of line (*k* – rate constant):

$$\frac{k_{exc}}{k_{relaxation}} = \frac{\sigma\,\Phi}{1/\tau} = \sigma\,\Phi\,\tau$$

$$\frac{\sigma\,\tau\,\Phi}{1+\sigma\,\tau\,\Phi}$$

- Calculated curves for AlexaFluor 488
- τ=4.1 ns
  ε=71000 $M^{-1}\,cm^{-1}$

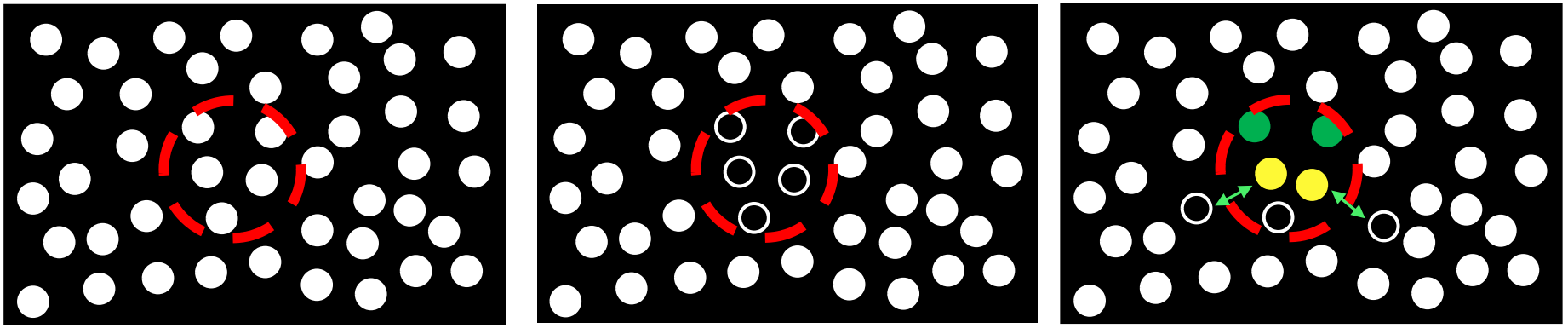
10% laser intensity on Zeiss LSM880

Intensity is linear when the excitation photon flux is much less than $1/\sigma\tau$

Things to consider: reversible dark states

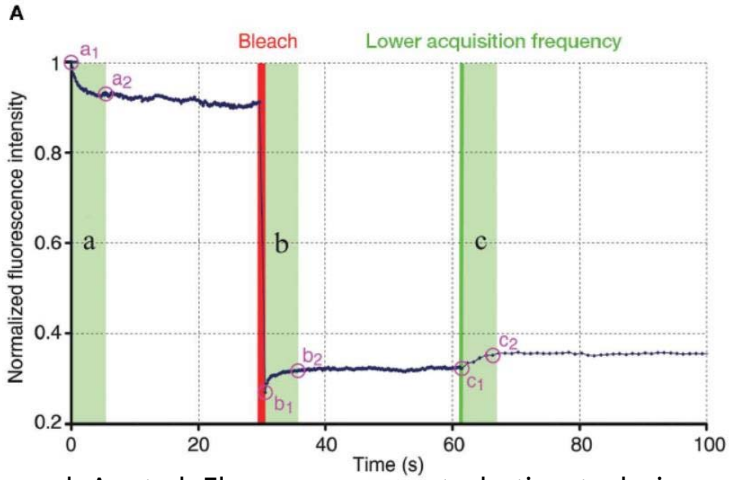
- FPs return from the “bleached” state to the fluorescent state
  - spontaneously or in a light-induced manner
  - on the second time scale

