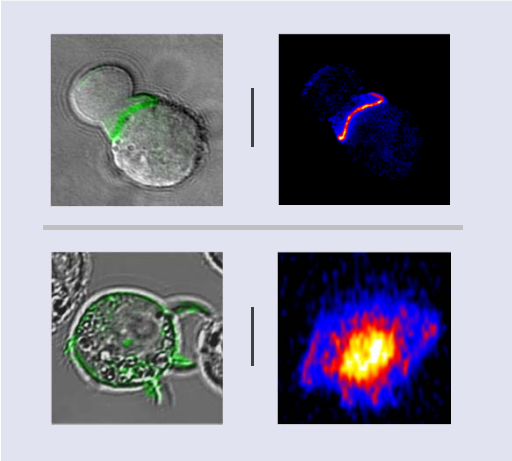
5th Course on Optical Microscopy Imaging for Biosciences

IBMC, Porto, Portugal

Molecular Imaging

FRAP, PA, PC, FRET, FLIM, FLIP, FLAP etc.

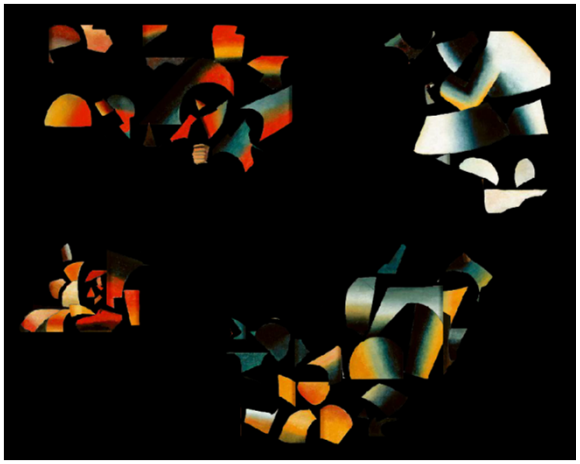


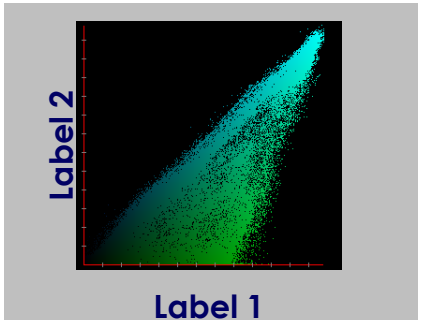
Martin Spitaler
FILM - Facility for Imaging by Light Microscopy
Imperial College London, UK

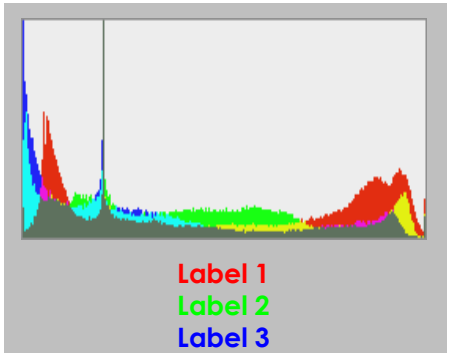
Imperial College London

FILM

Flow cytometry







Unknown sample

Results:

- Intensity distribution label 1
- Intensity distribution label 2
- Intensity distribution label 3
- Forward scatter (size)
- Sideward scatter (shape / granularity)
- …

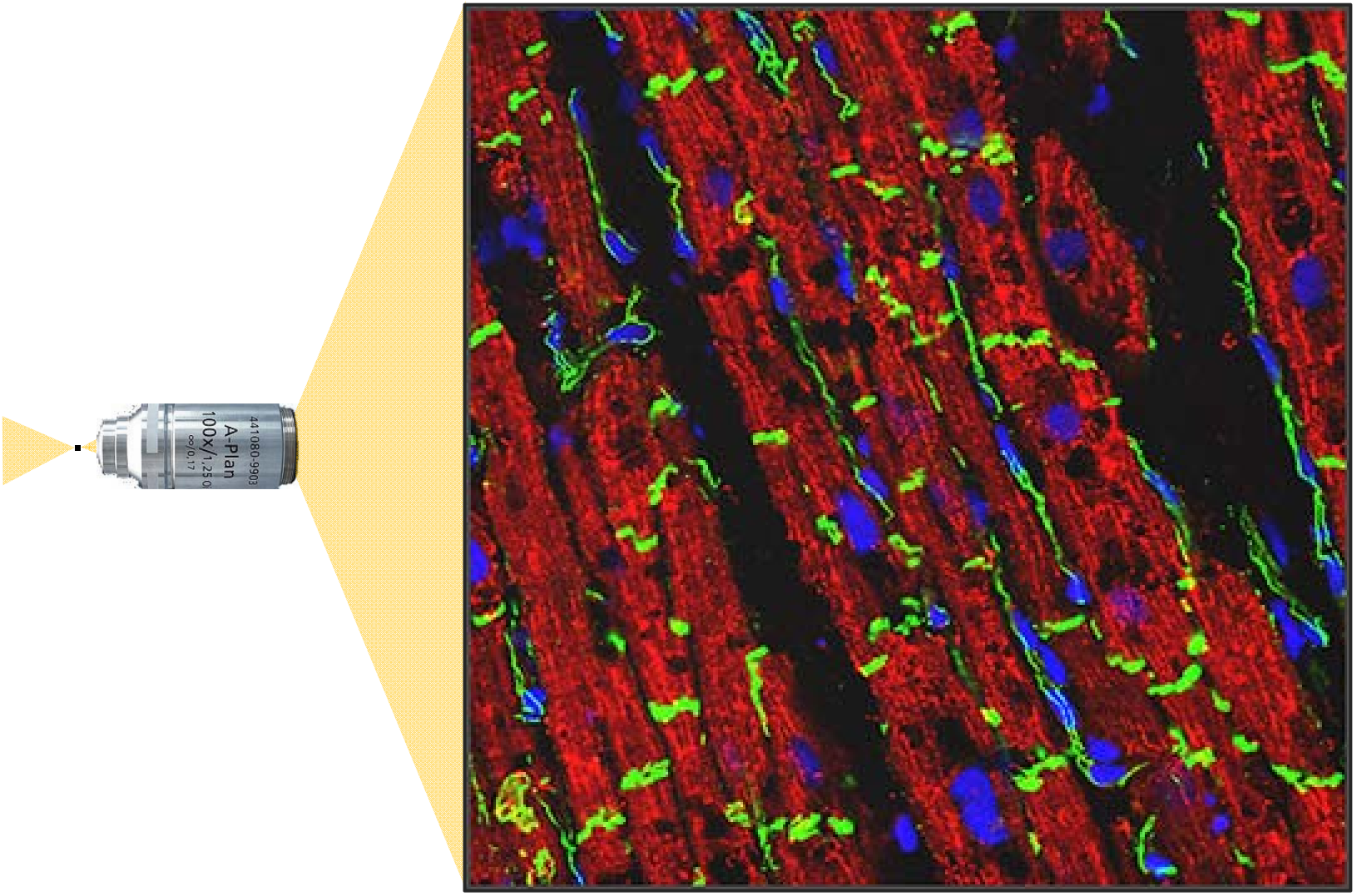
Source: Ursus Wehrl, www.kunstaufraeumen.ch

Microscopy

Result:

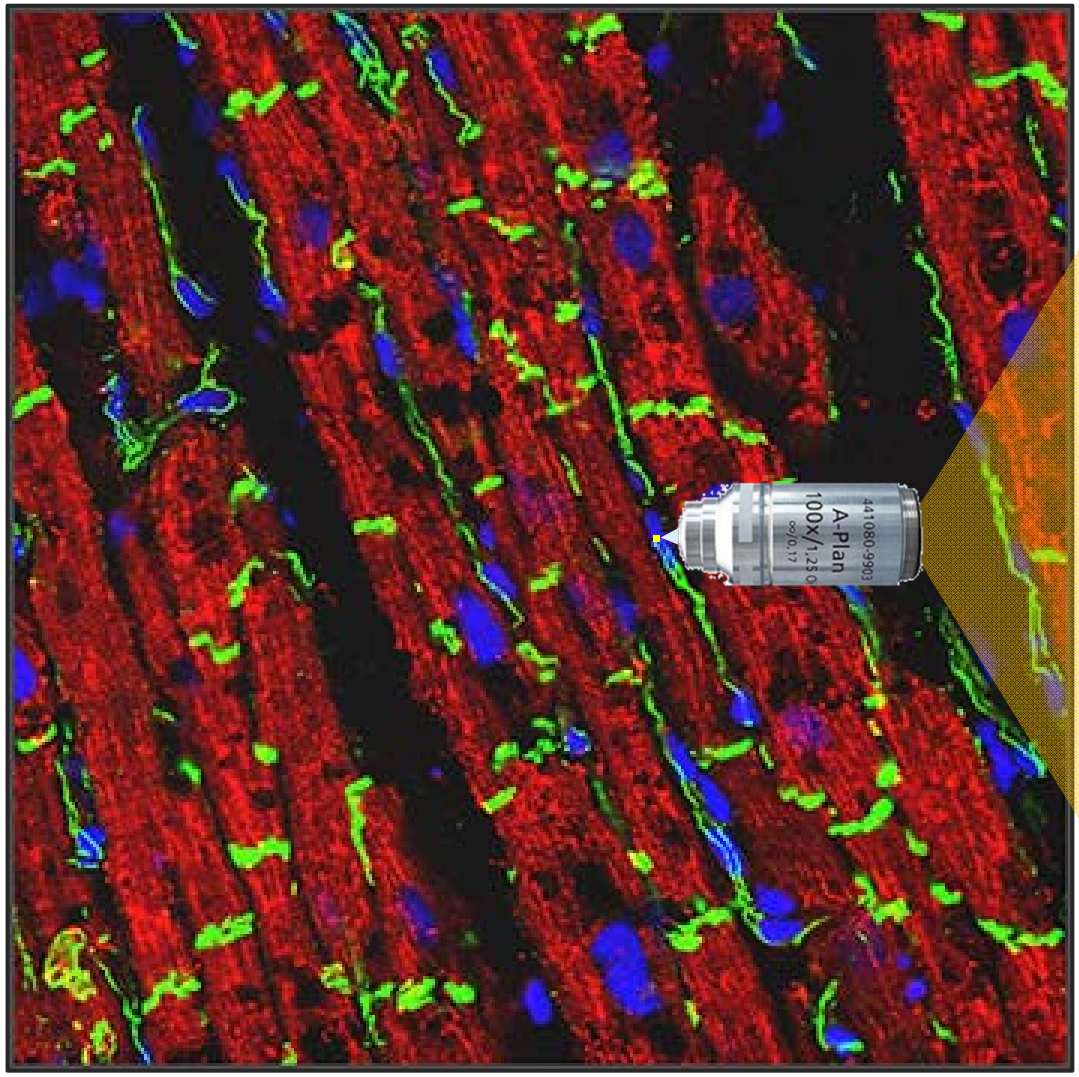
- The full picture

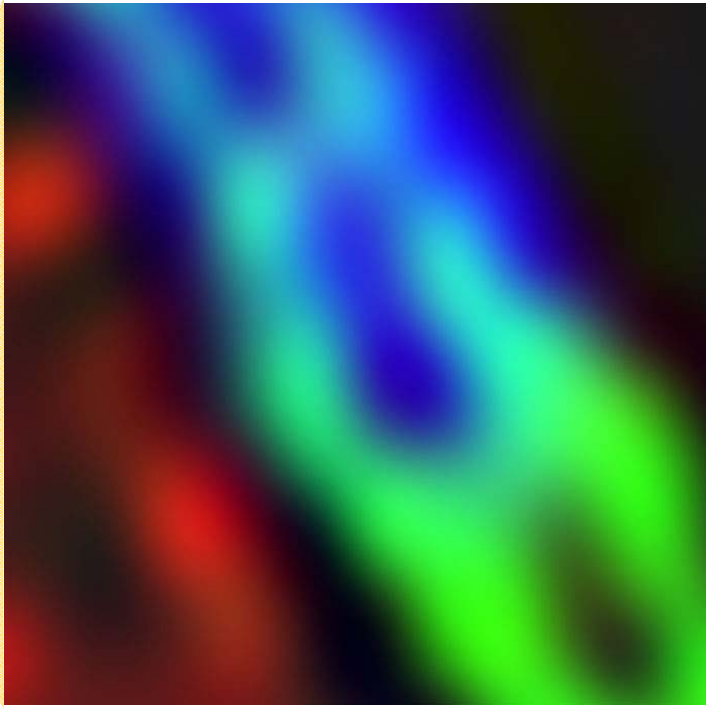
Kazimir Malevich: The Woodcutter

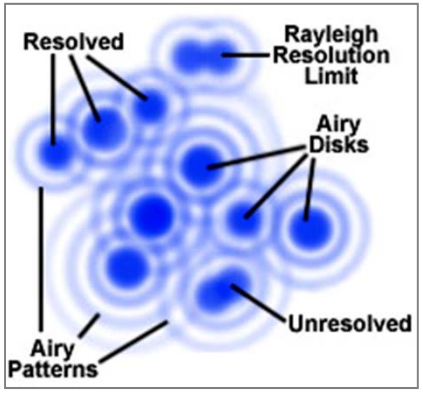


one cell

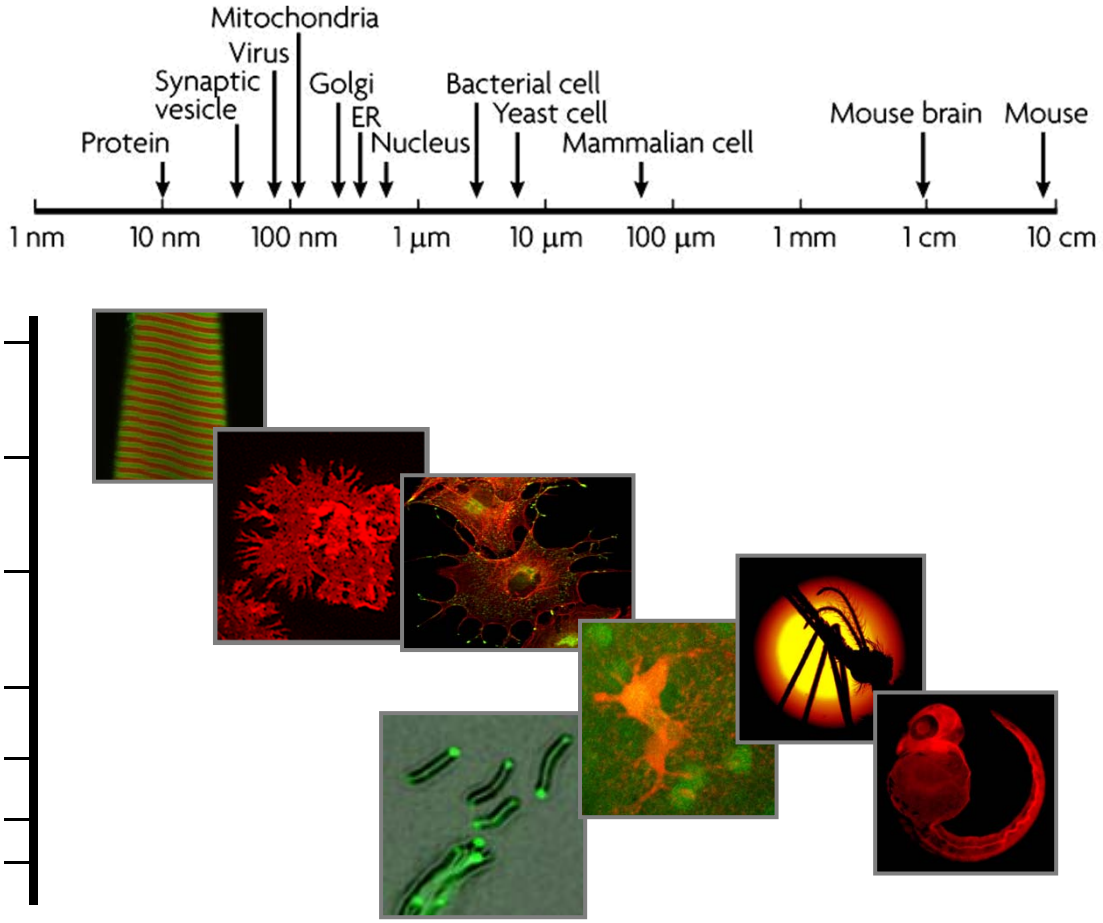
millions of details



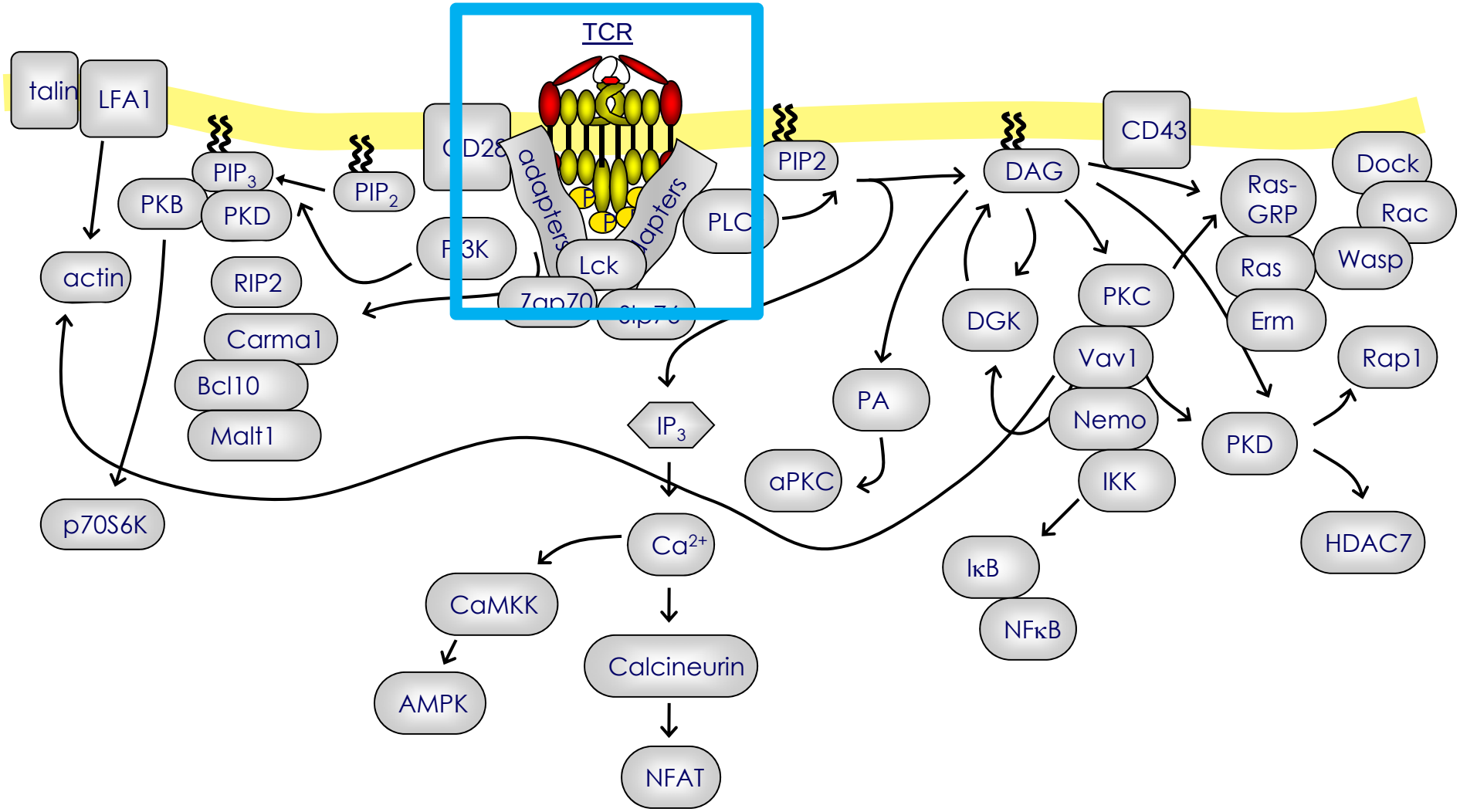




$$r = 0.61 * \frac{\lambda}{NA}$$

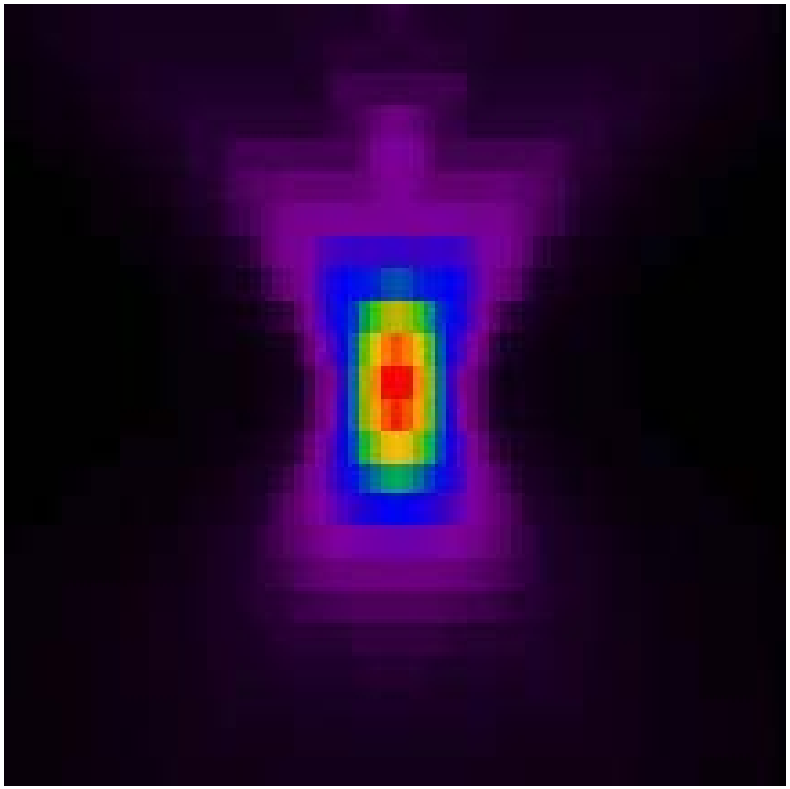

Mitochondria
Virus
Synaptic vesicle
Golgi
ER
Bacterial cell
Yeast cell
Protein
Nucleus
Mammalian cell
Mouse brain
Mouse
1 nm
10 nm
100 nm
1 µm
10 µm
100 µm
1 mm
1 cm
10 cm
nsec
µsec
msec
sec
min
h
d

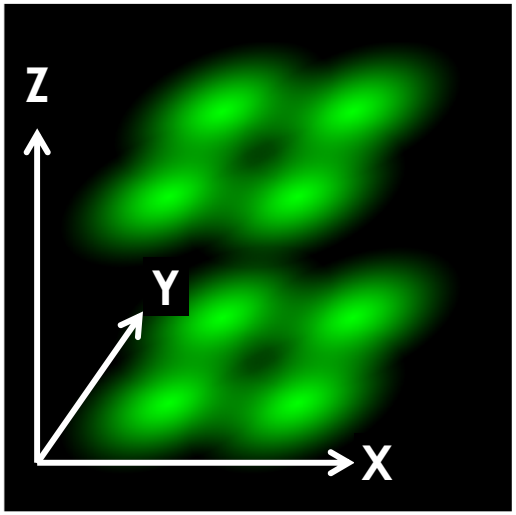
Molecular network in cell activation



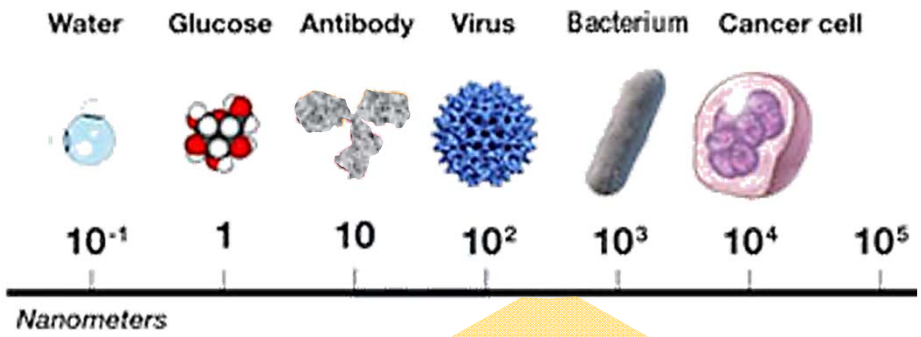
Molecular scales

	Wide-field		Confocal	
	Lateral resolution dx, y	Axial resolution dx, z	Lateral resolution dx, y	Axial resolution dx, z
Expression	$0.61\,\lambda_{em}/NA$	$2\,\lambda_{em}/NA^2$	$0.4\,\lambda_{em}/NA$	$1.4\,\lambda_{em}/NA^2$
Limit resolution of a 63× oil immersion objective with NA = 1.32 at λ_{em} = 500 nm	232 nm	574 nm	152 nm	402 nm
Minimal justified pixel size for this objective	101 nm	250 nm	66 nm	175 nm



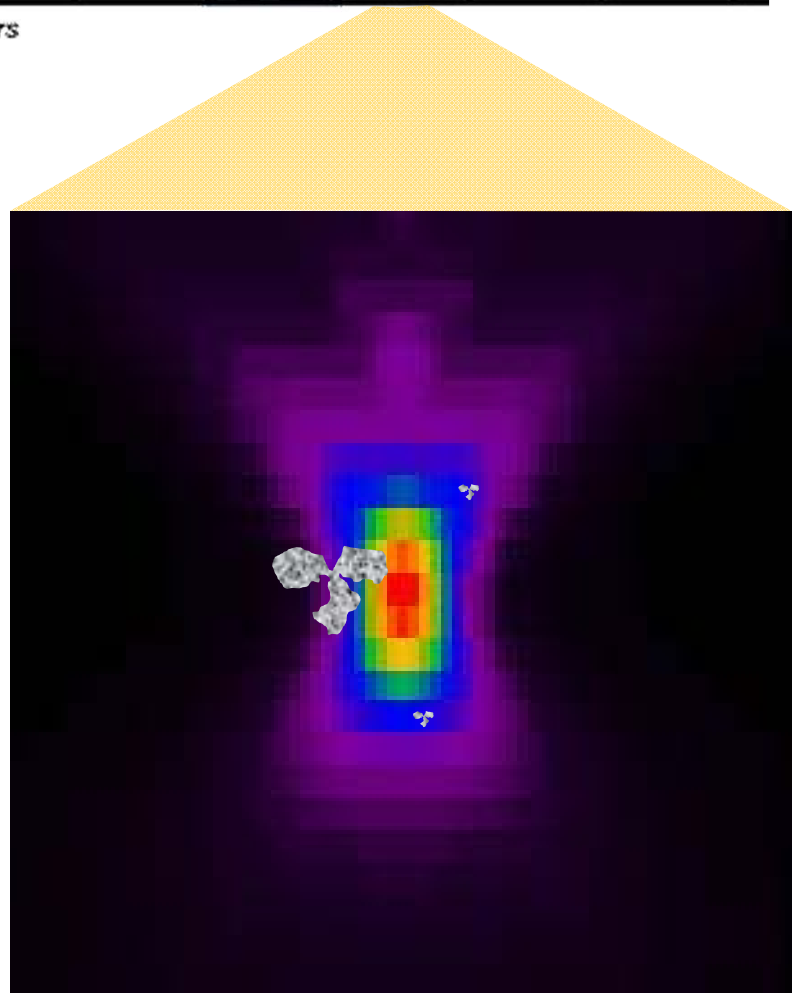


Molecular scales

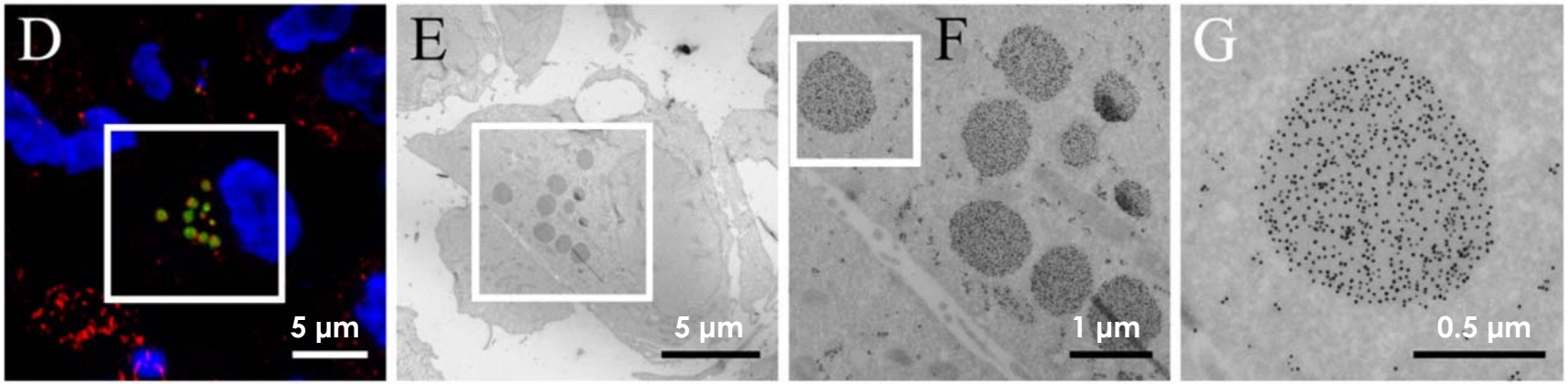


NIH
ChemHealthWeb

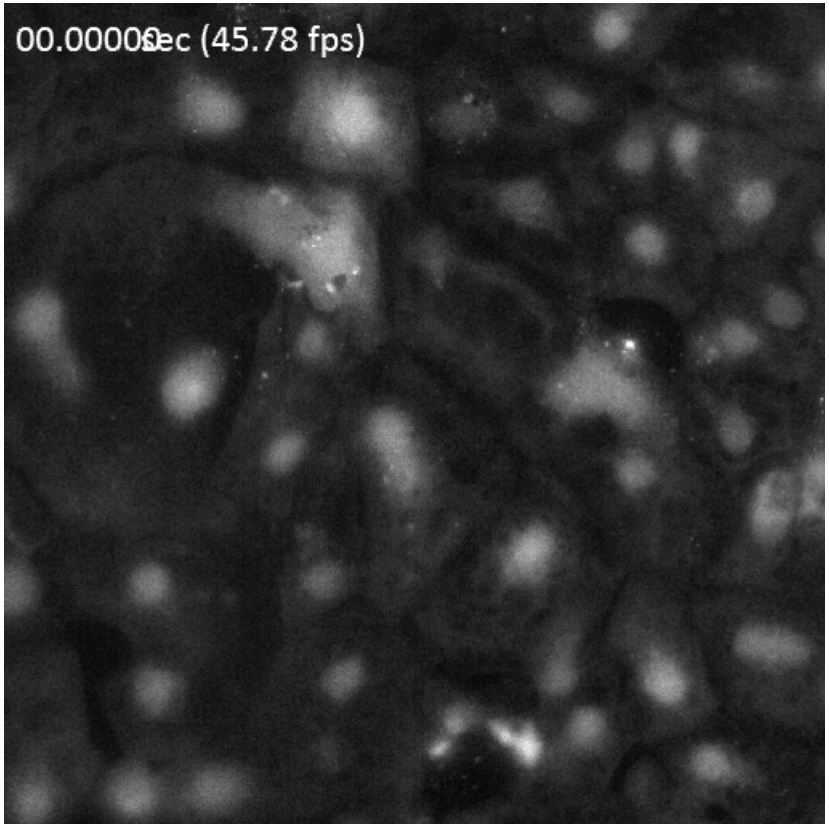
Confocal "voxel"



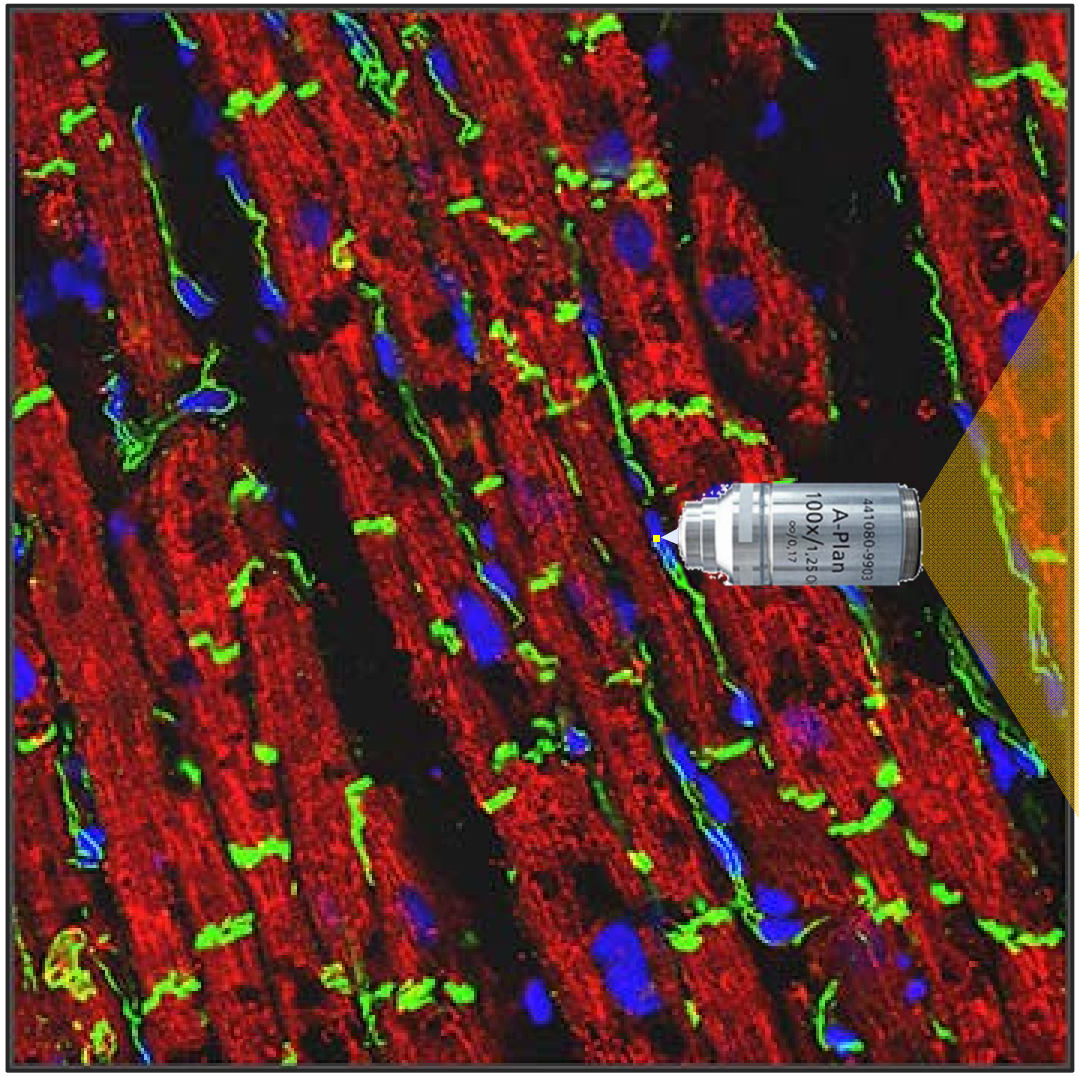
Molecular imaging by correlative light–electron microscopy

