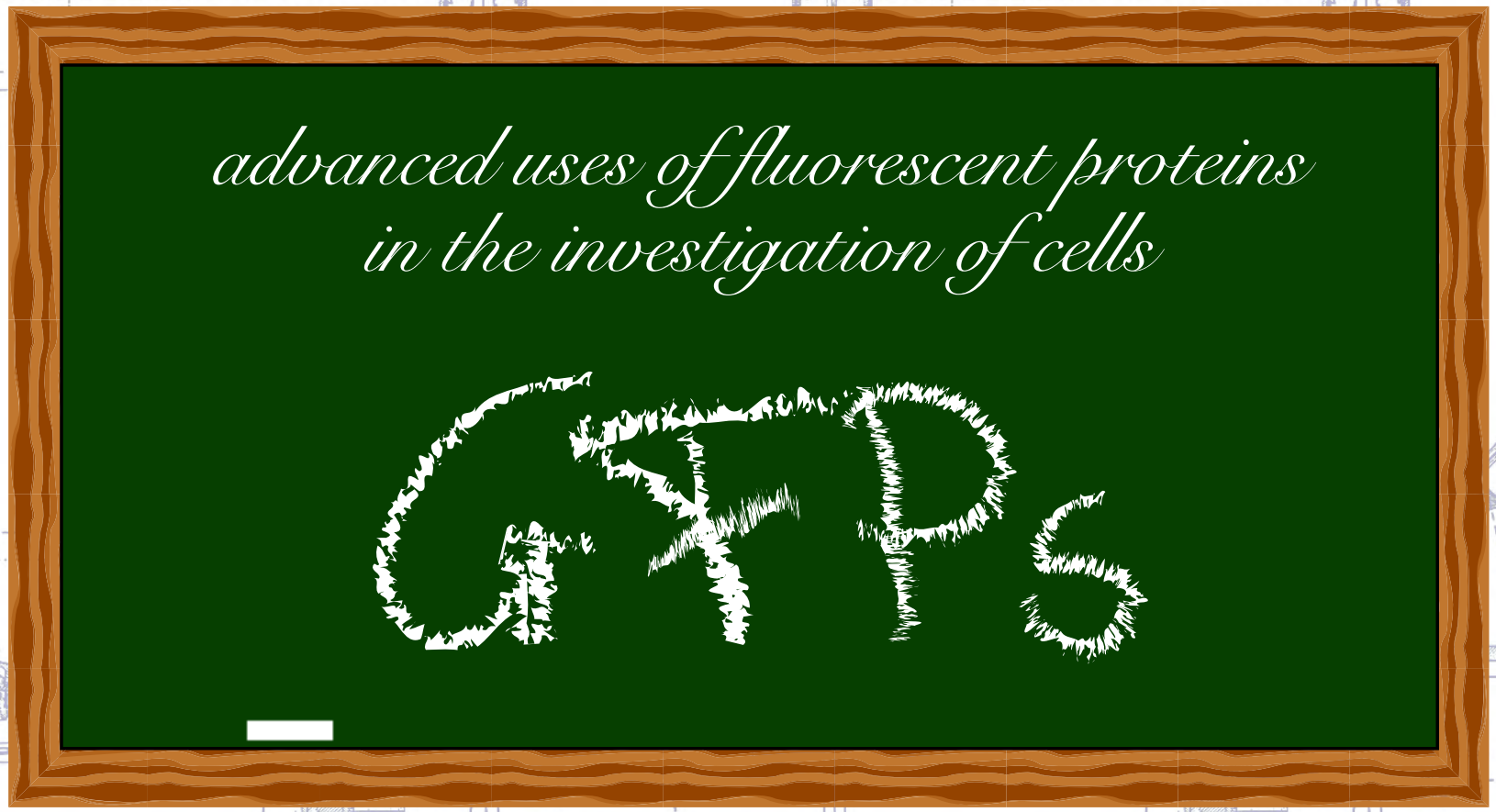


Principles of Fluorescence Techniques & CONFOCAL 9

Fred S. Wouters

Genoa, June 25-29, 2007

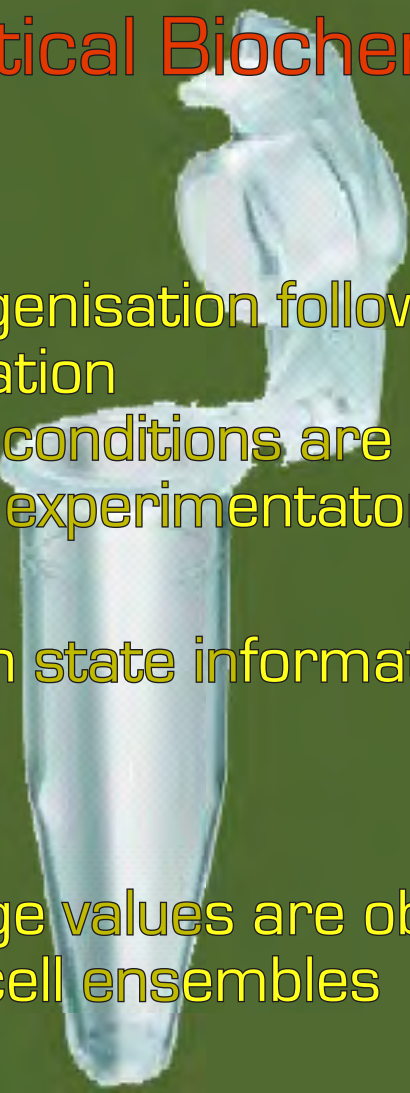
Biochemical Imaging

micro-environment in the cell is maintained

protein state and spatial information

asynchronous processes can be followed in single cells, enabling correlation with other cell state parameters

Analytical Biochemistry



homogenisation followed by separation
buffer conditions are chosen by the experimentator

protein state information

average values are obtained from cell ensembles

CBG

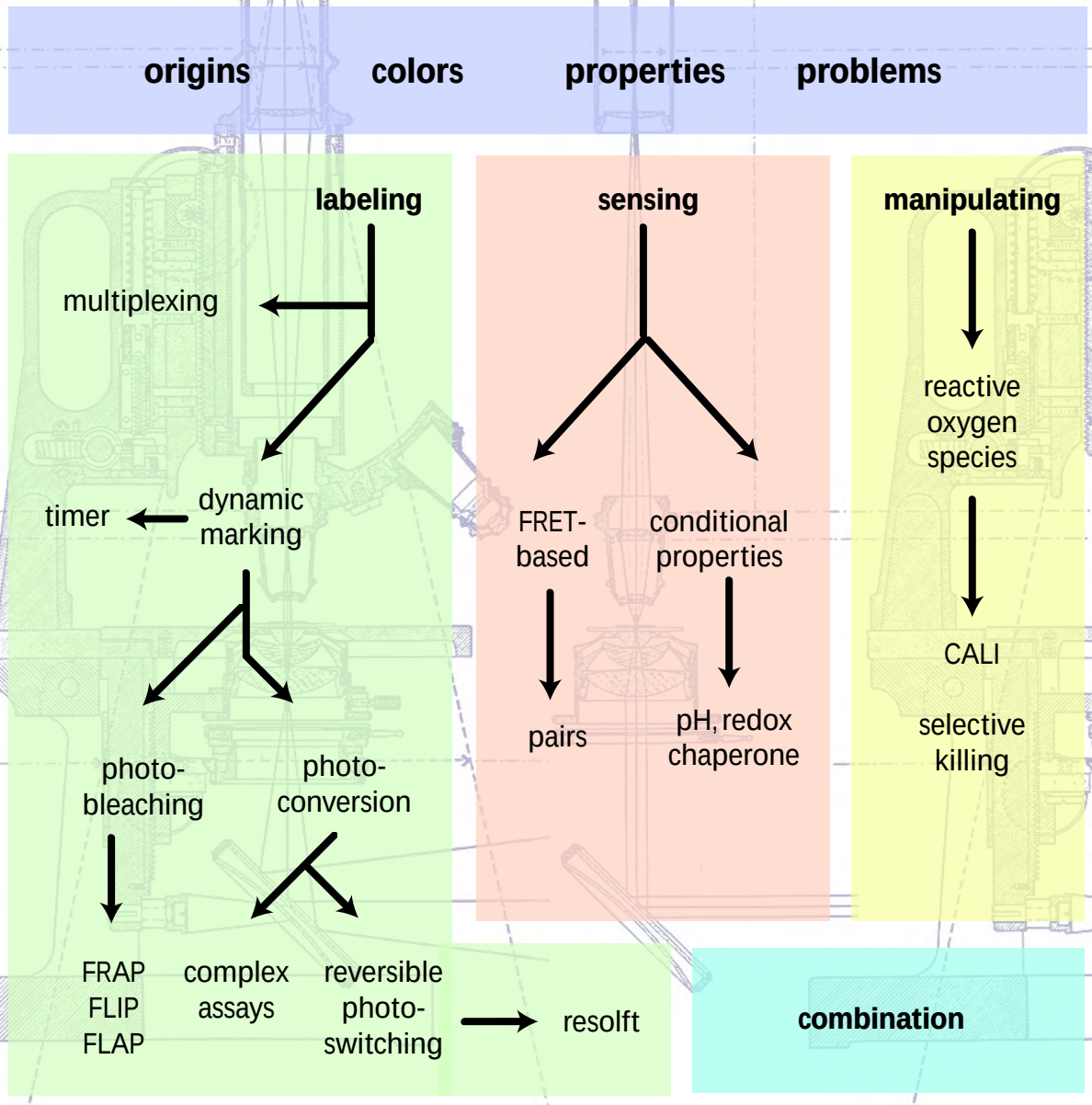
Göttingen

DFG Research Center
Molecular Physiology of the Brain
cmpb

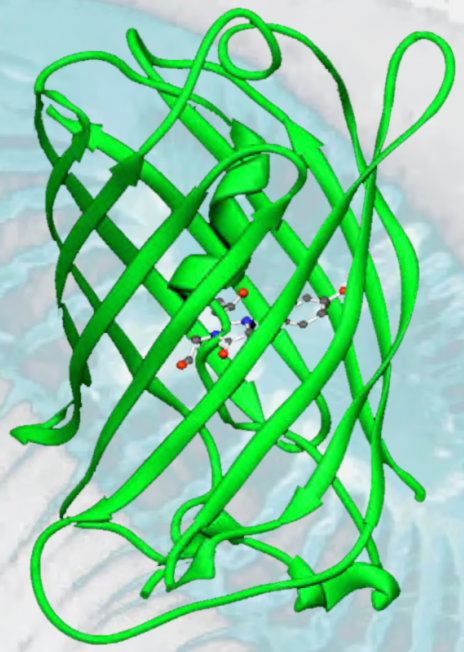
Molecular neurophysiology

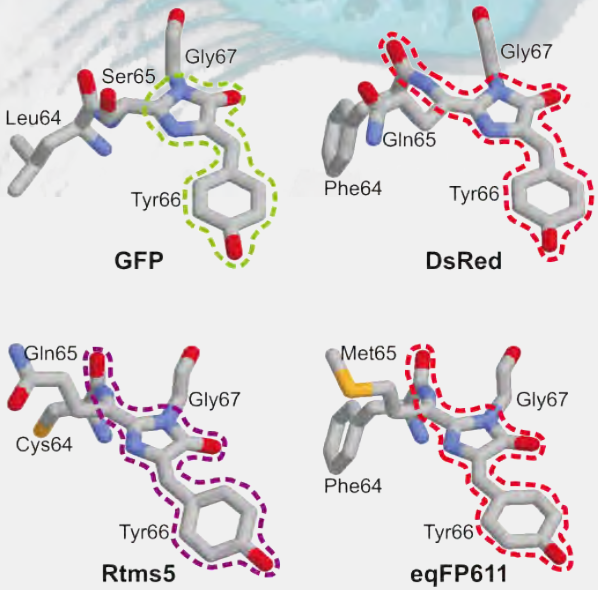
the Burgess nonsense book
being a complete collection of the humerous masterpieces of
Frank Gelett Burgess, Esq.
(published by Frederick A. Stokes Company, New York (1901)

Fluorescent Proteins



fluorescent proteins



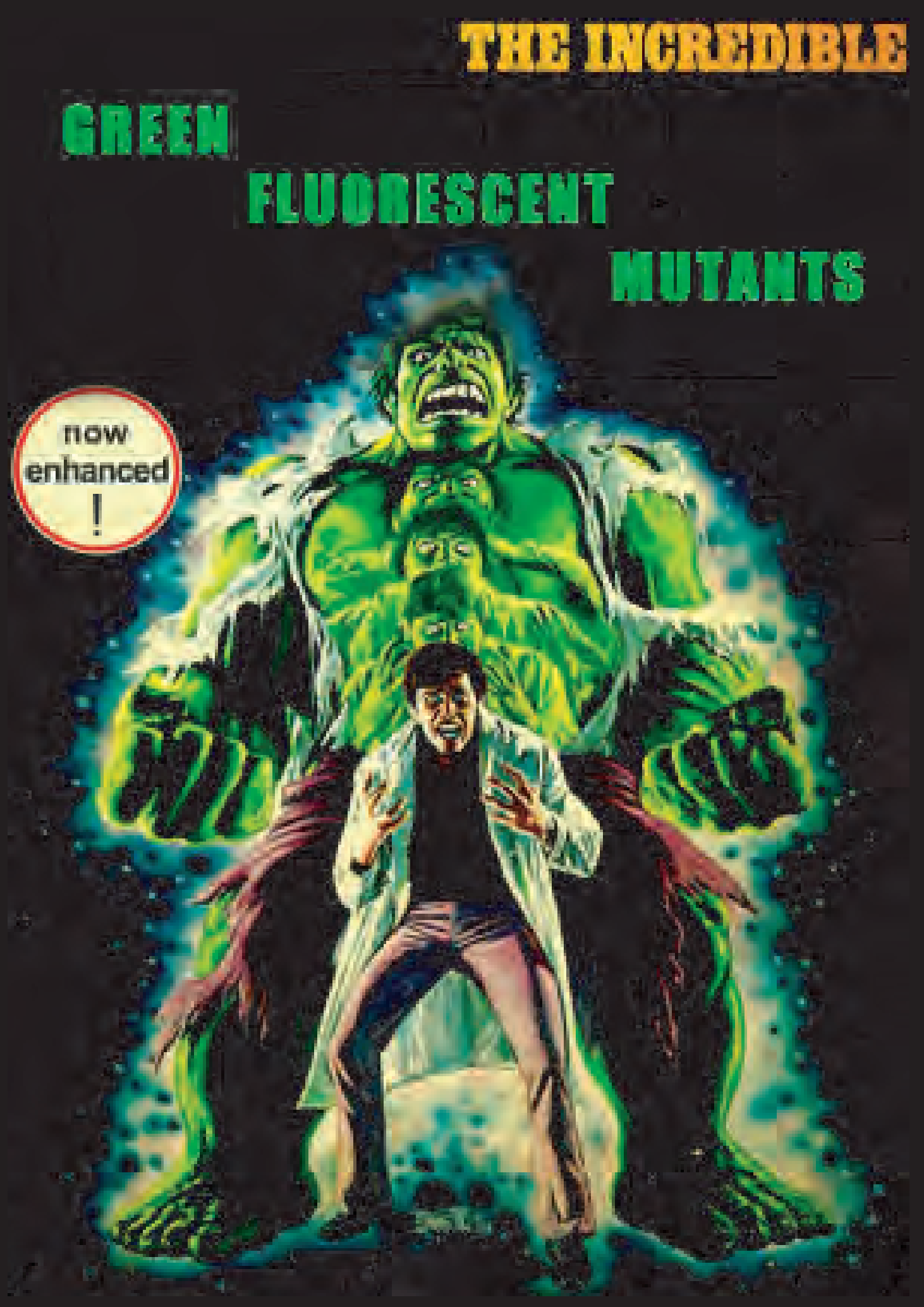


β-can structure

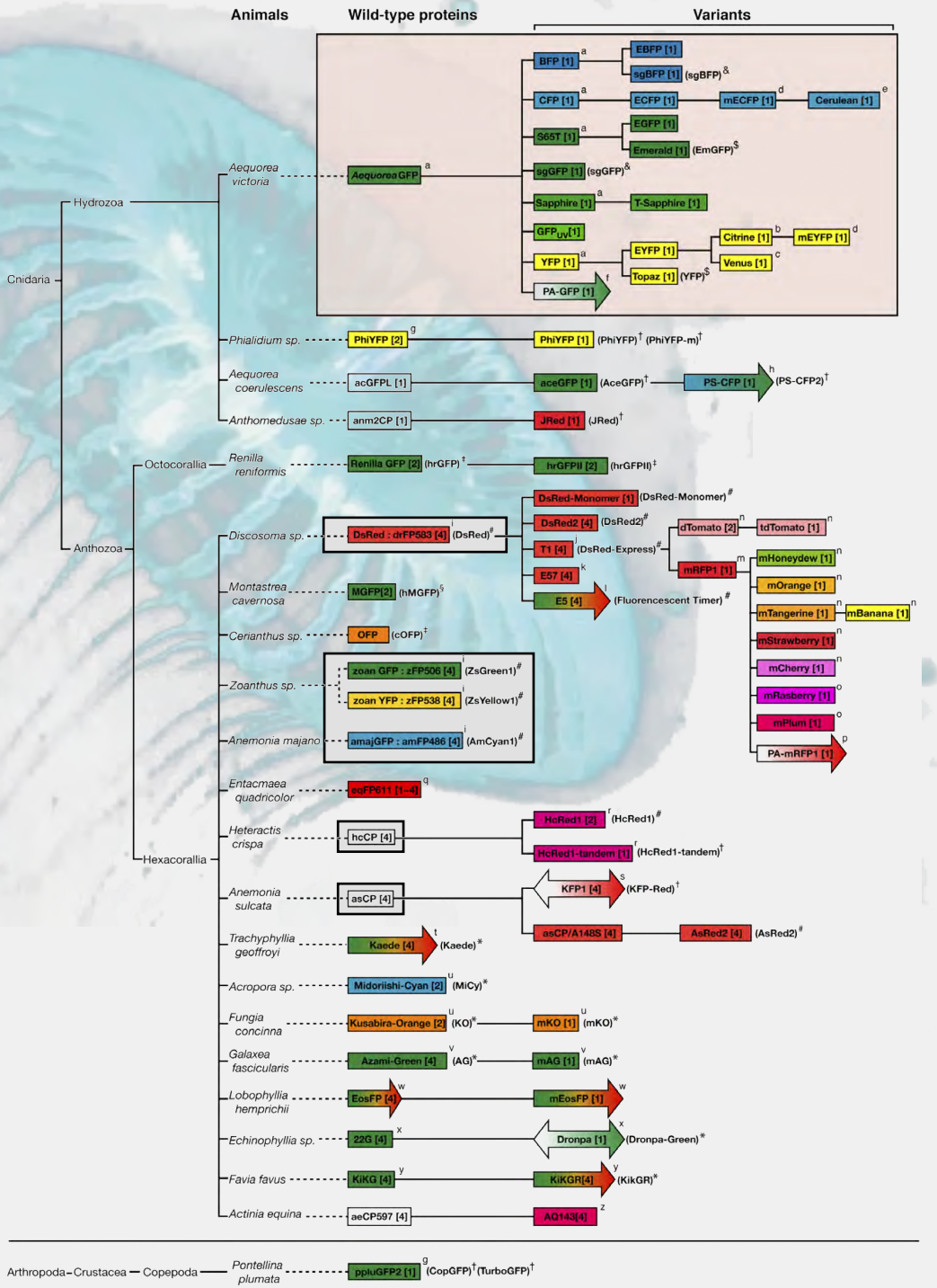
- rel. insensitive to environment
- proteolytically stable
- denaturation stable
- N- & C-terminus free
  allow fusion

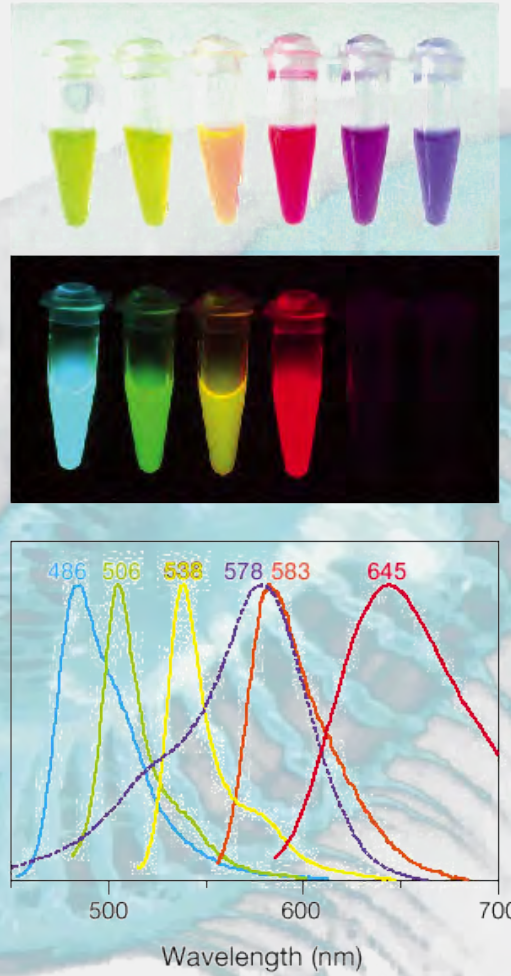
chromophore: aa 65-66-67

- post-translational oxidation
- single gene
- no co-factors required


THE INCREDIBLE
GREEN
FLUORESCENT
MUTANTS
now enhanced !

Rainbow



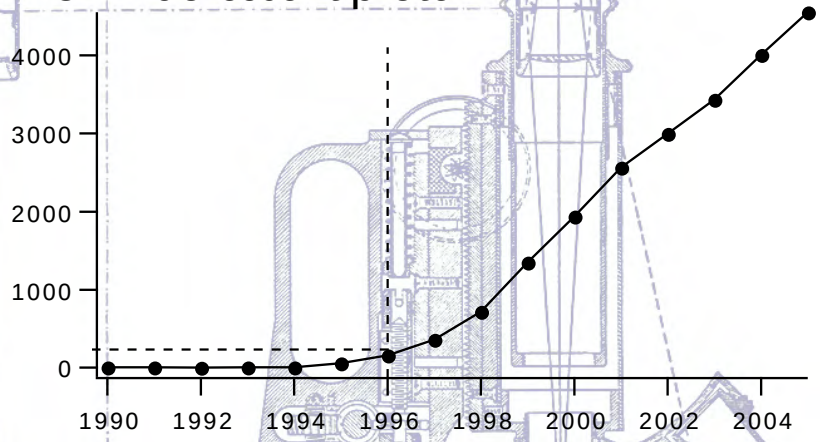


Miyawaki (2005) Neuron 48: 189-199

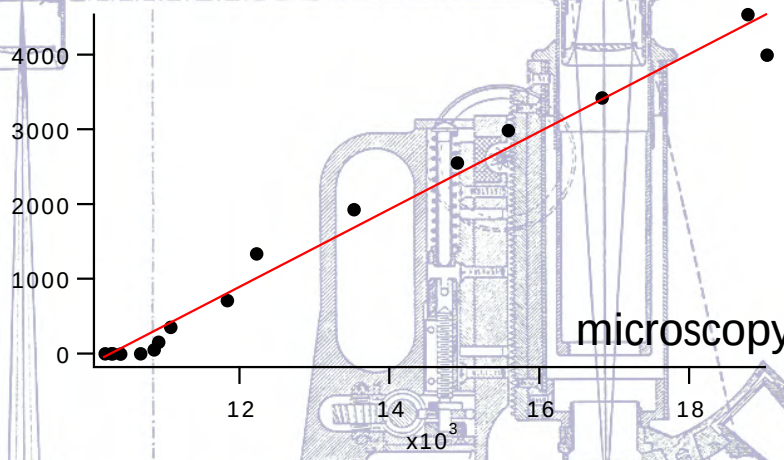
Verkhusha (2004) Nat. Biotechnol. 22: 289-296

popularity of GFP

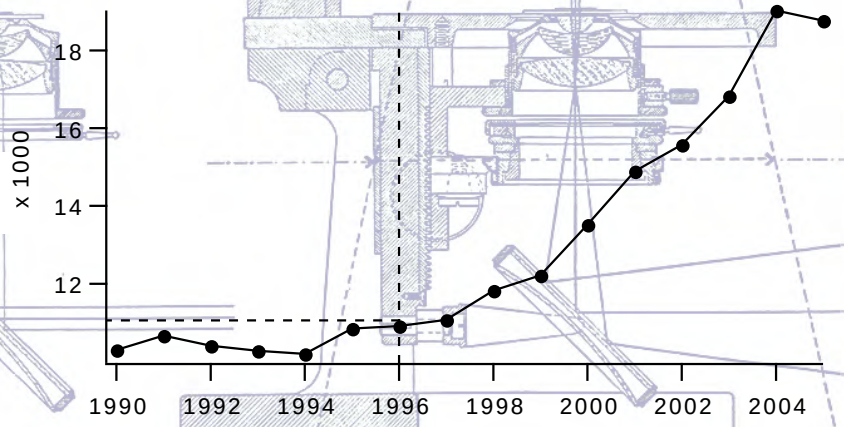
GFP OR "fluorescent protein"



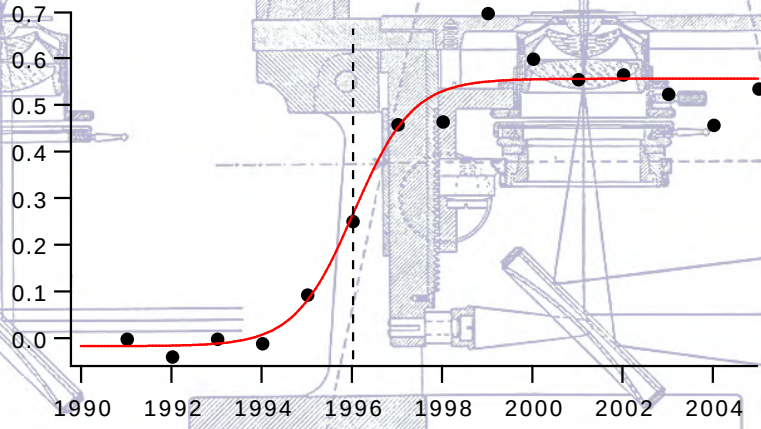
GFP OR "fluorescent protein"



