Introduction to Fluorescence Recovery after Photobleaching (FRAP)

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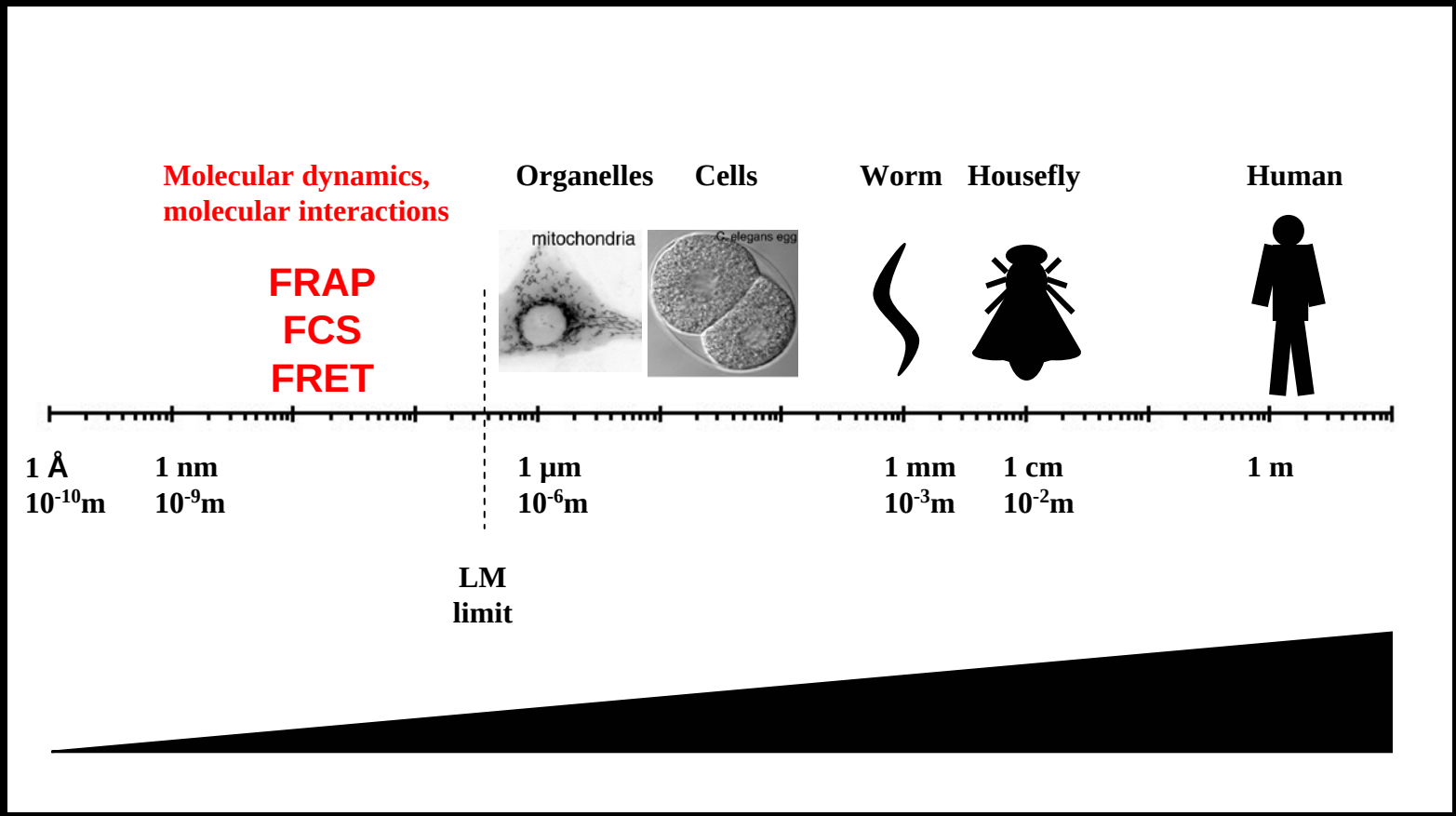
http://www.embl-heidelberg.de/almf/

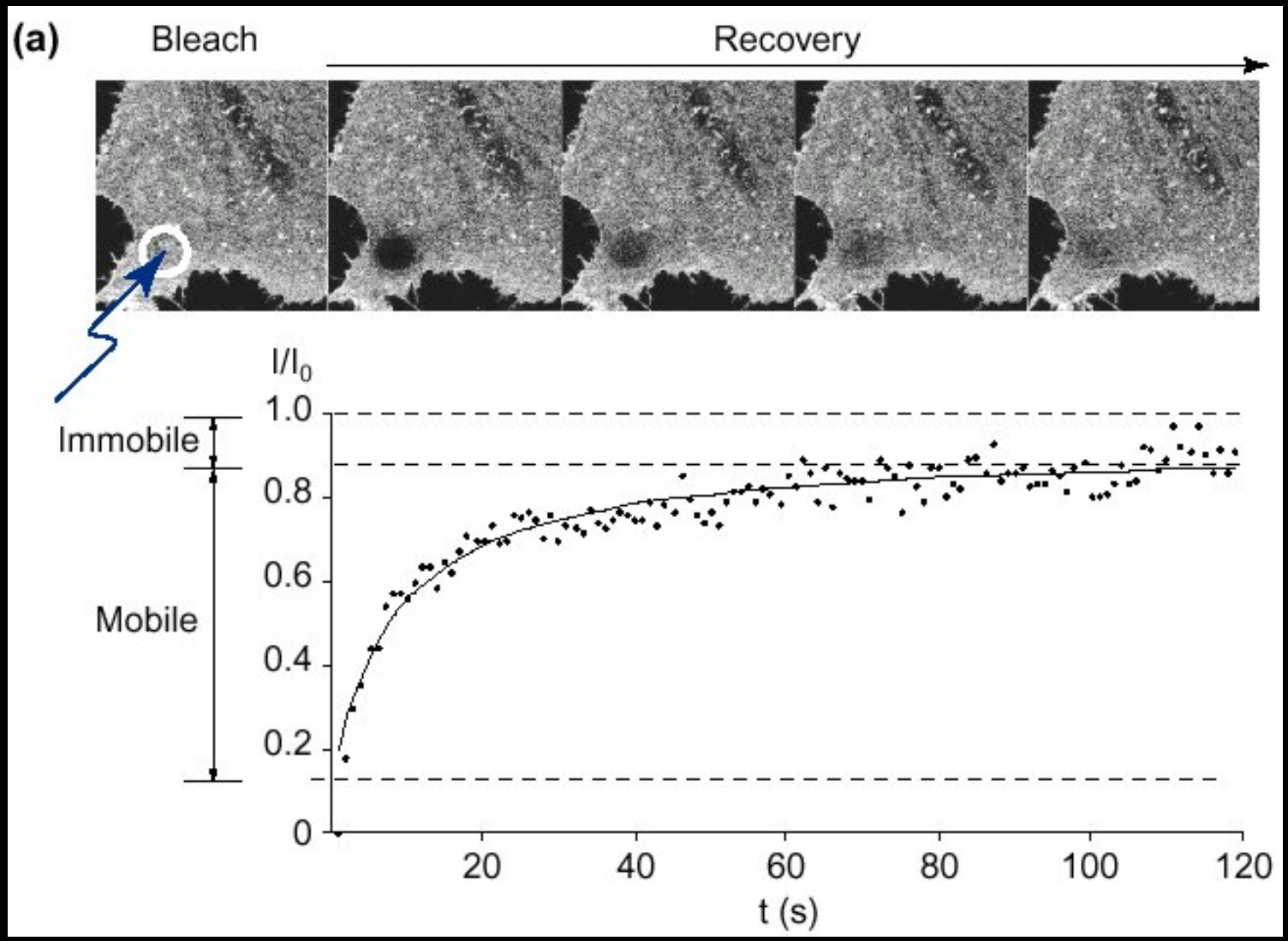
Overview

1) Introduction
2) FRAP principles
3) FRAP data analysis
4) Related techniques (FLIP, FLAP, Photoactivation, -conversion
5) Possible limitations
6) New technology developments

Resolution limit $R = \frac{\lambda}{2n (\sin \theta)}$



Fluorescence Recovery after Photobleaching (FRAP)



Bastiaens and Pepperkok (2000), TIBS 25/12

Timeline

1973: 1st application of the FRAP method (Poo and Cone)

1976: Mathematics for quantitative FRAP of focused laser spots in two dimensions (Axelrod et al.)

1996: Resurrection of FRAP using GFP and confocal microscopes (Cole et al., Lippincott-Schwartz..)

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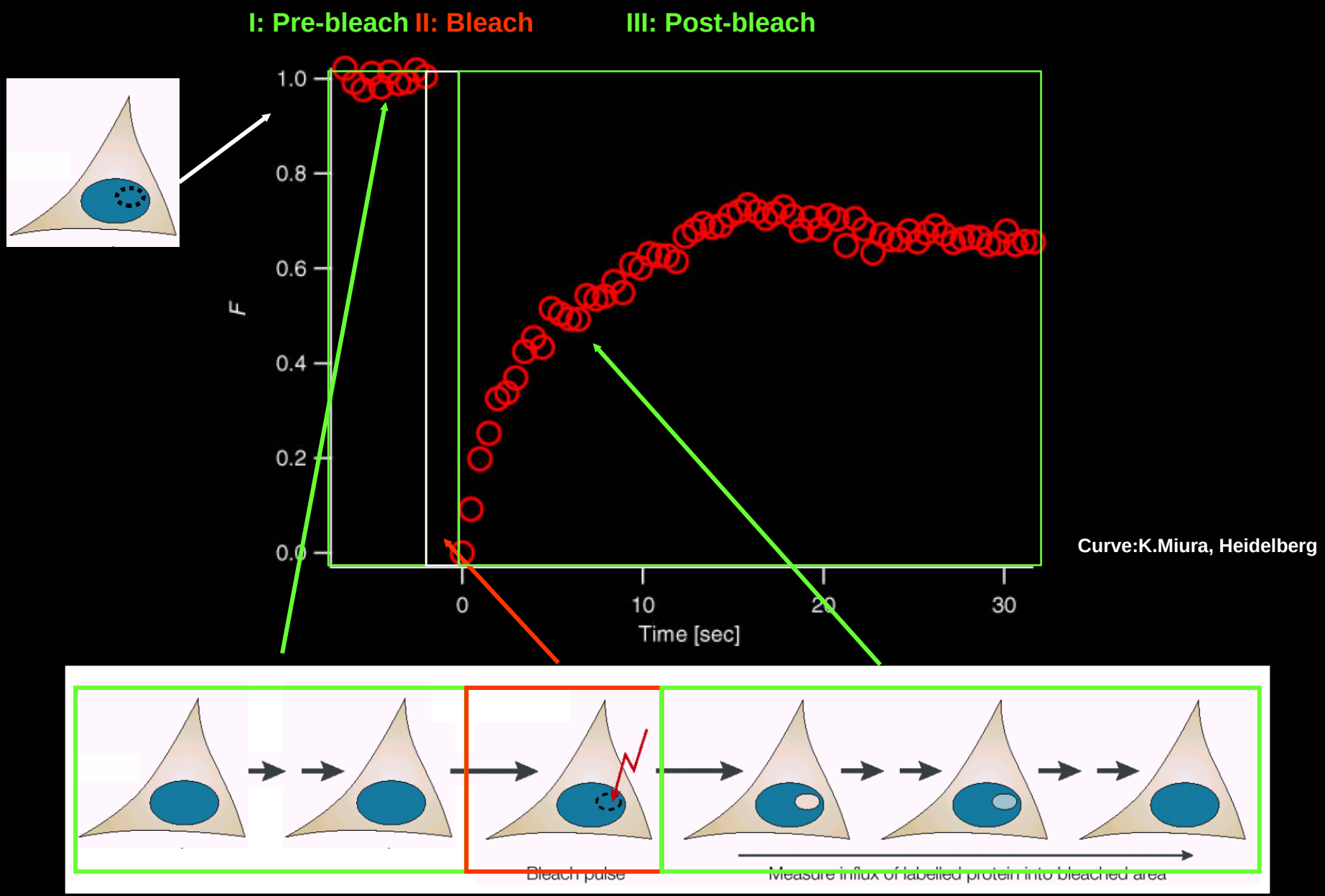
Schematic of a FRAP experiment



