How to visualize genes and their products

Genomics Lectures

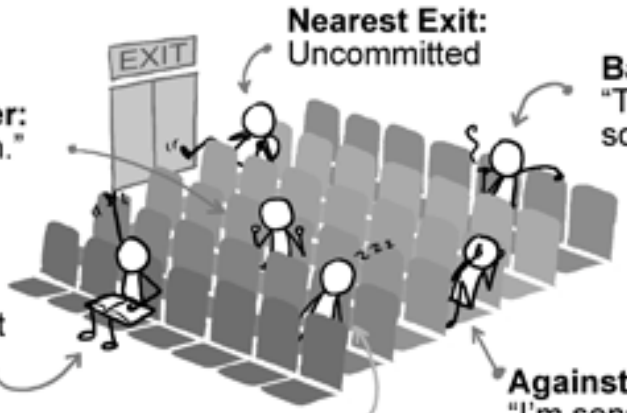
Kamil Růžička
FGP CEITEC MU

Outline

- reporter genes
- promoter fusions
- visualizing proteins
- visualizing RNA
- dynamics of protein imaging: FRAP, photoactivable proteins, FLIM, FCS

WHERE YOU SIT IN CLASS/SEMINAR

And what it says about you:



Nearest Exit:
Uncommitted

Back Row:
"Too cool for school"

Mid-Center:
"Bring it on."

Front Row:
Teacher's pet wannabes

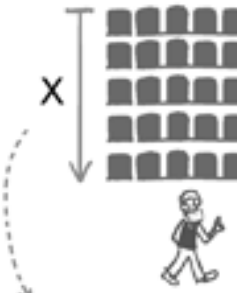
Against the wall:
"I'm sensitive. Please ignore me."

Second-row sleepers:
Good intentions, bad narcolepsy

WWW.PHDCOMICS.COM

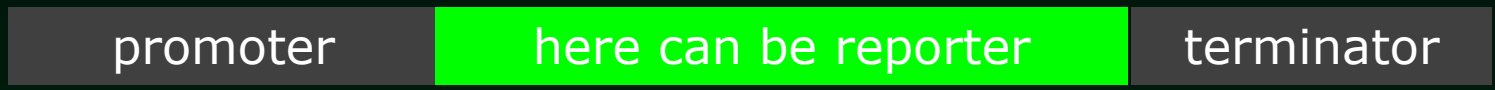
JORGE CHAM © 2008

Proximity to Lecturer:



$$X = \frac{\text{How much you care}}{\text{How sleepy you are}}$$

Promoter activity monitoring



1-10 kb prior to ATG

LacZ, GUS

Luciferase

GFP

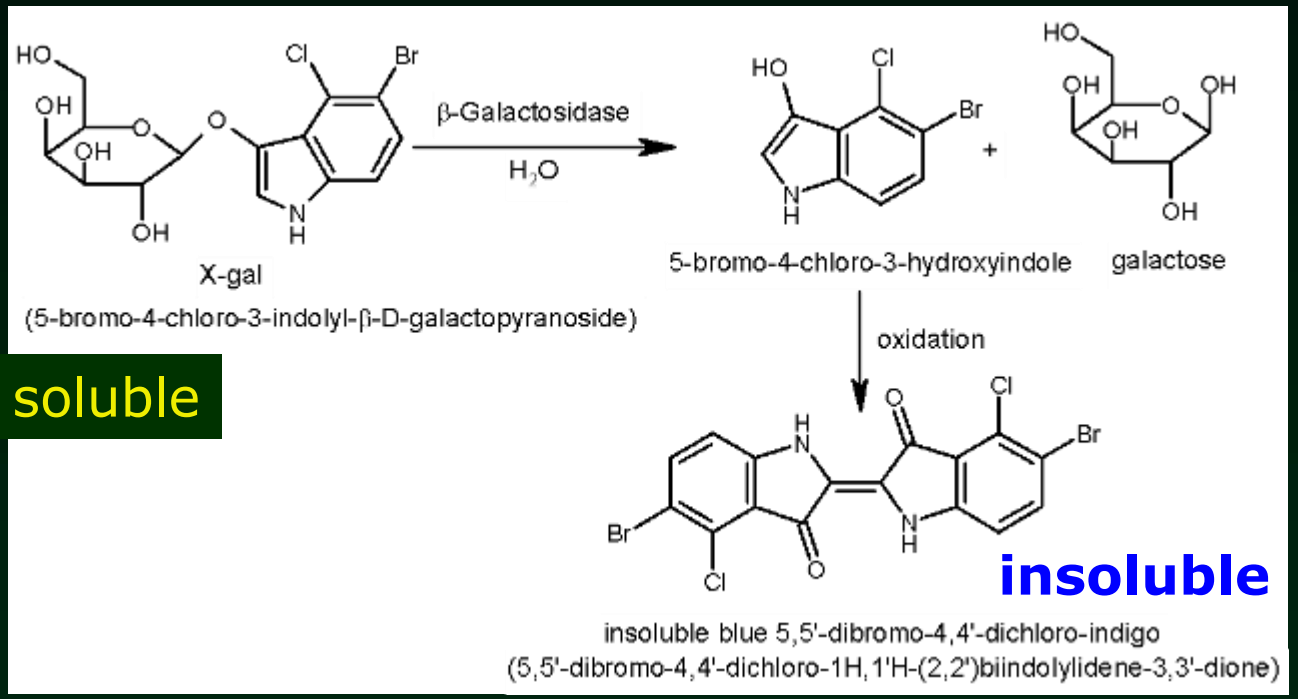
Reporter genes

- LacZ, GUS
- Luciferase
- GFP

some need external substrate, some not

LacZ, GUS – rhapsody in blue

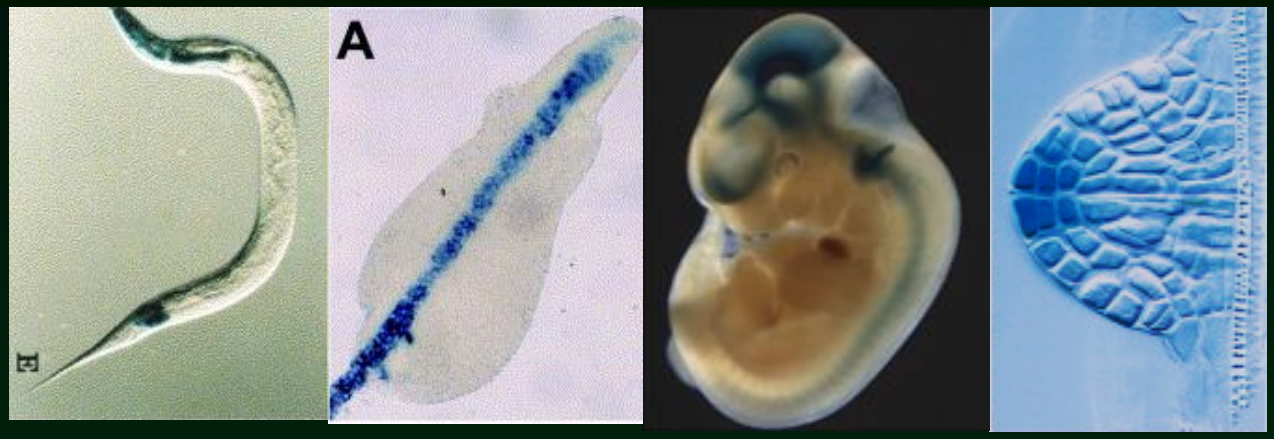
promoter	LacZ	terminator



(in case of GUS – X-Gluc)

LacZ, GUS

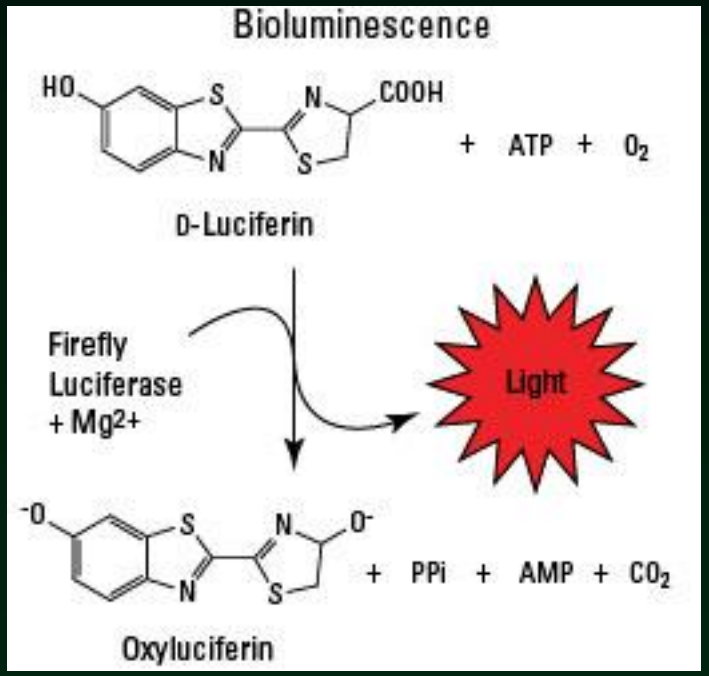
LacZ/ GUS:



worm, mouse – LacZ, plants - GUS

Luciferase

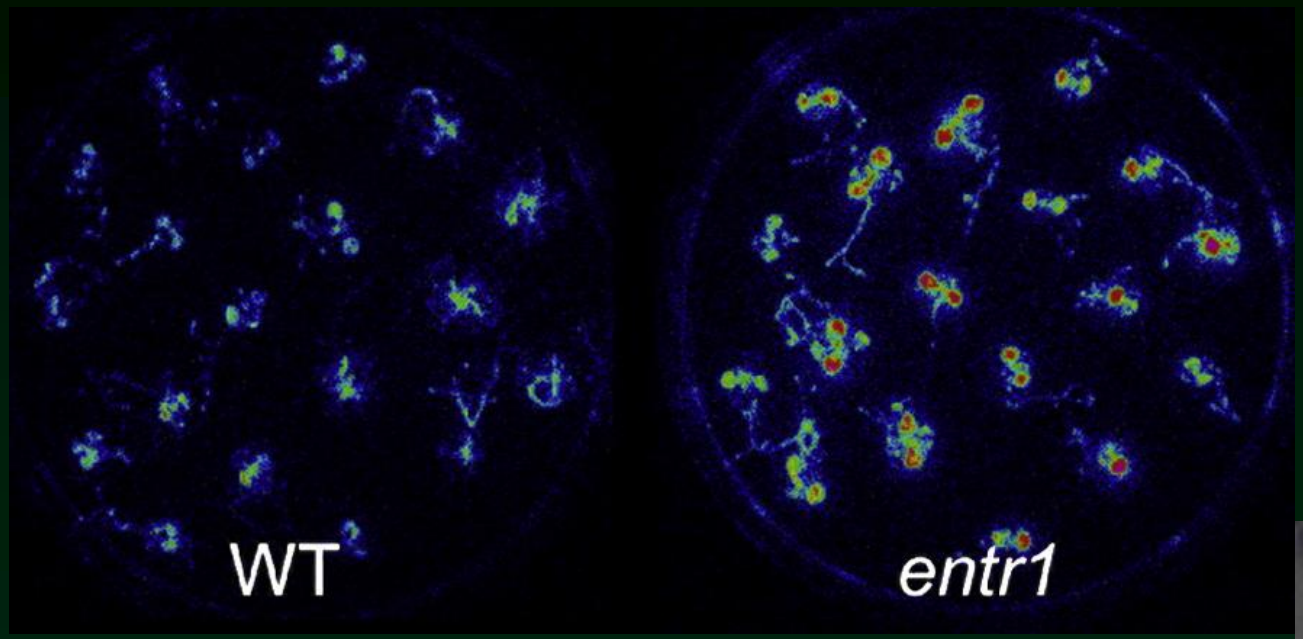
promoter	luciferase	terminator

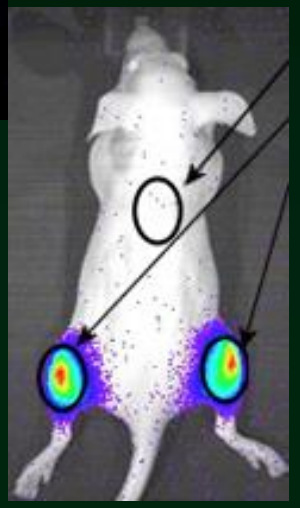


(used principle of bioluminiscence)

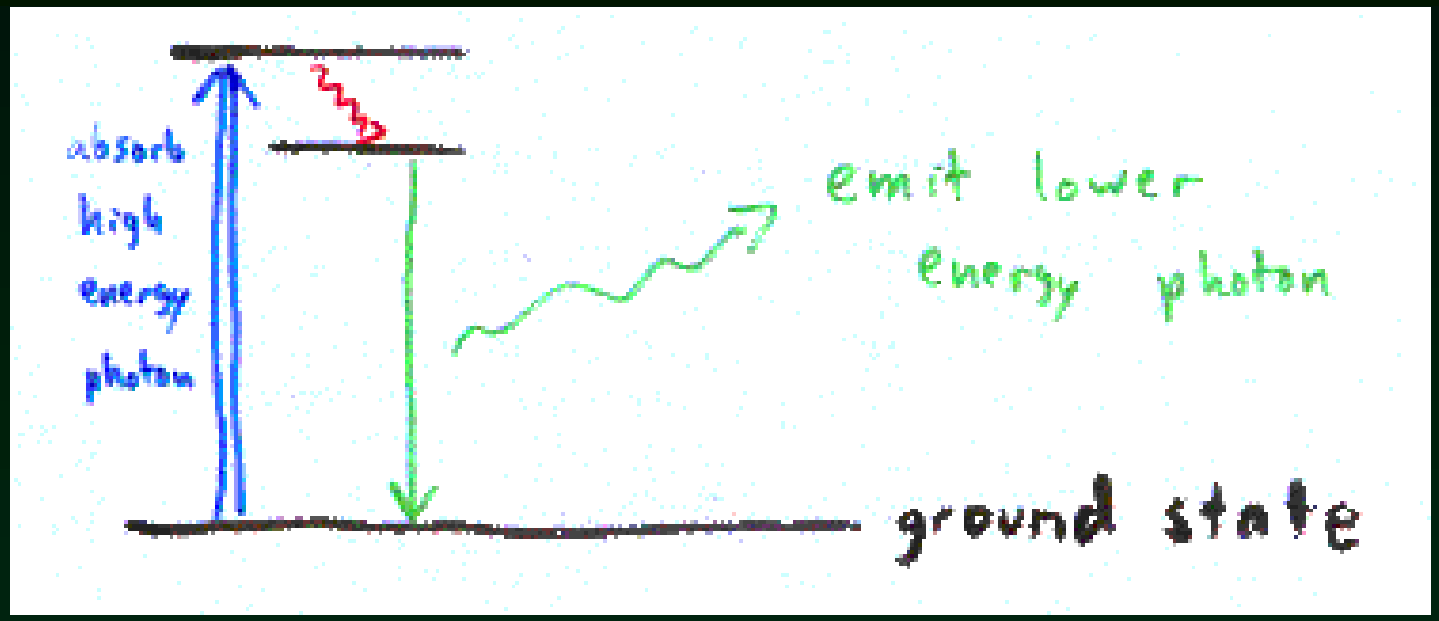
What's difference between flurescence and luminiscence?

Luciferase

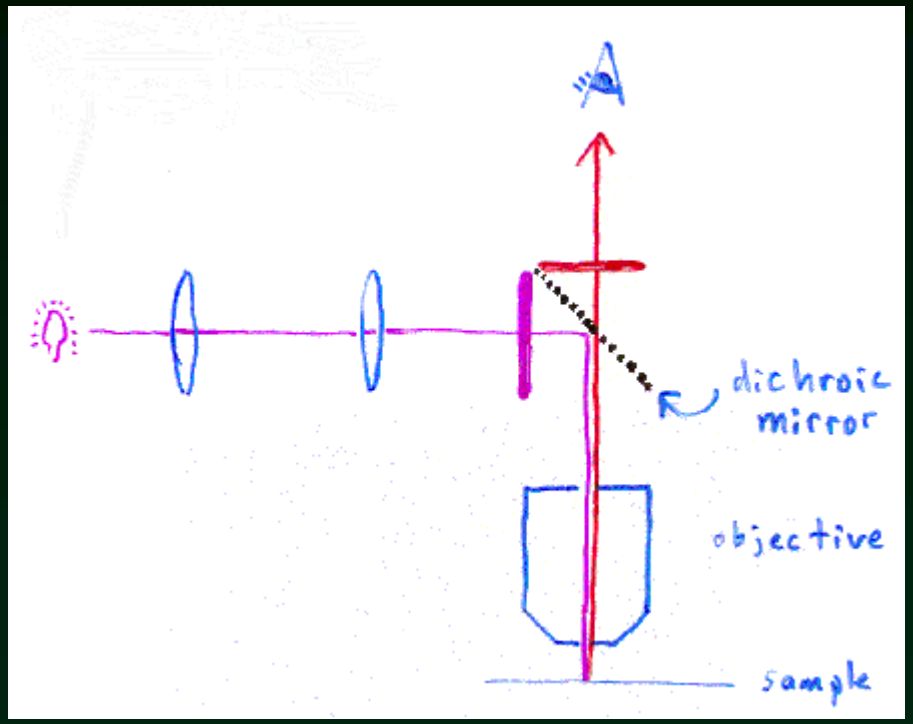




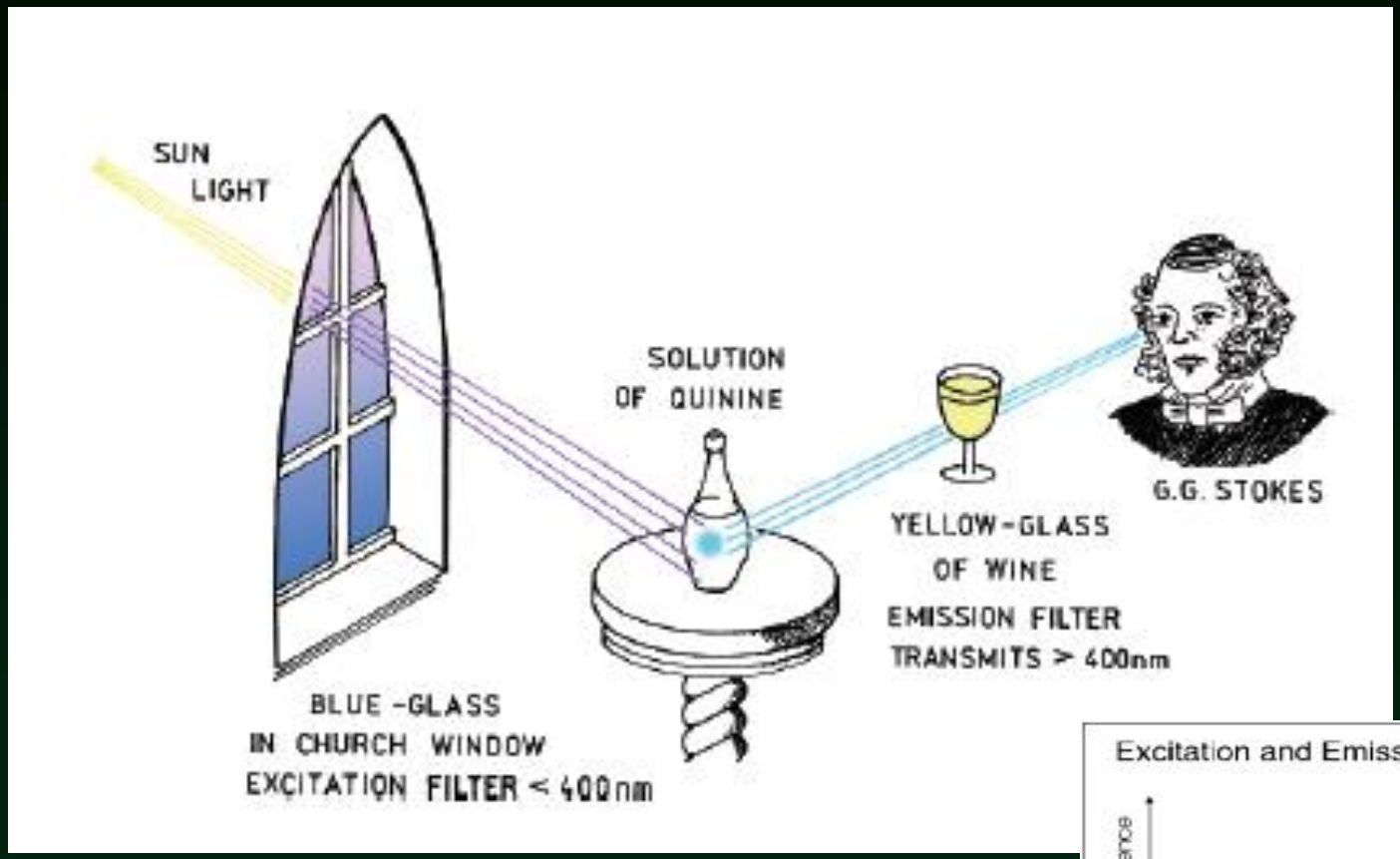
How does fluorescence work?

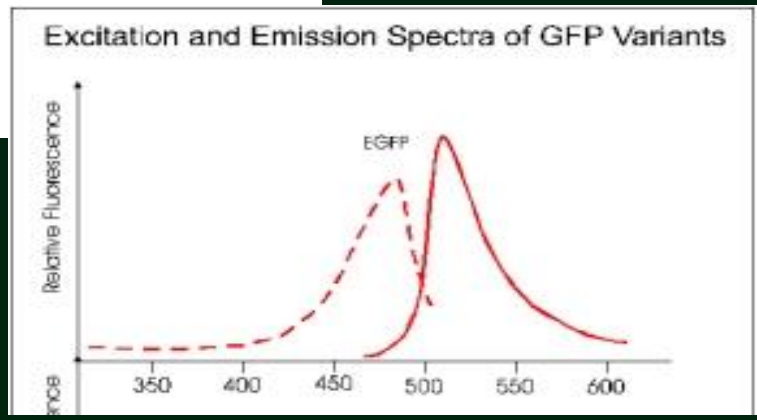


How does a fluorescence microscope work?