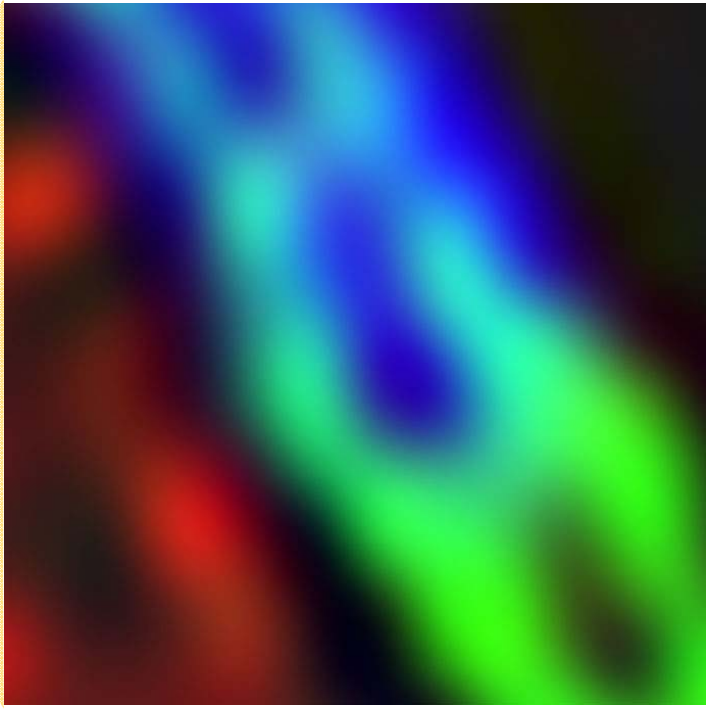


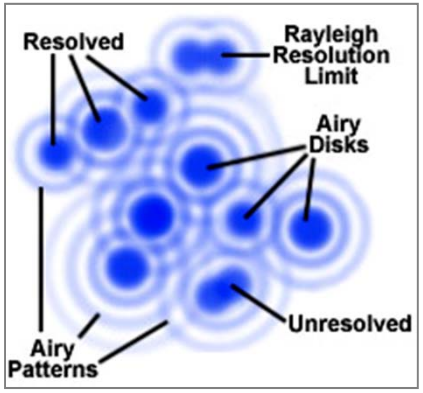
Katia Cortese, Alberto Diaspro, Carlo Tacchetti
J Histochem Cytochem December 2009 vol. 57 no. 12 1103-1112



Nicola Hellen: Ca”+ signal in cardiomyocytes





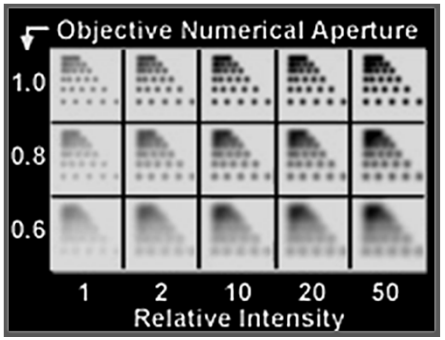


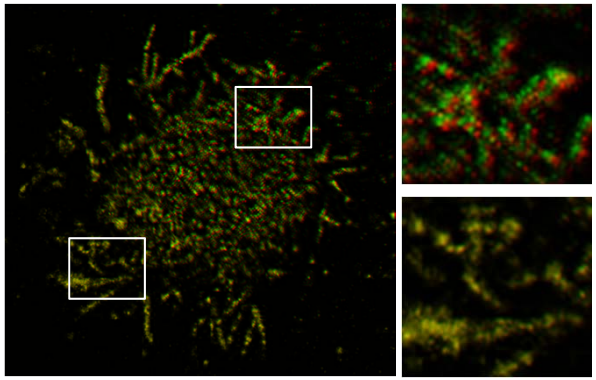
$$r = 0.61 * \frac{\lambda}{NA}$$

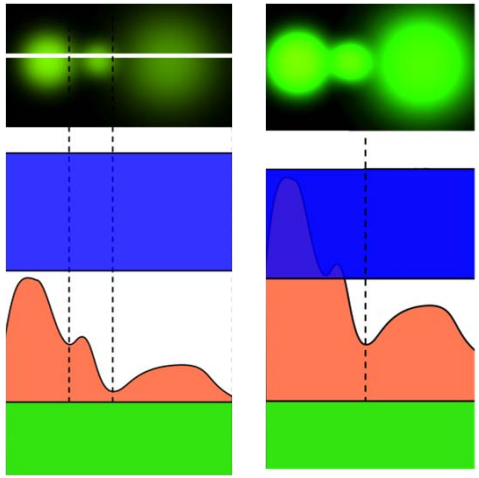
Molecular imaging:
Best possible image data!

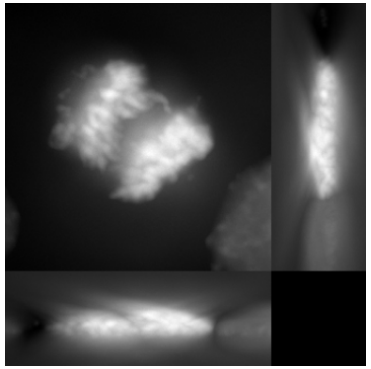
	Wide-field		Confocal	
	Lateral resolution dx, y	Axial resolution dx, z	Lateral resolution dx, y	Axial resolution dx, z
Expression	$0.61\,\lambda_{em}/NA$	$2\,\lambda_{em}/NA^2$	$0.4\,\lambda_{em}/NA$	$1.4\,\lambda_{em}/NA^2$
Limit resolution of a 63× oil immersion objective with NA = 1.32 at λ_{em} = 500 nm	232 nm	574 nm	152 nm	402 nm
Minimal justified pixel size for this objective	101 nm	250 nm	66 nm	175 nm

Nyquist

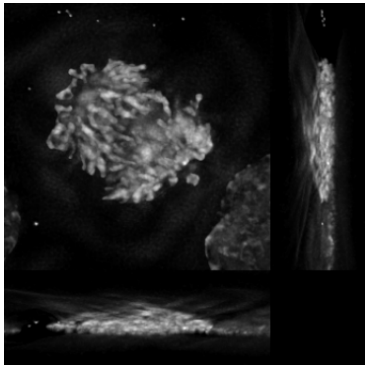






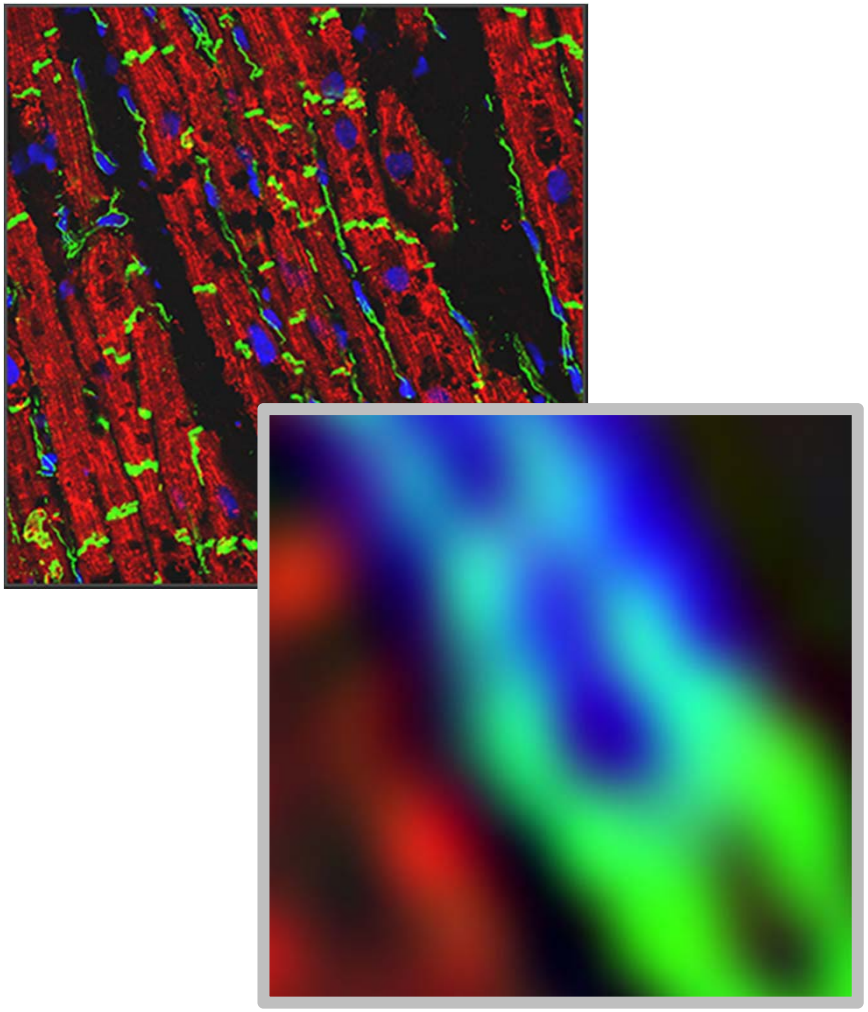


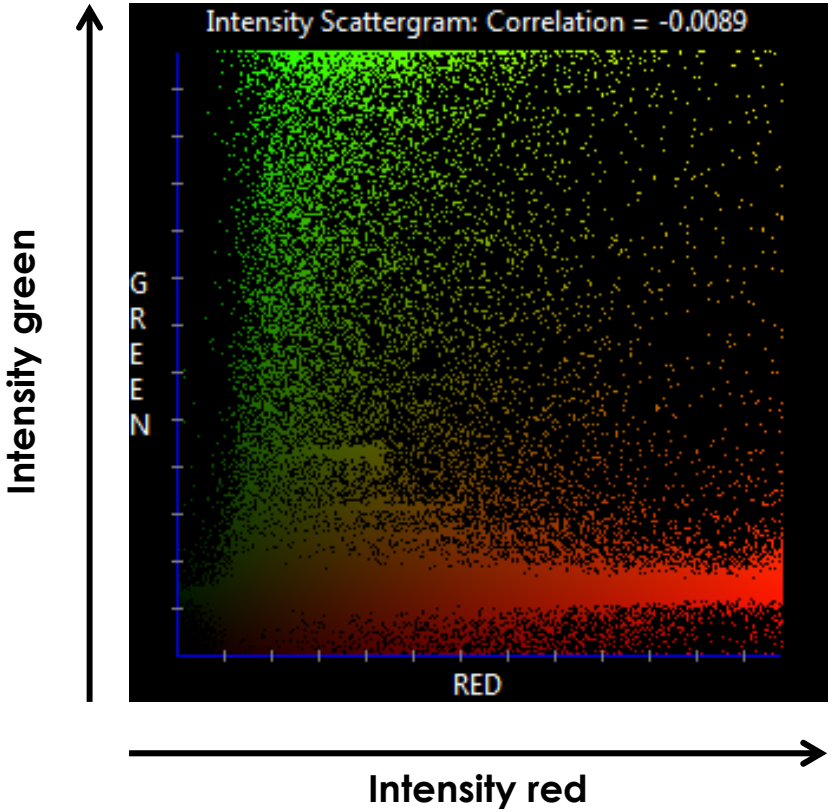
original



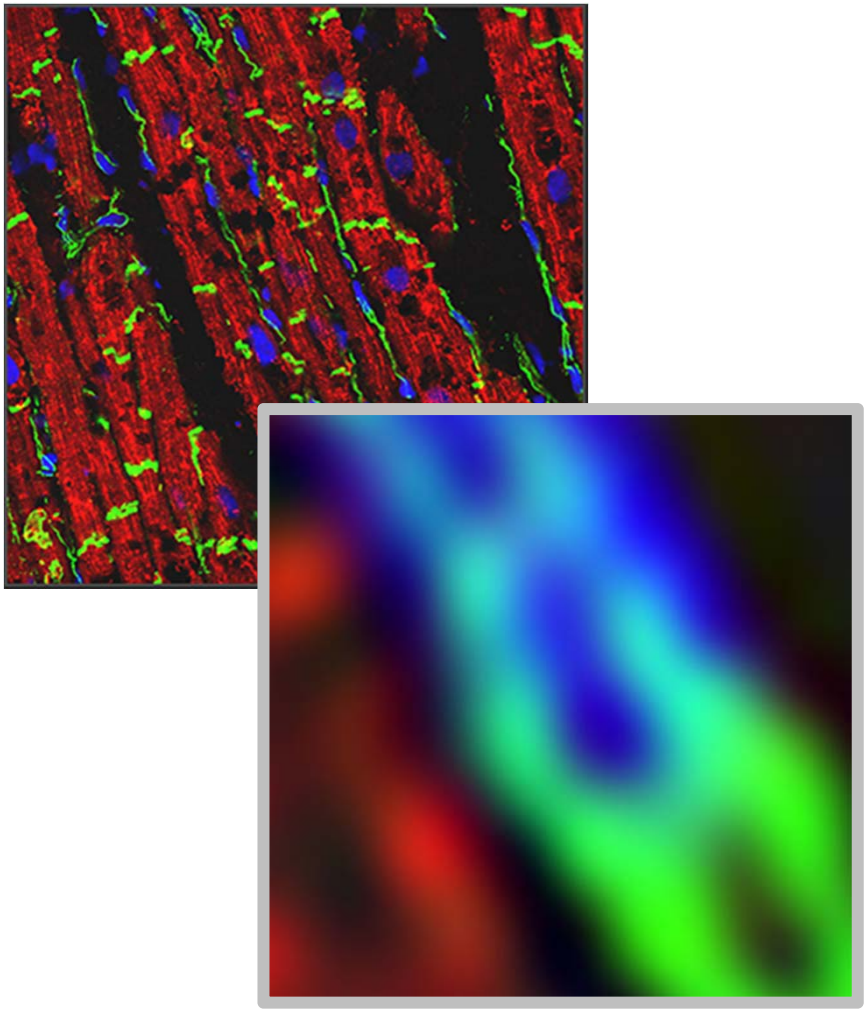
deconvolved

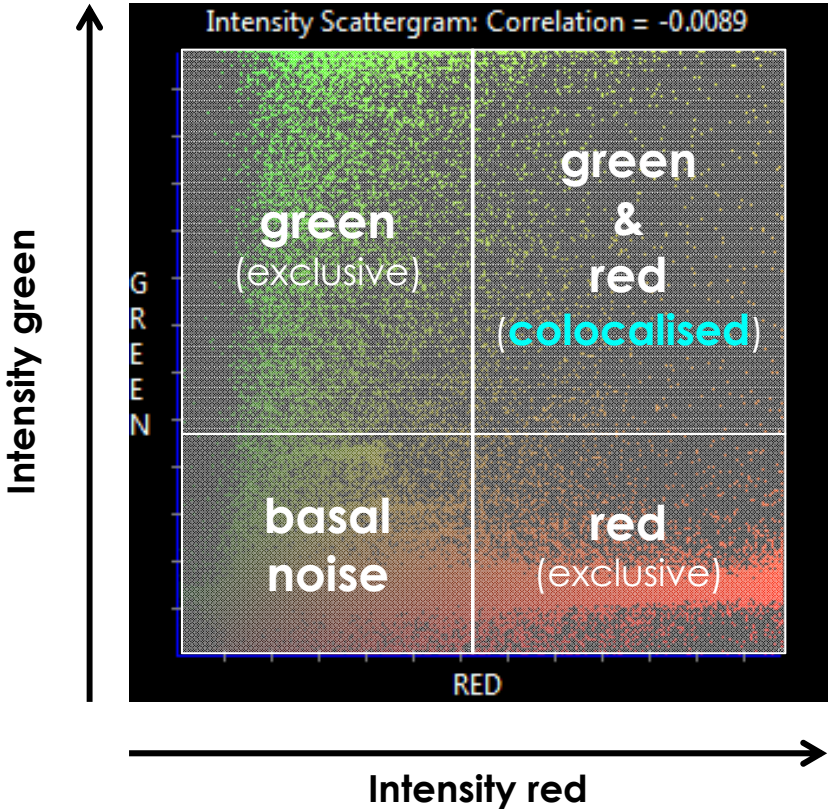
Colocalisation analysis





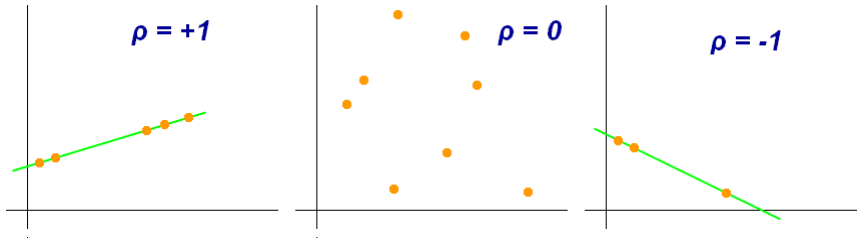
Colocalisation analysis





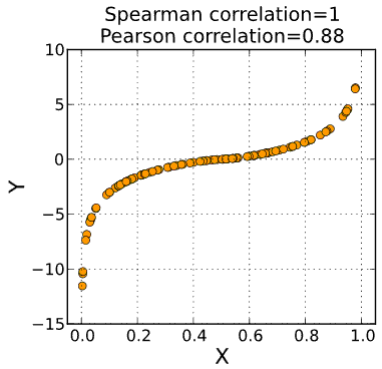
Colocalisation analysis:
Statistical analysis

Pearson's coefficient



linear correlation between
two single (homogeneous) populations

Spearson's coefficient



non-linear correlation
between two inhomogeneous populations

Overlap coefficient

coefficients M1 and M2 :
fraction of total area in one channel
coinciding with some intensity in the other channel

Manders' coefficient

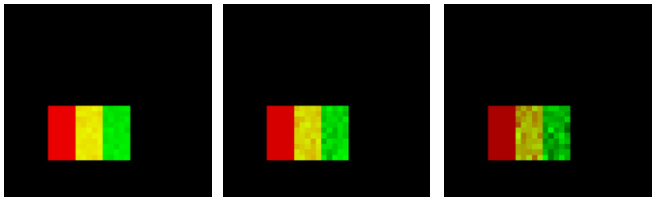
coefficients M1 and M2 :
fraction of total intensity in one channel
coinciding with some intensity in the other channel