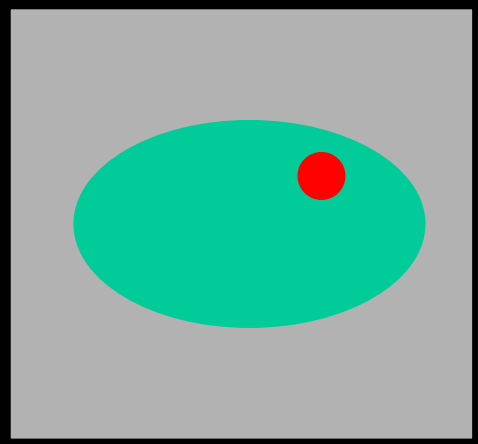
Execution of a FRAP experiment

1) Take a series of images before bleach (same settings as after the bleach)
2) Apply short local bleach
3) Take images after bleach until the recovery in the bleached area reaches a plateau

Intensity of bleaching light

AOTF upregulation (0-100%):

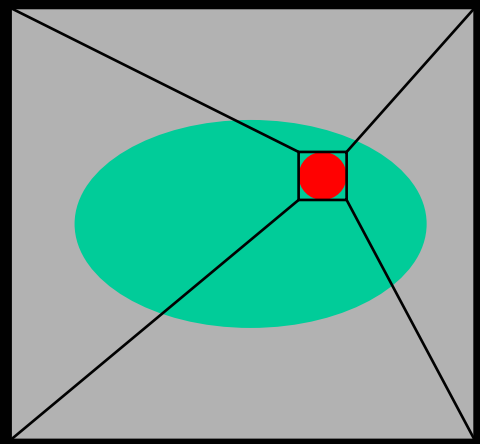
Linear



Zoom In:

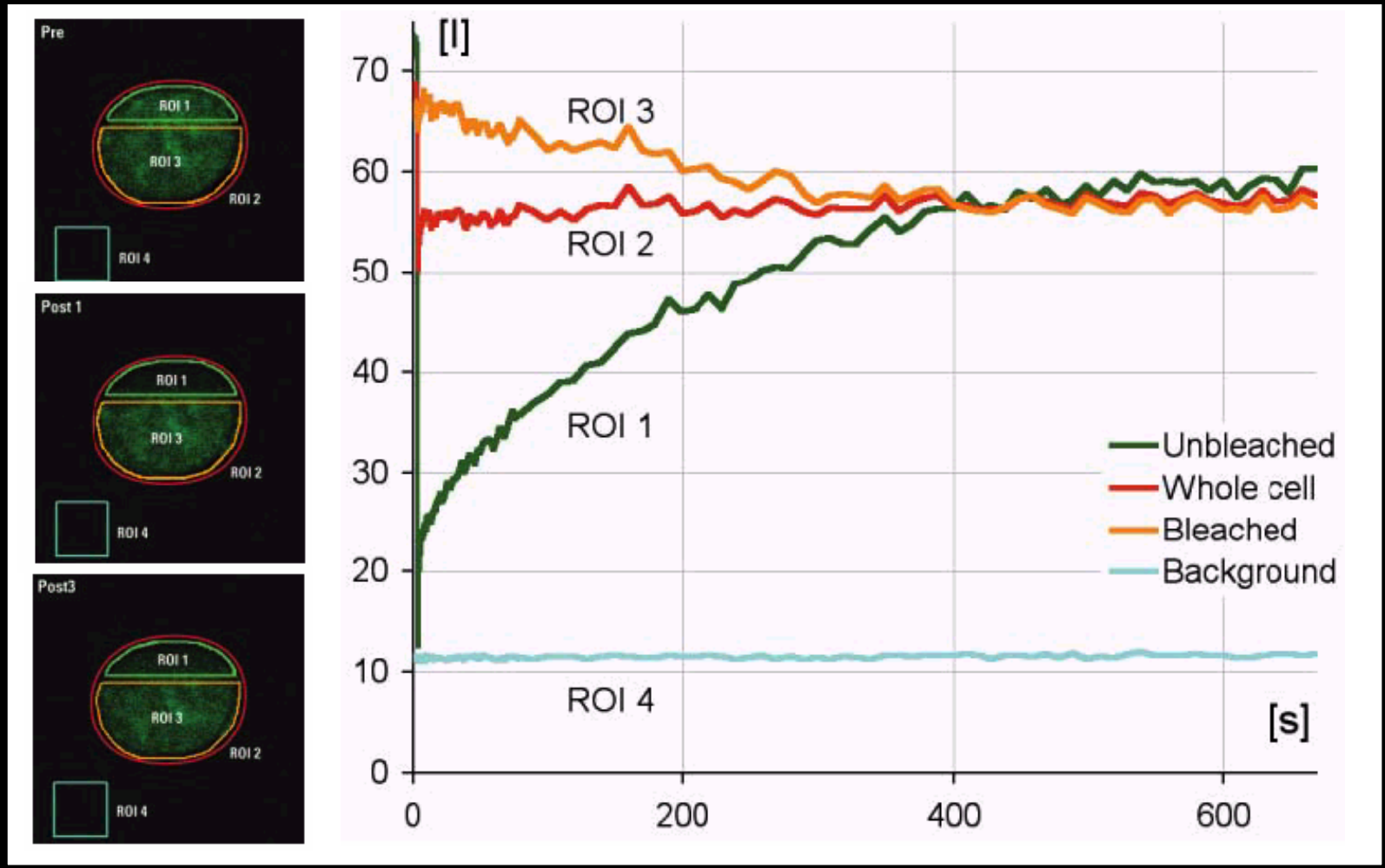
Exponential

$2^{zoomfactor}$



Speed limitation due to switching of the scanfield

FRAP experimental data



Kappel and Eils, Leica App.Letter 2004

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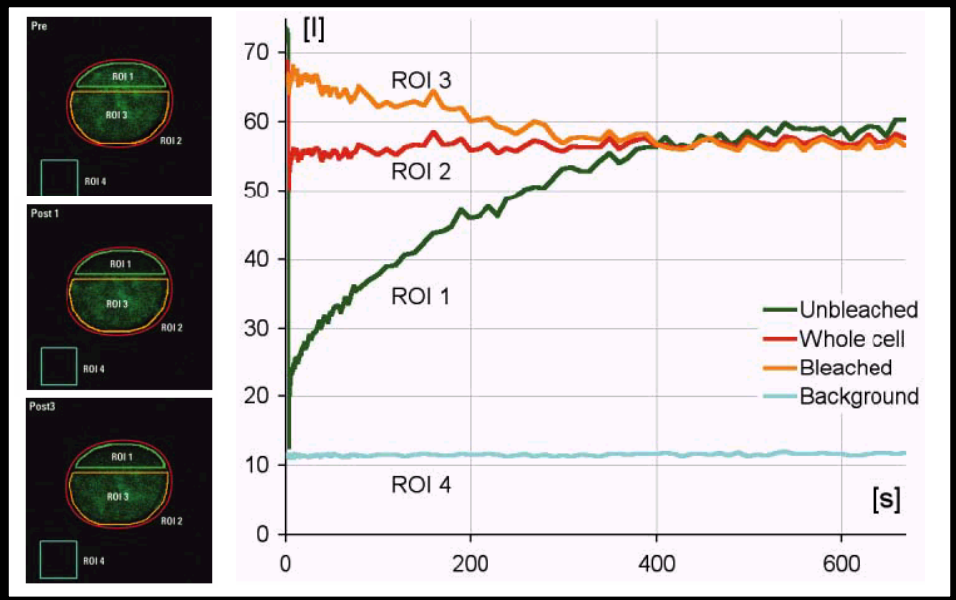
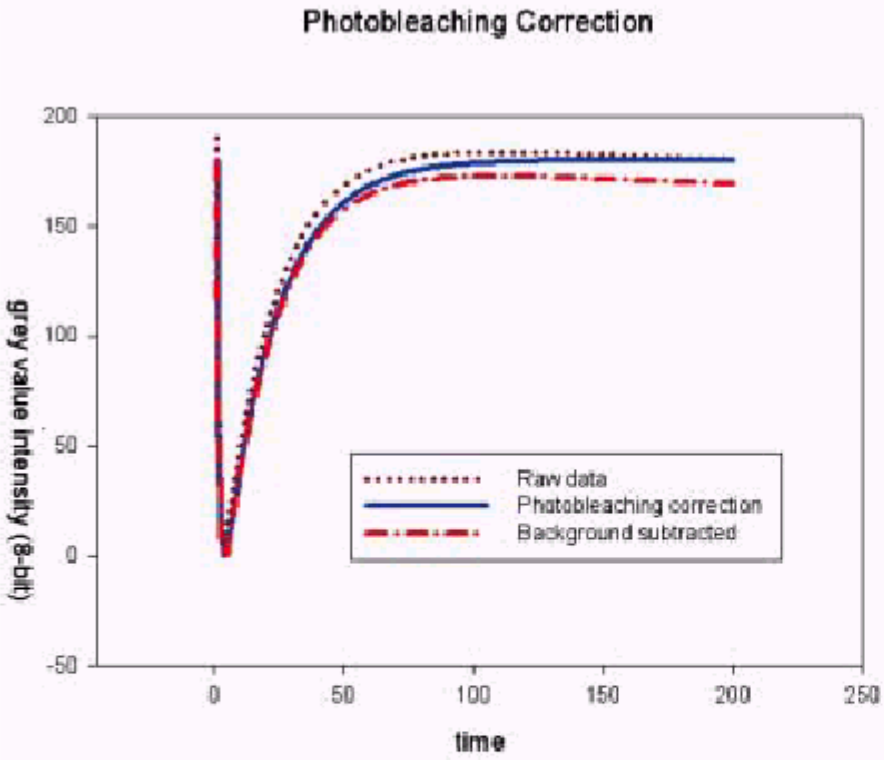
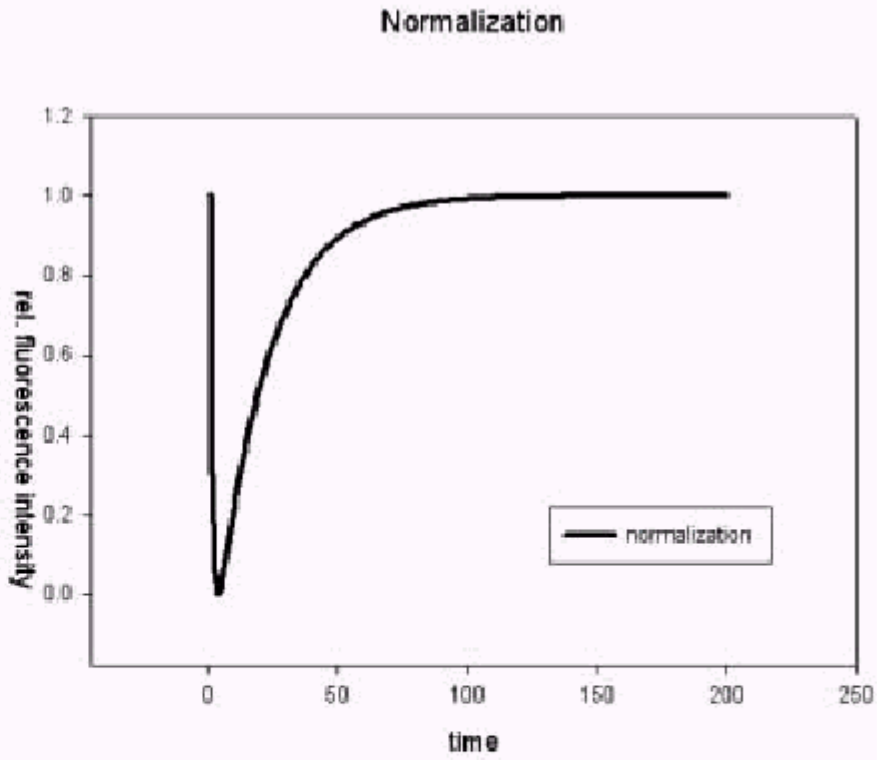
Correction of the experimental data