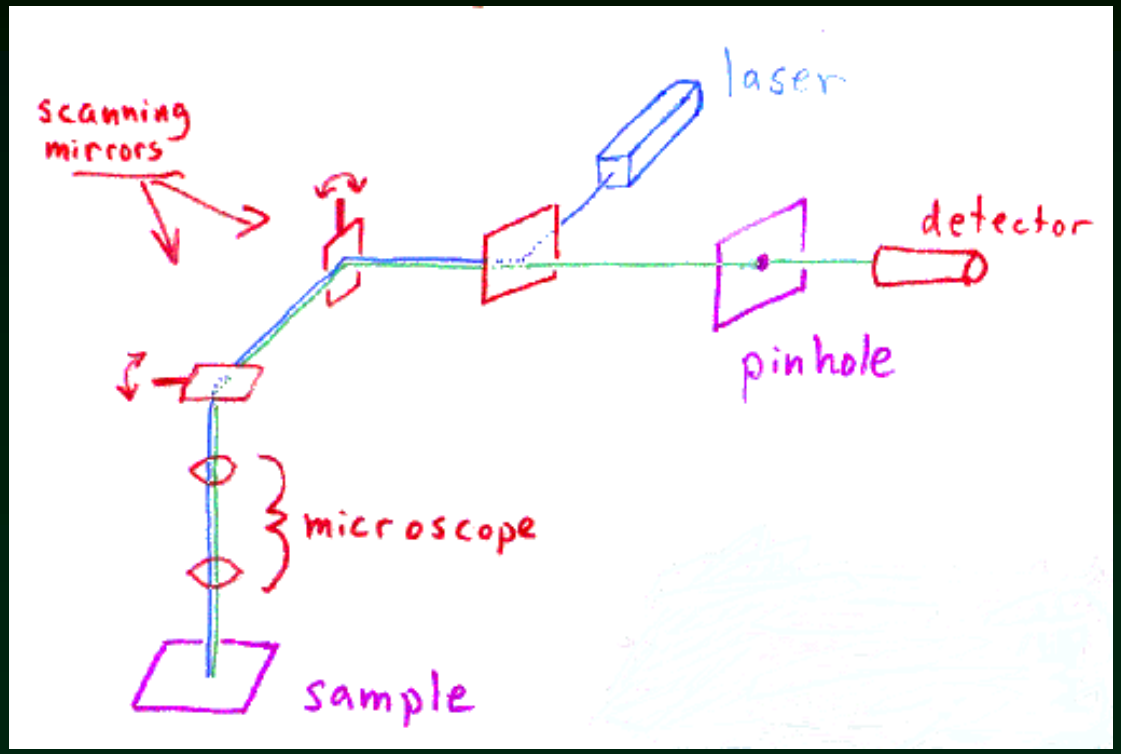
Stokes shift





George G. Stokes

How does a confocal microscope work?



What are advantages of confocal microscopy?

Live imaging

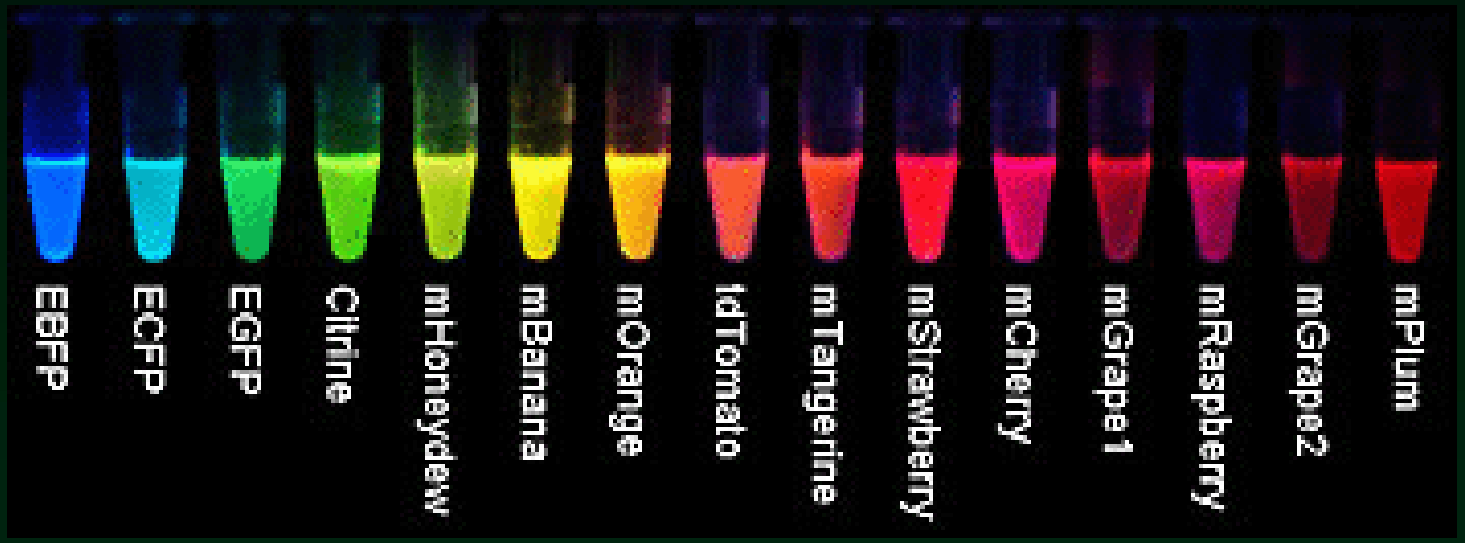
GFP discovery - Nobel Prize 2008

Osamu Shimomura

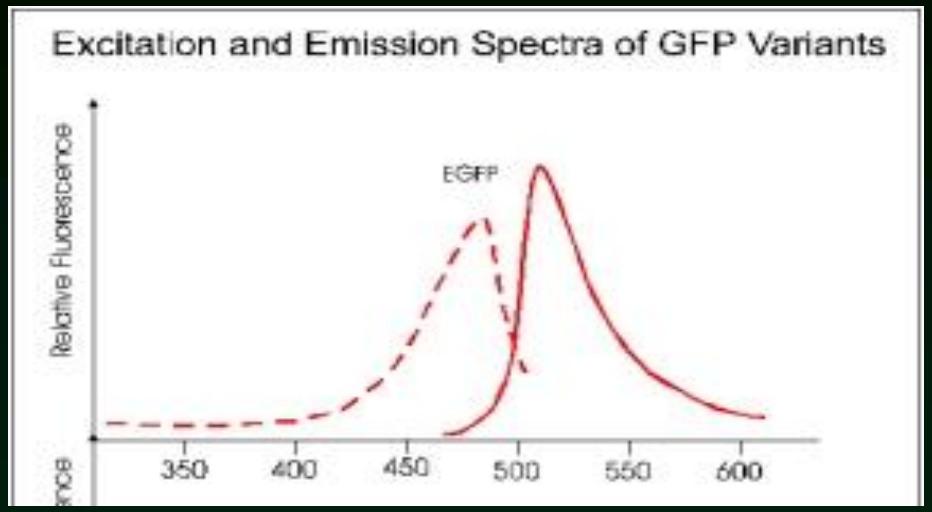
Martin Chalfie

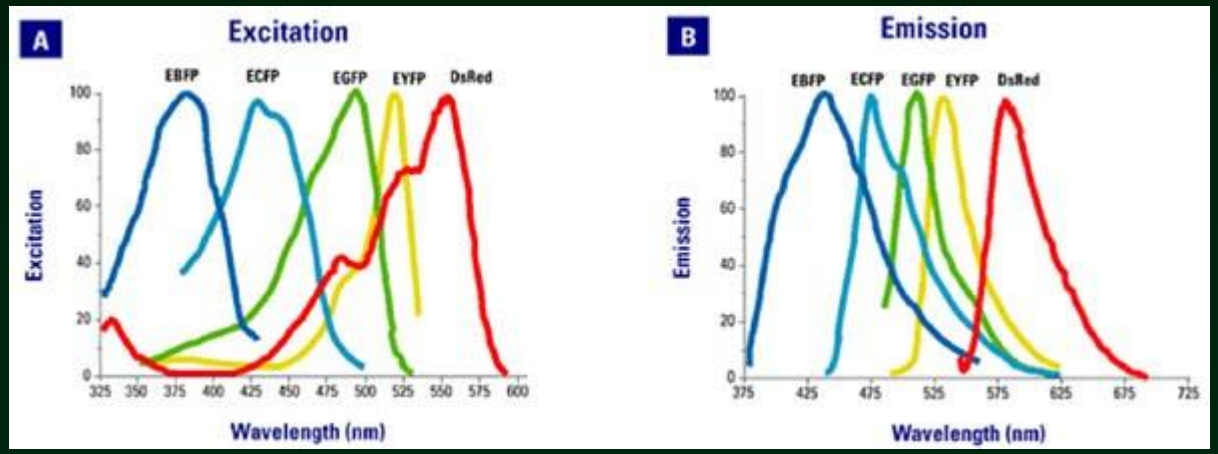
Roger Tsien

Fluorescent proteins on the market (Tsien's fruits)

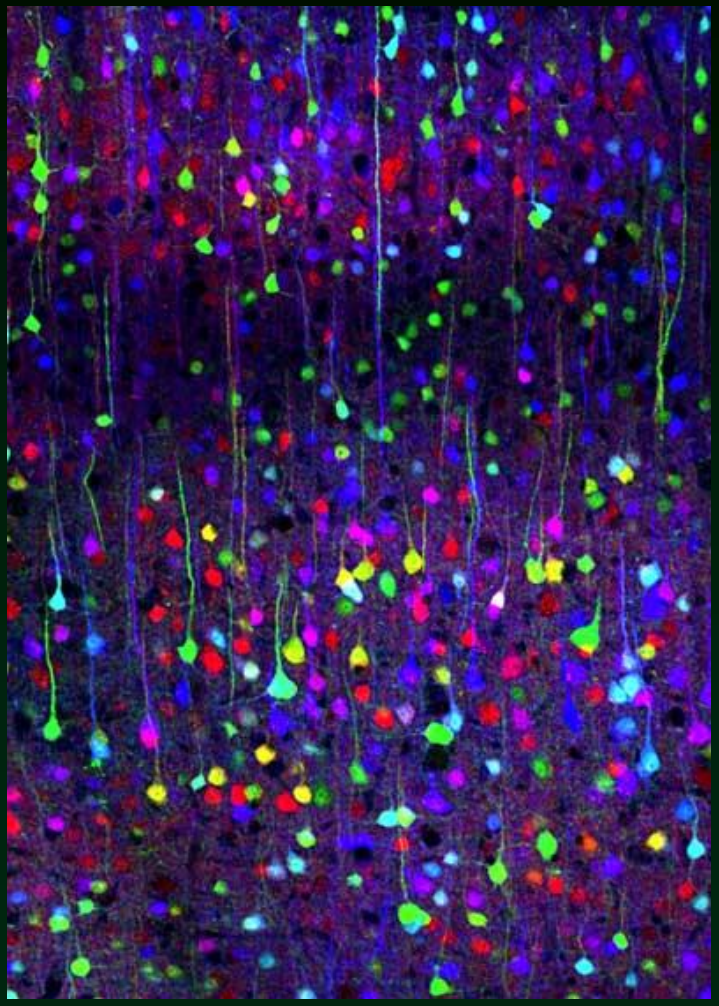


Excitation and emission



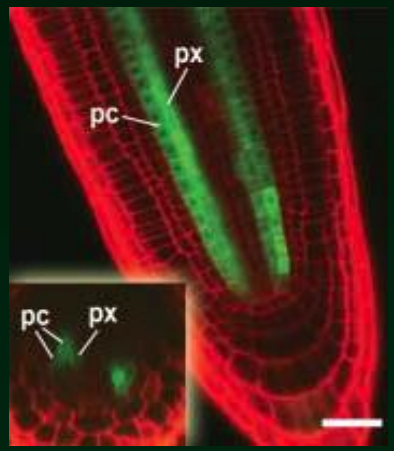


Multicolored fluorescent protein image (neurones)



Promoter-GFP

promoter	GFP	terminator



Promoter activity monitoring

choice of suitable reporter

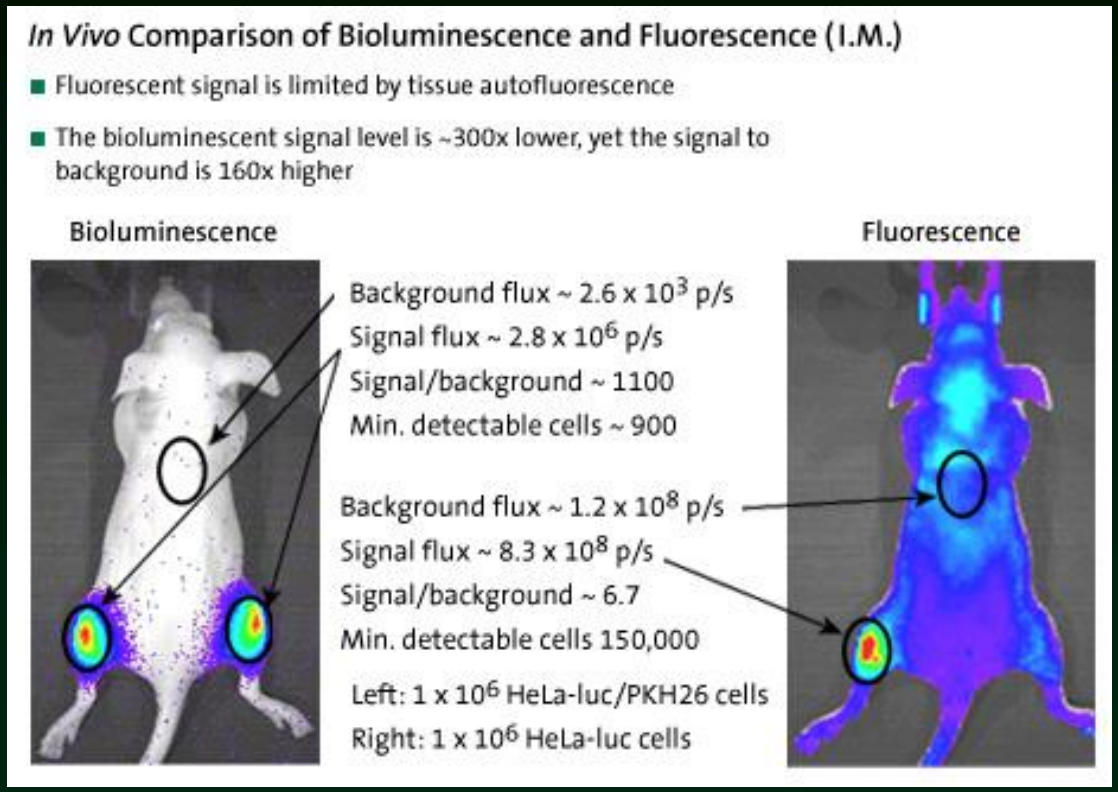
- LacZ, GUS
- Luciferase
- GFP

accessibility, sensitivity, accuracy…

Promoter activity monitoring

- LacZ, GUS
  - easy assay, also on sections, easy imaging
  - substrate must diffuse, kills the organism
- luciferase
  - good quantification, very sensitive, no autophluorescence
  - substrate must diffuse, special machine, dark
- GFP
  - good sensitivity, colocalization with other dyes/promoters possible, no substrate needed
  - only in vivo, autophluorescence, thin transparent sample

Luminiscent mouse better than phluorescent mouse



Promoter activity monitoring

Pros:

-

Cons:

-

Promoter activity monitoring

Pros:

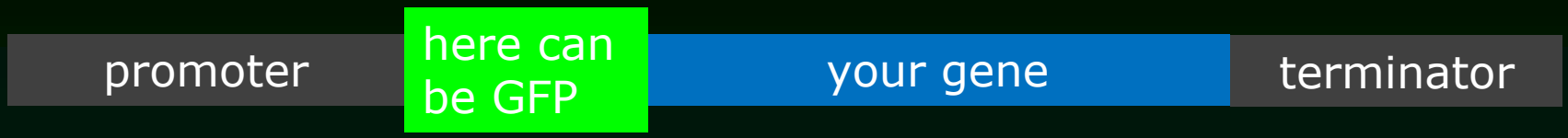
- easy to clone, easy to visualize
- “always works”
- can be used in less accessible organs

Cons:

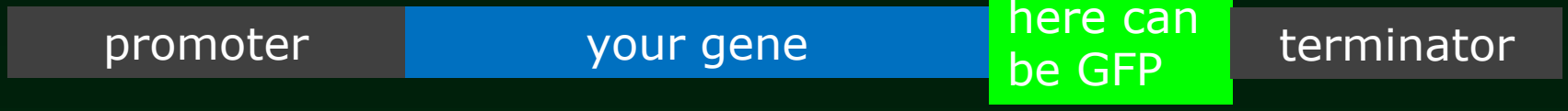
- limited information about gene product (mRNA, protein etc)
- needs cloning and transformation
- neglects regulatory elements (introns, UTRs etc.)

Translational GFP fusions

N-terminal fusion

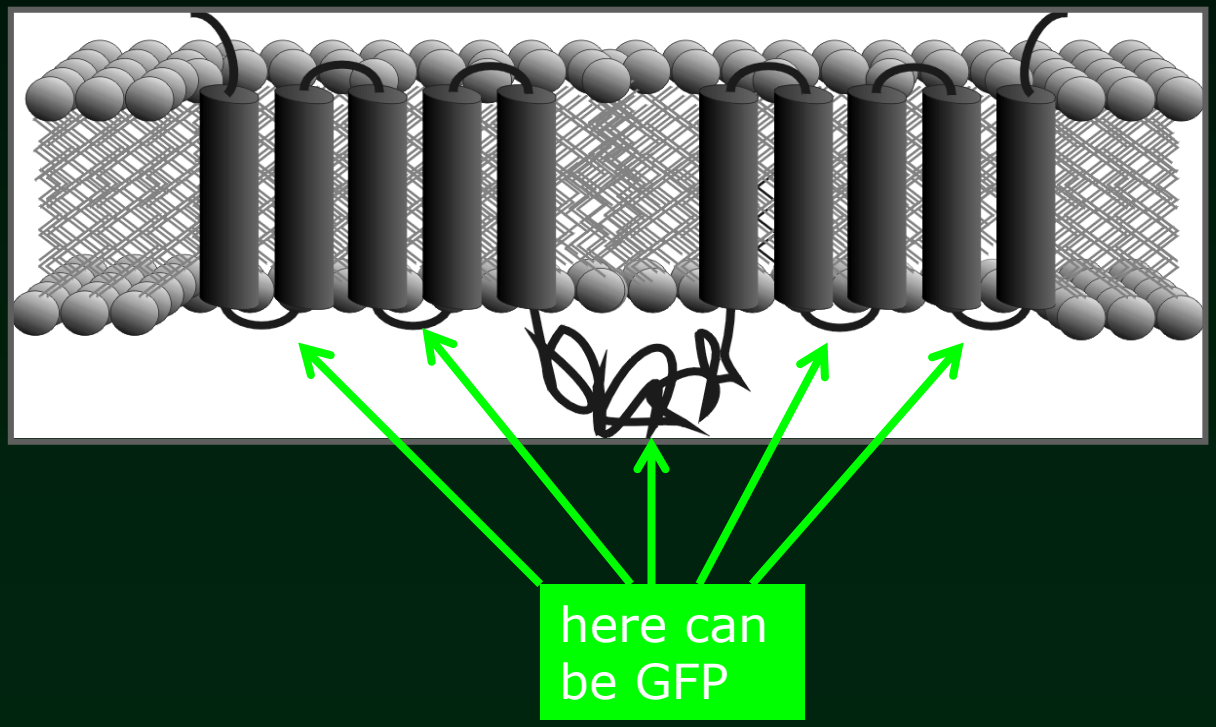


C-terminal fusion



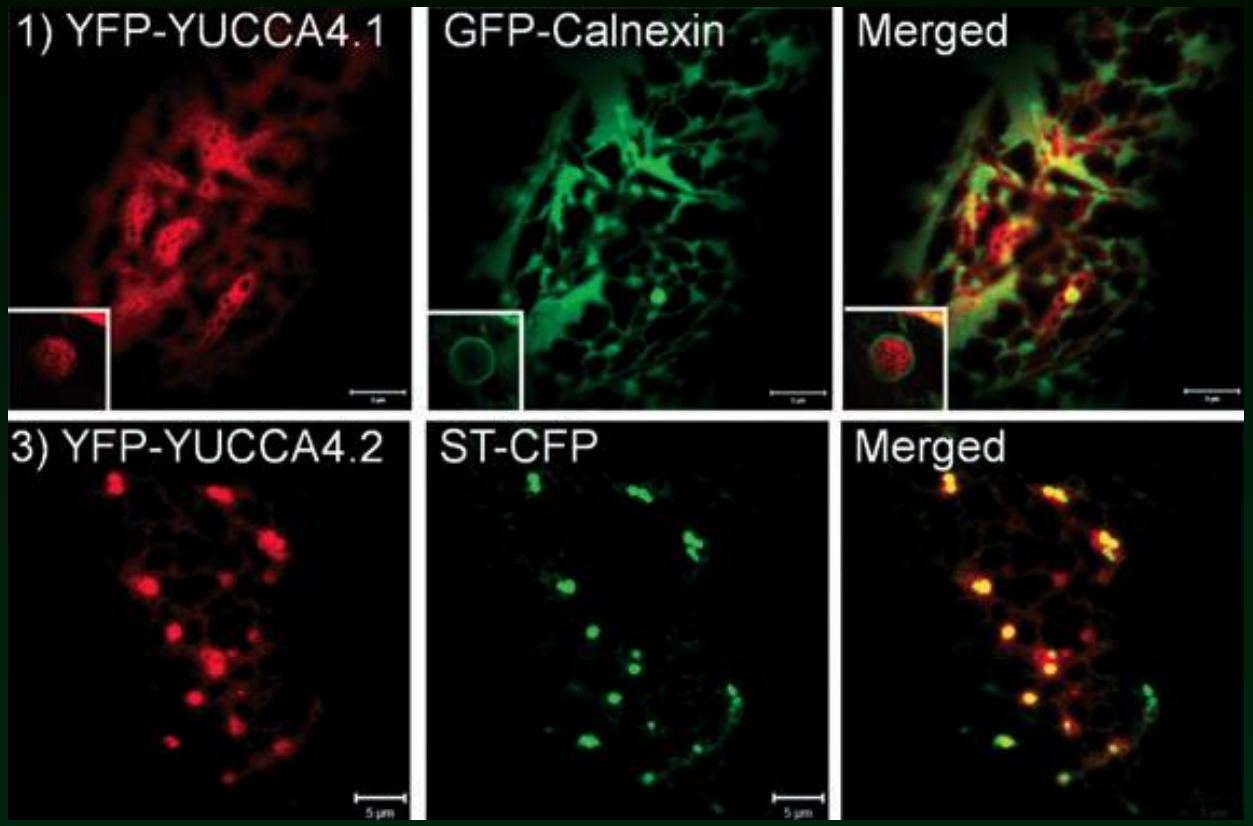
fusion inside the coding sequence

GFP and membrane proteins



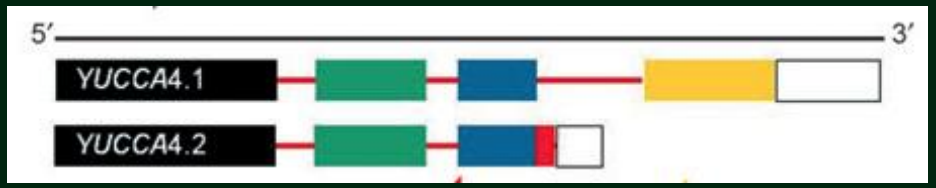
It is good to have GFP tag localized inside the cell (plants)