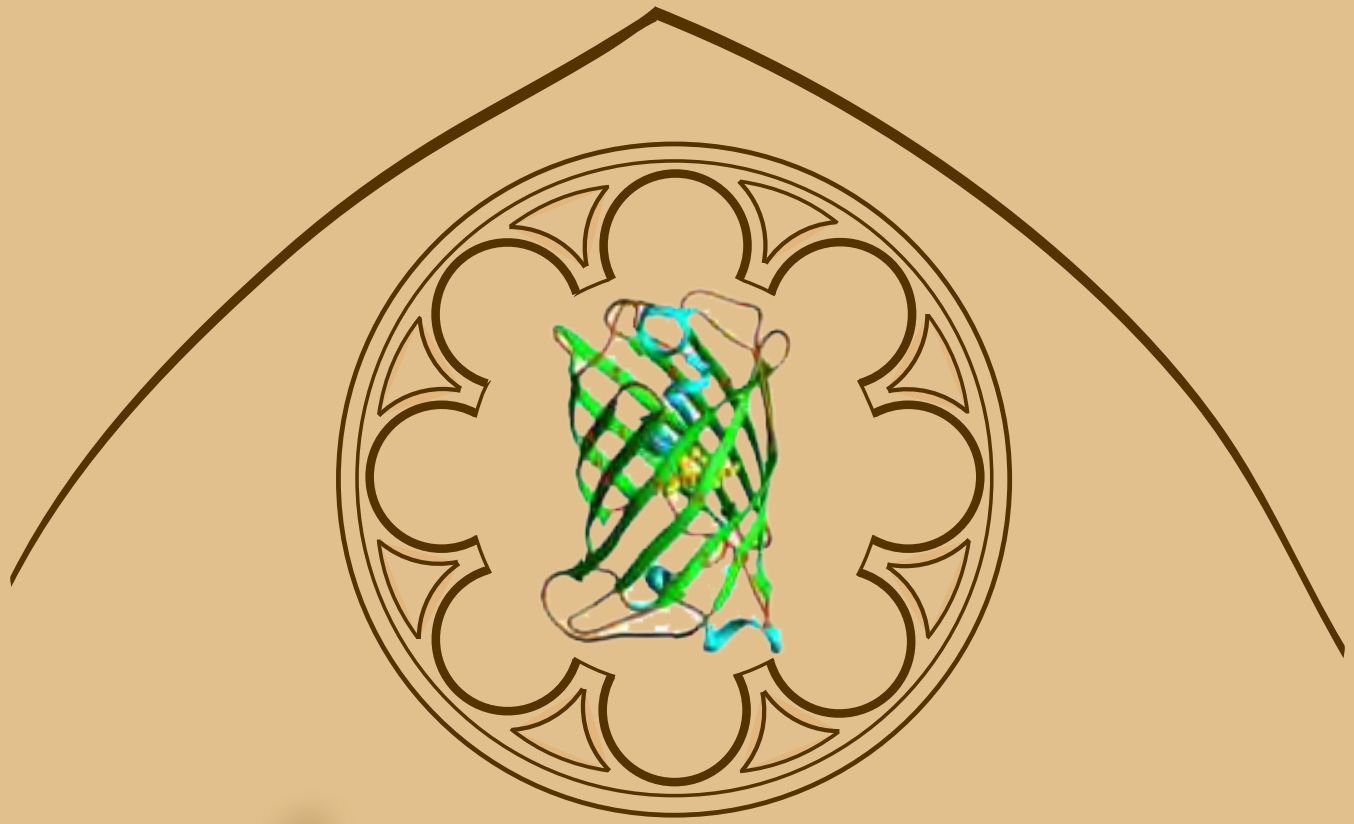
microscopy



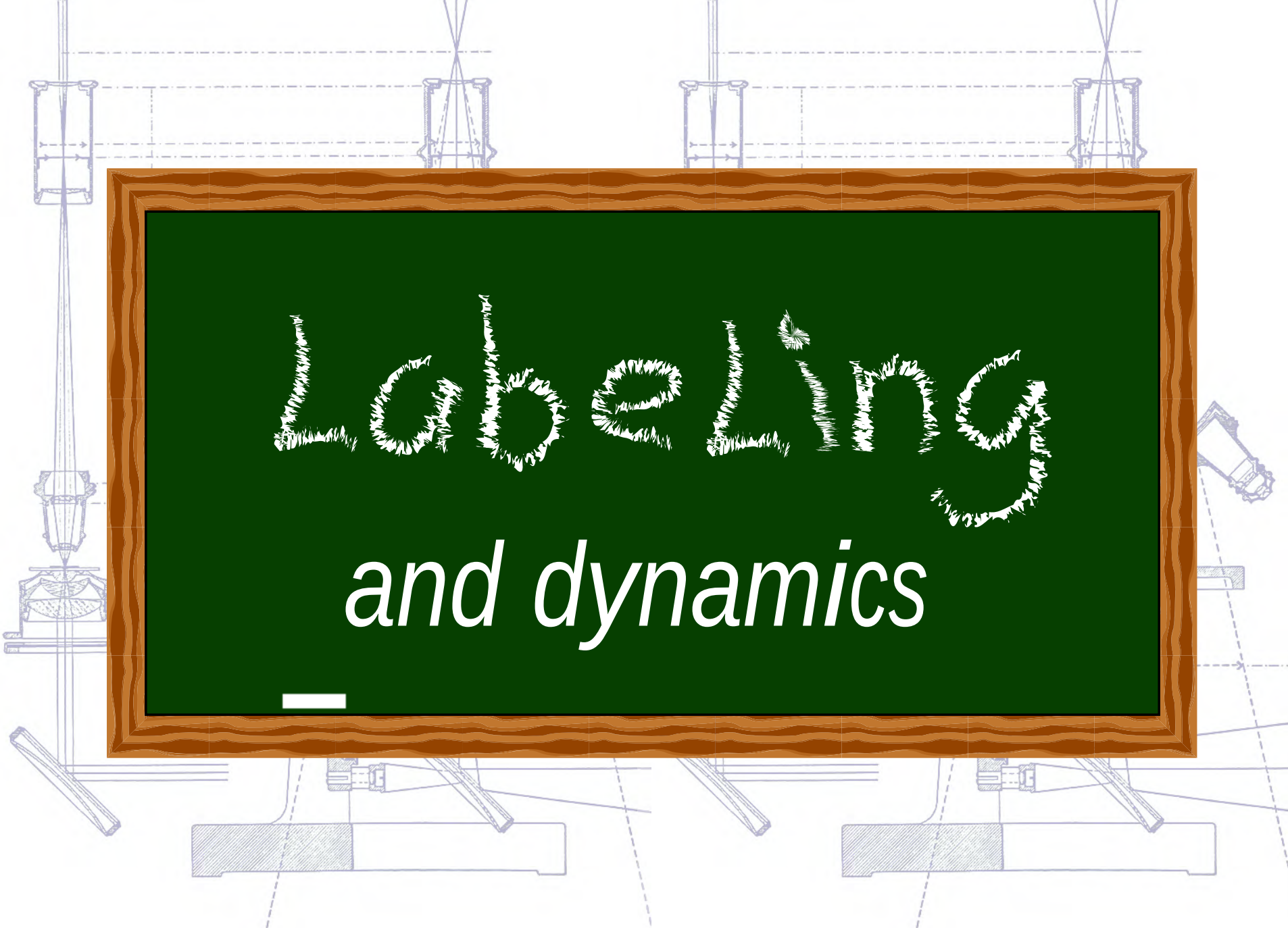
increase in microscopy by GFP



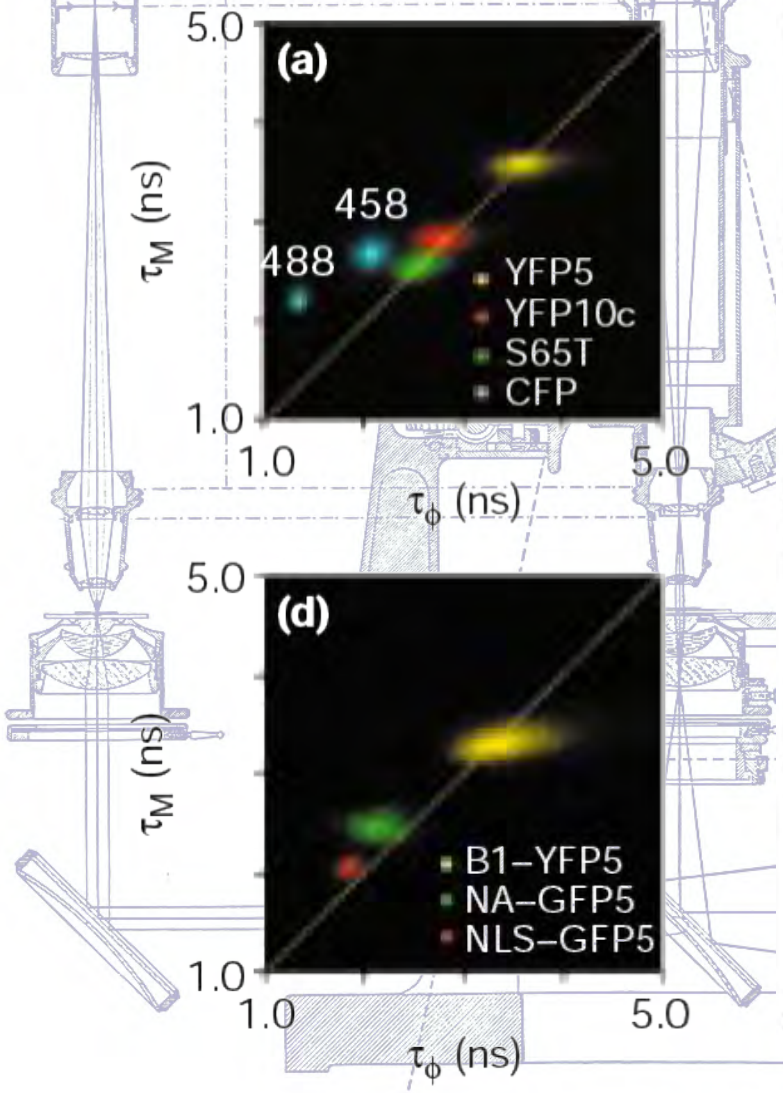
Wouters (2006) Contemporary Physics 47: 239-255

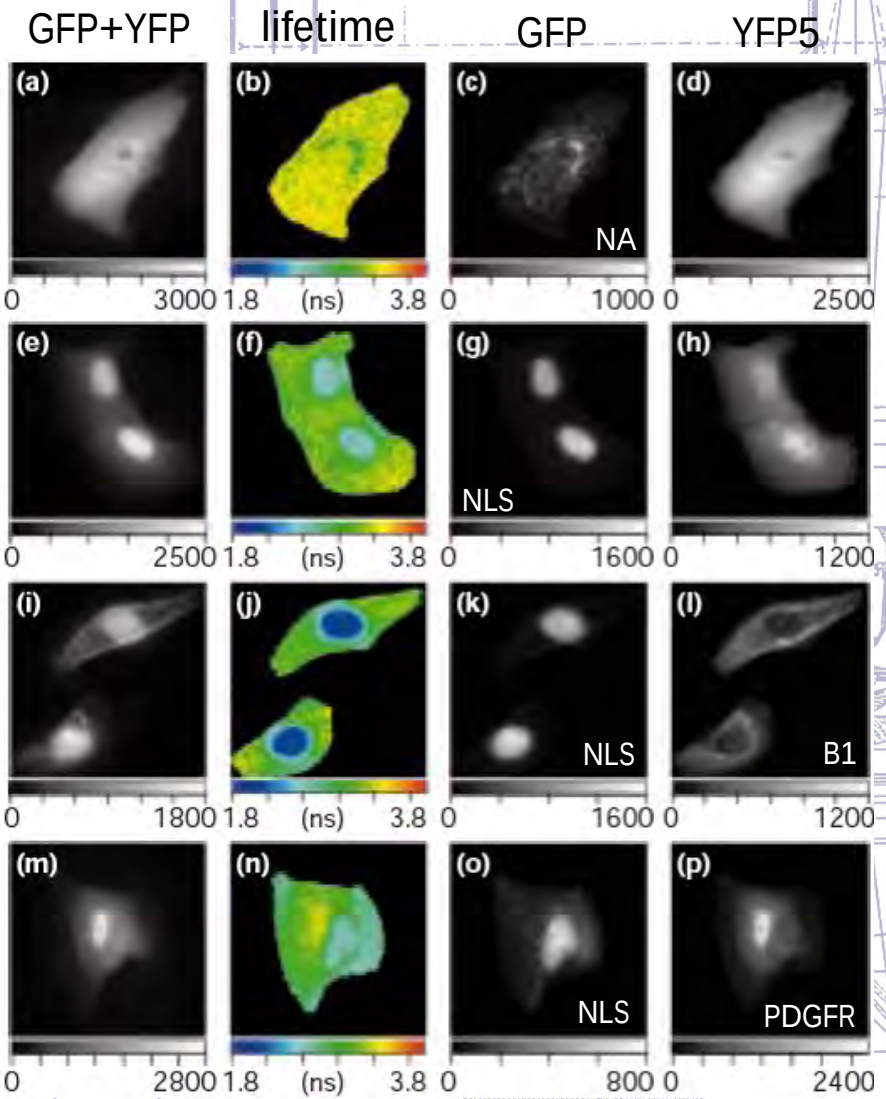


Fluoraissance


Labeling
and dynamics

Doing more with less colors



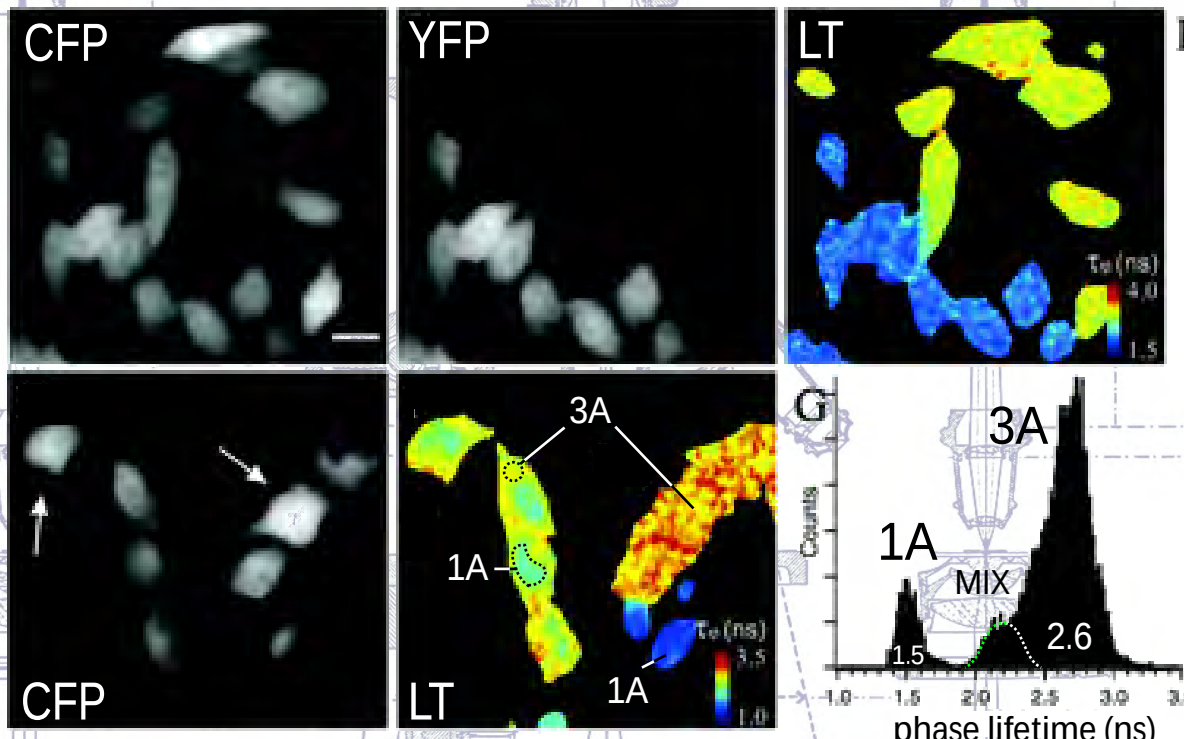


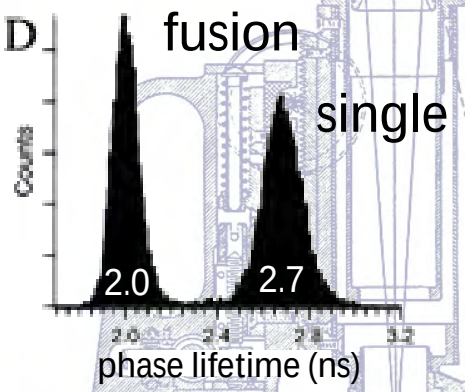
NA: N-acetylglucosaminyltransferase (Golgi+ER)
B1: Cyclin B1 (cytosol, microtubules)

Pepperkok (1999) Curr. Biol. 9: 269-272

Lifetime contrasting

SYFP2-SCFP3A





$$QY_{FRET} = (1-E) \bullet Q_{non\text{-}FRET}$$

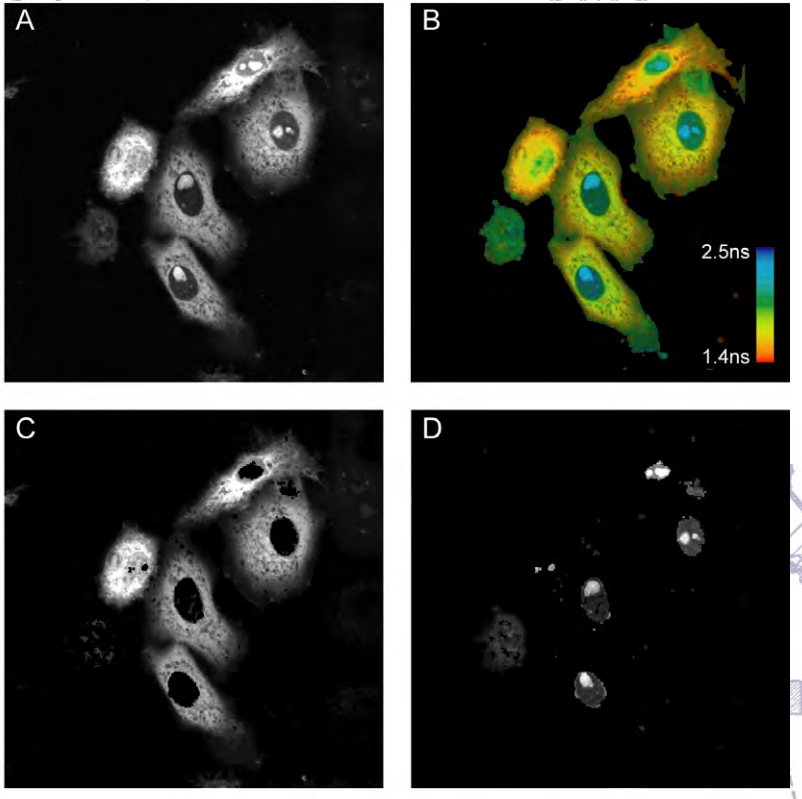
SCFP3A-NES (τ_Φ=2.6 ns)

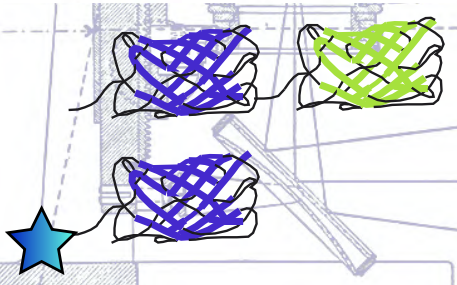
SCFP1A-NLS (τ_Φ=1.5 ns, QY=0.7)

$$QY = \frac{\tau_{obs}}{\tau_{rad}}$$

Kremers (2006) Biochemistry 45: 6570-6580

changing lifetime by FRET



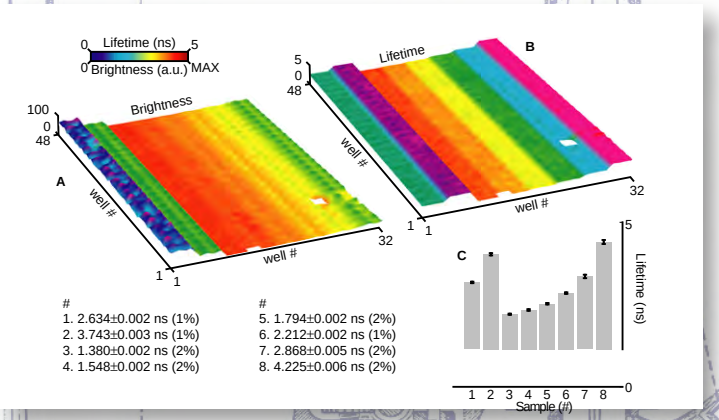


cytoplasmic

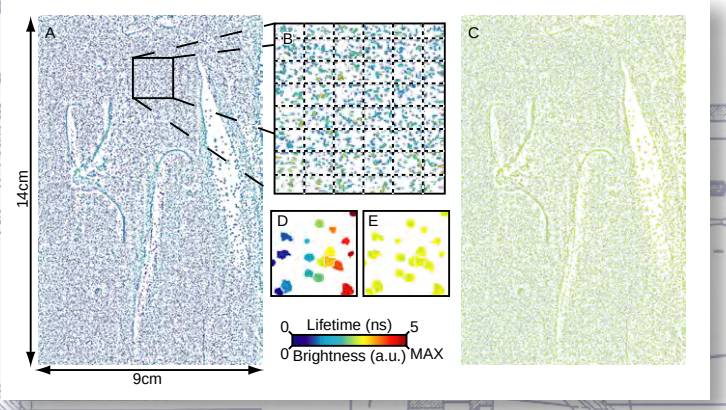
nucleolar
targeting signal

Esposito A & Wouters FS (2004) Fluorescence Lifetime Imaging (Unit 4.14). In Current Protocols in Cell Biology. J. Wiley & Sons, NY, USA.

mutational screening for changed lifetime



8 groups of 96 wells:
EGFP, R6G+EGFP, R6G+KI (63, 50, 38, 25, 13,0 mM) 20 min.

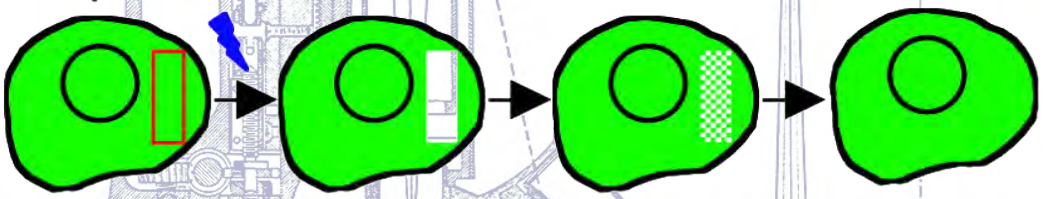


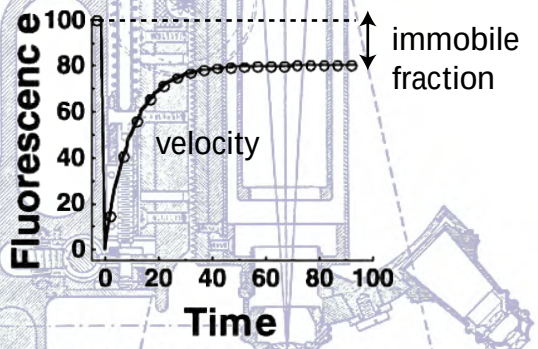
±20,000 colonies of *E. coli* expressing EYFP on a 9x14 cm agar plate. 1900 FOV, ±1 h.

Photobleaching tools: synchronizing populations

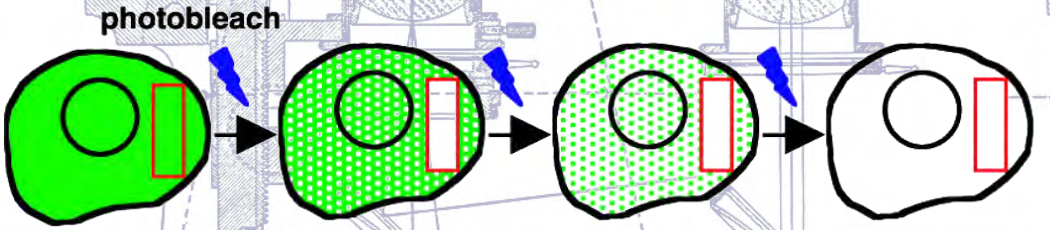
Diffusion rates

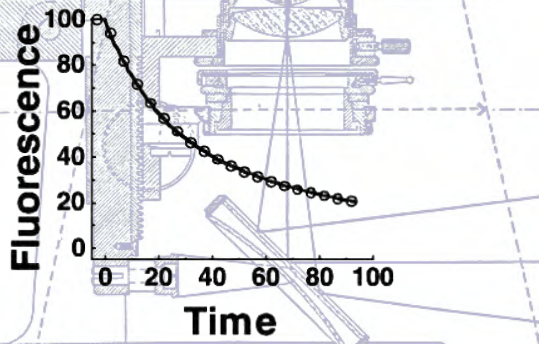
Fluorescence Recovery After Photobleaching (FRAP)





Fluorescence Loss in Photobleaching (FLIP)

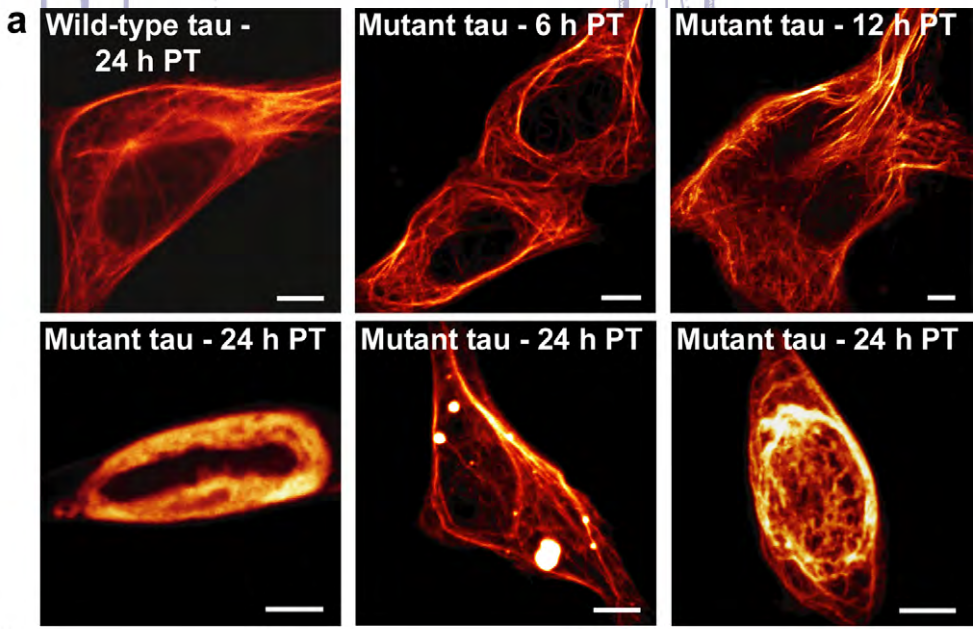


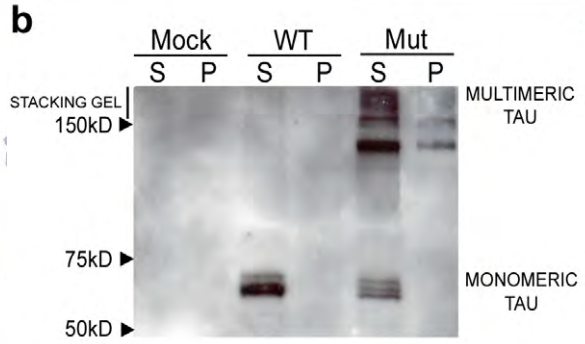


Diffusion continuities

Lippincott-Schwartz (2003) Science. 300: 87-91

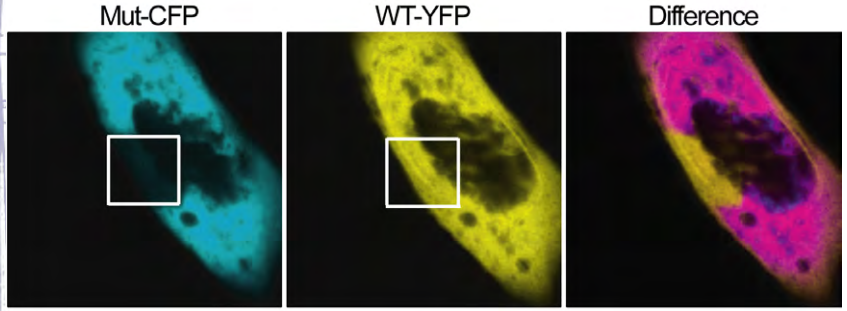
aggregation of structurally-optimized tau

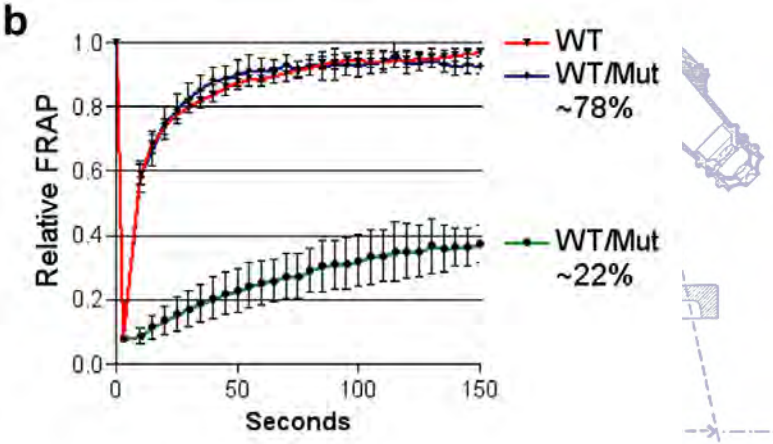




c

Hyper-aggregating tau mutant was created by optimization of internal oligomerization sequences





±1/4 cells expressing hyper-aggregating tau exhibit WT tau immobilization

Iliev (2006) J. Biol. Chem. 281(48): 37195-204.

