FRAP Tutorial

EMBO course, Debrecen August 2011

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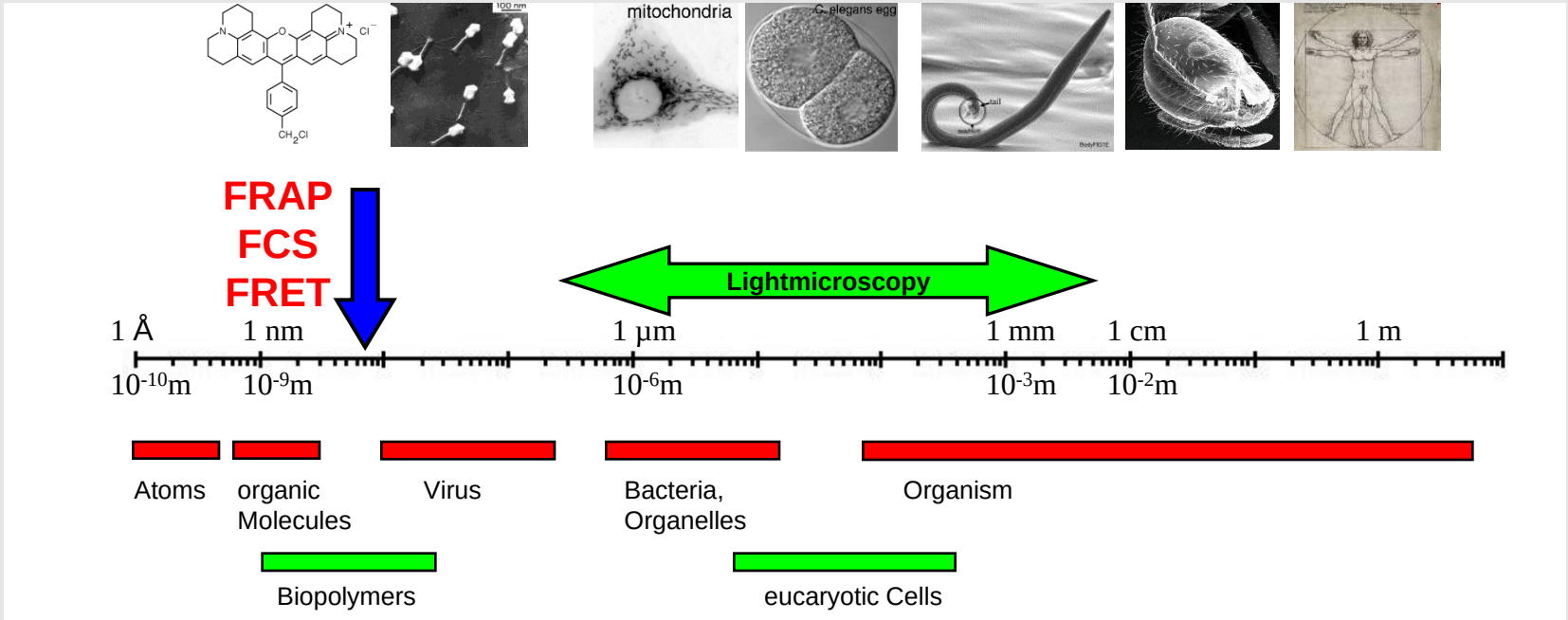
www.embl.de/almf

EMBL

Overview

- Introduction
- Application examples and related techniques
- Instrument setups for FRAP
- Basic FRAP analysis procedures

Molecular Dynamics and Interactions



Brief FRAP history

1970s: 1st applications of the FRAP method
mathematics for Gaussian beam profile bleaching
(Poo & Cone, Axelrod et al.)

→ Custom built systems, mainly diffusion in membranes

1980s: first commercial confocal microscopes

1990s: revival of FRAP using GFP and confocal microscopes with ROI-scanning (AOTF)
(Tsien, Cole et al., Lippincott-Schwartz..)

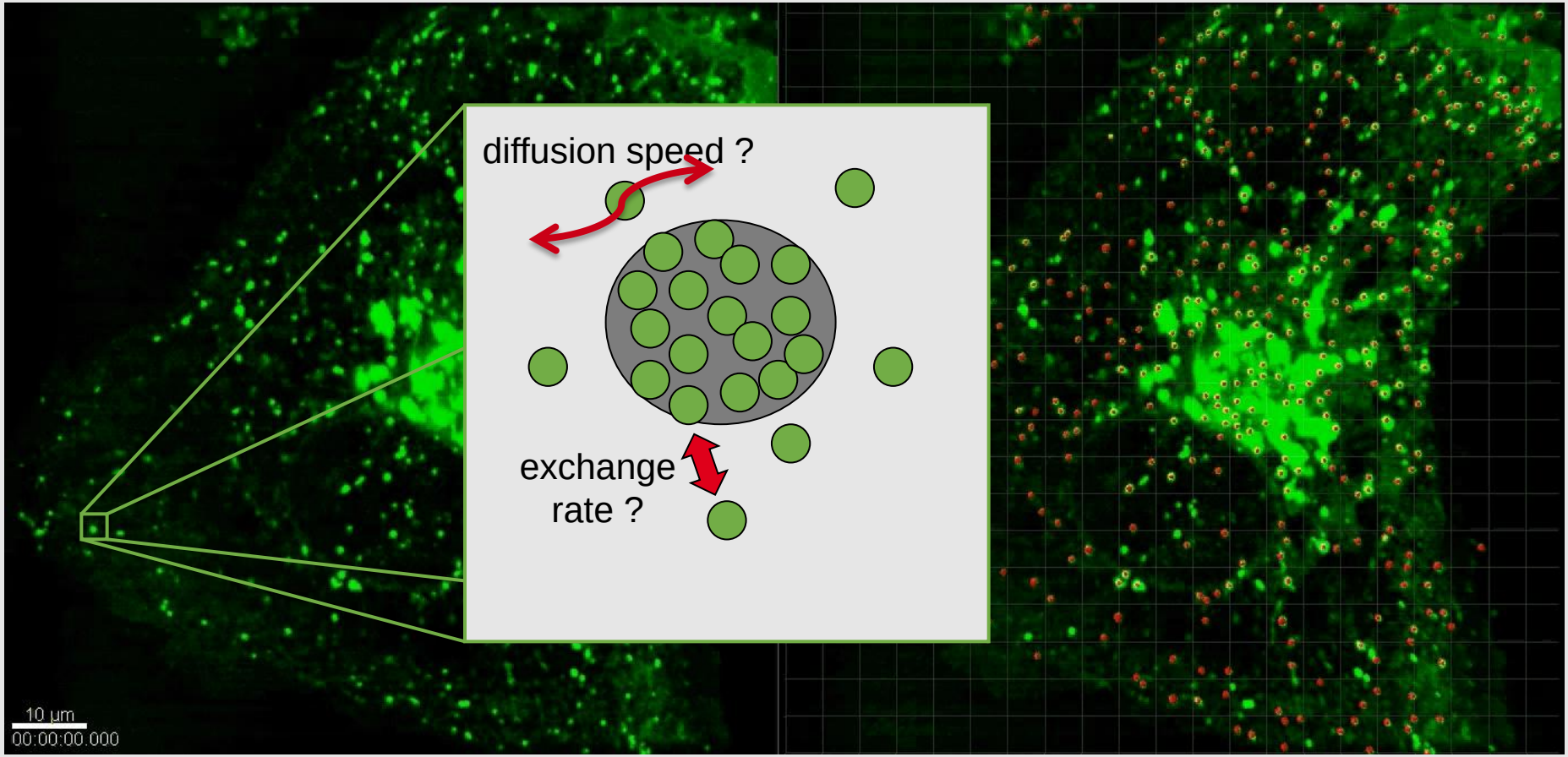
→ Commercial confocal systems, virtually any protein fused to a fluorescent protein

2000s: Computer modeling for quantitative FRAP analysis
Improvements of microscope systems for FRAP

Questions addressed by FRAP

- How fast do proteins move ?
- Which compartments are connected ?
- How long do proteins bind other proteins ?
- What is the percentage of proteins ‘immobilized’ by binding to larger structures (e.g. DNA, Actin) ?
- How do drugs or mutations alter the binding of a protein ?
- How fast are molecules transported between compartments (e.g. nuclear import) ?

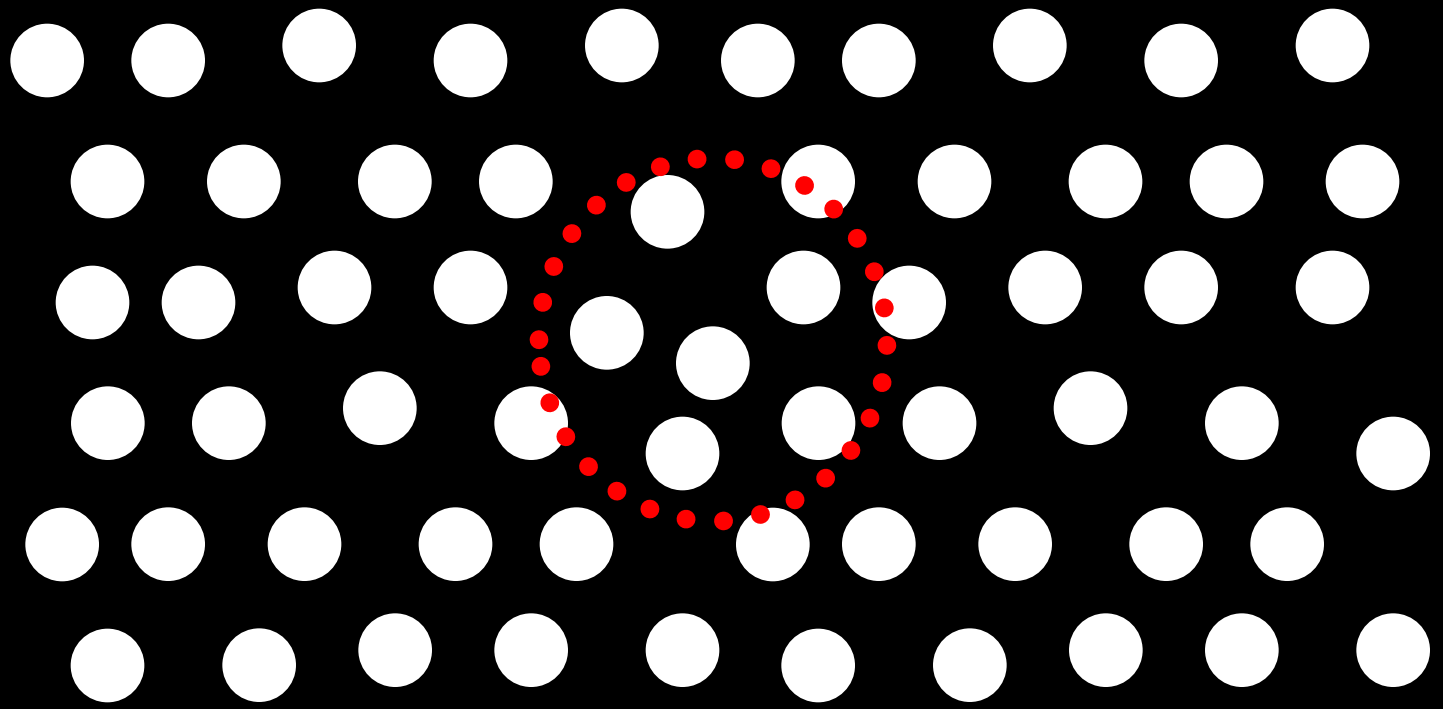
Molecular Dynamics



tsO45G-YFP traffic in HeLa cell

1. What is FRAP?

Fluorescence Recovery After Photobleaching (FRAP)



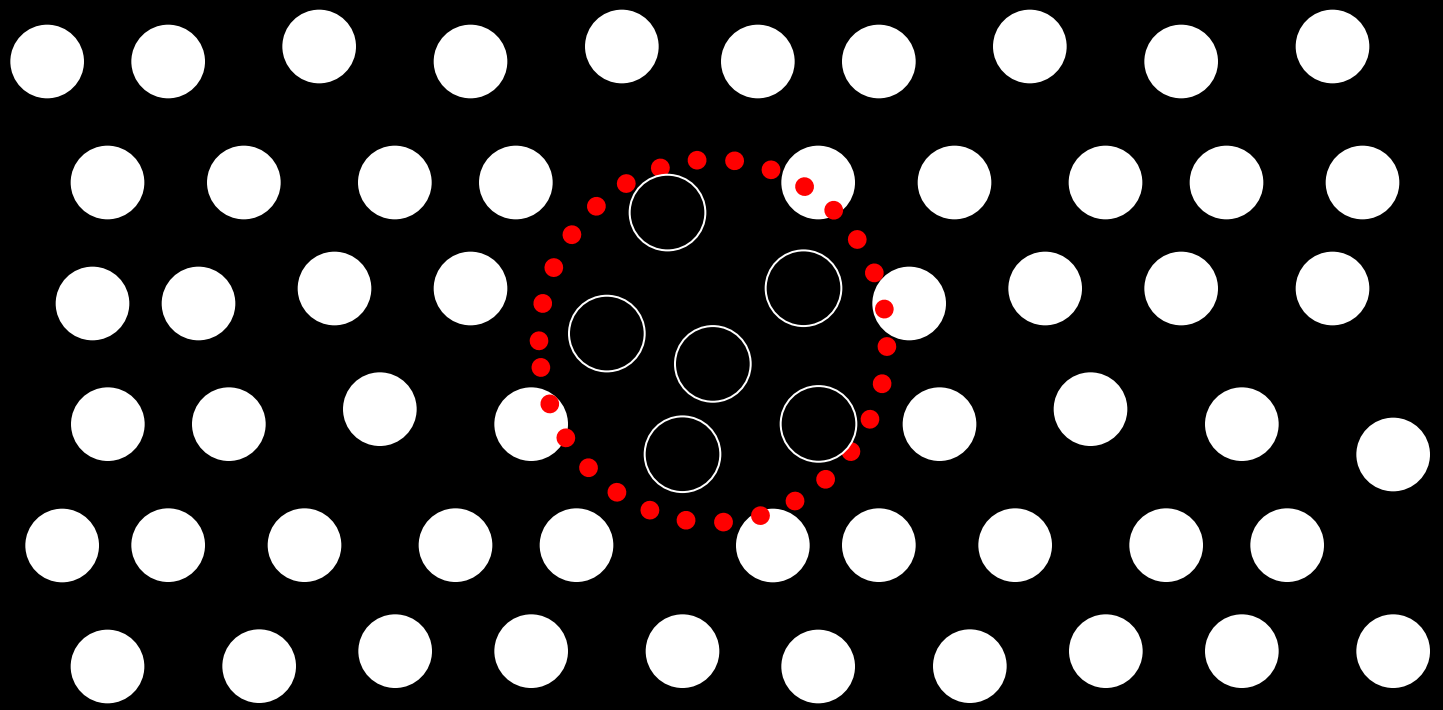
Let’s think of fluorescence molecules dispersed in a field. White circles represent the molecules.

We focus a strong laser beam to a spot (red dotted circle).

Kota Miura

1. What is FRAP?

Fluorescence Recovery After Photobleaching (FRAP)

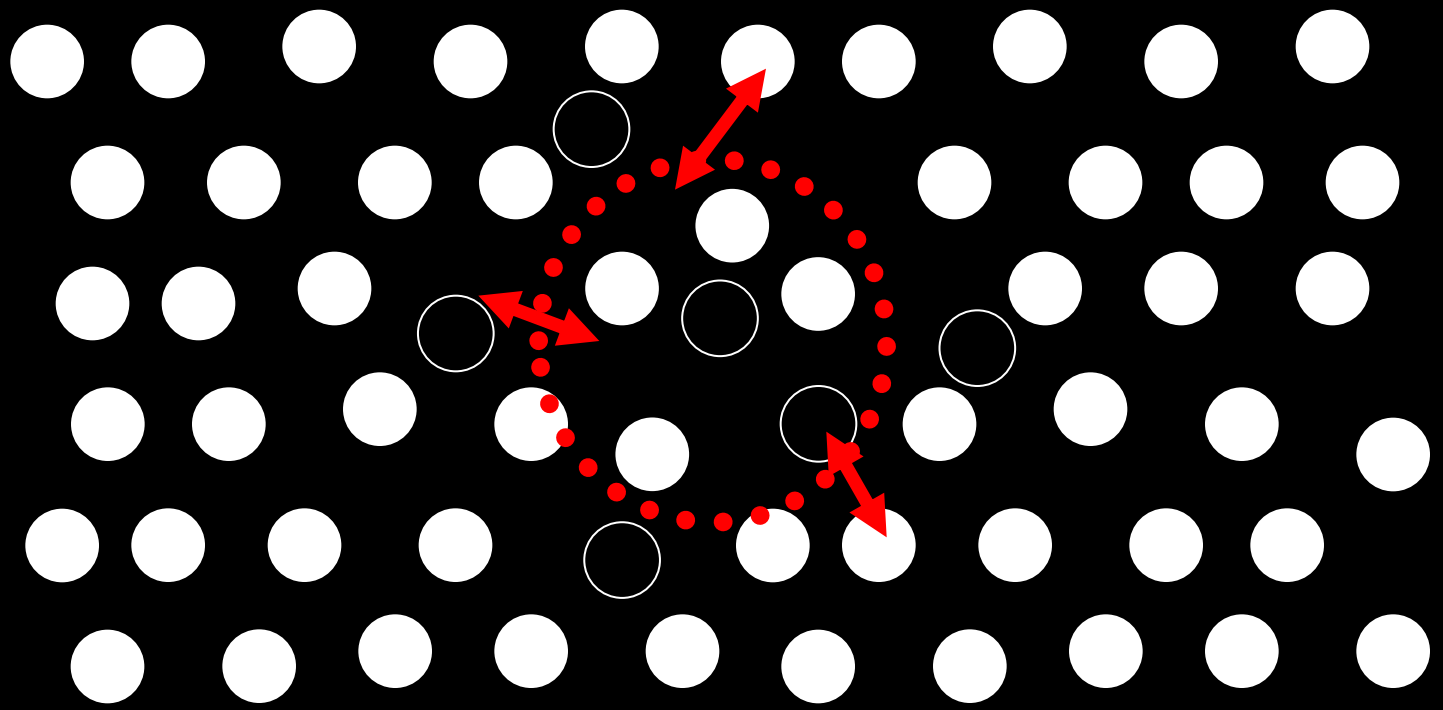


Then the strong irradiation BLEACHES the fluorescence at that spot.