1) Background subtraction
2) Correction for photobleaching during the measurement (whole cell or neighboring cell as reference)
3) Data normalization (alternative methods)







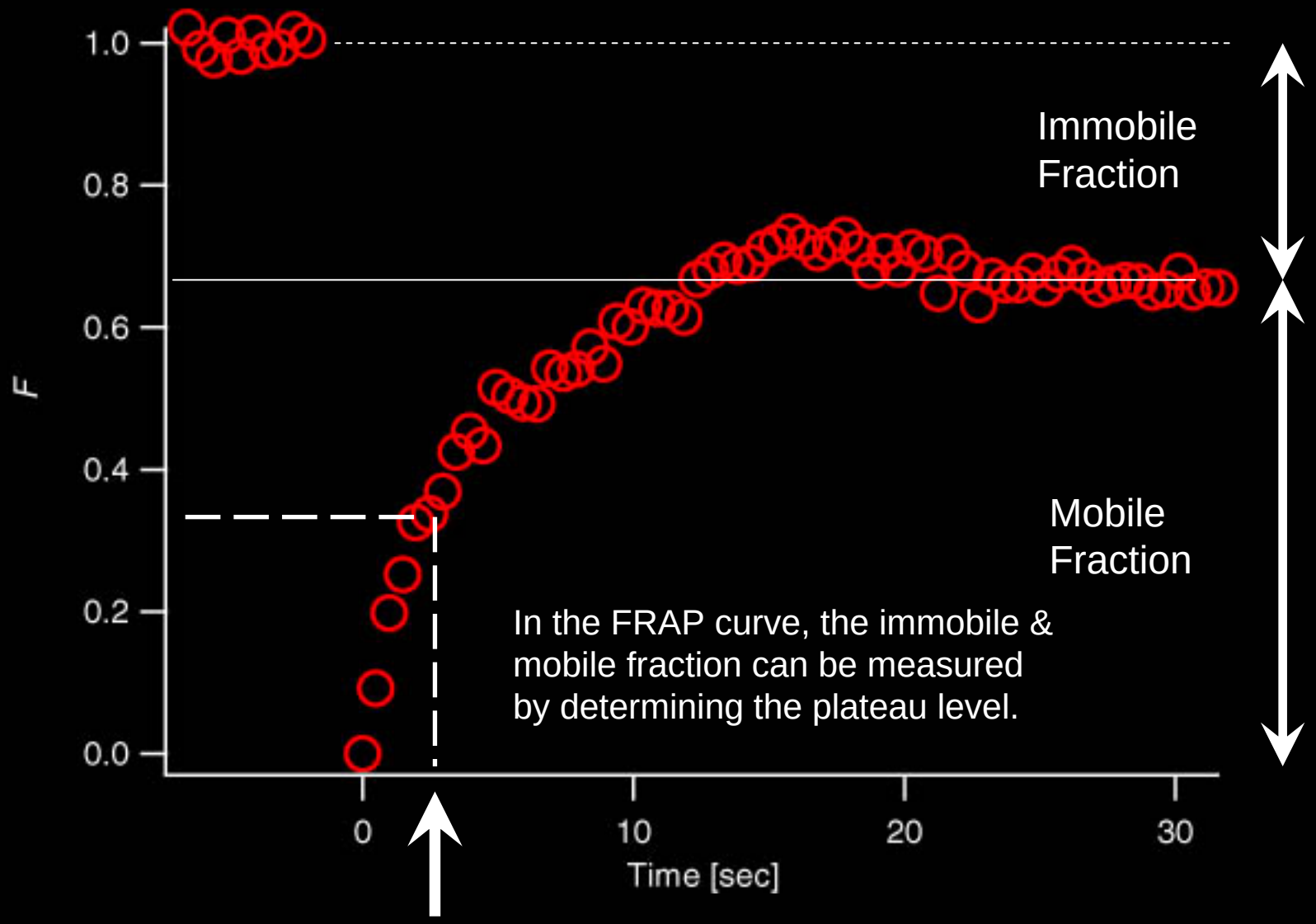
$$F_b = F(t) - background$$

$$F_{b,corr,normAxelrod}(t) = \frac{F_{b,corr}(t) - F_{b,corr}(0)}{F_{b,corr}(inf) - F_{b,corr}(0)}$$

$$F_{b,corr}(t) = F_b(t)\frac{F_{precell}}{F_{infcell}}$$

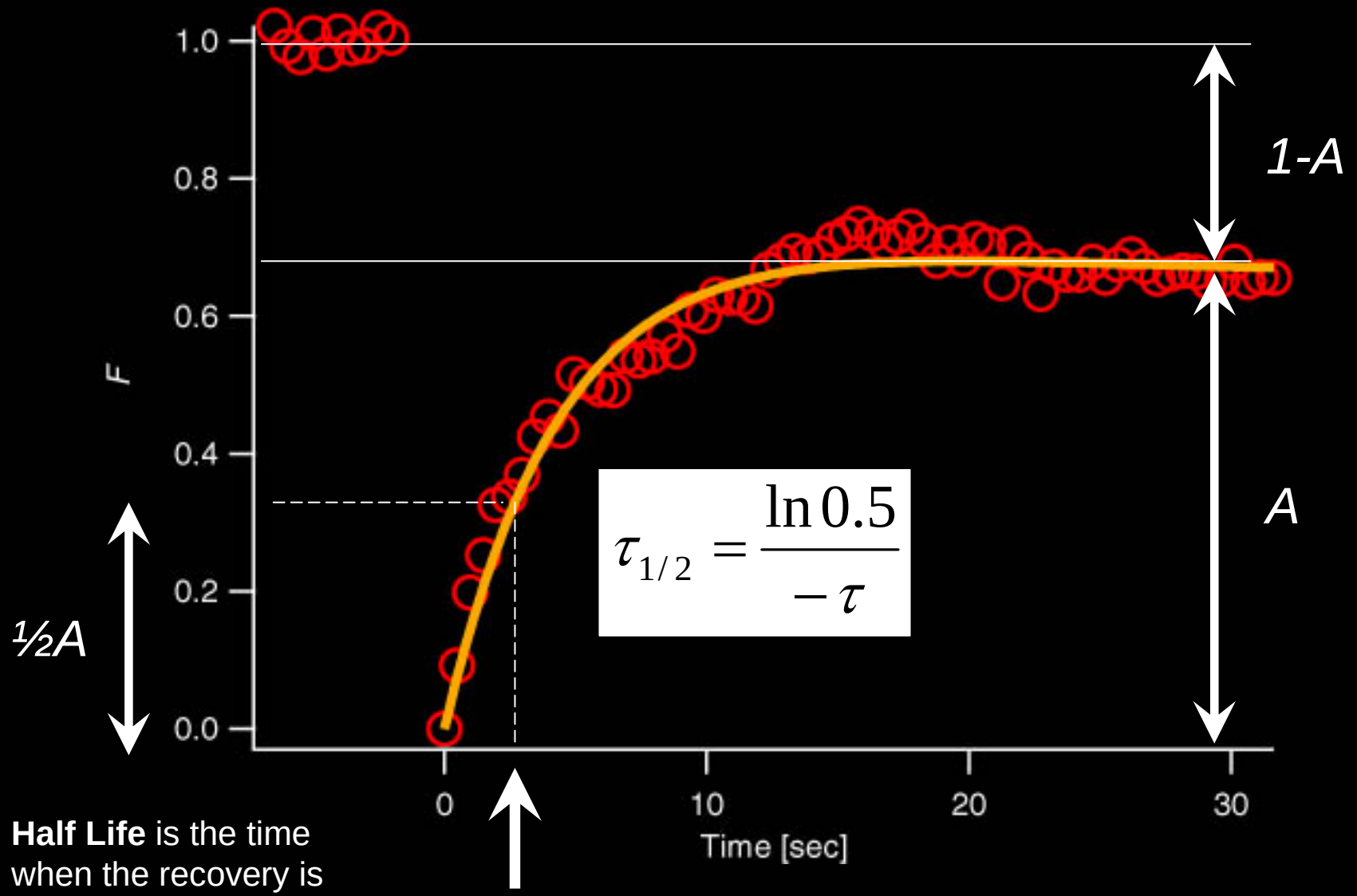
Kappel and Eils, Leica App.Letter 2004

The time constant and mobile / immobile fractions



Slide:K.Miura, Heidelberg

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



Half Life is the time when the recovery is the half of A, by definition.

Half Life ($\tau_{1/2}$)

Slide:K.Miura, Heidelberg

Estimated parameters by exponential fit:

1) **Mobile and immobile fraction**
2) **Recovery half-time**

Estimation of diffusion coefficient (Axelrod et al.)