Co-localisation analysis: effects of various image content

degree of overlap

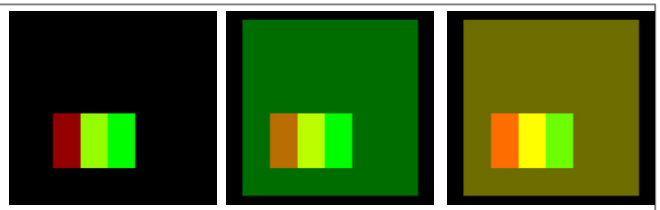
P = -0.108	P = 0.446	P = 0.169
M1 = 0.0	M1 = 0.500	M1 = 0.25
M2 = 0.0	M2 = 0.500	M2 = 0.25
O = 0	O = 0.500	O = 0.25

noise



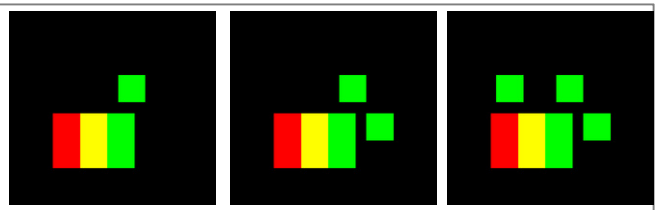
P = 0.445	P = 0.437	P = 0.425
M1 = 0.500	M1 = 0.500	M1 = 0.500
M2 = 0.499	M2 = 0.495	M2 = 0.493
O = 0.499	O = 0.491	O = 0.480

intensity of object and background



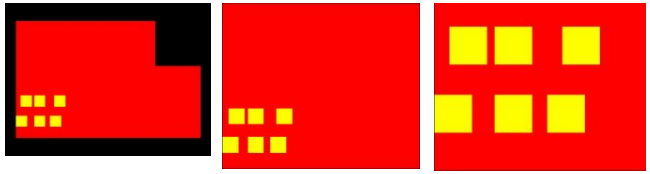
P = 0.446	P = 0.446	P = 0.446
M1 = 0.500	M1 = 1.000	M1 = 1.000
M2 = 0.500	M2 = 0.163	M2 = 1.000
O = 0.500	O = 0.468	O = 0.890

object area



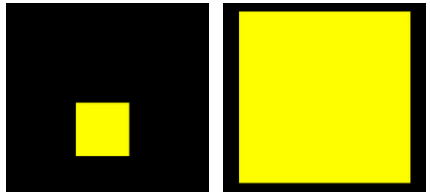
P = 0.380	P = 0.329	P = 0.288
M1 = 0.500	M1 = 0.500	M1 = 0.500
M2 = 0.400	M2 = 0.333	M2 = 0.286
O = 0.447	O = 0.408	O = 0.378

object area (zooming)



P = 0.446	P = 0.446	P = 0.446
M1 = 0.500	M1 = 1.000	M1 = 1.000
M2 = 0.500	M2 = 0.163	M2 = 1.000
O = 0.500	O = 0.468	O = 0.890

object area (cropping)

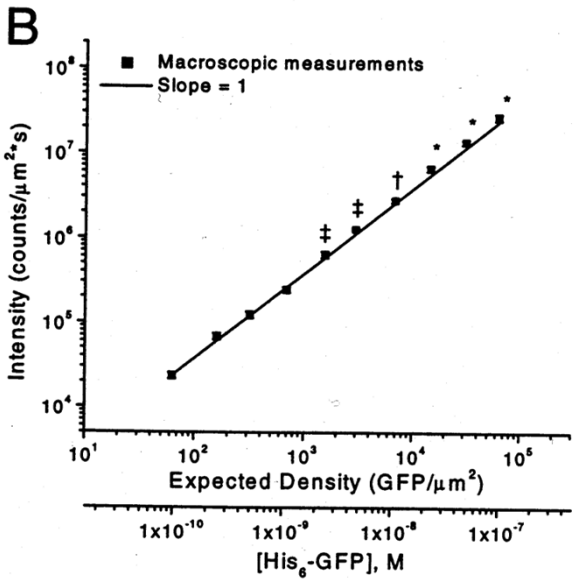


P = 0.380	P = 0.329
M1 = 0.500	M1 = 0.500
M2 = 0.400	M2 = 0.333
O = 0.447	O = 0.408

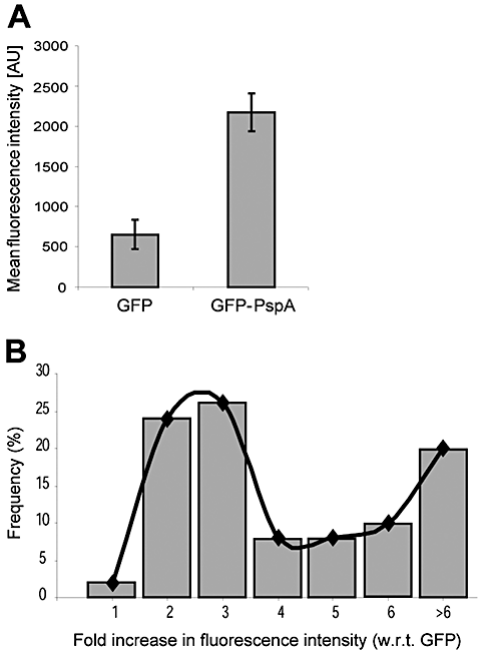
P = Pearson
M = Manders
O = Overlap

http://www.svi.nl/ColocalizationCoefficients

Single-molecule analysis by fluorescence calibration

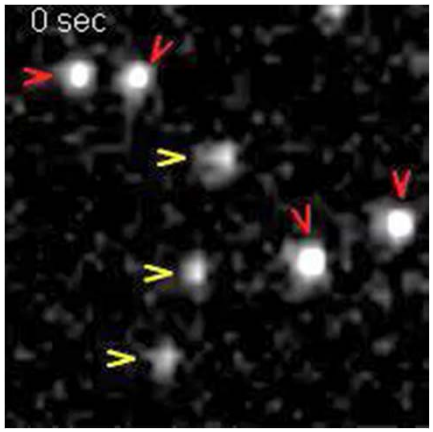


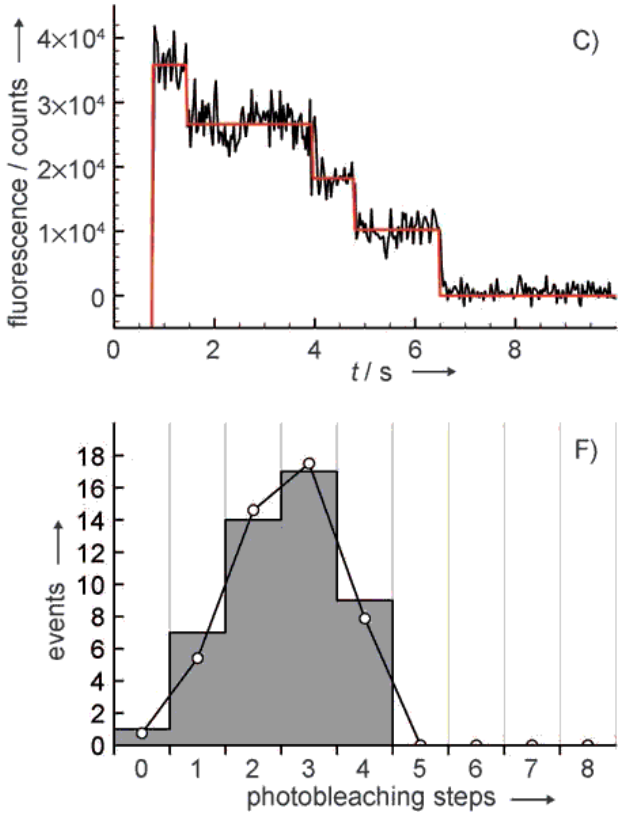
Chiu CS, Kartalov E, Unger M, Quake S, Lester HA.
J Neurosci Methods. 2001; 105(1): 55-63.



Engl C, Jovanovic G, Lloyd LJ, Murray H, Spitaler M, Ying L, et al.
Mol Microbiol. 2009.

Single-molecule analysis by step-wise photobleaching

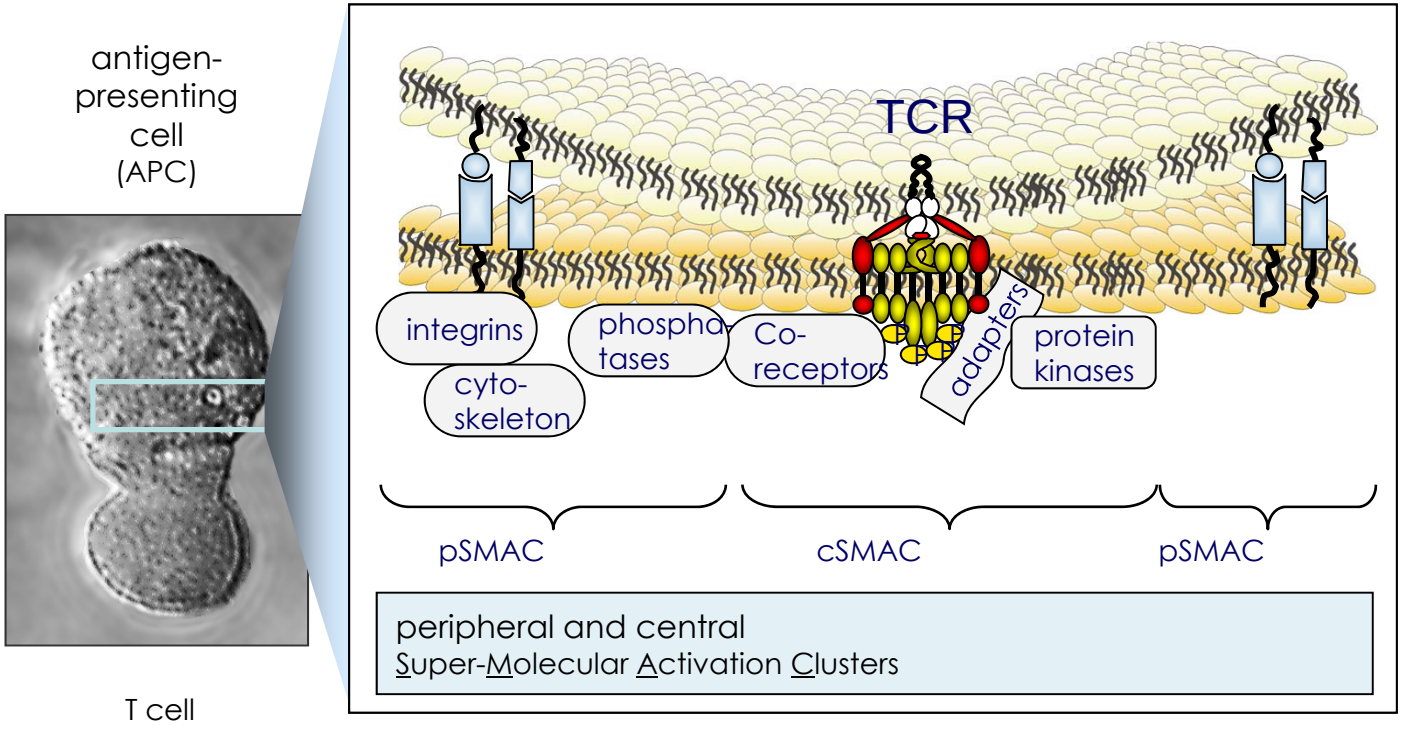




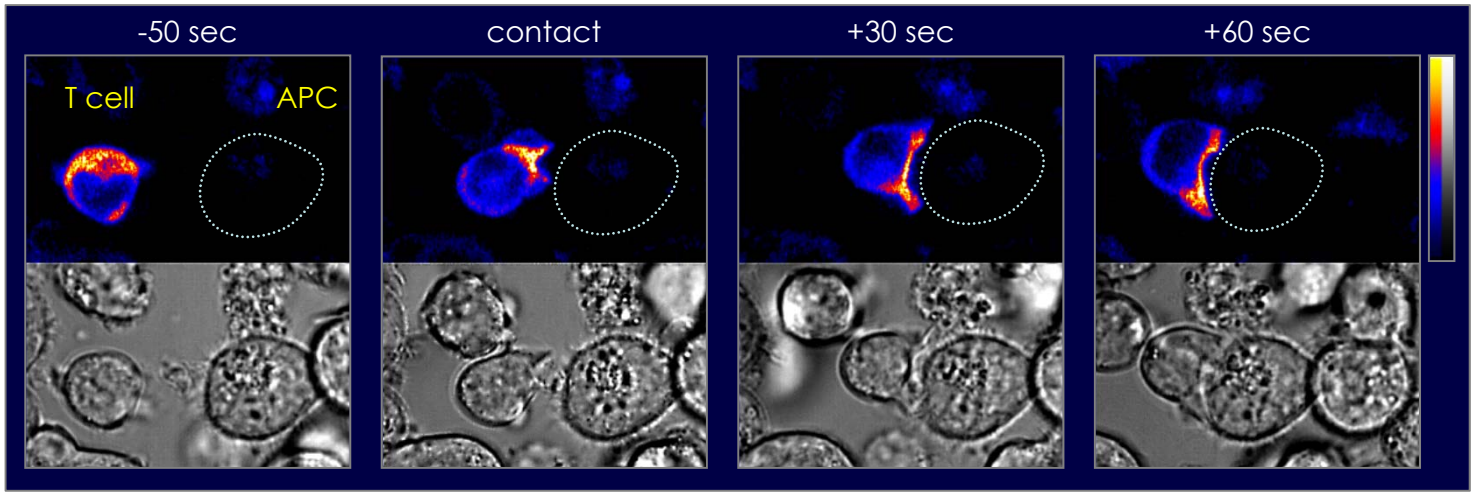
Breitsprecher D, Jaiswal R, Bombardier JP, Gould CJ, Gelles J, Goode BL. Science. 2012; 336(6085): 1164-8.

Das et al., ChemBioChem Volume 8, Issue 9, pages 994–999, June 18, 2007

Measuring molecular motility:
Fluorescence Recovery After Photo-bleaching (FRAP)

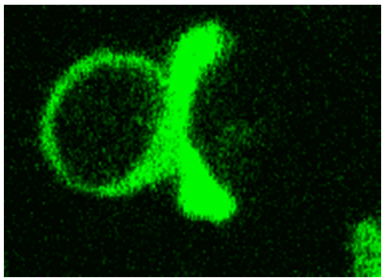


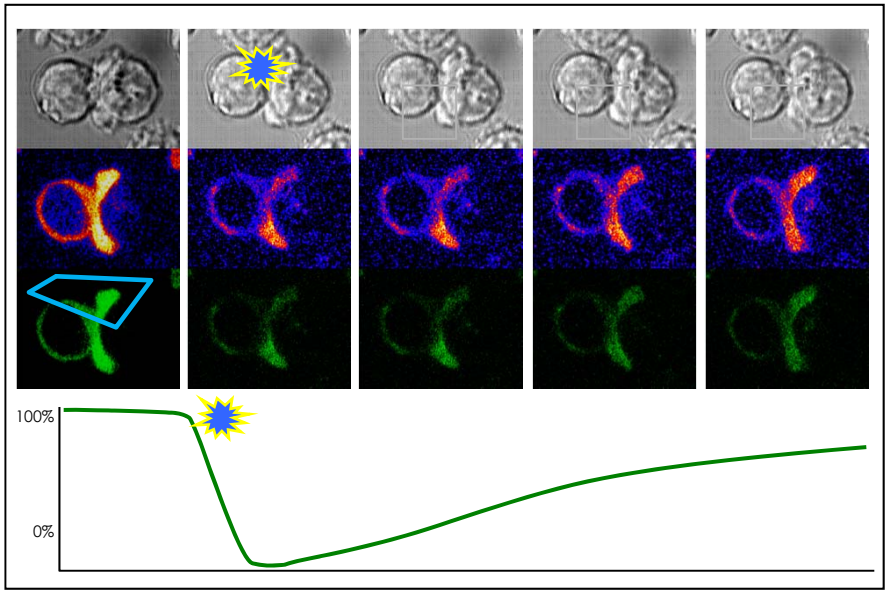
Measuring molecular motility:
Fluorescence Recovery After Photo-bleaching (FRAP)



Spitaler M et al.,
Immunity. 2006; 24(5): 535-46.