Kota Miura

1. What is FRAP?

Fluorescence Recovery After Photobleaching (FRAP)

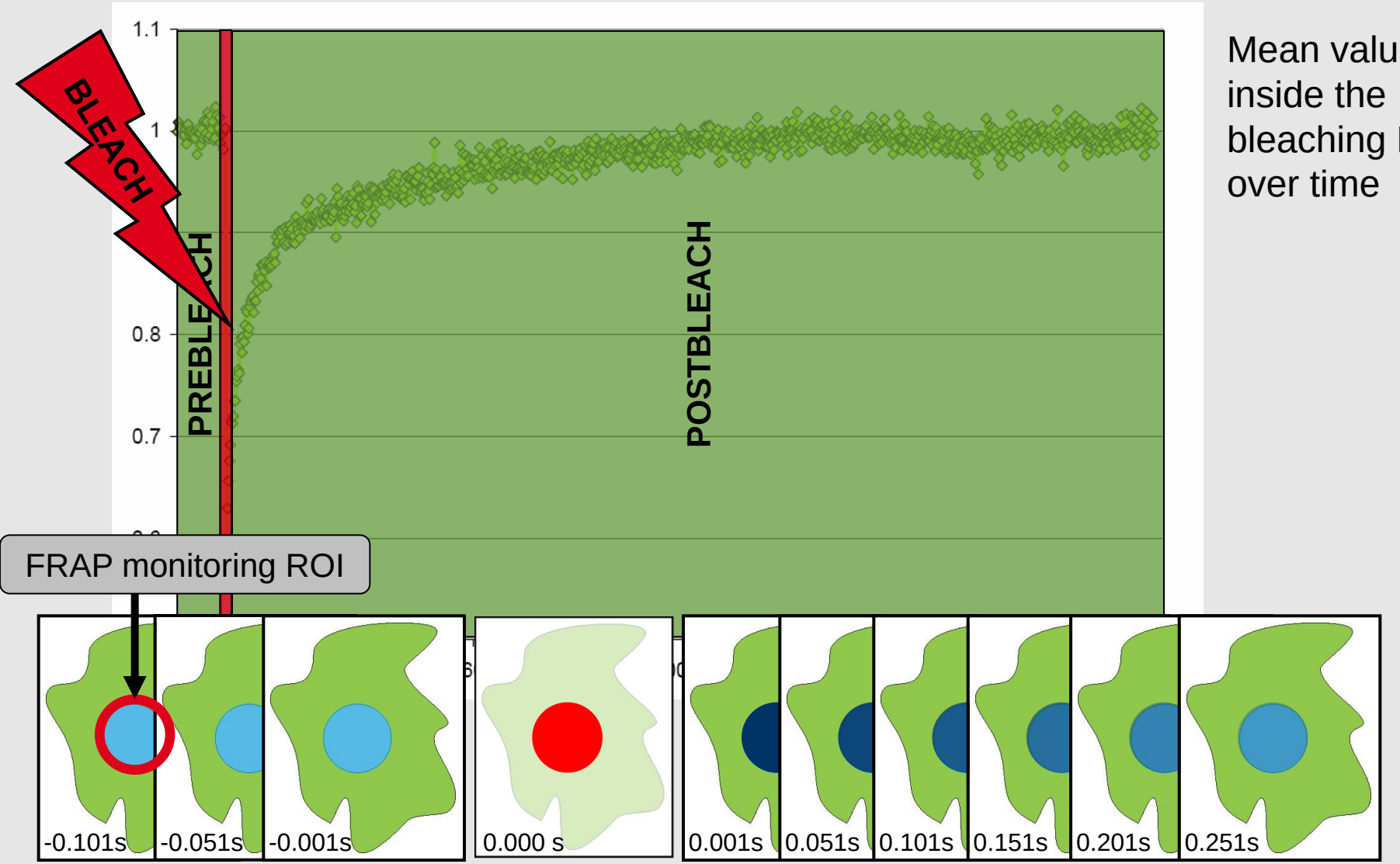


Since molecules are moving driven by diffusion or active transport,
bleached molecules exchange their place with un-bleached molecules.

Then the average intensity at the bleached spot recovers.

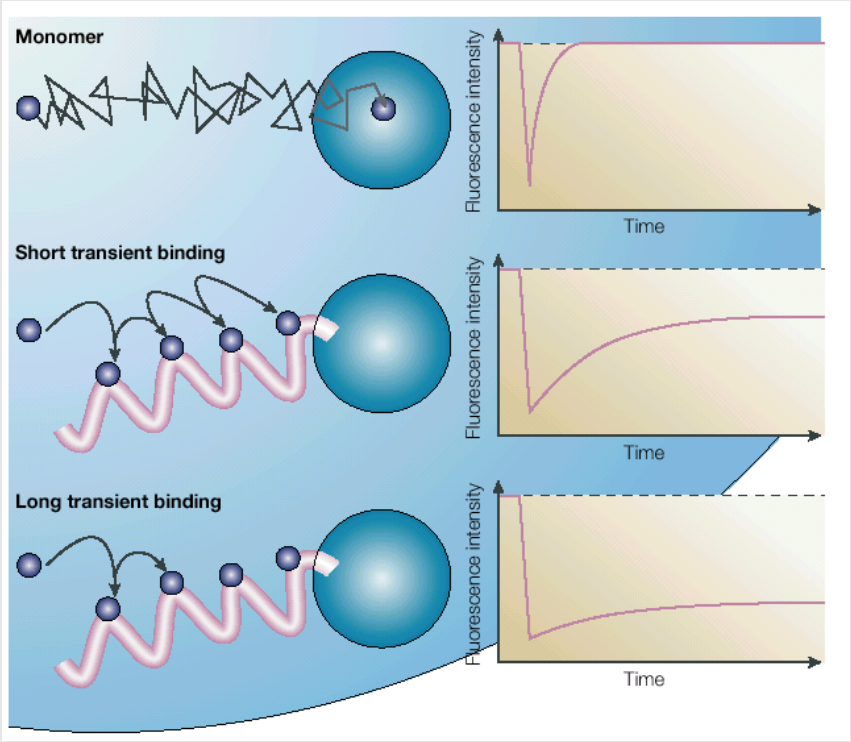
Kota Miura

Scheme of a FRAP experiment

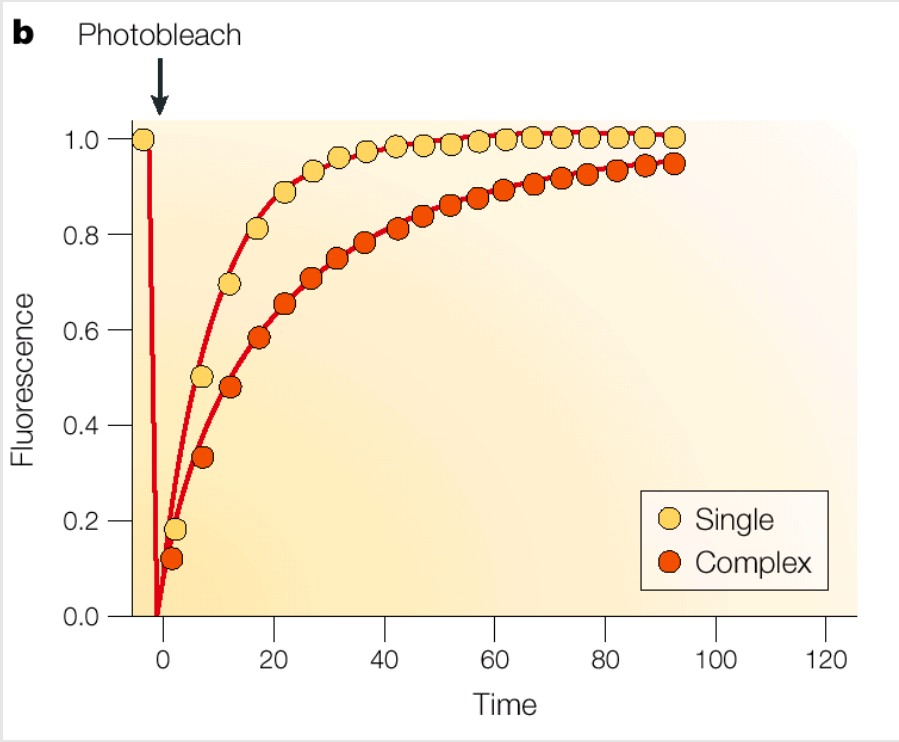


Mean value inside the bleaching ROI over time

Free diffusion vs. binding



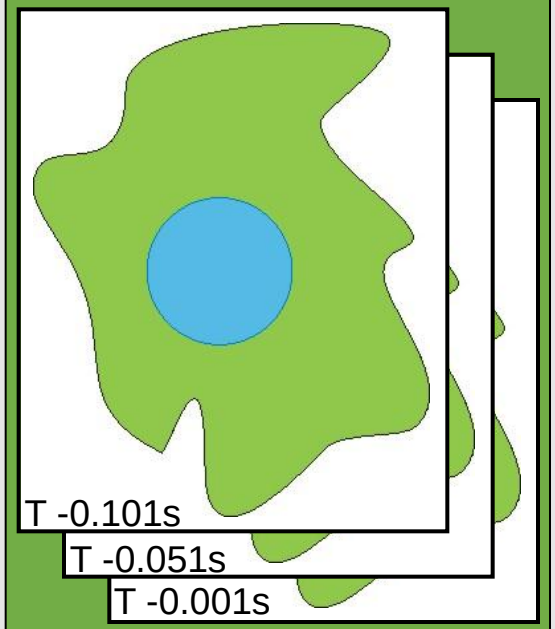
Phair and Mistelli, Nature Reviews MolCellBio, 2001



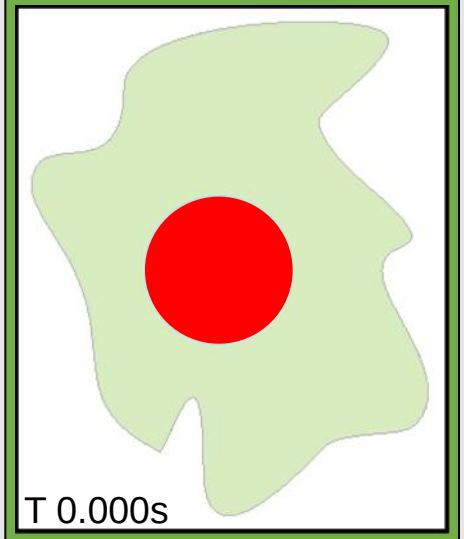
Lippincott-Schwartz et al. Nature CellBio Supp. 2003

Multiple populations with differing diffusion rates
→ multi-component equations

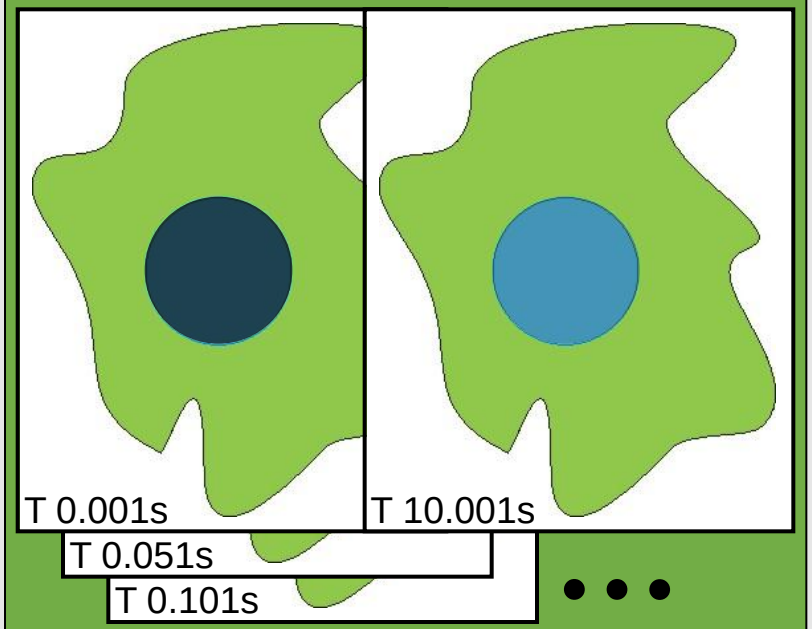
The ‘Ideal’ FRAP Acquisition



Prebleach Series
(“normal time-lapse”)

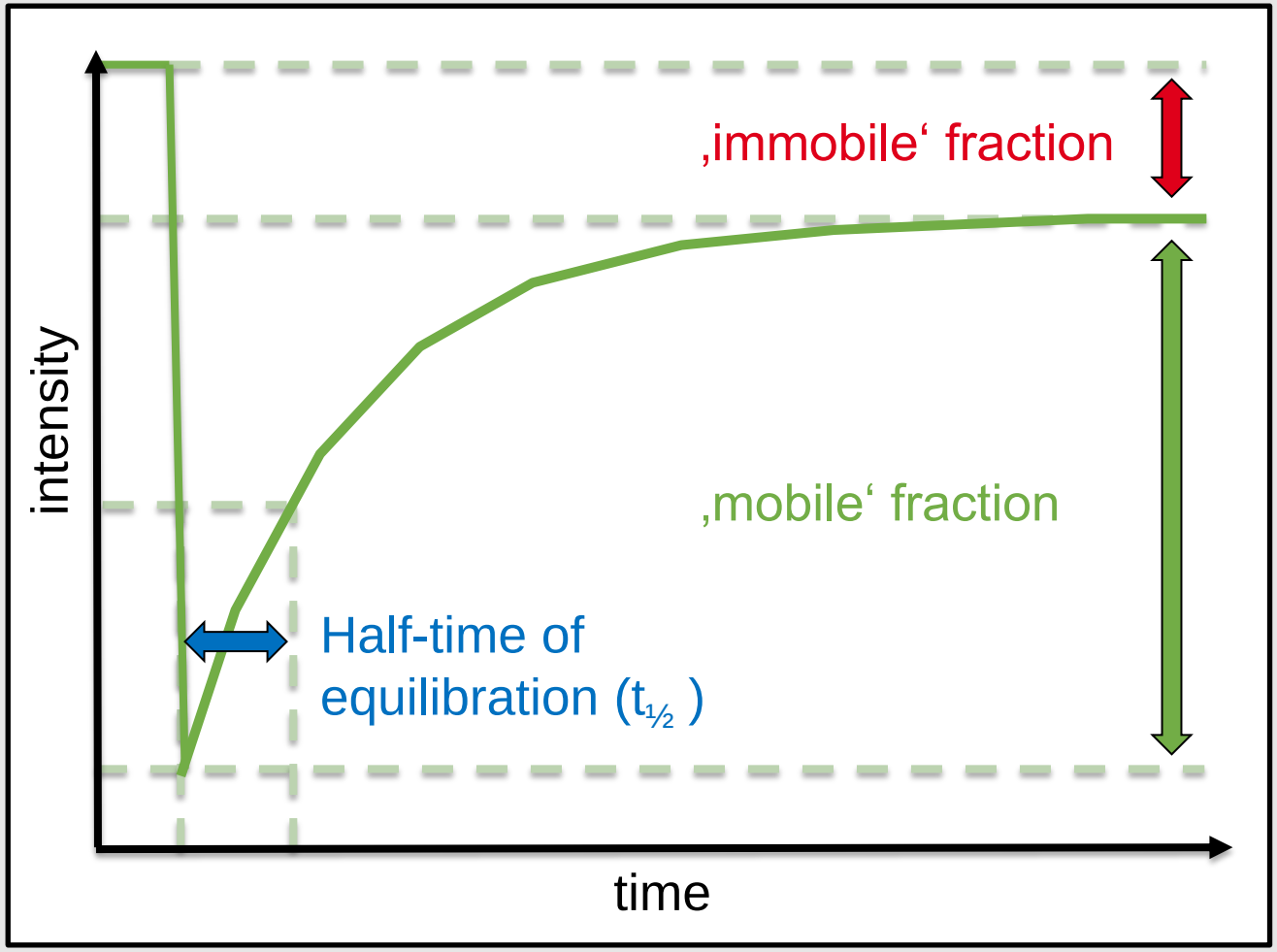


Instantaneous Bleach pulse



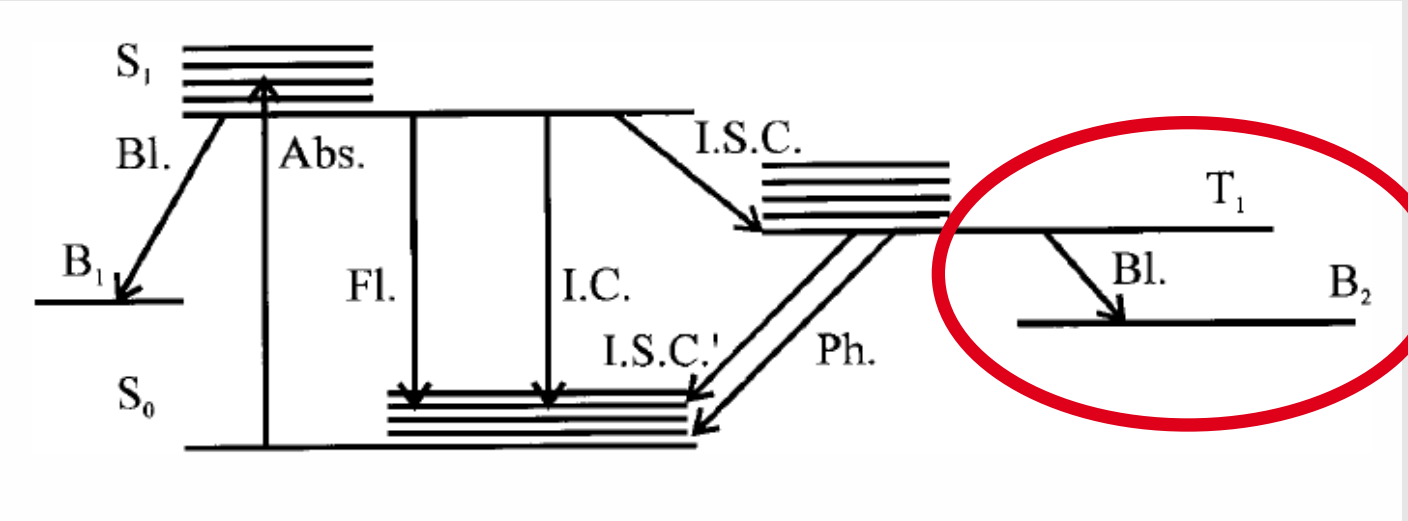
Postbleach Series
(“normal time-lapse” starting without delay after the bleach)
>20 data points until half-recovery

FRAP – basic parameters

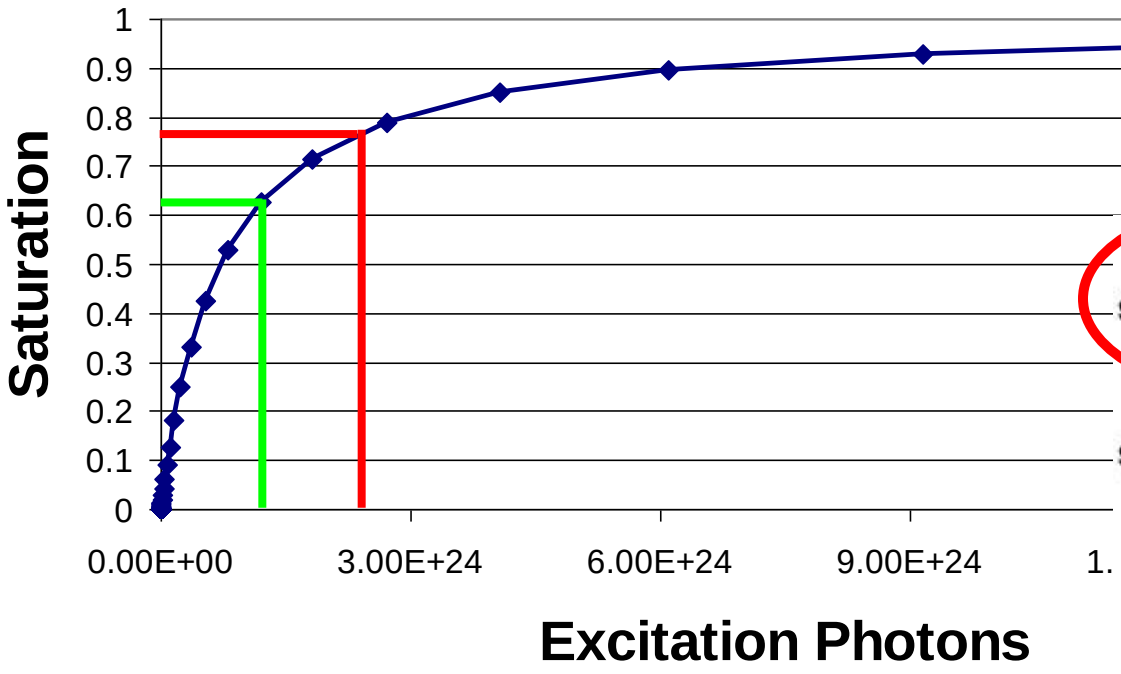


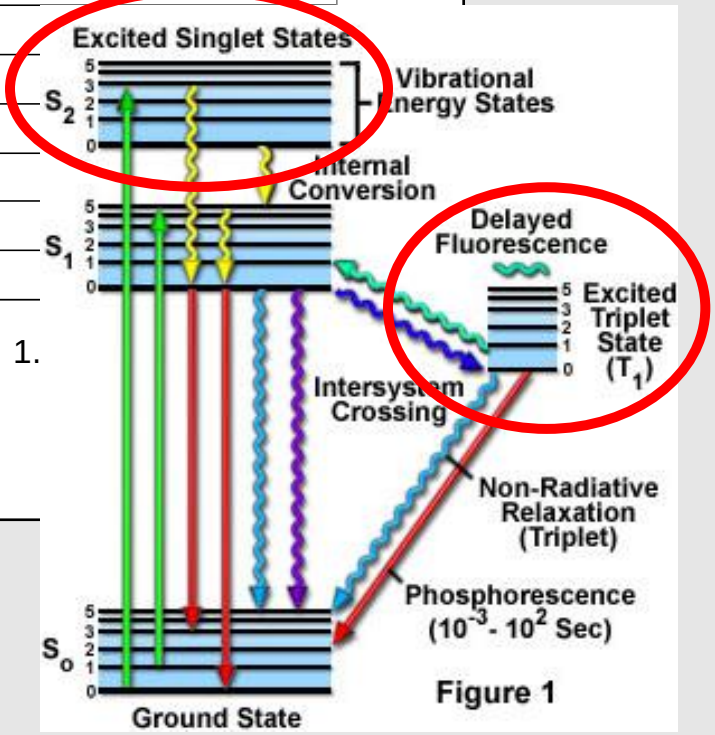
Bleaching

- The amount of fluorophore bleaching is dependent on the excitation power
- Bleaching should be avoided during acquisition, but should be sufficient and ‘instantaneous’ for the bleach pulse of a FRAP experiment
- Bleaching can also cause **photodamage**
  → bleach only to ~50% of initial intensity
  → repeat bleaching at same spot to check for differences due to photodamage)



