# How to visualize genes and their products

Genomics Lecture Series

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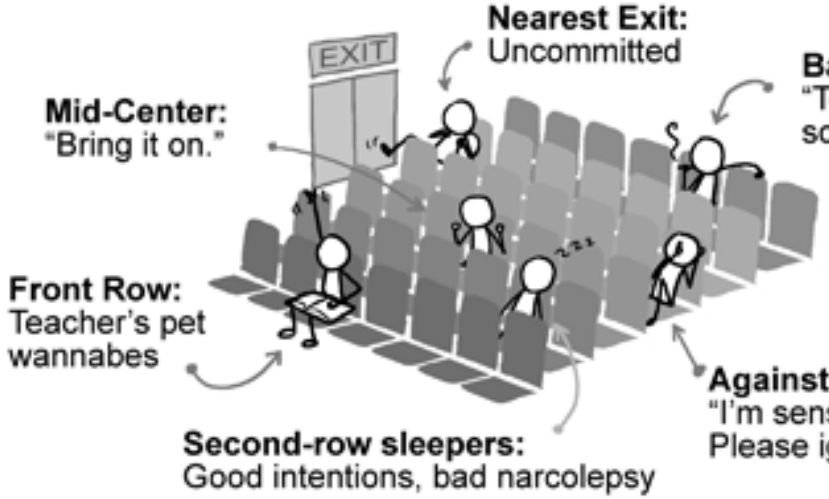
# Outline

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

# WHERE YOU SIT IN CLASS/SEMINAR

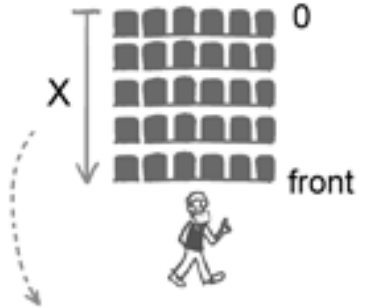
And what it says about you:




WWW.PHDCOMICS.COM

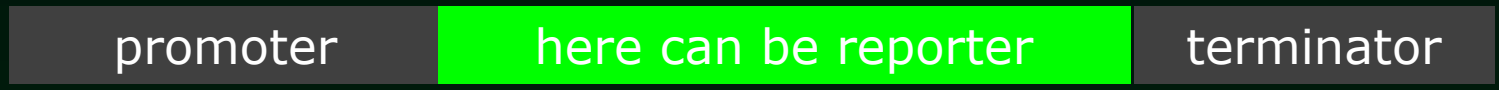
JORGE CHAM © 2008

**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

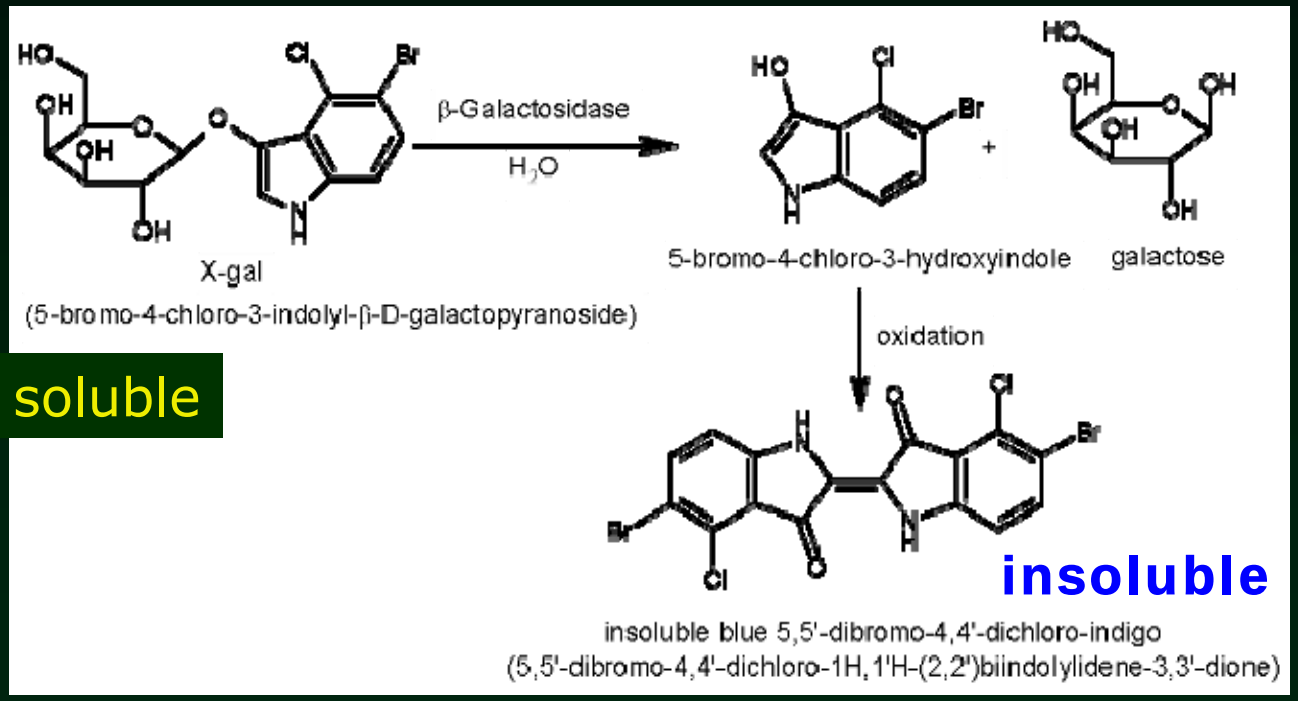
# Reporter genes

- LacZ, GUS
- Luciferase
- GFP