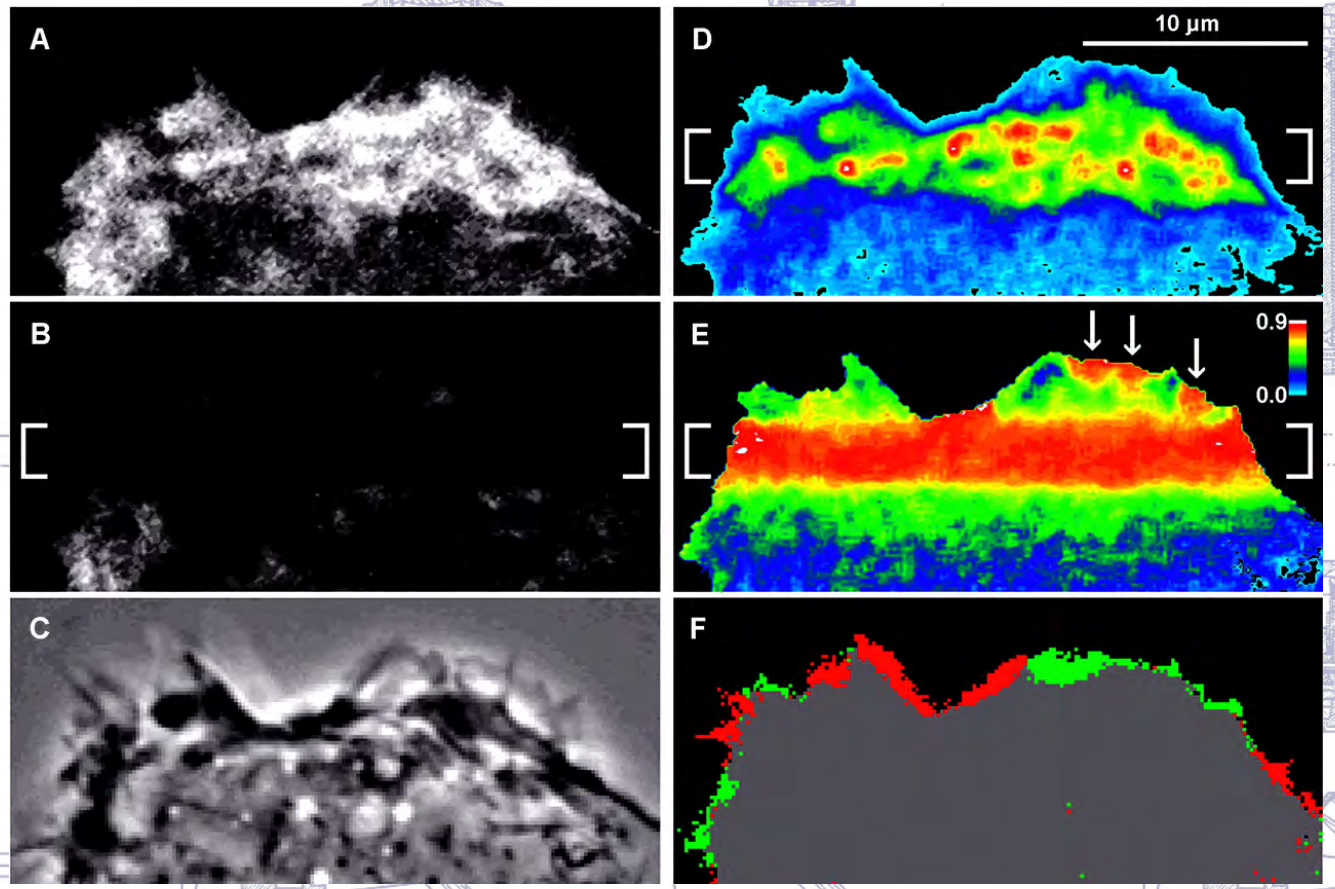
Fluorescence Localization After Photobleaching

rapid actin transport during cell protrusion

CFP-actin

YFP-actin

phase contrast



FLAP

(CFP-YFP)

rFLAP

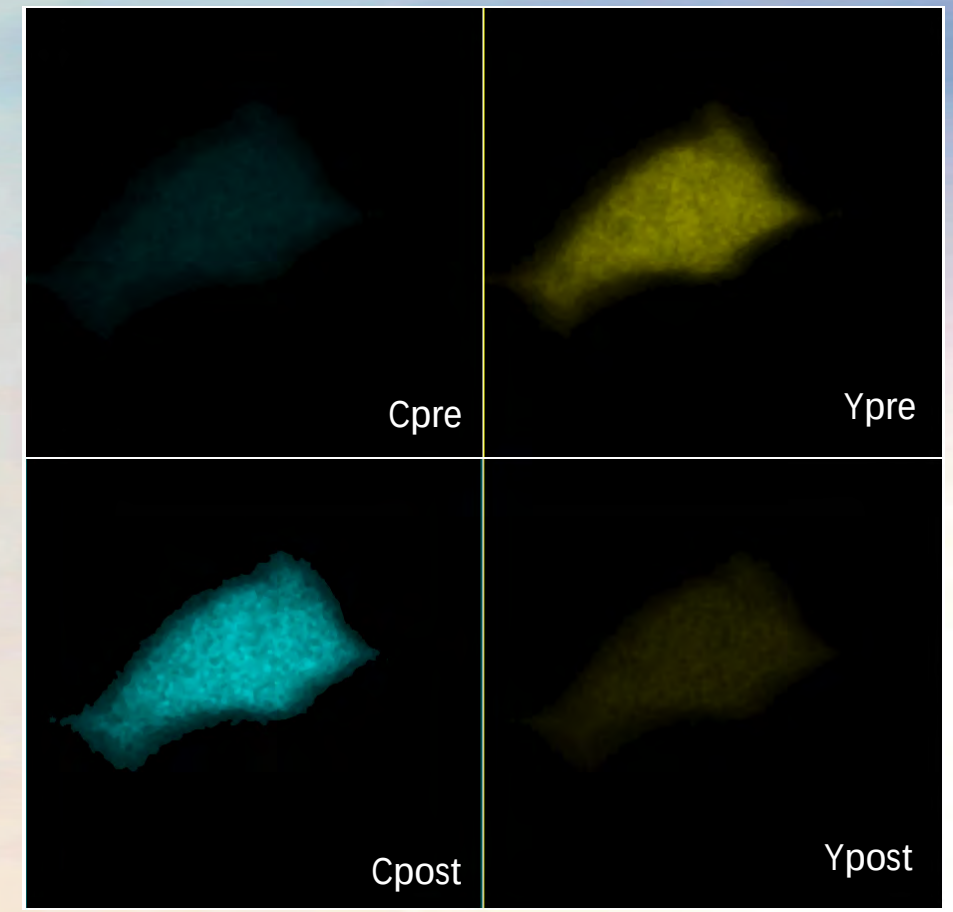
$1 - \frac{YFP}{CFP}$

protrusion

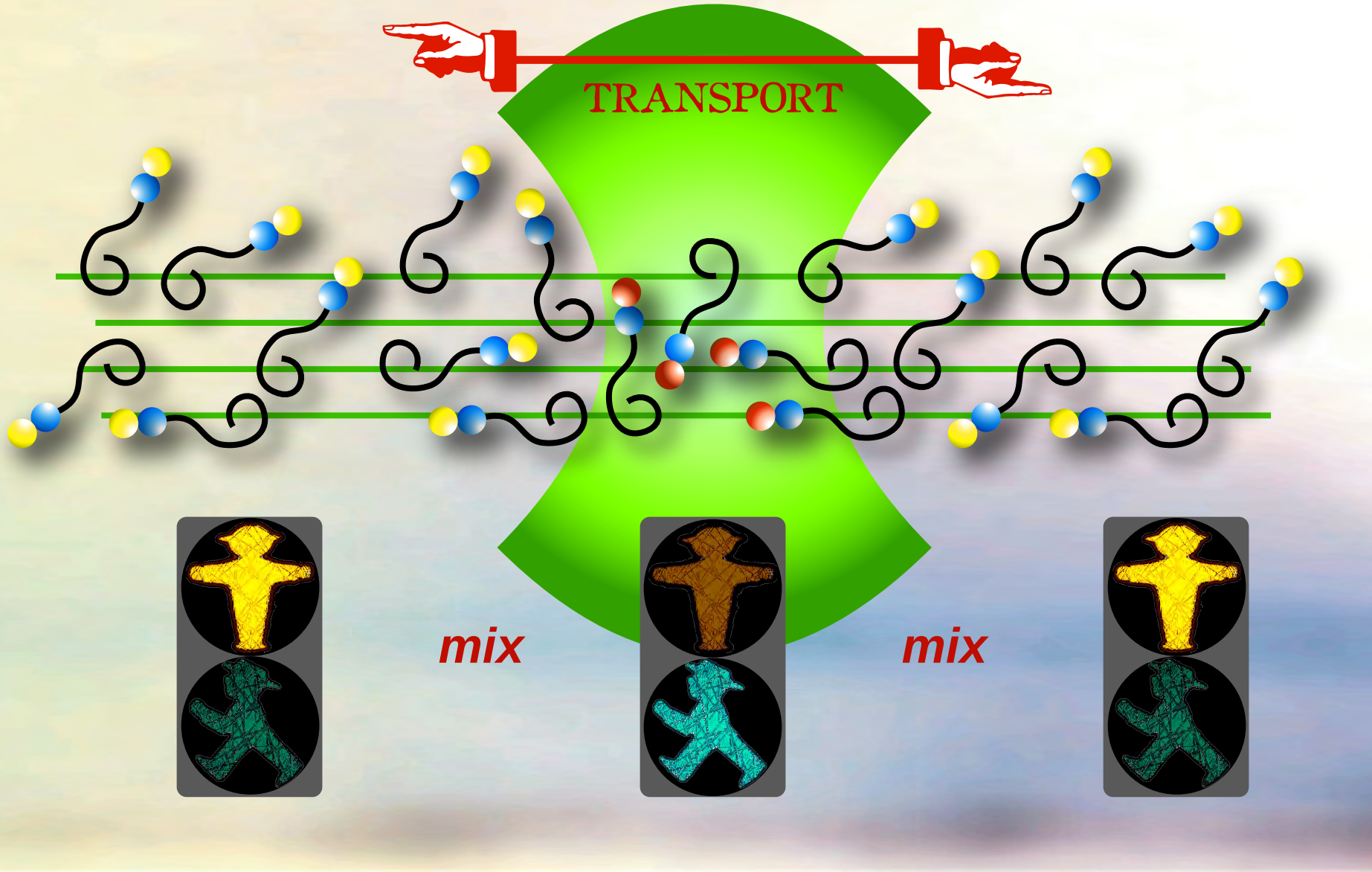
retraction

Zicha (2003) Science 300: 142-145

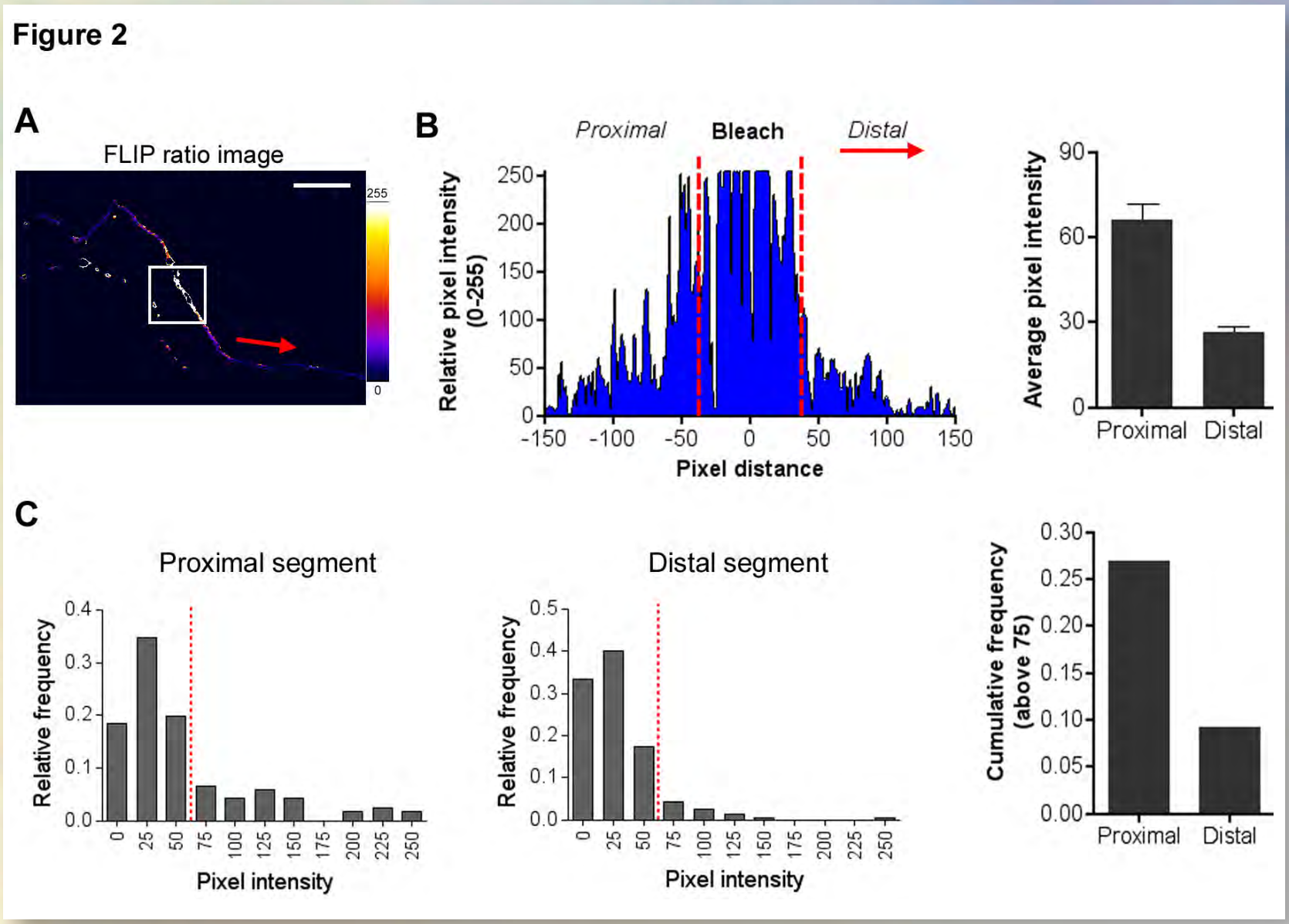
FLIPping the switch



FRAP, FLIP, FLAP WITH A FRET DYE

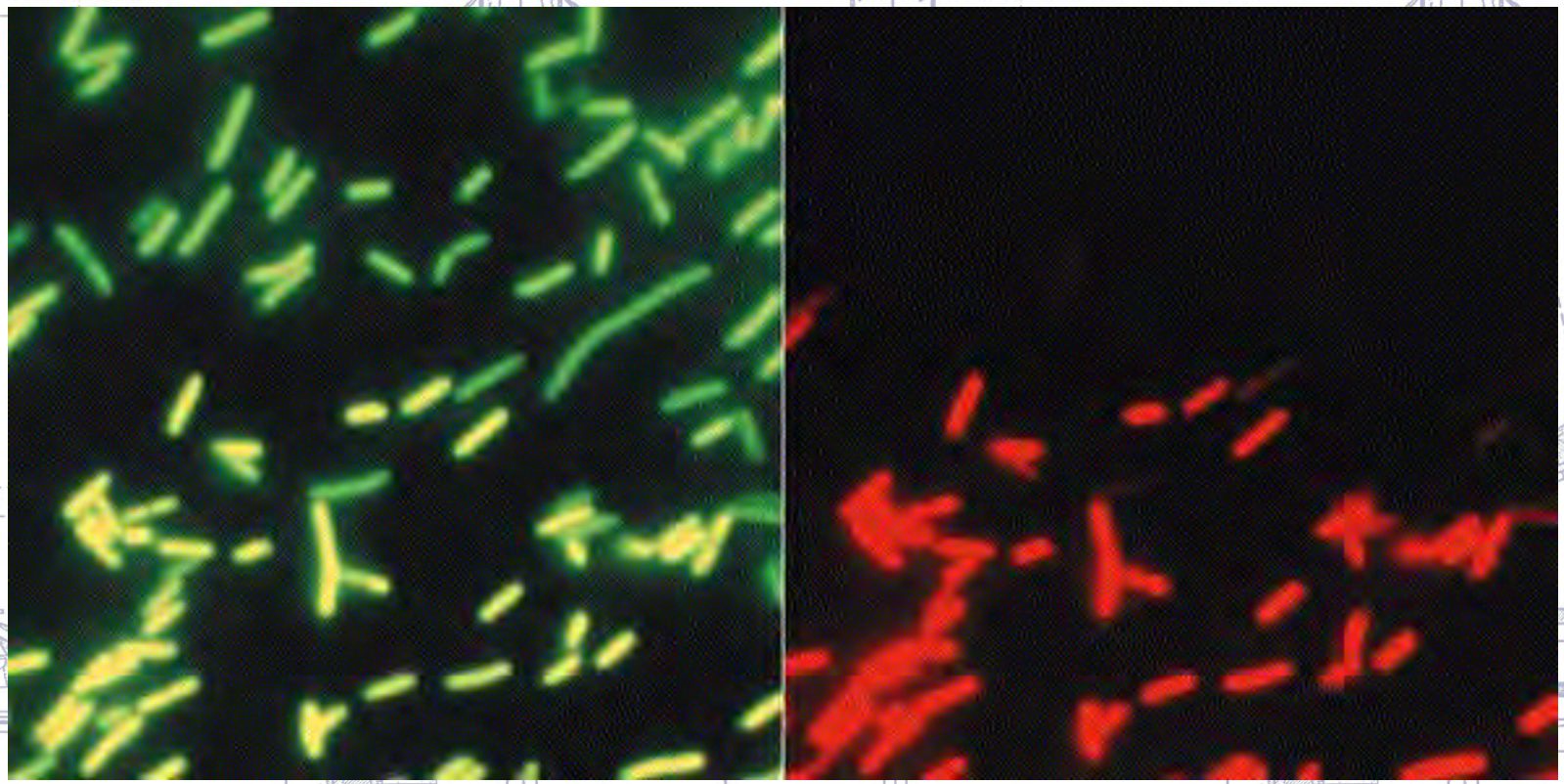


Directional transport of Synaptophysin-FLAP



Iliev (2007) J. Neurosci. Methods Nov 21 Epub ahead of print

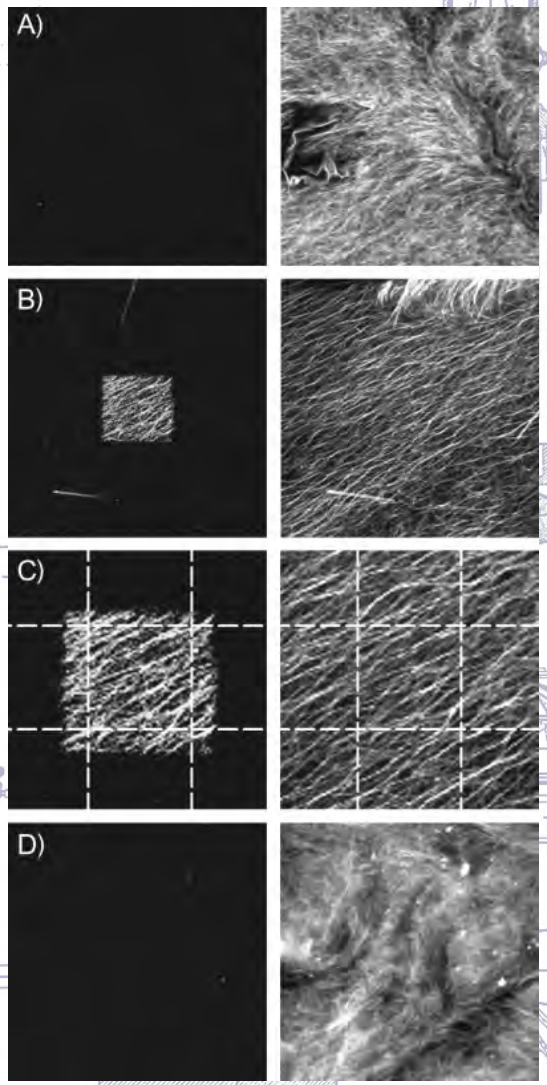
photoconversion

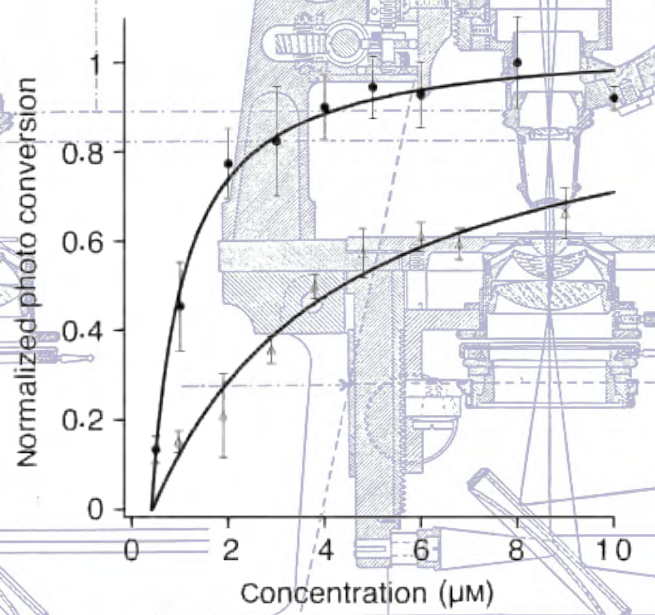


GFP bleaching during observation under anearobic conditions produces a red-emitting form

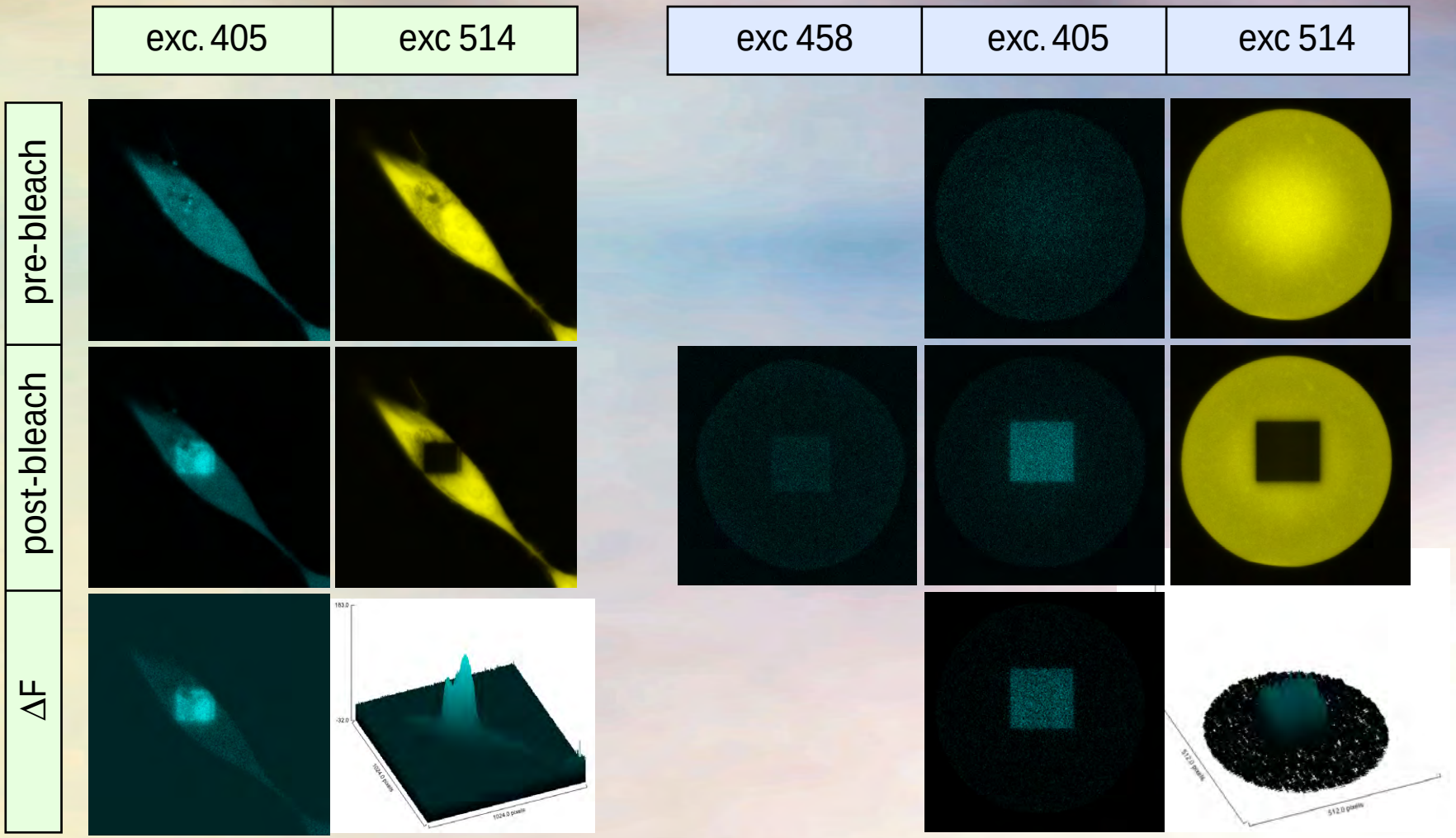
Elowitz (1997) Curr. Biol. 7: 809-812

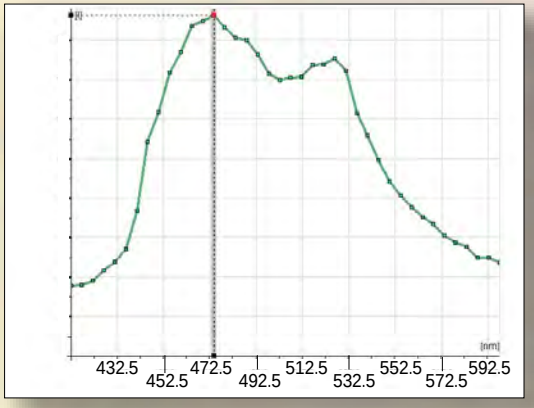
GFP photoconversion put to (limited) use





Brunet (2006) Traffic. 7: 1283-1289

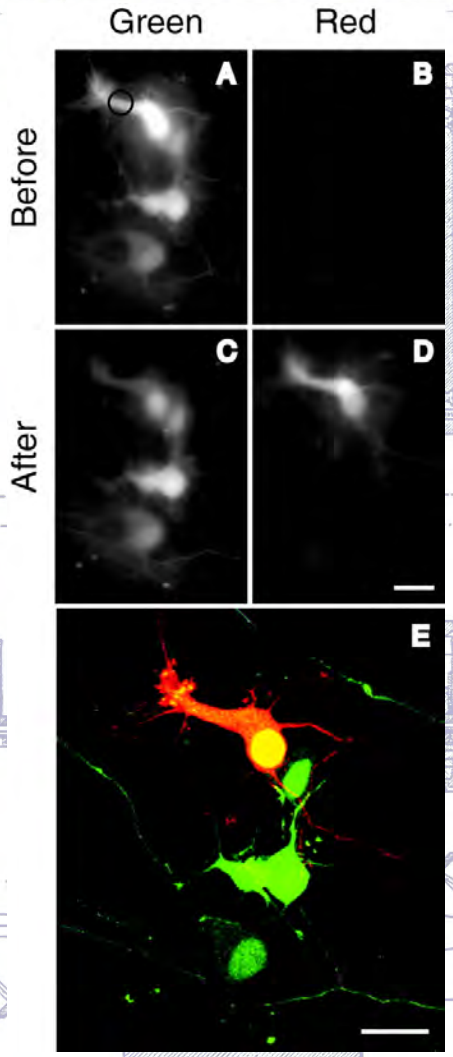




photoconversion of YFP:
bleaching of YFP leads to the formation of a CFP-like photoproduct

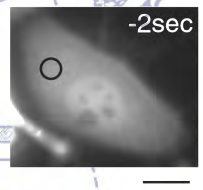
Gertrude Bunt (Stuttgart University) and Valentin (2005) Nat. Meth. 2: 801.

Photoconversion: another lucky observation

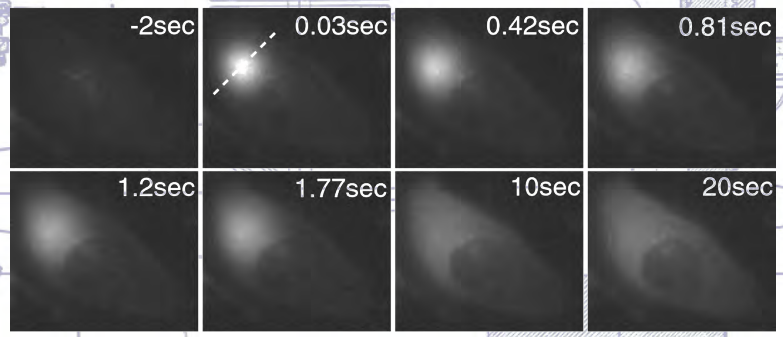


Kaede

We happened to leave one of the protein aliquots on the laboratory bench overnight. The next day, we found that the protein sample on the bench had turned red, whereas the others that were kept in a paper box remained green. Although the sky had been partly cloudy, the red sample had been exposed to sunlight through the south-facing windows.