Measuring molecular motility:
Fluorescence Recovery After Photo-bleaching (FRAP)





0 1min

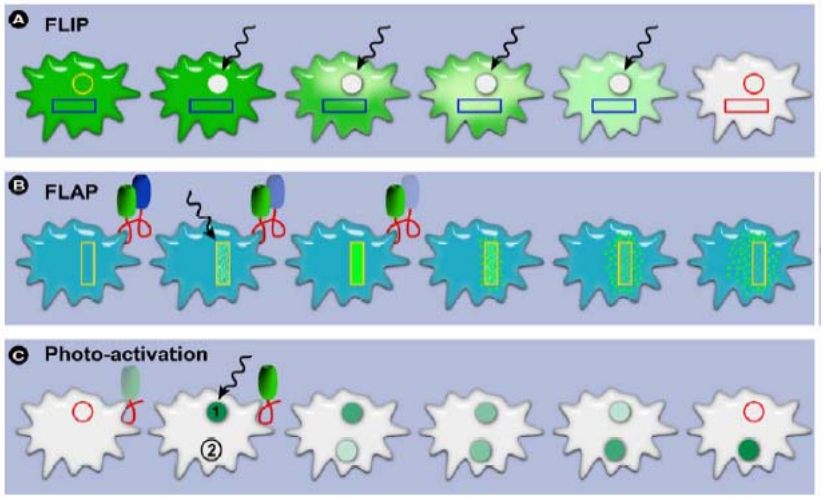
Problems:

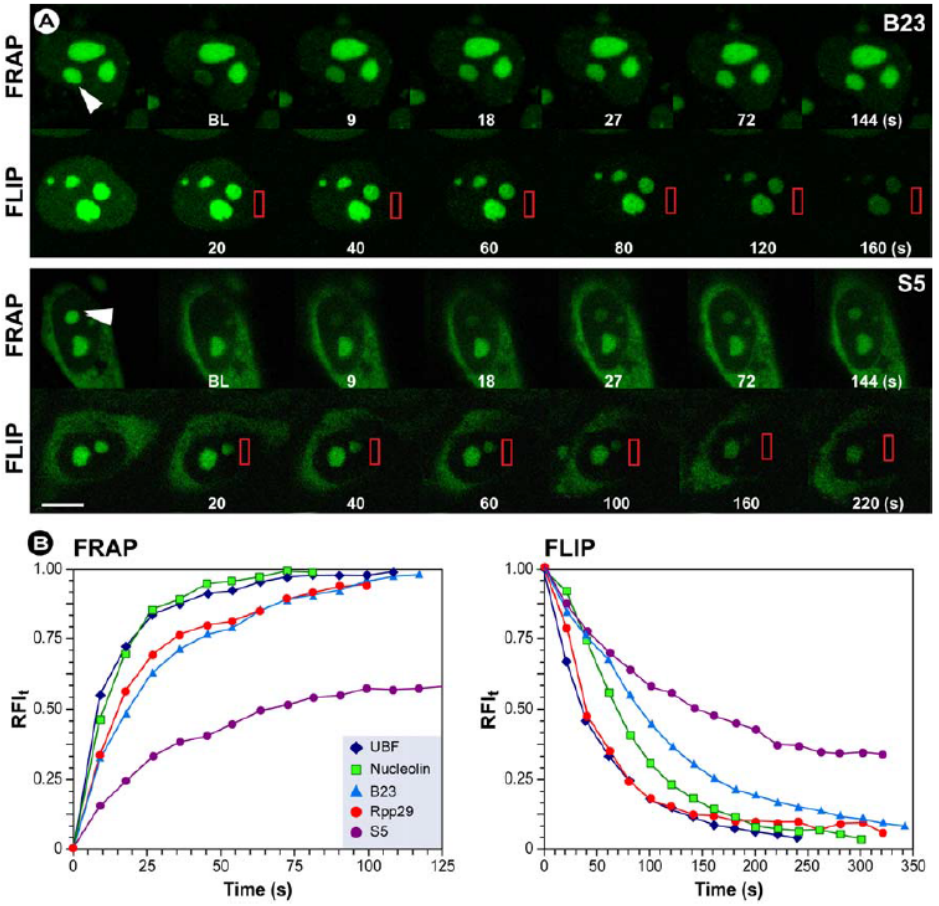
- high **phototoxicity**
- **slow** (sample movement)
- incomplete bleaching

$$N(t) = \frac{F(t) - F(0)}{F(\infty) - F(0)}$$

- N(t) = normalised signal
- F(t) = fluorescence at time t
- F(0) = fluorescence before bleaching
- F(∞) = fluorescence at full recovery

Measuring molecular motility:
Fluorescence Loss In Photobleaching (FLIP)
and Fluorescence Localisation after Photobleaching (FLAP)



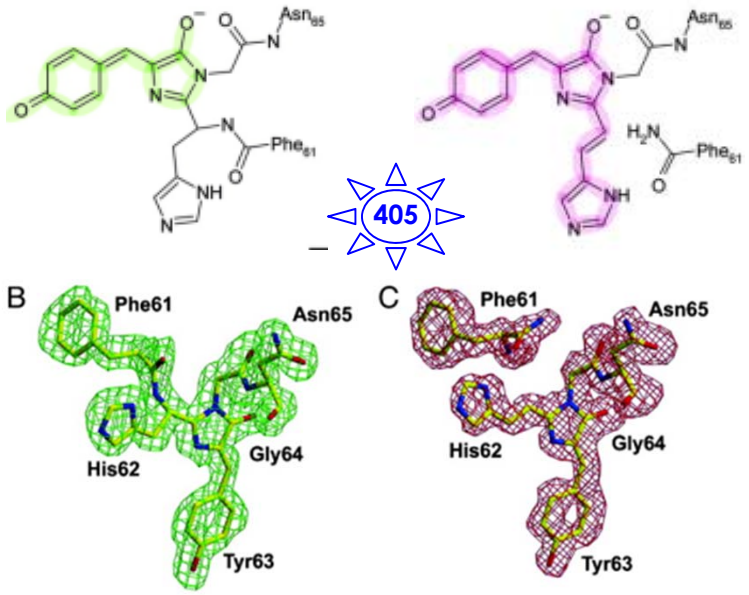


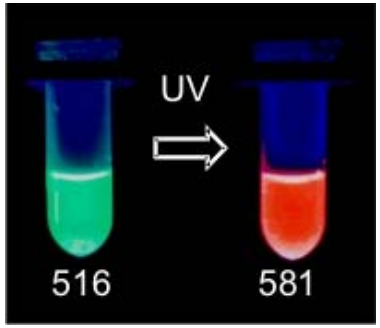
FLIP:
- prevents recovery during bleaching
- measures motile vs. stationary fraction

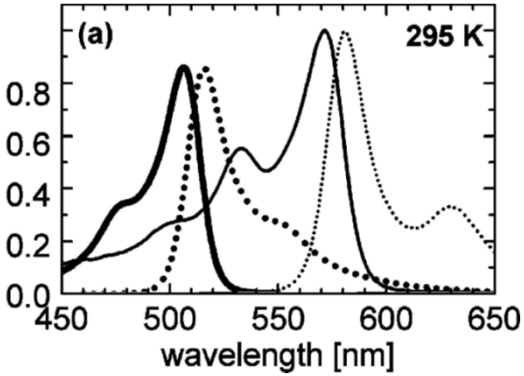
FLAP:
- measuring fast molecules (ratiometric rather than absolute intensities)
- can be applied to photo-switching and photo-conversion

Ishikawa-Ankerhold HC, Ankerhold R, Drummen GP
Molecules. 2012; 17(4): 4047-132

Measuring molecular motility: Photo-switchable proteins



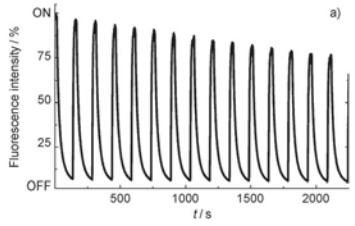


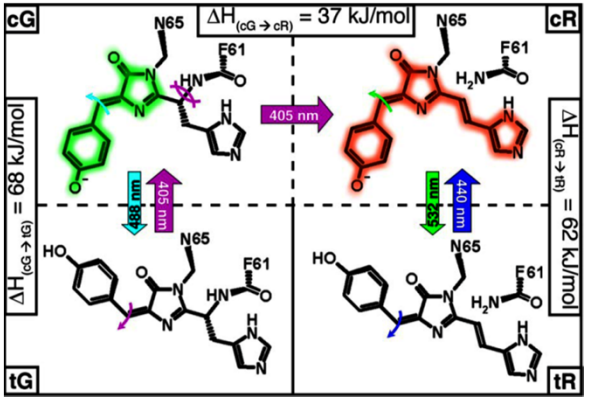


Example: EOS-FP

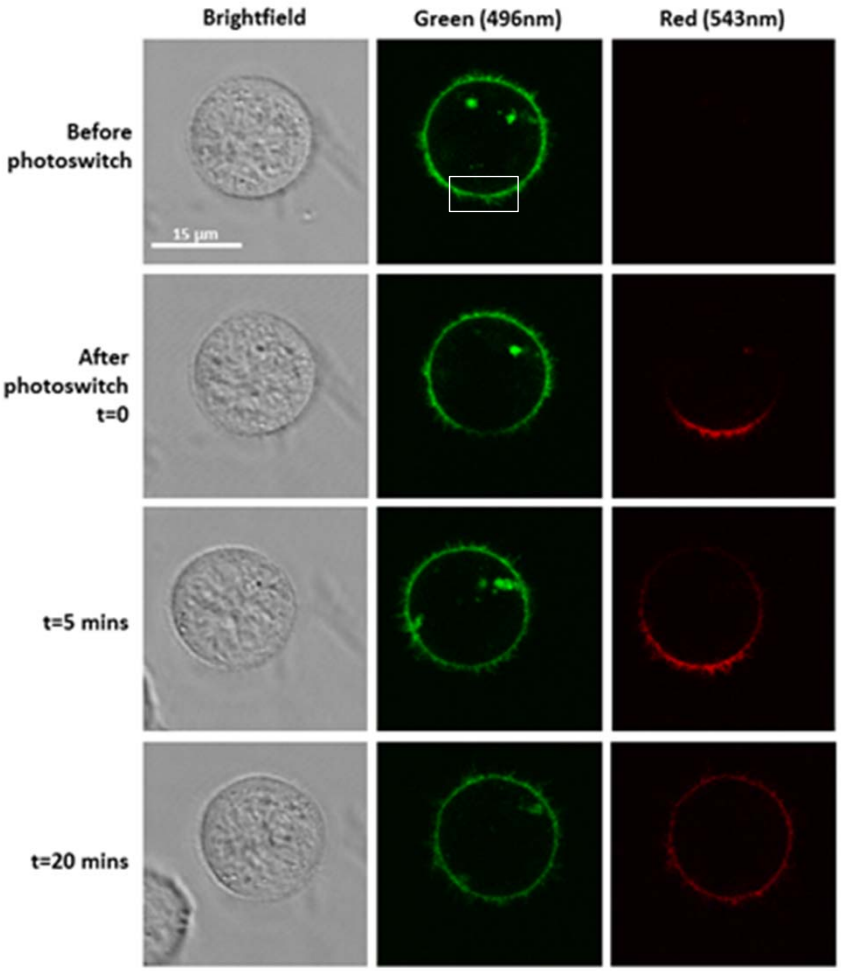
Measuring molecular motility: Photo-switchable proteins

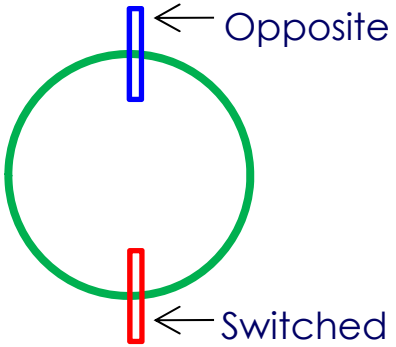
Protein (Acronym)	Switch from	(emission) to	Photostability	Quaternary Structure	Brightness (% EGFP)
Photoactivatable					
PS-CFP2	n/a	511	++	Monomer	32
PA-GFP	n/a	517	++	Monomer	41
Phamret	n/a	517	++	Monomer	41
PA-mCherry1	n/a	595	++	Monomer	25
PA-mRFP1	n/a	605	+	Monomer	3
Photoactivatable (reversible)					
mTFP0.7	n/a	488	+	Monomer	89
bsDronpa	n/a	504	++	Monomer	67
Dronpa-3	n/a	514	++	Monomer	56
Dronpa	n/a	517	+++	Monomer	240
rsFastLime	n/a	518	++	Monomer	89
Padron	n/a	522	++	Monomer	82
E2GFP	n/a	523	++	Monomer	79
KFP1	n/a	600	+++	Tetramer	12
rsCherryRev	n/a	608	++	Monomer	1
rsCherry	n/a	610	++	Monomer	5
Photoconvertible					
Dendra2 (G)	507	573	++	Monomer	67
mKikGR (G)	515	591	+	Monomer	101
wtEosFP (G)	516	581	++	Tetramer	150
dEos (G)	516	581	++	Dimer	165
tdEos (G)	516	581	++	Tandem Dimer	165
wtKikGR (G)	517	593	++	Tetramer	112
Kaede	518	580	++	Tetramer	259
mEos2 (G)	519	584	++	Monomer	140
Photoconvertible (reversible)					
IrisFP (G)	516	580	++	Tetramer	67
Fluorescent Protein Timers					
Slow-FT	465	604	++	Monomer	35
Medium-FT	464	600	++	Monomer	55
Fast-FT	466	606	++	Monomer	44
DsRed-E5	500	583	+++	Tetramer	ND*
Source: http://zeiss-campus.magnet.fsu.edu					

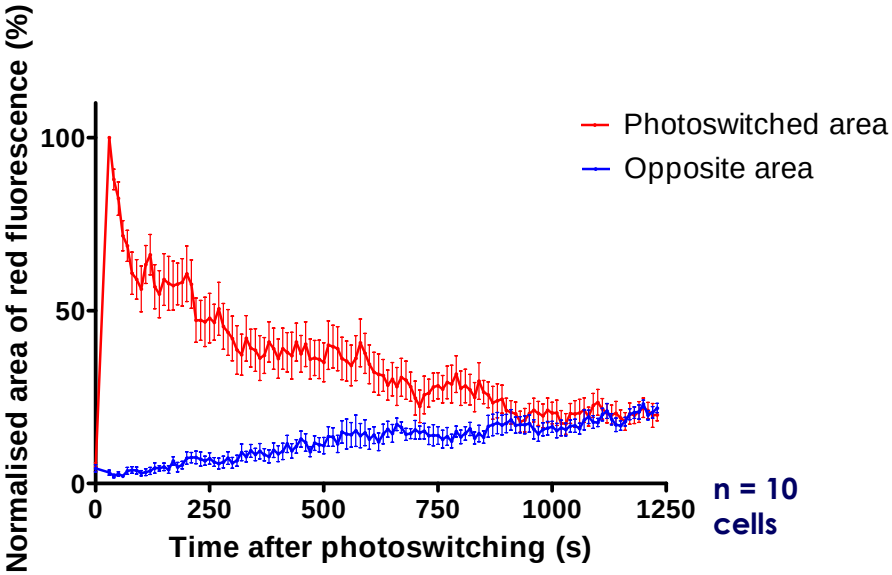




Measuring molecular motility: Photo-switchable proteins

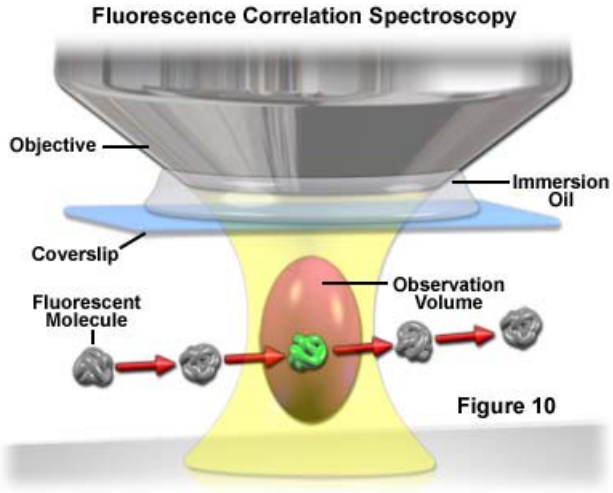


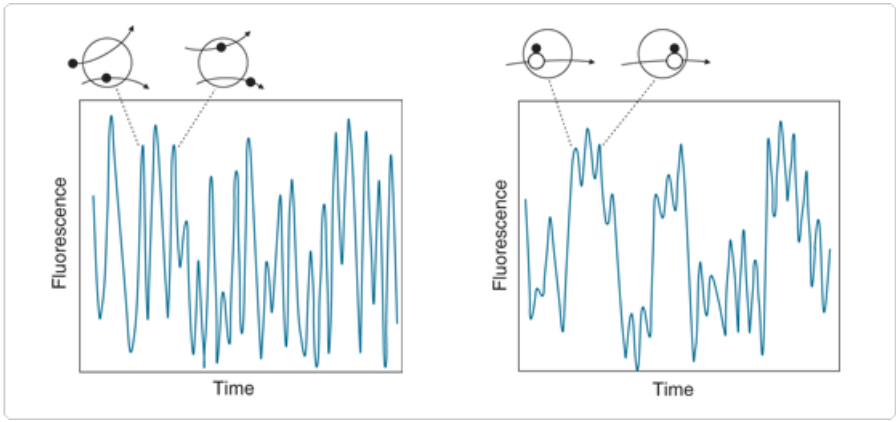




Sophie Pageon: Molecular signalling in NK cell activation measured with EOS-FP

Measuring molecular motility: Fluorescence Correlation Spectroscopy





- *Observation volume: <1 femtoliter (confocal volume, ~ volume of an E.coli bacterial cell)*
- *nanomolar molecule concentrations*

$$G(\tau) = \frac{\langle \delta F(t) \bullet \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

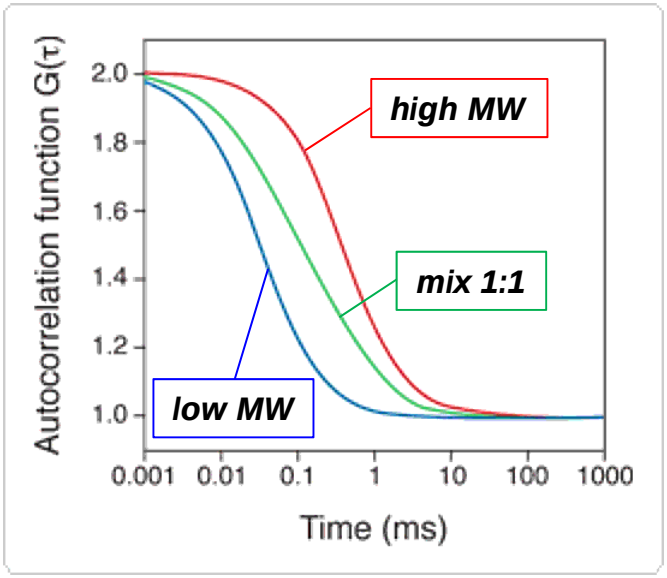
G (τ) = autocorrelation function

F(t) = fluorescence intensity at time t
F(t+T) = intensity at (t + τ), where τ is a variable time interval