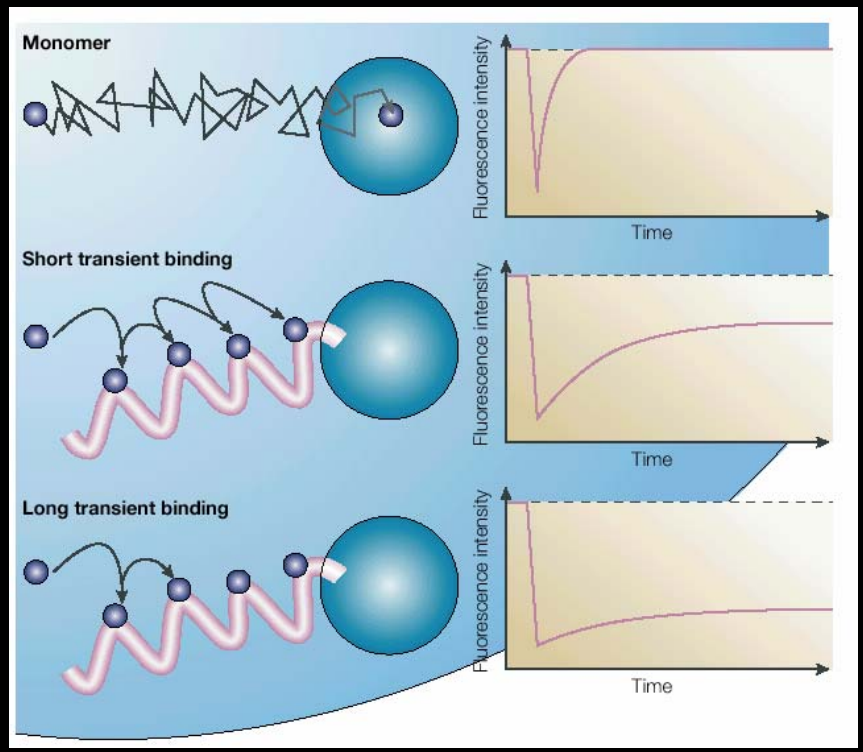
$$D = 0.88^{*} w^{2}/(4\, t_{1/2})$$

w: bleach radius

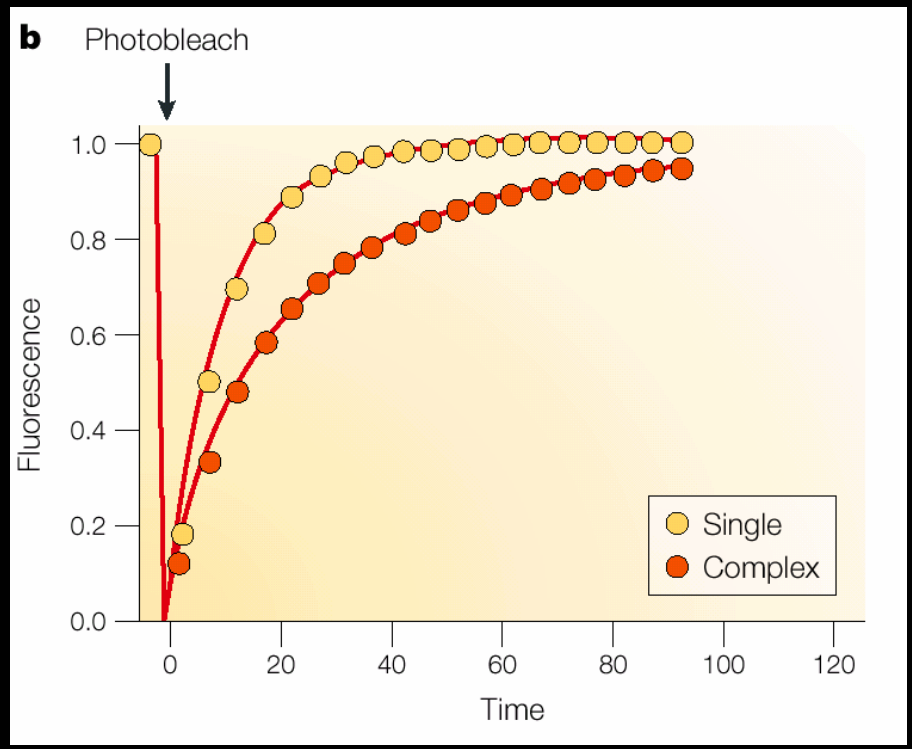
Assumptions:

- **bleached area is disk shaped**
- **diffusion occurs only in 2D**

Free diffusion vs. binding



Phair and Mistelli, Nature Reviews MolCellBio, 2001

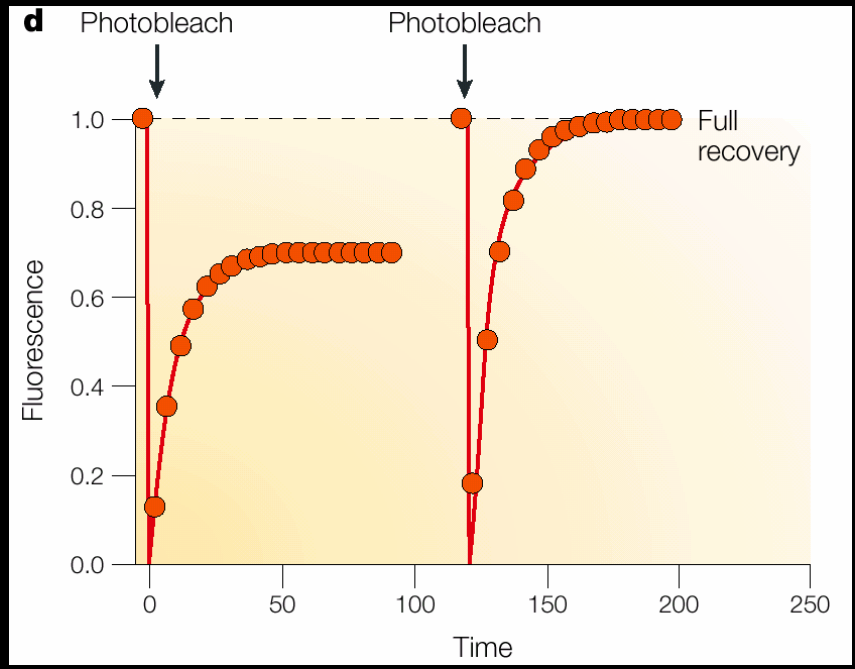


Lippincott-Schwartz et al. Nature CellBio Supp. 2003

Multiple populations with differing diffusion rates => multi-component equations

Possible FRAP artifacts

Photo-induced immobile fraction



Lippincott-Schwartz et al. Nature CellBio Supp. 2003

Problem:	**Potential explanation**
Partial recovery:	**e.g. immobile fraction, physical separation**
Reversible photobleaching:	**fixed samples, varition of the bleach spot size**
Non-diffusive behaviour:	**binding, active transport => modelling**
Different values in consecutive measurements:	**photodamage**

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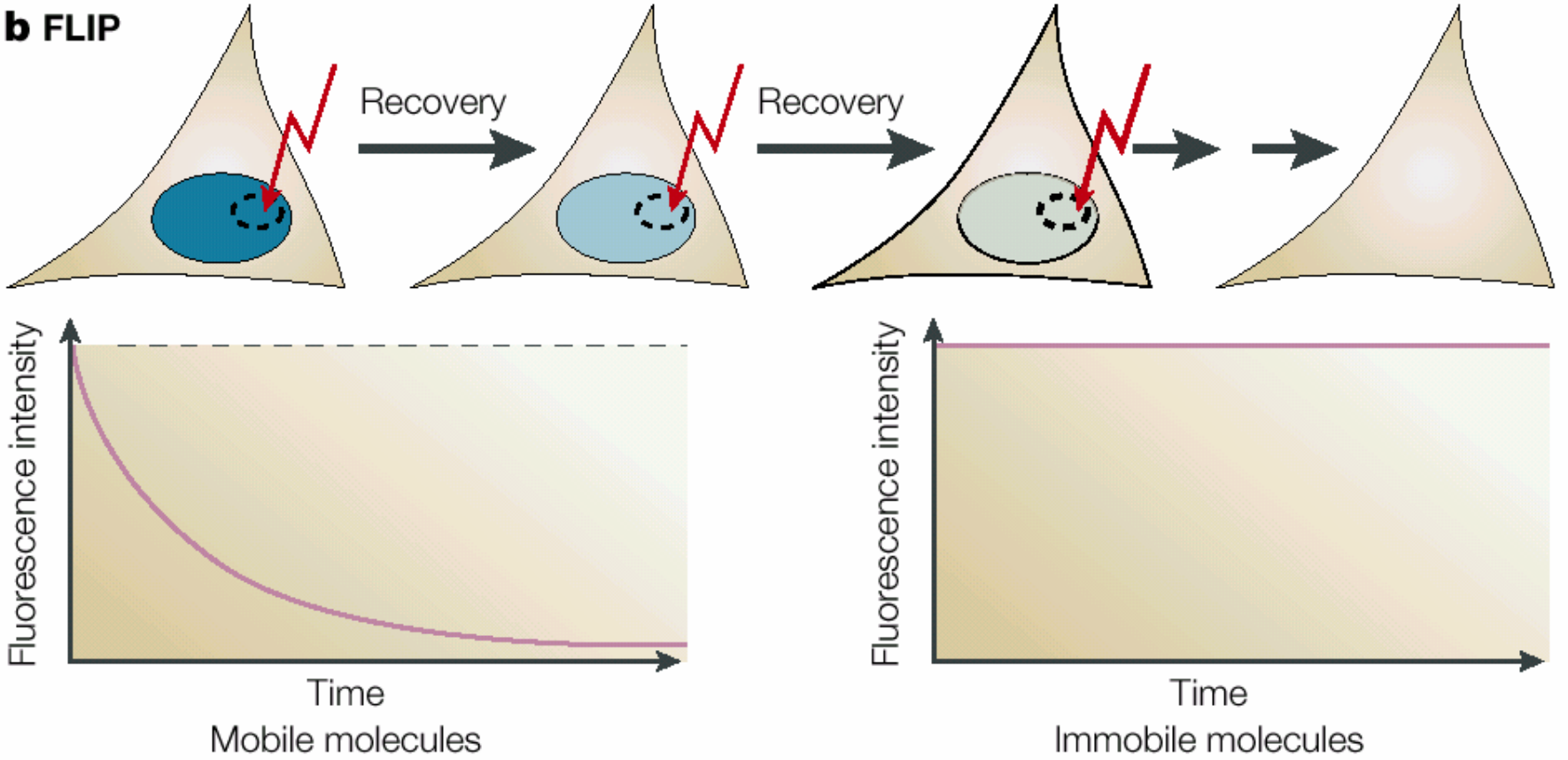
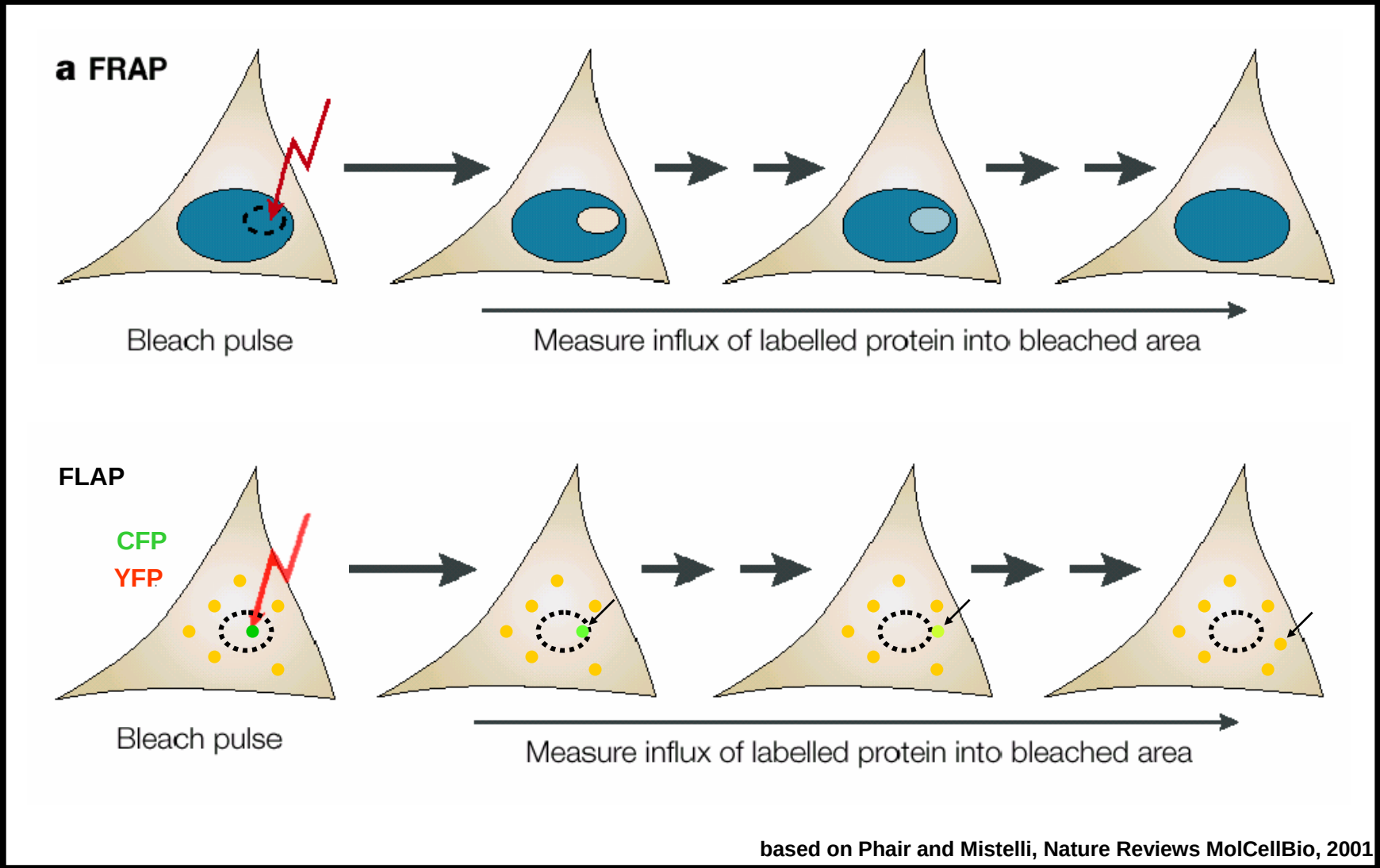
Fluorescence Loss in Photobleaching (FLIP)