Expression of isoforms



endoplasmic reticulum

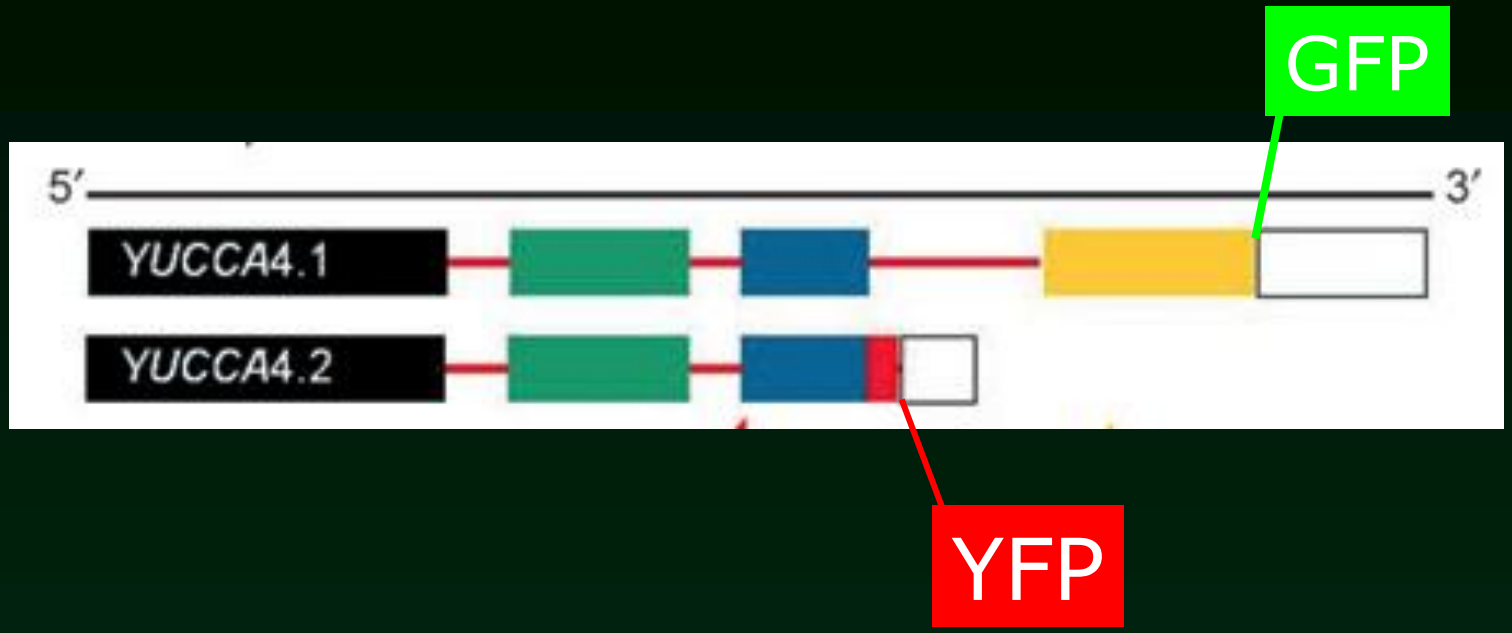
cytosol



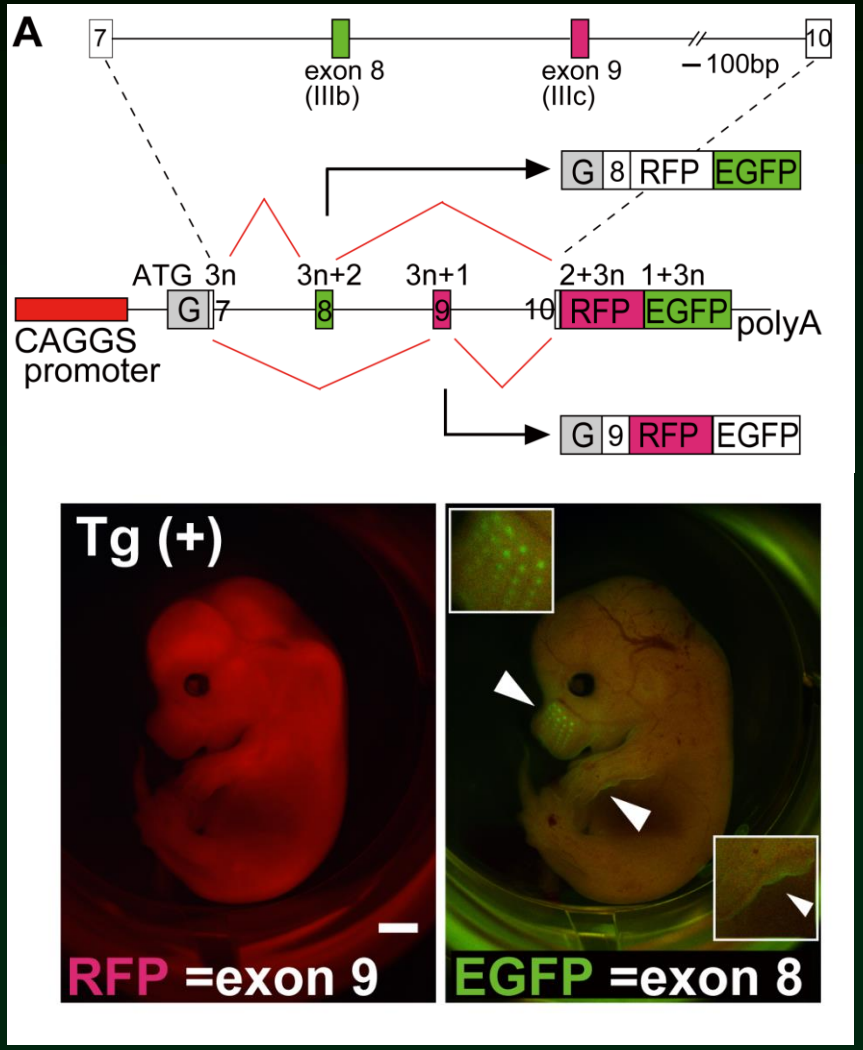
Not the best option available – can you guess?

Kriechenbaumer et al 2011

Isn’t this better?



Expression of isoforms



Fluorescent protein fusion

Pros:

-

Cons:

Fluorescent protein fusion

Pros:

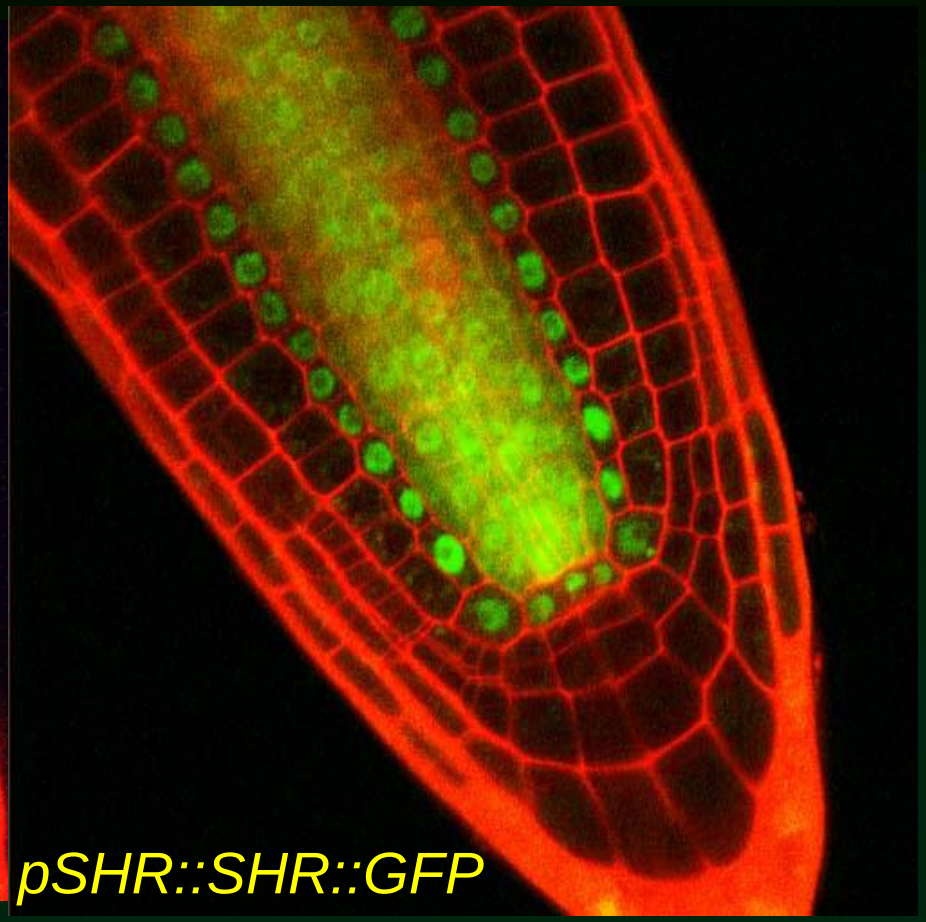
- in vivo imaging

Cons:

- not always functional
- transformation needed
- transparent material (you can sometimes fix GFP signal, however)
- sometimes GFP artifacts (tag doesn’t allow proper targeting)

Why to visualize all this stuff

promoter

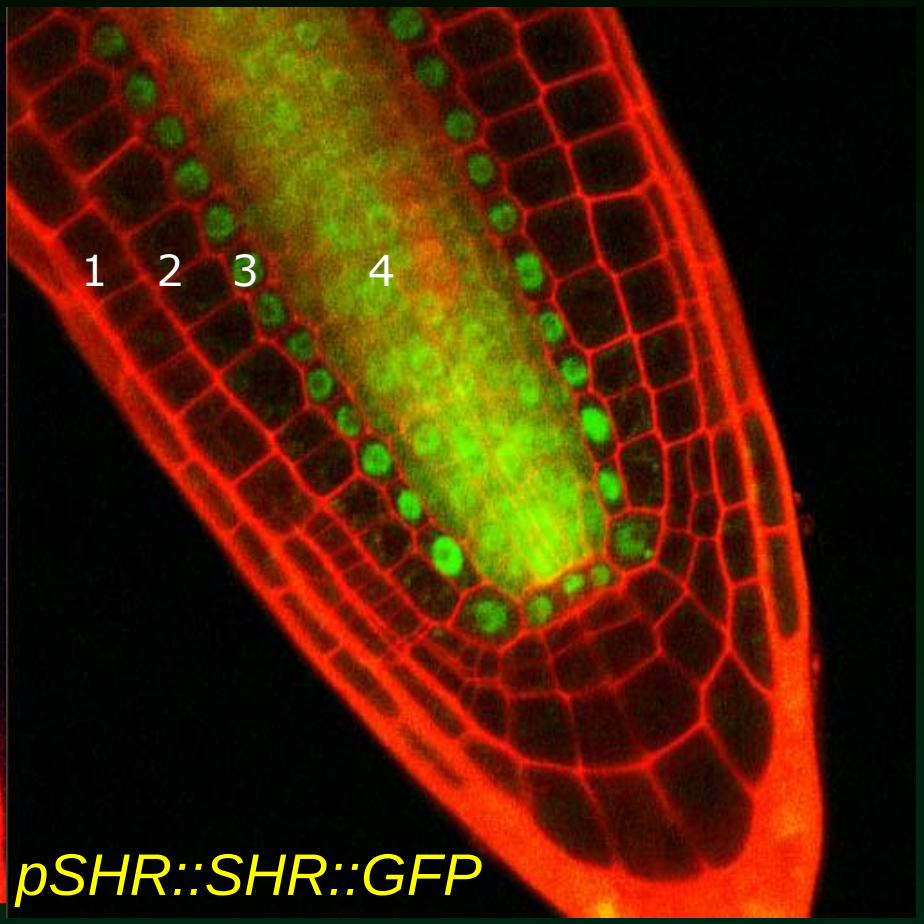


translational

Nakajima et al, Nature 2001

Why to visualize all this stuff

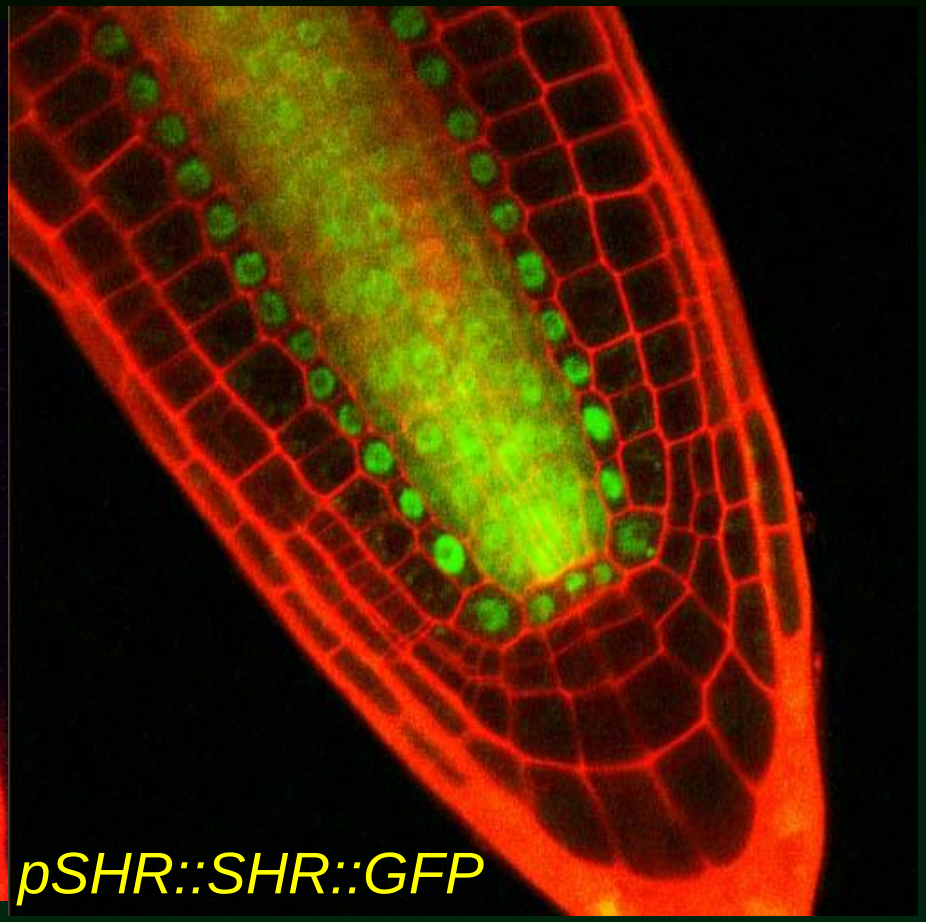
promoter



translational

1 – epidermis
2 – cortex
3 – endodermis
4 – stele

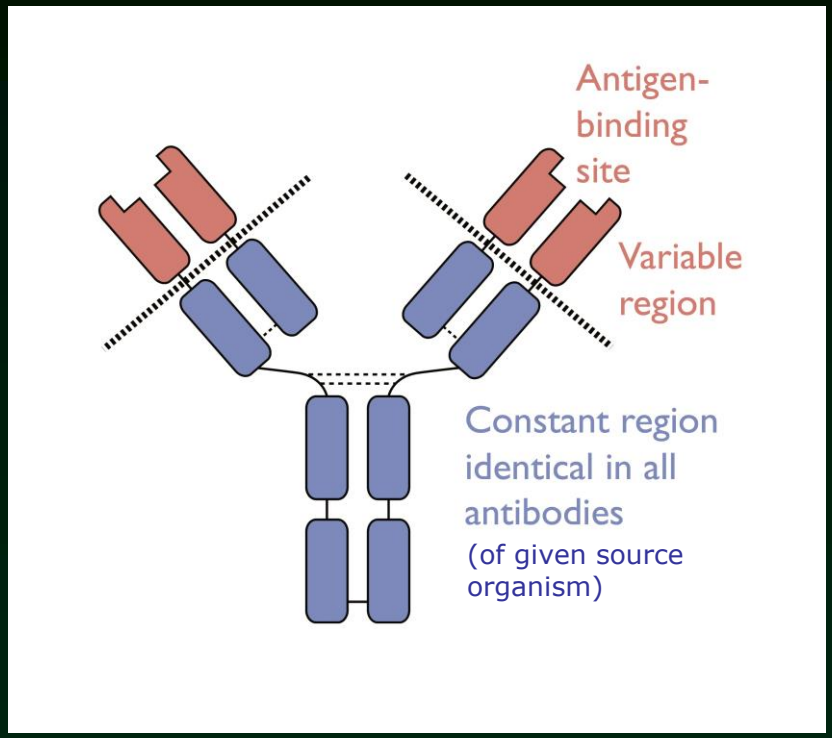
Why to visualize all this stuff



BANG! SHR moves from stele to endodermis

Nakajima et al, Nature 2001

Protein immunolocalization

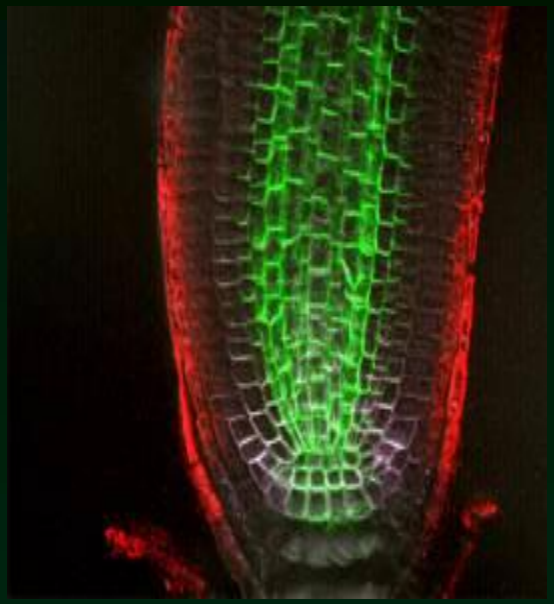


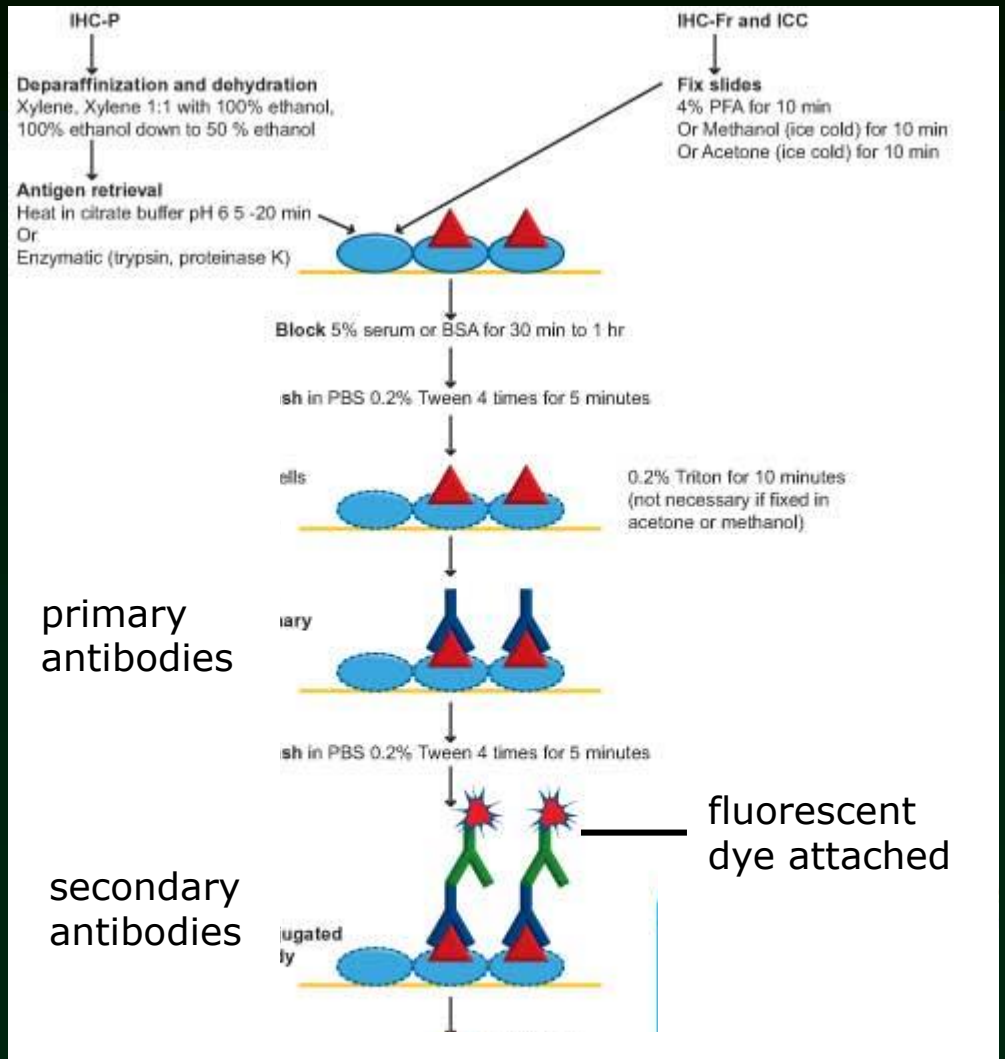
Most favorite animals:
-rabbit (too many rabbits)
-mouse (low volume)
-goat
-chicken
-rat
-sheep
-donkey
-guinea pig

2ndary: antirabbit from no-rabbit, antimouse from no-mouse, etc.