some need external substrate, some not

# LacZ, GUS – rhapsody in blue

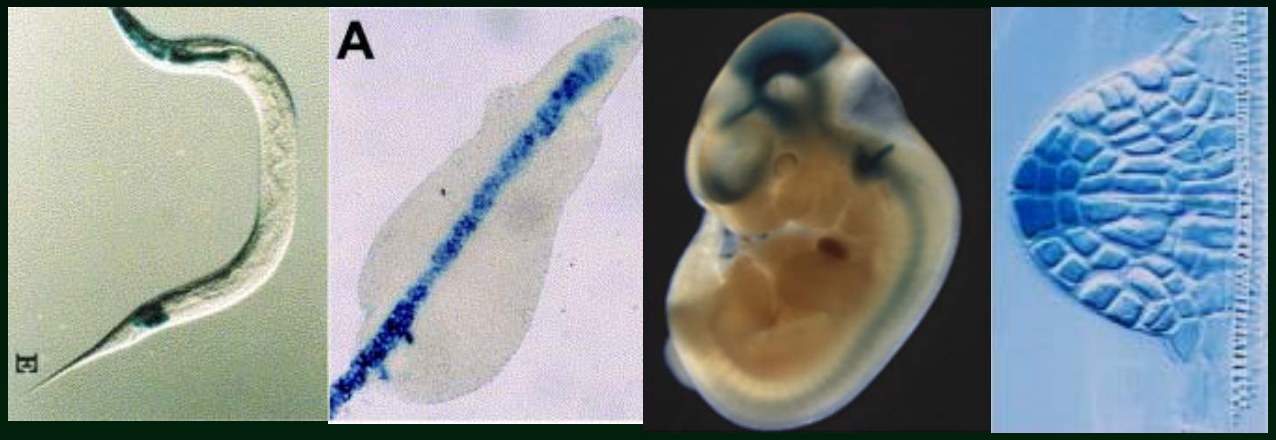
| promoter | LacZ | terminator |
|---|---|---|




(in case of GUS – X-Gluc)

# LacZ, GUS

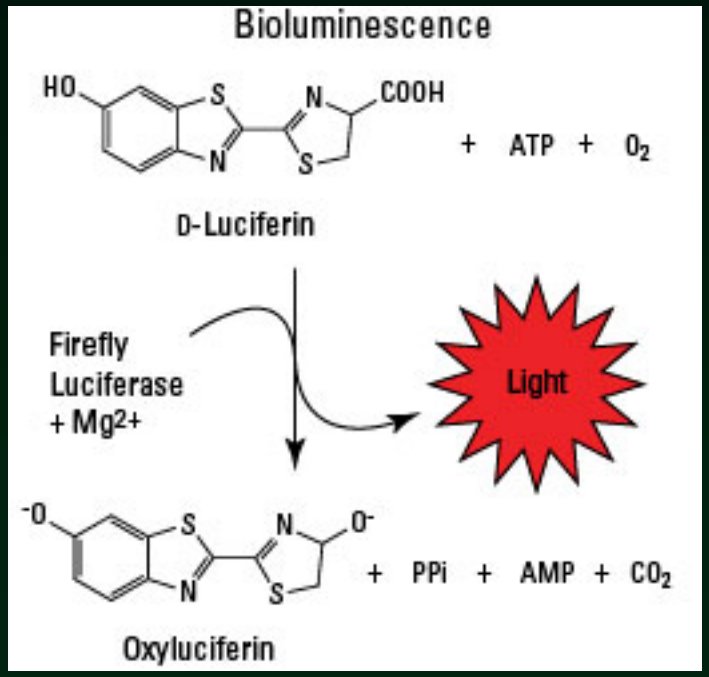
LacZ/ GUS:




worm, mouse – LacZ, plants - GUS

# Luciferase

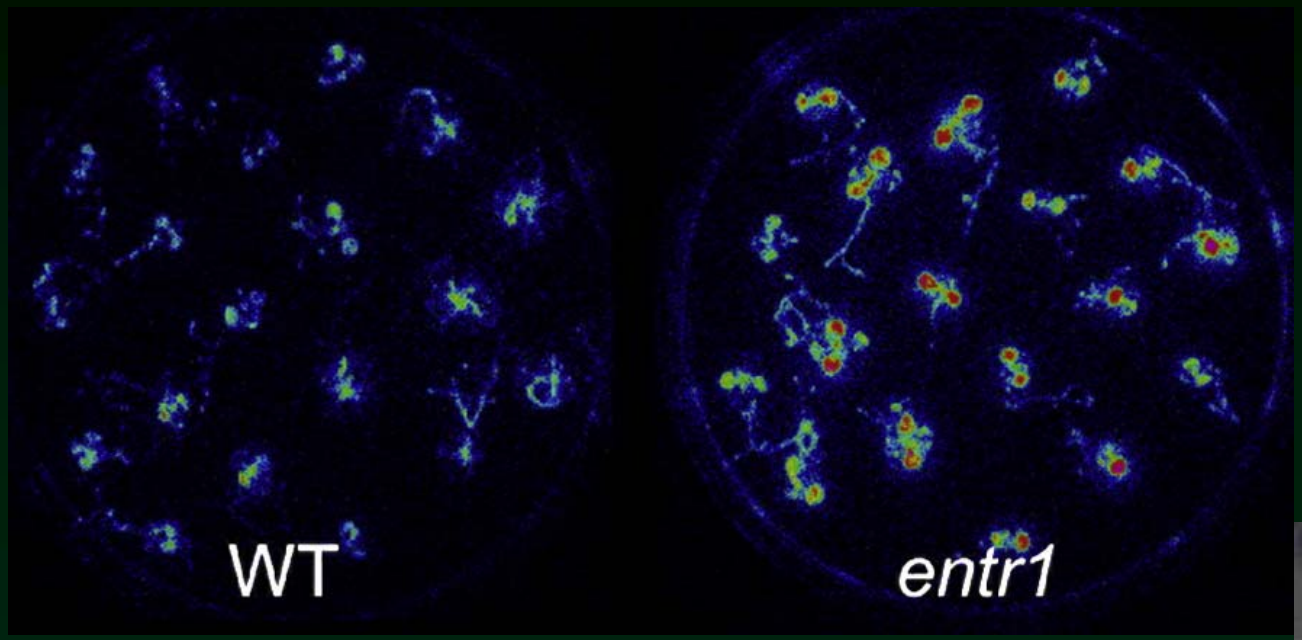
| promoter | luciferase | terminator |
|---|---|---|

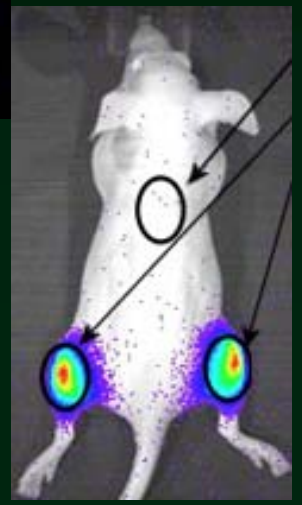



(principle of chemiluminiscence)