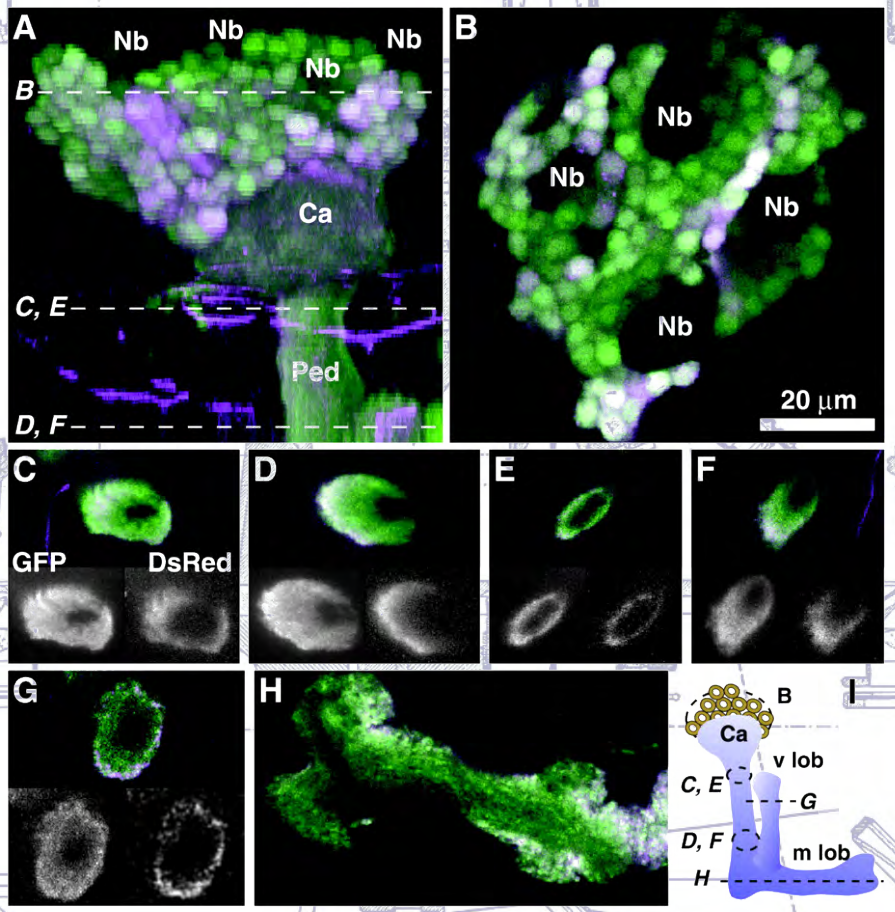


Ando (2002) PNAS 99: 12651-12656



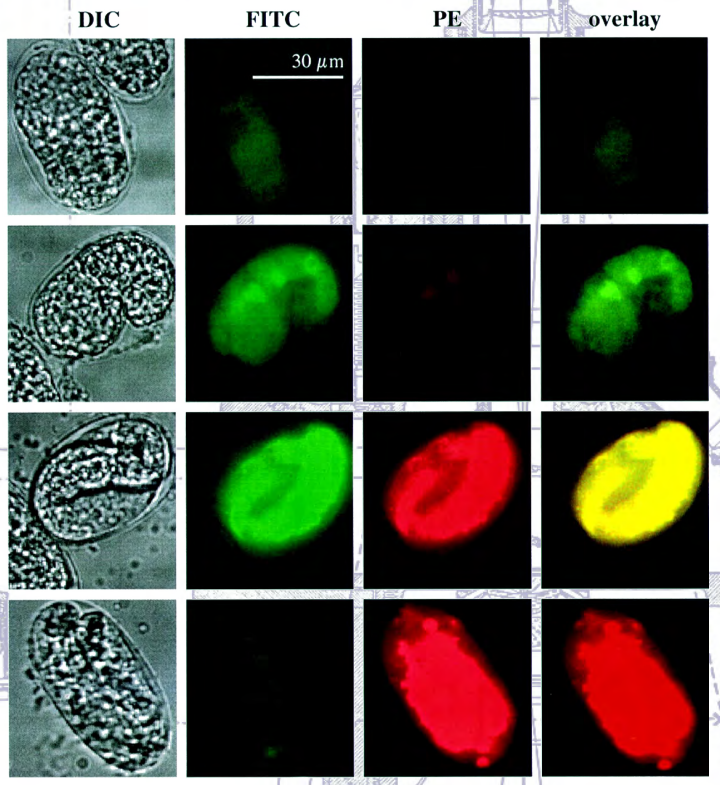
dsRed maturation mutant: timer

Drosophila neural fiber bundle formation



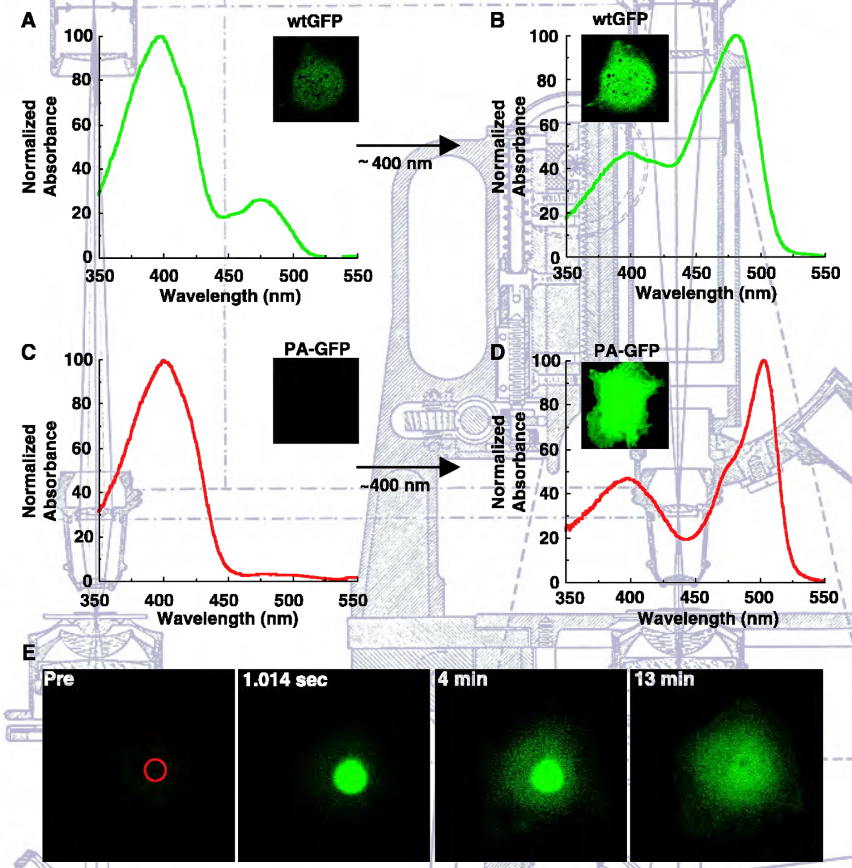
Verkushka (2001) J. Biol. Chem. 276: 29621-29624

expression in C. elegans

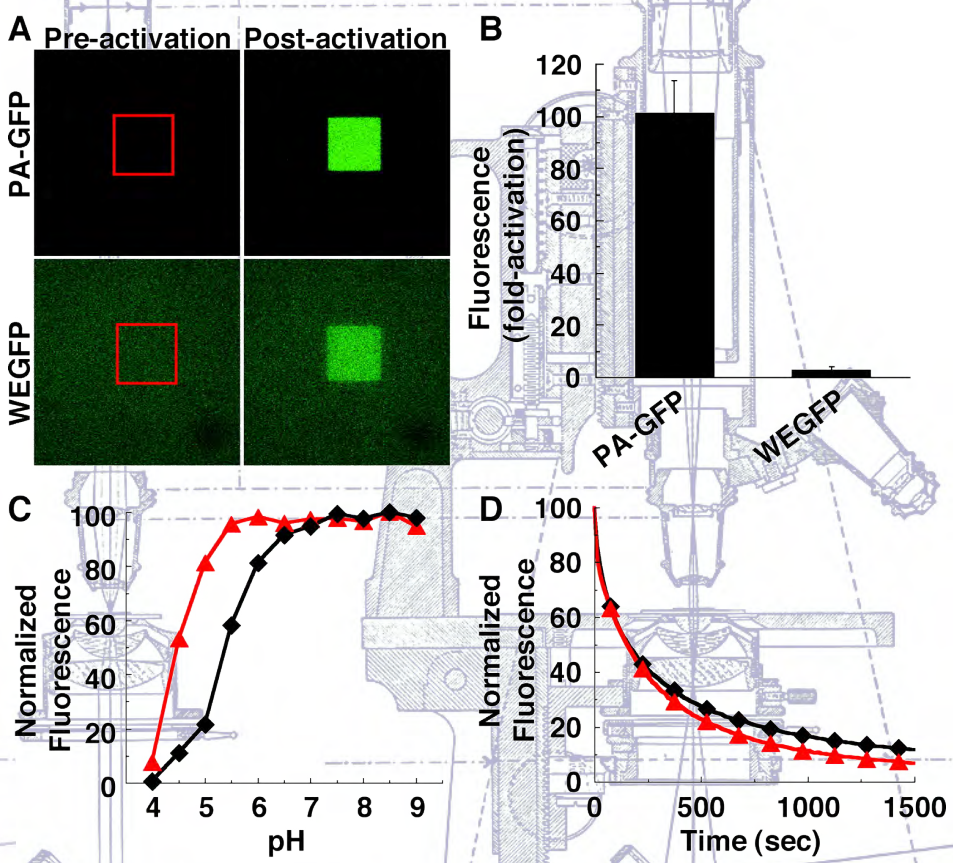


Terskikh (2000) Science 290: 1585-1588

Photoactivation of GFP

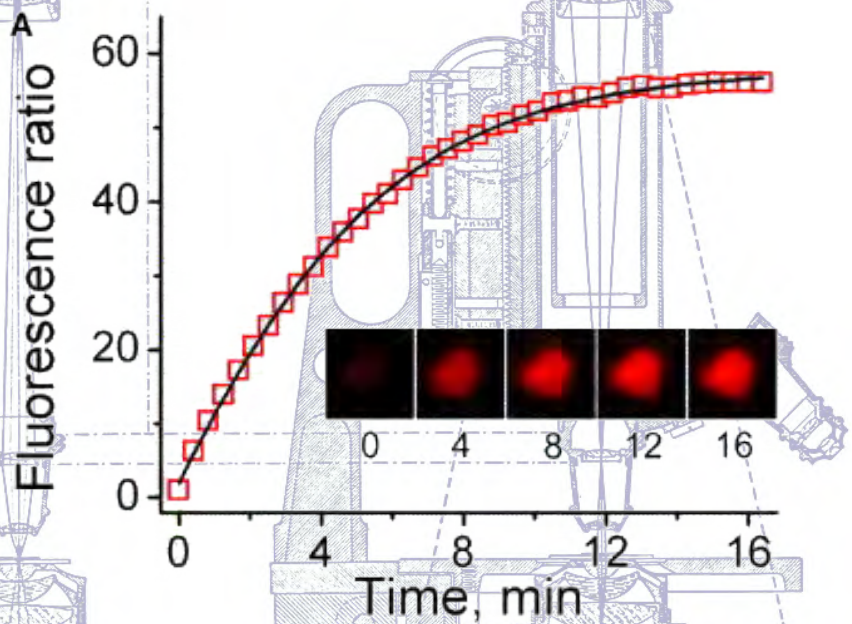


Lippincott-Schwartz (2003) Science. 300: 87-91

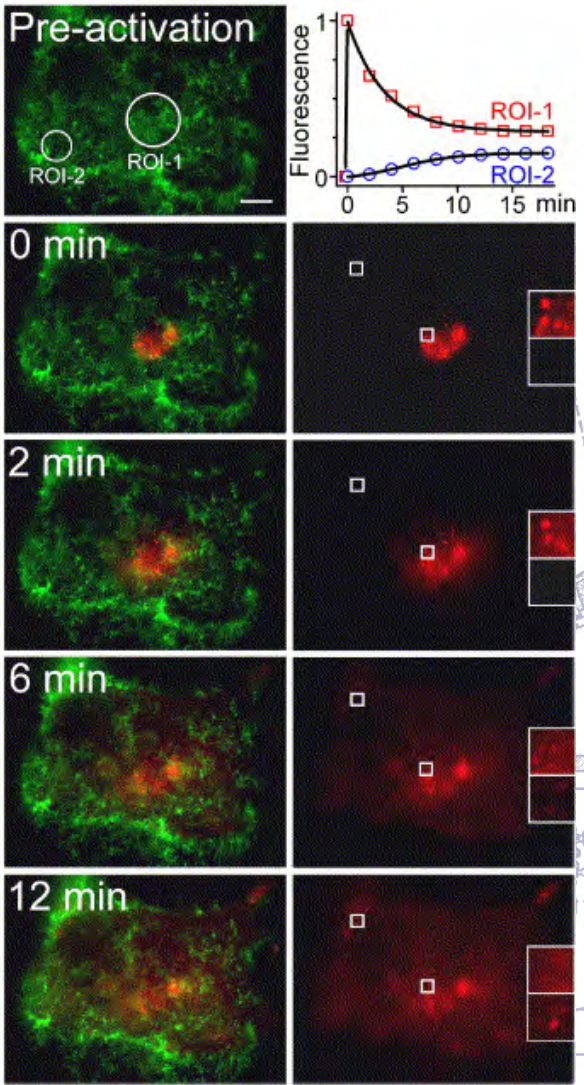


Patterson (2002) Science. 297: 1873-1877

photoactivatable mRFP



allows highly complex multi-color dynamic bioassays



EYFP-Dopamine transporter (plasma membrane) and PA-mRFP-Rab5 (endosomal surface)