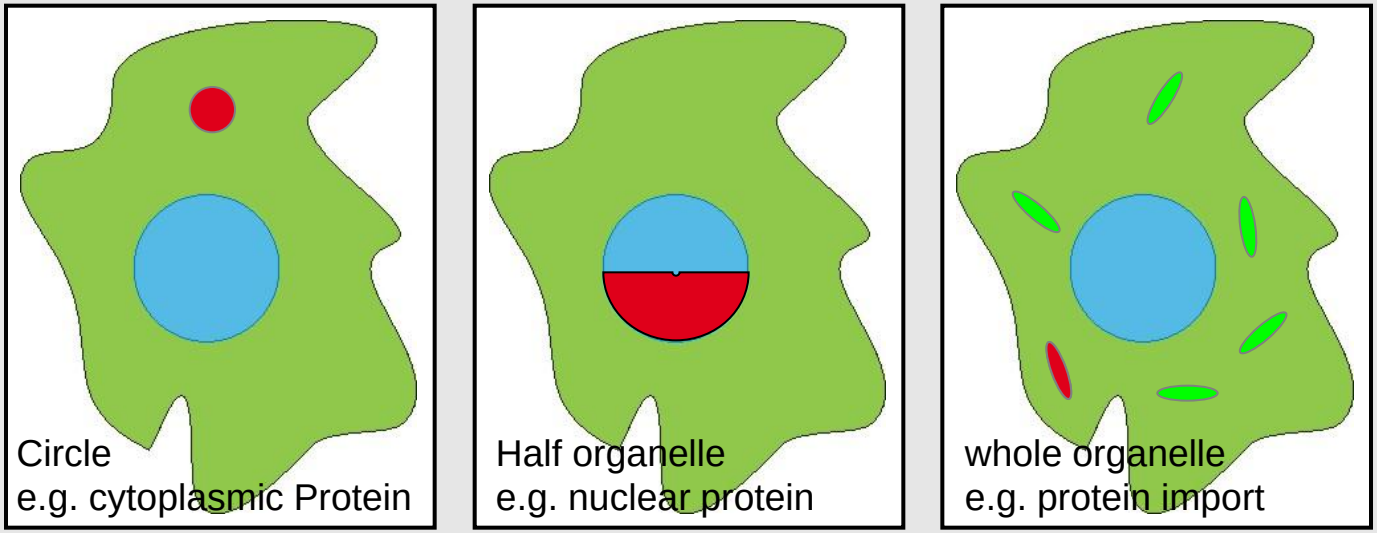
Bleaching depends on excitation intensity





FRAP conditions depend on the sample

- The bleaching ROI has to be matched to the protein/structure to FRAP



- Conditions are highly instrument dependent (laser power, % AOTF, PMT Voltage…) and cannot (easily) be compared between systems, but should be kept constant on one system to compare different conditions.

Controls

- Calibrate bleached volume in x,y and z
- Check dependence of recovery rate on different bleaching power (cross-linking ?)
- Repeat FRAP on same spot (difference due to photodamage ?)
- Dependence of recovery on ROI size ?
- Compare wiltype with mutant (e.g. non-binding mutant)
- Check for dark states / reversibility

Overview

- Introduction
- Application examples and Related techniques
  - FLIP: Fluorescence Loss In Photobleaching
  - iFRAP: inverse FRAP
  - Photoactivation (paGFP…)
  - Photoconversion (Kaede, Dendra, EOS…)

- Instrument setups for FRAP
- Basic FRAP analysis procedures

FRAP: Comparison of 2 conditions
ER-export with and without sterol depletion

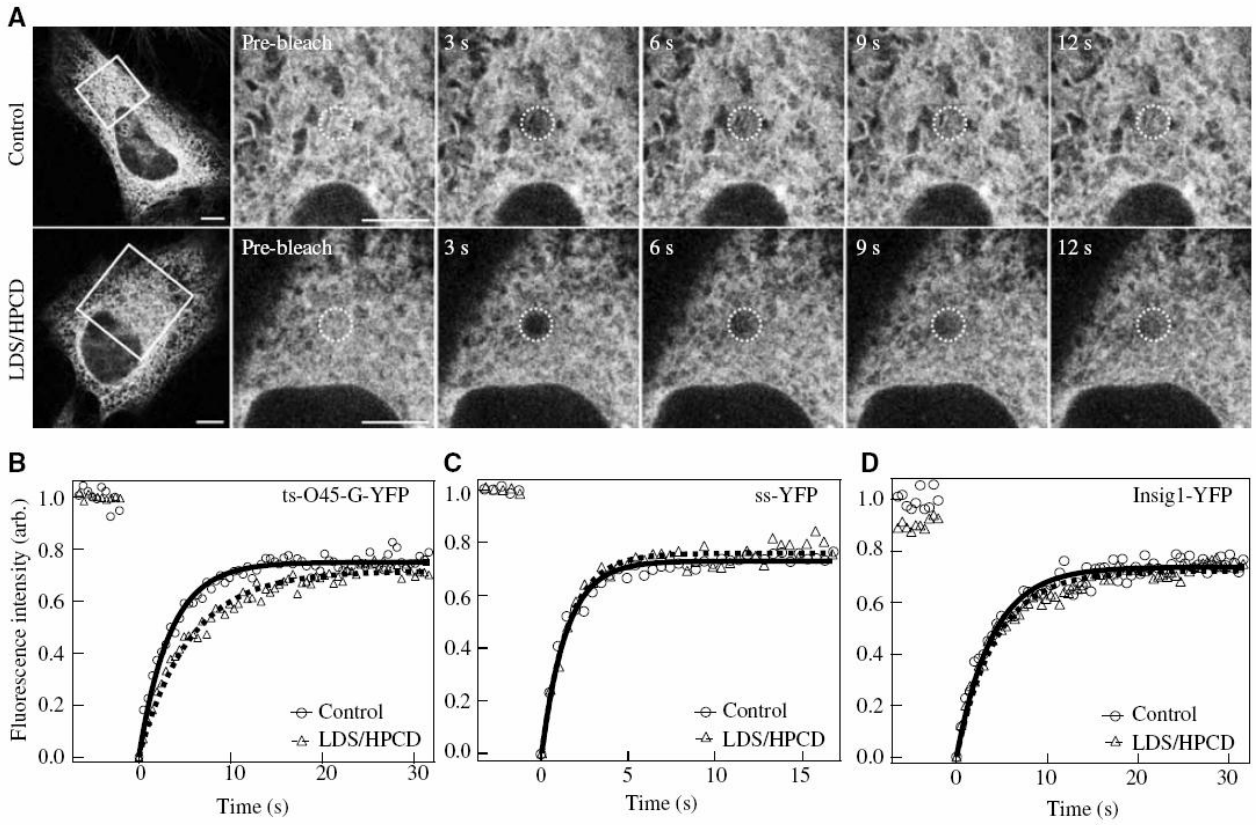
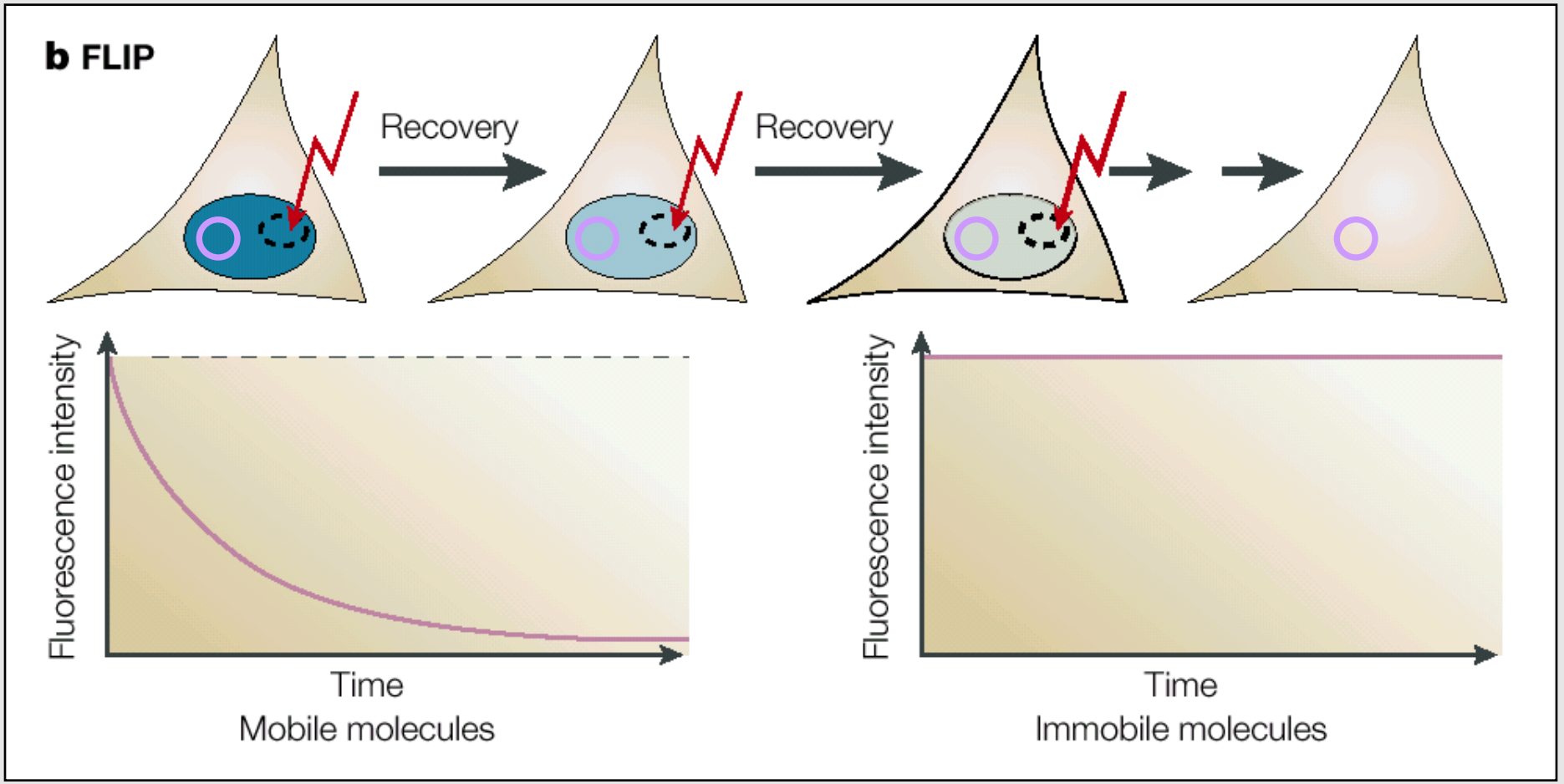


Figure 5 Lateral mobility of ts-O45-G-YFP in the ER is reduced upon sterol depletion. (**A**) FRAP analysis of ER-localized ts-O45-G-YFP in HeLa-cells incubated at 39.5°C under control (upper panel) or sterol-depleted conditions (lower panel). Images were obtained before photobleaching and at the indicated time points thereafter. The cell area acquired during the time course of the experiment is outlined by a box with the photobleached area (circle) in its centre. Bars: 5 μm. (**B–D**) FRAP analysis of ts-O45-G-YFP in the ER at 39.5°C (B), or ss-YFP (C) and Insig1-YFP (D) at 37°C under control (●, curve fit: full line) or sterol-depleted conditions (▲, curve fit: dashed line). Relative fluorescence intensities from one representative cell per condition were plotted against time (in s). Images were taken at 500 ms intervals (ss-YFP: 250 ms). For details on recovery curve analysis see Supplementary data.

Runz H et. al 2006: Sterols regulate ER-export dynamics of secretory cargo protein ts-O45-G. *EMBO Journal* **25:** 2953-2965

EMBL ALMF

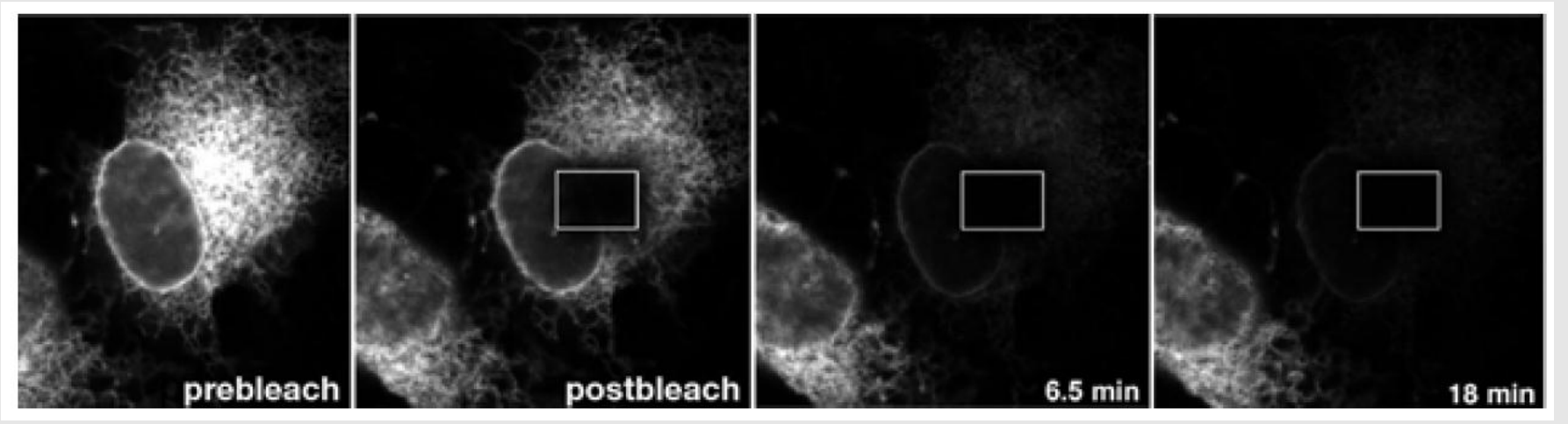
Fluorescence Loss in Photobleaching (FLIP)



Phair and Mistelli, Nature Reviews MolCellBio, 2001

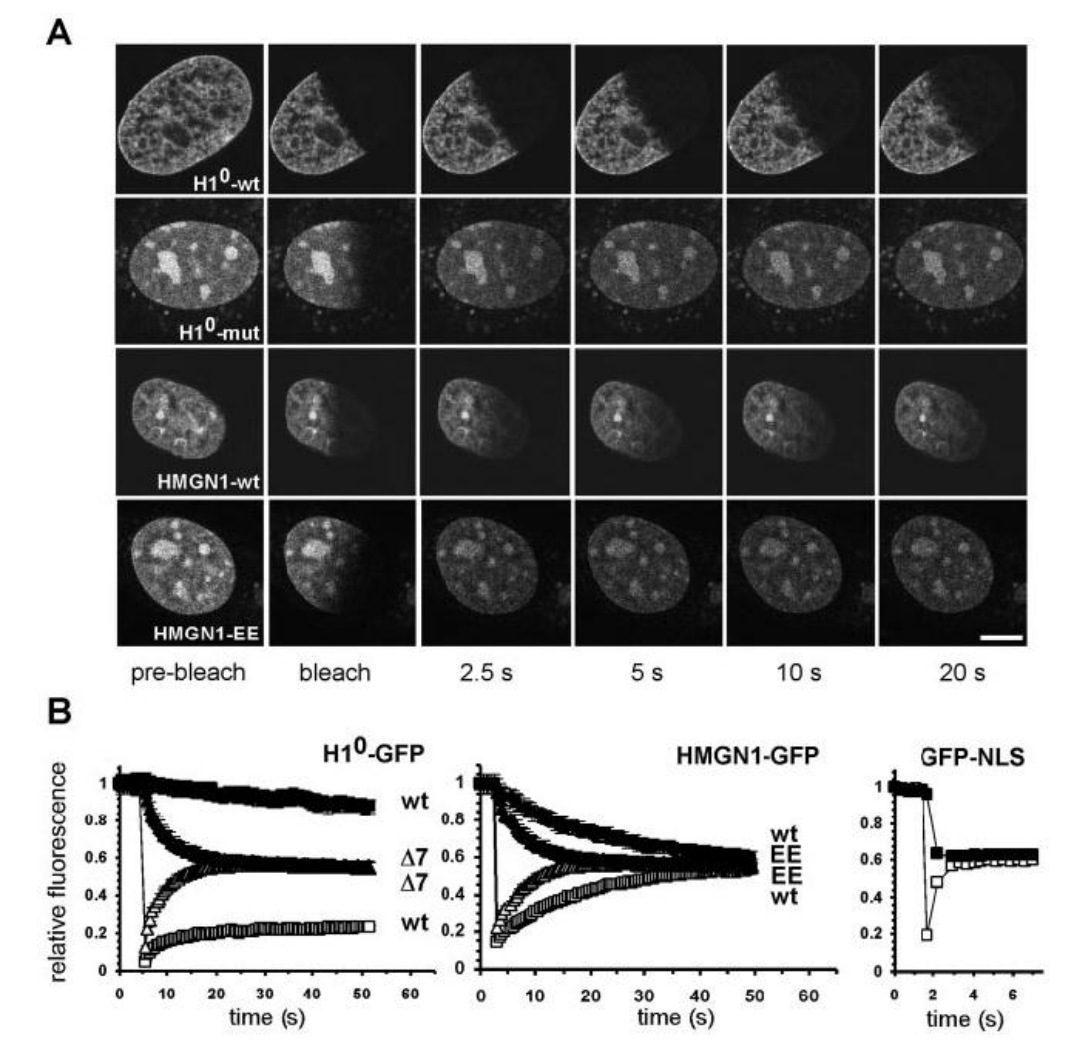
FLIP

- Probing organelle continuity by repetitive bleaching



GFP in the ER
Snapp E, Altan N, Lippincott-Schwartz J (2003) Measuring protein mobility by photobleaching GFP chimeras in living cells. In *Current Protocols Cell Biology*, 21. Wiley