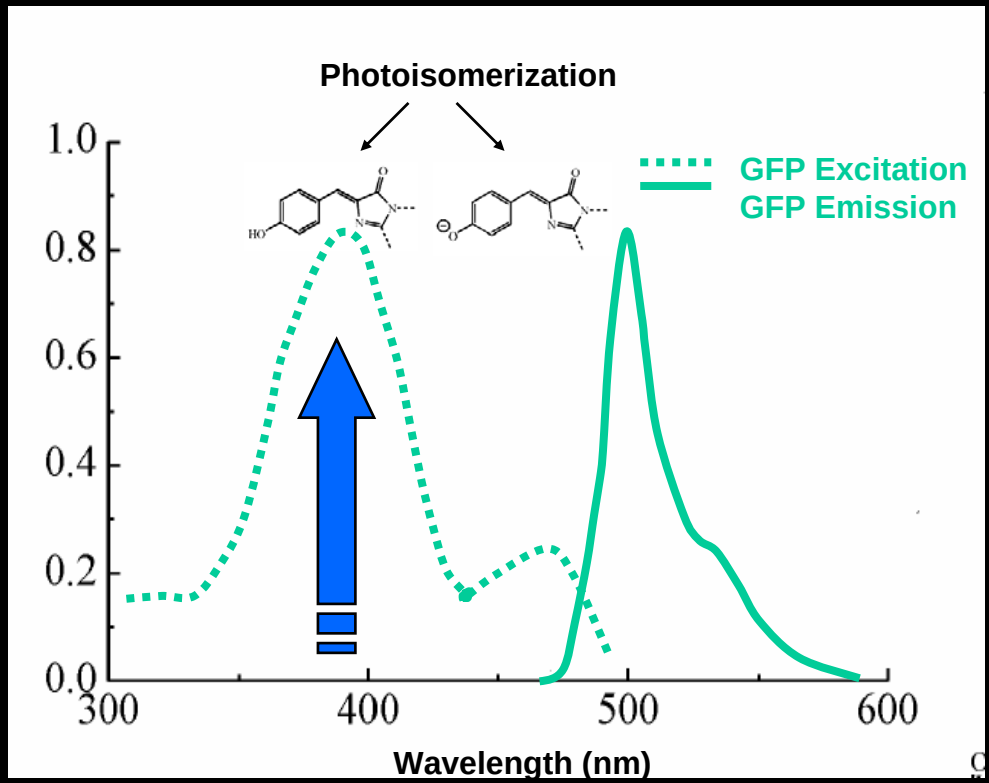
Phair and Mistelli, Nature Reviews MolCellBio, 2001

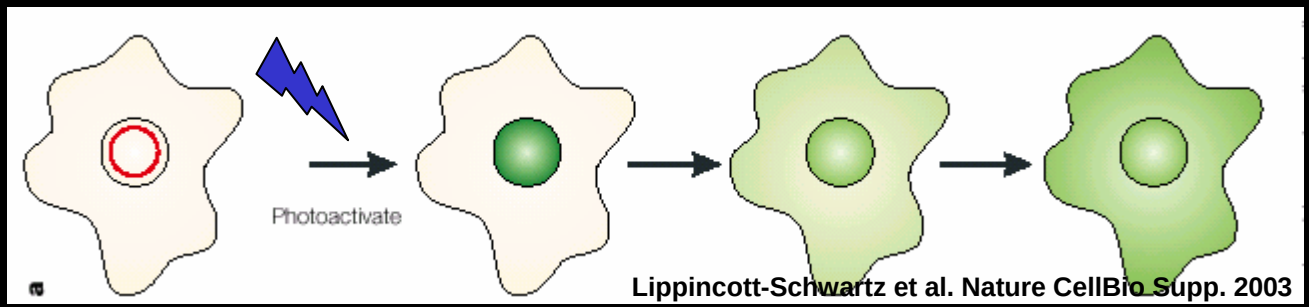
Fluorescence Loacalisation After Photobleaching (FLAP)



based on Phair and Mistelli, Nature Reviews MolCellBio, 2001

Photoactivatable GFP



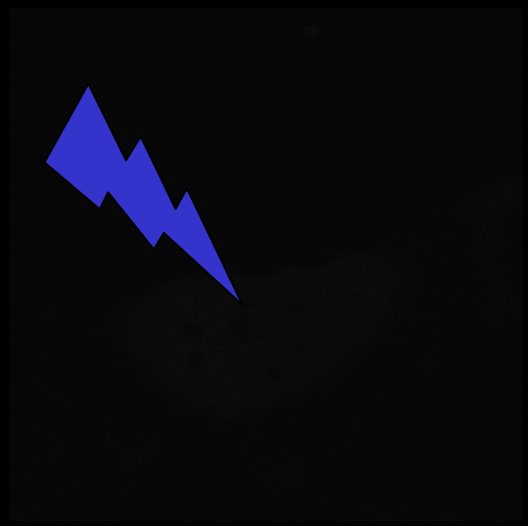


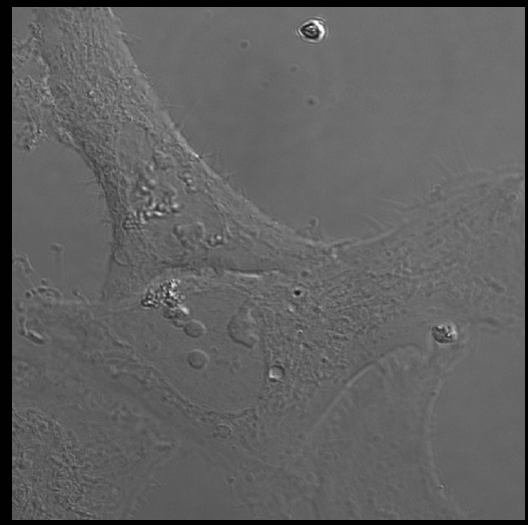
Lippincott-Schwartz et al. Nature CellBio Supp. 2003

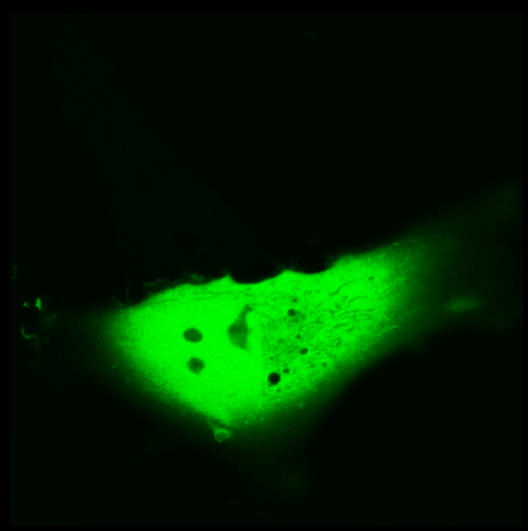
Photoactivatable GFP (PA-GFP)