Protein immunolocalization

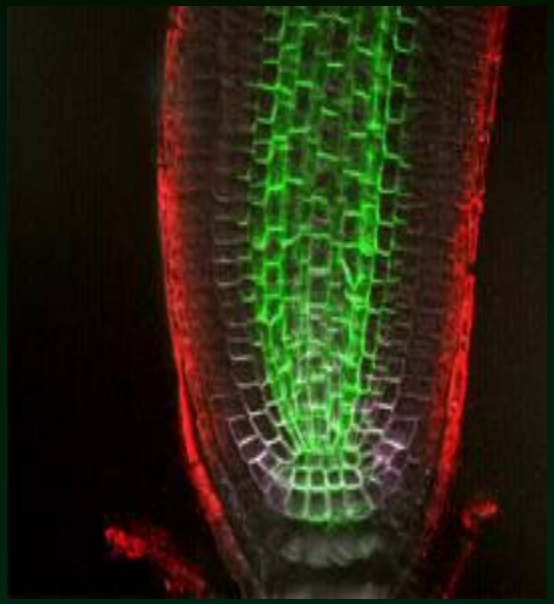
immunolocalization - fluorescently





Protein immunolocalization

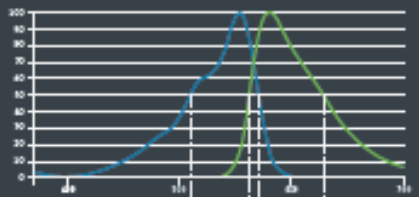
immunolocalization



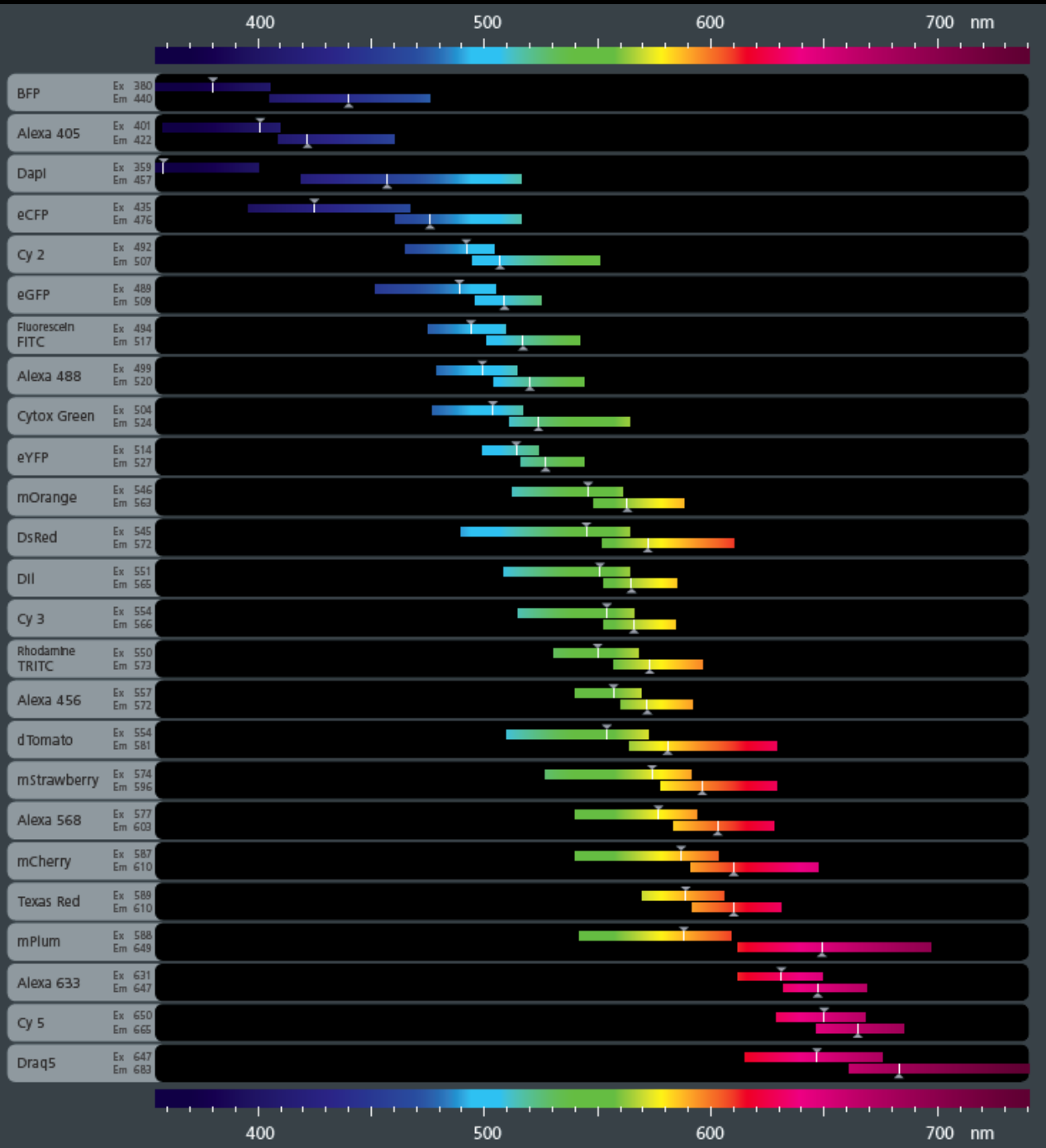
Fluorescent dyes conjugated to 2ndary (examples):

- FITC (obsolete)
- CY3, CY5
- Alexa (488, 568, 633)

Fluorescent Dyes and Proteins



Dye Name
Excitation Max
Emission Max



www.zeiss.com/microscopy

Protein immunolocalization

Pros:

-
-

Cons:

-
-

Protein immunolocalization

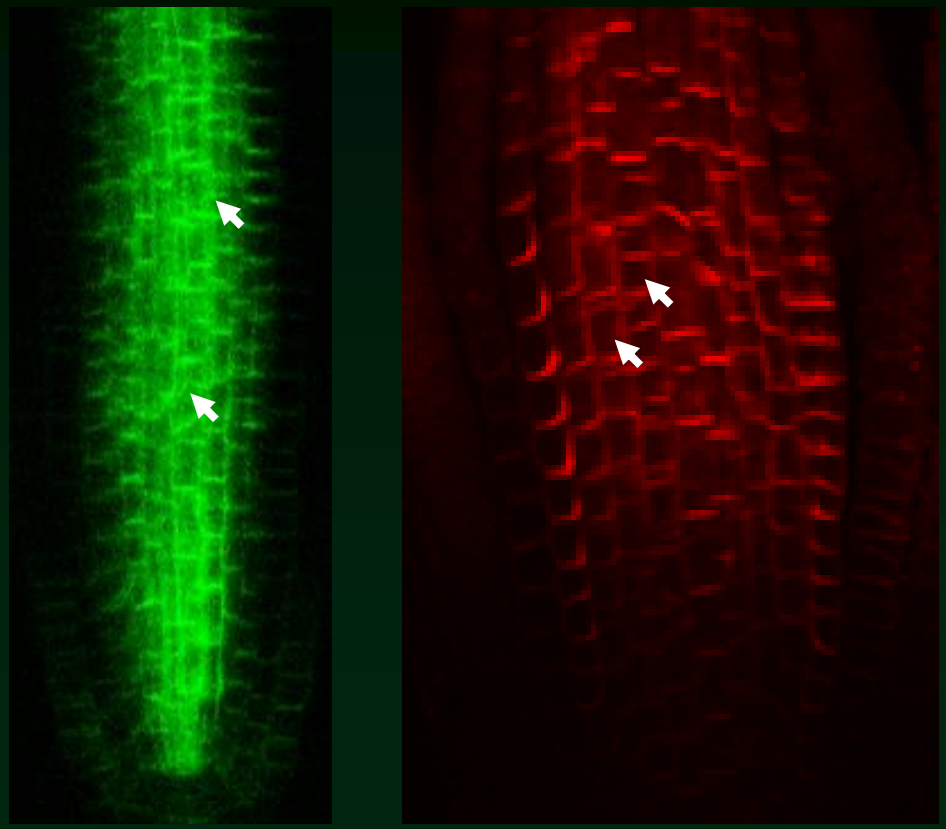
Pros:

- no need to clone or transform or cross
- direct (if no tag used)
- allows sectioning (less accessible tissues)

Cons:

- fixed material only
- excellent antibodies only, sometimes tricky

GFP tag partially retains PIN1 in endoplasmic reticulum (-> artifact)

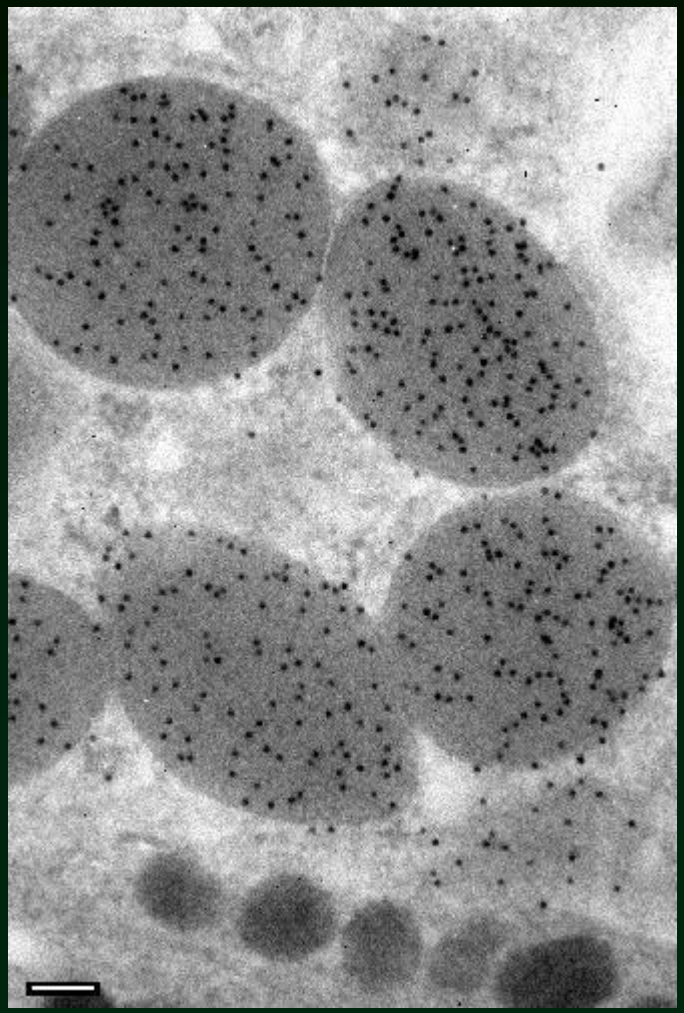


PIN1-GFP

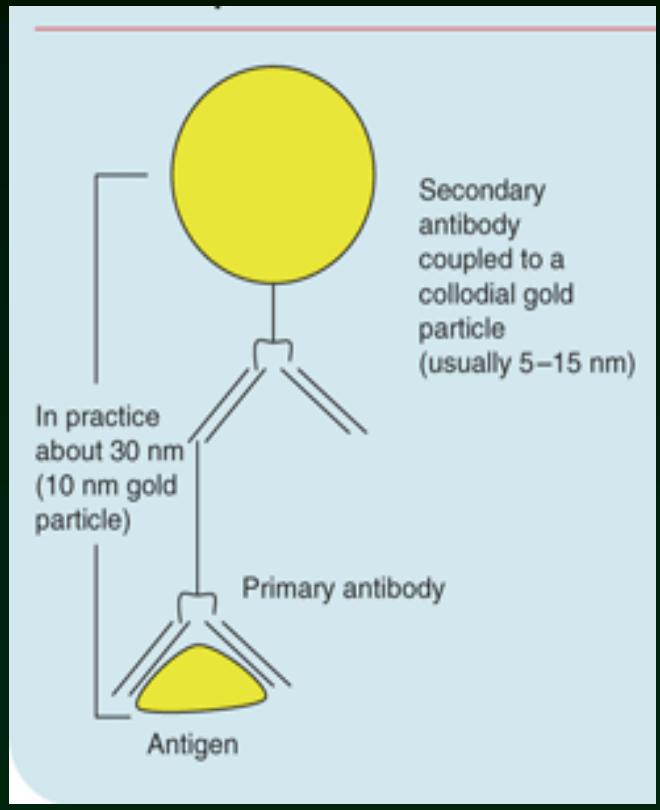
anti-PIN1

Protein localization - immunogold

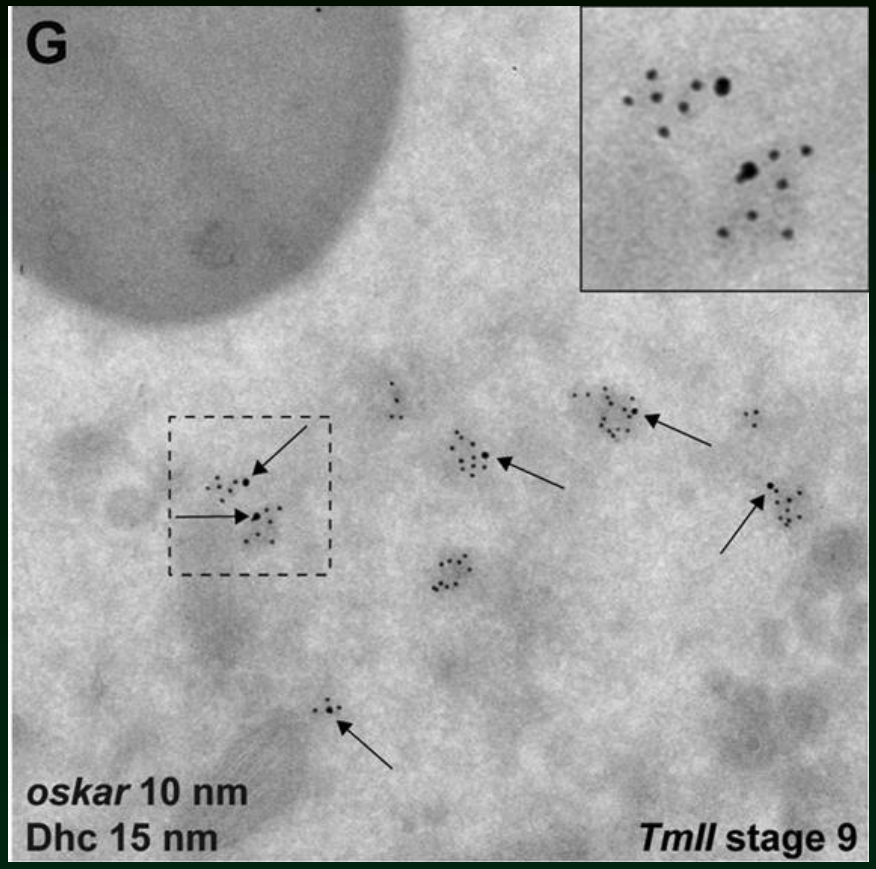
immunolocalization - immunogold



electron microscope



Immunogold collocalization



Pros/cons

Pros:

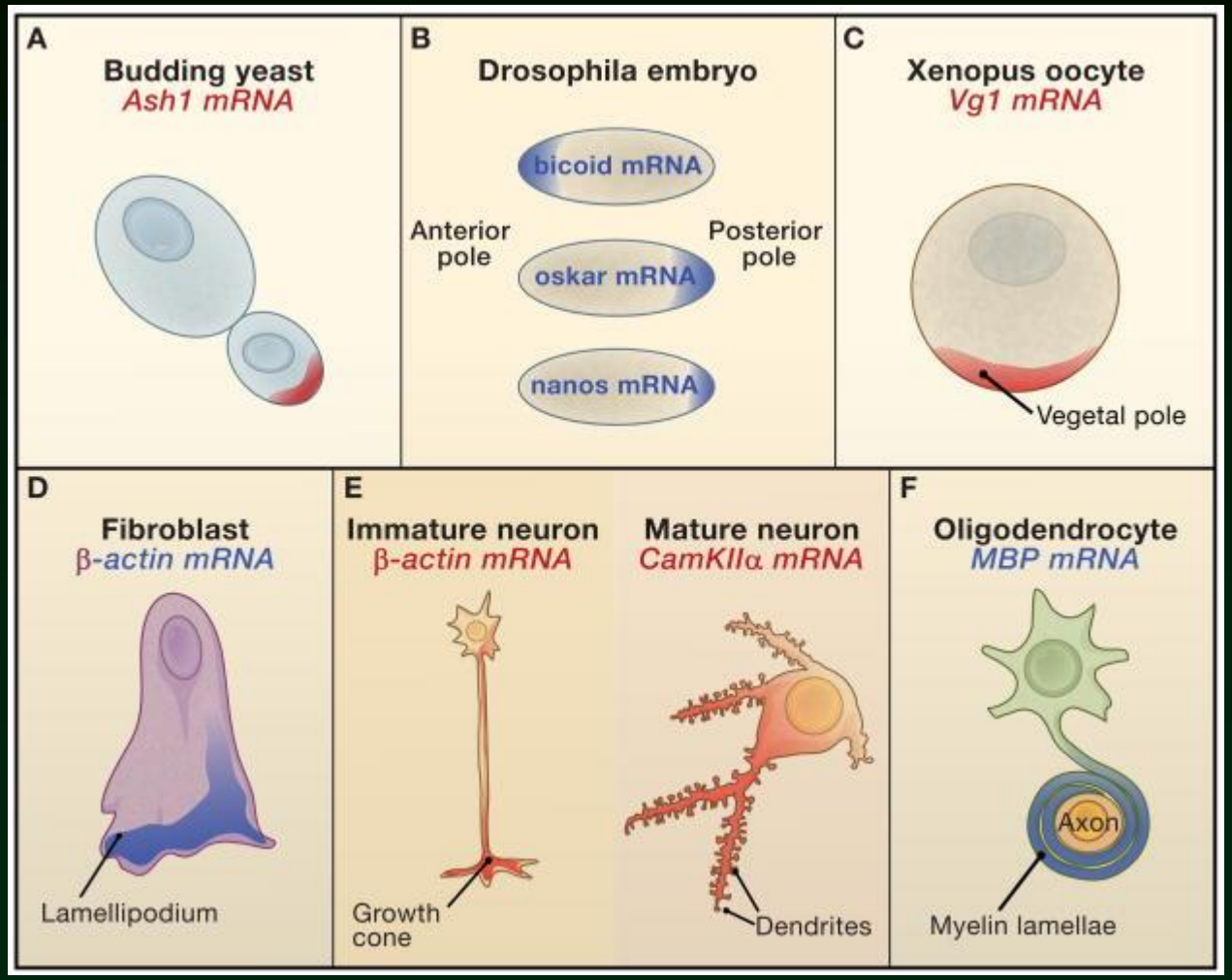
- direct
- nothing can beat the resolution

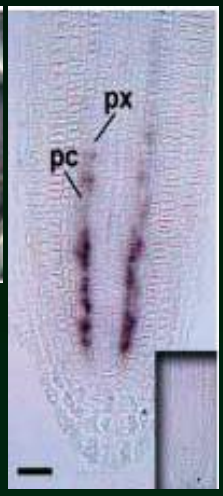
Cons:

- very tricky (few labs, mainly specialized)
- huge experience for interpretation needed
- immunogold colocalization – only theoretical?

Can we visualize postranslational modifications?