FRAP-FLIP

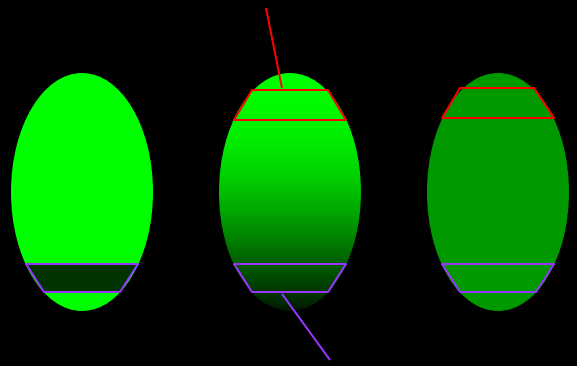


Phair et al. 2004
Mol Cell Biol **24:** 6393-6402

Combined FRAP and FLIP

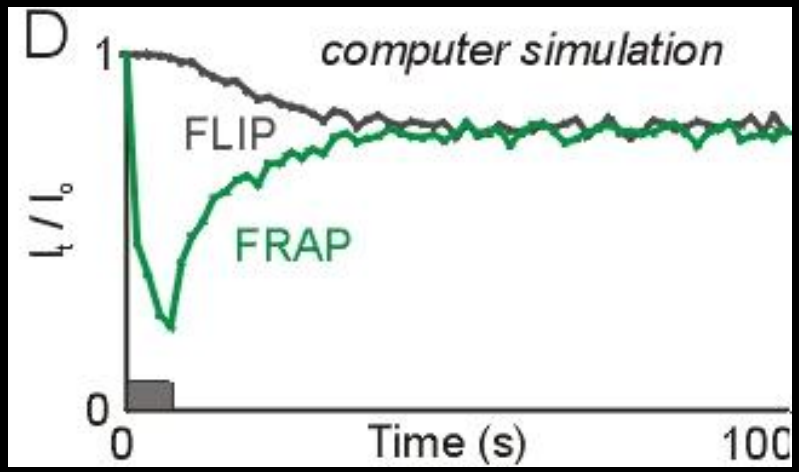
fluorescence loss in photobleaching

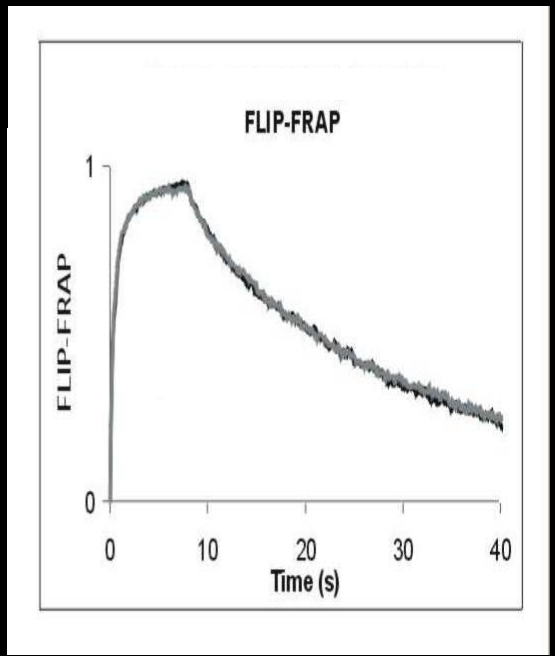
(FLIP)



(FRAP)

fluorescence redistribution
after photobleaching





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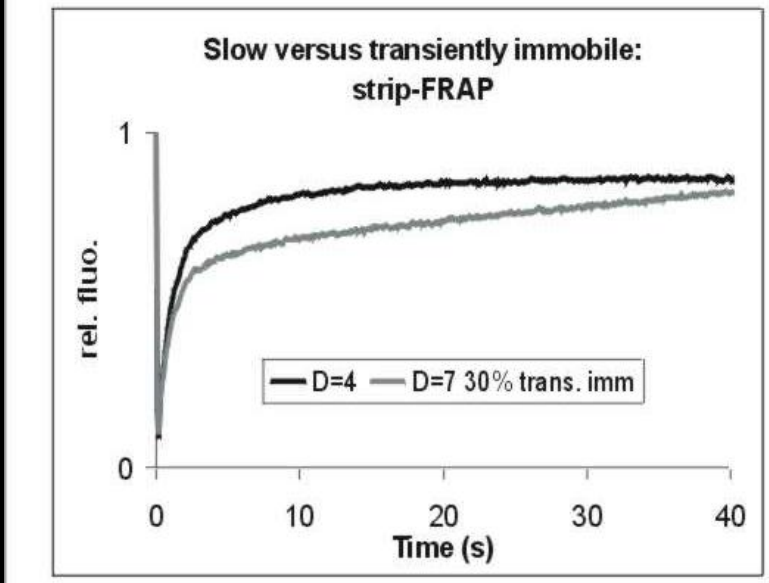
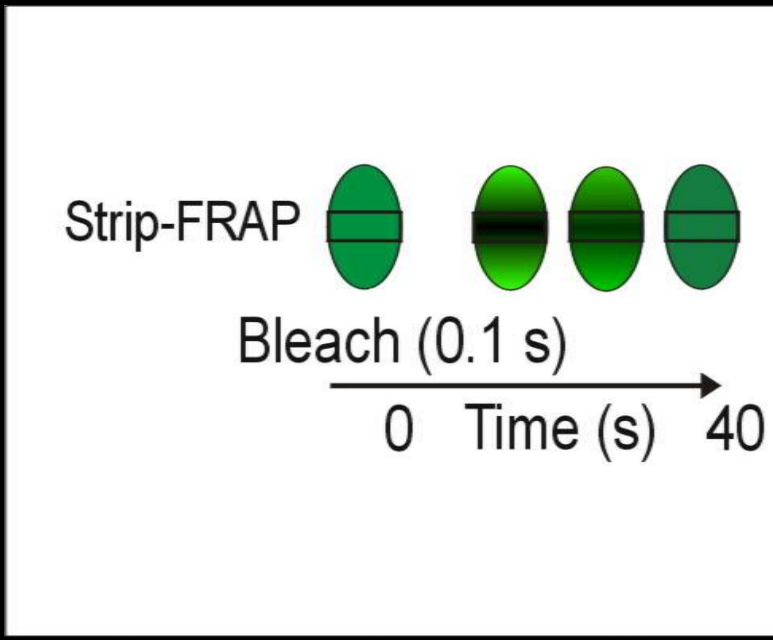
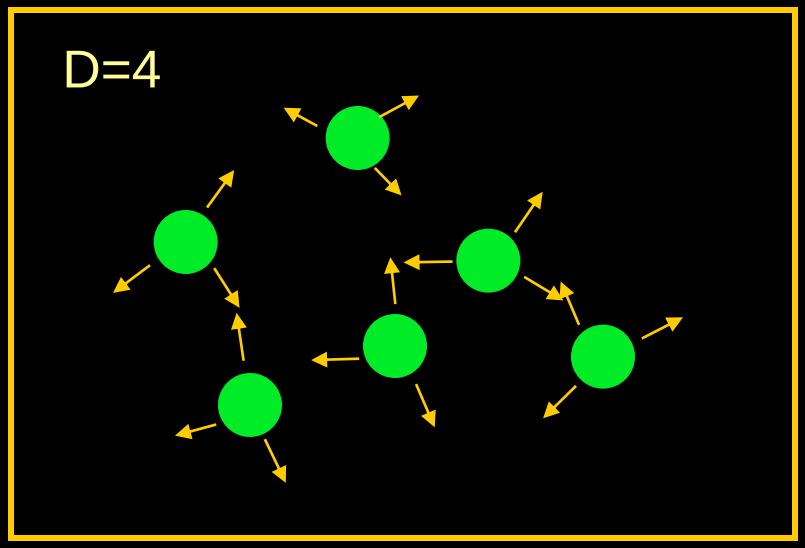
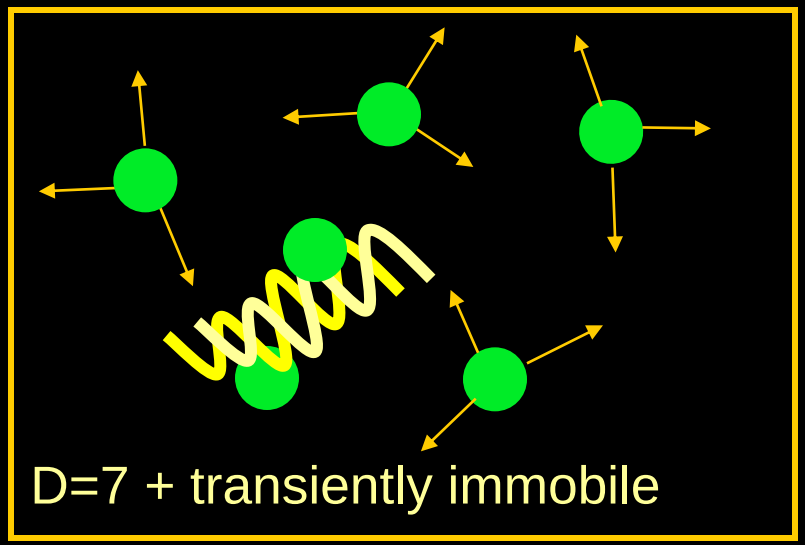
Houtsmuller









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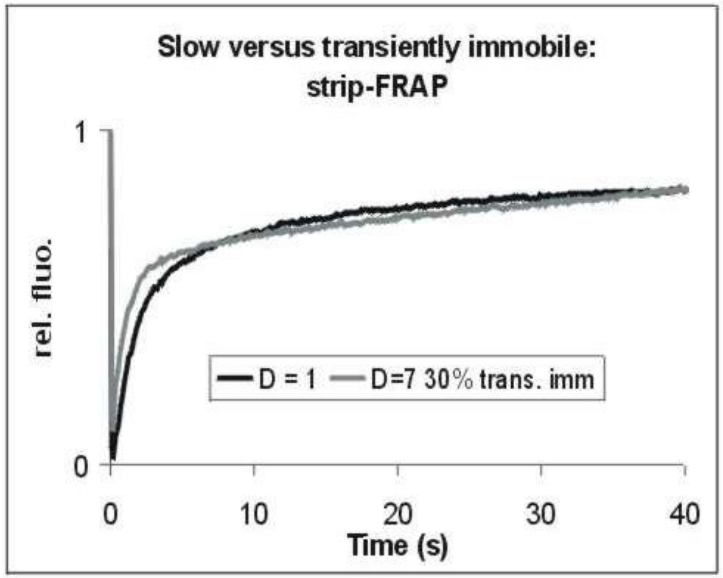

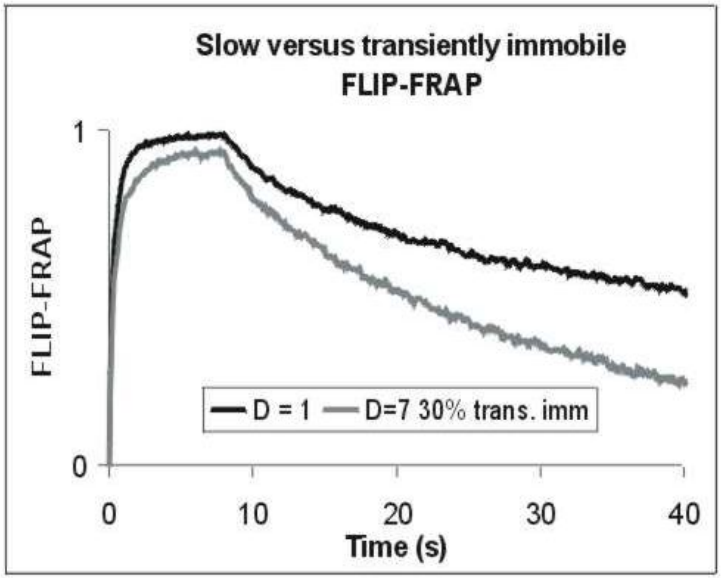

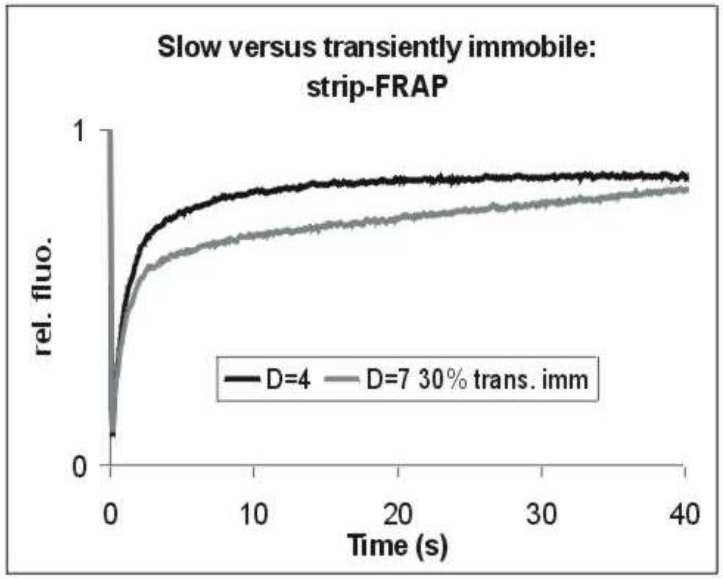

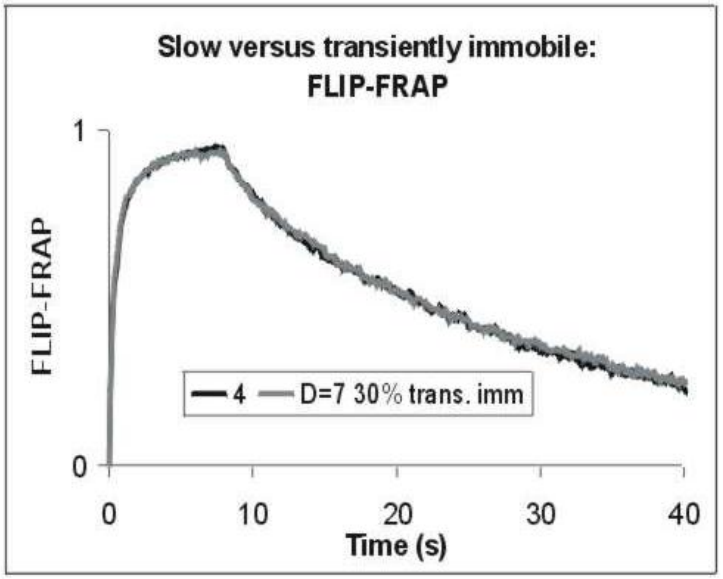

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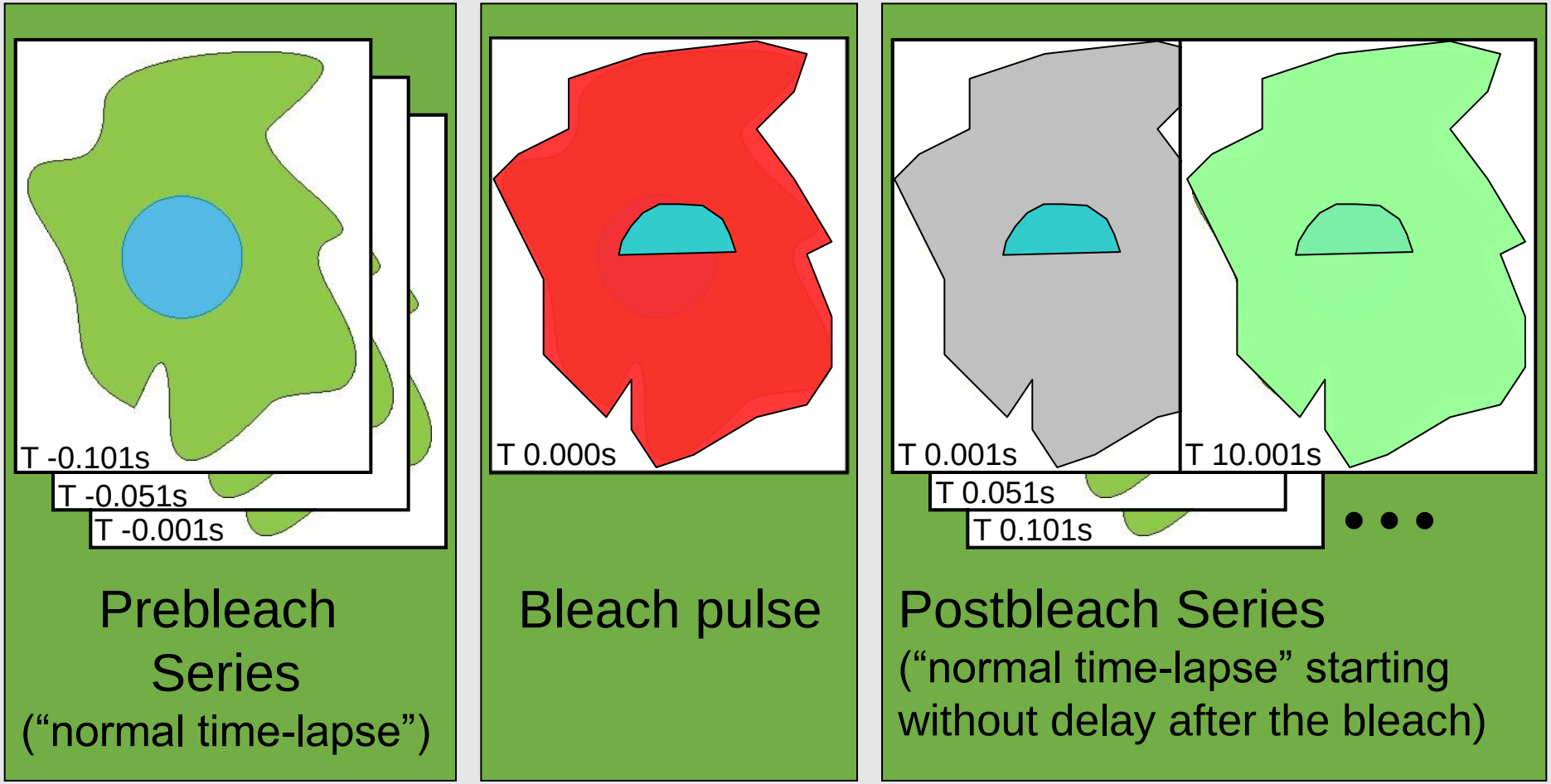
iFRAP

Inverse FRAP (iFRAP): All fluorescence except the region of interest is bleached and the loss of fluorescence is monitored.

