Introduction to Fluorescence Recovery after Photobleaching (FRAP)

Timo Zimmermann, Advanced Light Microscopy Facility
European Molecular Biology Laboratory, Heidelberg

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 $R = \frac{\lambda}{2n \sin \theta}$

Molecular dynamics, molecular interactions

FRAP  FCS  FRET

1 Å  1 nm  1 µm  1 mm  1 cm  1 m
10^{-10}m  10^{-9}m  10^{-6}m  10^{-3}m  10^{-2}m  1 m

Organelles  Cells  Worm  Housefly  Human

LM limit
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

I: Pre-bleach  II: Bleach  III: Post-bleach

Curve: K. Miura, Heidelberg
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)
\[ F_b = F(t) - \text{background} \]

\[ F_{b,\text{corr}}(t) = F_b(t) \frac{F_{\text{presell}}}{F_{\text{inf cell}}} \]

\[ F_{b,\text{corr, norm Azetrod}}(t) = \frac{F_{b,\text{corr}}(t) - F_{b,\text{corr}}(0)}{F_{b,\text{corr}}(\infty) - F_{b,\text{corr}}(0)} \]

Kappel and Eils, Leica App.Letter 2004
The time constant and mobile / immobile fractions

Mobile Fraction

Immobile Fraction

In the FRAP curve, the immobile & mobile fraction can be measured by determining the plateau level.
Half Life is the time when the recovery is the half of A, by definition.

\[ f(t) = A \left( 1 - e^{-\tau t} \right) \]

\[ \tau_{1/2} = \frac{\ln 0.5}{-\tau} \]
Estimated parameters by exponential fit:

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

Estimation of diffusion coefficient (Axelrod et al.)

\[ D = \frac{0.88^* w^2}{4 t_{1/2}} \]

w: bleach radius

Assumptions:

- bleached area is disk shaped
- diffusion occurs only in 2D
Free diffusion vs. binding

Multiple populations with differing diffusion rates \implies multi-component equations

Phair and Mistelli, Nature Reviews MolCellBio, 2001

Possible FRAP artifacts

Photo-induced immobile fraction

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 Localisation After Photobleaching (FLAP) based on Phair and Mistelli, Nature Reviews MolCellBio, 2001

a FRAP

Bleach pulse  Measure influx of labelled protein into bleached area

FLAP

CFP  YFP

Bleach pulse  Measure influx of labelled protein into bleached area
Photoactivatable GFP

Wavelength (nm)

GFP Excitation
GFP Emission

Photoisomerization

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

$\text{O}_2$
488 nm

O₂
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
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: UPlanApo0x01
Advantages of photoactivation

⇒ Direct measurement of the Off-Rate
Negligible background

Off + On
Background of unbound molecules
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
  \[ 2^{\text{zoomfactor}} \]

Speed limited, does not work with ‘Fly’-Mode
Leica AOBS SP2

Available laser lines

Argon Laser

- 405
- 458
- 476
- 488
- 496
- 514
- 543
- 633

Argon laser

100 mW => 500 mw
Olympus FluoView 1000

FV1000 scan unit with SIM Scanner

Tornado scanning
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)