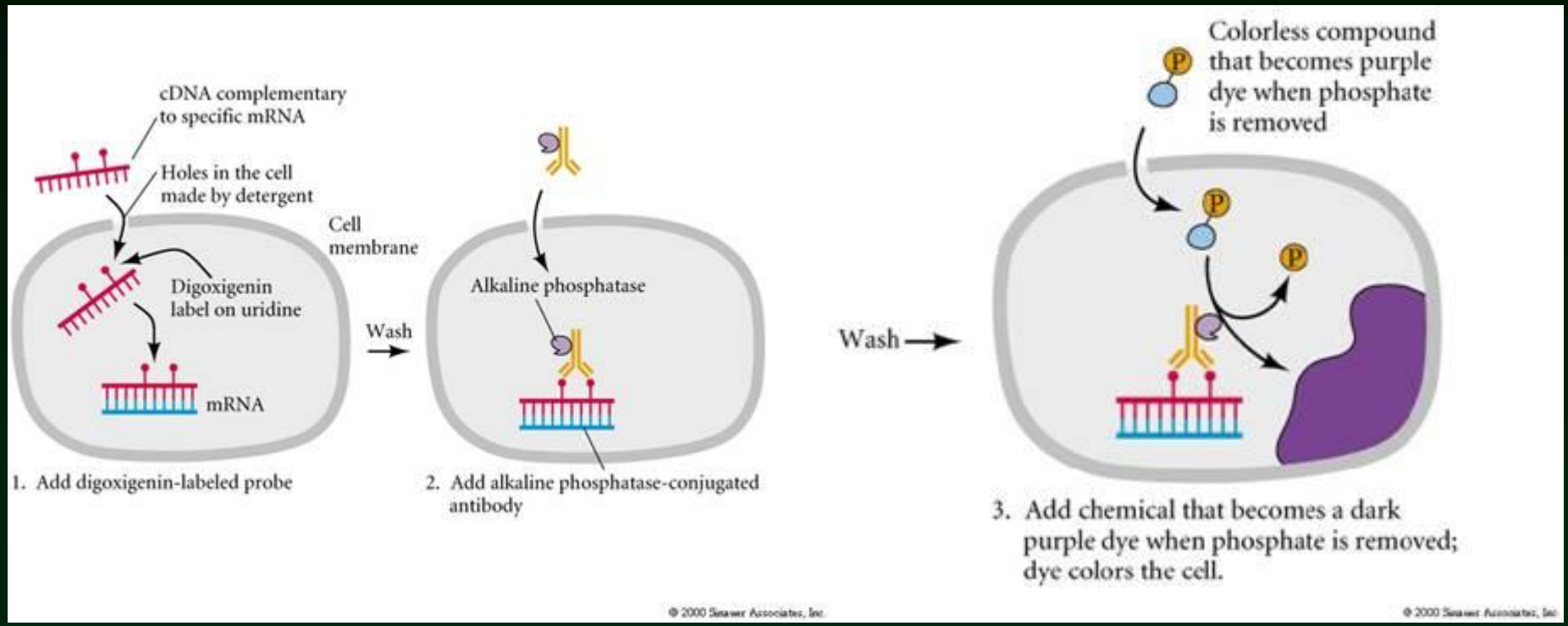
Also RNA can be visualized





Localization of mRNA

RNA hybridization *in situ*



Visualization of mRNA

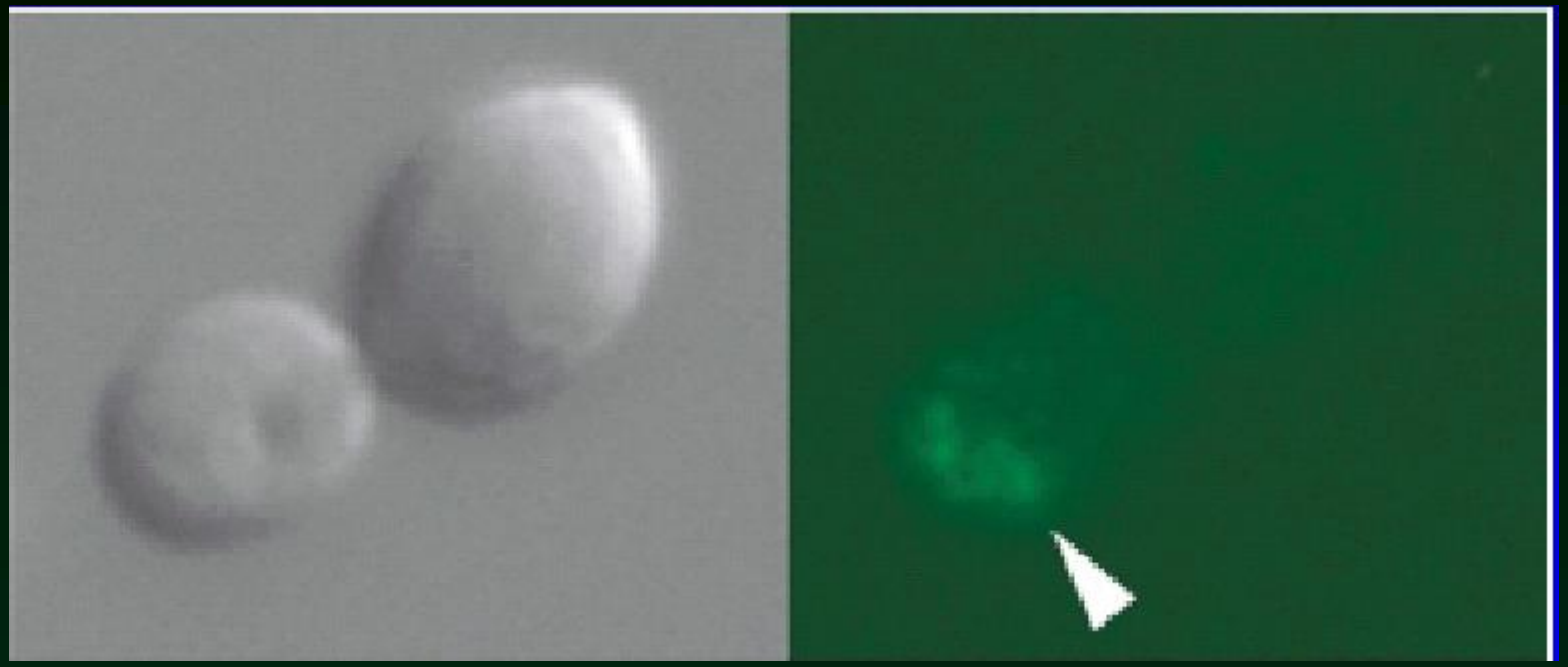
RNA hybridization *in situ*

Pros
- classical technique in developmental biology
- no transgenes needed

Cons
- tedious, tricky, no success guaranteed
- only on fixed samples

For shorter RNAs (miRNA etc.):
- LNA probes needed

Also mRNA can be visualized in vivo

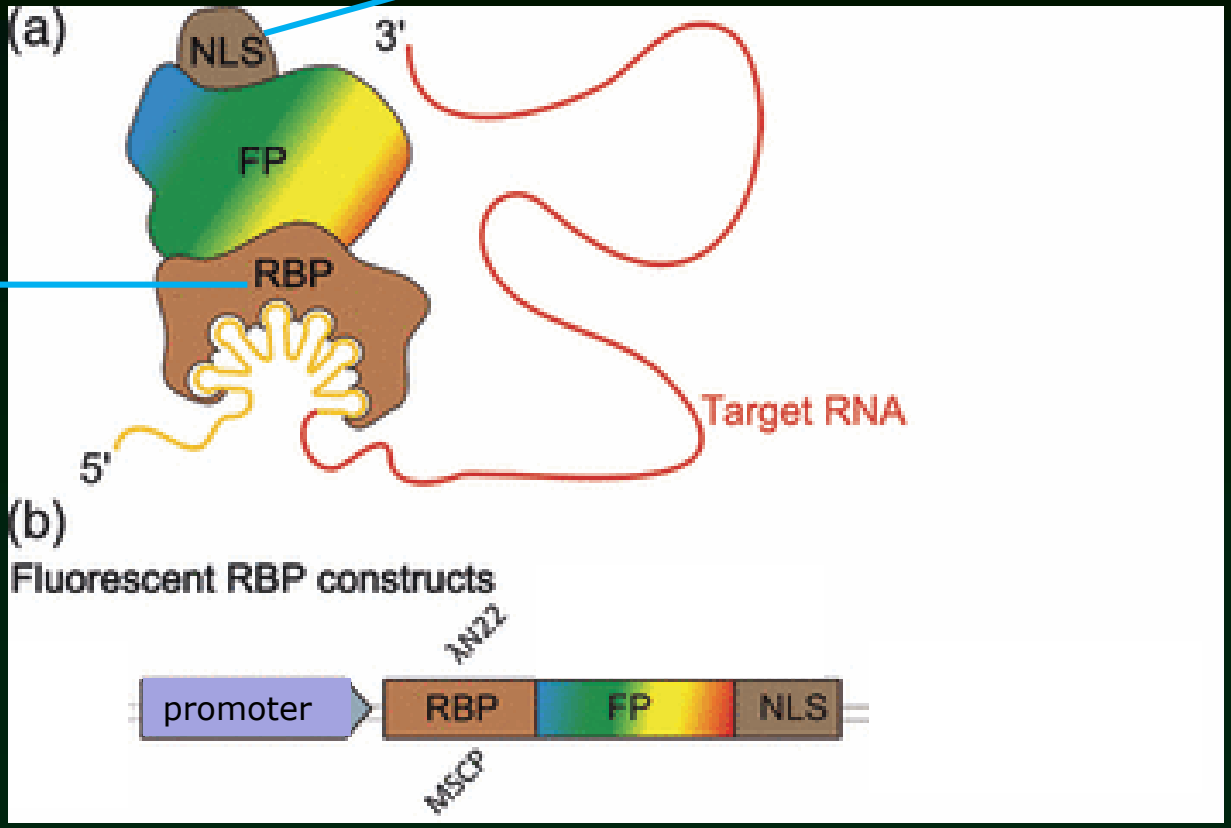


Ash1 mRNA localized to the tip of the daughter cell

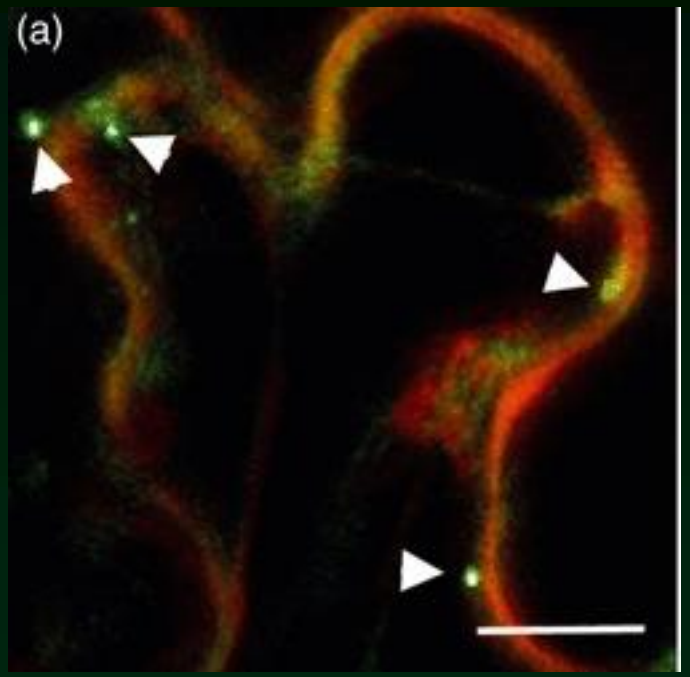
λN_{22} system – RNA imaging *in vivo*

nuclear localization signal

viral RNA binding protein



Also mRNA can be visualized in vivo

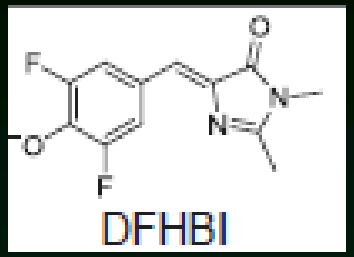


Drawbacks of λN_{22} system - we have SPINACH

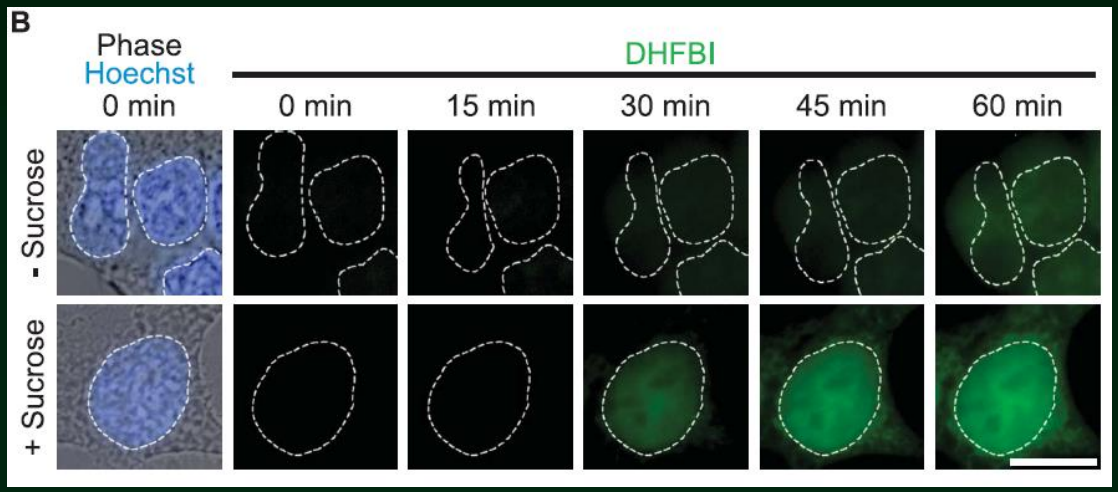
GACGCAACUGAAUGAAA
UGGUGAAGGACGGGUCC
AGGUGUGGCUGCUUCGG
CAGUGCAGCUUGUUGAG
UAGAGUGUGAGCUCCGU
AACUAGUCGCGUC