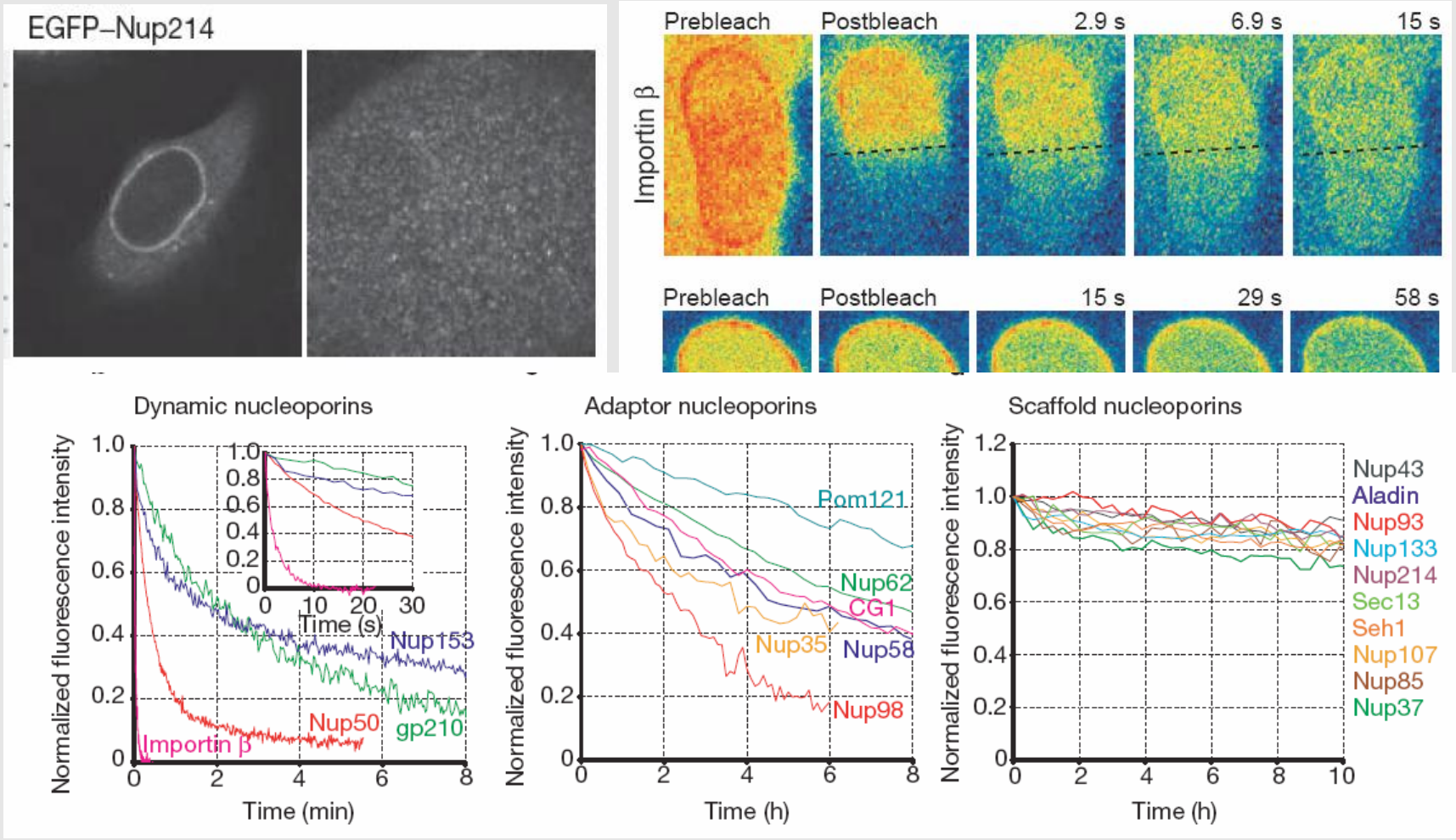


Prebleach Series
("normal time-lapse")

Bleach pulse

Postbleach Series
("normal time-lapse" starting without delay after the bleach)

iFRAP: Nuclear Pore Complex



Rabut G, Doye V, Ellenberg J (2004) Nat. Cell Biology 6: 1114-1121

Photoactivation and Conversion

Protein tracking

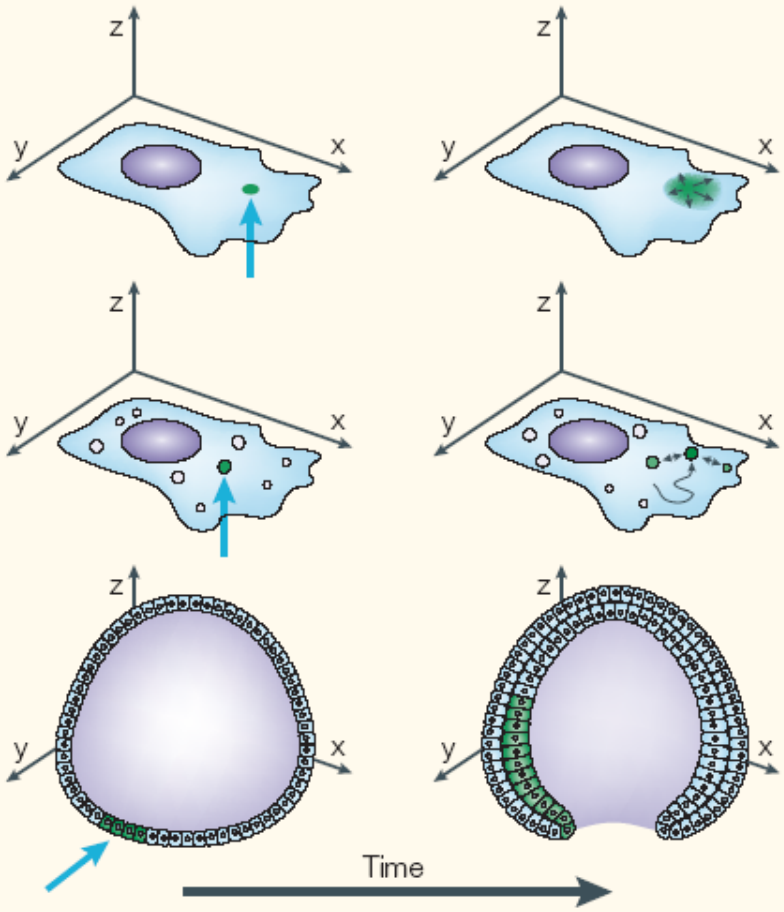
Parameters determined:
- Movement rate and direction
- Diffusion coefficient
- Mobile and immobile fractions
- Time parameters of compartmental residency and exchange between compartments
- Rate of turnover

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



From: Lukyanov, K. A. et al. (2005) Nat Rev Mol Cell Biol **6**(11): 885-890.

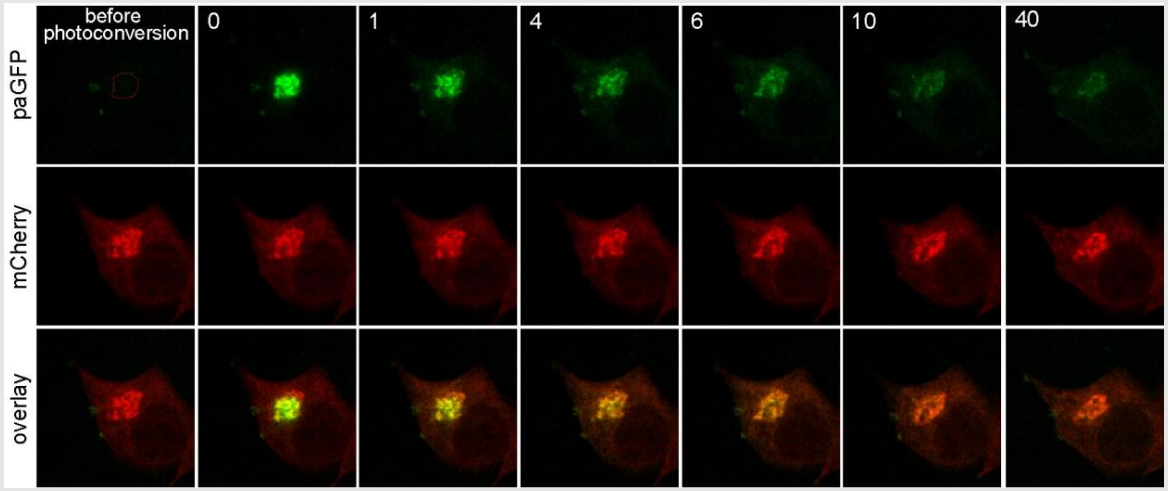
Photoactivation and Conversion

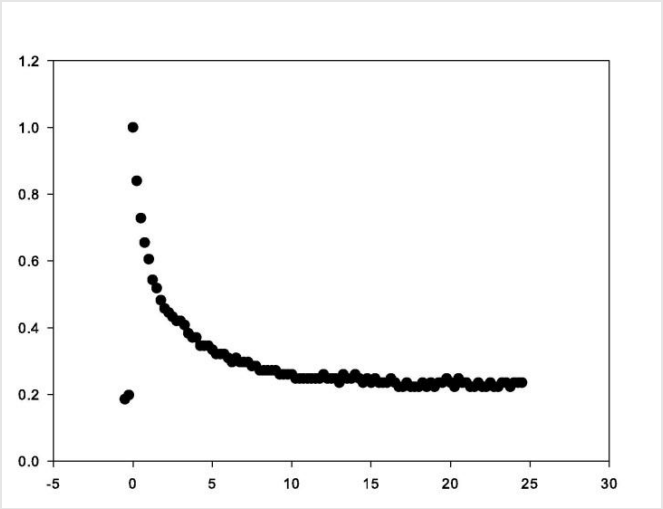
Fluorescent Protein (Acronym-Color)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Molecular Structure	Relative Brightness (% of EGFP)	pKa
PA-GFP (G/NA)	400	515	20,700	0.13	Monomer	8	4.5
PA-GFP (G)	504	517	17,400	0.79	Monomer	41	-
PS-CFP (C)	402	468	34,000	0.16	Monomer	16	4.0
PS-CFP (G)	490	511	27,000	0.19	Monomer	15	6.0
PS-CFP2 (C)	400	468	43,000	0.20	Monomer	26	4.3
PS-CFP2 (G)	490	511	47,000	0.23	Monomer	32	6.1
PA-mRFP1 (R)	578	605	10,000	0.08	Monomer	3	4.4
Kaede (G)	508	518	98,800	0.88	Tetramer	259	5.6
Kaede (R)	572	580	60,400	0.33	Tetramer	59	5.6
Kikume (KikGR; G)	507	517	53,700	0.70	Tetramer	112	7.8
Kikume (KikGR; R)	583	593	35,100	0.65	Tetramer	68	5.5
wtEosFP (G)	506	516	72,000	0.70	Tetramer	150	-
wtEosFP (R)	571	581	41,000	0.55	Tetramer	67	-
dEosFP (G)	506	516	84,000	0.66	Dimer	165	-
dEosFP (R)	569	581	33,000	0.60	Dimer	59	-
tdEosFP (G)	506	516	84,000	0.66	Monomer	165	-
tdEosFP (R)	569	581	33,000	0.60	Monomer	59	-
mEosFP (G)	505	516	67,200	0.64	Monomer	128	5.5
mEosFP (R)	569	581	37,000	0.62	Monomer	68	5.5
Dendra (G)	488	505	21,000	0.70	Monomer	44	6.6
Dendra (R)	556	575	20,000	0.72	Monomer	43	6.9
Dendra2 (G)	490	507	45,000	0.50	Monomer	67	6.6
Dendra2 (R)	553	573	35,000	0.55	Monomer	57	6.9
Kindling (R)	580	600	59,000	0.07	Tetramer	12	-
Dronpa (G)	503	518	95,000	0.85	Monomer	240	5.0

www.olympusconfocal.com/applications/opticalhighlighters.html

Photoactivation + Reference

Transport speed between compartments: Golgi ↔ plasma membrane

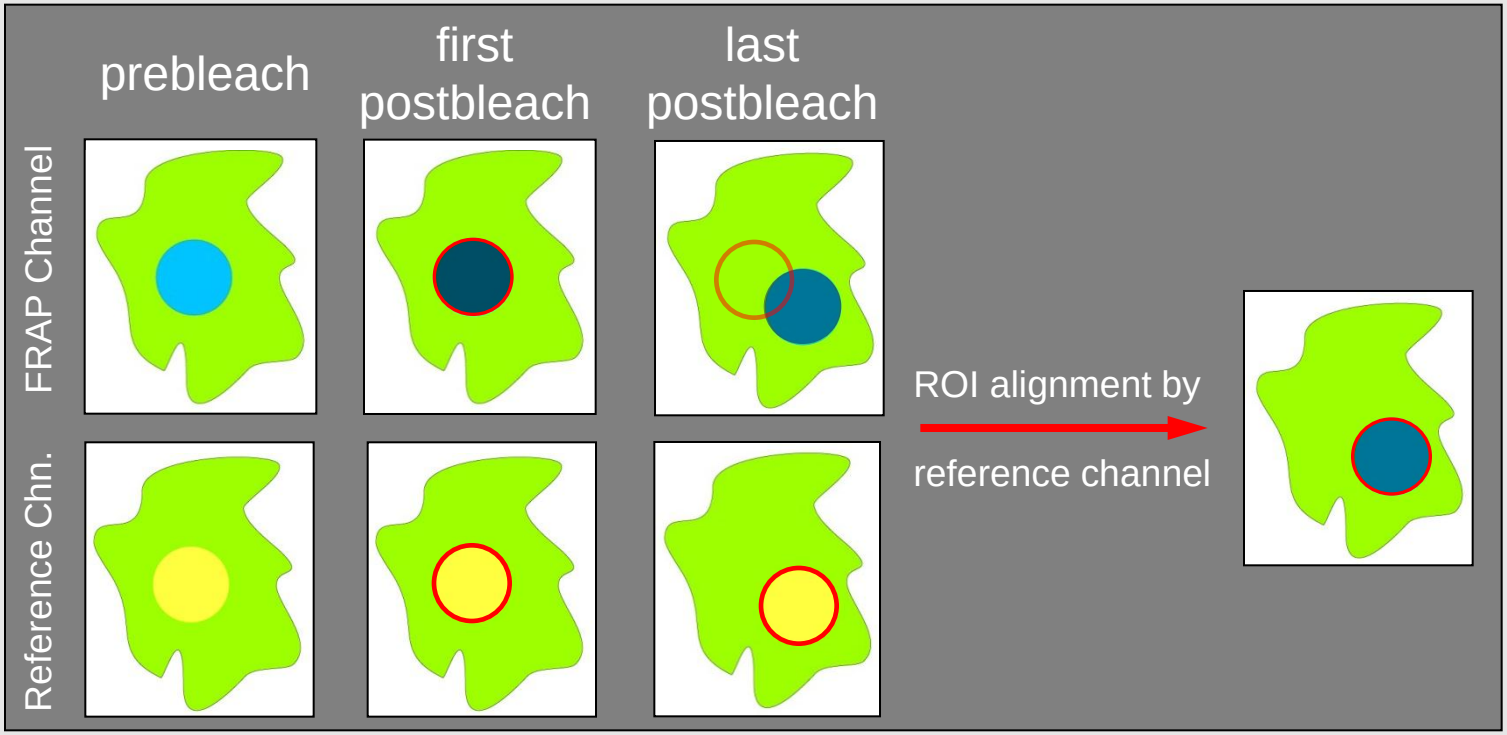




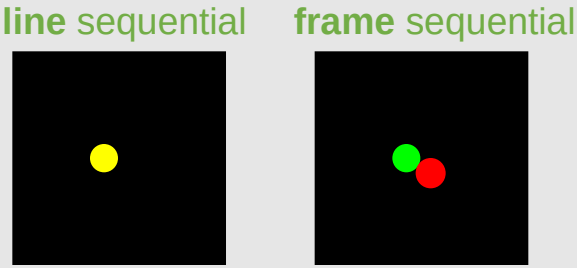
RAS (localized to plasma membrane and Golgi) tagged with paGFP (green) and mCherry (red)
O.Rocks (Bastiaens Group)

Reference Channel

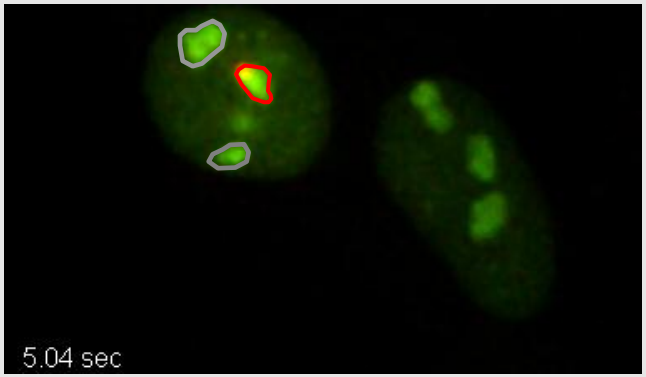
- For long-term FRAP experiments it might be necessary to include a reference channel, which shows the bleached structure.

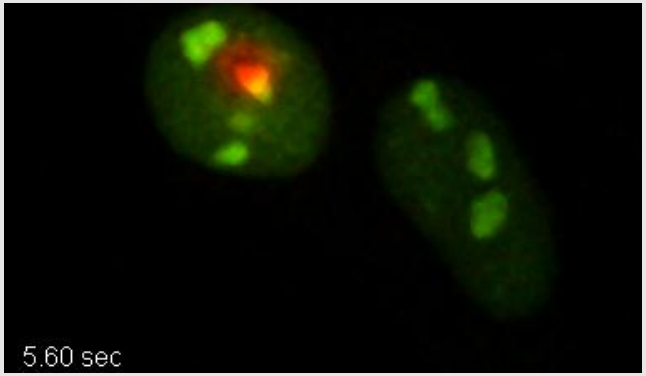


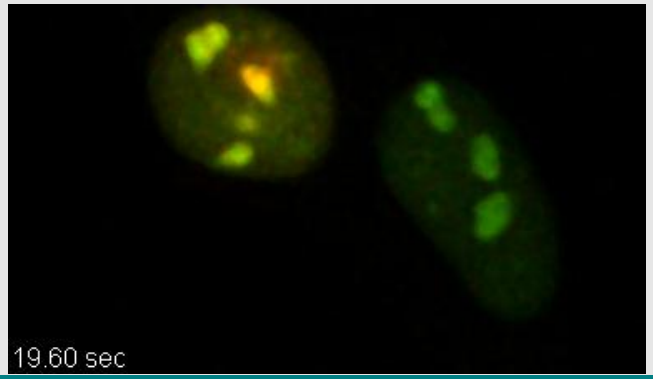
- If sequential acquisition is necessary, use line-by-line sequential!



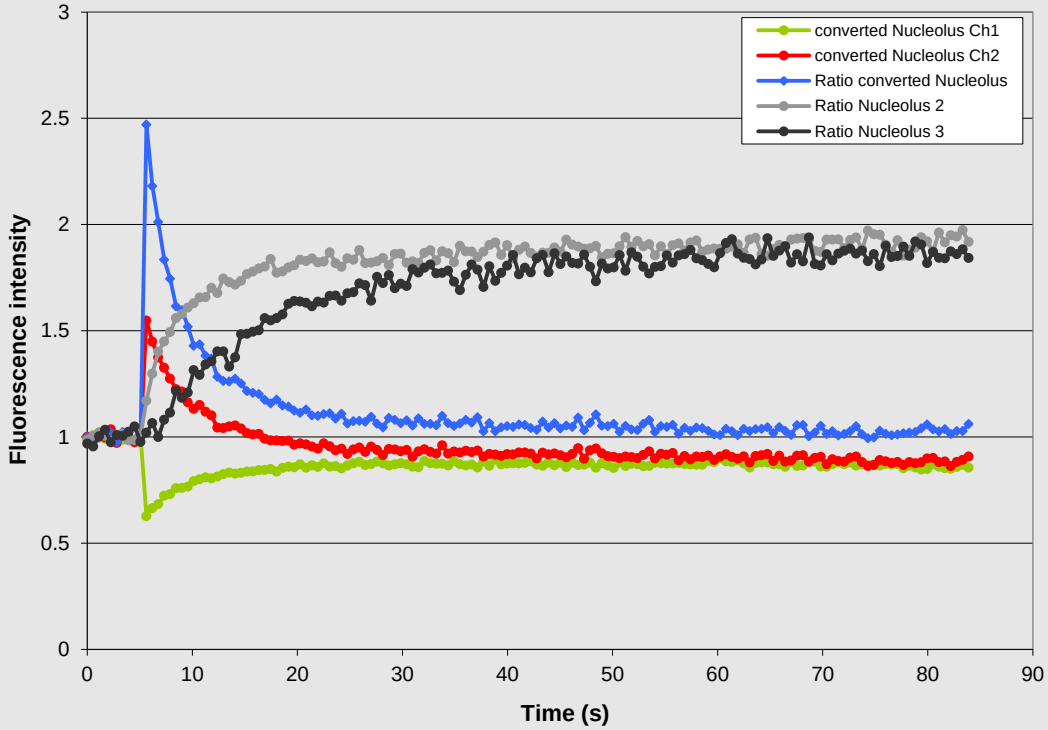
Dendra2-Fibrillarin – Example from a course







Nucleolus highlighted with a Dendra 2 fibrillarin fusion protein is converted with tornado scan (Olympus FV1000)



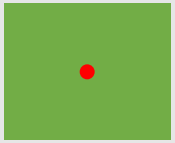
EMBL ALMF

Overview

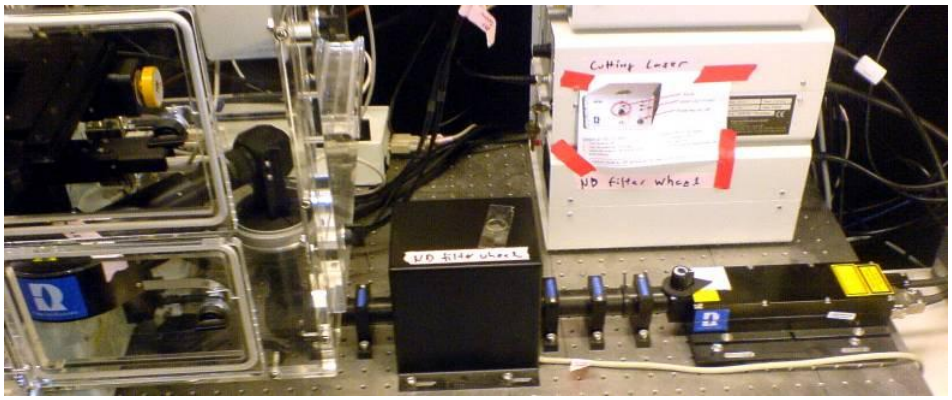
- Introduction
- Application examples
- Related techniques
- Instrument setups for FRAP
- Basic FRAP analysis procedures