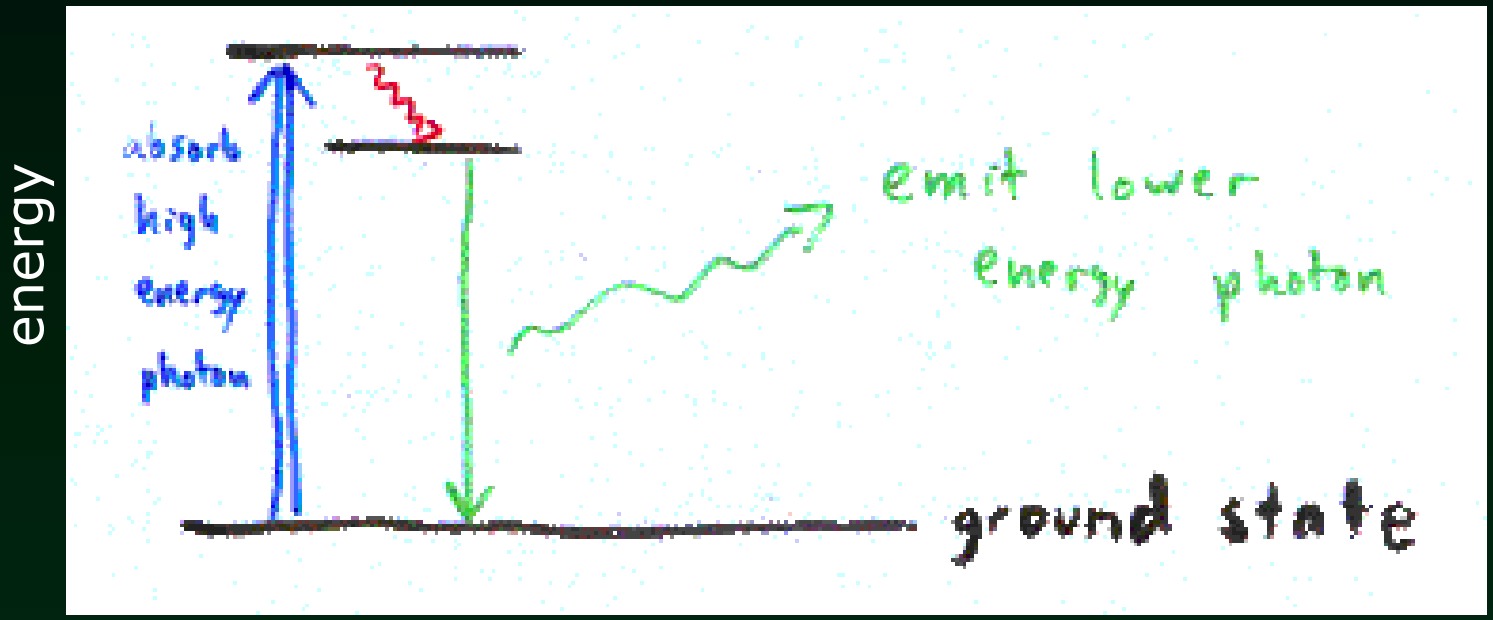
What's difference between flurescence and luminiscence?