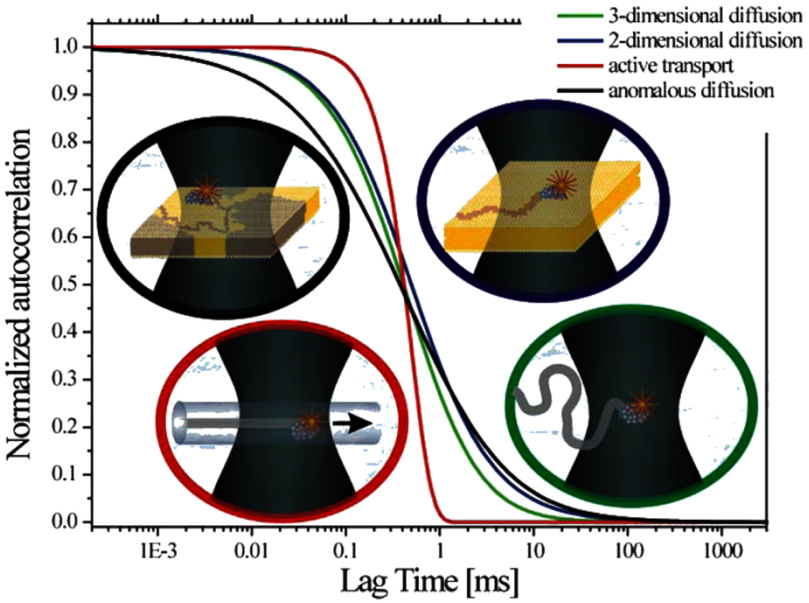
http://zeiss-campus.magnet.fsu.edu

http://www.invitrogen.com

Measuring molecular motility:
Fluorescence Correlation Spectroscopy



http://www.invitrogen.com

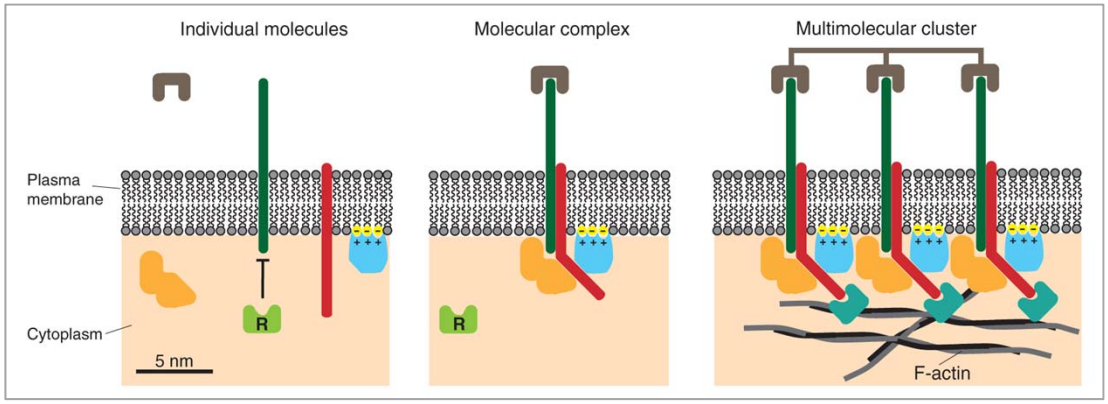


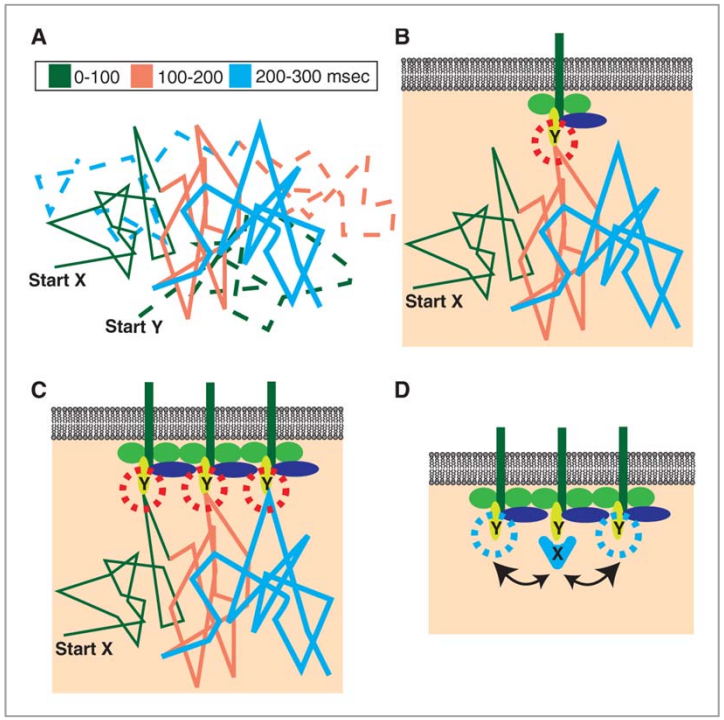
Petra Schwille and Elke Haustein:
Fluorescence Correlation Spectroscopy

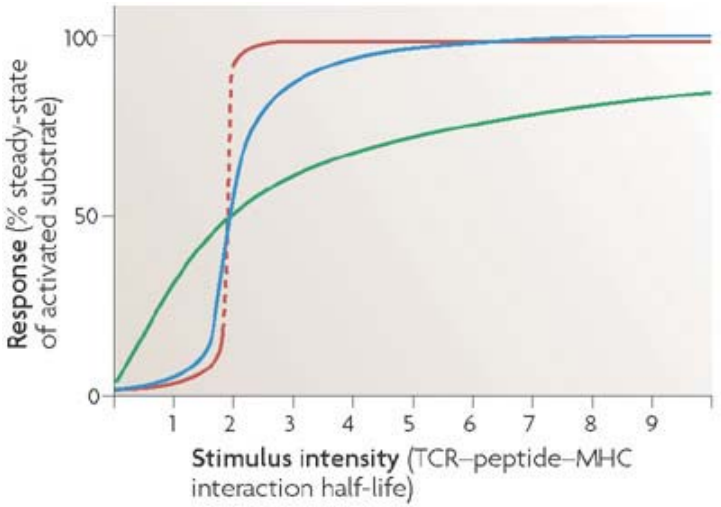
Capabilities of FCS:

- fluctuations quantified in strength and duration by temporally autocorrelating the recorded intensity signal
- high-resolution spatial and temporal analysis of extremely low concentrated biomolecules
- can measure any physical parameters that give rise to fluctuations in the fluorescence signal (local concentrations, mobility coefficients, inter- or intramolecular reactions)

Implications of molecular motility and clustering

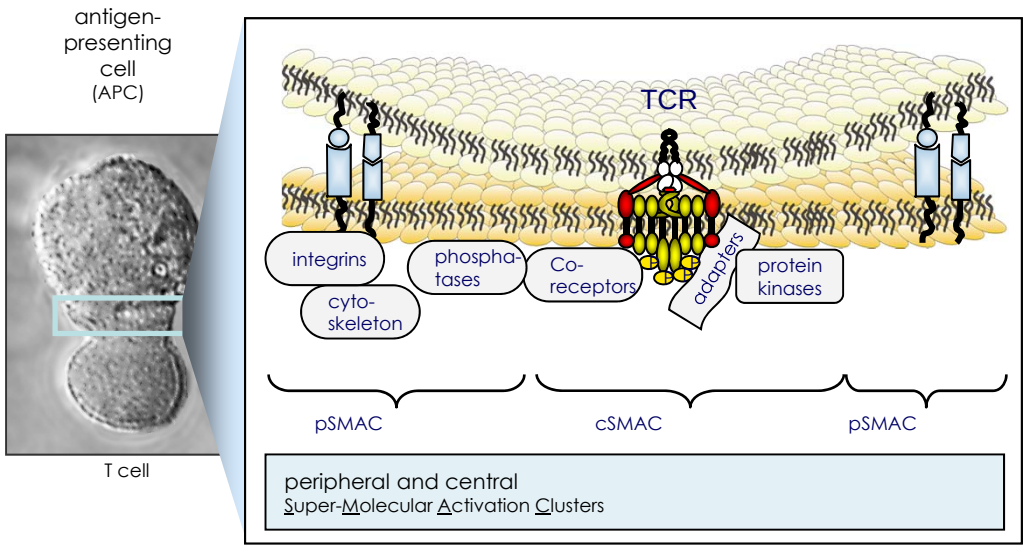


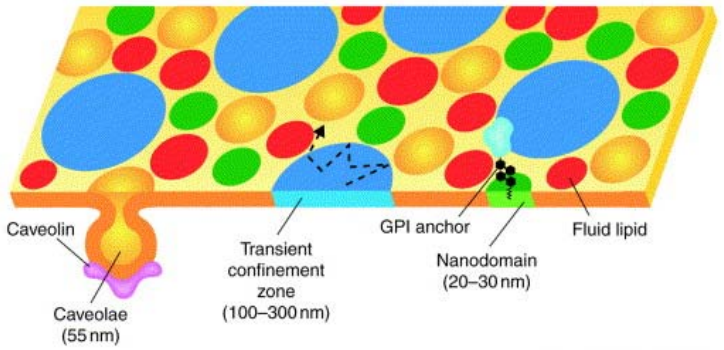




Cebecauer M, Spitaler M, Sergé A, et al,
J Cell Sci, 2010, Vol:123

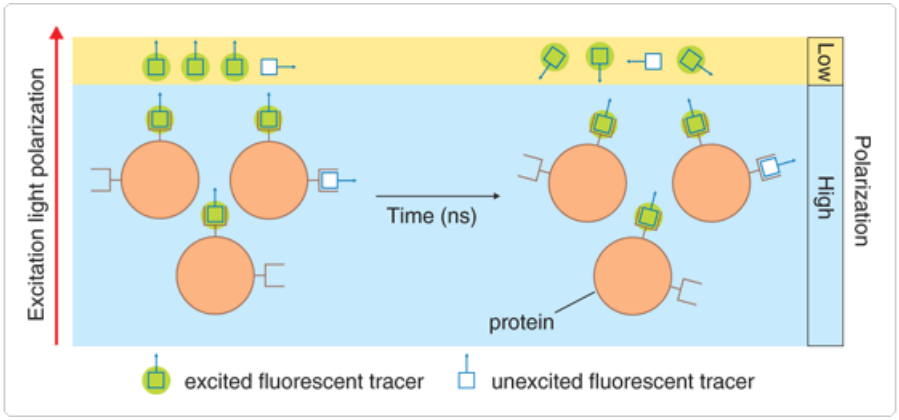
Implications of molecular motility and clustering





Frederick R Maxfield
Current Opinion in Cell Biology,
Volume 14, Issue 4, 1 August 2002,
Pages 483–487

Measuring molecular motility: Fluorescence anisotropy



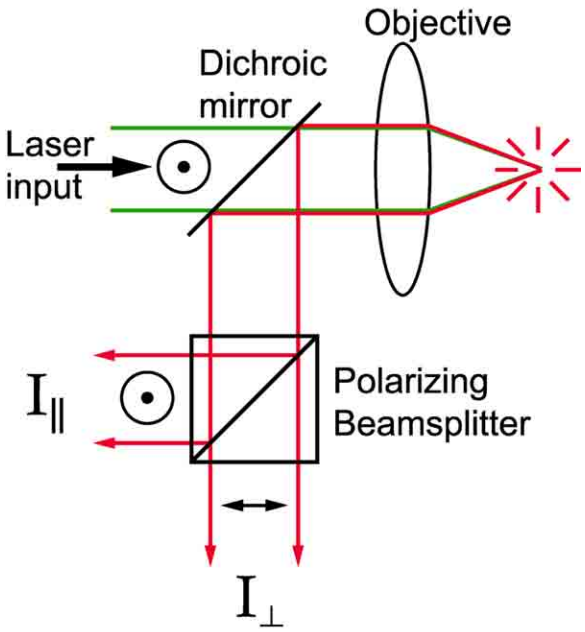
http://www.invitrogen.com

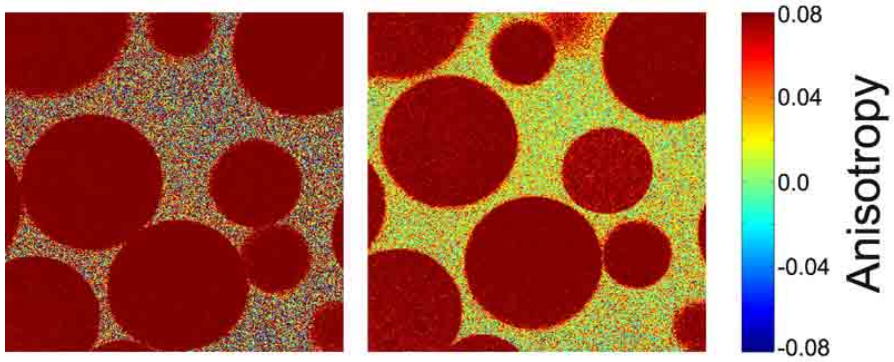
$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

$I_{\parallel}$ = *fluorescent intensity parallel to the excitation plane*

$I_{\perp}$ = *fluorescent intensity perpendicular to the excitation plane*

Measuring molecular motility: Fluorescence anisotropy





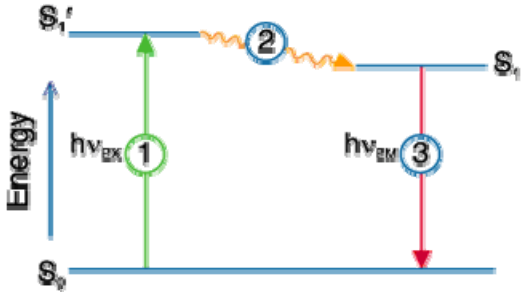
Anisotropy images acquired 40 seconds (left) and 4 minutes (right) after mixing the enzyme protease k with sepharose beads containing albumin conjugated to the fluorophore Bodipy-FL

http://www.urmc.rochester.edu/smd/rad/foster

Capabilities of Fluorescence Anisotropy:

- binding constants and kinetics of reactions that cause a change in the rotational time of the molecules
- dynamics of protein folding
- local viscosity of the cytosol or membranes

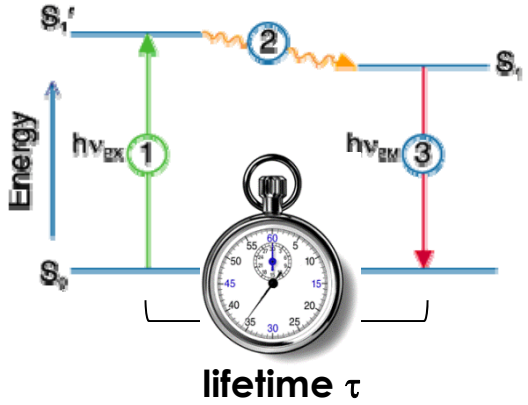
Sensing the molecular environment: Fluorescence Lifetime Imaging (FLIM)

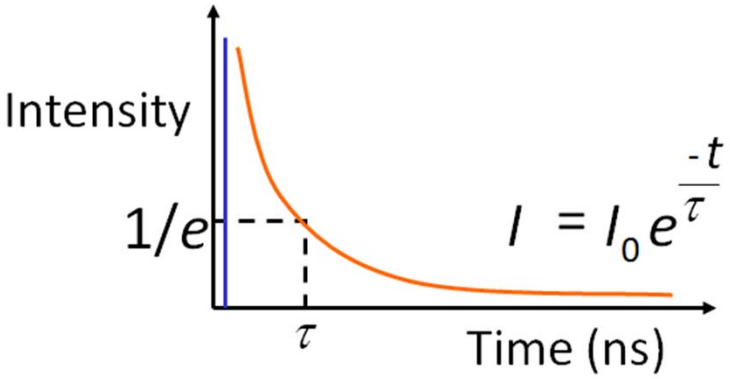


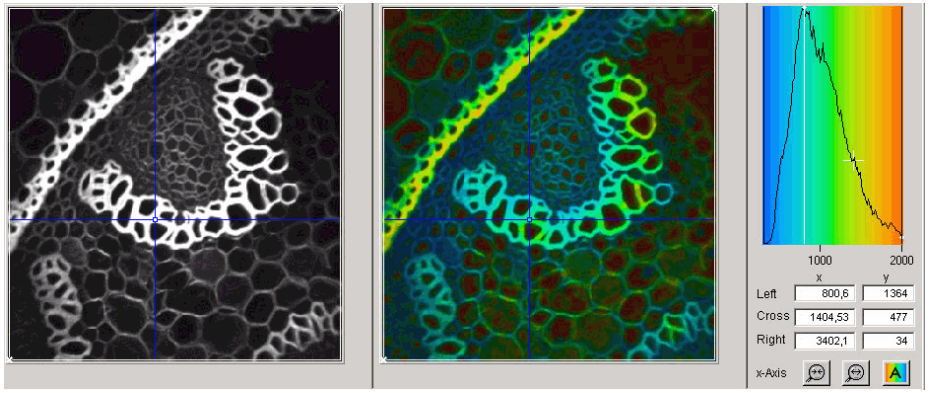
Jablonksi diagram of fluorescence excitation

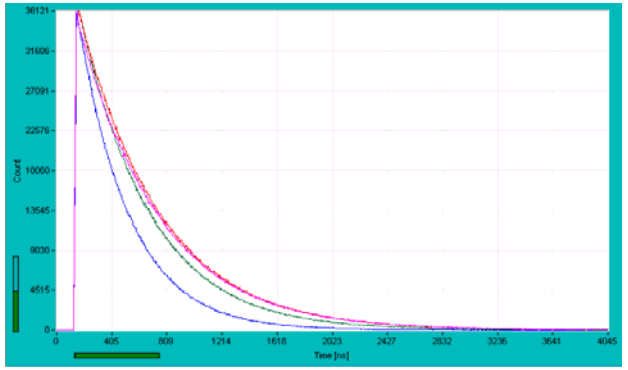
Sensing the molecular environment: Fluorescence Lifetime Imaging (FLIM)