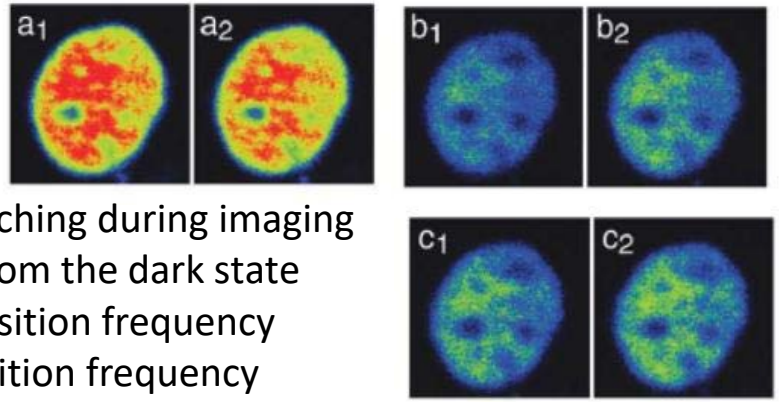
before bleaching | after bleaching | during recovery



○ fluorescent state
● bleached state
● fluorescent protein entering the ROI by diffusion
● fluorescent protein returned from the reversible dark state

- As a result **recovery is overestimated**
- Difficult to correct for, therefore to be avoided



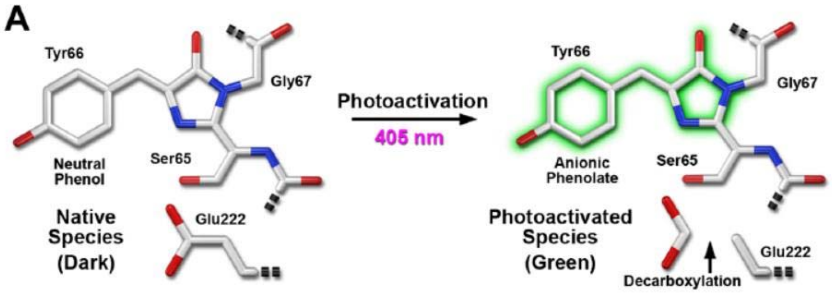


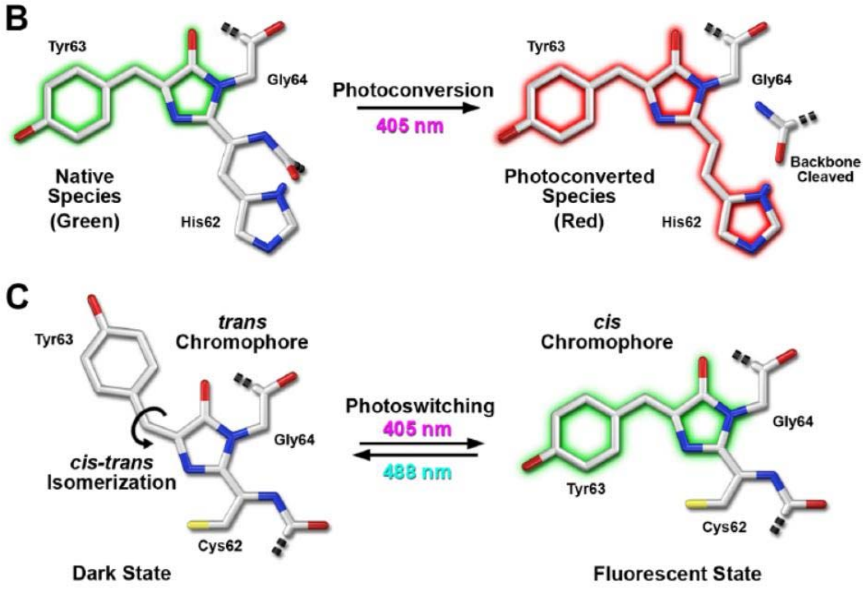
a – photobleaching during imaging
b,c – return from the dark state
b – high acquisition frequency
c – low acquisition frequency

Bancaud, A. et al, Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, photoactivation, photoconversion, and FLIP. *Cold Spring Harb Protoc* **2010**, doi:10.1101/pdb.top90 (2010).