RNA fusion

+



aptamer

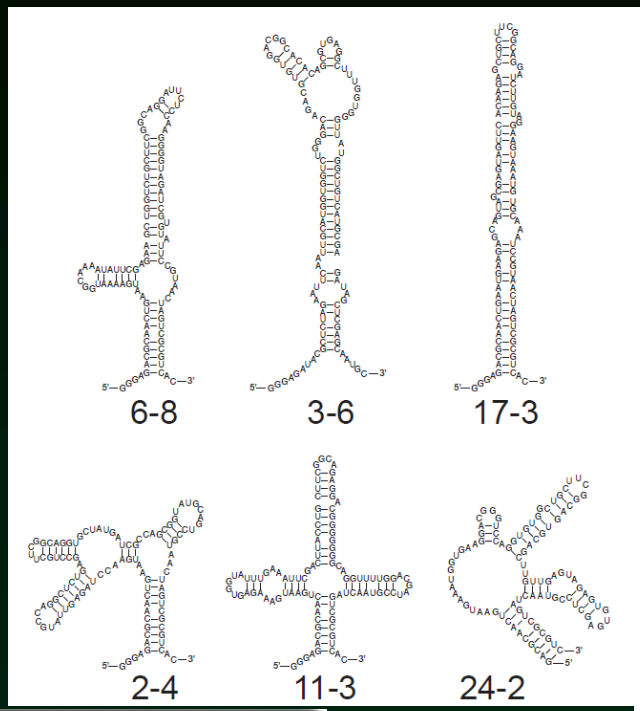


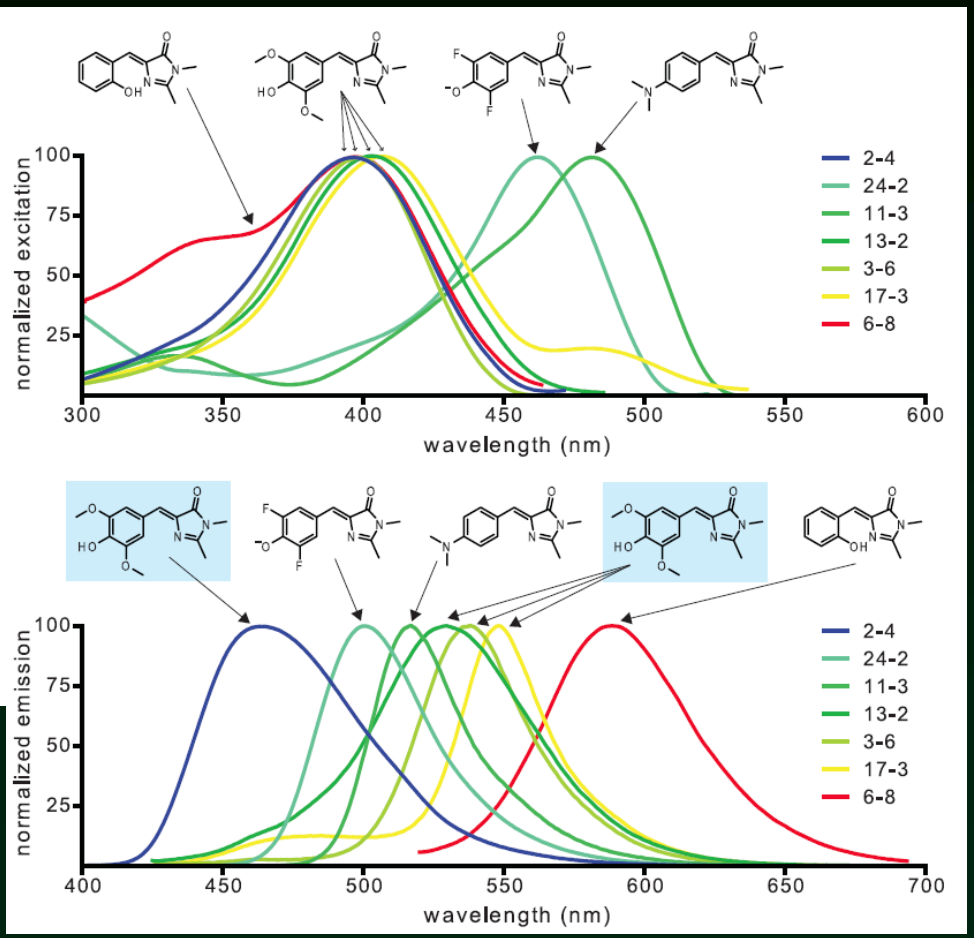
blue-DNA

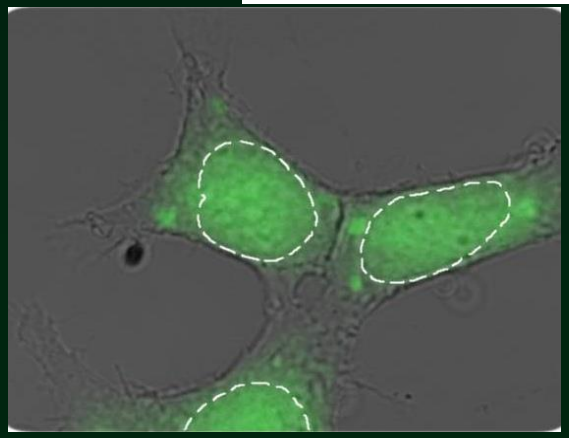
green-RNA

Paige et al. 2012

Other vegetables than SPINACH

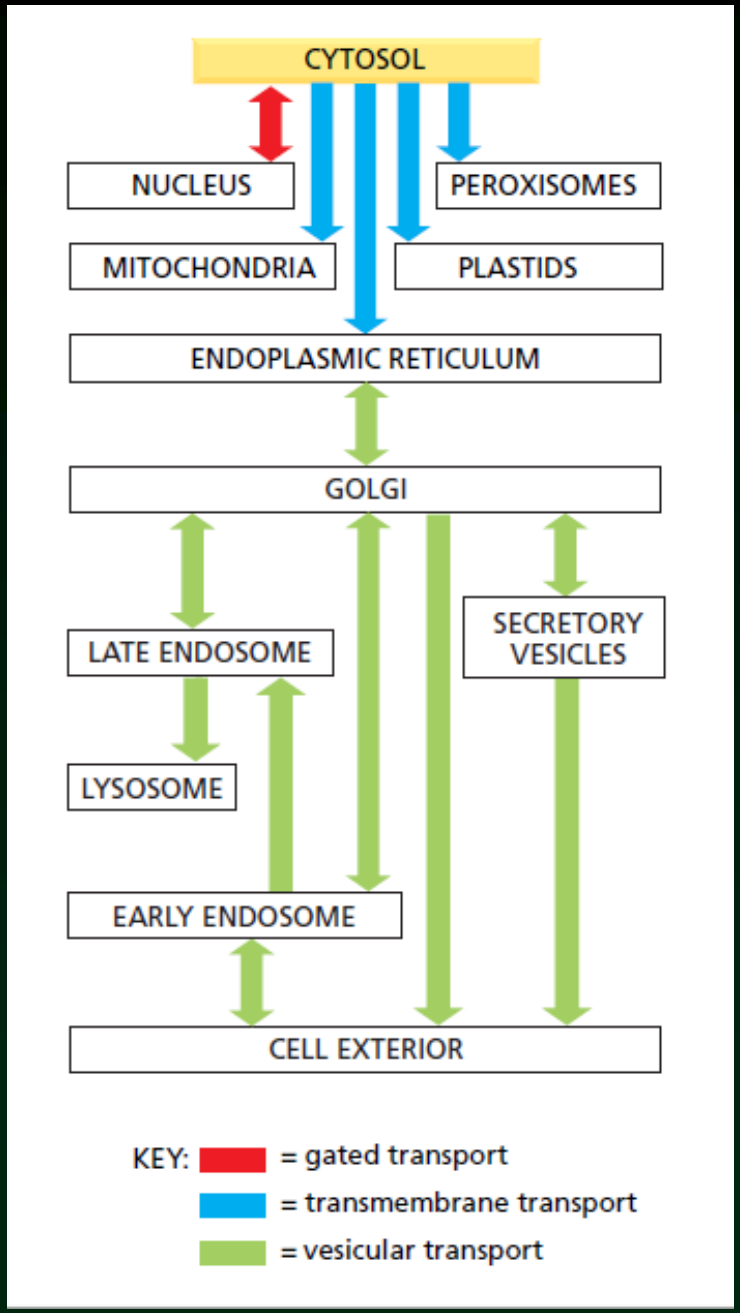






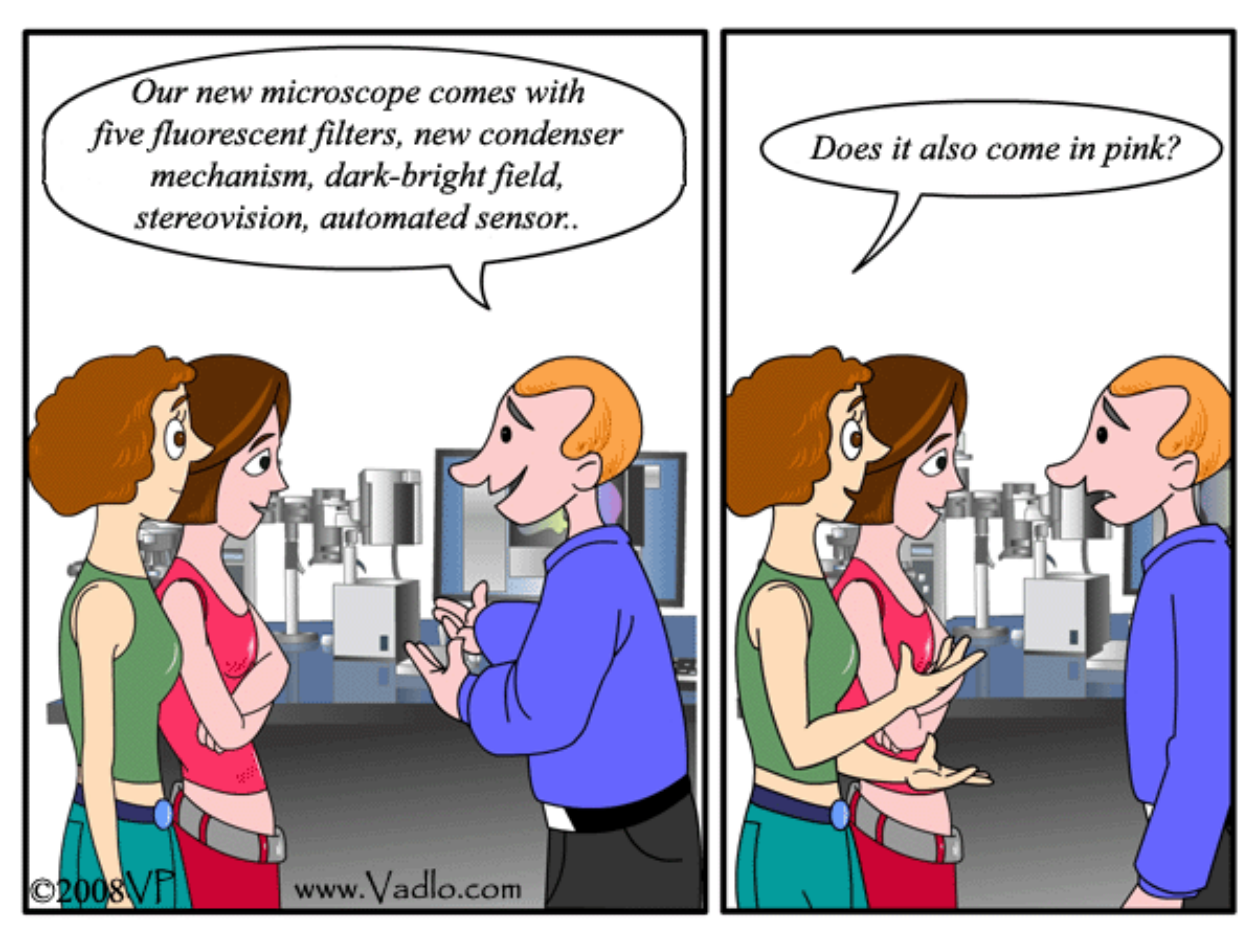
Paige et al. 2012; Song et al. 2014

Transport among compartments



Alberts et al. 2008

Advanced confocal techniques



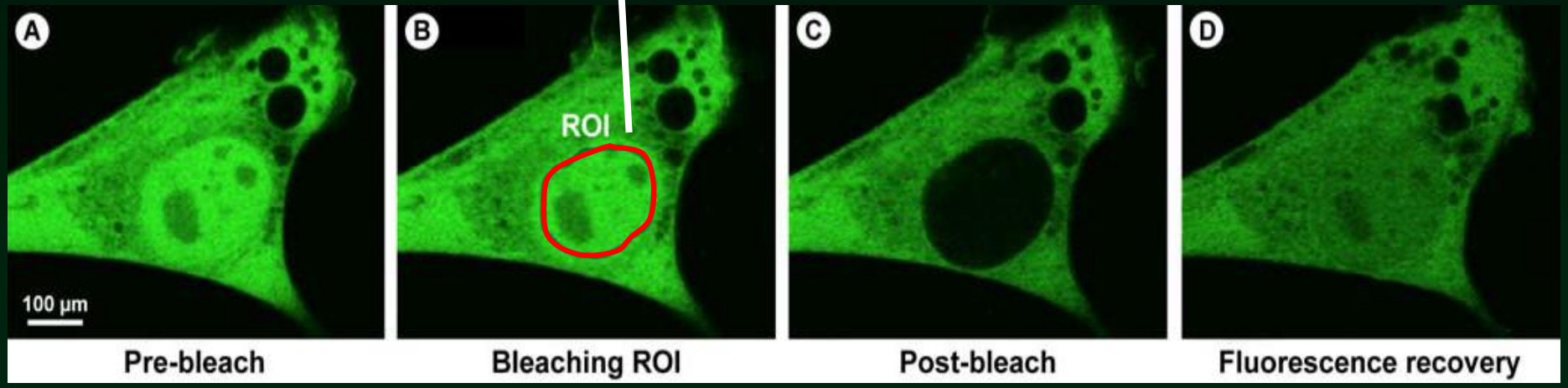
(slightly) Advanced confocal techniques

- FRAP
- photoactivatable FP
- FCS

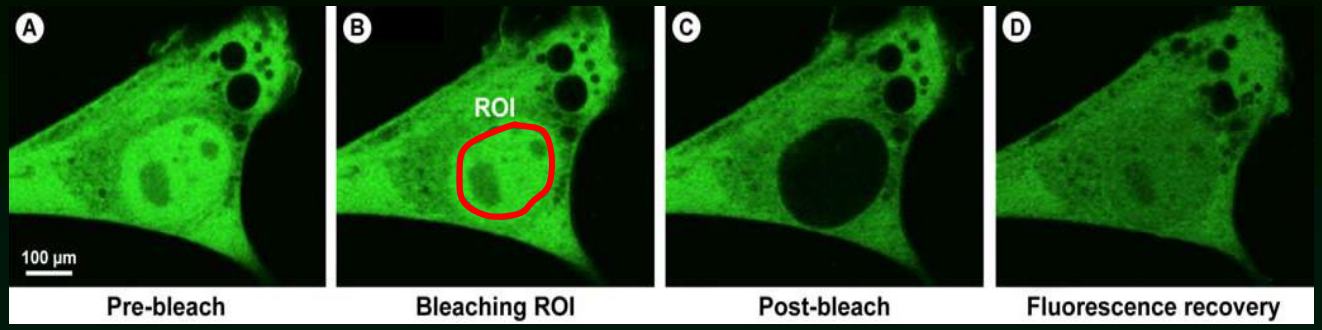
FRAP

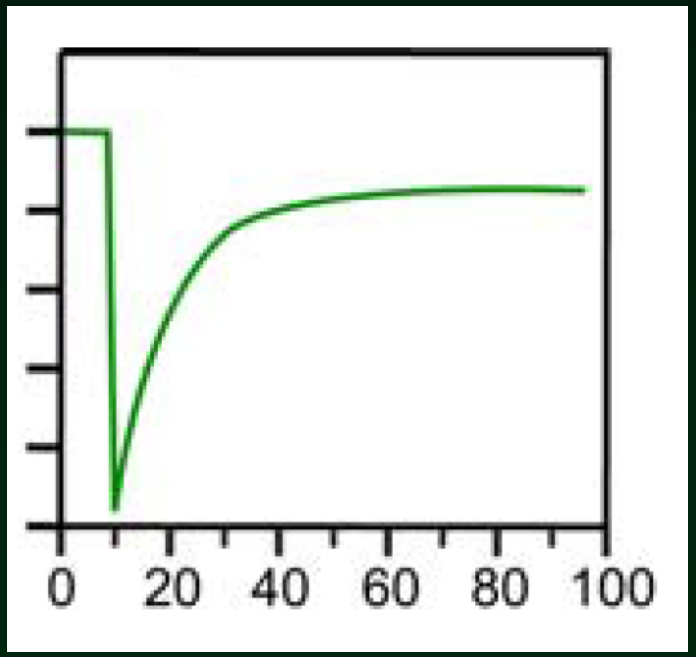
Fluorescence Recovery After Photobleaching

region of interest (ROI)

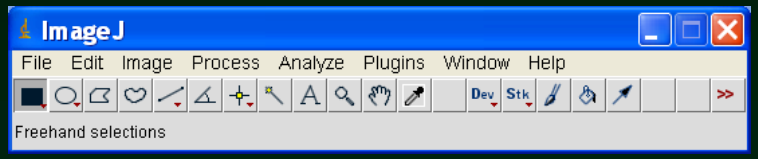


FRAP



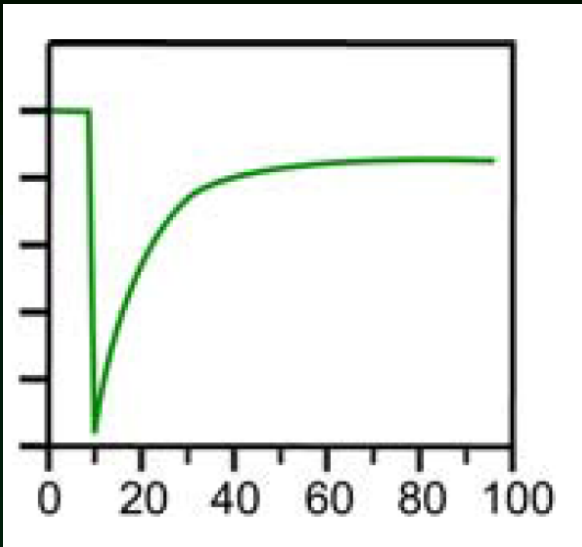


you can quantify fluorescence..
(ImageJ is our friend)

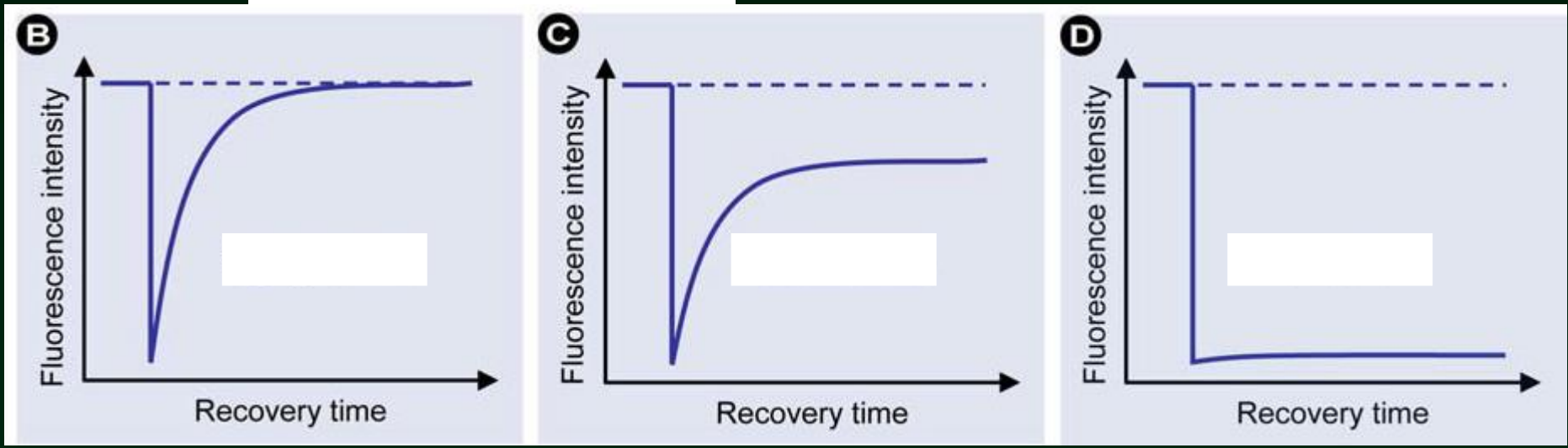


	mean	min	max
A	90.404	49	113
C	8.556	3	8
D	39.934	19	63

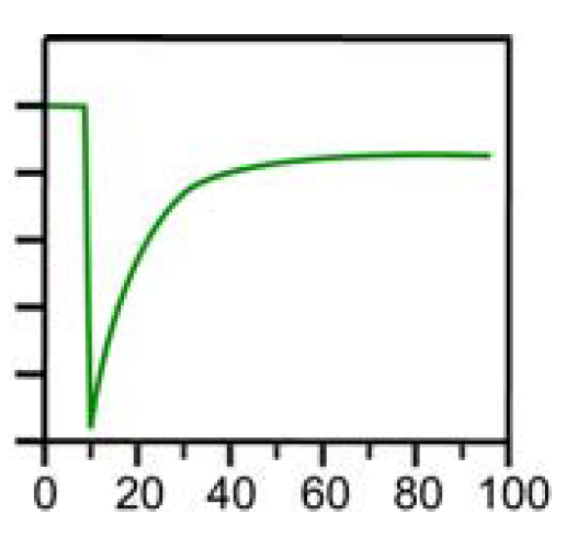
FRAP – bleaching curve

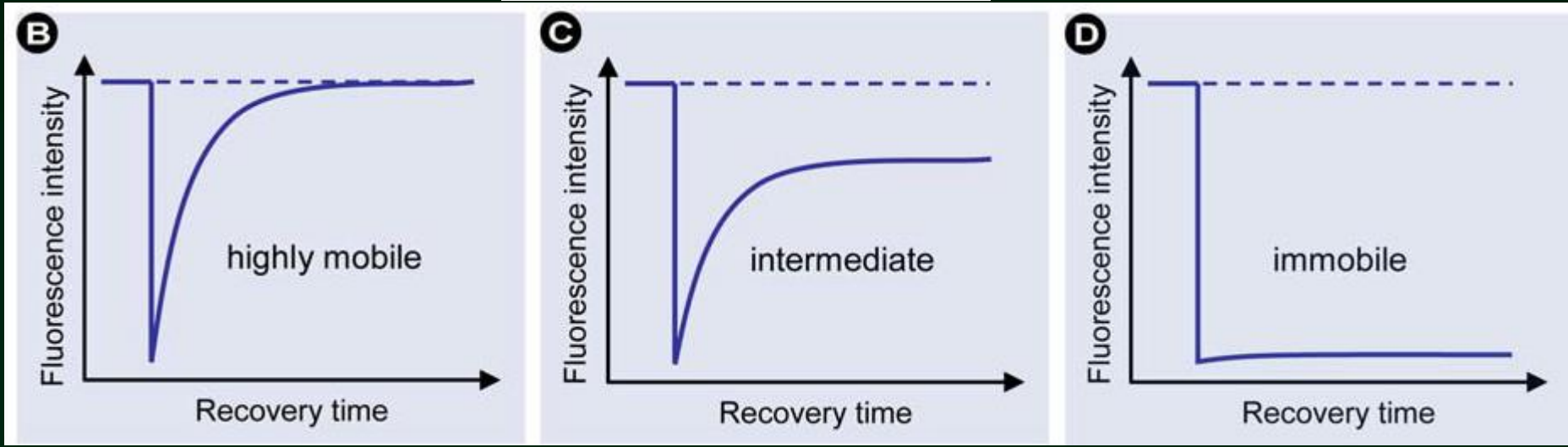


What does the curve tell?



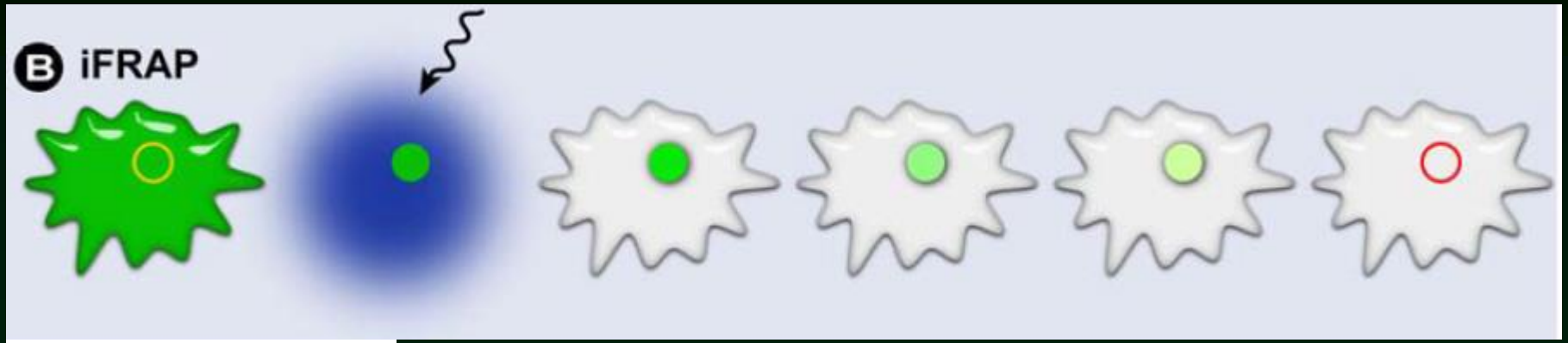
FRAP – bleaching curve

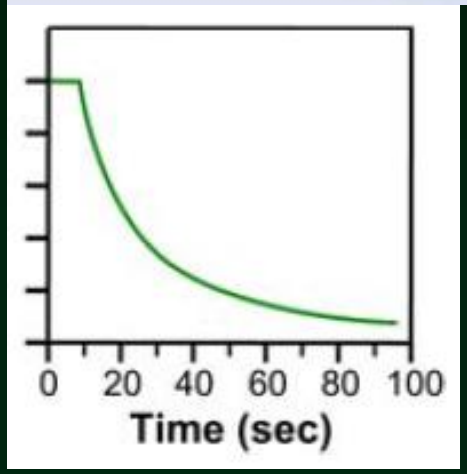




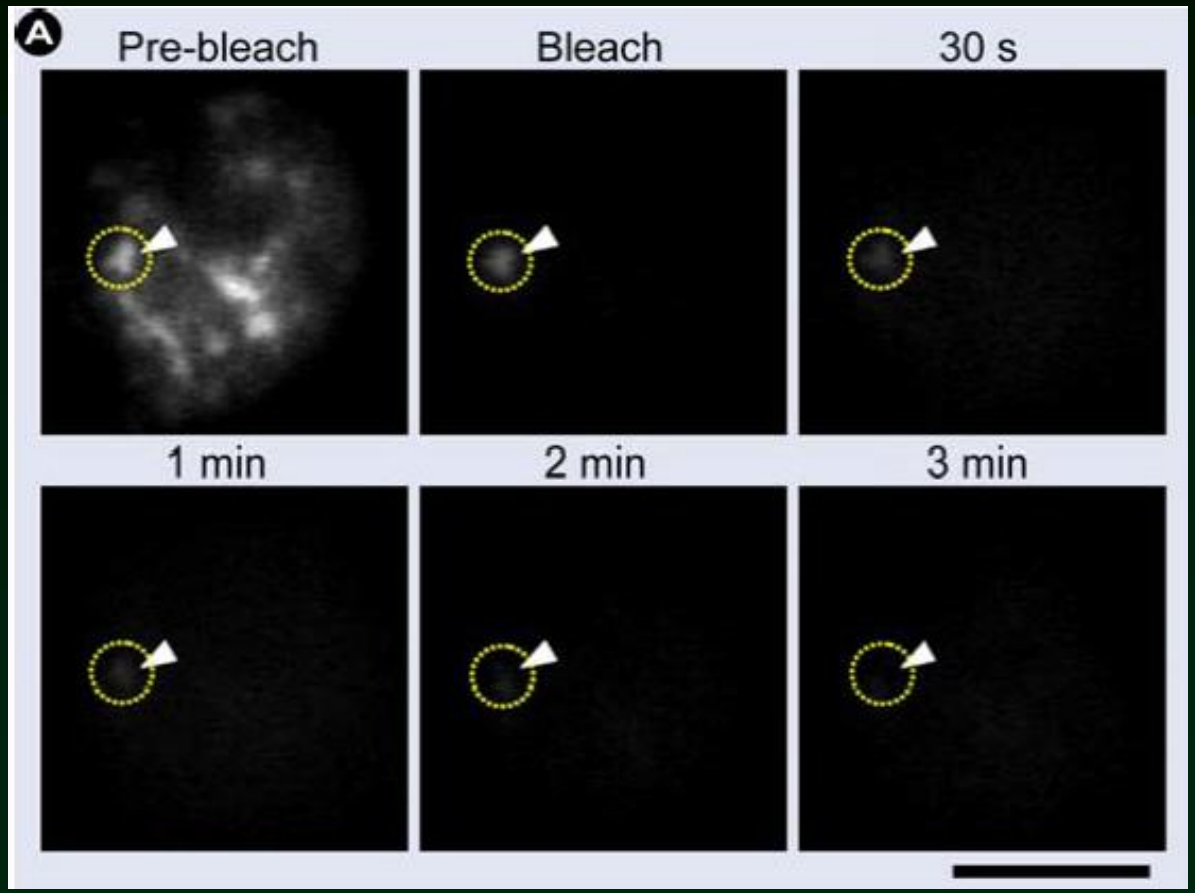
iFRAP

inverse FRAP





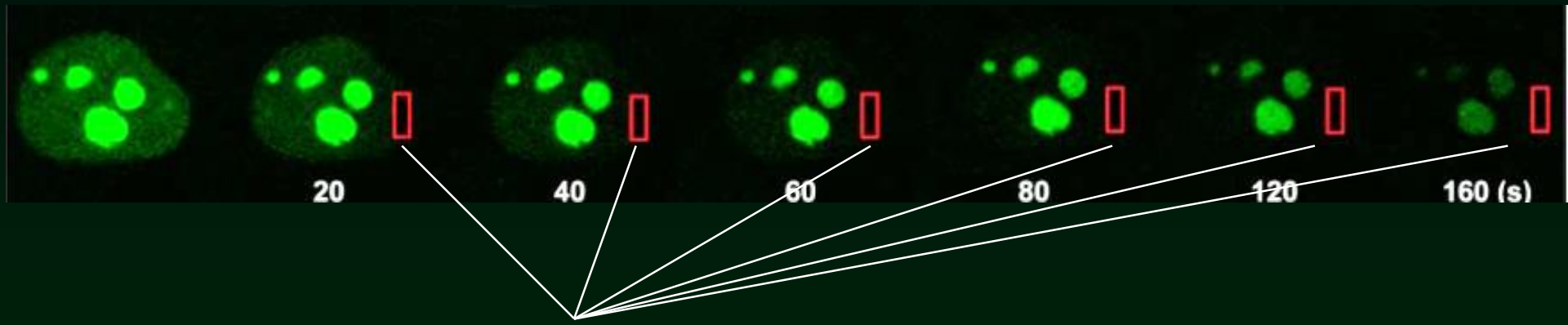
iFRAP – dissociation of premRNA from specles



FRAP derivatives

FLIP

Fluorescence Loss After Photobleaching



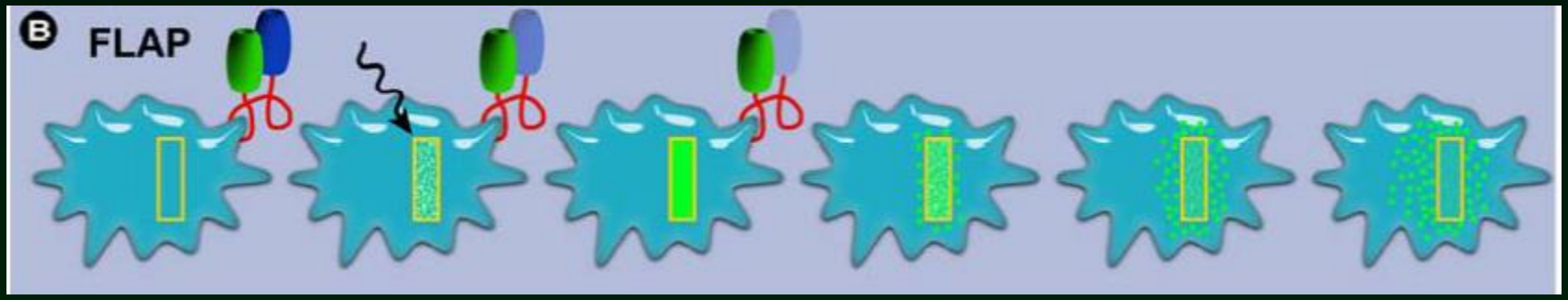
continuous bleaching here

- bleaching process is repeated during the experiment
- for studying general protein turnovers in compartments
- is there a fraction of protein which does not leave the bright green patches?