Time domain FLIM









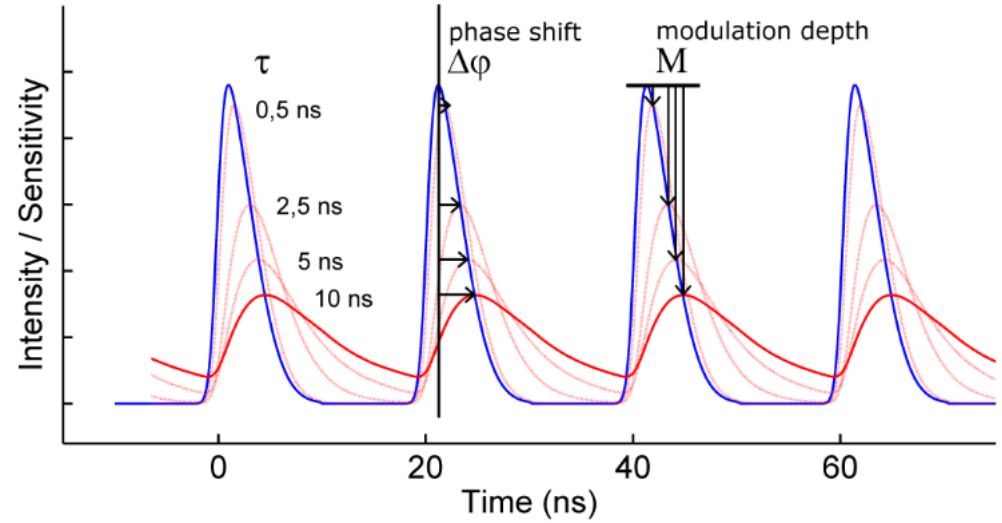
Sensing the molecular environment: Fluorescence Lifetime Imaging (FLIM)

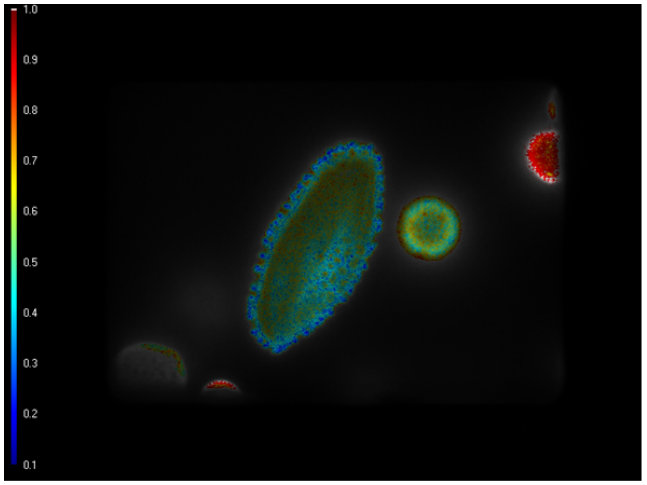
Time domain FLIM

- pulsed laser, e.g. diode laser or two-photon laser (80MHz, <psec pulses)
- single-photon counting detector (PMT + photon counting card)
- direct lifetime measurement
- acquisition speed:
  ~10-60 sec per frame

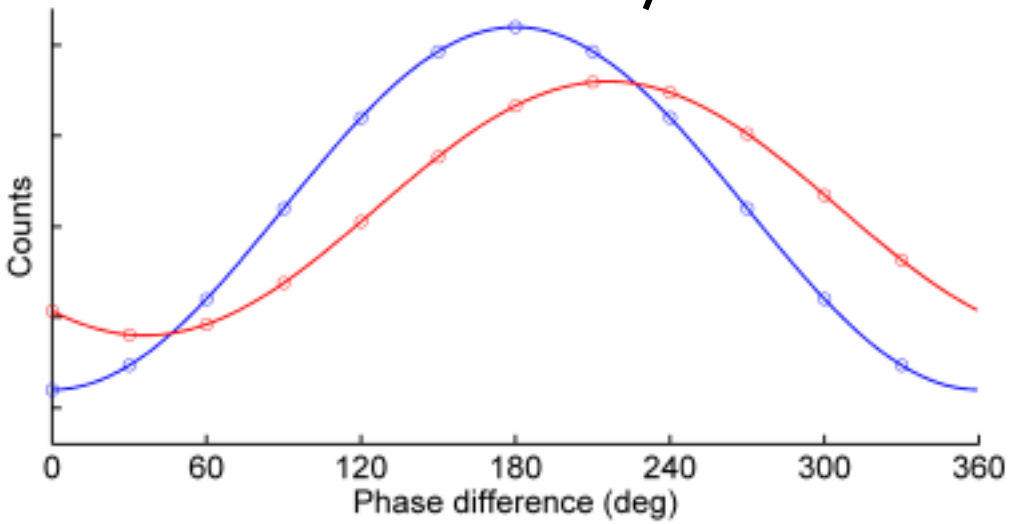
Sensing the molecular environment: Fluorescence Lifetime Imaging (FLIM)

Frequency domain FLIM





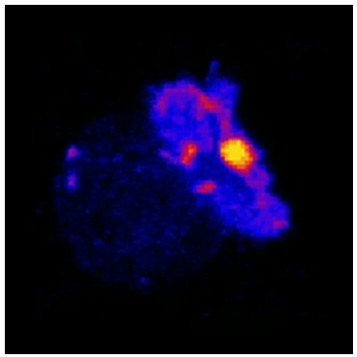
detector phase shift



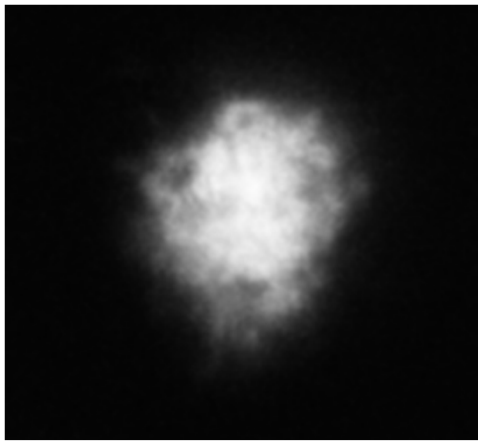
Sensing the molecular environment: Fluorescence Lifetime Imaging (FLIM)

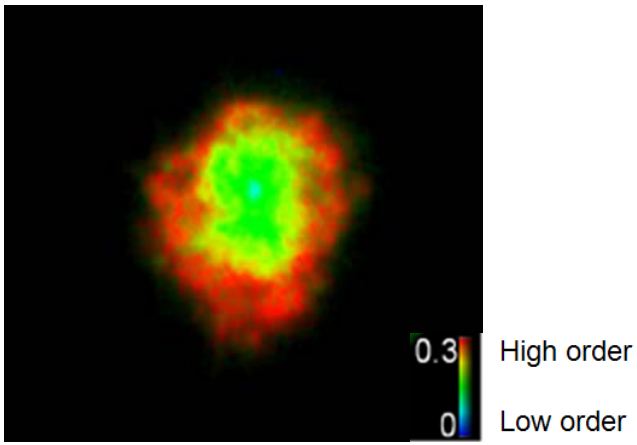
Time domain FLIM	Frequency domain FLIM
• pulsed laser, e.g. diode laser or two-photon laser (80MHz, <psec pulses) • single-photon counting detector (PMT + photon counting card) • direct lifetime measurement • acquisition speed: ~10-60 sec per frame	• Pulsed laser • pulsed, synchronised detector (e.g. CCD camera) • fast • acquisition speed: ~0.1-10 sec per frame

Sensing the molecular environment: Fluorescence Lifetime Imaging (FLIM)



Spitaler M et al.,
Immunity. 2006; 24(5): 535-46.

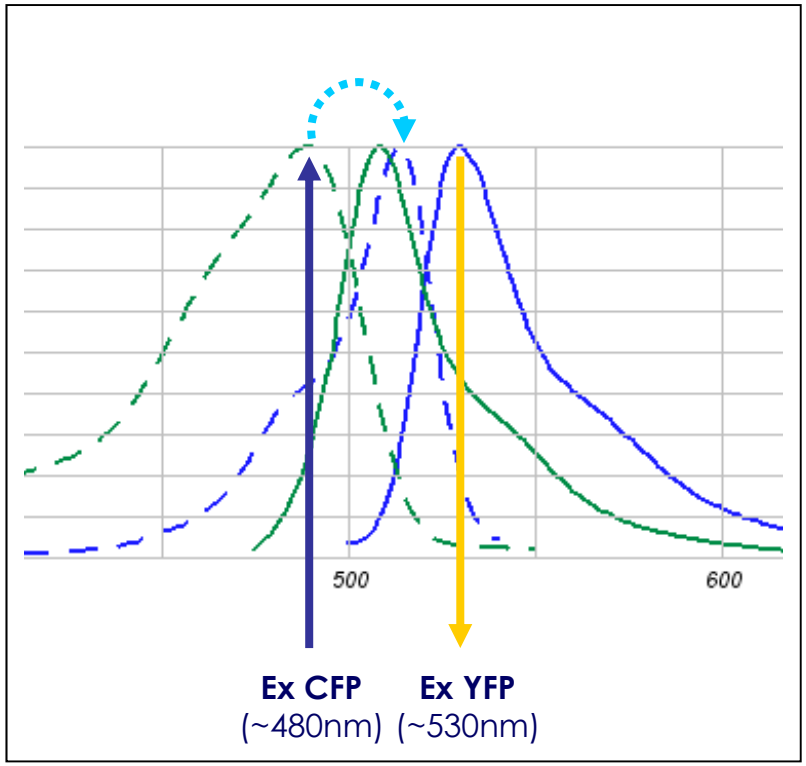


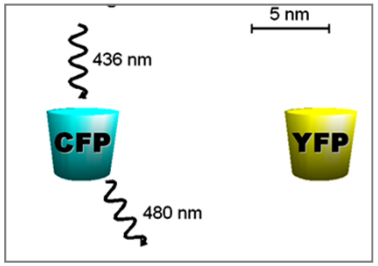


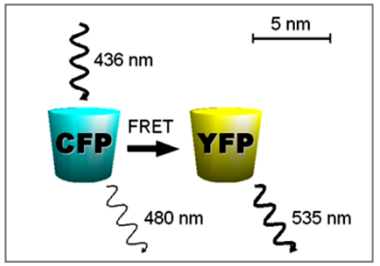
Visualisation of membrane fluidity by FLIM of di-4-ANEPPDHQ

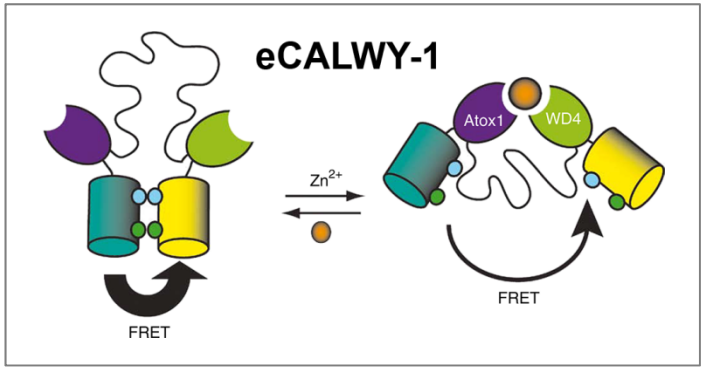
Dylan Owen, Mark Neil , Paul French, Anthony Magee,
Seminars in Cell & Developmental Biology 18 (2007) 591–598

Visualising molecular interaction:
Fluorescence (Förster) Resonant Energy Transfer







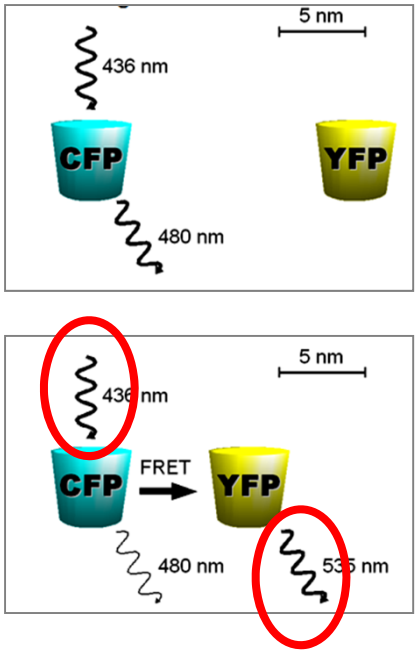


FRET

FRET efficiency: $E = \frac{1}{1 + (r/R_0)^6}$

Vinkenborg JL, Nicolson TJ, Bellomo EA, Koay MS, Rutter GA, Merkx M. Nat Methods. 2009; 6(10): 737-40.

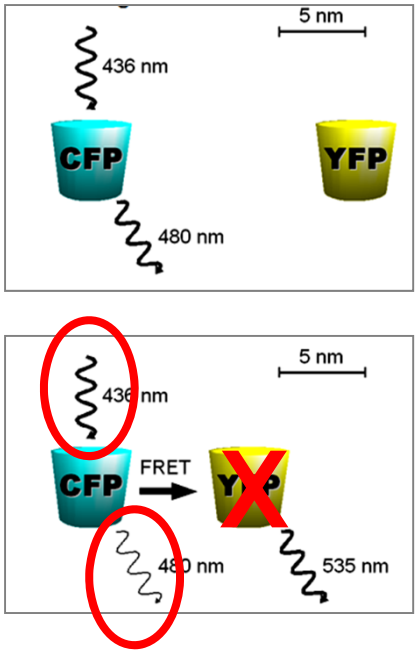
Visualising molecular interaction:
Fluorescence (Förster) Resonant Energy Transfer



Methods to measure FRET:

- **1) Stimulated emission:**
  excitation of **donor**, **visualisation** of **acceptor**
  - advantage: technically simple (any fluorescent microscope)
  - disadvantage: hard to quantify (spectral overlap, uneven staining, bleaching)
  - best used: relative, fast changes (ratiometric, live)

Visualising molecular interaction:
Fluorescence (Förster) Resonant Energy Transfer



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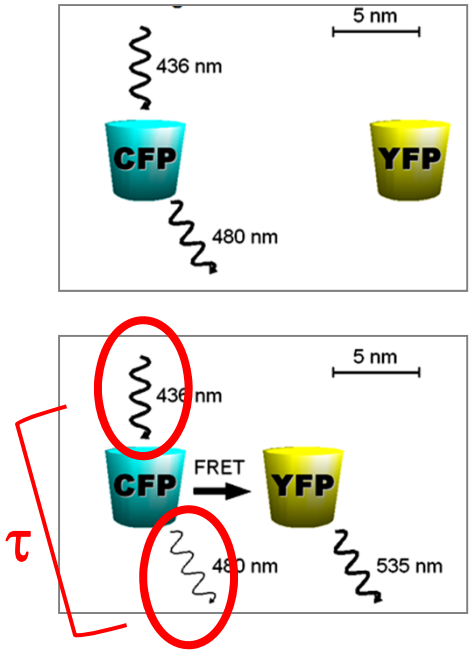
  - advantage: technically simple (any fluorescent microscope)
  - disadvantage: hard to quantify (spectral overlap, uneven staining, bleaching)
  - best used: relative, fast changes (ratiometric, live)

- **2) Acceptor photobleaching:**
  visualisation of **donor** before and after **bleaching** of the **acceptor**
  - advantage: relatively simple (any confocal microscope), less affected by bleaching, not affected by spectral overlap
  - disadvantage: high phototoxicity, motion artefacts
  - best used: additional control for (1) and (3)

Visualising molecular interaction:
Fluorescence (Förster) Resonant Energy Transfer



Methods to measure FRET:

- **1) Stimulated emission:**
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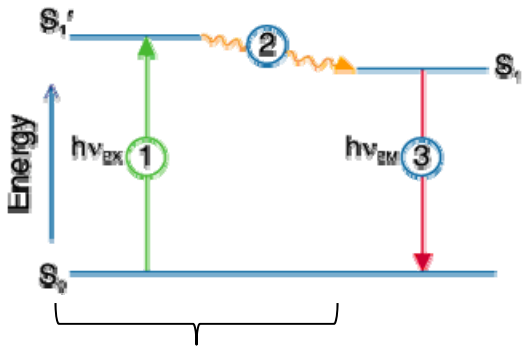
  - advantage: technically simple (any fluorescent microscope)
  - disadvantage: hard to quantify (spectral overlap, uneven staining, bleaching)
  - best used: relative, fast changes (ratiometric, live)

- **2) Acceptor photobleaching:**
  visualisation of **donor** before and after **bleaching** of the **acceptor**
  - advantage: relatively simple (any confocal microscope), less affected by bleaching, not affected by spectral overlap
  - disadvantage: high phototoxicity, motion artefacts
  - best used: additional control for (1) and (3)

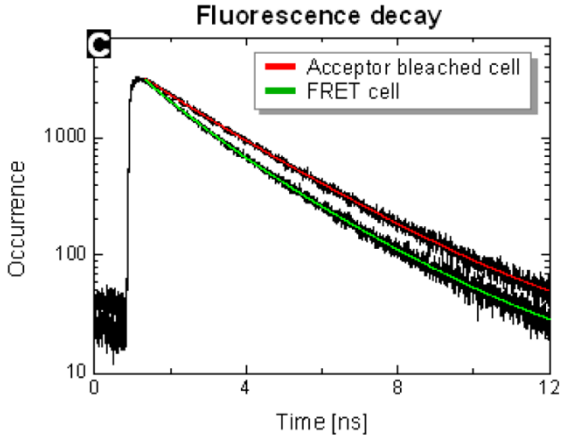
- **3) FLIM-FRET**
  (acceptor photobleaching can be used as a control)
  - advantage: least affected by artefacts (independent of intensity, not affected by spectral overlap)
  - disadvantage: technically most demanding
  - best used: quantitative, low-speed imaging