FRAP-related techniques: photoactivatable FPs

- Some FPs undergo photoactivation, photoconversion or photoswitching





Examples (a huge number of variants available, consult the references below)

Name	Type	λ_{ex} / λ_{em}	QY	ε
PA-GFP	photoactivatable	504 / 517	0.79	17400
Kaede	photoconvertible	508 / 518	0.88	98800
Dronpa	photoswitchable	503 / 518	0.85	95000

- Bancaud, A. et al, Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, photoactivation, photoconversion, and FLIP. *Cold Spring Harb Protoc* **2010**, doi:10.1101/pdb.top90 (2010).
- Shaner, N. et al, Advances in fluorescent protein technology. *J Cell Sci* **120**, 4247-4260, doi:10.1242/jcs.005801 (2007).
- Lukyanov, K. A. et al, Photoactivatable fluorescent proteins. *Nat Rev Mol Cell Biol* **6**, 885-891, doi:10.1038/nrm1741 (2005)
- https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/confocal/applications/opticalhighlighters/

FRAP-related techniques: photoactivatable FPs

Protein tracking

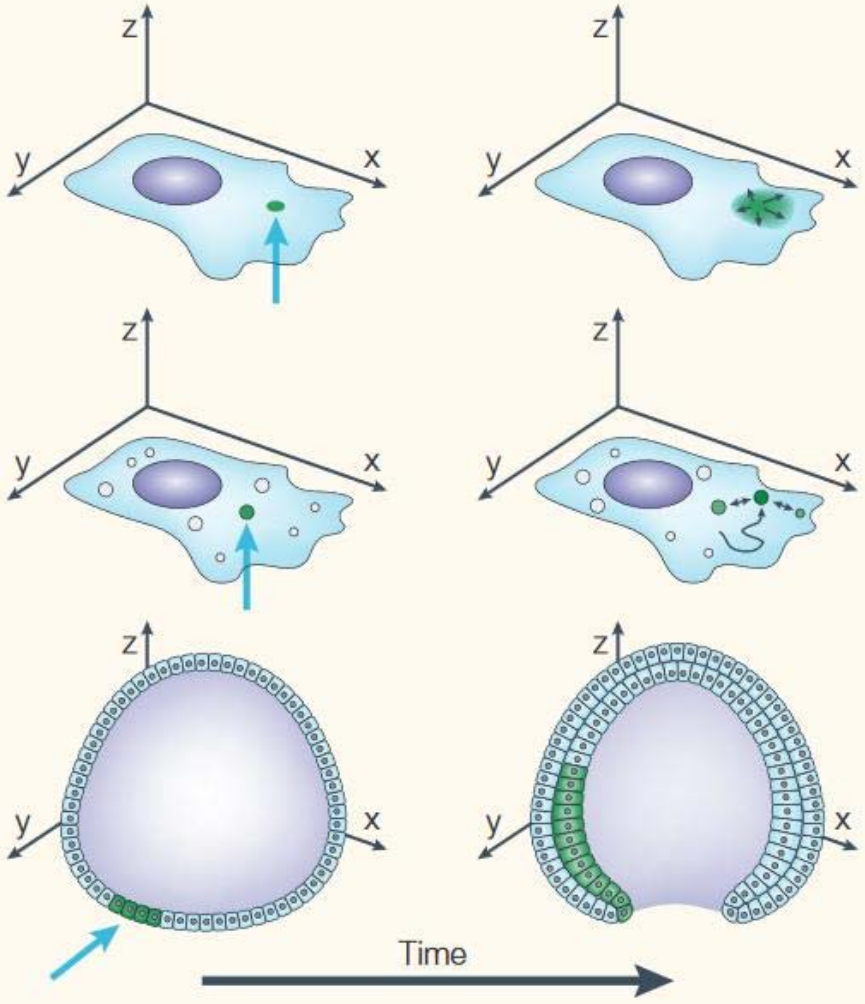
Parameters determined:
- Movement rate and direction
- Diffusion coefficient
- Mobile and immobile fractions
- Time parameters of compartmental residency and exchange between compartments
- Rate of turnover