Verkusha (2006) Chem. Biol. 12: 279-285

photolabeling

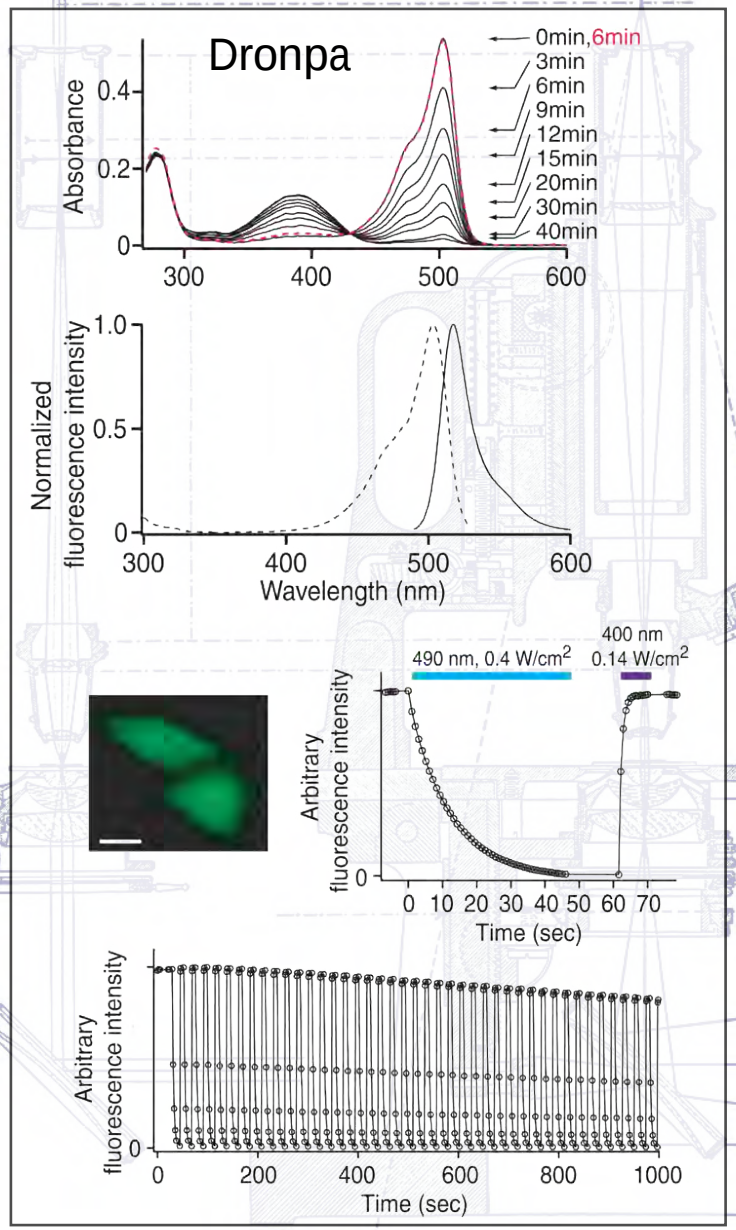
photoconversion

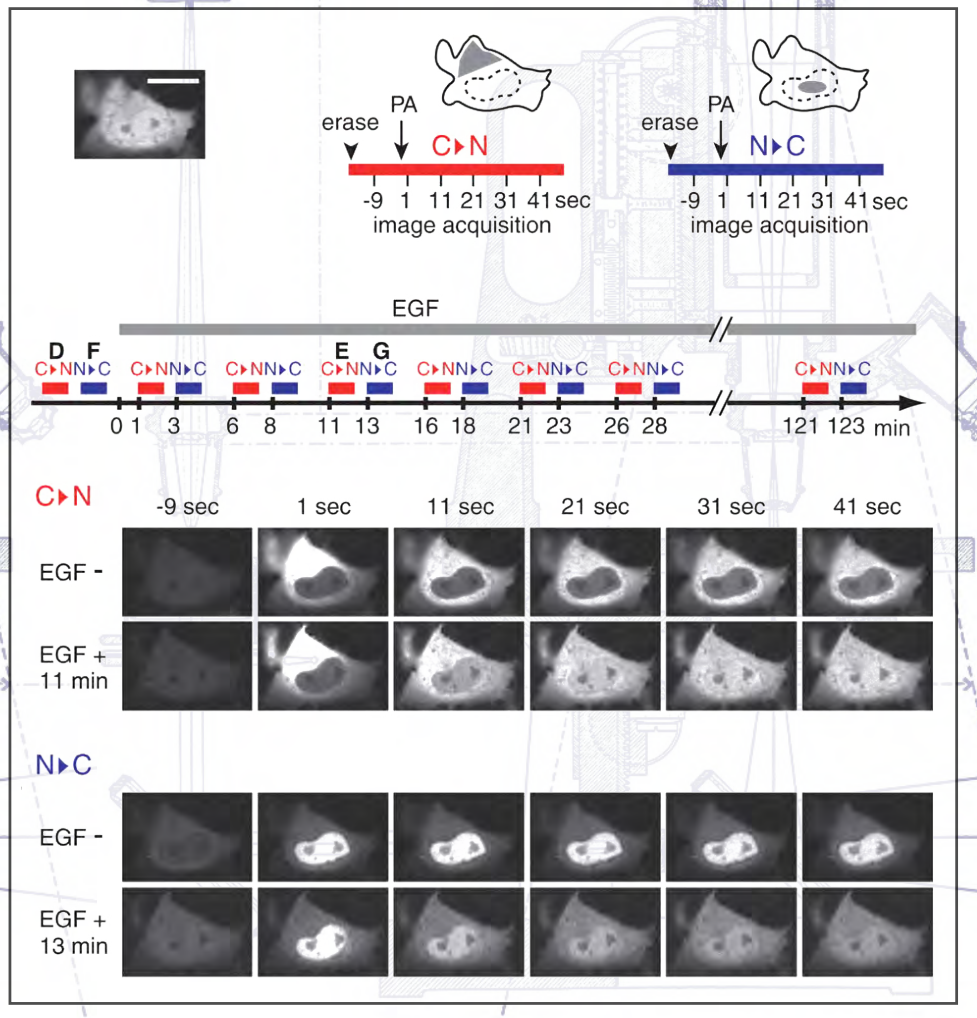
photo-
activation

photoswitching

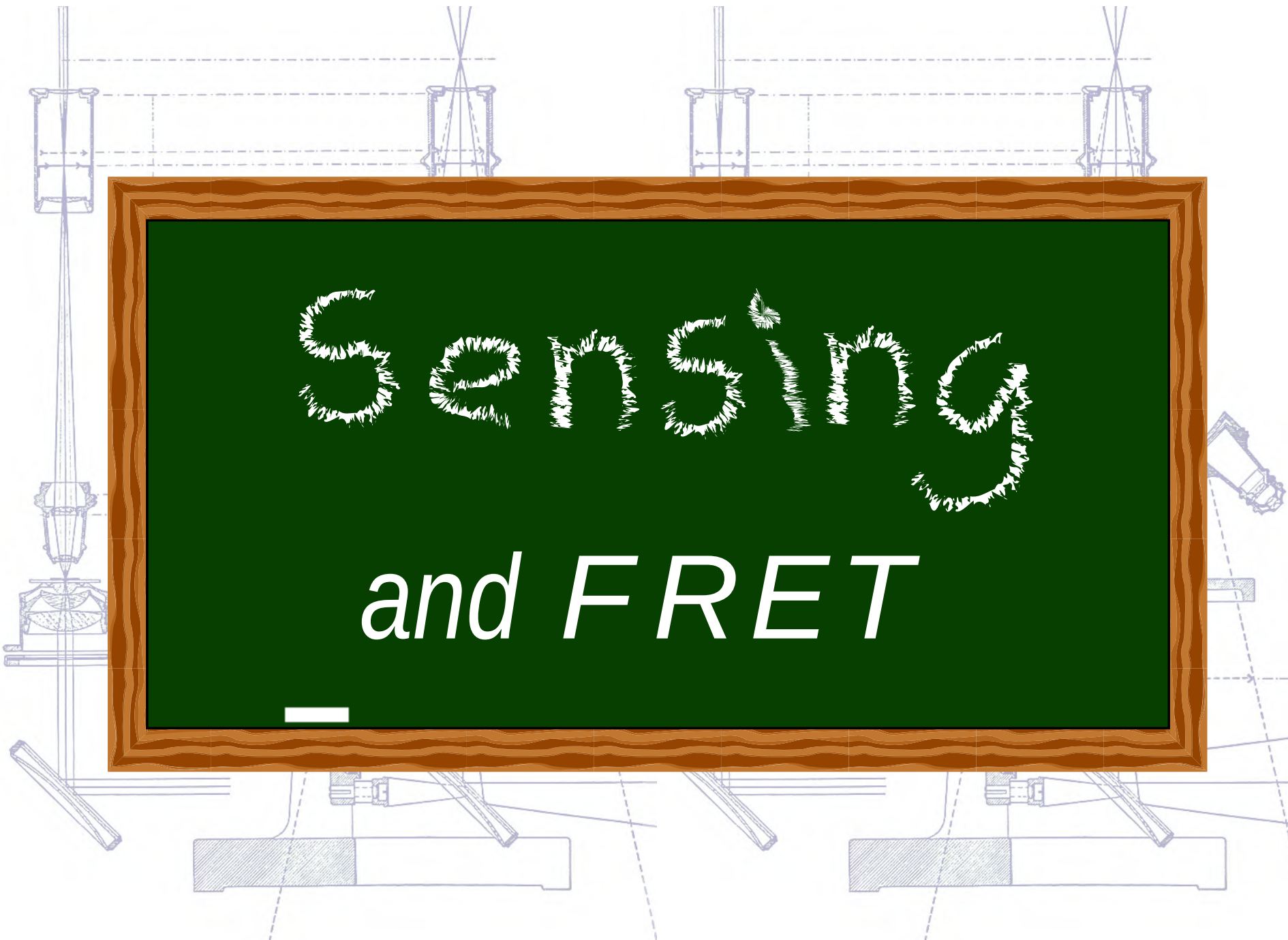
photochromism

photoswitching

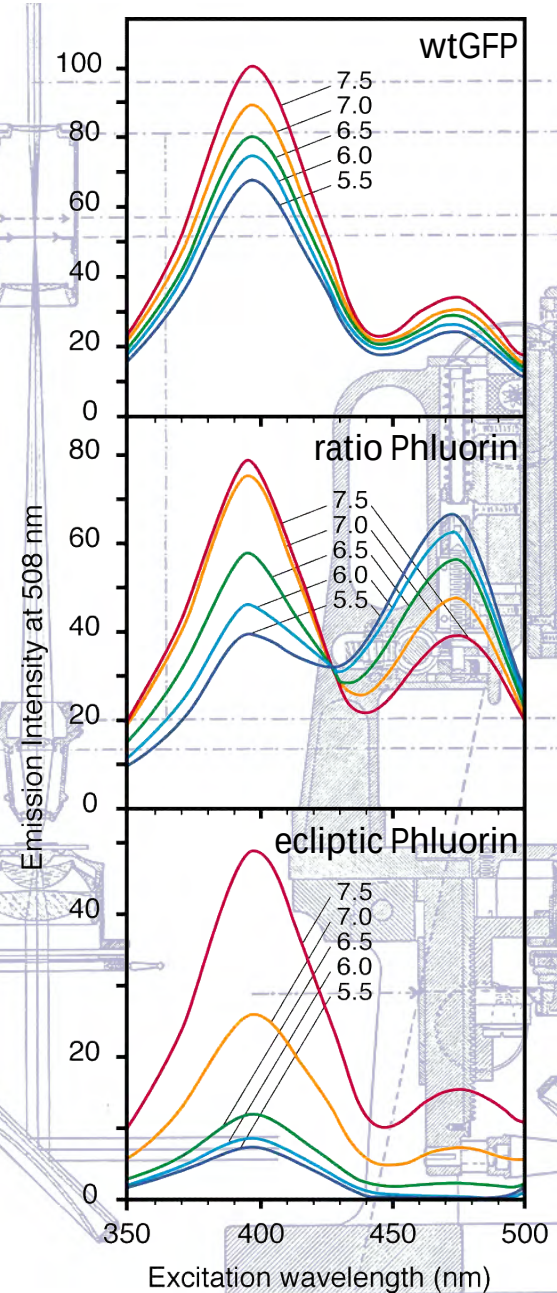


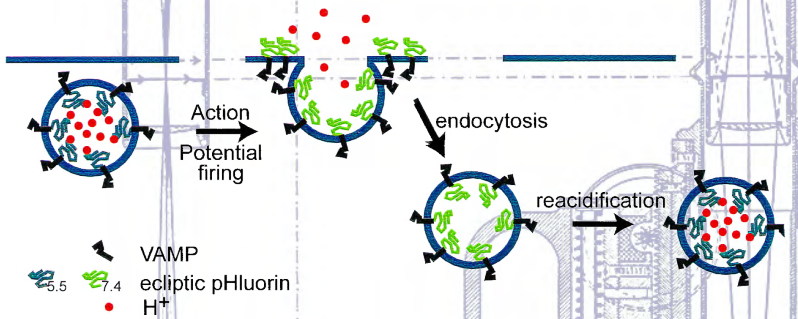


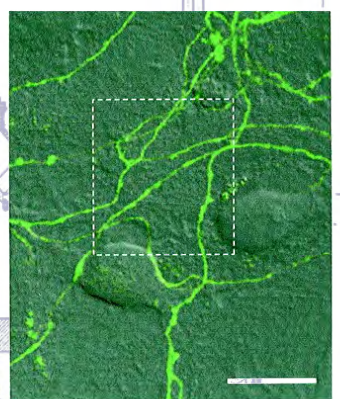
Ando (2004) Science 306: 1370-1373

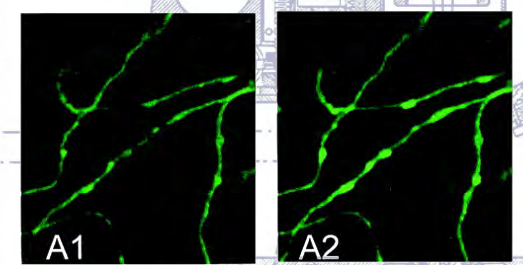

Sensing
and FRET

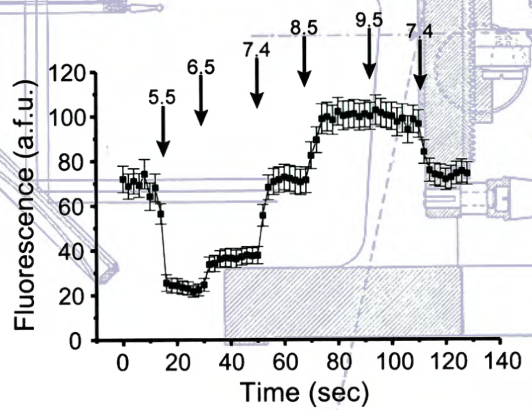
protonation sensing







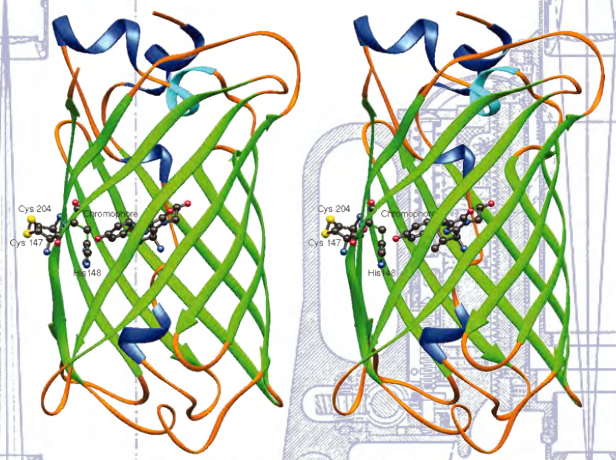


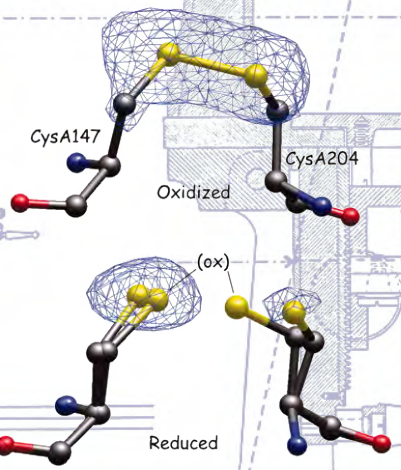


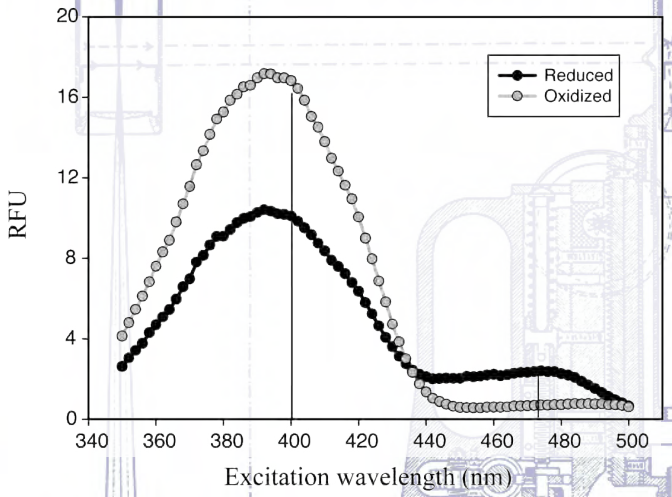
Miesenböck (1998) Nature 394:192-195

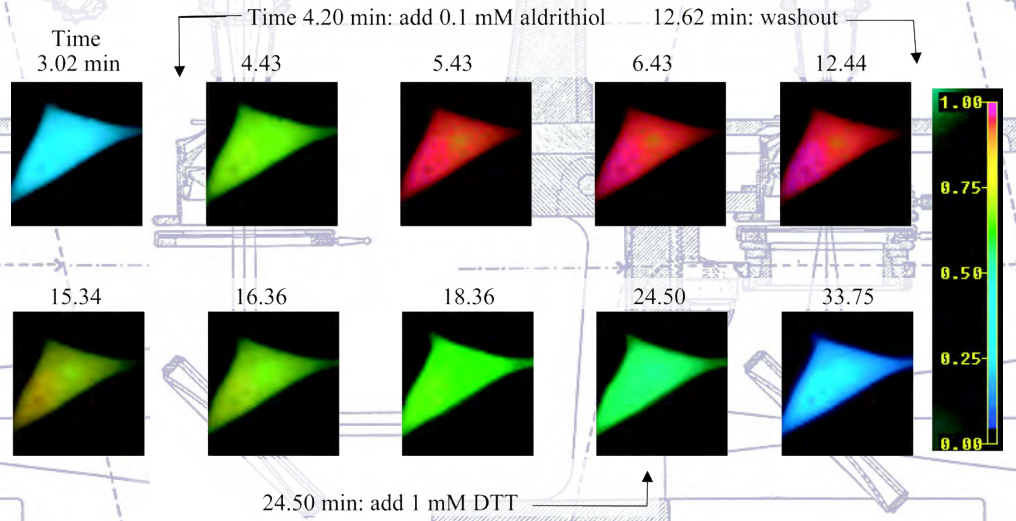
sensing redox potentials

barrel destabilization





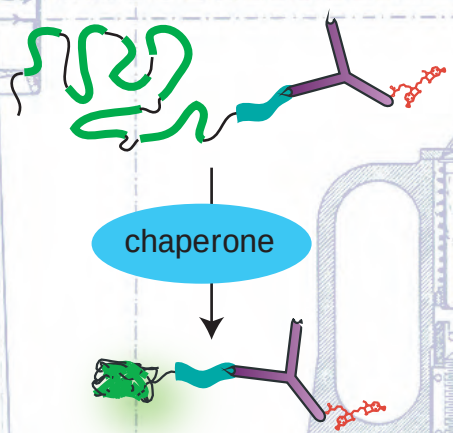


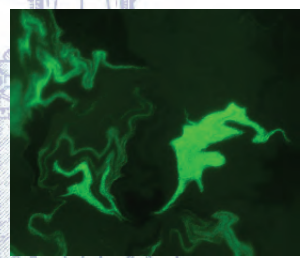


Hanson (2004) J. Biol. Chem. 279: 13044-13053

Dooley (2004) J. Biol. Chem. 279: 22284-22293

chaperone folding biosensor

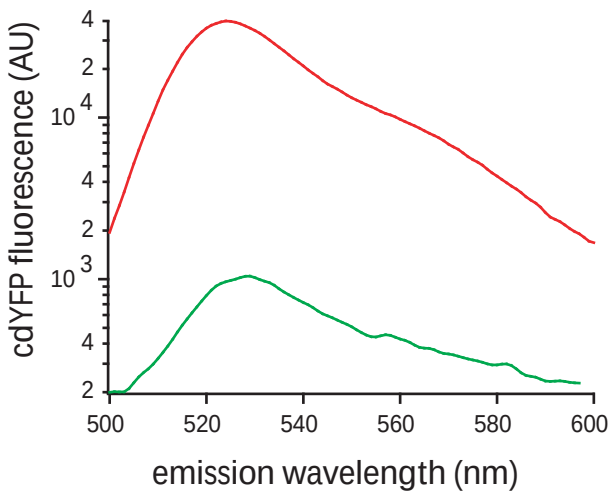


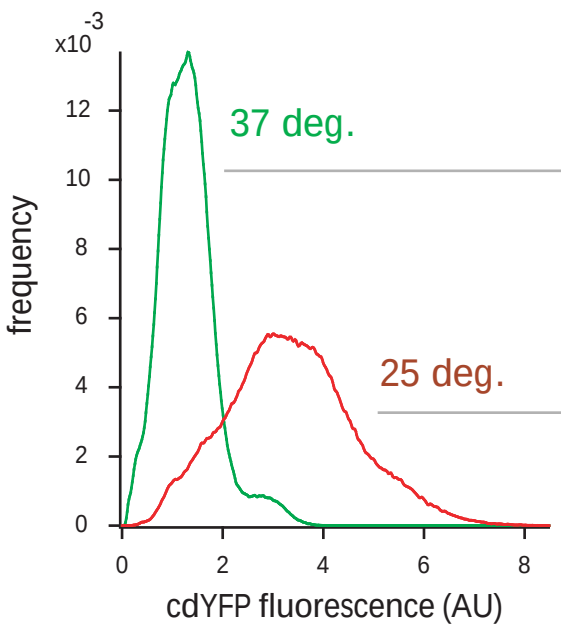


bacterial phenotype

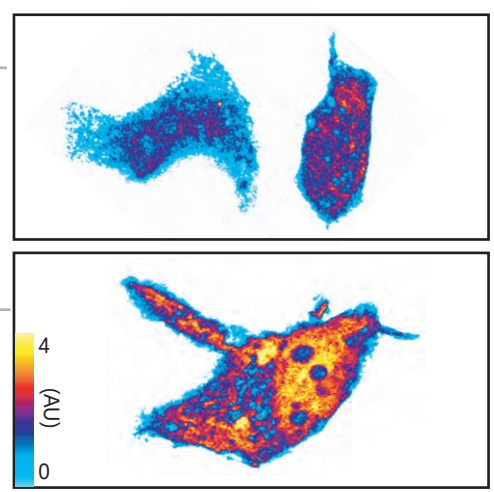
Liman (2005) Mol. Cell Biol. 25: 3715-3725

cold ethanol shock in bact. culture

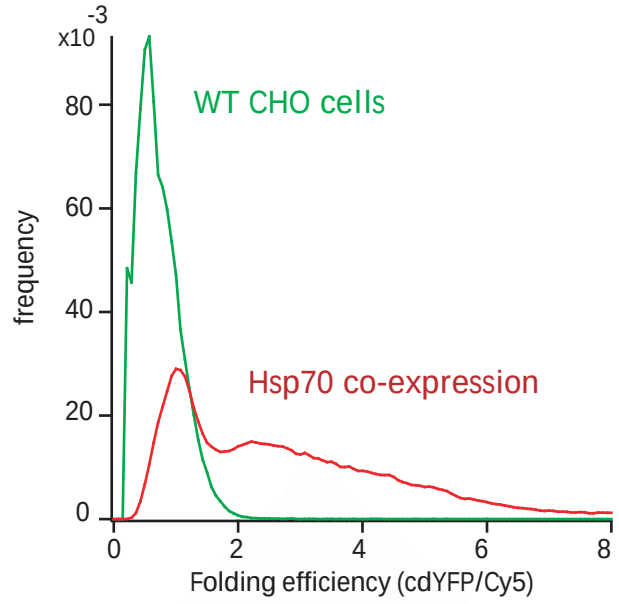


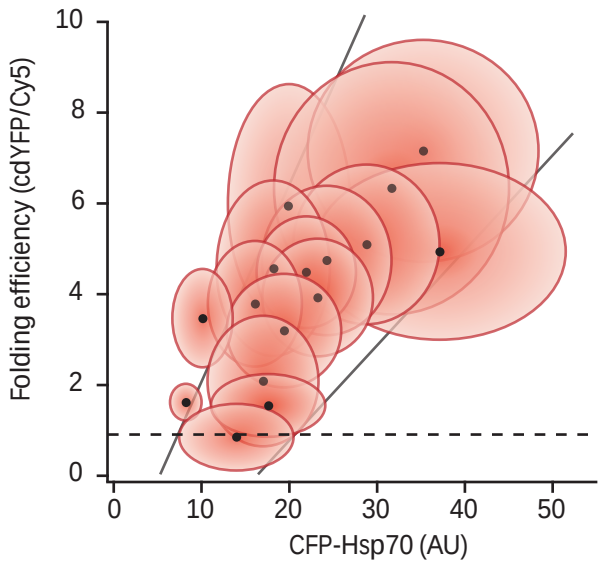


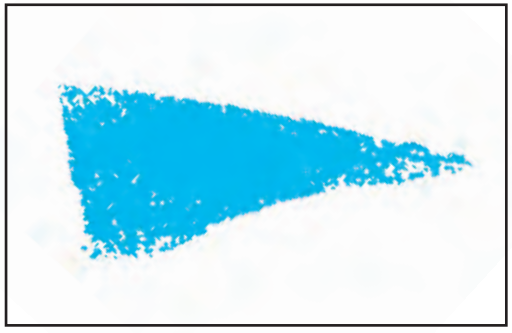
expression in Cos cells



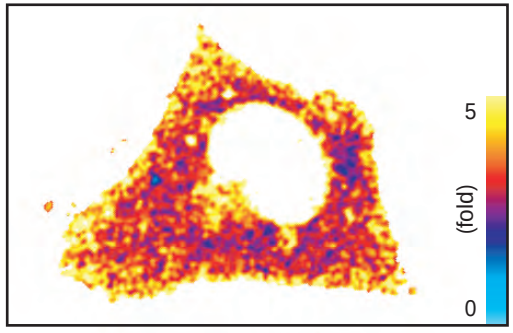
the folding biosensor is sensitive for Hsp70





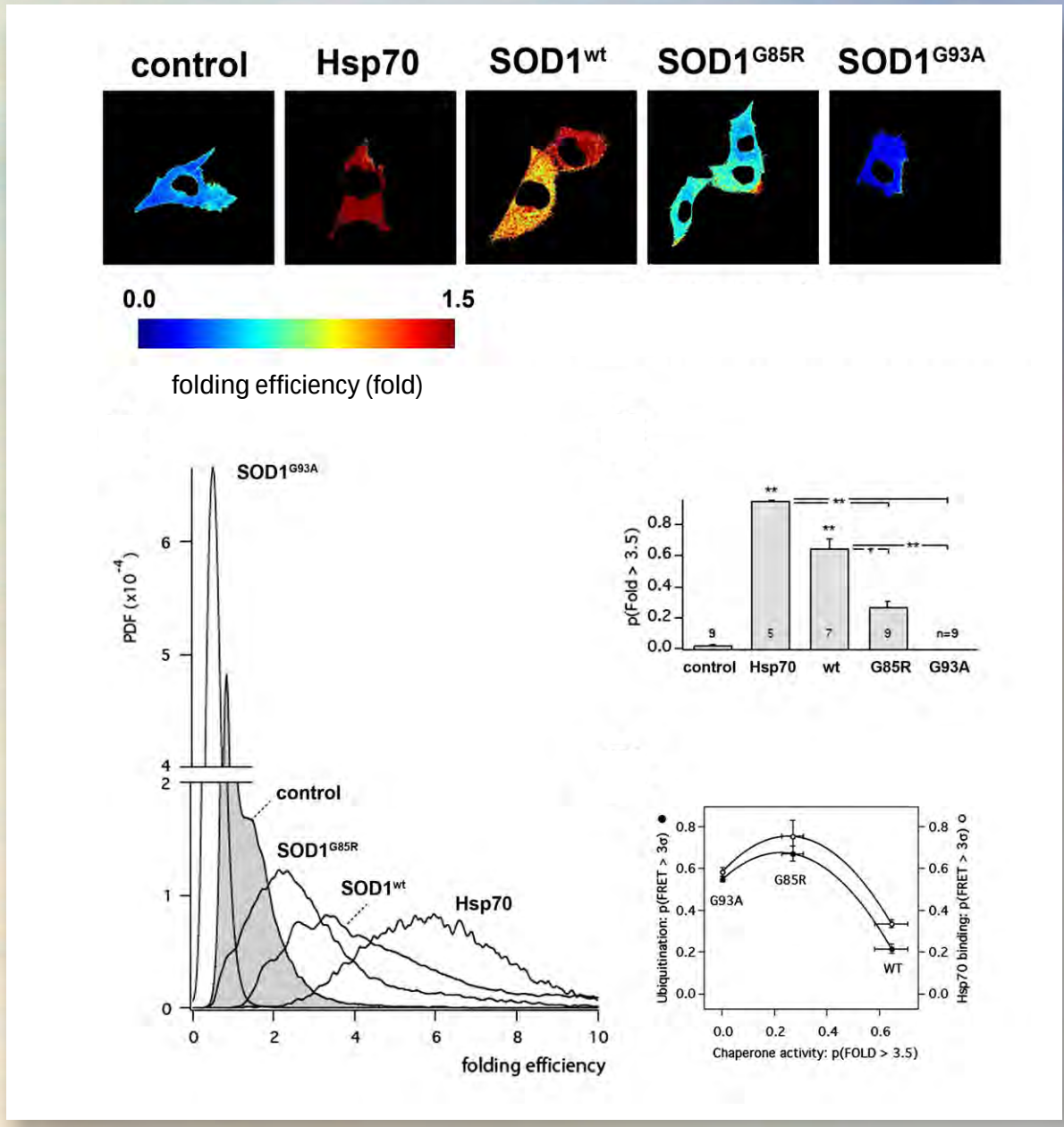


WT- CHO

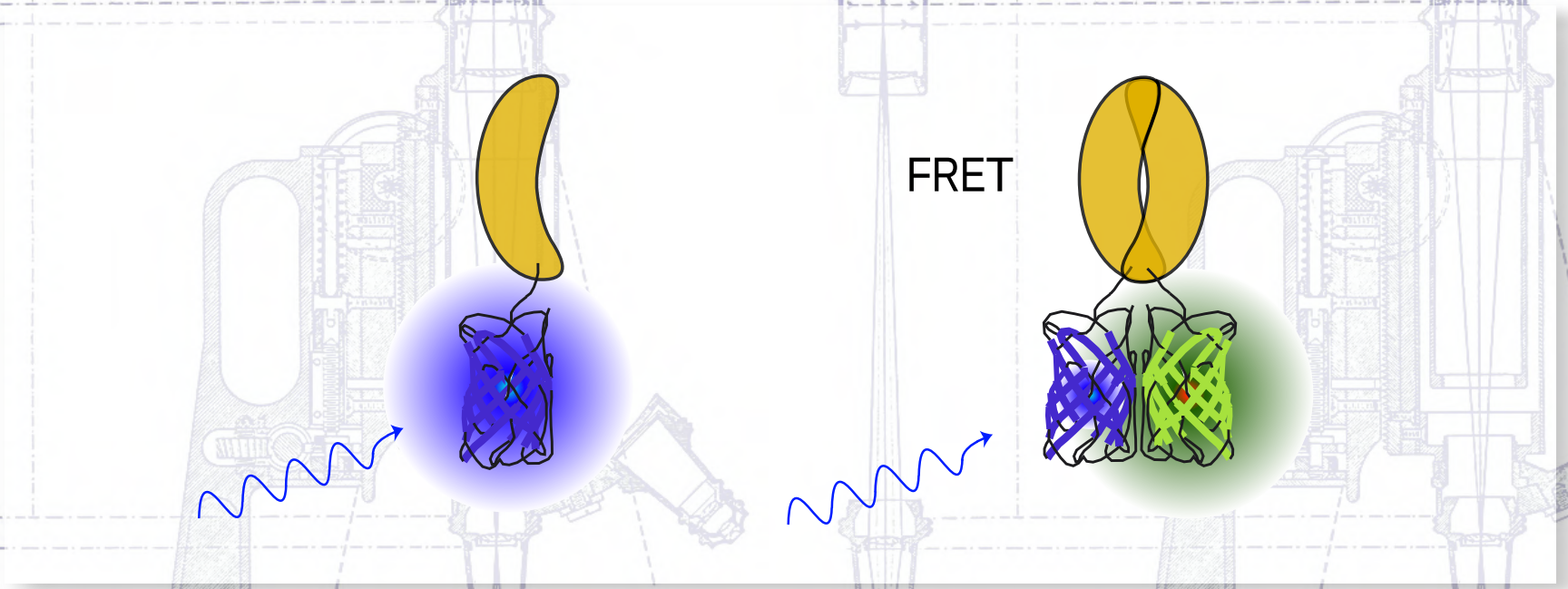


CHO
transient overexpression of
Hsp70 chaperone

Cellular folding capacity in cellular ALS models



Förster Resonance Energy Transfer (FRET)



Methods

ratio/ SE imaging
Acceptor PB

intensities

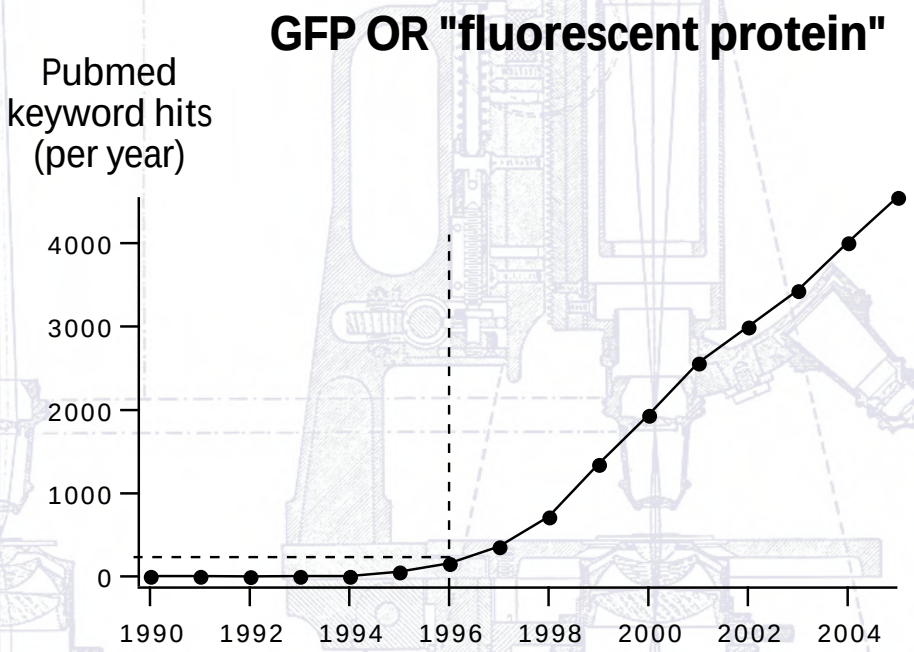
"quenched" donor emission

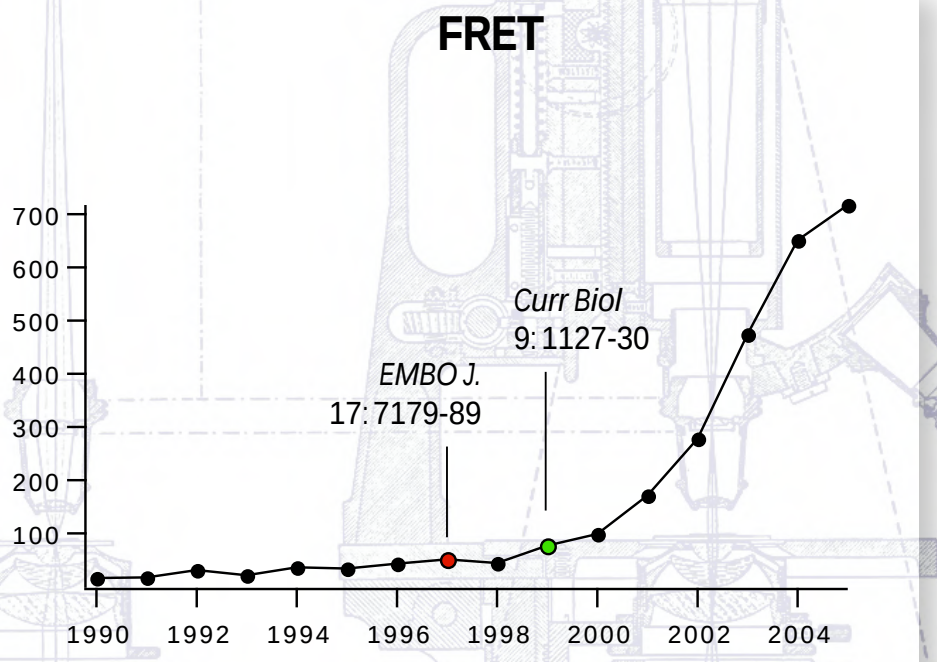
"sensitized" acceptor emission

FLIM

photons are emitted sooner

Molecular microscopy in modern biology



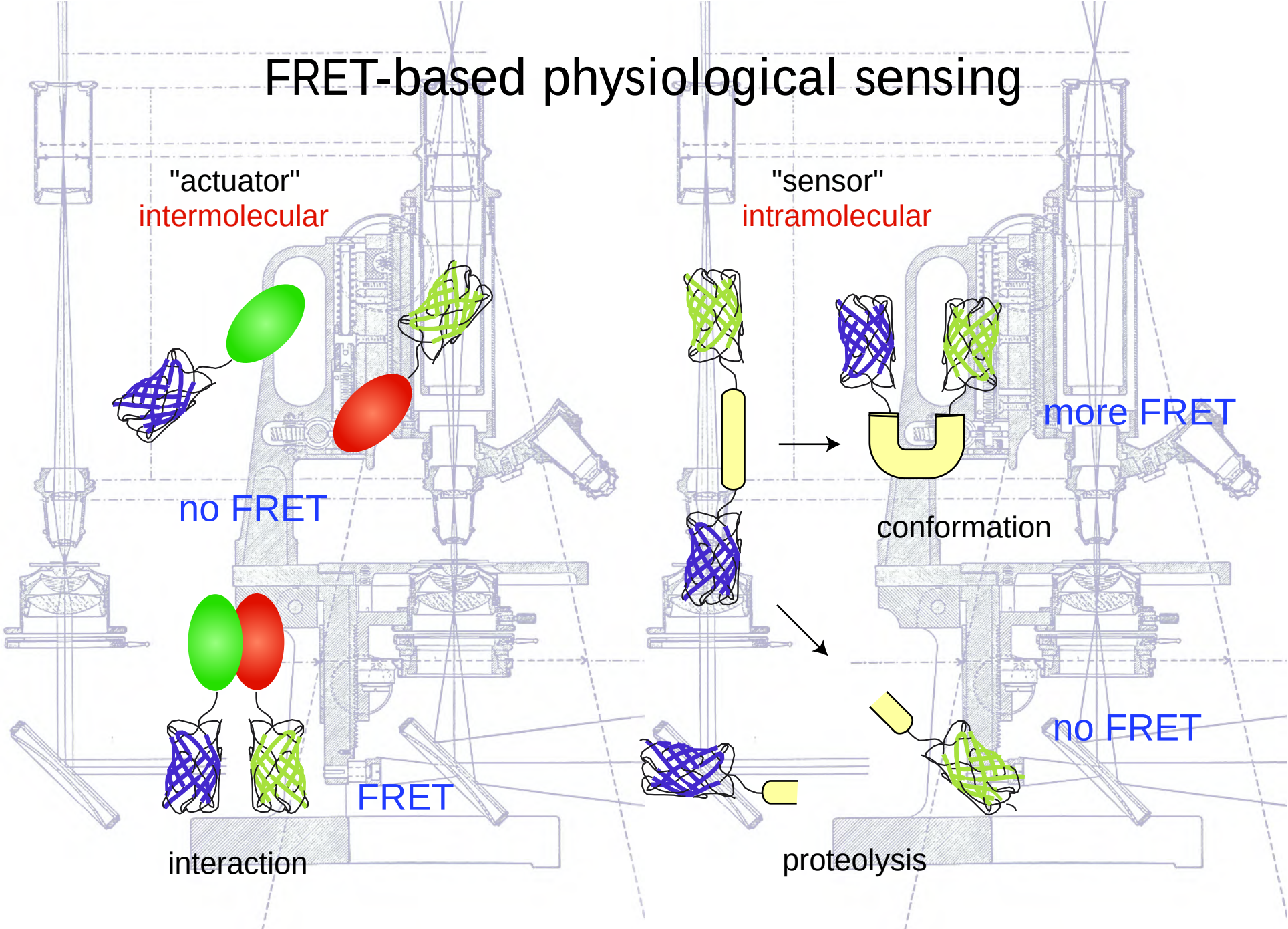


Localization
Dynamics

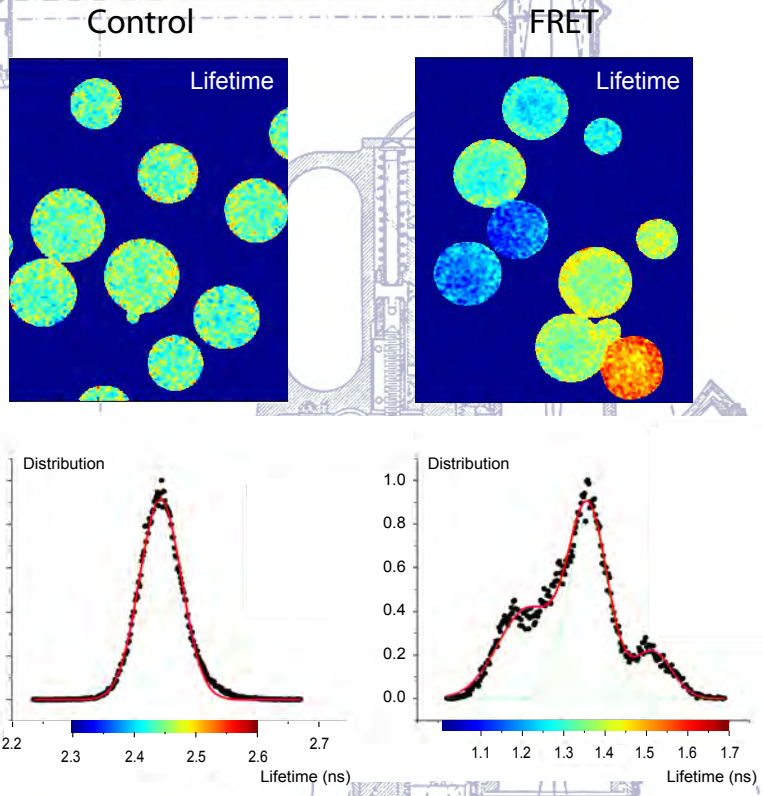
+ Biological function

= Cellular physiology

FRET-based physiological sensing



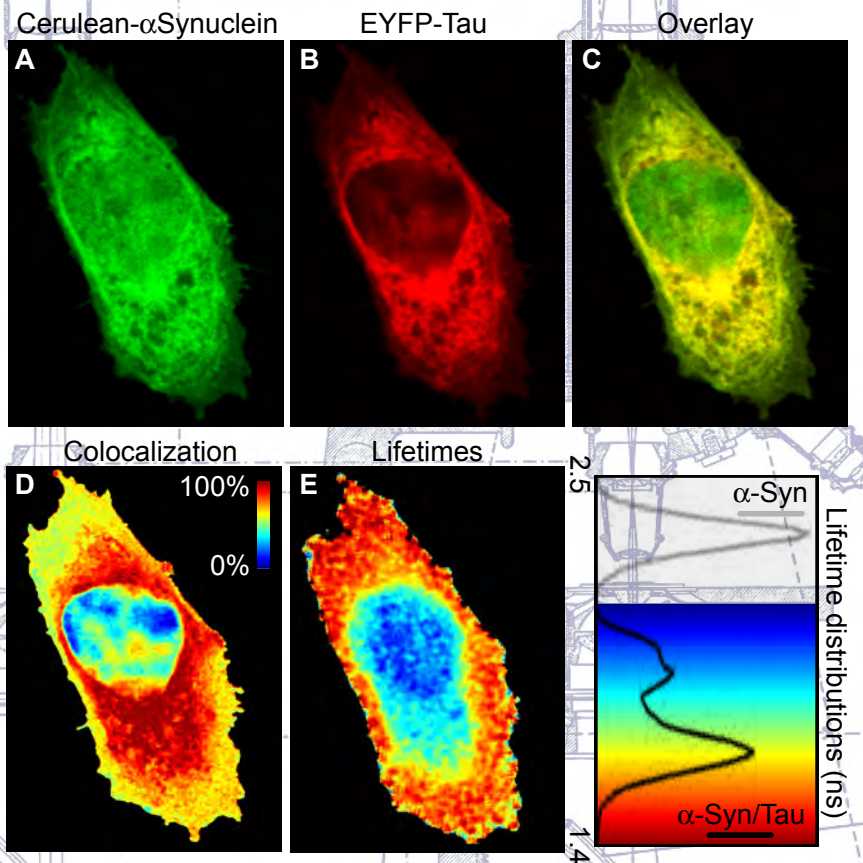
FRET examples



favorite FRET test subject: polystyrene beads, covalently with GFP protein, chemically labeled with Cy3