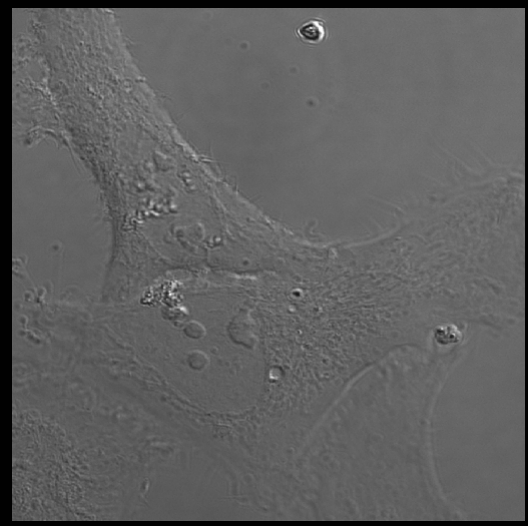
Excitation at 488 nm

Irradiation at 405 nm



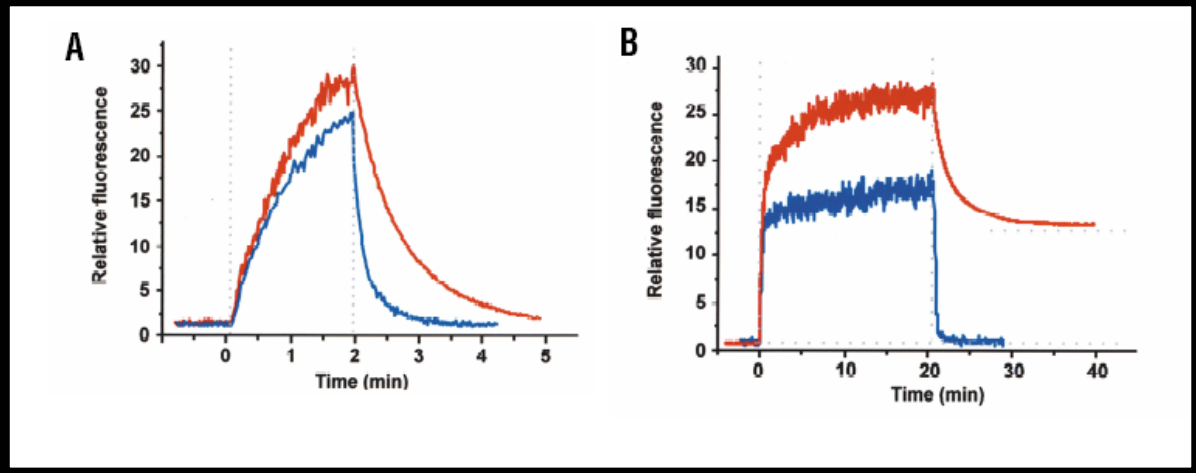


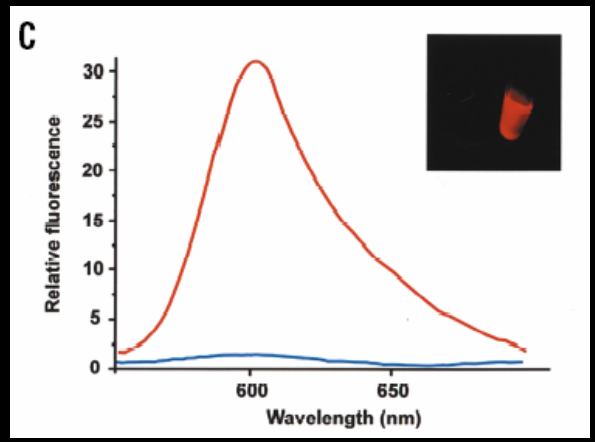




Patterson and Lippincott-Schwartz (2002), Science 297:1873-1877

Kindling (KFP)





488 nm

O_2

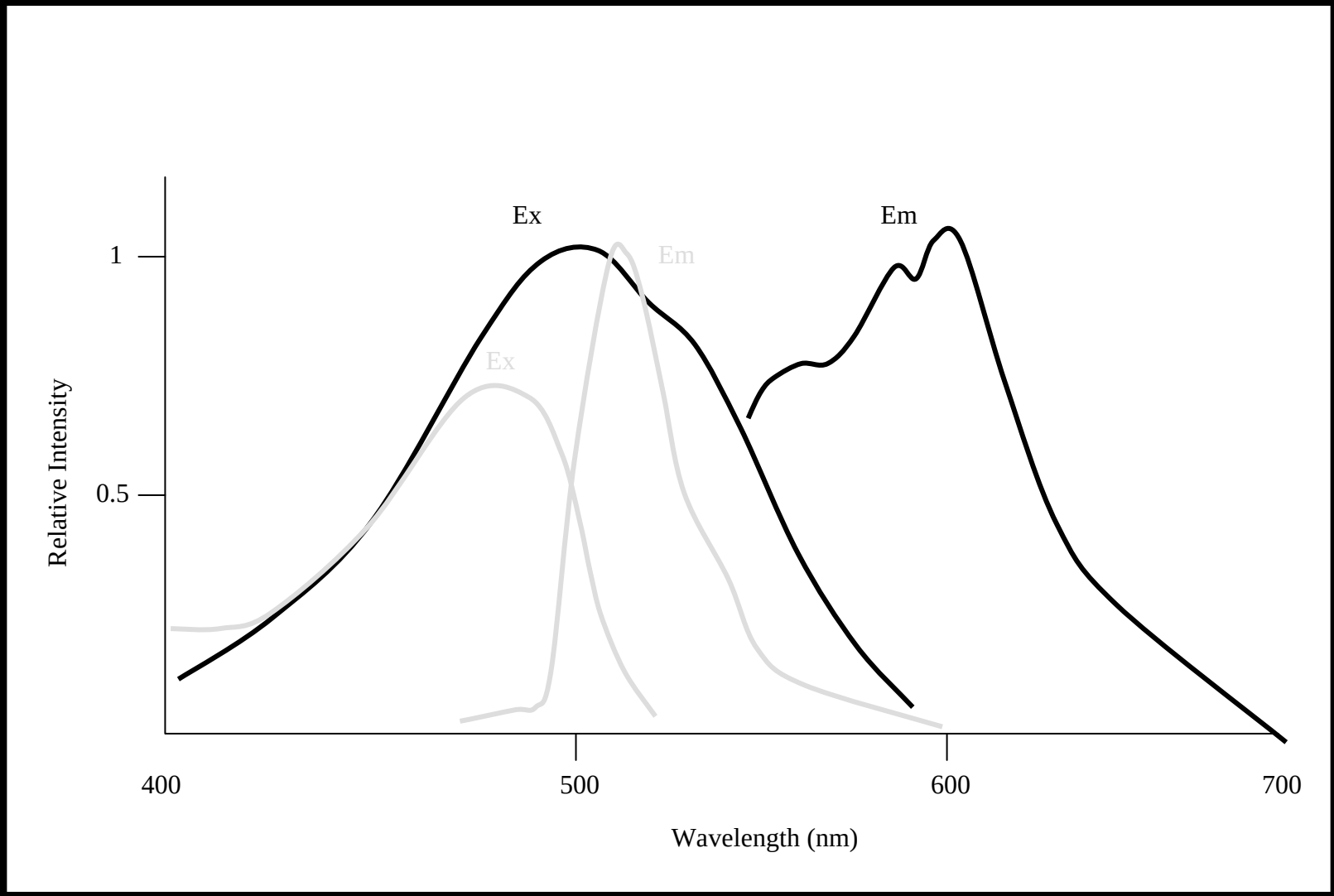
488 nm

O_2

543 nm

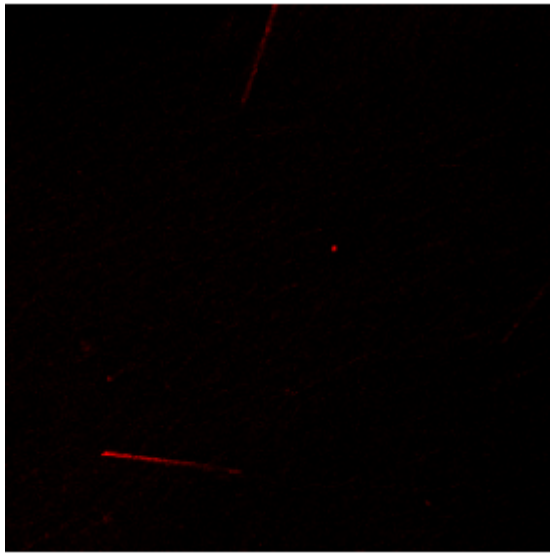
O_2

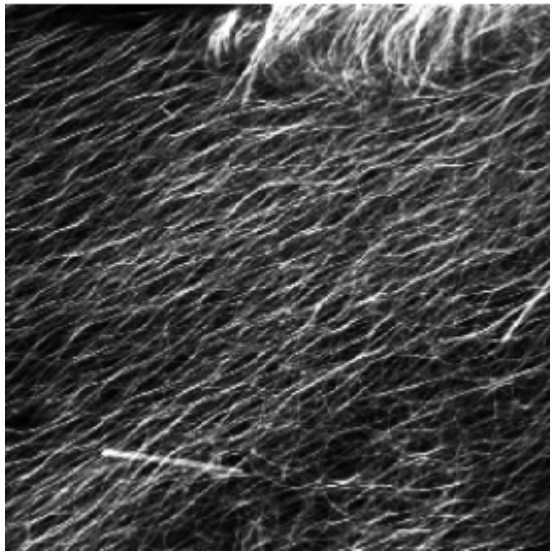
Spectral change after photoconversion

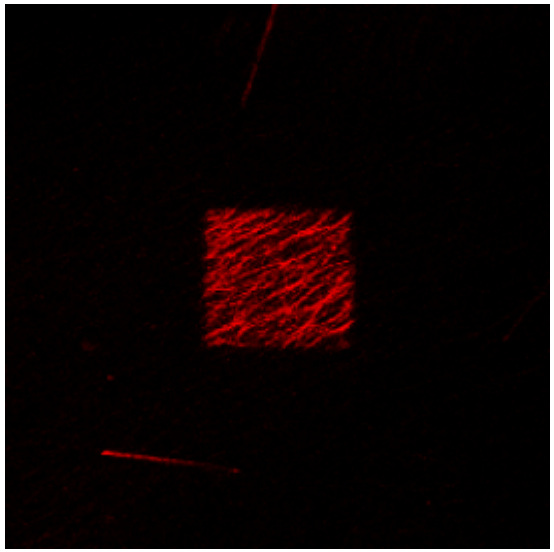


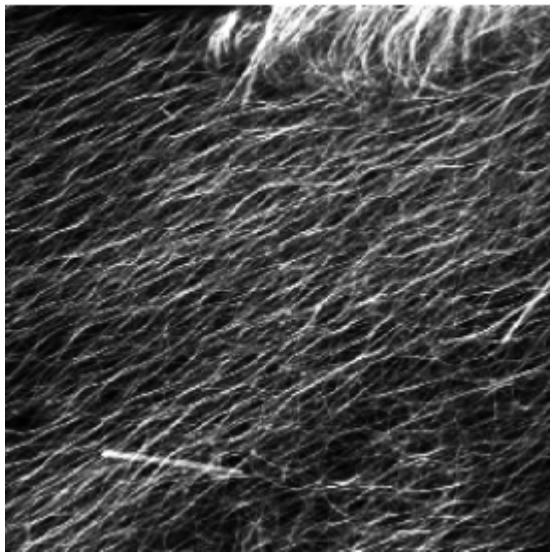
after Elowitz et al. (1997), Curr. Biol. 7:809-812

Microtubule binding proteins (TPX2)