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



- Bancaud, A. et al, Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, photoactivation, photoconversion, and FLIP. *Cold Spring Harb Protoc* **2010**, doi:10.1101/pdb.top90 (2010).
- Shaner, N. et al, Advances in fluorescent protein technology. *J Cell Sci* **120**, 4247-4260, doi:10.1242/jcs.005801 (2007).
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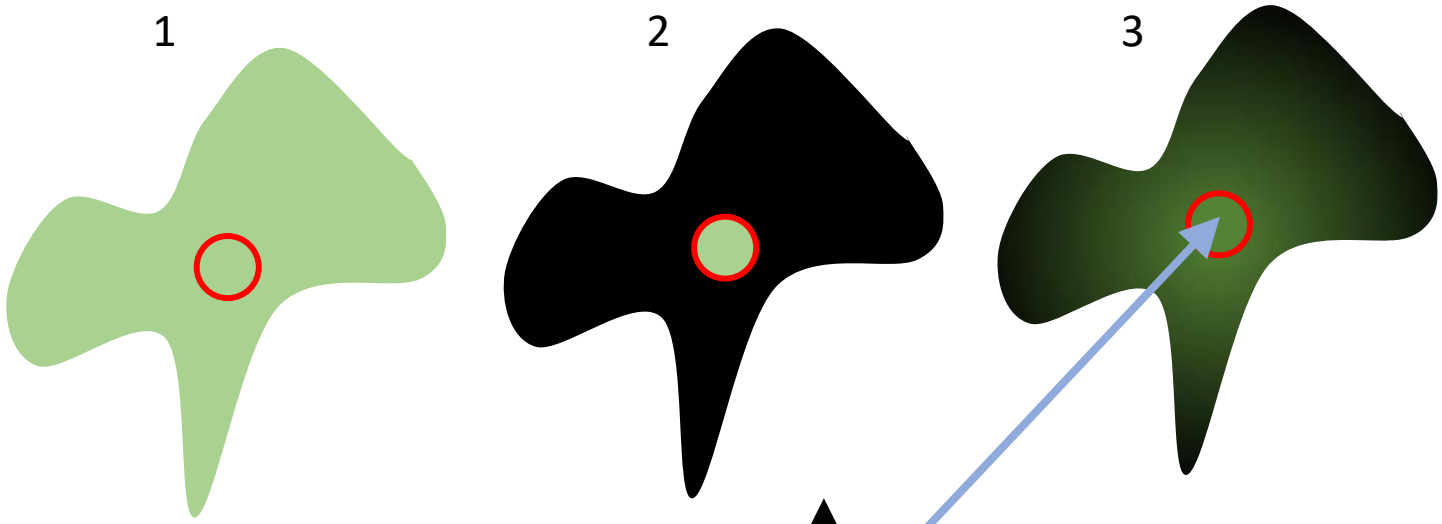
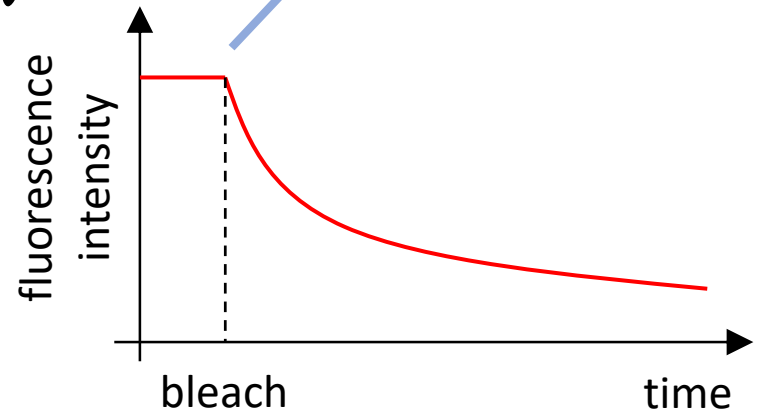
FRAP-related techniques: iFRAP (inverse FRAP)

1. Fluorescent cell
2. The entire cell is bleached except for a ROI
3. Decrease in the intensity of the ROI is monitored
   The intensity of the ROI decreases because unbleached molecules diffuse out.