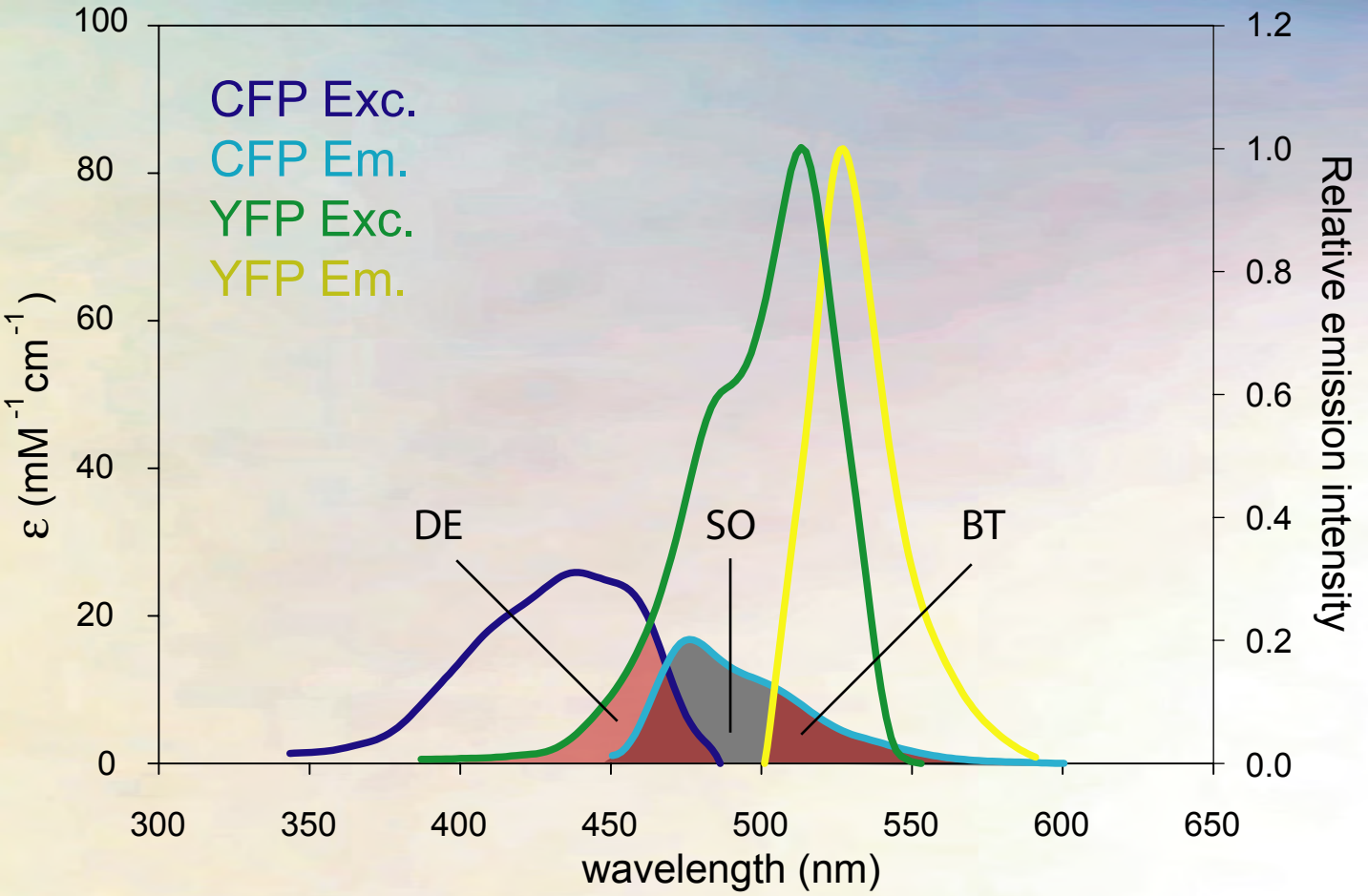
FRET resolution translates to sub-nanometre resolution!!

Wouters (2006) Contemporary Physics 47: 239-255
see also: Bunt (2004) Int. Rev. Cytol. 237: 205-277



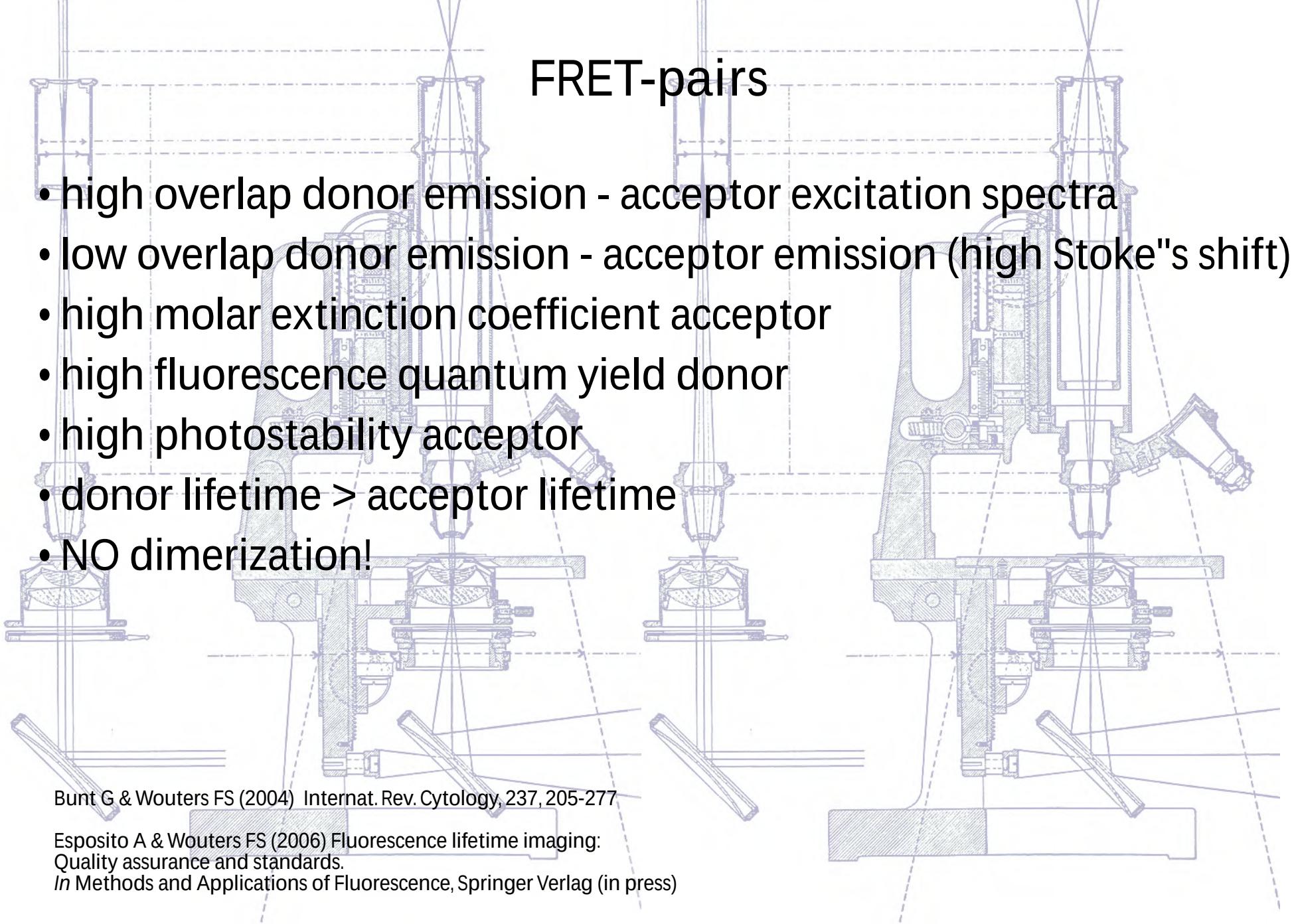
Esposito (2007) Neurobiol. Disease, *in press*

Spectral Issues with FRET



FRET-pairs

- high overlap donor emission - acceptor excitation spectra
- low overlap donor emission - acceptor emission (high Stoke"s shift)
- high molar extinction coefficient acceptor
- high fluorescence quantum yield donor
- high photostability acceptor
- donor lifetime > acceptor lifetime
- NO dimerization!



Bunt G & Wouters FS (2004) Internat. Rev. Cytology, 237, 205-277

Esposito A & Wouters FS (2006) Fluorescence lifetime imaging:
Quality assurance and standards.
In Methods and Applications of Fluorescence, Springer Verlag (in press)

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die Eierlegende Wollmilchsau!
(the egg-laying woolly milk-pig)

Bunt G & Wouters FS (2004) Internat. Rev. Cytology, 237, 205-277

Esposito A & Wouters FS (2006) Fluorescence lifetime imaging:
Quality assurance and standards.
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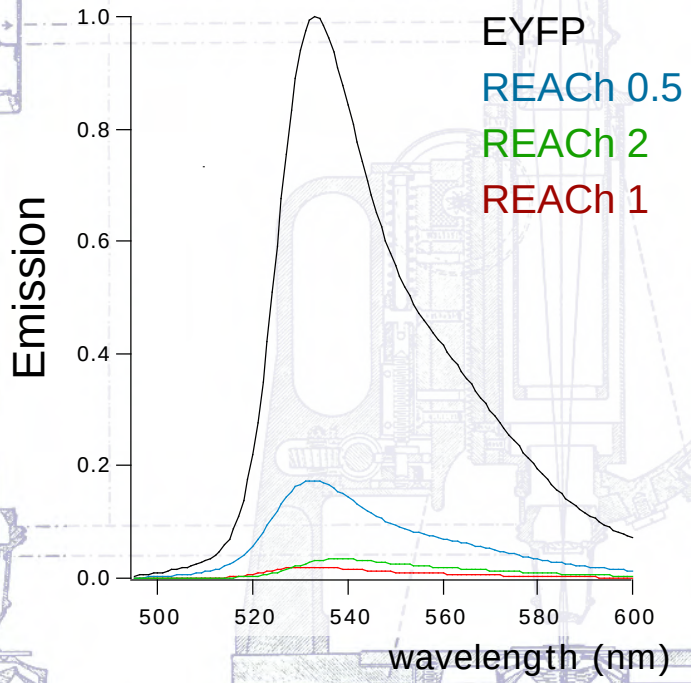
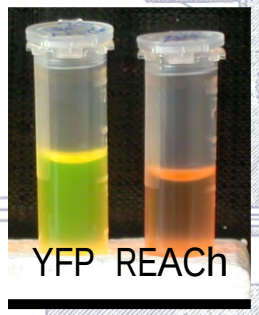
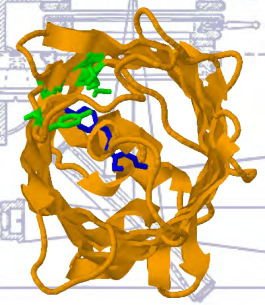
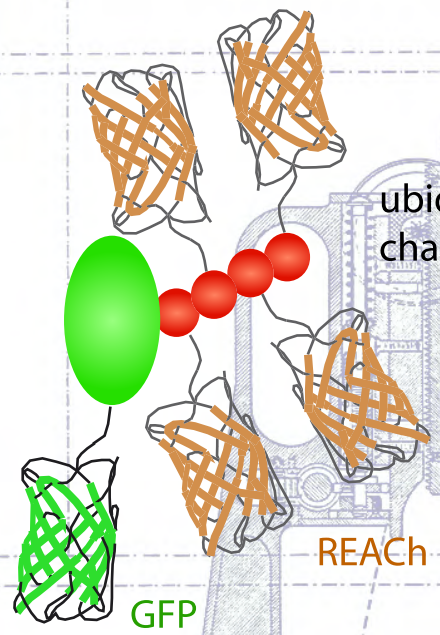
chromoprotein FRET acceptor

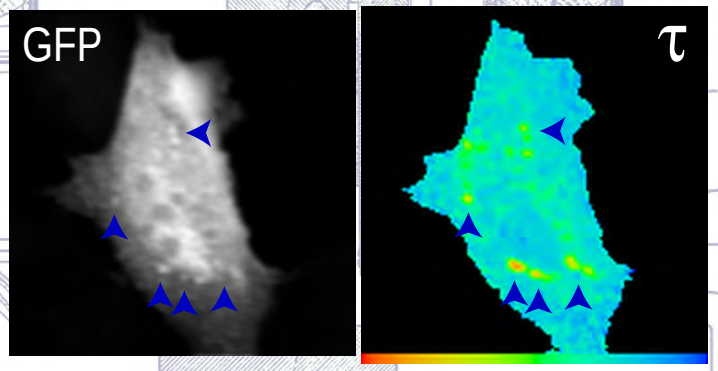




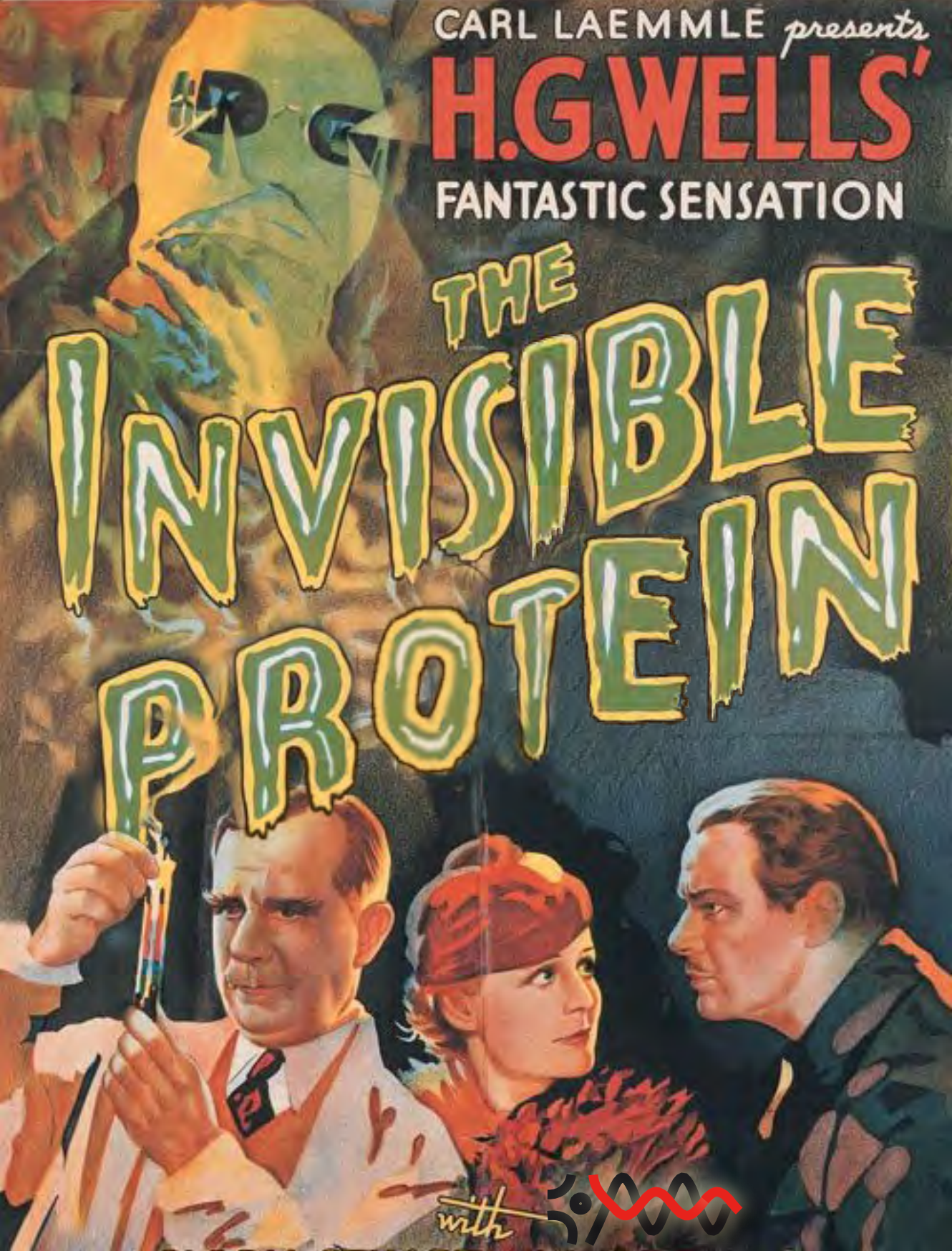




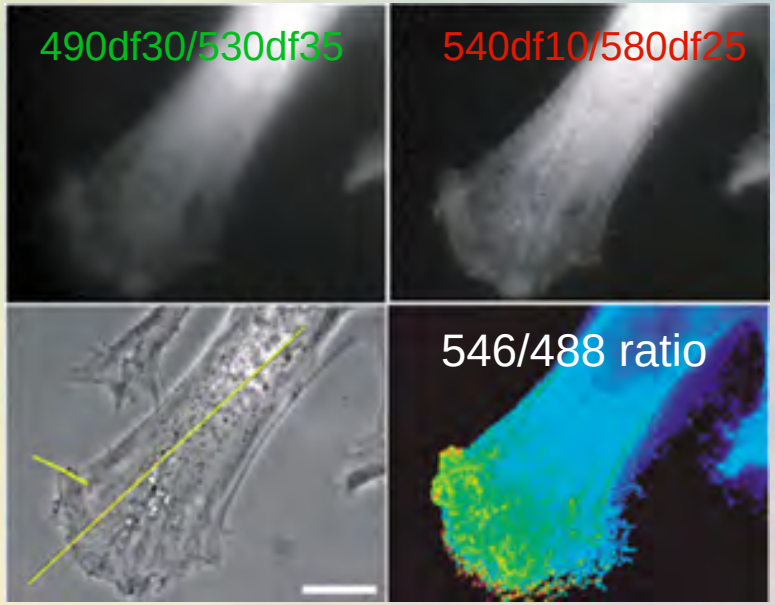
GFP-PEST + Ubq-REACh1



Ganesan (2006) PNAS 103: 4089-4094


CARL LAEMMLE presents
H.G.WELLS'
FANTASTIC SENSATION
THE
INVISIBLE
PROTEIN
with

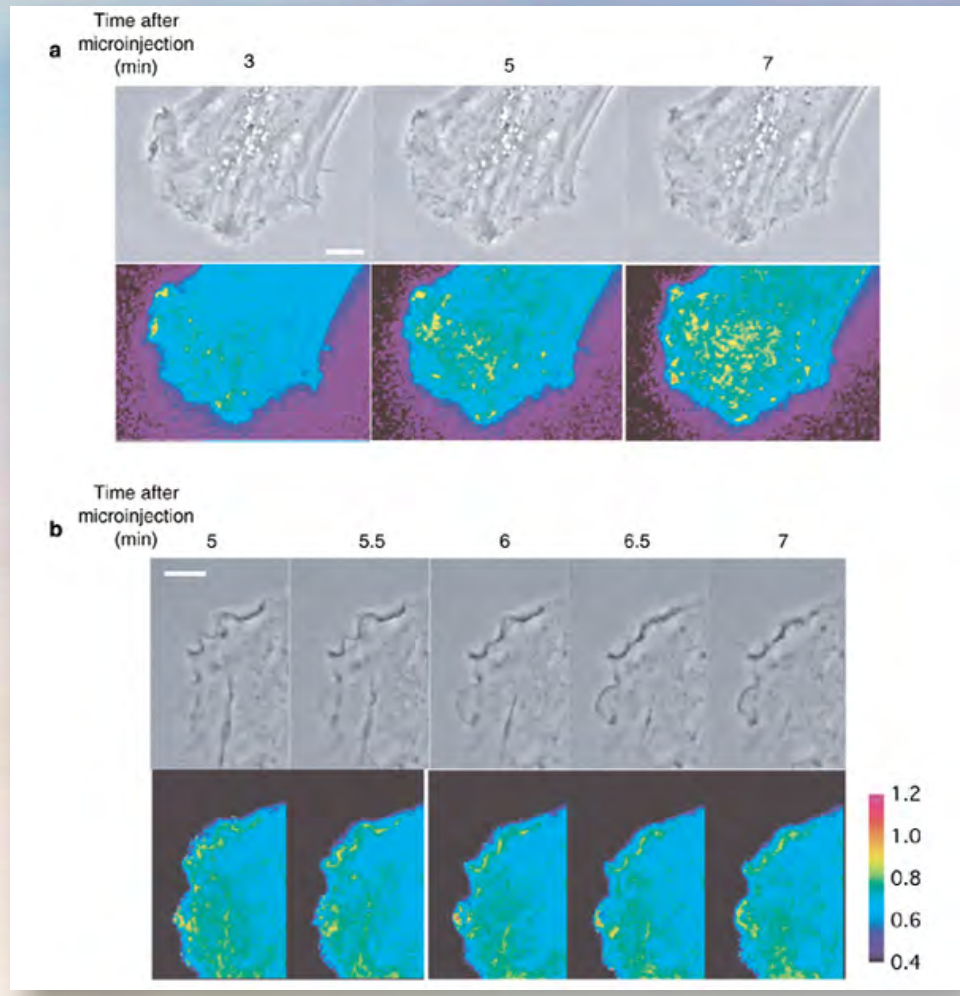
FqRET: donor quenching judged by an optically inert concentration reference



Alexa-488 Actin Rhodamine-Actin

NO FRET
reference

FRET
emission quenched

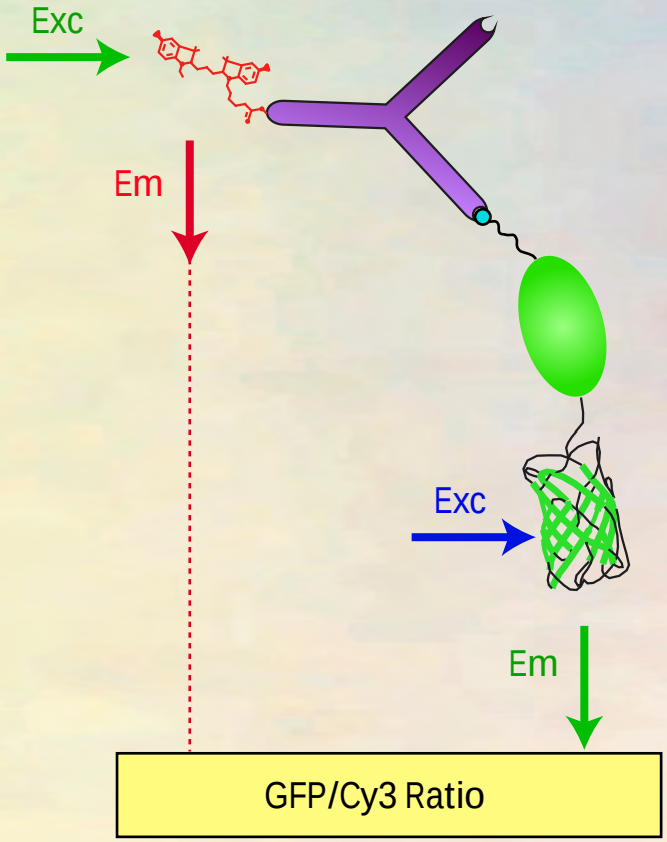


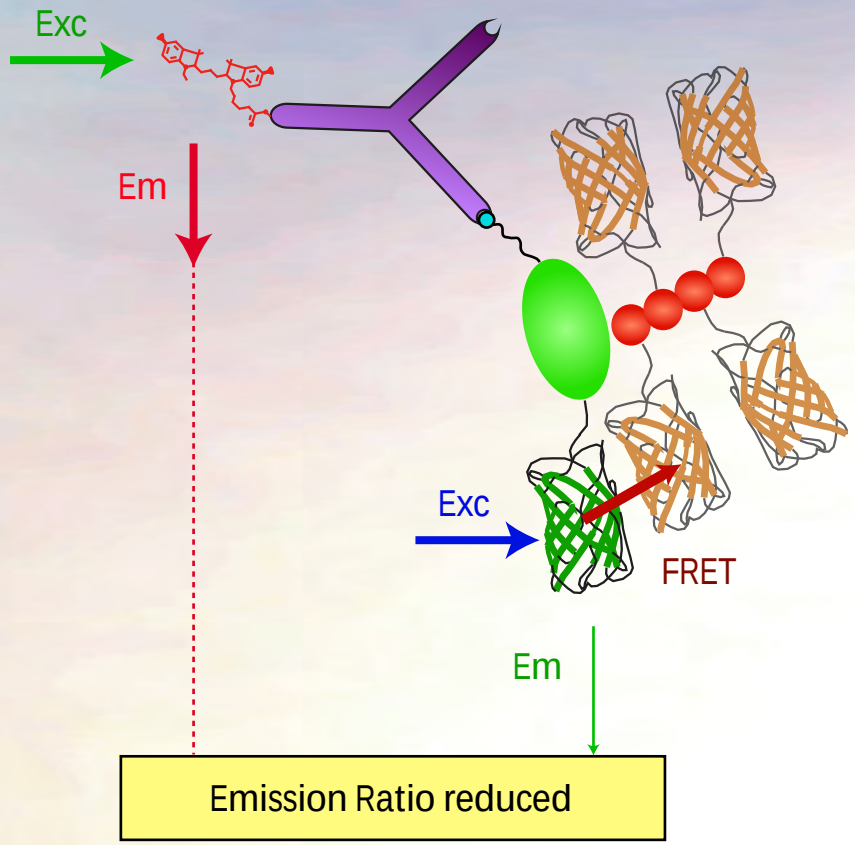
Gelsolin actin uncapping at ruffles

Allen P.G. *Nat. Cell Biol*. **5**, 972-979 (2003)