FRAP on Epifluorescence microscope

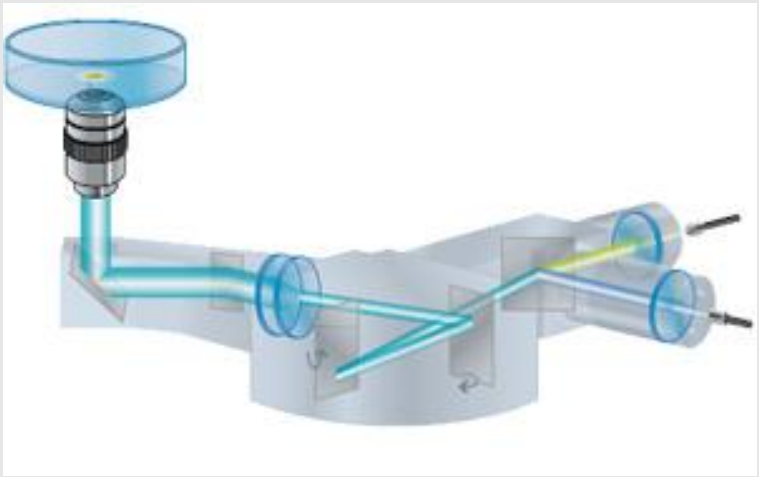
- Fixed laser spot incoupling



- Laser incoupling via scanner

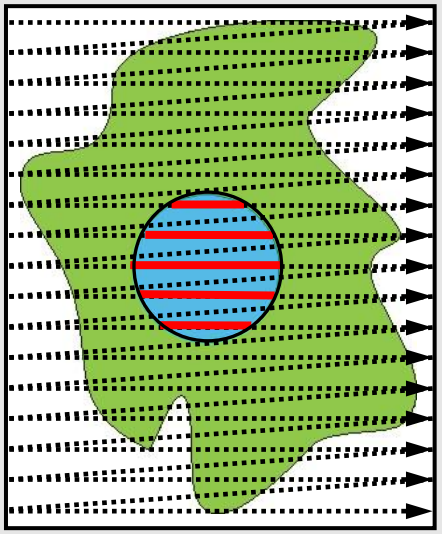


Olympus / Rapp OptoElectronic, 355 nm pulsed laser (cutting)



FRAP on CLSM

- current commercial CLSMs can control the laser intensity pixel by pixel with AOTFs
  → scanning of Regions of interest (ROI)



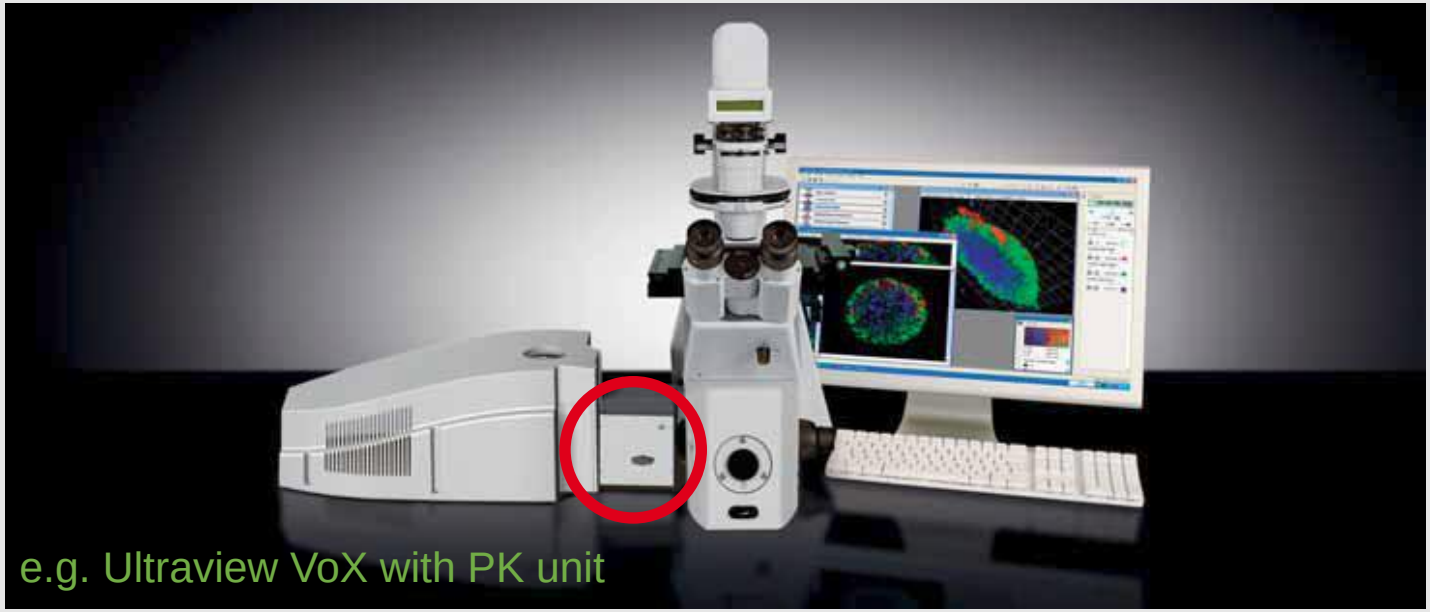
- Bleach regions are very flexible

- Together with GFPs the commercial availability of ROI bleaching with CLSMs was important for FRAP revival
- Pinhole can be adjusted appropriately

FRAP on Spinning disk

- Laser coupling via scanner

- Low bleaching during acquisition
- Fast frame rate
- A flipping mirror switches between imaging and bleaching



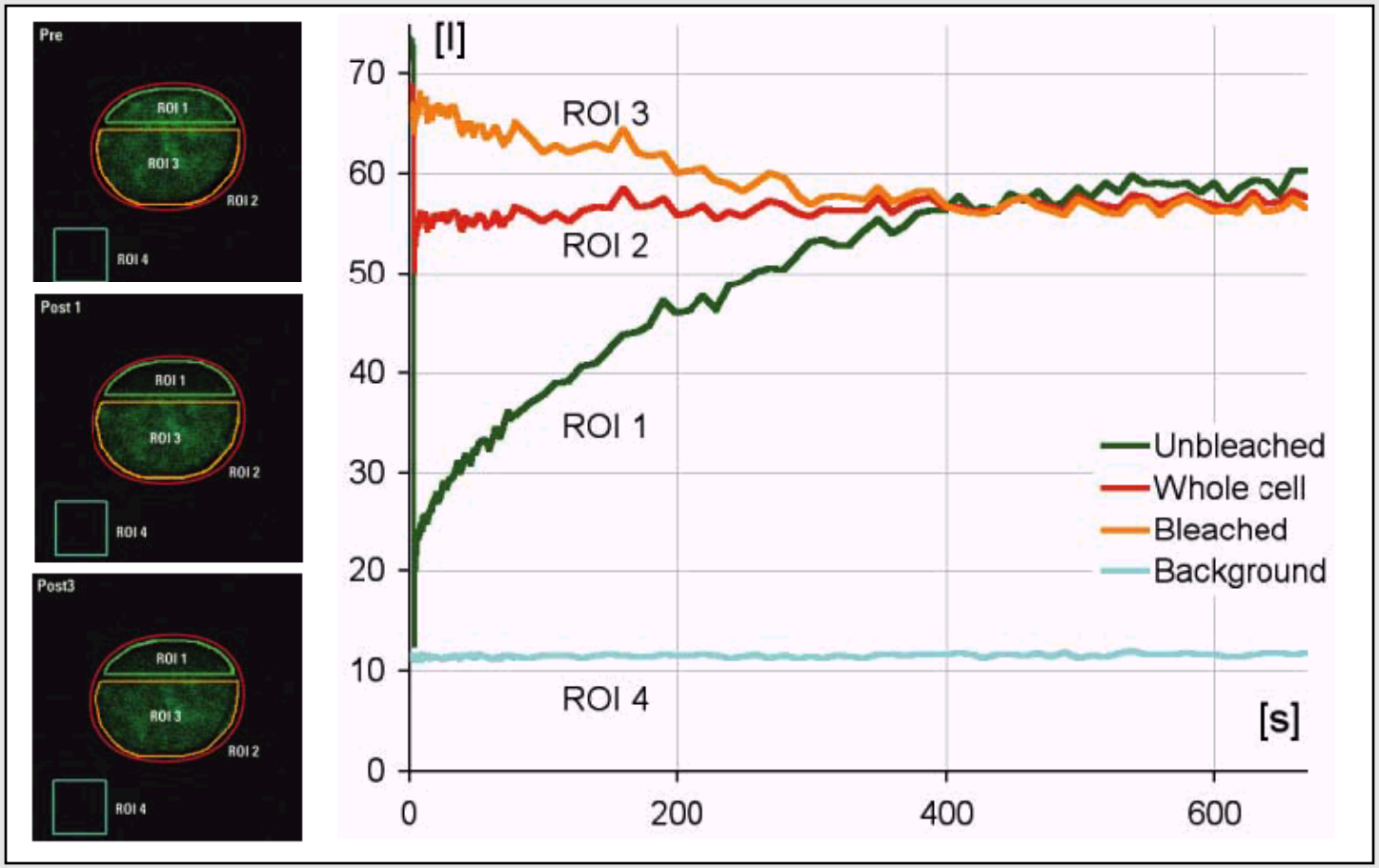
e.g. Ultraview VoX with PK unit

Overview

- Introduction
- Application examples and related techniques
- Instrument setups for FRAP
- Basic FRAP analysis procedures
  - Retrieving raw data
  - Qualitative vs. quantitative analysis
  - Recovery time
  - Mobile / immobile fraction
  - Photobleaching correction
  - Data normalization
  - Curve fitting

Basic Frap analysis: retrieving raw data

Mean intensities inside regions of interest (ROI) are measured over time.



Kappel and Eils, Leica App.Letter 2004

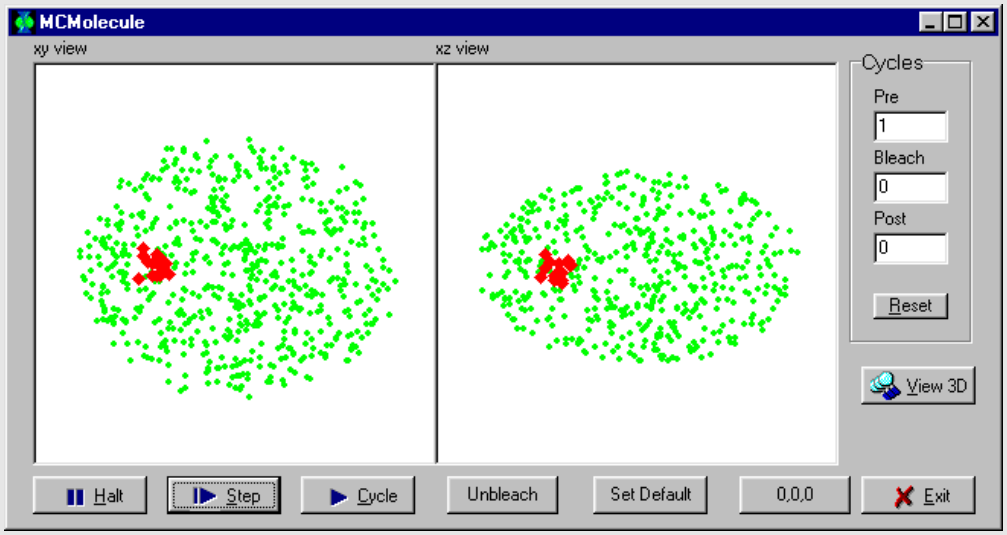
Qualitative vs. quantitative analysis

- It is relatively easy to compare two conditions qualitatively by FRAP, e.g. recovery of a wildtype vs. mutant protein.
  → use exactly the same imaging/bleaching conditions!

- To determine quantitative data like the diffusion coefficient (inside cells) or binding coefficients more information about the investigated system is necessary.
  → modeling can be used to compare the experimental data with simulated data

Modeling + Simulation

Monte Carlo Simulation



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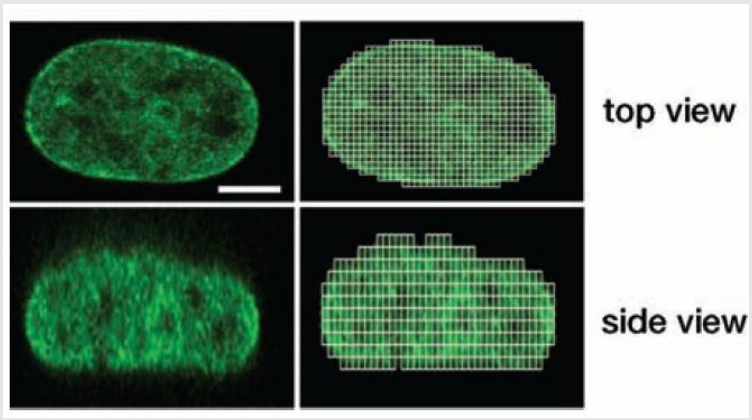
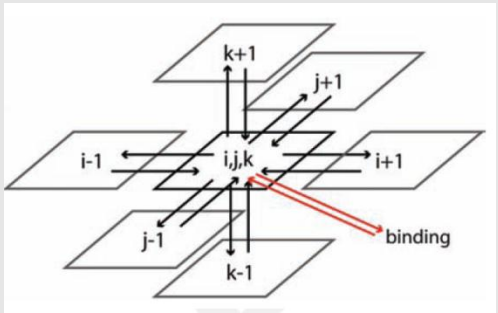
www.erasmusmc.nl/pathologie/research/houtsmuller/?lang=en

Spatial modeling



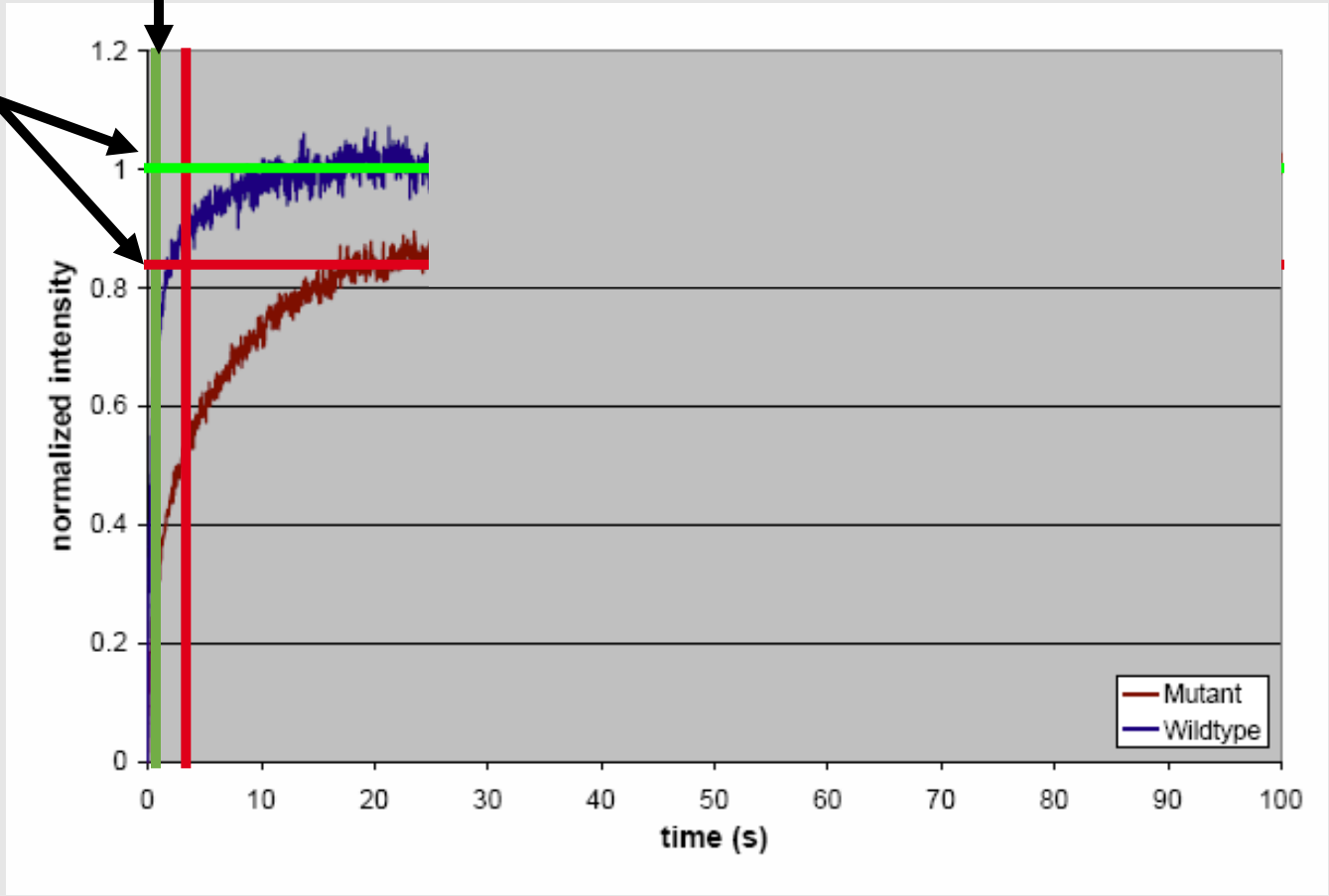


A. Bancaud, S. Huet et al. (2010)
Live Cell Imaging: A Laboratory Manual, Second Edition
Cold Spring Harbor Laboratory Press