# Luciferase

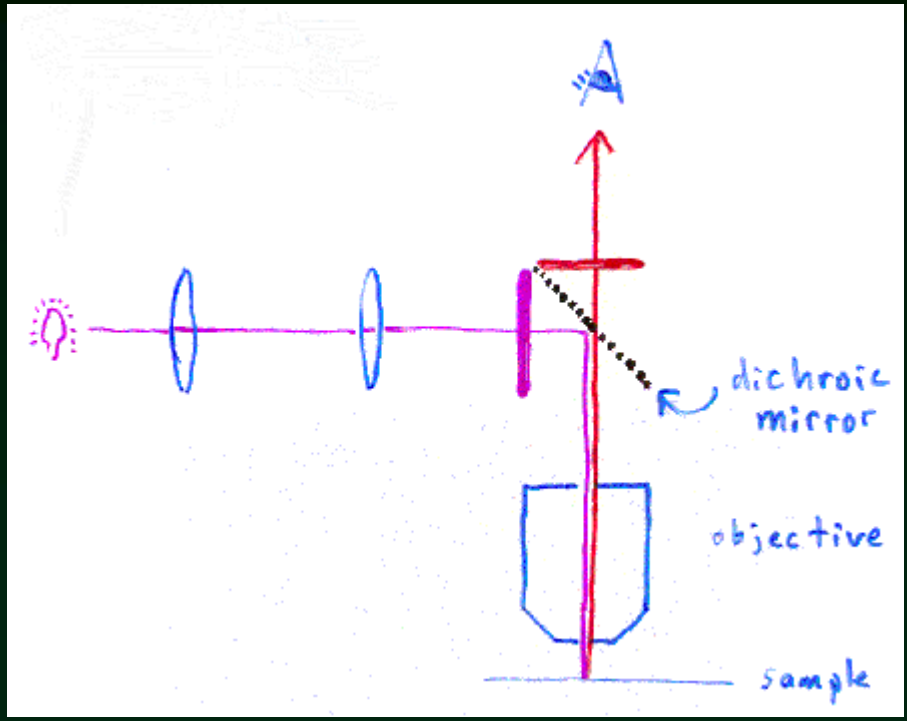


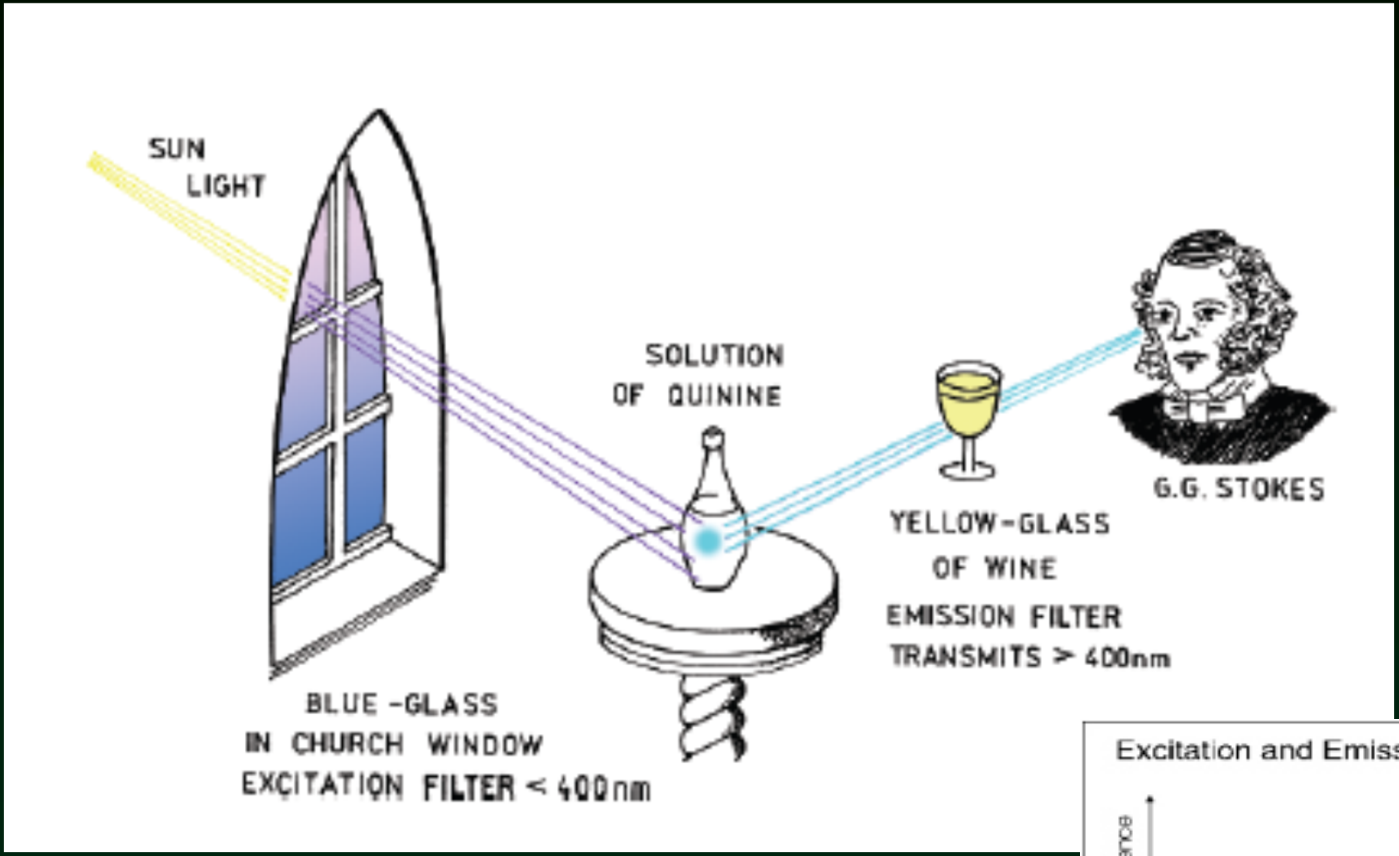
# How does fluorescence work?