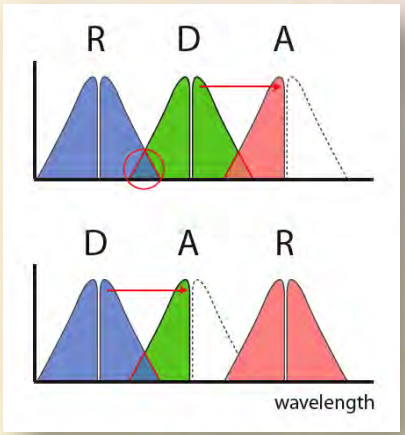
Intensity-based FRET using a dark acceptor

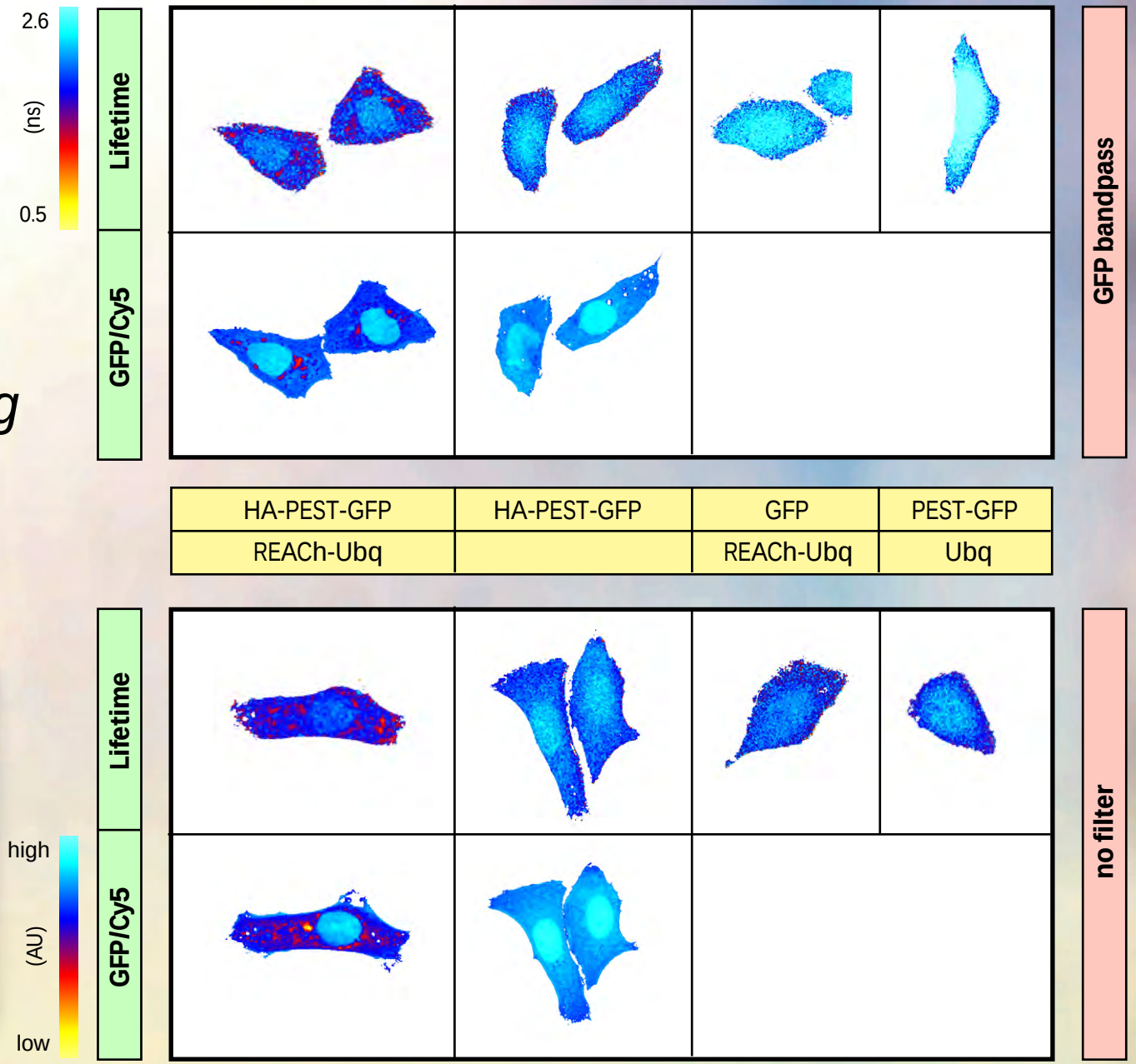
by application of a concentration reference dye





Protein ubiquitination by lifetime (FLIM) and normalised donor quenching (FqRET)





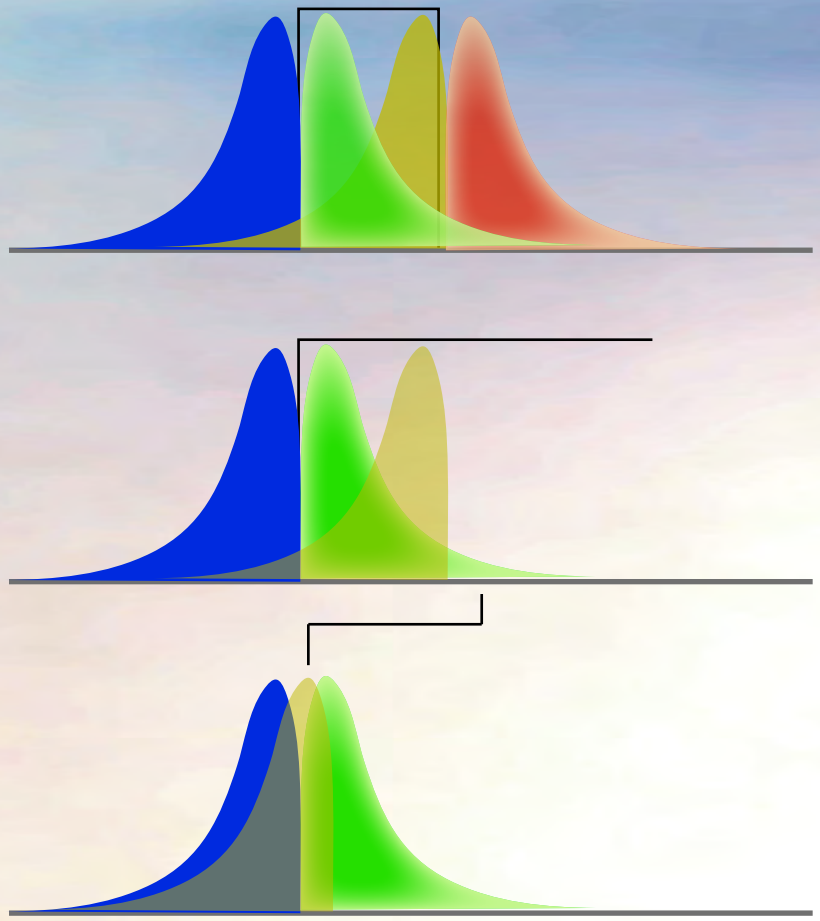
Why have a dark acceptor?

1: spectral advantages

conventional

collect all fluorescence photons

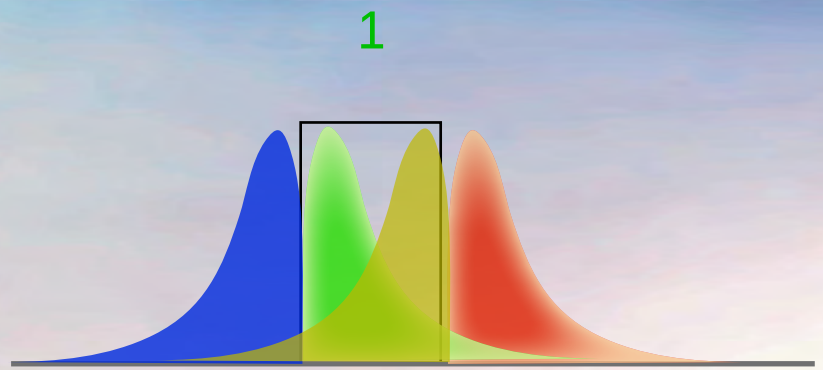
increase spectral overlap: bigger R_0



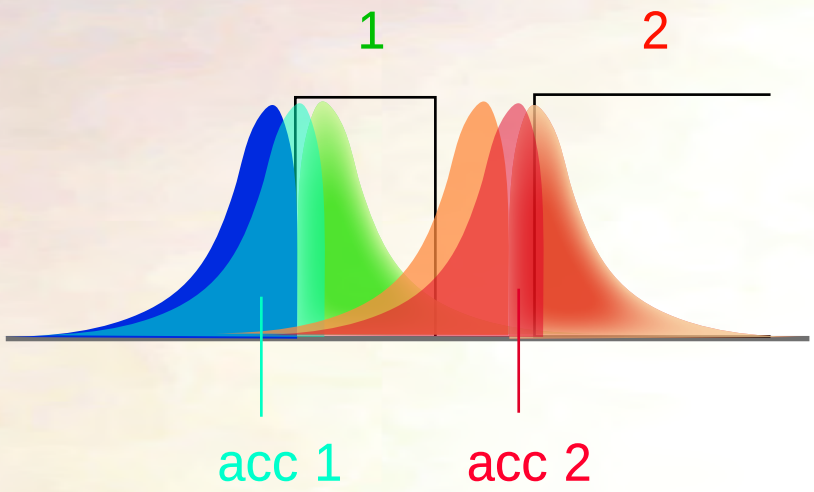
Why have a dark acceptor?

2: multiplexing FRET pairs

conventional: 1 FRET pair
CFP-YFP

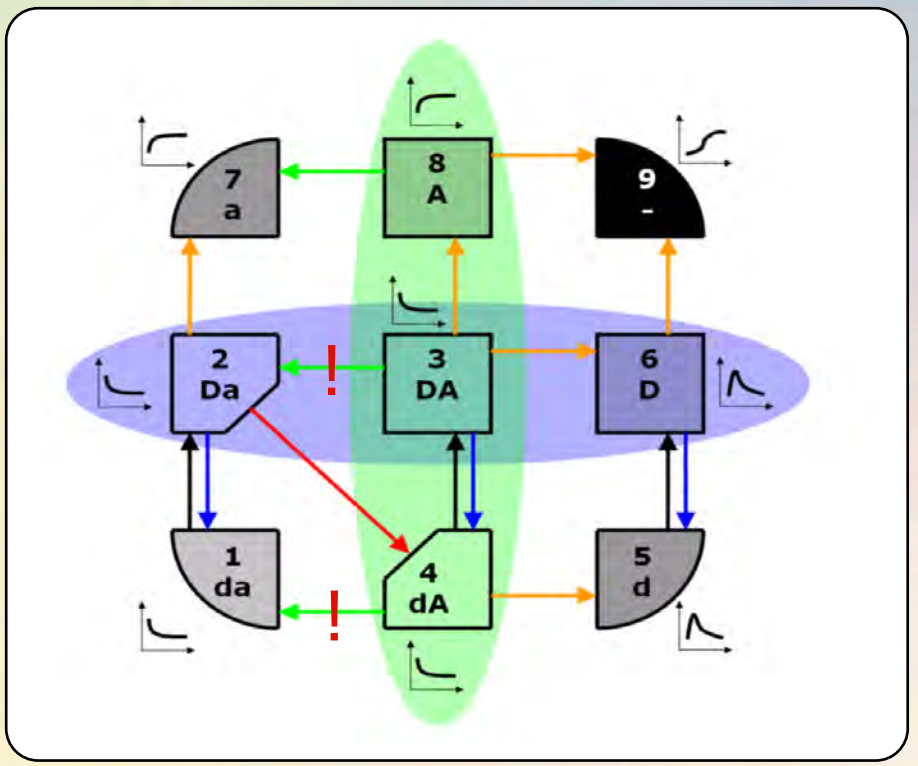


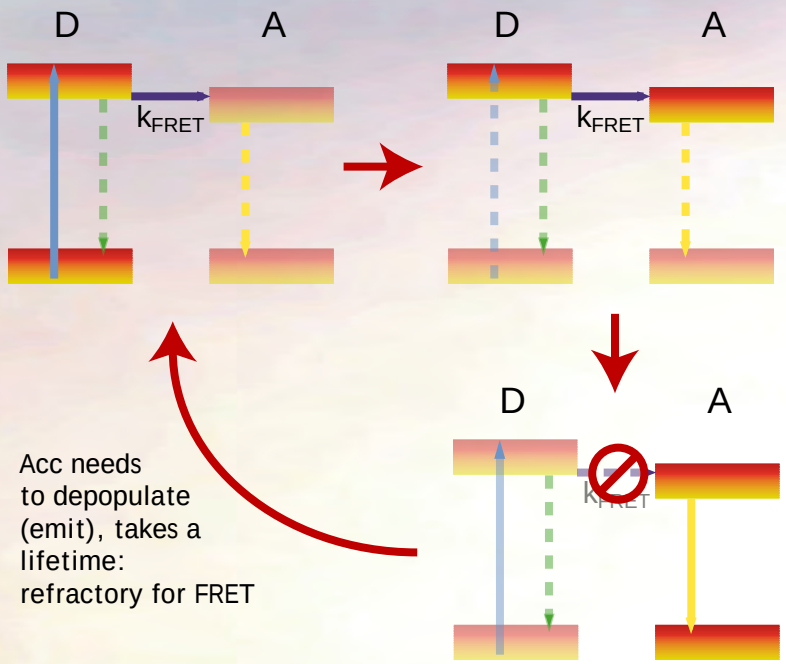
2 FRET pair with
dark acceptors



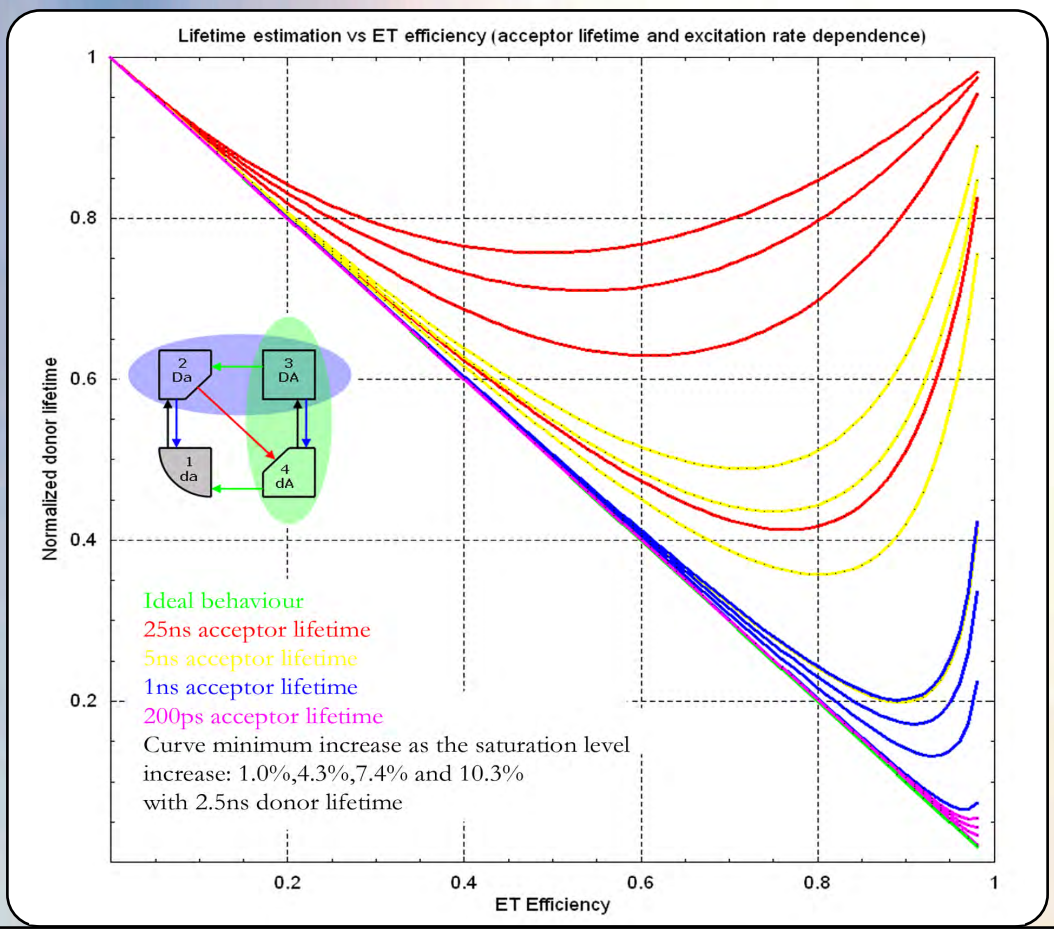
Why have a dark acceptor?

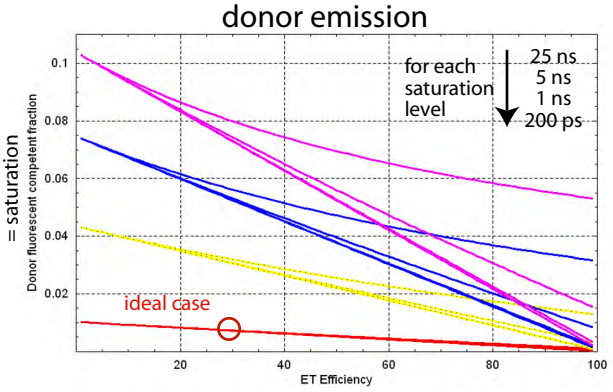
3: no frustration of FRET (no build-up of FRET-incompetent DA species, AND no contamination of FRET donor lifetime with non-FRET donor decay lifetime)

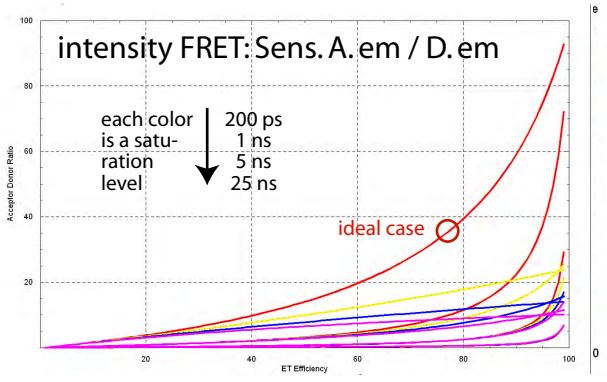




FRET incompetent fraction effects







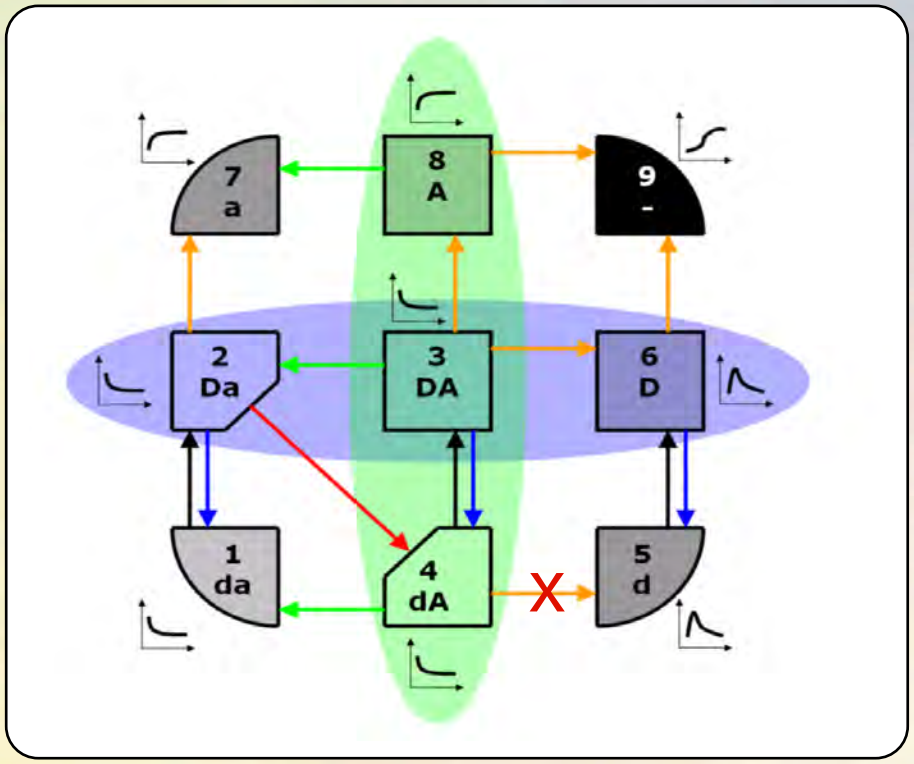
Build-up of the FRET-incompetent DA state causes :

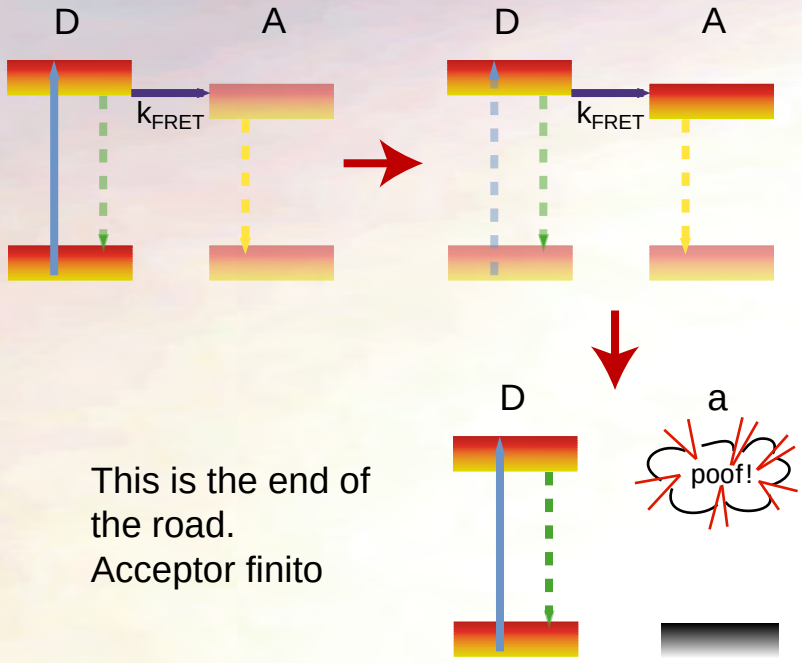
- the emission of photons with normal, unreduced, lifetime
  this has consequence for FLIM
- the emission of photons that should have been quenched by FRET
  this has consequence for FLIM, but more so for intensity-based ratio methods
  because the ratio is highly unlinear and the error thus "explodes"

this effect increases with acceptor lifetime and saturation-level of the donor!

Why have a dark acceptor?

4: no sensitized acceptor photobleaching therefore no loss of FRET and persistent protection of donor for photobleaching





This is the end of the road.
Acceptor finito

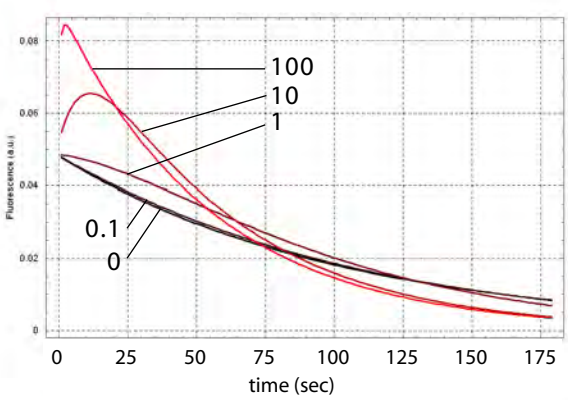
The problem of sensitized acceptor photobleaching in FRET

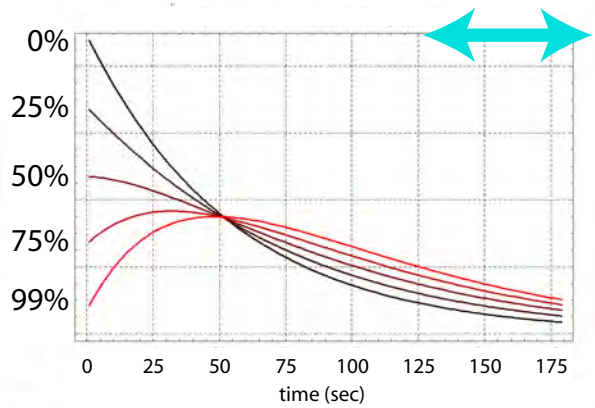
photostability ratio donor/acceptor

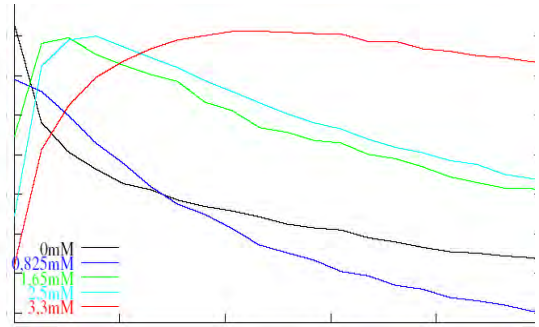
FRET efficiency

GFP-labeled beads incubated with increasing concentrations of Rhodamin-6G leads to concentration-dependent FRET as the average distance between molecules decreases

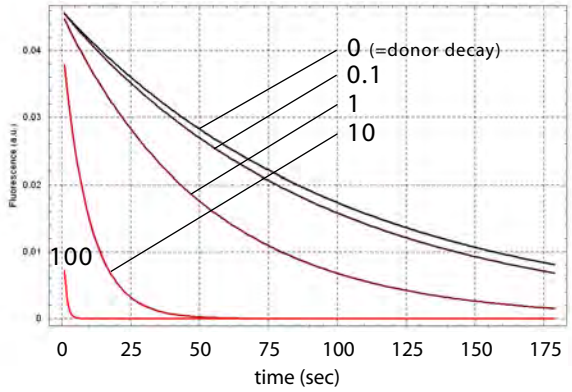
Donor emission

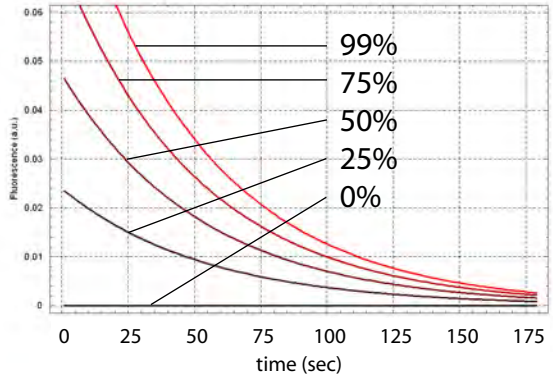


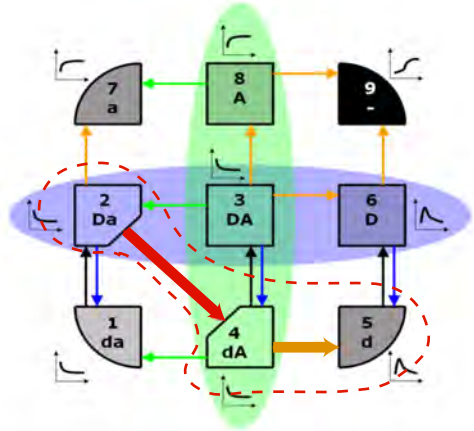




sensitized Acceptor emission

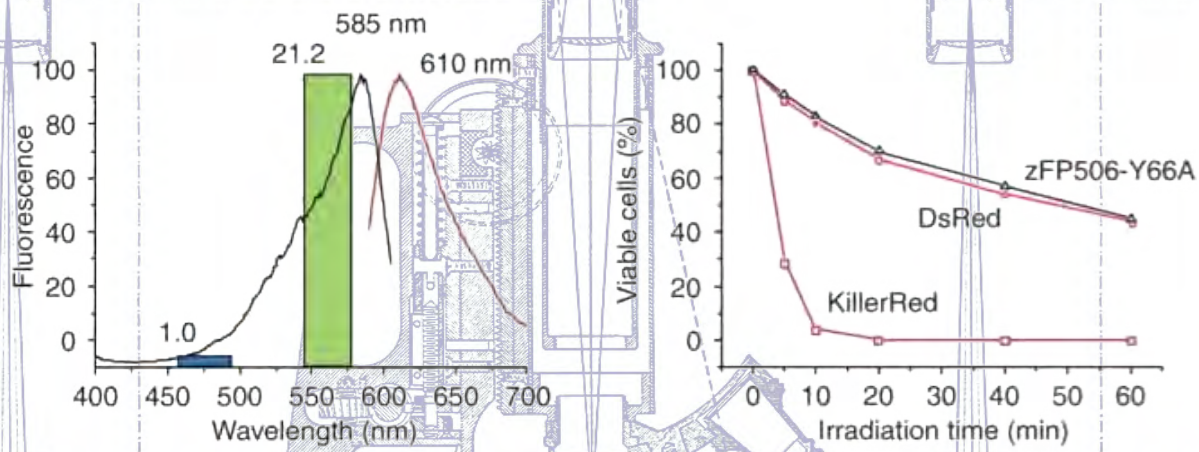


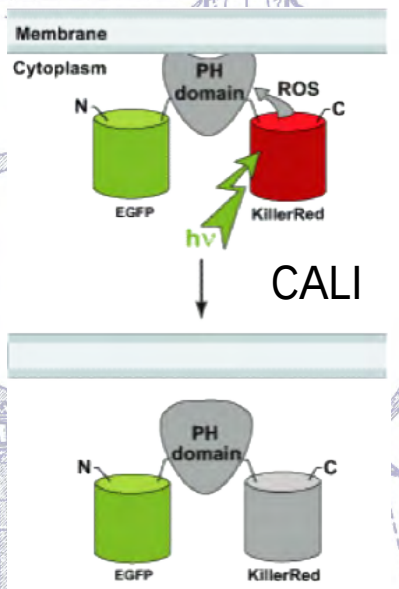





Manipulating
and Other

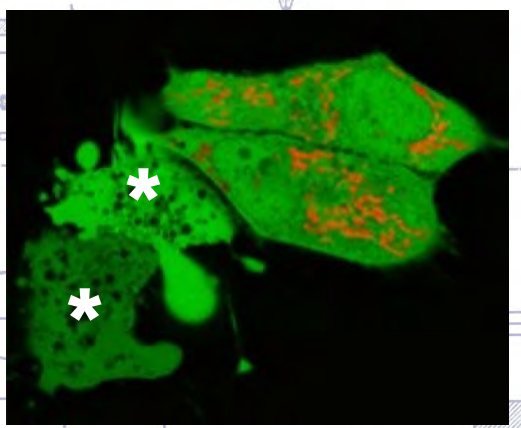
even more scary: Killer Red FP



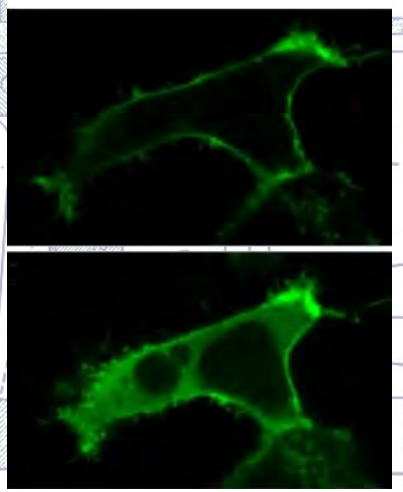


- genetically-encoded photosensitizer
- generates singlet oxygen ROS mechanism ?