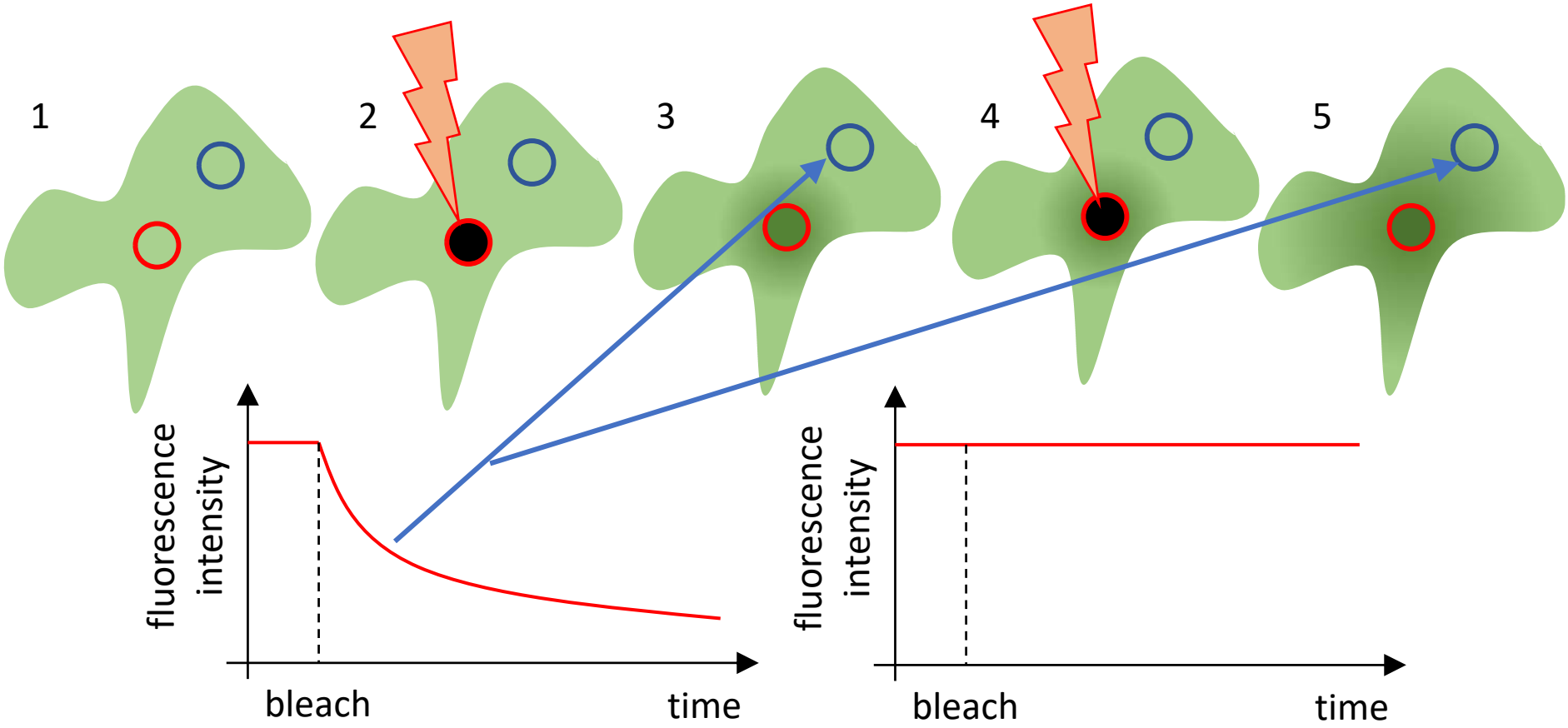


- Principle similar to photoactivation of FPs (decrease of fluorescence of a spot is monitored)
- Limitations:
  - harsh on cells (photodamage to a large area)
  - takes a long time to bleach the entire cell



FRAP-related techniques: FLIP (fluorescence loss in photobleaching)

1. Fluorescent cell
2. Bleach a ROI (red)
3. Follow the fluorescence decrease in another ROI (blue)
4-5. Repeated bleaching followed by monitoring the fluorescence in the blue ROI