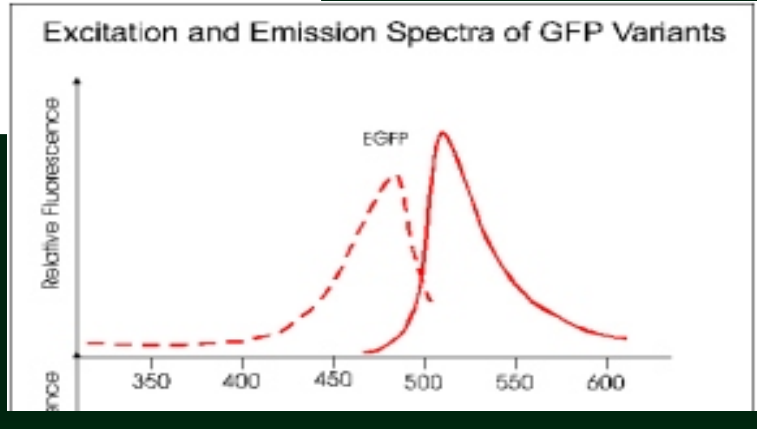


# How does a fluorescence microscope work?



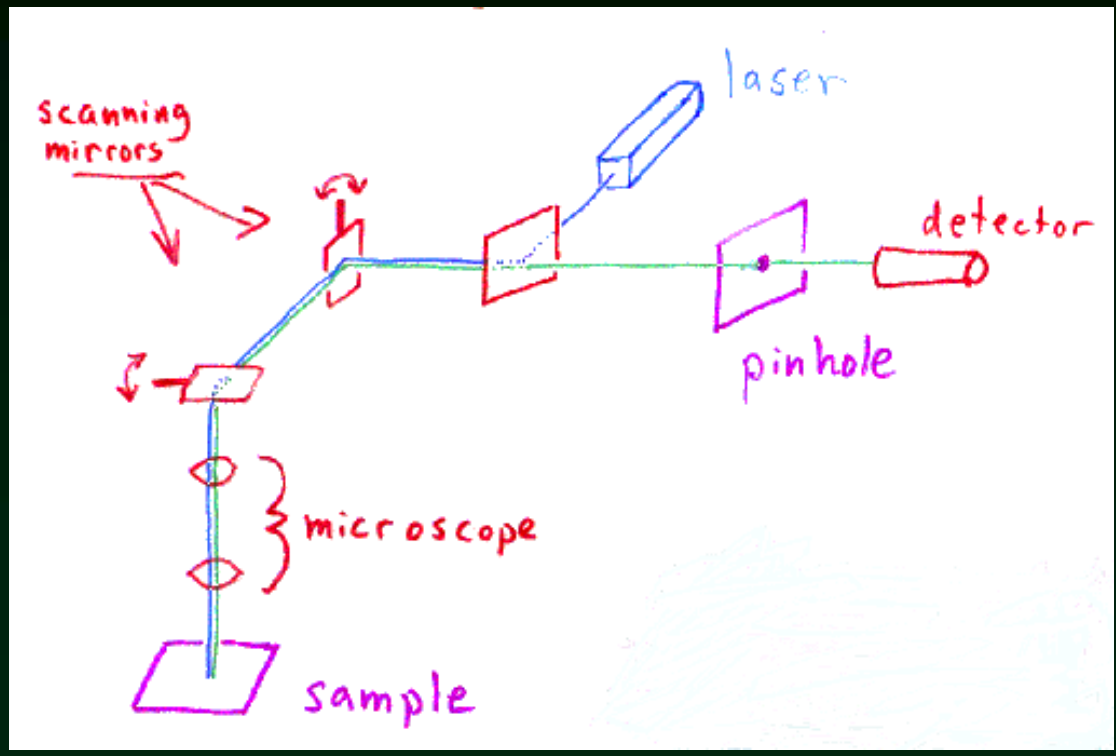
# Stokes shift




George G. Stokes

# How does a confocal microscope work?



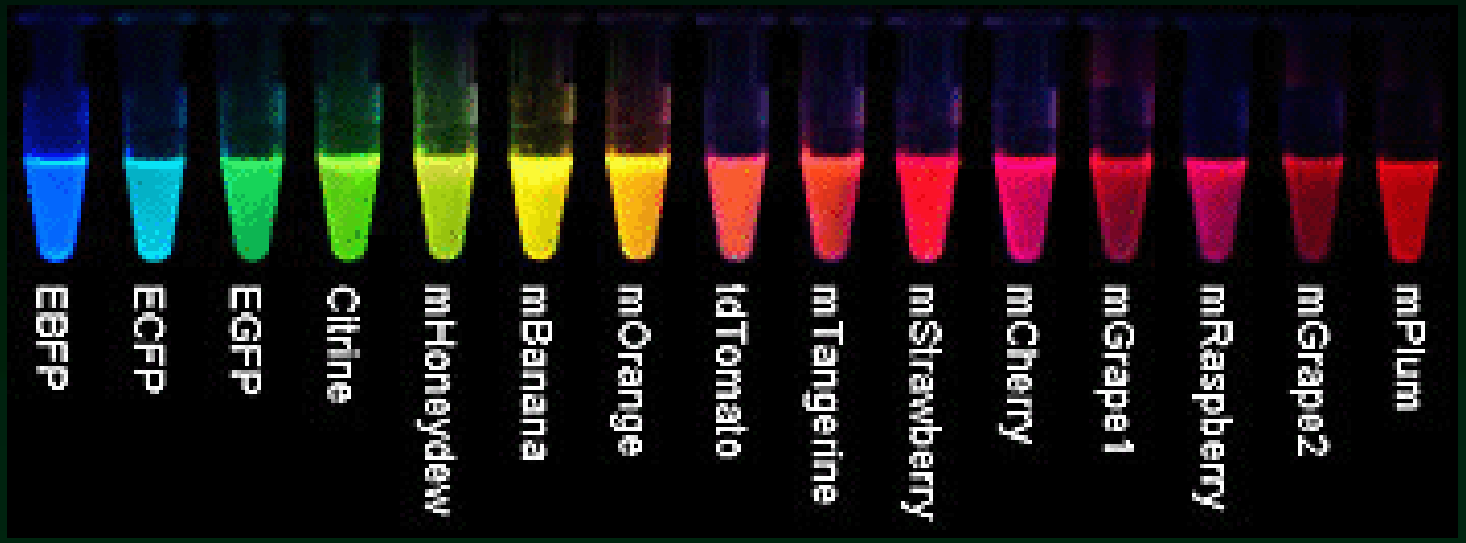

What are advantages of confocal microscopy?