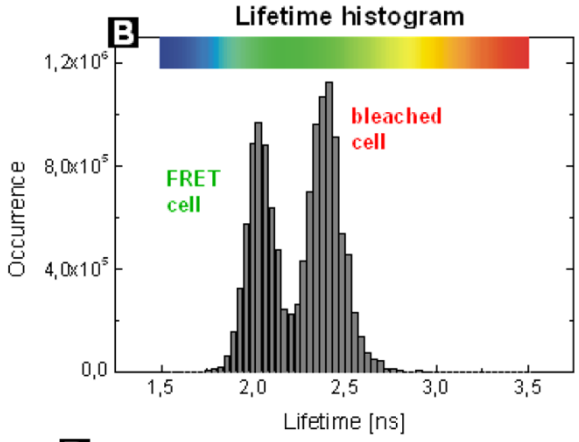
Visualising molecular interaction:
Fluorescence (Förster) Resonant Energy Transfer

FRET measurement by FLIM-FRET



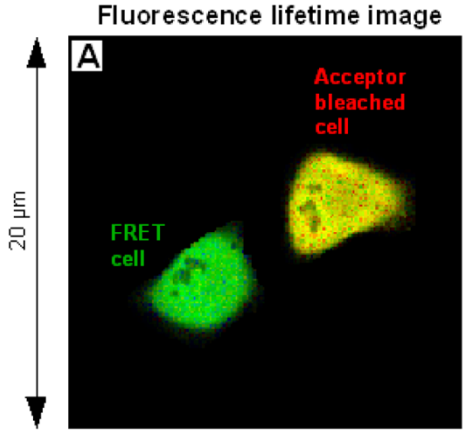
lifetime τ





D

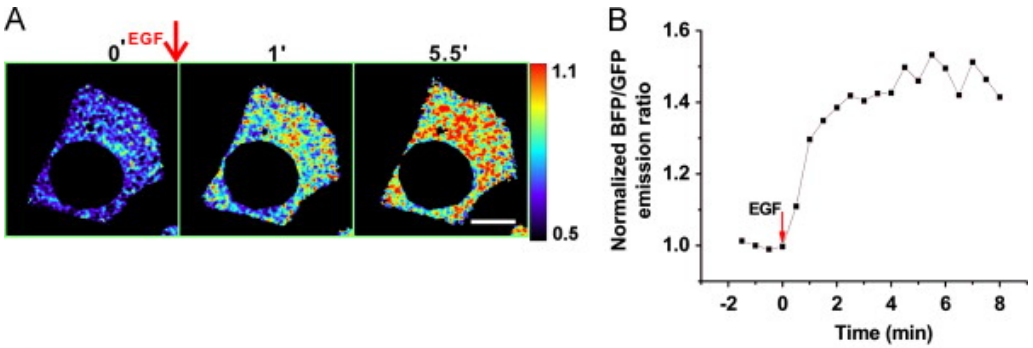
	FRET (D+A)	Bleach (D)
Lifetime 1	2.4 ns	2.4 ns
Lifetime 1	1.2 ns	1.2 ns
Amp. 1	51%	85%
Amp. 2	49%	15%



Visualising molecular interaction:
Fluorescence (Förster) Resonant Energy Transfer

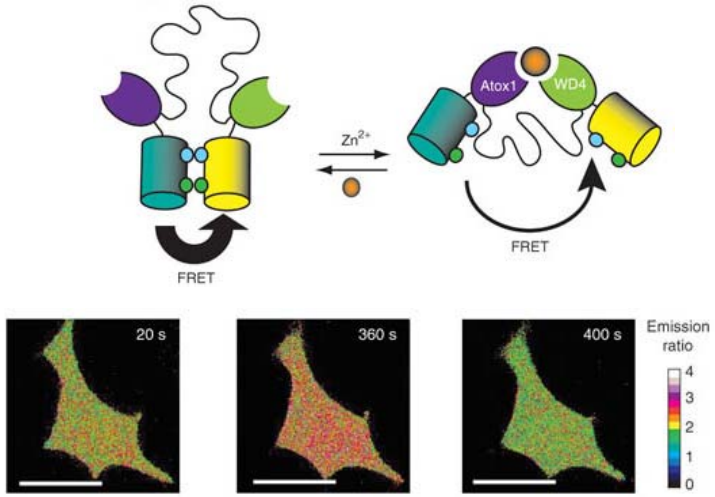
Applications of FRET:

- protein - protein interactions
- protein cleavage (apoptosis)
- protein modifications (e.g. phospho-specific antibodies)
- ion sensors (e.g. Ca^{2+}, Zn^{2+}, ...)



BFP/GFP ratio images of BG-Src upon EGF (100 ng/ml) stimulation of HeLa cells

Biosensors and Bioelectronics Volume 46, 15 August 2013, Pages 97–101
Monitoring of dual bio-molecular events using FRET biosensors based on mTagBFP/sfGFP and mVenus/mKOκ fluorescent protein pairs
Ting Sua, Shaotao Pana, Qingming Luoa, Zhihong Zhanga



INS-1(832/13) cells after Zn2+ stimulation

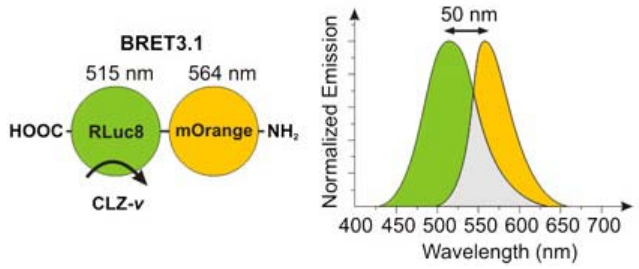
Vinkenborg JL, Nicolson TJ, Bellomo EA, Koay MS, Rutter GA, Merkx M.
Nat Methods. 2009; 6(10): 737-40.

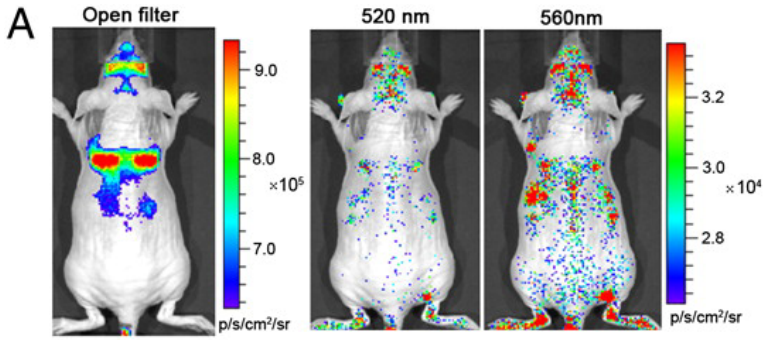
Visualising molecular interaction:
Fluorescence (Förster) Resonant Energy Transfer

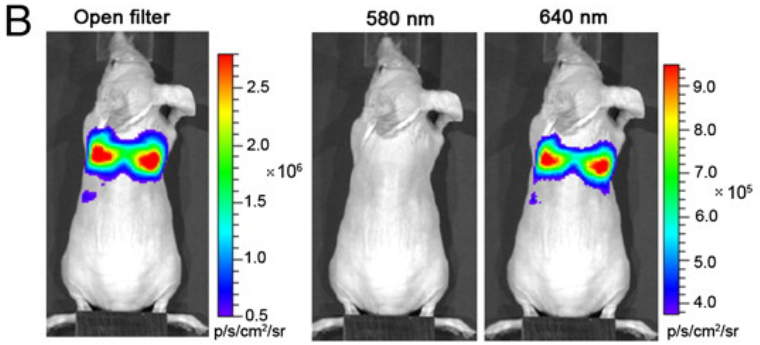
Limitations of FRET:

- the donor fluorophore needs to be excited by shorter wavelength:
  - difficult in vivo (intravital)
  - photobleaching
  - autofluorescence
  - fluorescence crosstalk

Visualising molecular interaction: Bioluminescence Resonant Energy Transfer (BRET)



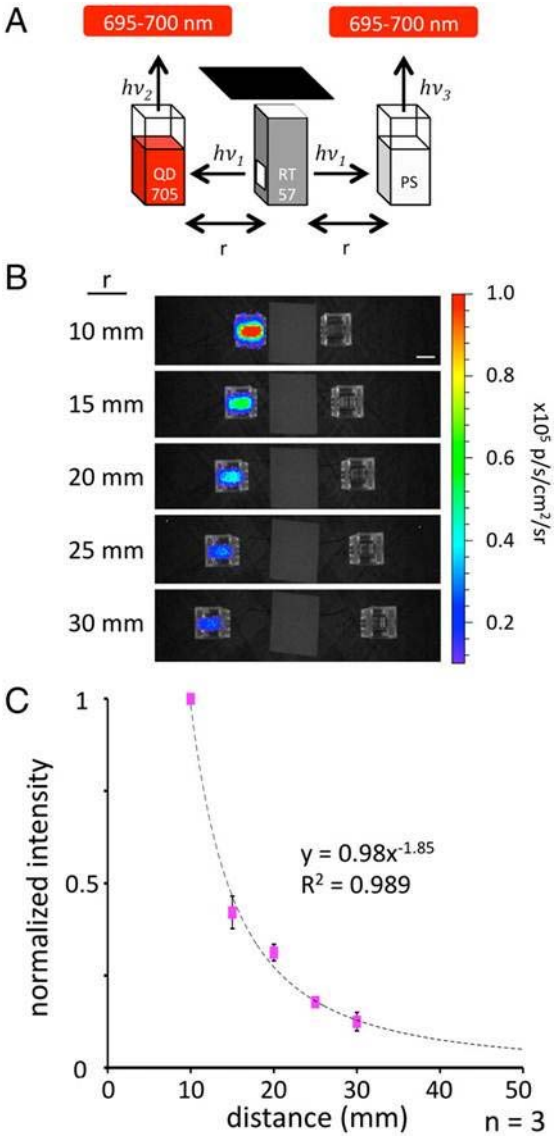


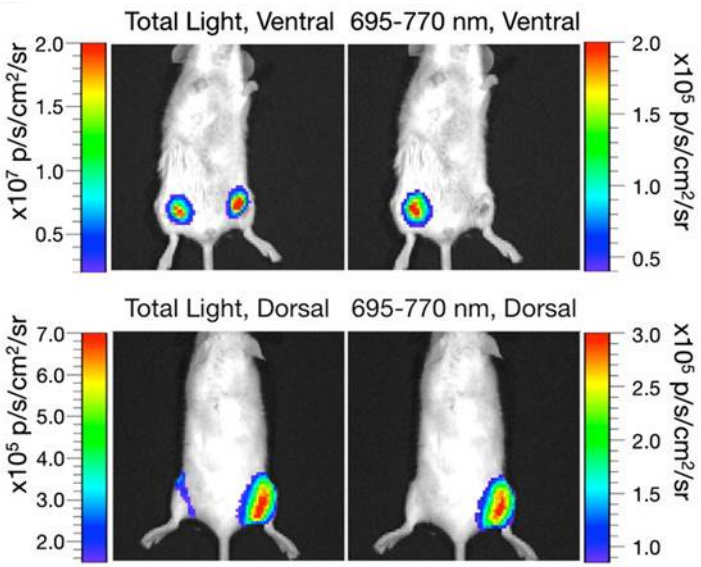


- natural exciation of GFP in the jellyfish Aequorea victoria
- only emitted light needs to pass through tissue → deeper penetration depth
- no autofluorescence
- only one wavelength passes through the sample

Dragulescu-Andrasi A et al. PNAS 2011;108:12060-12065

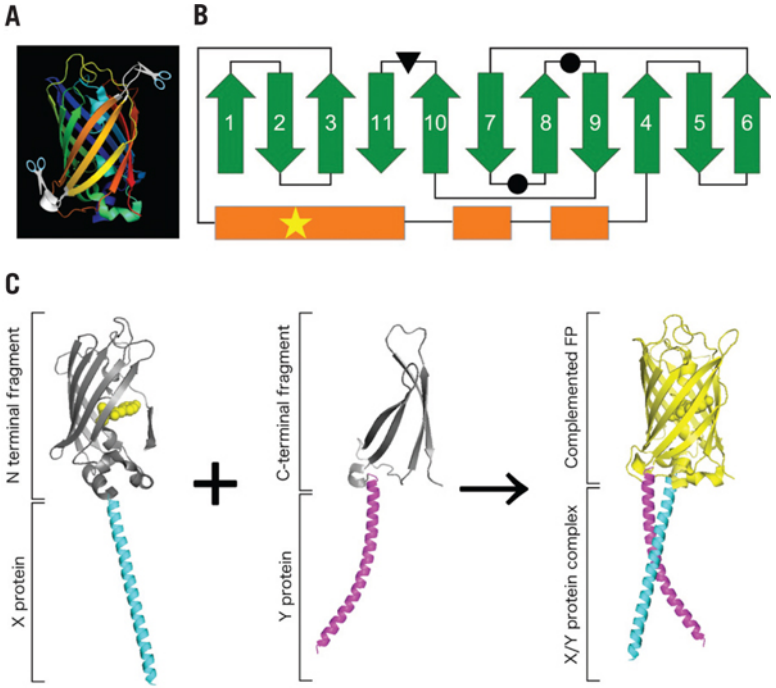
Long-distance BRET-like fluorescence: “Fluorescence by Unbound Excitation from Luminescence” (FUEL)



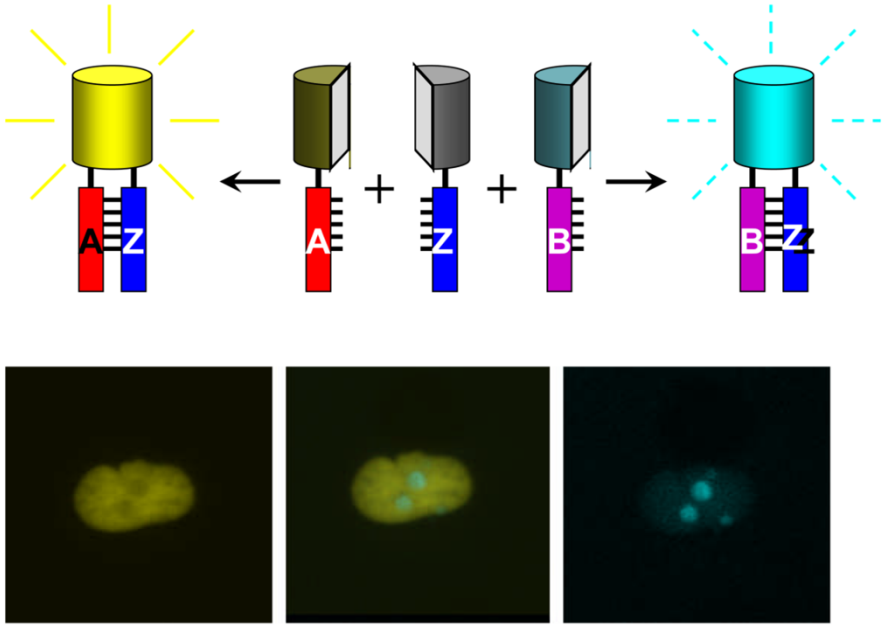


Dragavon J, …, Spencer Shorte
Proc Natl Acad Sci U S A 109: 8890-8895

Visualising molecular interaction:
Bi-molecular Fluorescence Complementation (BiFC)



Kodama Y, Hu CD.
Biotechniques. 2012; 53(5): 285-98

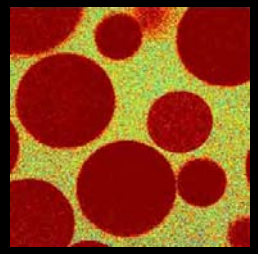


Kerppola TK.
Annu Rev Biophys. 2008; 37: 465-87

Tools for molecular imaging

FLAP

FRAP



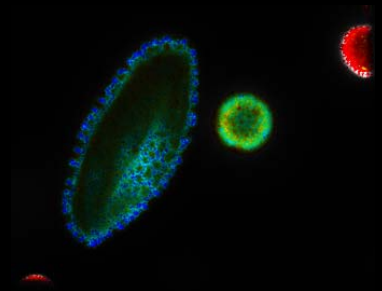
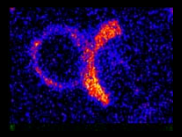
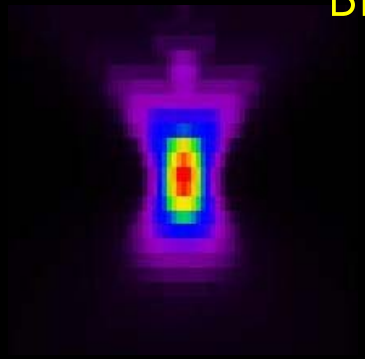


Photo-switching

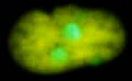


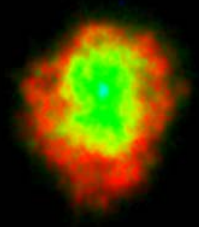
BRET



Co-localisation

FCS





FLIP

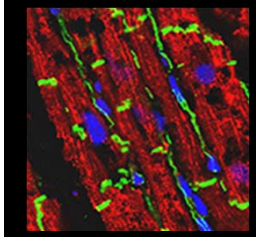
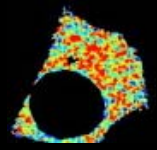


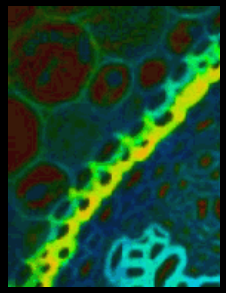
Photo-conversion



FLIM

Fluorescence Anisotropy

FUEL



FRET