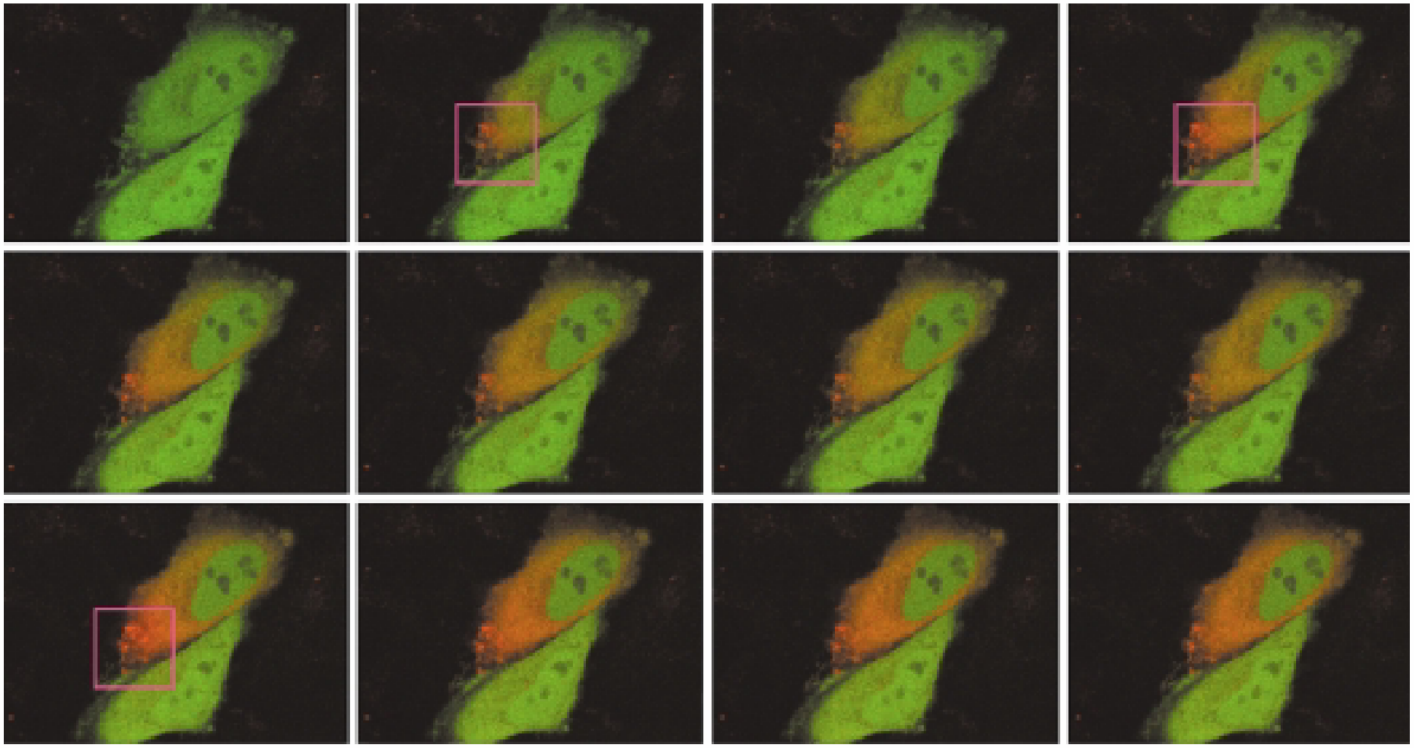


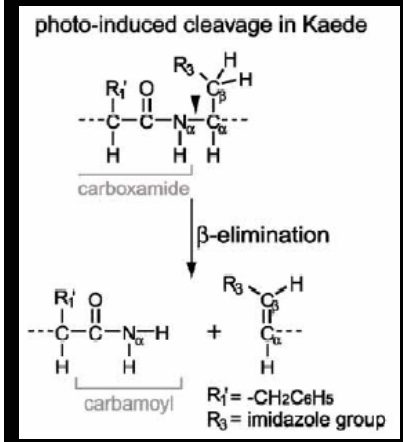
Photoconverted
GFP

Cy5-labelled
microtubules

Kaede

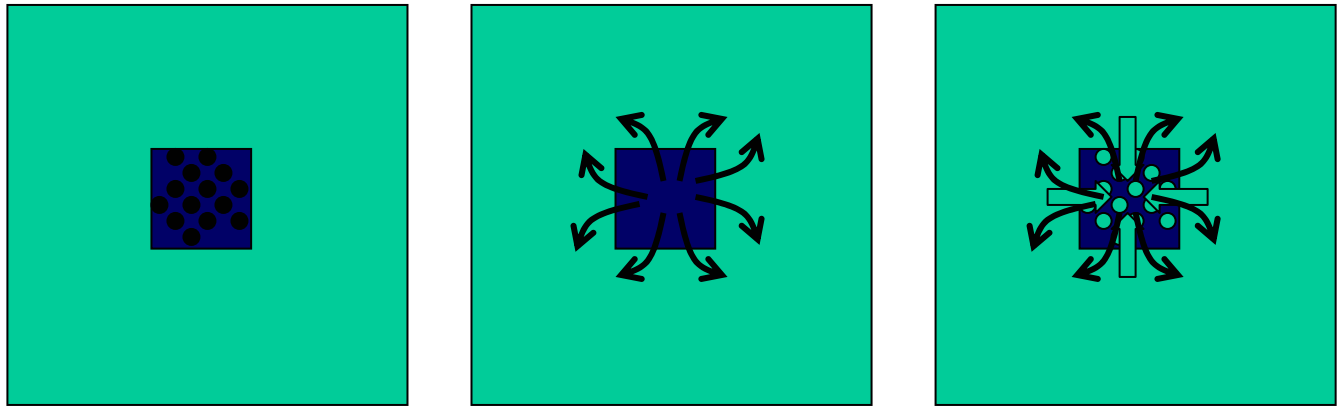
Acquiring images of Kaede-expressed HeLa cells while exciting with a 488nm/543nm laser every 3 seconds, and observing the reddening processes via 405nm laser illumination with SIMS scanners.
Data courtesy of :Ms. Ryoko Ando, Dr. Atsushi Miyawaki, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics
Objective: UPlanApo60xoil





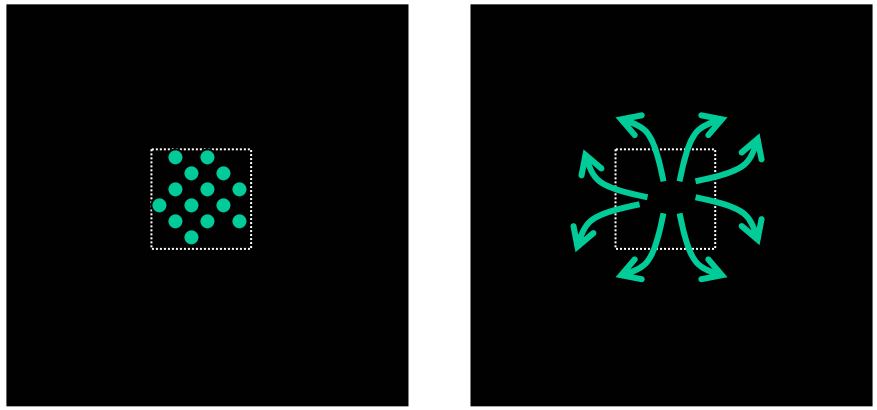
Advantages of photoactivation

FRAP



Off + On
Background of unbound molecules

iFRAP, Photoactivation, Photoconversion



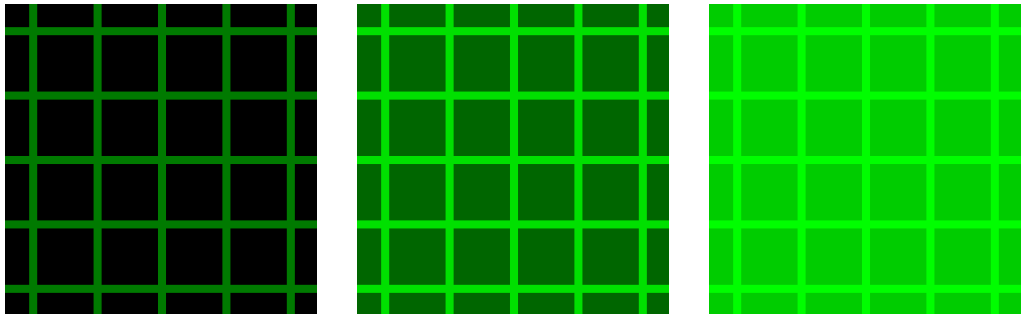
Off

⇒Direct measurement of the Off-Rate
Negligible background

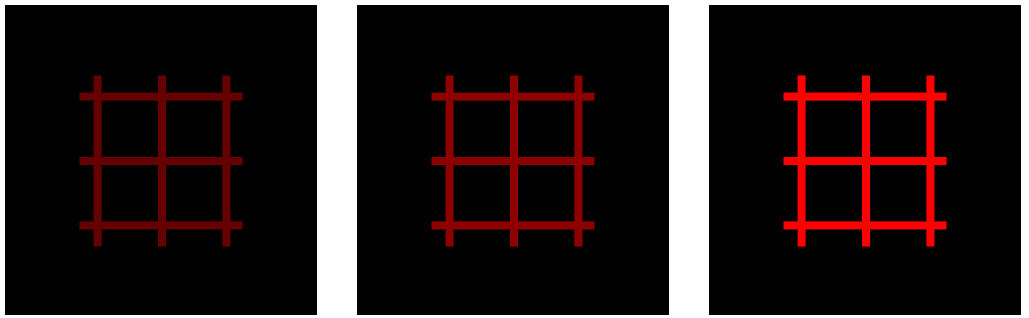
Advantages of photoconversion

Binding measurements

GFP channel



Photoconverted GFP channel



[C]

⇒No background correction
High signal to background

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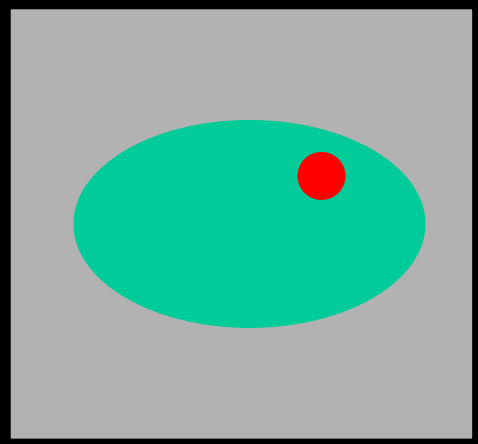
Present limitations of quantitative FRAP analysis

- The experimental system does not correspond to a 2D diffusion model => 3D FRAP models have been developed
- Diffusion during the bleach period is neglected, leading to underestimation of diffusion coefficients => calculation models, technical solutions

Intensity of bleaching light

AOTF upregulation (0-100%):

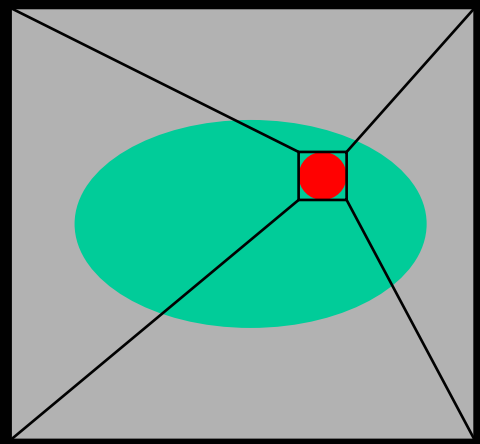
Linear



Zoom In:

Exponential

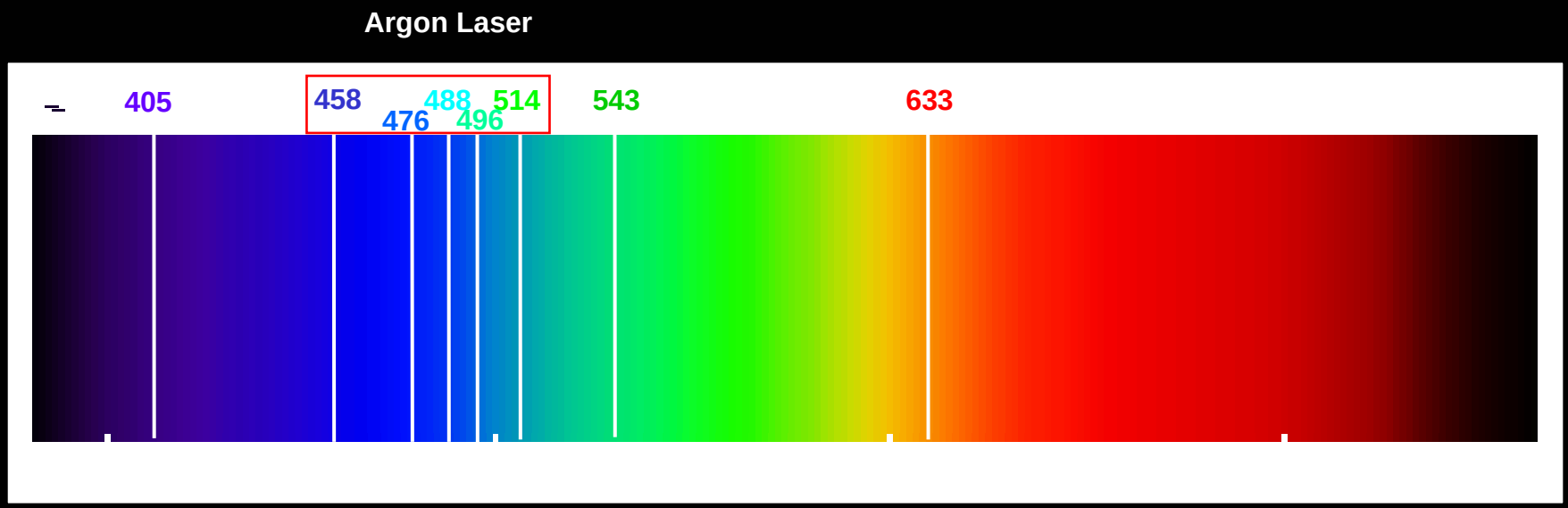
$2^{zoomfactor}$



Speed limited, does not work with ‘Fly’-Mode

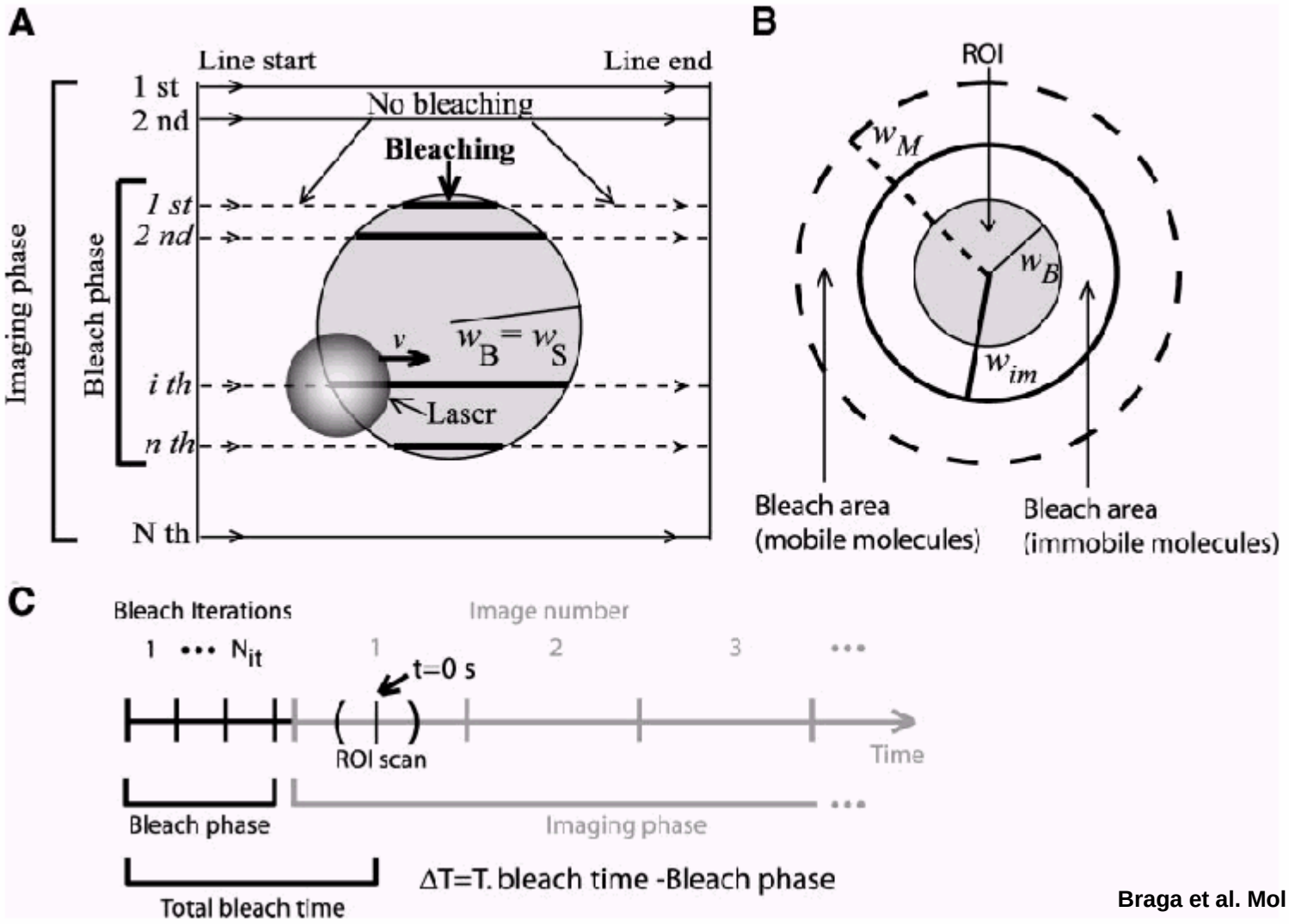
Leica AOBS SP2

Available laser lines



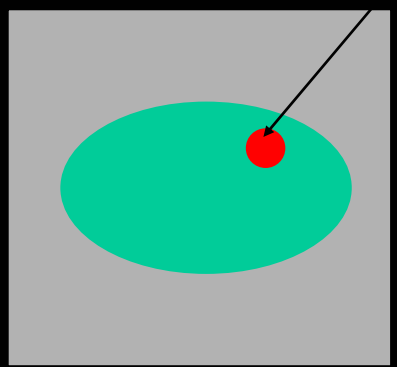
Argon laser

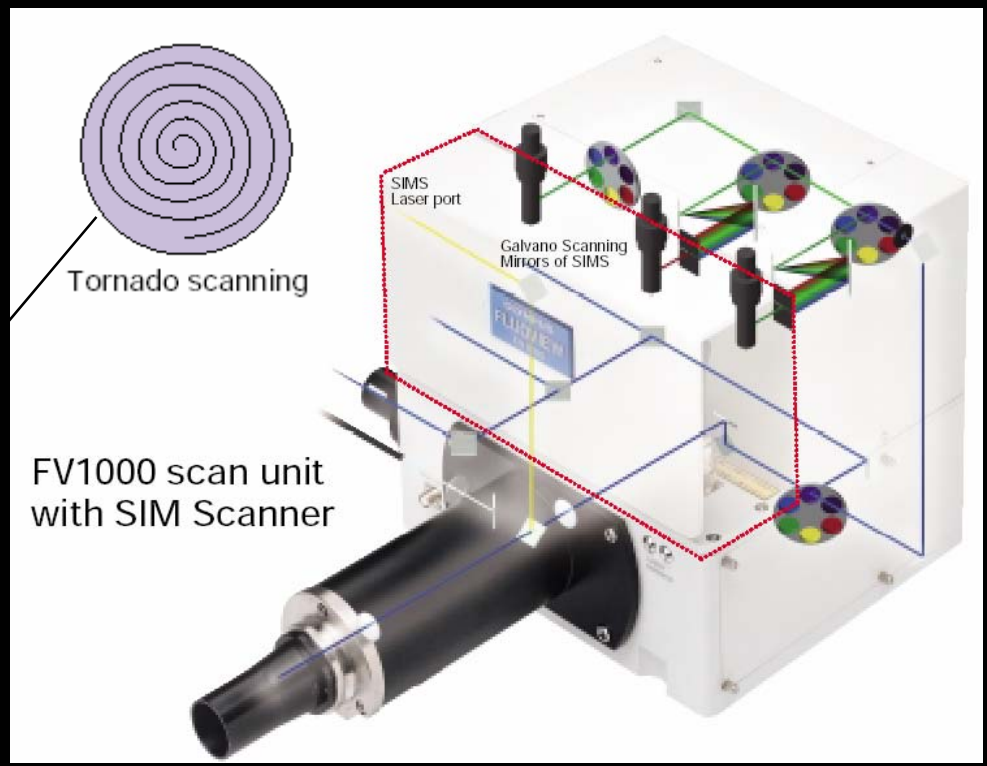
100 mW => 500 mw



Braga et al. Mol Biol Cell, 2004

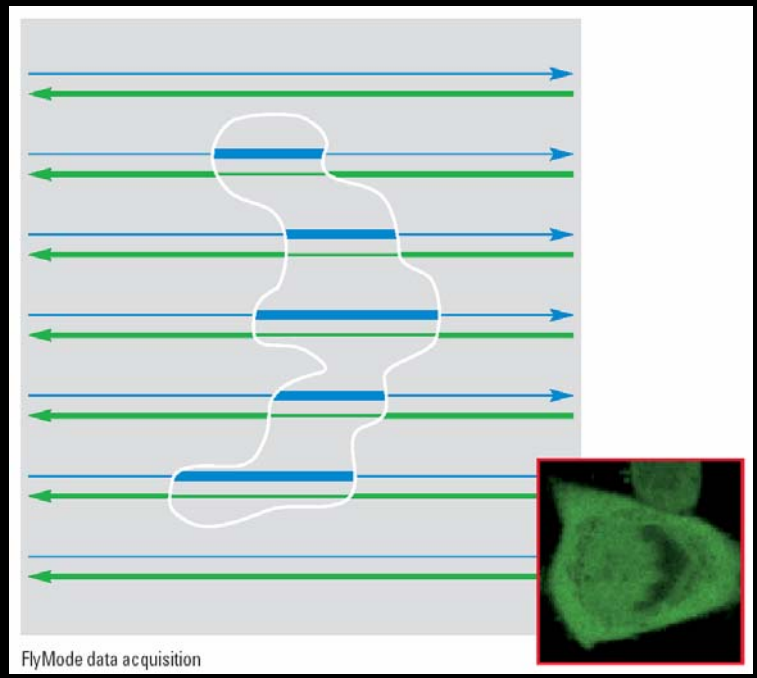
Olympus FluoView 1000





Leica AOBS SP2

‘Fly-back’ FRAP detection



=> readout within milliseconds of bleaching

Renaissance of widefield microscopes with sensitive CCD cameras and laser bleaching modules (Deltavision RT Quantifiable Laser module)