Qualitative vs. quantitative analysis

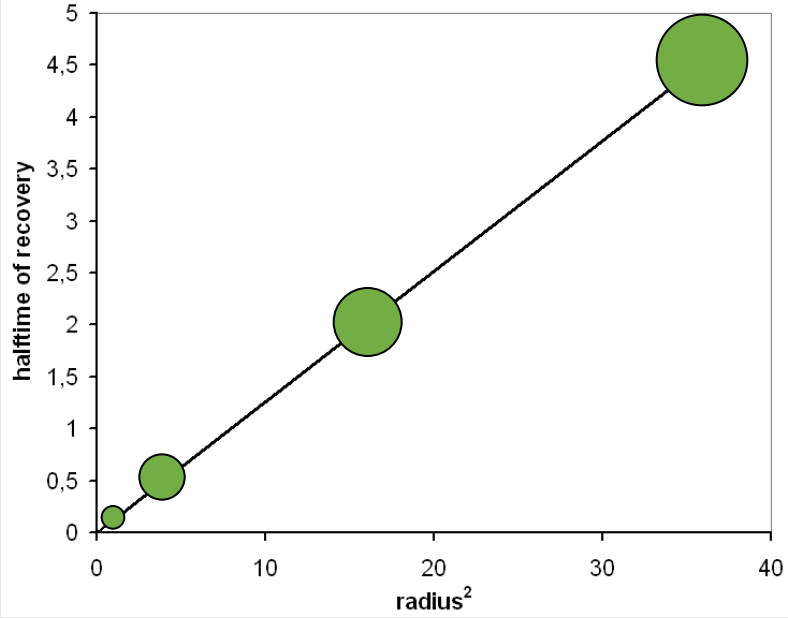
Halftime of recovery

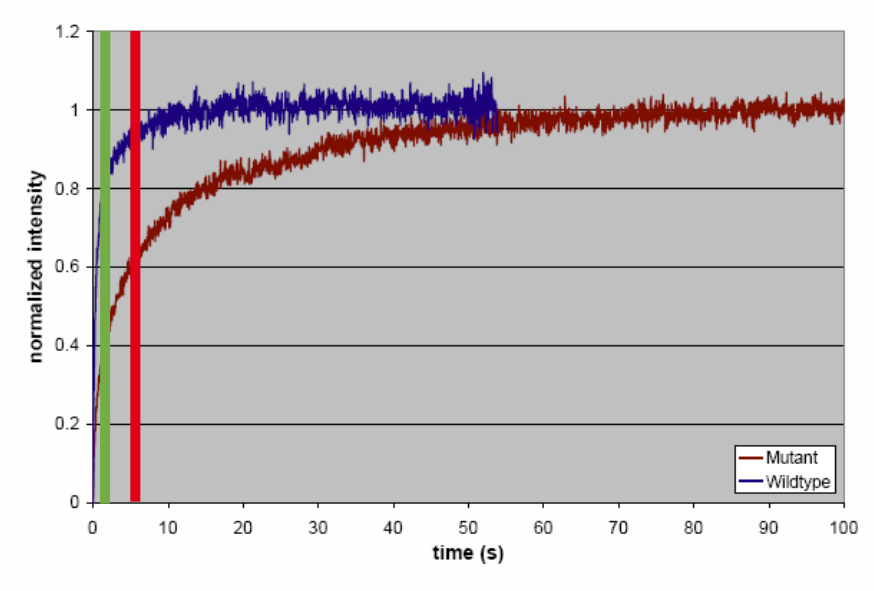
Amount of ‘mobile’ molecules



Recovery time

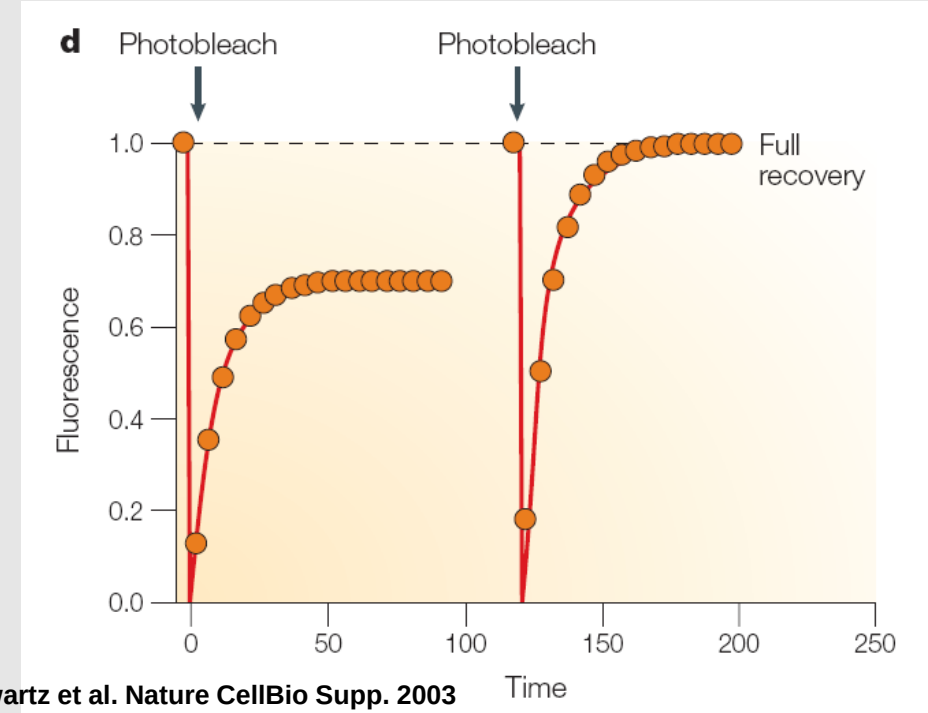
- Halftime of recovery $\tau_{1/2}$ is a qualitative measure of the recovery speed under constant experimental conditions.
- The Halftime of recovery is proportional to the bleach area size, if the recovery is limited by diffusion.
  diffusion coefficient D [$\mu m^2 s^{-1}$]





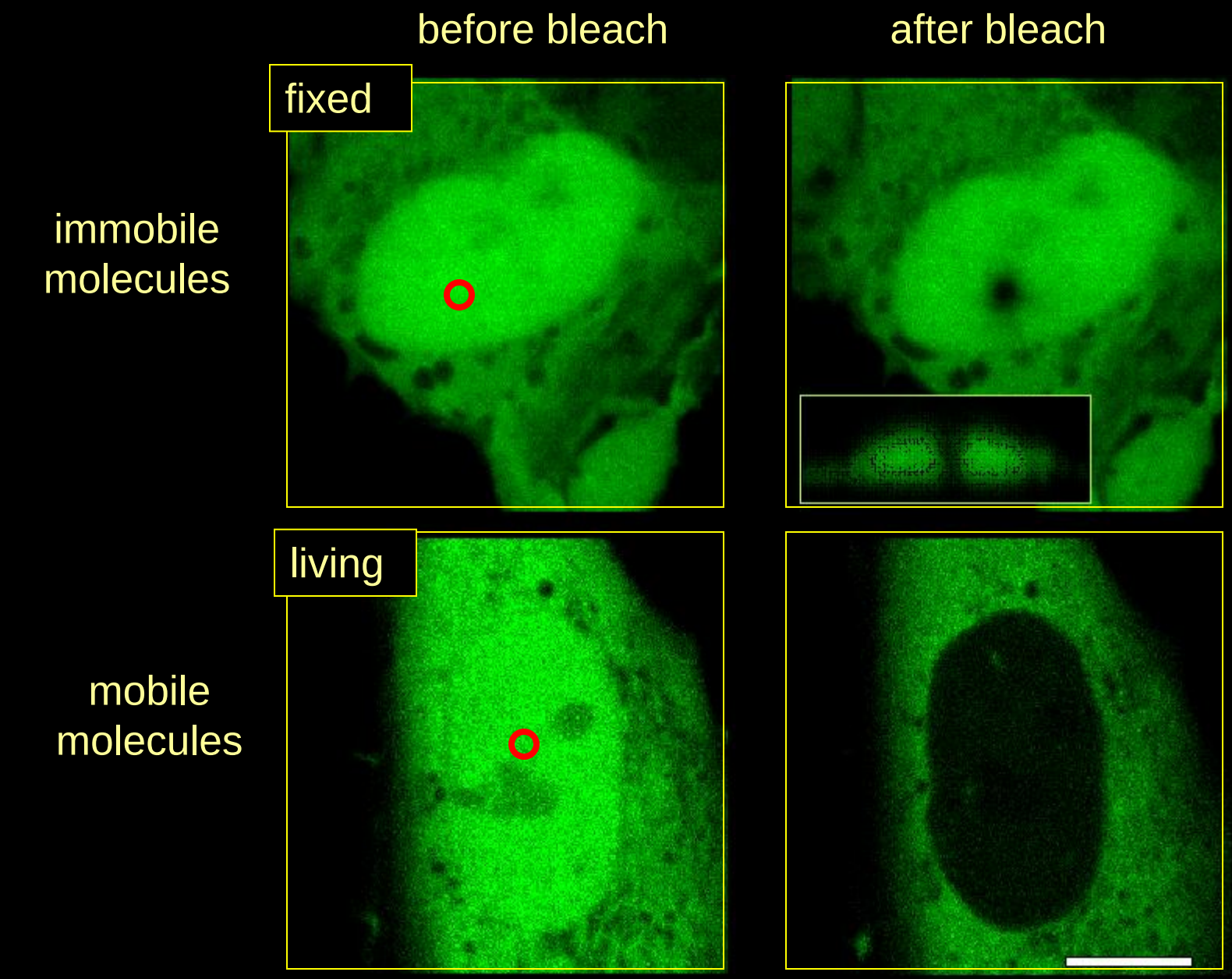
Mobile / immobile fraction

- Photodamage can also create an ‘immobile’ fraction
  → bleaching creates radicals, which can cause crosslinking between proteins by chemical reactions
- A simple test for photo-induced ‘immobile’ fraction is repeating the FRAP experiment at the same position:
  → higher recovery indicates a real ‘immobile’ fraction
  → similar ‘immobile’ fraction indicates potential photodamage



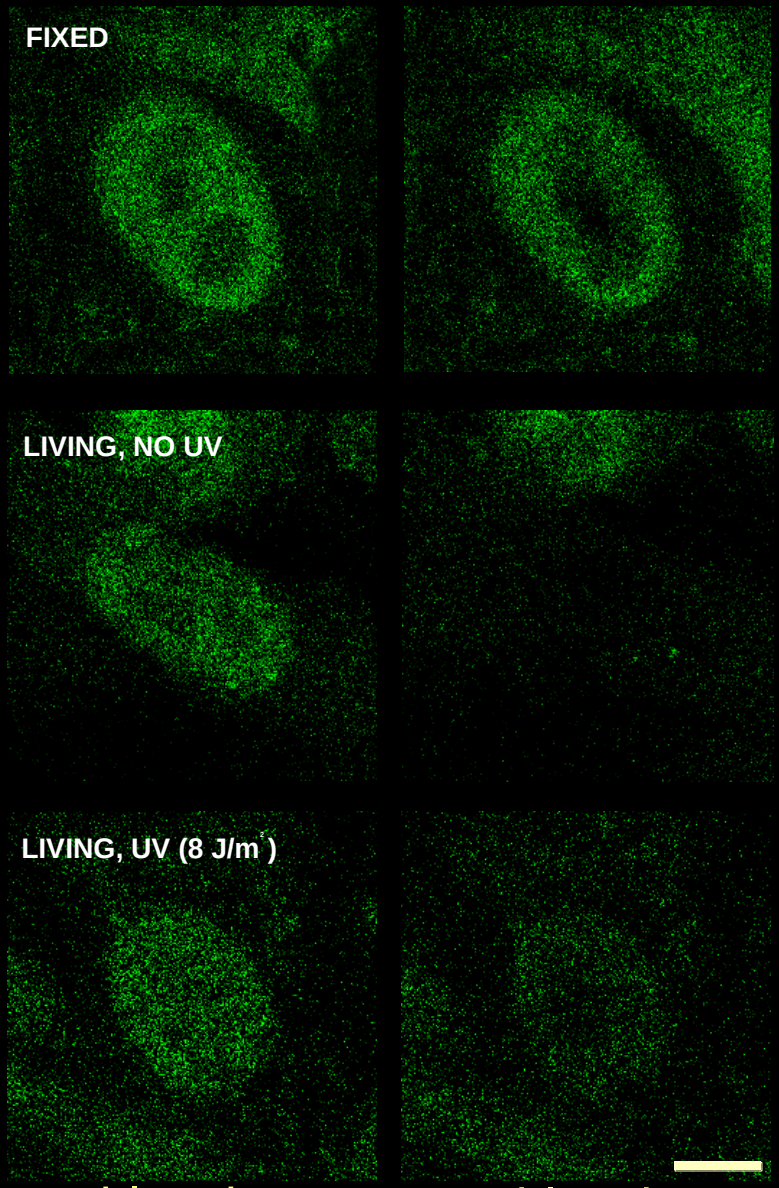
Lippincott-Schwartz et al. Nature CellBio Supp. 2003

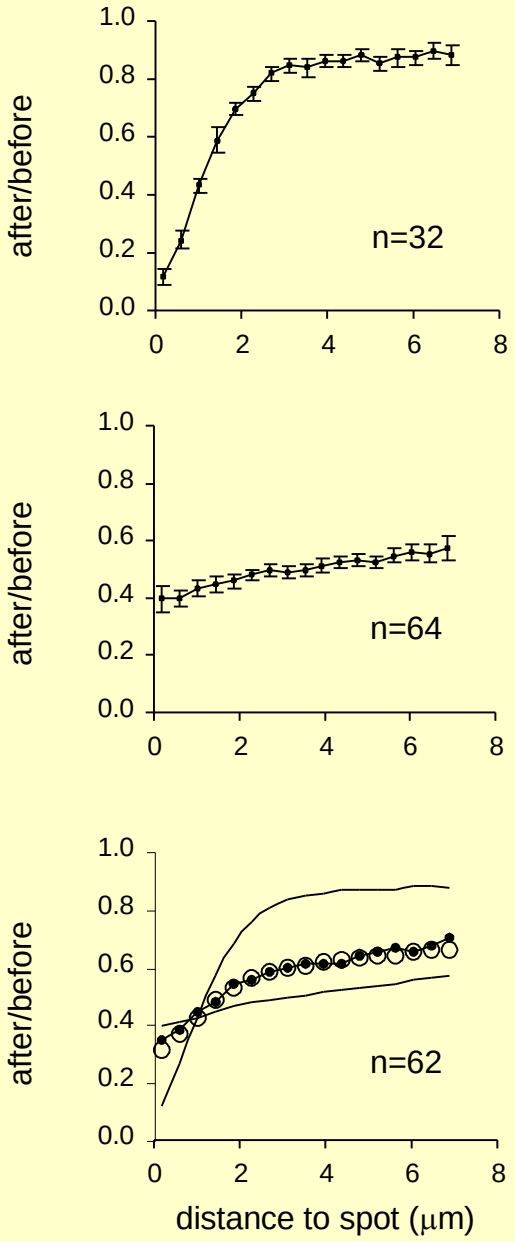
Spot bleaching of GFP in nuclei



Adriaan Houtsmuller

Spot-FRAP on ERCC1-GFP expressing cells





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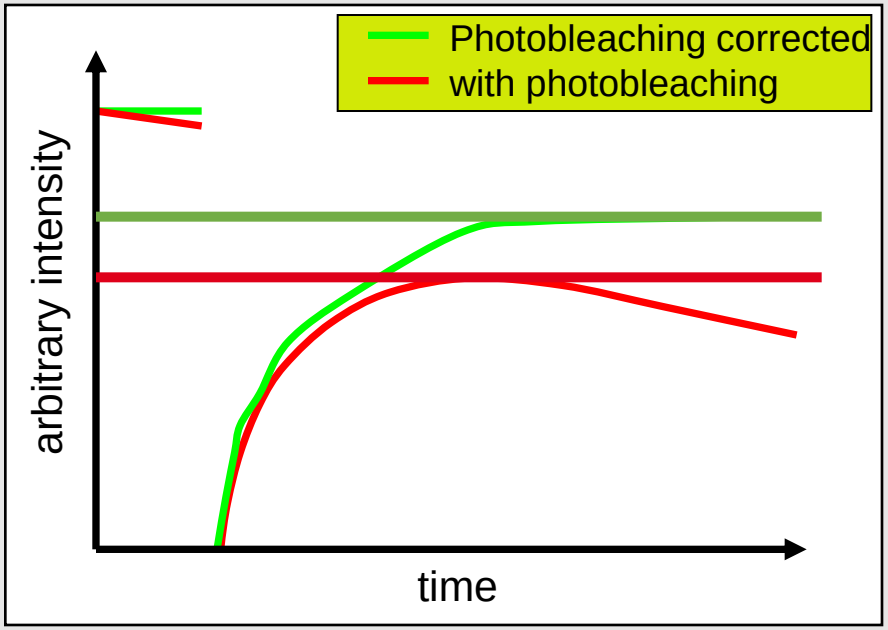
Photobleaching correction

Photobleaching correction is important:

- to determine the ‘right’ relation of mobile/immobile fraction
- Photobleaching influences the recovery time determination since it changes the plateau of recovery



Photobleaching correction

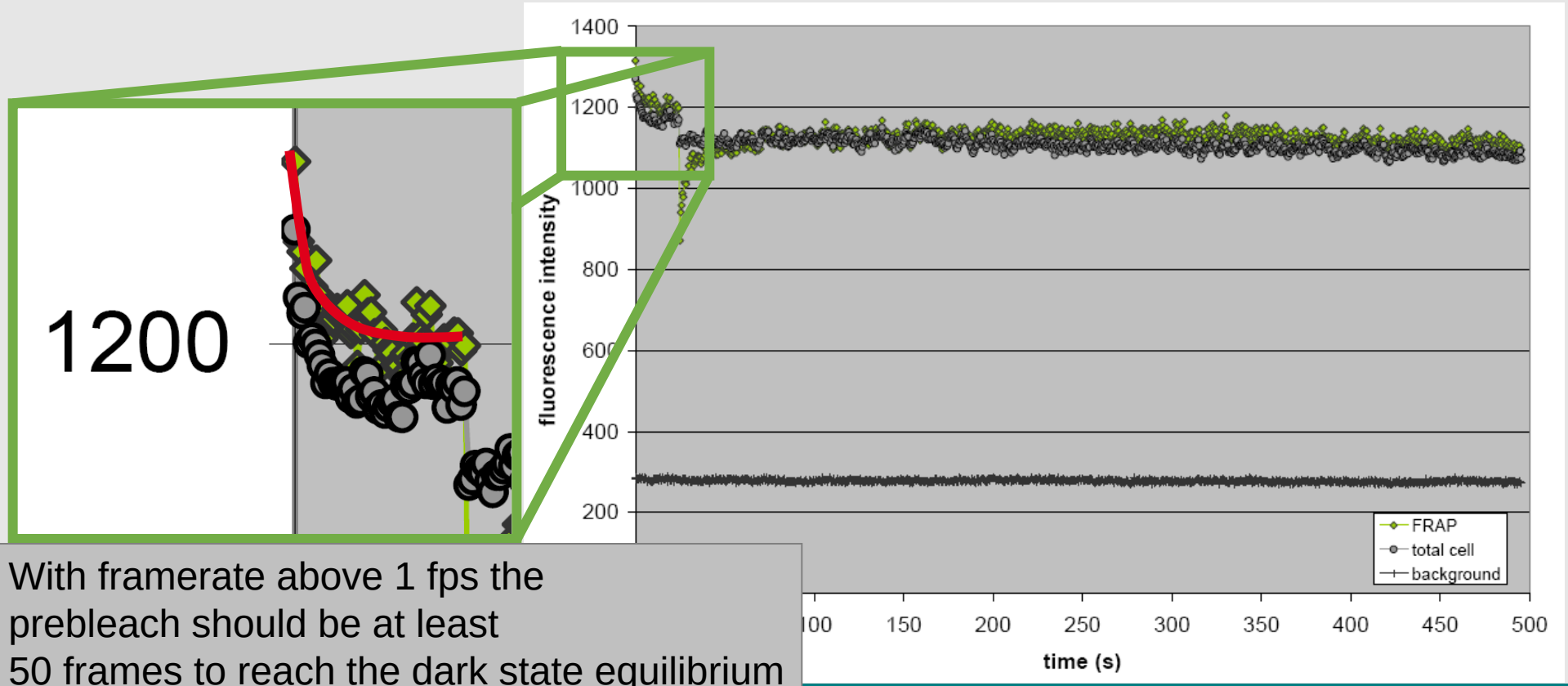
Methods for photobleaching correction:

1. Acquisition photobleaching can also be corrected by using the **total cell intensity**. This also corrects for fluorescence loss due to the bleach pulse and laser fluctuations.
2. The amount of acquisition photobleaching can be determined by measuring the **intensity decay of neighboring cells** (not ‘FRAPed’ cells).
3. Measuring bleaching in a **time-lapse without FRAP**, after the fluorescence is back in steady state. This bleaching curve can be fitted with an exponential decay function to correct the FRAP data.
4. Using the **prebleach** data to determine the acquisition bleach rate.