FRAP derivatives

FLAP

Fluorescence Localization after Photobleaching

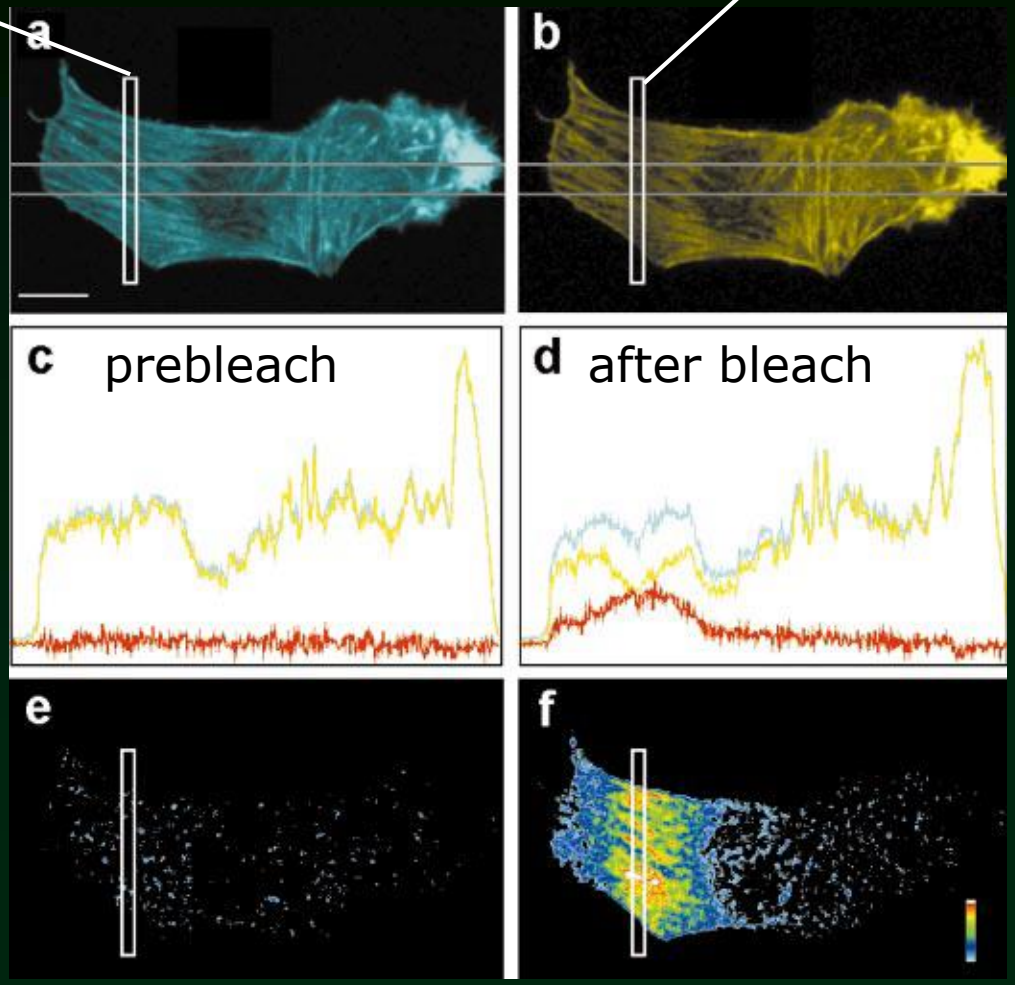


- two fluorochromes on one protein– one bleached, non bleached as control

Perhaps better scheme than previous

CFP not bleached

YFP bleached



RED=CFP-YFP

Dunn et al. 2002

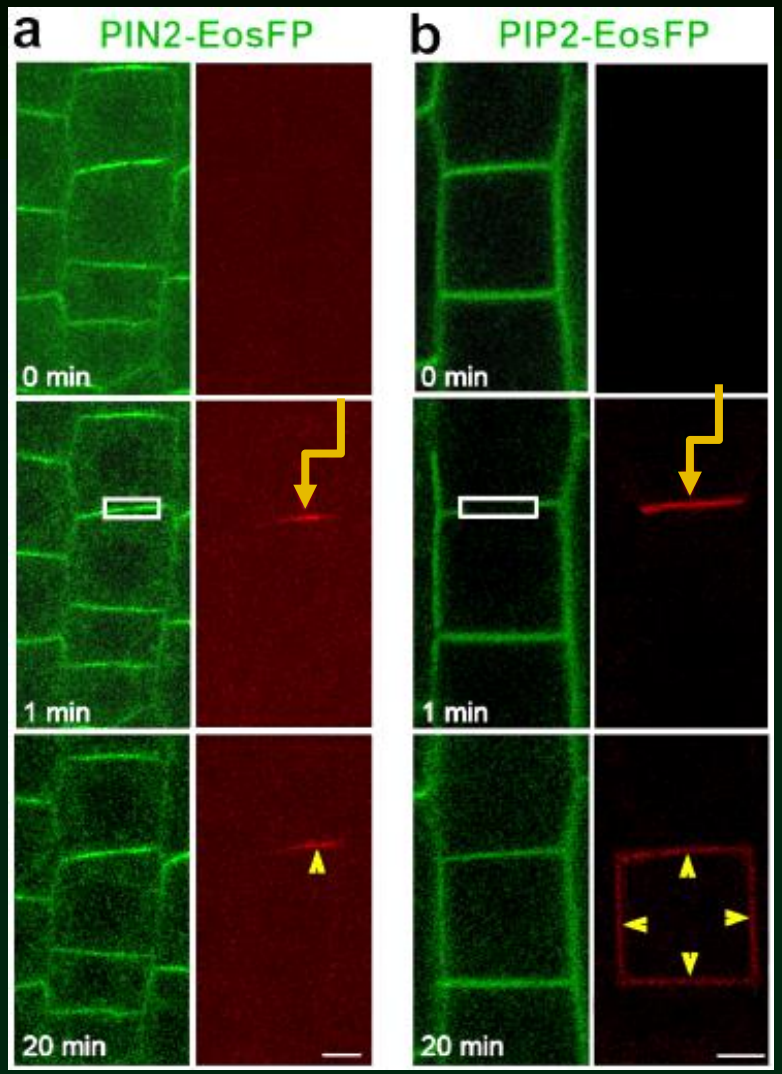
FRAP - advantages

- not only proteins (also other dyes)
- tells you more than simple life imaging movie

FRAP – pitfalls

- your cells are moving
- high energy needed to bleach the ROI
  - long time needed to bleach
  - can damage your material
- usually only one ROI can be observed – time consuming
- for gourmets perhaps awkward (although more reliable and robust)

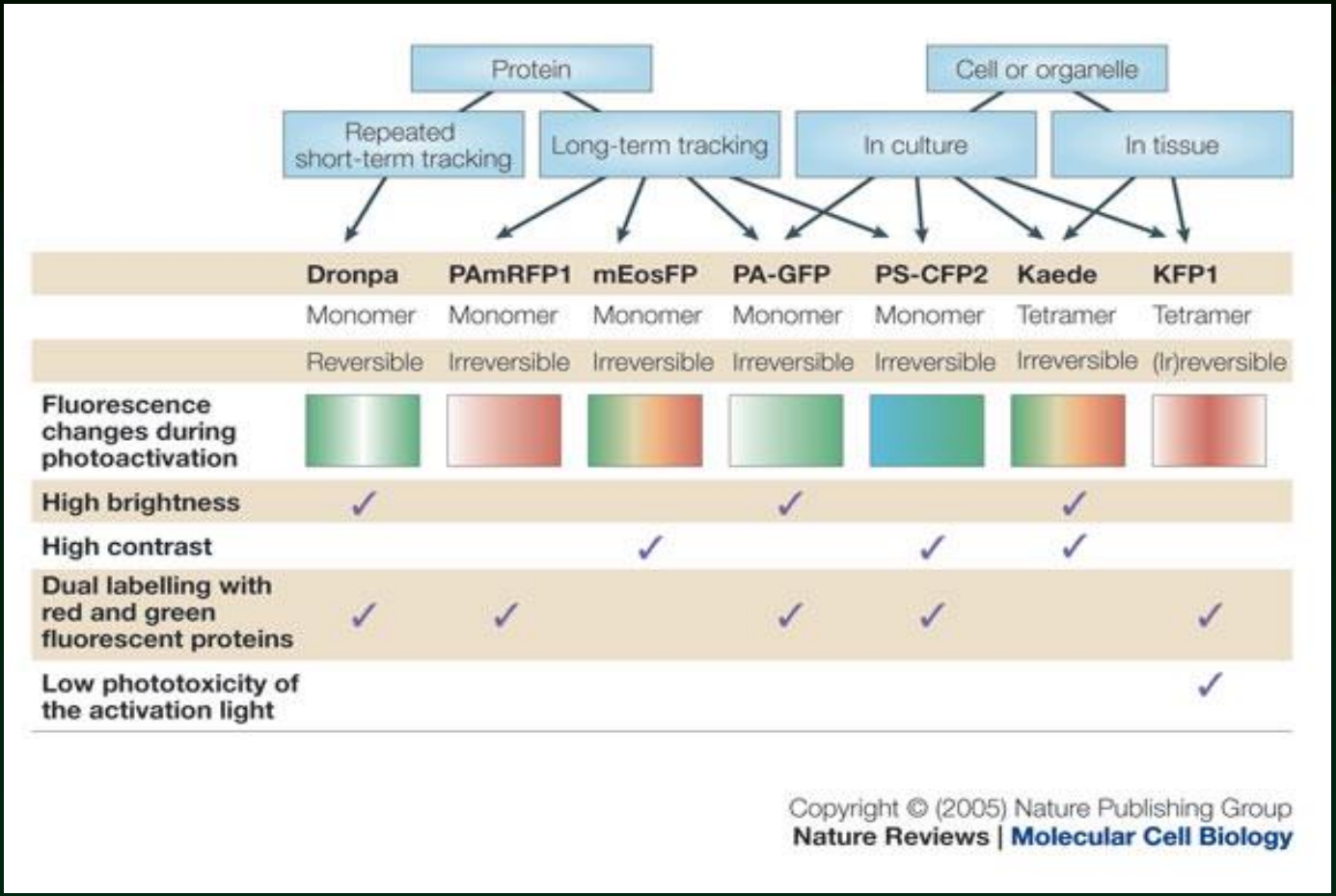
Photoactivable fluorescent proteins



photoactivation (UV)

aquaporin PIP2 undergoes lateral difussion

Photoactivable proteins



	Dronpa	PAmRFP1	mEosFP	PA-GFP	PS-CFP2	Kaede	KFP1
	Monomer	Monomer	Monomer	Monomer	Monomer	Tetramer	Tetramer
	Reversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	(Ir)reversible
Fluorescence changes during photoactivation							
High brightness	✓			✓		✓	
High contrast			✓		✓	✓	
Dual labelling with red and green fluorescent proteins	✓	✓		✓	✓		✓
Low phototoxicity of the activation light							✓

Dronpa, Kaede, Eos – probably most popular

Photoactivable proteins

Advantages:
-elegant, can be convincing

Disadvantages:
-very weak signal
-each material needs optimization

Remarks

- your material is 3D
- protein *de novo* synthesis in some experiments (e.g. cycloheximide stops translation)

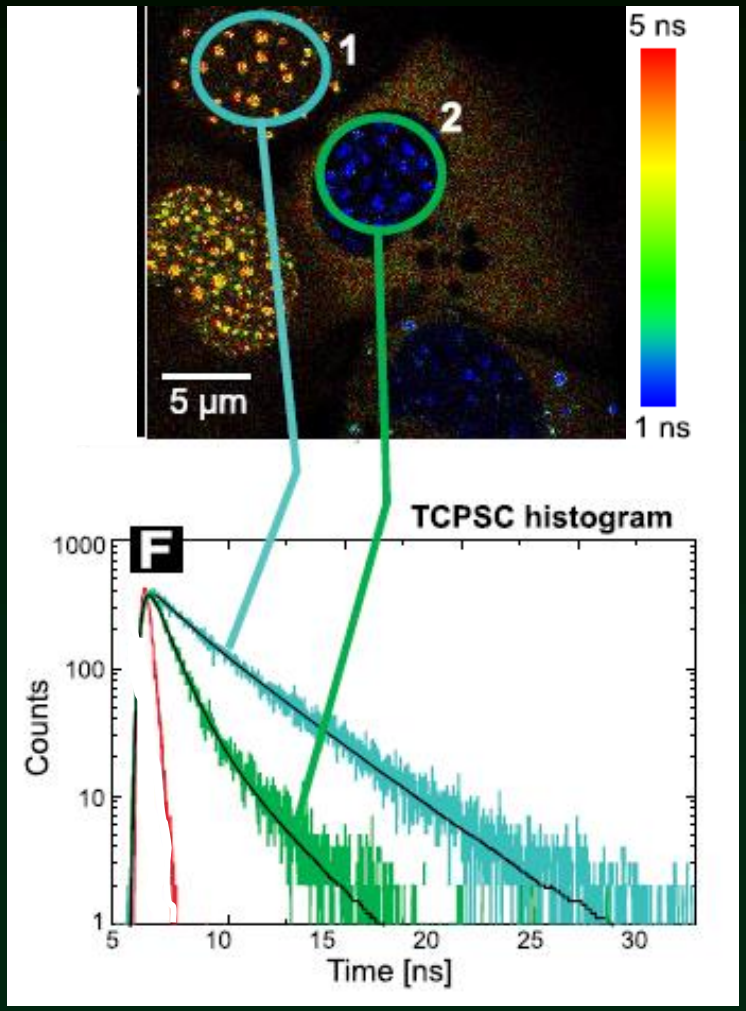
FLIM

Fluorescence Life Time Imaging Microscopy

Fluorochromes

- excitation spectra
- emission spectra
- unique lifetime

FLIM - applications



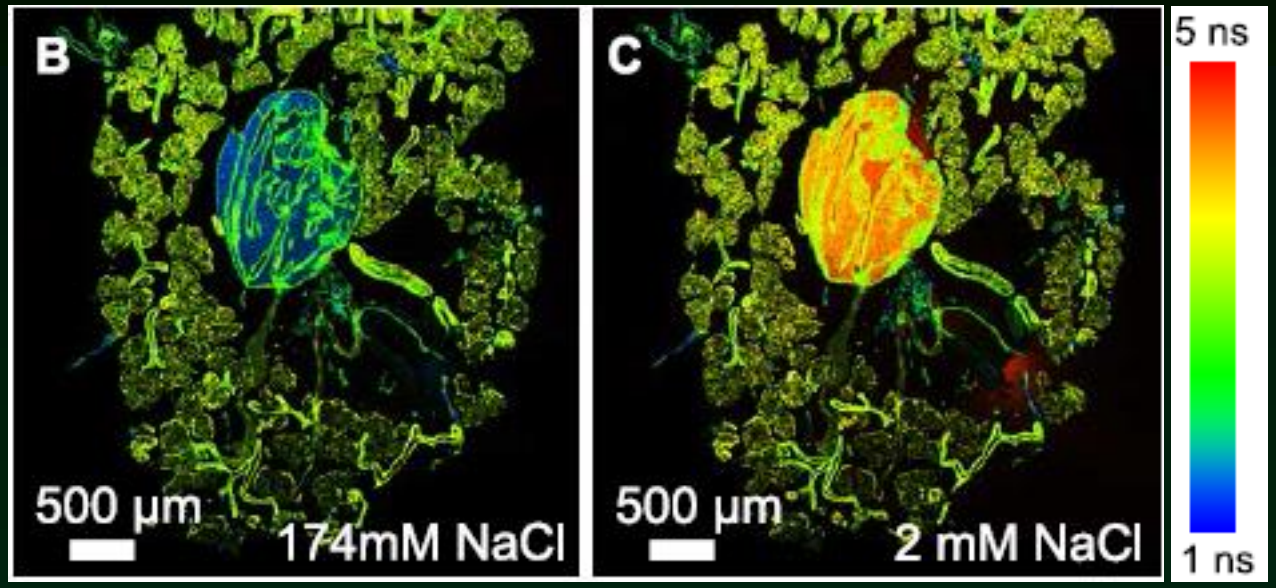
FLIM - applications

Lifetime sensitive to almost everything:

- pH
- ionic strength
- solution polarity
- other fluorochrome

Protein-protein interactions
(FRET-FLIM) (other lecture)

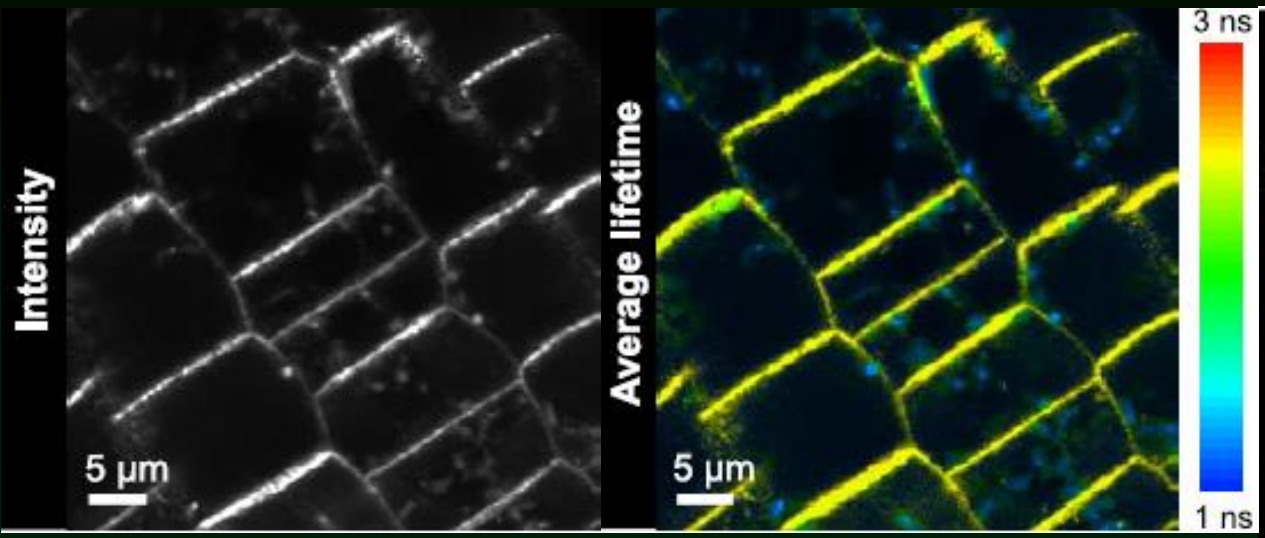
FLIM

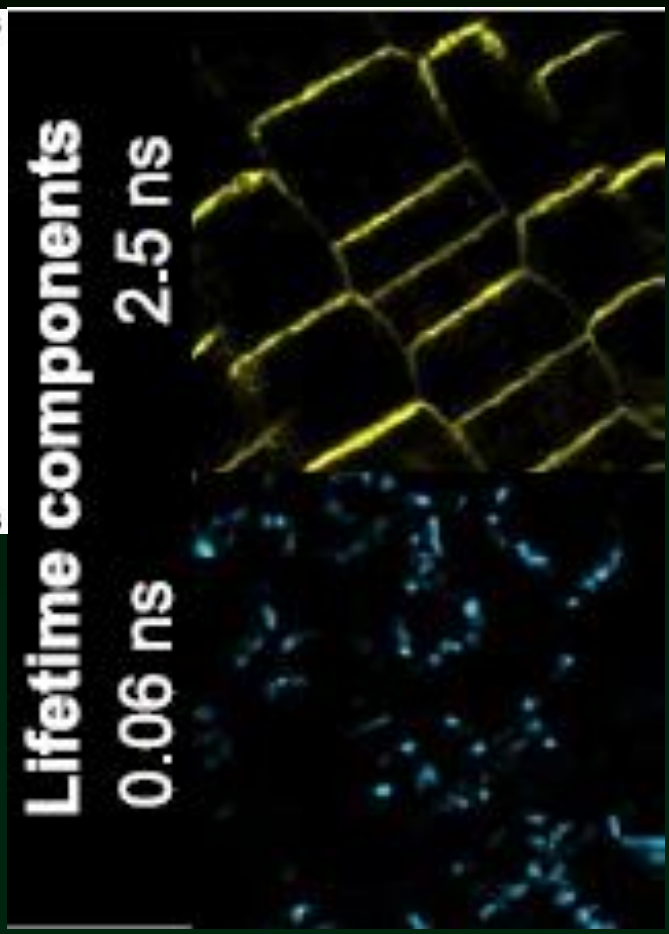


indeed, salt changes fluorophore life time
(American cockroach glands)