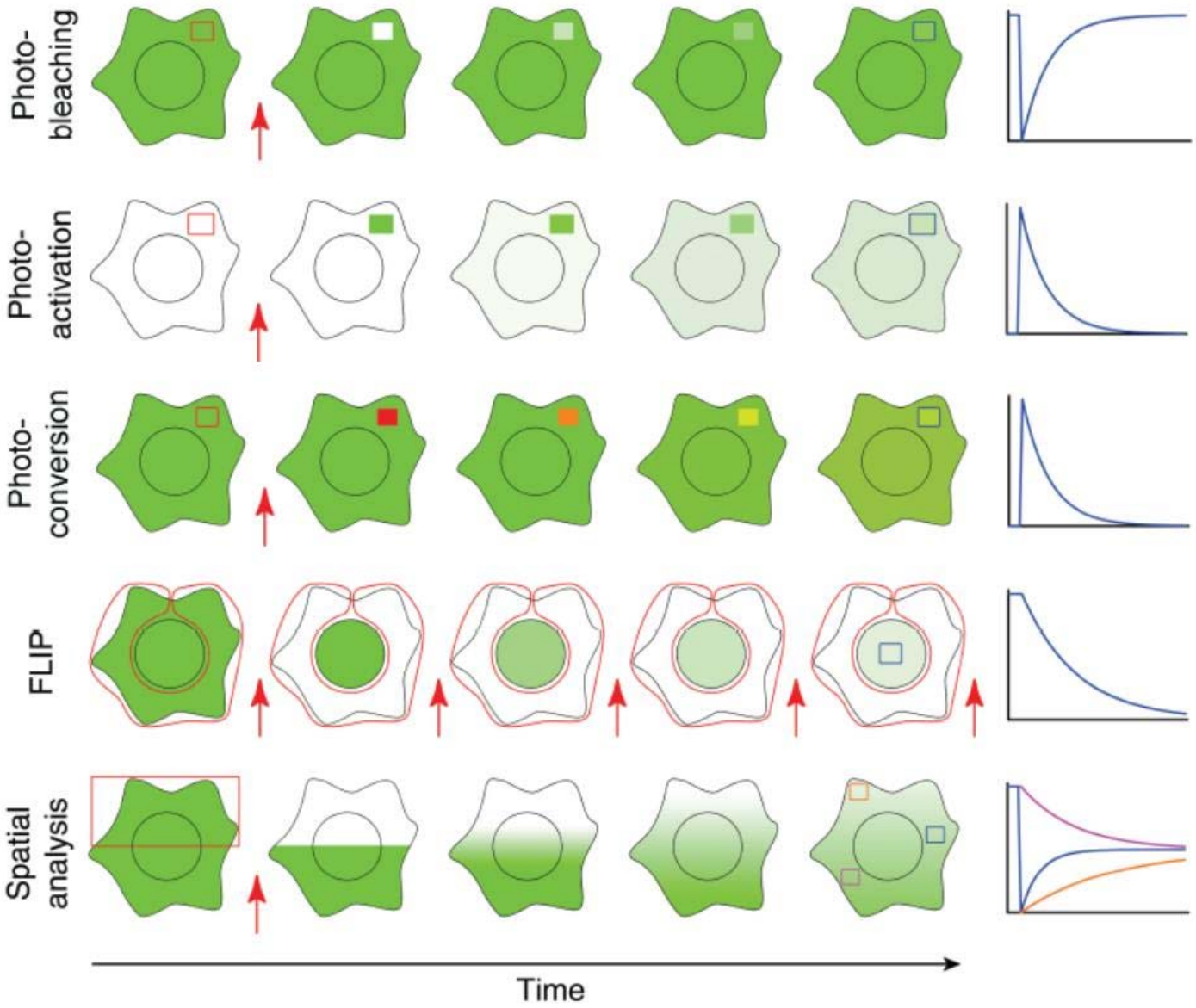


- mobile molecules
- ROIs communicating with each other

- immobile molecules
- or ROIs NOT communicating with each other

Phair, R. D. & Misteli, T. Kinetic modelling approaches to in vivo imaging. *Nat Rev Mol Cell Biol* **2**, 898-907, doi:10.1038/35103000 (2001).

Comparison of FRAP and related techniques



Bancaud, A., Huet, S., Rabut, G. & Ellenberg, J. Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, photoactivation, photoconversion, and FLIP. *Cold Spring Harb Protoc* **2010**,doi:10.1101/pdb.top90 (2010).

Summary of the FRAP part

- FRAP is easy to perform and interpret in a qualitative way
- It is much more difficult to get physical parameters (e.g. diffusion coefficient) in the complex environment of the cell (quantitative analysis)
- FRAP related techniques give insight to transport between organelles
- Alternatives for FRAP:
  - SPT (single particle tracking)
  - FCS (fluorescence correlation spectroscopy)

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