Data normalization

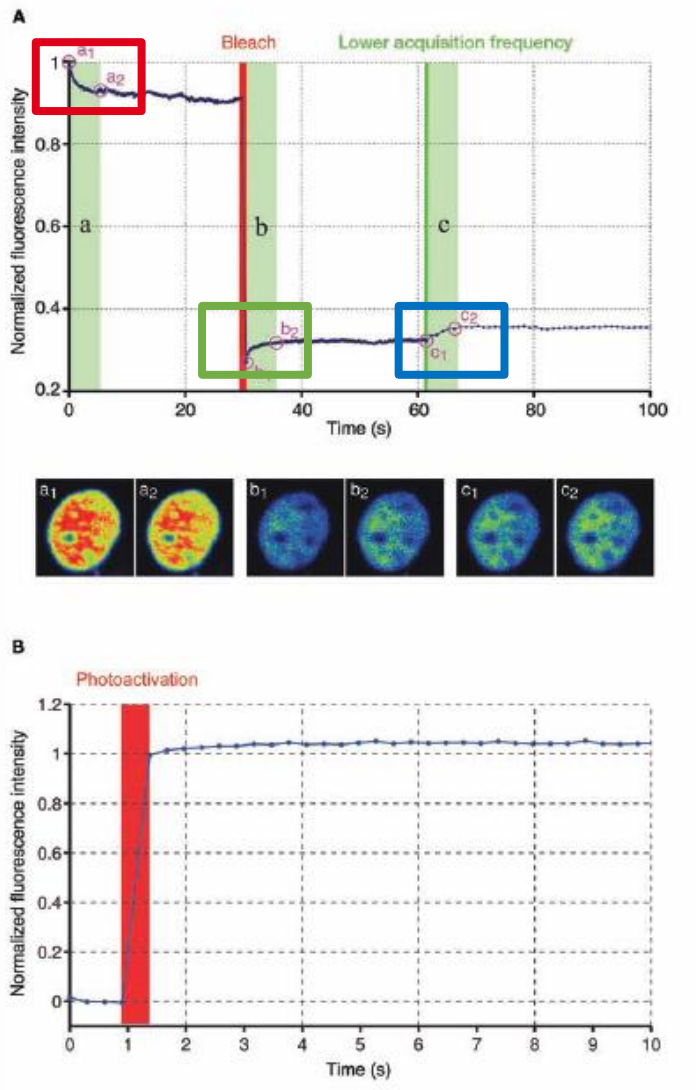
- Raw Data

Normalization is important to compare different experiments

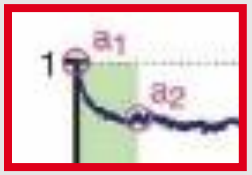


With framerate above 1 fps the prebleach should be at least 50 frames to reach the dark state equilibrium

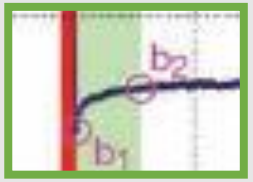
Reversible Dark states



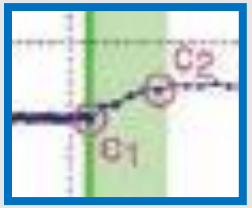
Living cell expressing H2b-GFP



Driving FPs into dark state equilibrium.



Returning FPs into fluorescent state after bleach pulse.



Returning FPs into fluorescent state at slower frame rate.

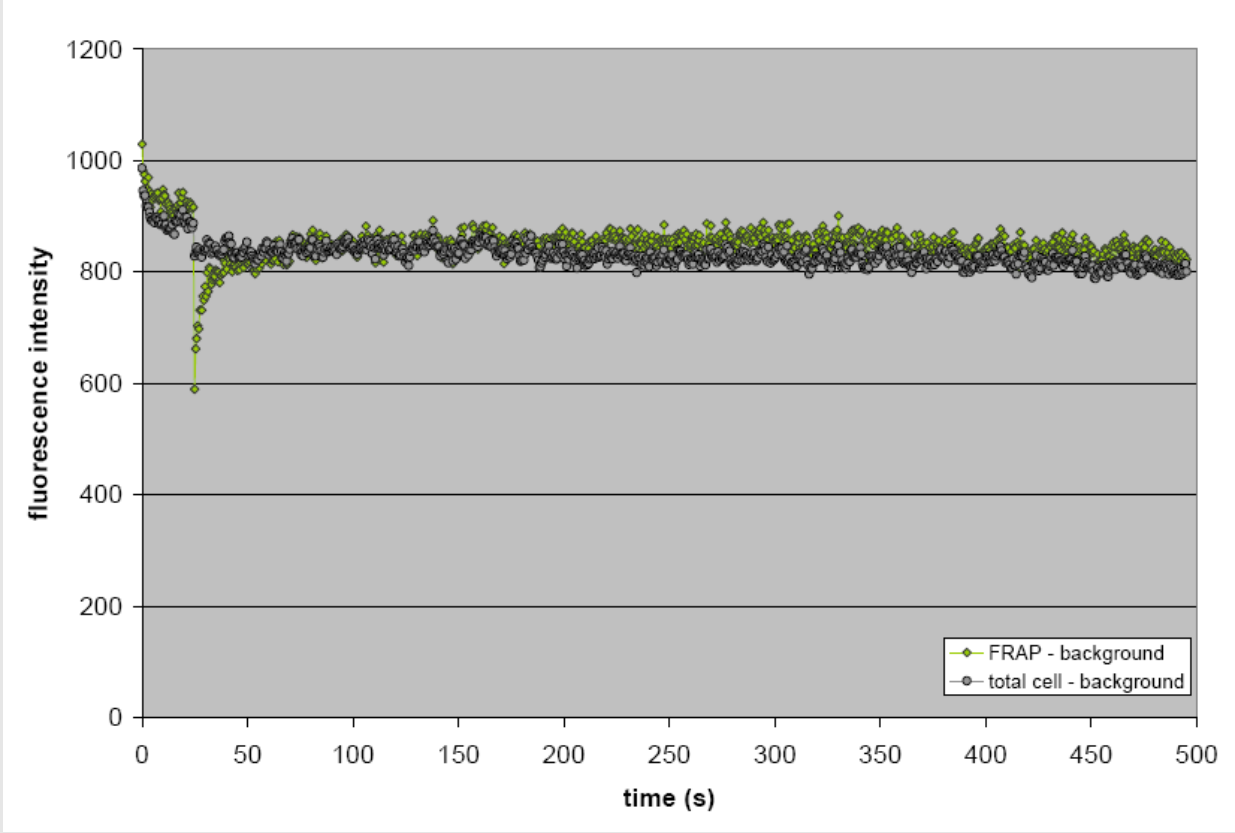
Living cell expressing H2b-paGFP

Bancaud, A., Huet, S., RABUT, G., and Ellenberg, J. 2010 *Live Cell Imaging: A Laboratory Manual, Second Edition* Cold Spring Harbor Laboratory Press

Data normalization

- Raw Data
- background subtracted

$$T(t) - I_{back}(t) \qquad I(t) - I_{back}(t)$$

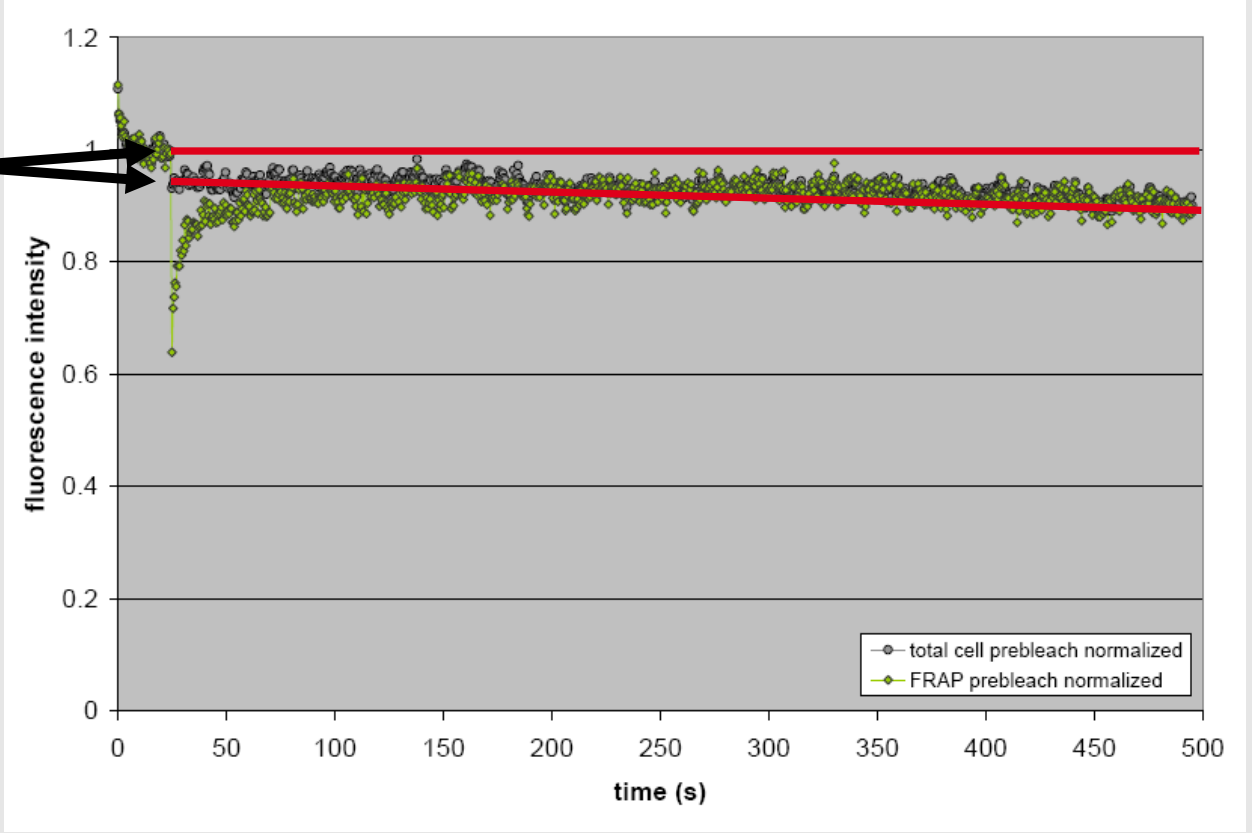


Data normalization

- Raw Data
- background subtracted
- normalized to prebleach (single normalization)

$$\frac{T(t) - I_{back}(t)}{T_{prebleach} - I_{back}} \qquad \frac{I(t) - I_{back}(t)}{I_{prebleach} - I_{back}}$$

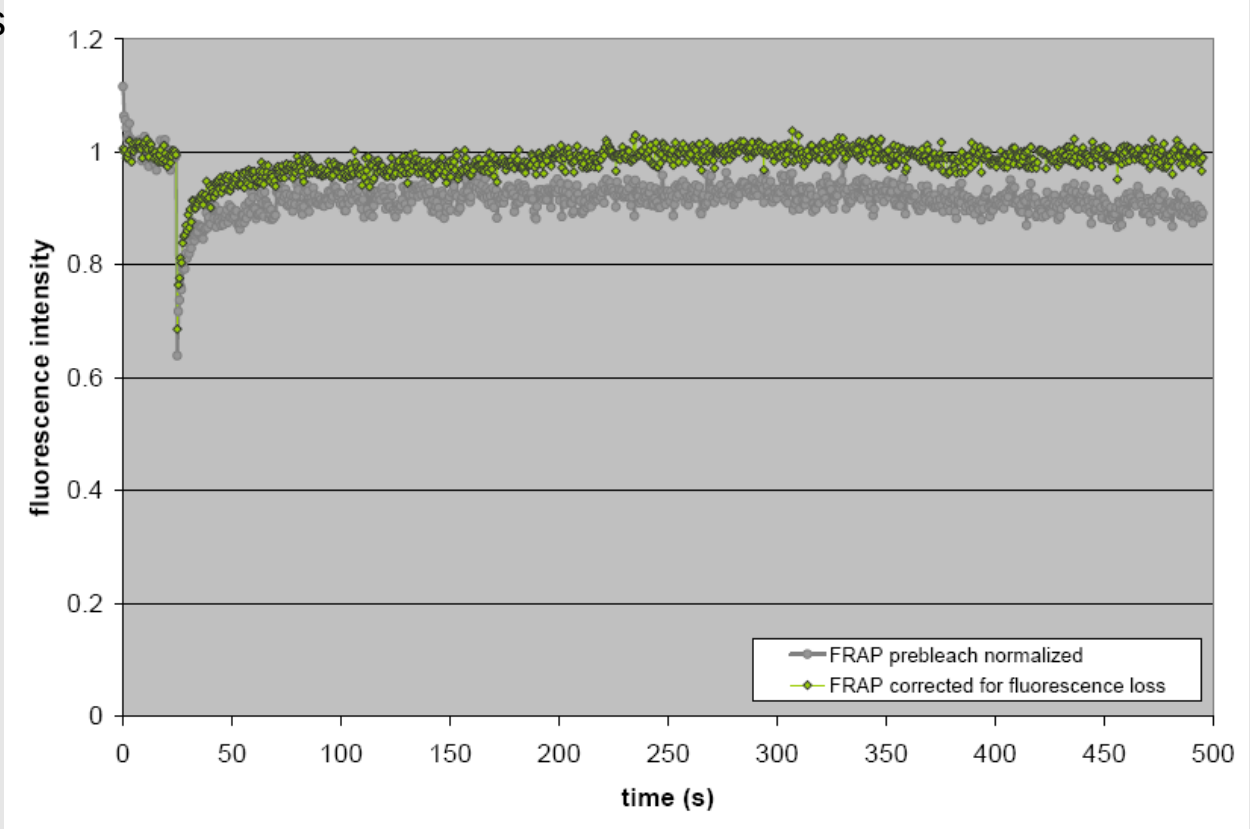
Fluorescence loss due to bleach pulse and acquisition bleaching



Data normalization

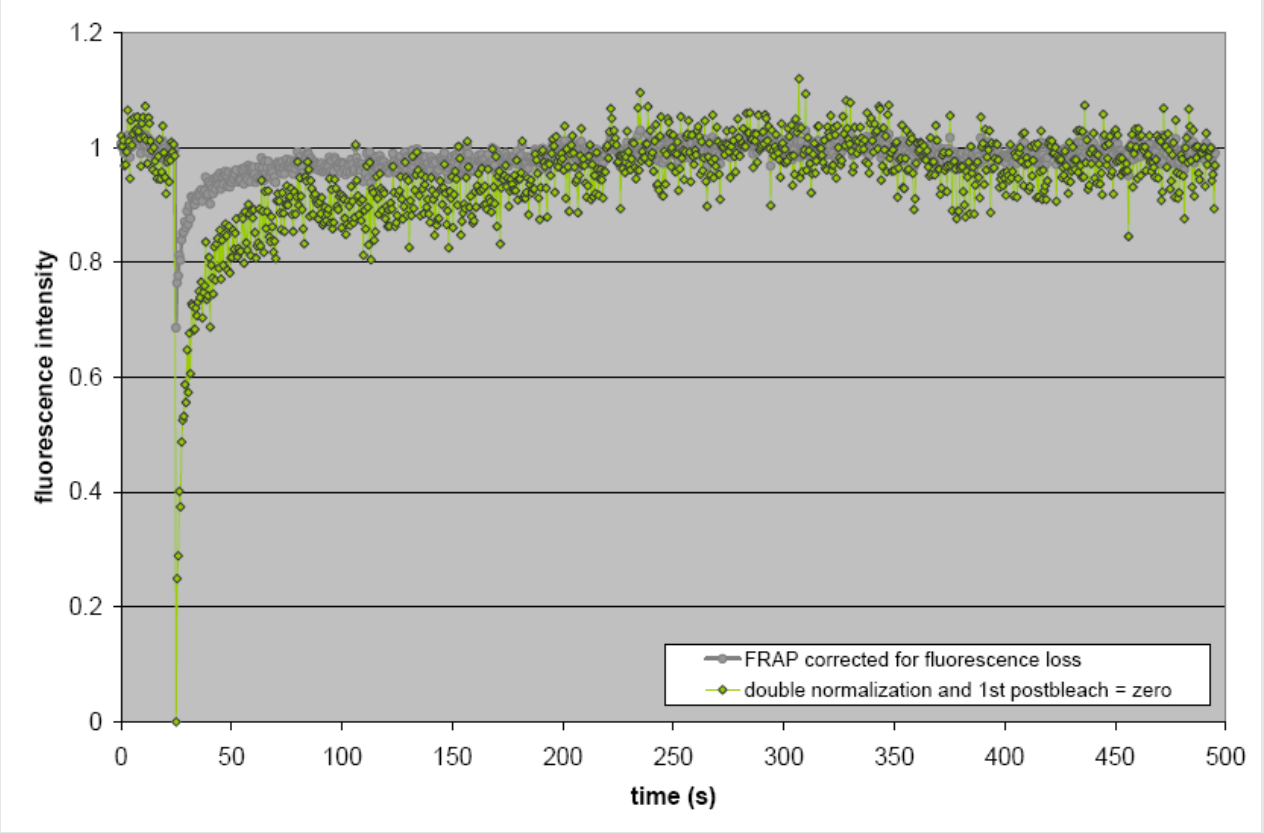
- Raw Data
- background subtracted
- normalized to prebleach (single normalization)
- Corrected for fluorescence loss (double normalization; Phair et al. 2003)

$$I_{norm}(t) = \frac{I(t) - I_{back}(t)}{I_{prebleach} - I_{back}} \bullet \frac{T_{prebleach} - I_{back}}{T(t) - I_{back}(t)}$$

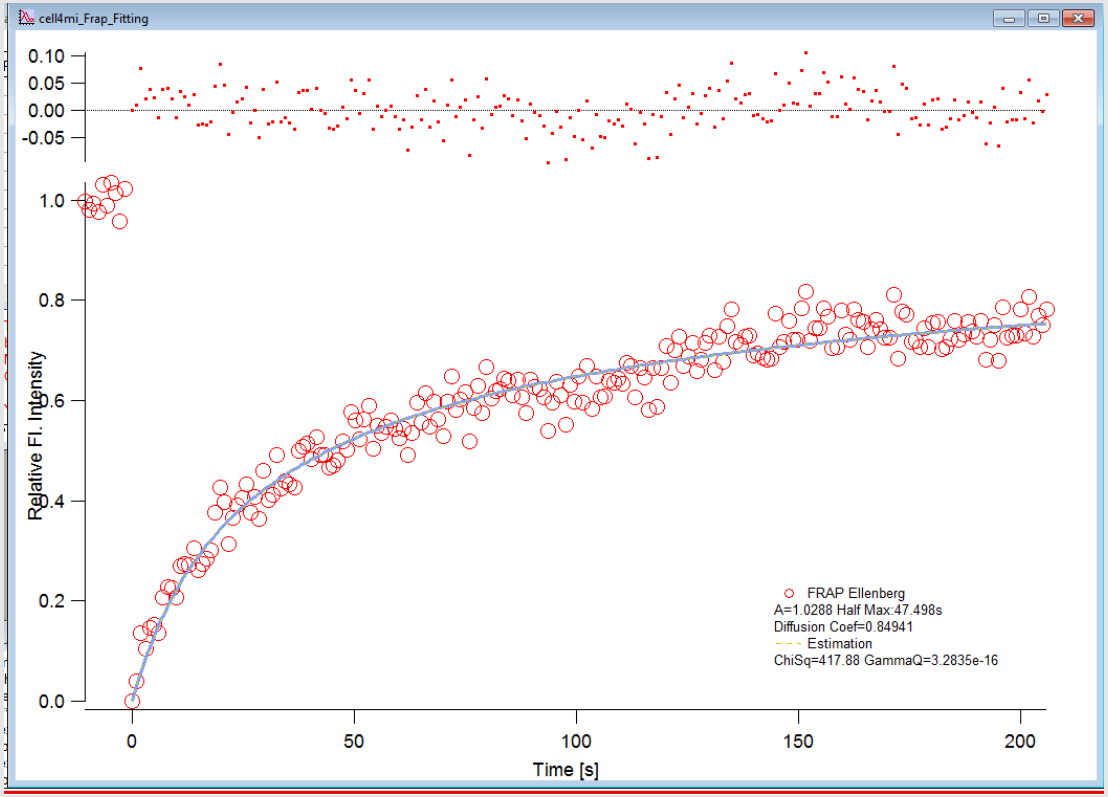


Data normalization

- Raw Data
- background subtracted
- normalized to prebleach (single normalization)
- Corrected for fluorescence loss (double normalization; Phair et al. 2003)
- Additional normalization to 1st postbleach set to zero → experiments can easily be compared with different bleach depth.



Curve fitting



Using the appropriate equations, data can be fitted to obtain e.g. the effective diffusion coefficient (D_{eff}).

$$FRAP(t) = a_0 + a_1 \cdot e^{-\frac{\tau}{2(t-t_{bleach})}} \cdot \left(I_0\left(\frac{\tau}{2(t-t_{bleach})}\right) + I_1\left(\frac{\tau}{2(t-t_{bleach})}\right)\right)$$

Here $I_0(x)$, $I_1(x)$ – modified Bessel functions.

Circular ROI: Soumpasis 1983

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

Stripe ROI: Ellenberg 1997

Acknowledgements

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→ IgorPro FRAP analysis Macro:
http://cmci.embl.de/downloads/frap_analysis

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Arne Seitz, EPFL, Lausanne

Jens Rietdorf, FMI, Basel

FRAPAnalyser http://actinsim.uni.lu/eng/Downloads

www.embl.de/almf/