Trautmann et al. PicoQuant Application note 2013

FLIM - discrimination of autofluorescence

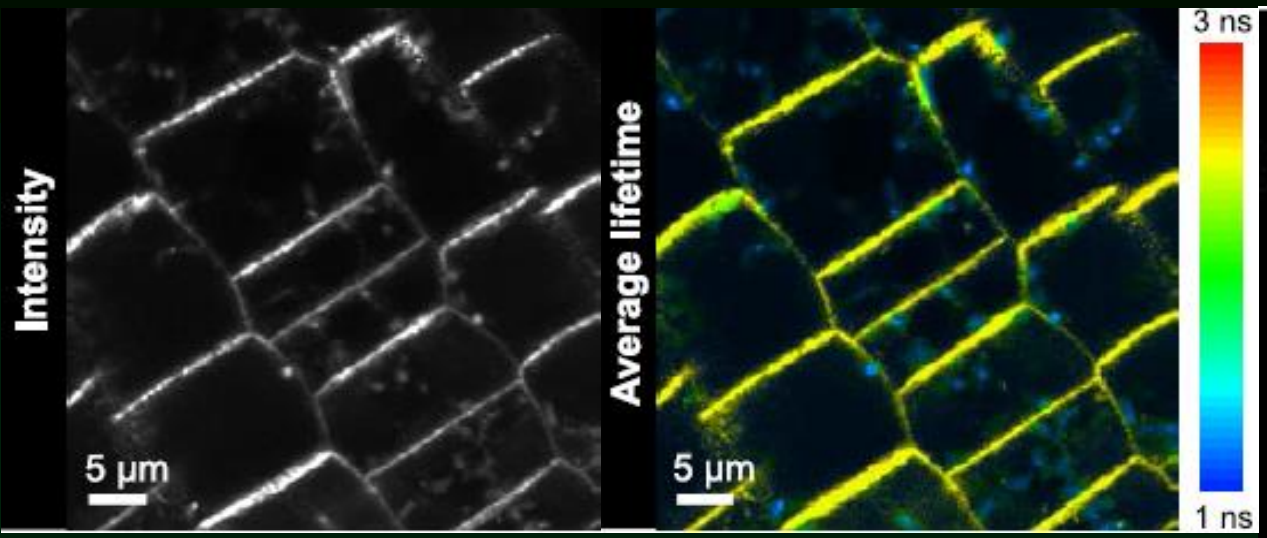


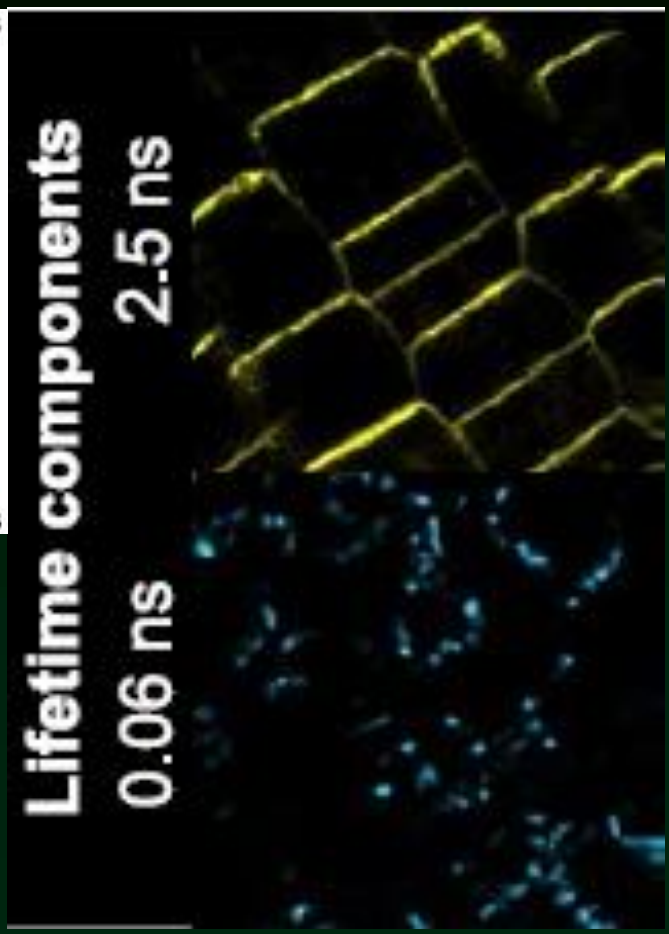


(be careful with the interpretation)

Dovzhenko, Trautmann, PicoQuant Application note 2013

FLIM - discrimination of autofluorescence





Q: What is easier experiment to confirm autofluorescence?

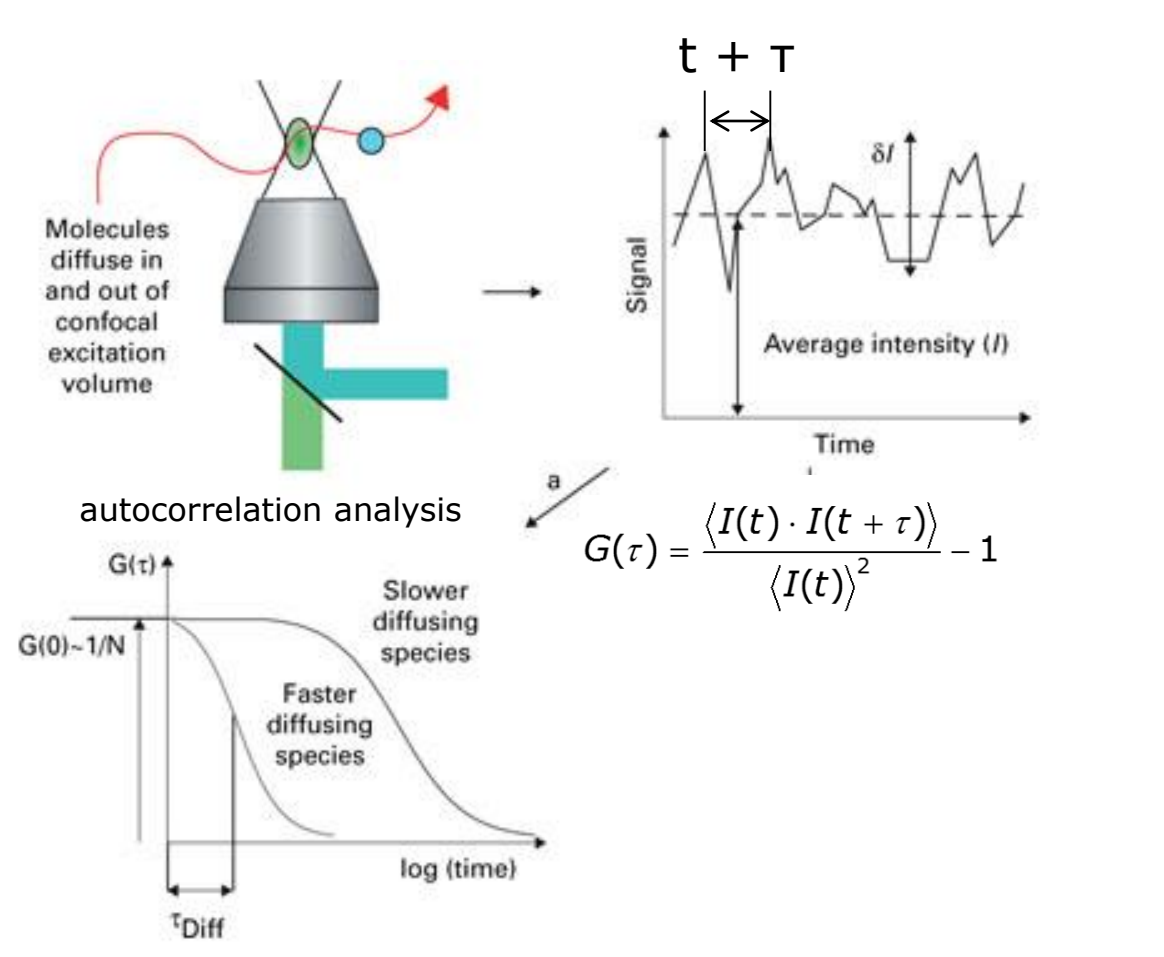
Dovzhenko, TrautmannPicoQuant Application note 2013

FLIM

- need to have experience
- need to have special module on your confocal

FCS

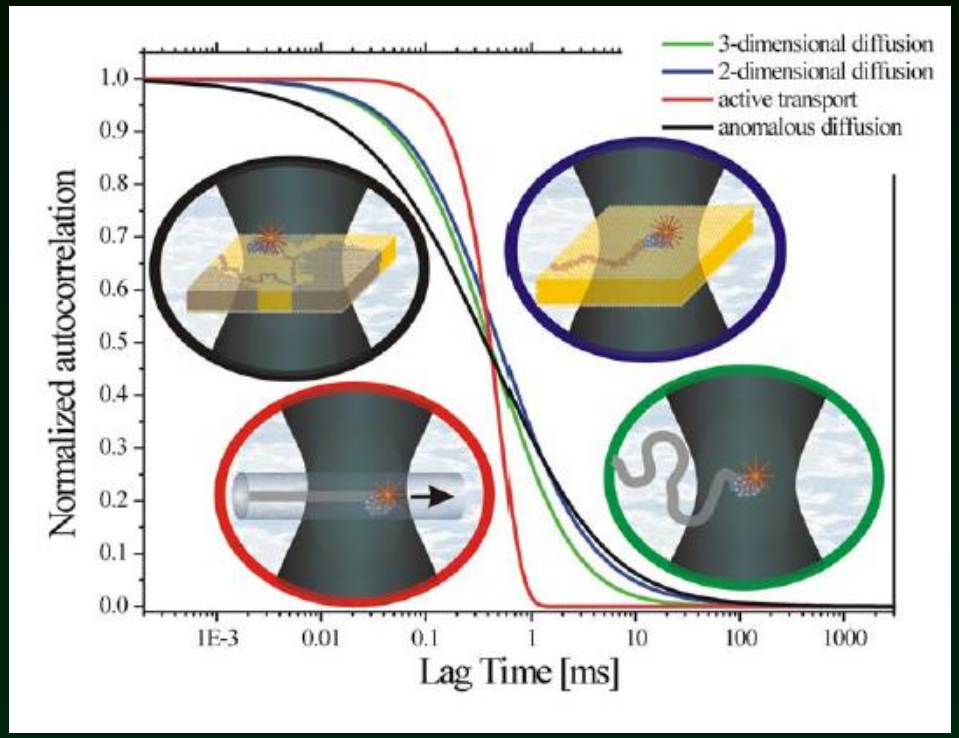
Fluorescence Correlation Spectroscopy



It is counted, how many times the fluorescent molecule comes through the focal plane.

Autocorrelation analysis: the way how to discriminate the diffusions speeds of particles.

FCS

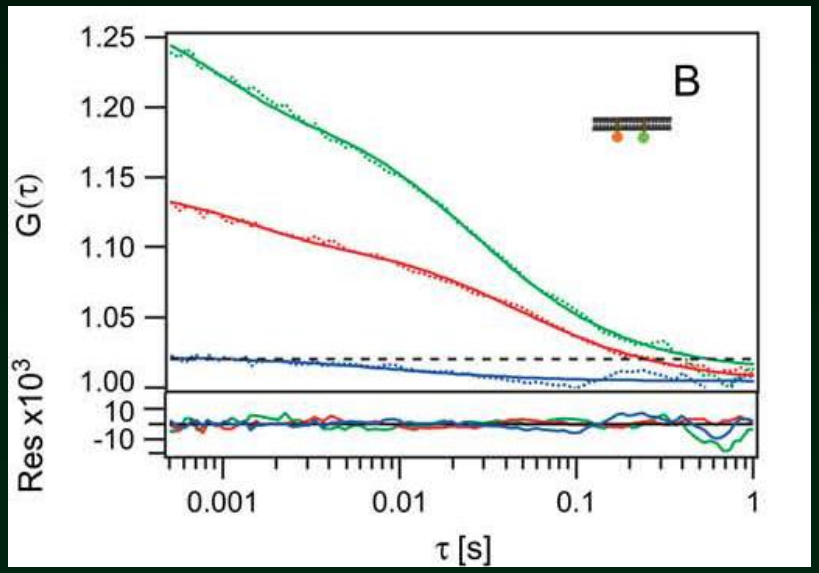


Schwille und Haustein

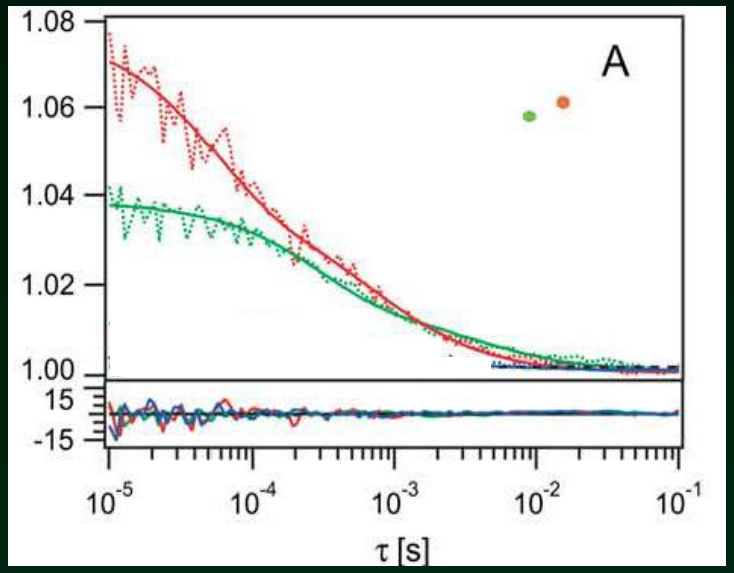
FCS (FCCS)

fluorescence cross-correlation spectroscopy

$$G_{CC}(\tau) = \frac{\langle I_A(t) \cdot I_B(t+\tau) \rangle}{\langle I_A(t) \rangle \langle I_B(t) \rangle} - 1$$

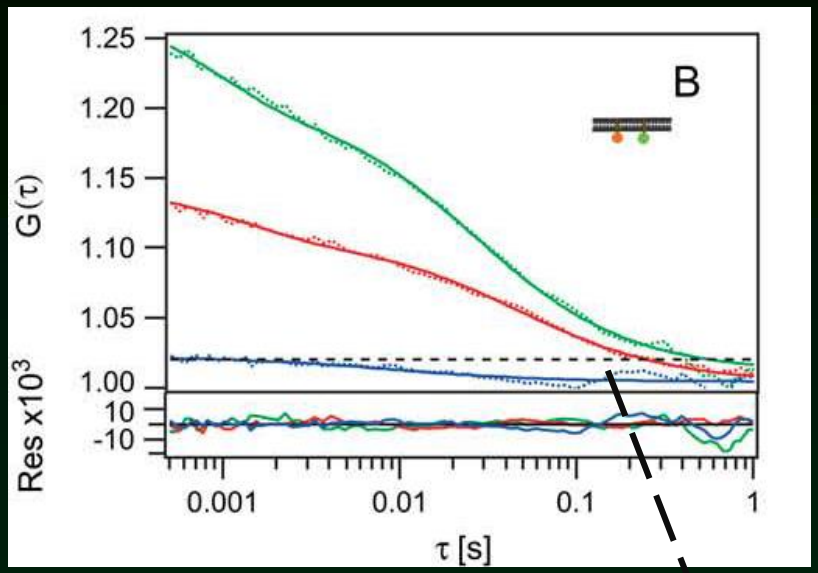


control membrane bound GFP and RFP (crosscorrelation curve)



free GFP and RFP

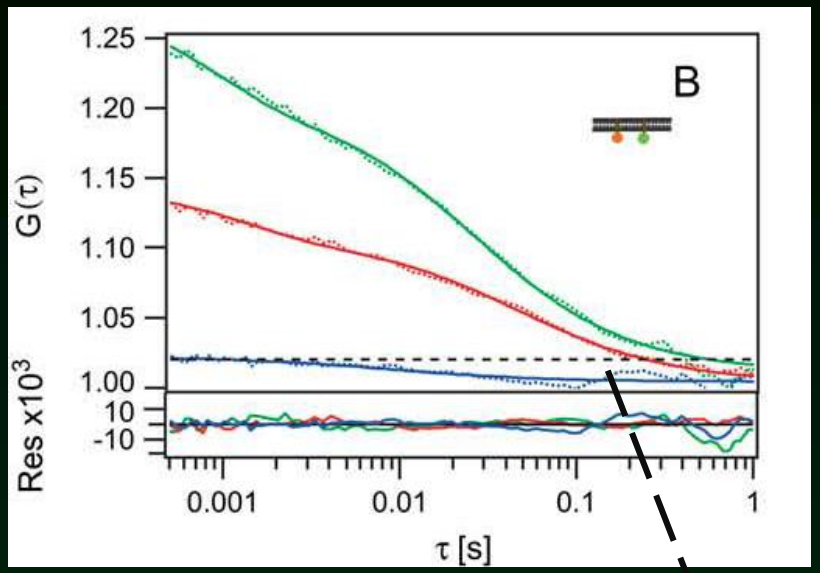
FCCS



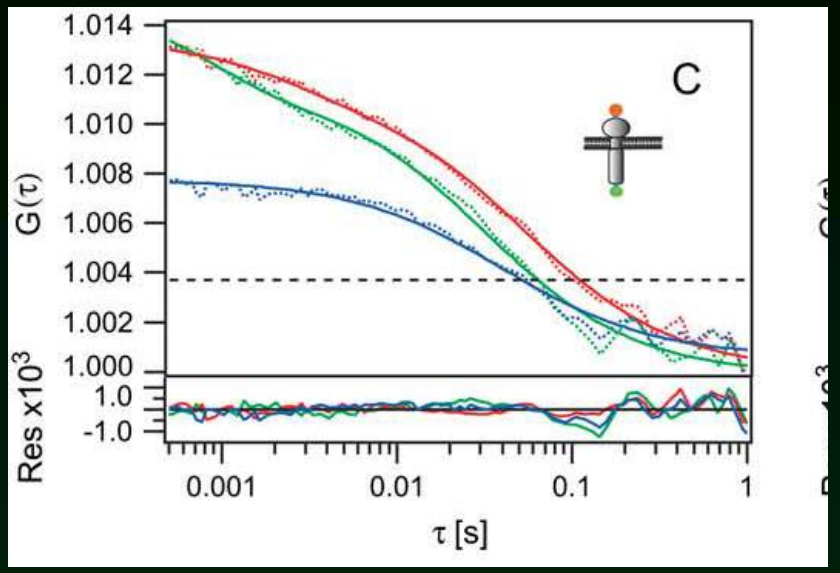
control membrane bound
GFP and RFP
(crosscorrelation curve)

channel crosstalk threshold

FCCS



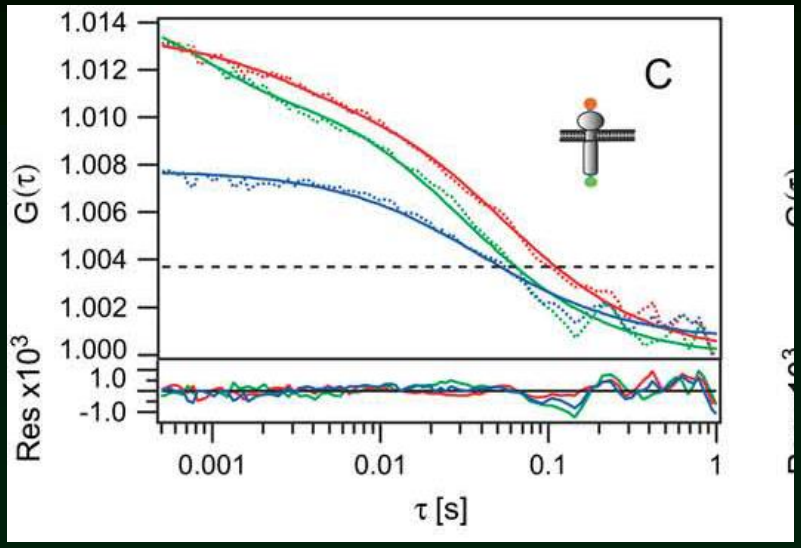
control membrane bound GFP and RFP (crosscorrelation curve)



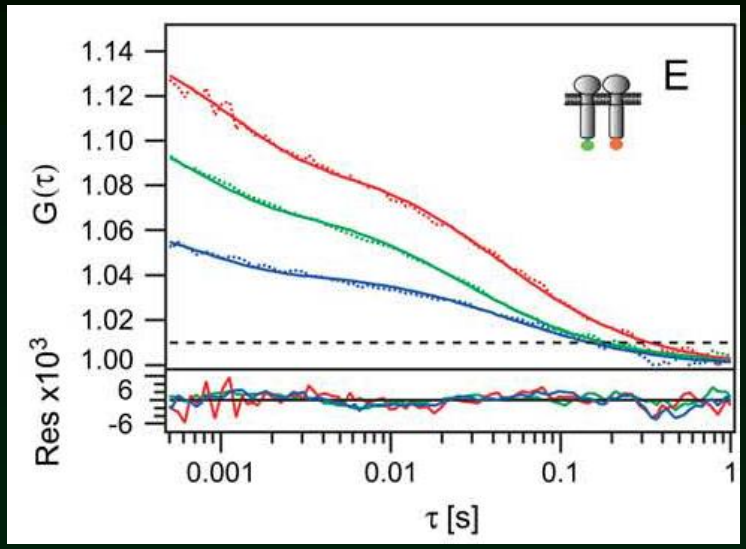
receptor with two labels

channel crosstalk threshold

FCCS



receptor with two labels



the crosscorelation curve is above threshold -> EGFR protein dimerizes

Liu et al., 2007

FCS

- special confocal module and objectives needed
- interpretation tricky

Literature

- Paige et al., RNA Mimics of Green Fluorescent Protein, Science 333, 642-646, 2011 (SPINACH and other vegetables)
- https://www.micro-shop.zeiss.com/index.php?s=452278238ae52c&l=en&p=de&f=f&a=d (comprehensive and broad list of phluorochromes)
- http://www.illuminatedcell.com/ - nicely done pages about plant cell imaging
- Ishikawa-Ankerhold et. al. Advanced Fluorescence Microscopy Techniques — FRAP, FLIP, FLAP, FRET and FLIM, Molecules 2012, 17, 4047-4132
- Sambrook & Russell Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press; 3rd edition (January 15, 2001), 13.5 pounds weight
- Ctirad Hofr – Pokročilé biofyzikální metody v experimentální biologii (přednáška)