selective killing

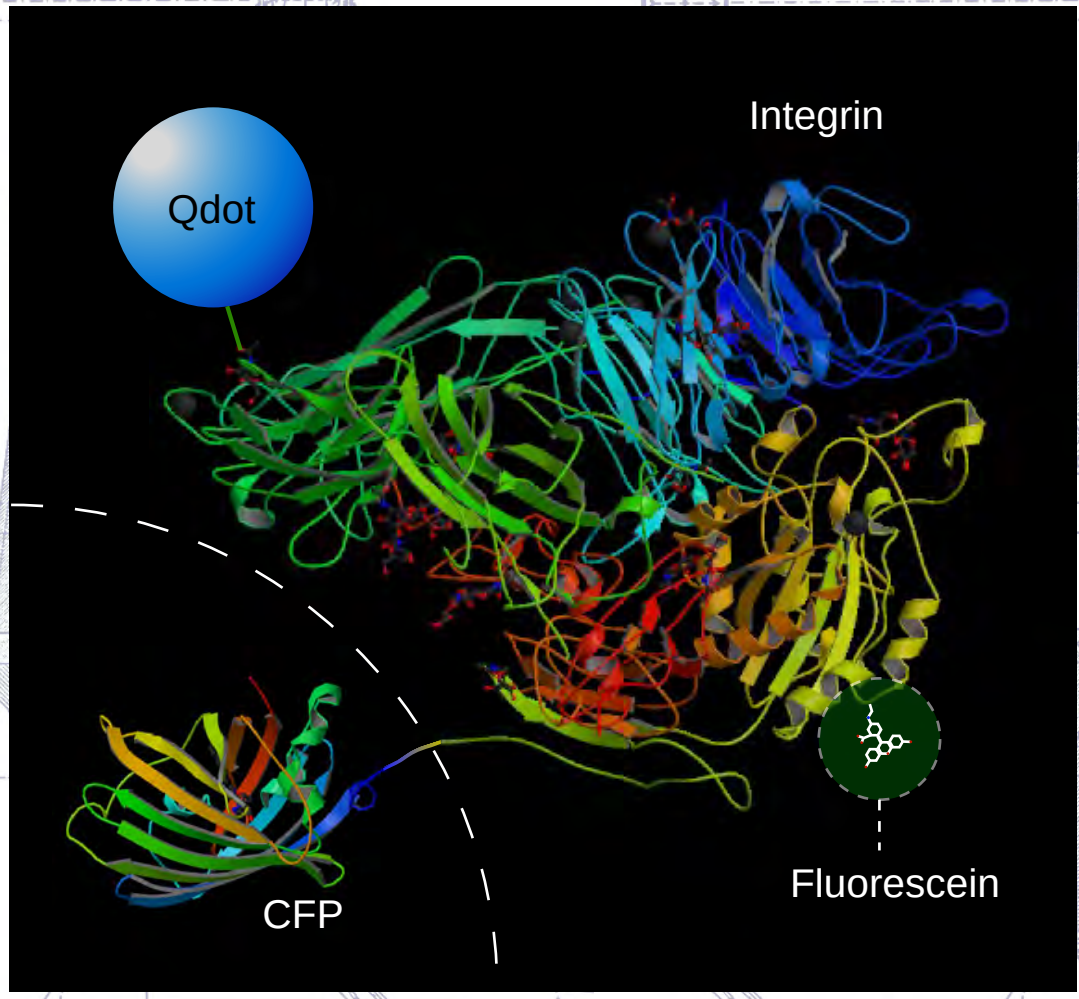


killer-red Mito ±1 h.
Turbo-GFP cytoplasma



Bulina (2005) Nat. Biotechnol. 24: 95-99

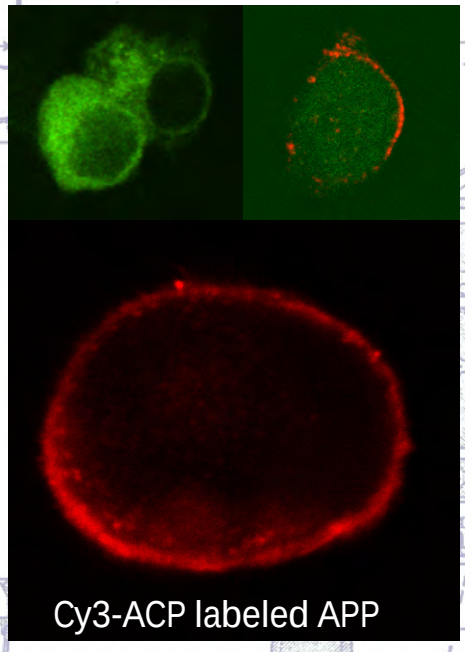
GFP and synthetic fluorophores

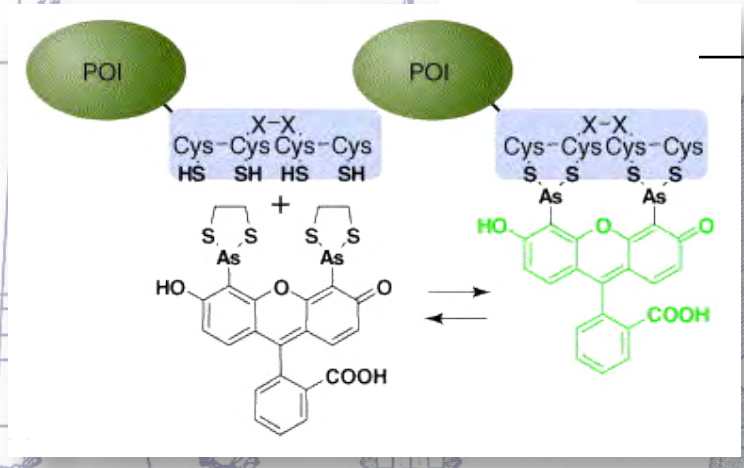


Wouters (2006) Contemporary Physics 47: 239-255

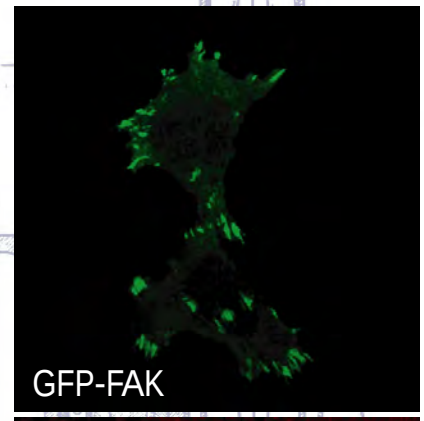
bioorthogonal chemical labeling

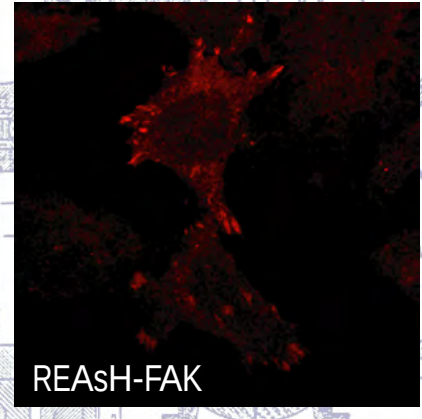
GFP-APP, red Qdot-labeled sAPP

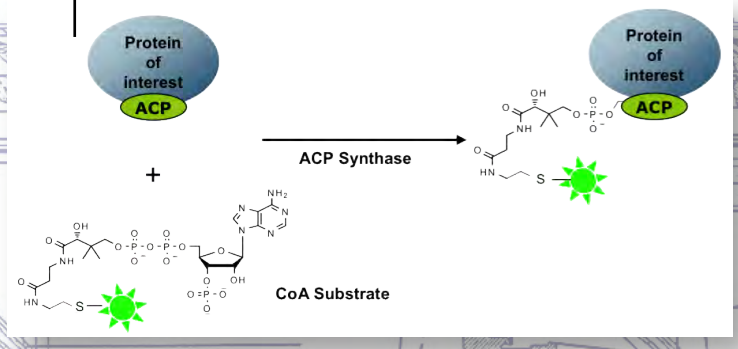


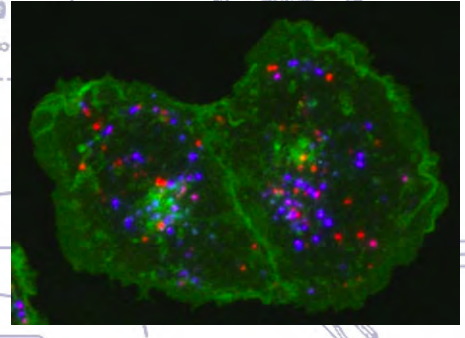


(G. Bunt, Stuttgart University)

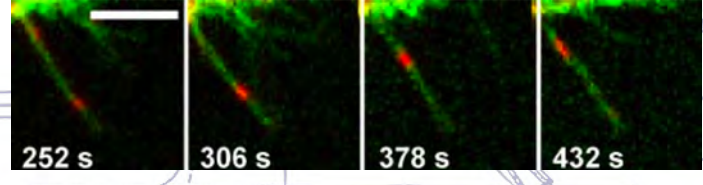








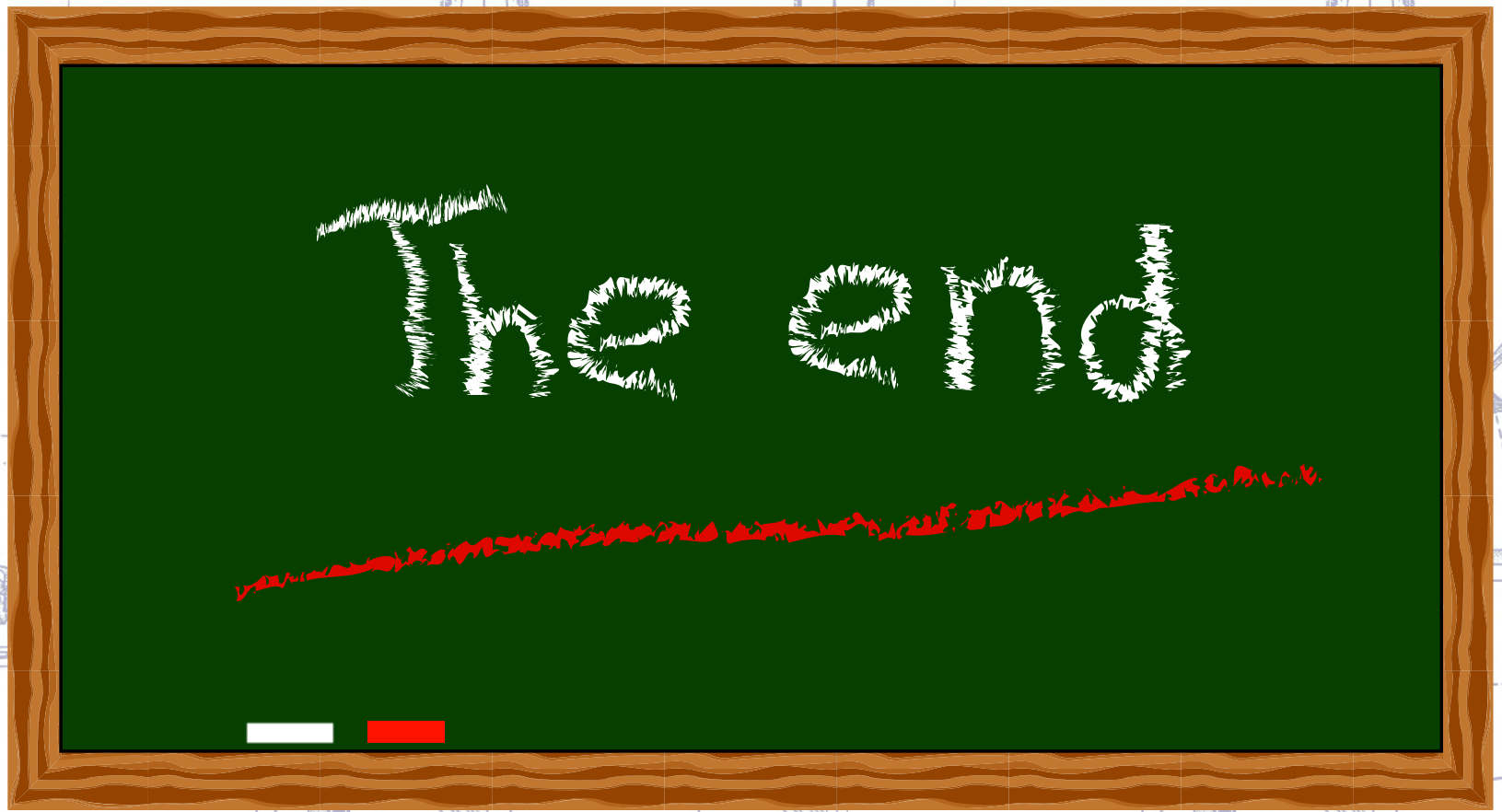
GFP-EGFR, Qdot-EGF orange and blue Qdots followed each other by 20 min.



Qdot-EGF during actin-driven retrograde filopodial transport

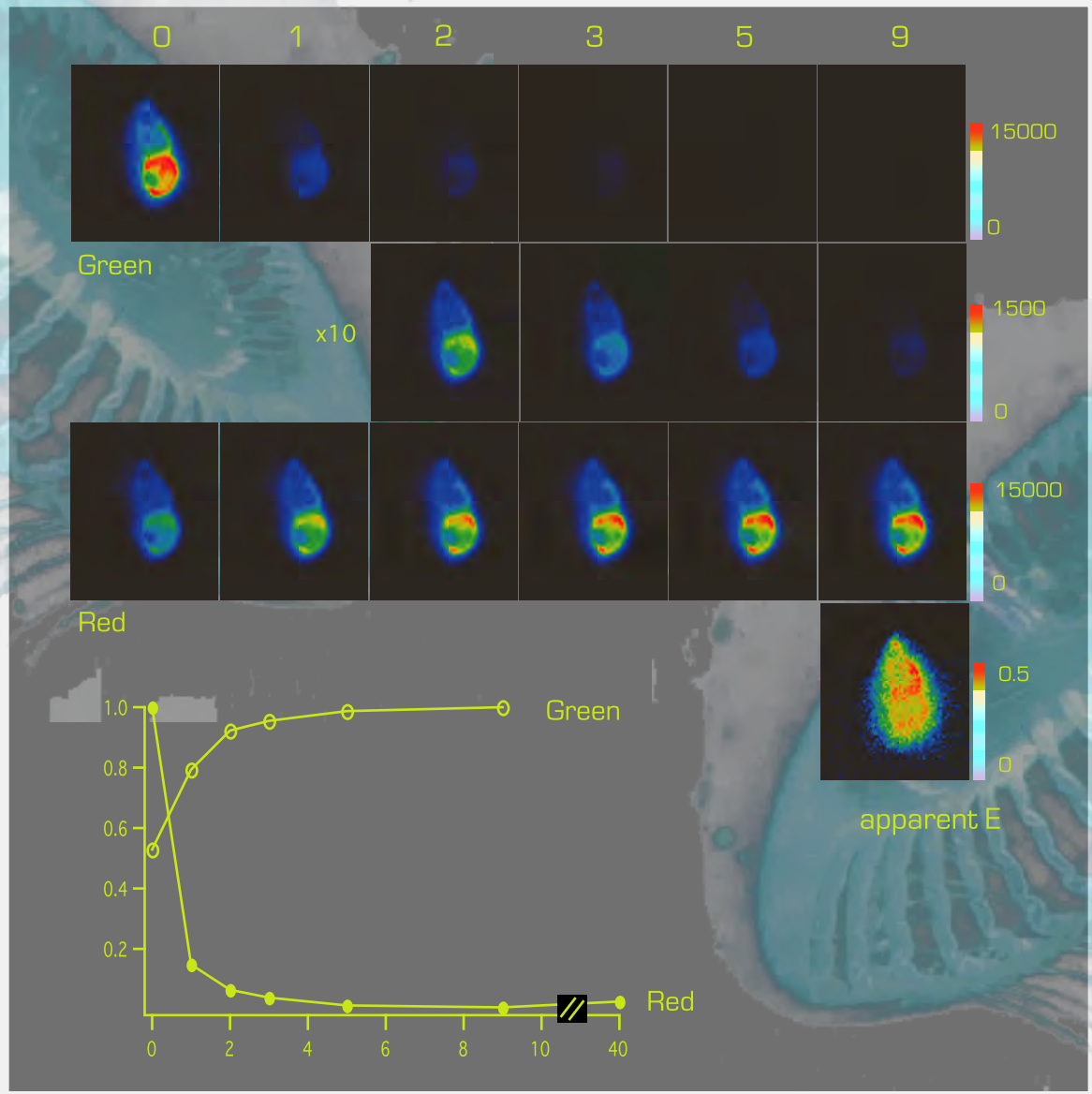
Arndt-Jovin (2003) Proc. SPIE 6096: 60960

reviews: Prescher (2005) Nat. Chem. Biol. 1: 13-21
Ting (2005) Curr. Opin. Biotechnol. 16: 35-40
Miller (2005) Curr. Opin. Chem. Biol. 9: 56-61

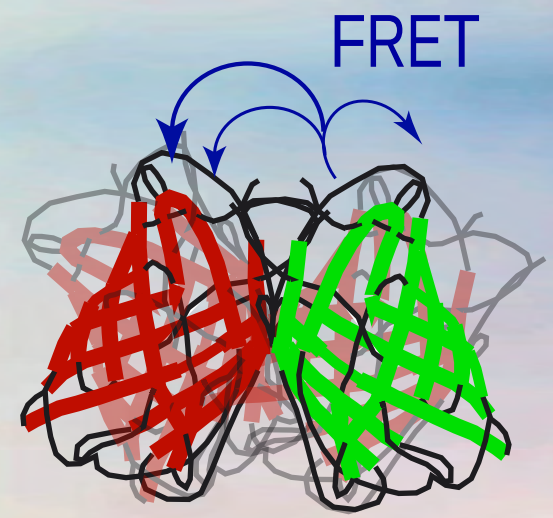


fred.wouters@gwdg.de

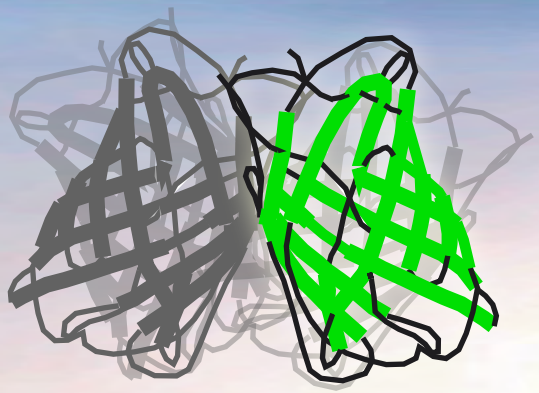
more Green Fluorescent Problems

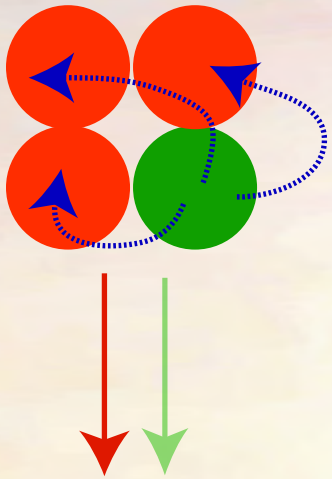


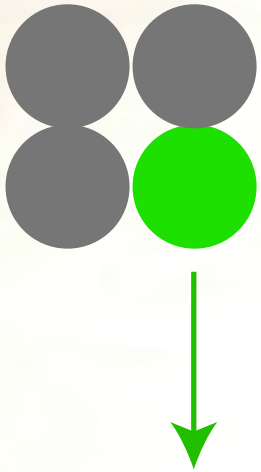
Seeing Red & Green



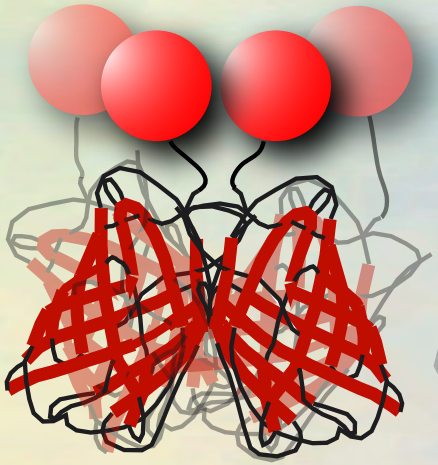
upon photobleaching

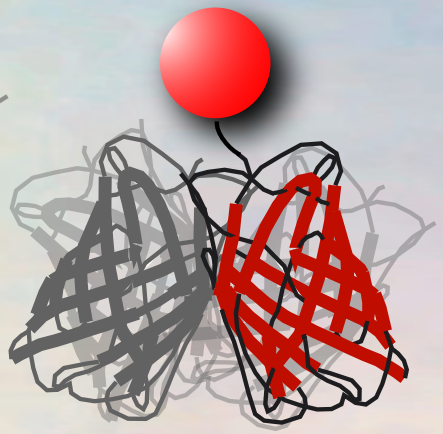


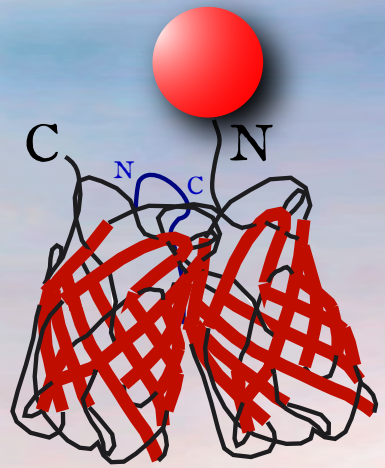




Coping with Tetramerisation







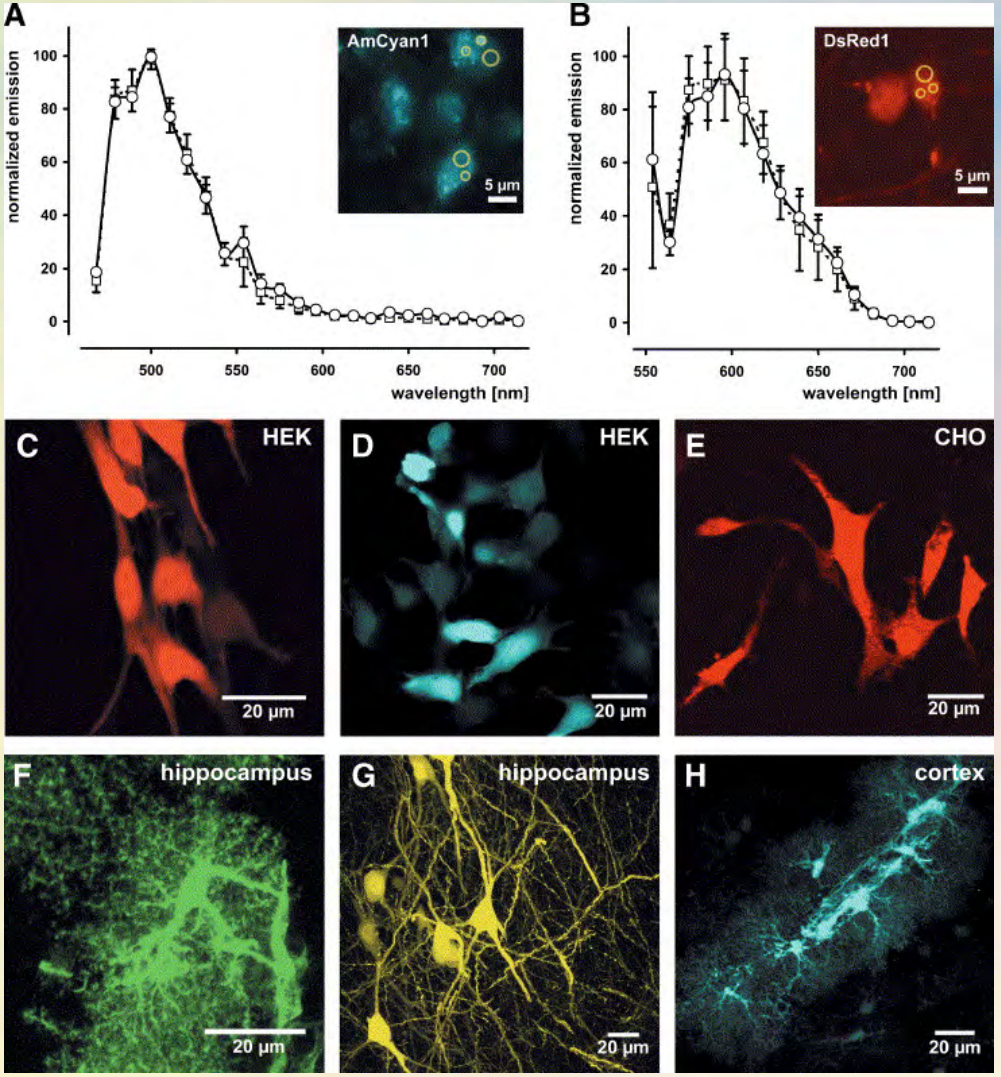
1. BIOLOGICAL SELECTION
   CHOOSE PROTEINS
   OR DOMAINS THAT
   DON'T CARE ABOUT
   BEING TETRAMERIC OR
   THAT ARE TERTRAMERIC
   (ION CHANNELS!!)

2. DILUTION WITH
   NON-FLUORESCENT
   "HELPER RED"

3. HEAD-TO-TAIL
   DIMER OF
   DS-RED OR
   HC-RED

4. HEAVY
   MUTAGENESIS
   MONOMERIC
   DS-RED

even the "monomerized" forms aggregate



when expressed
in transgenic mice

as compared to

their transient expression

or their *Aequoria*
counterparts

Hirrlinger (2005) Mol. Cell. Neurosci. 30:291-303