# Live imaging

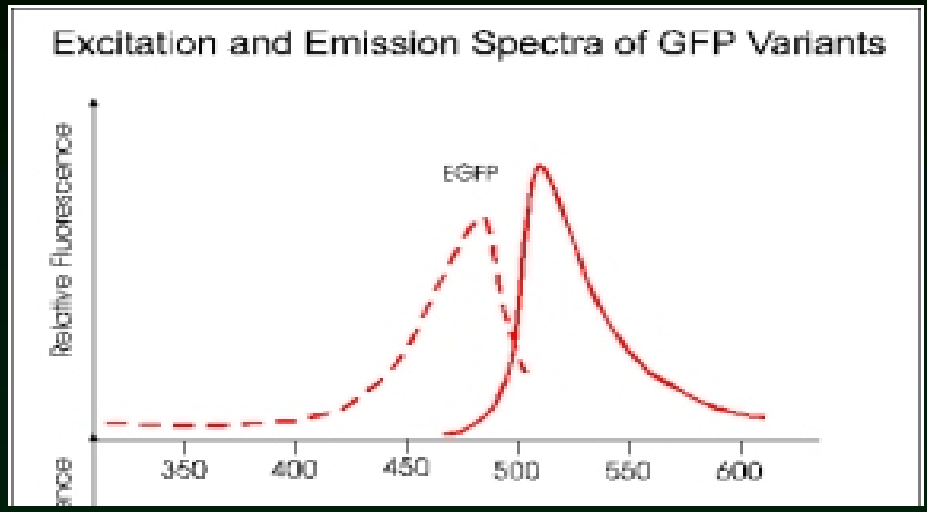
GFP discovery - Nobel Prize 2008

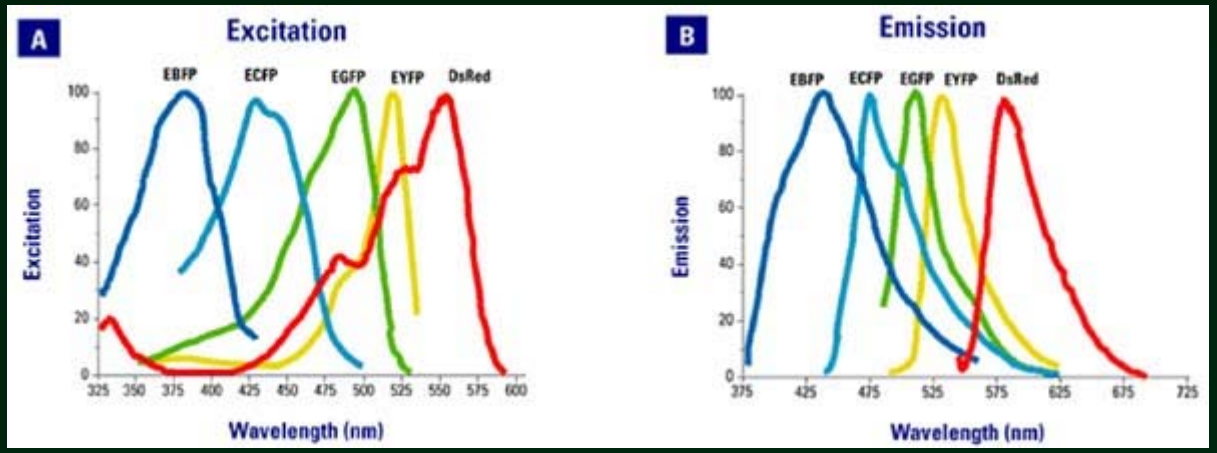

Osamu Shimomura Martin Chalfie Roger Tsien

# Many fluorescent proteins on the market (Tsien's fruits)

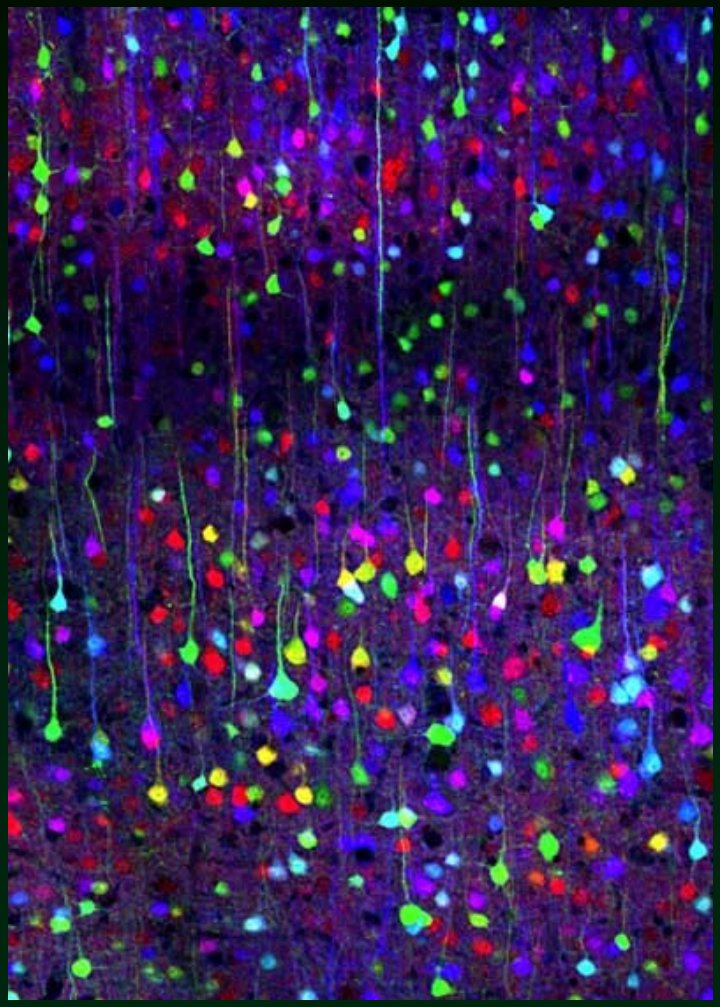


# Excitation and emission

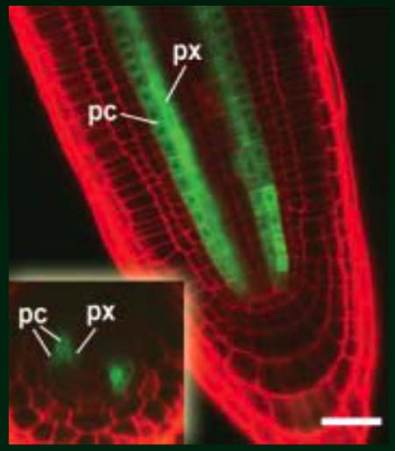


# Multicolored fluorescent protein image (neurones)



# Promoter-GFP

# Promoter activity monitoring

choice of suitable reporter

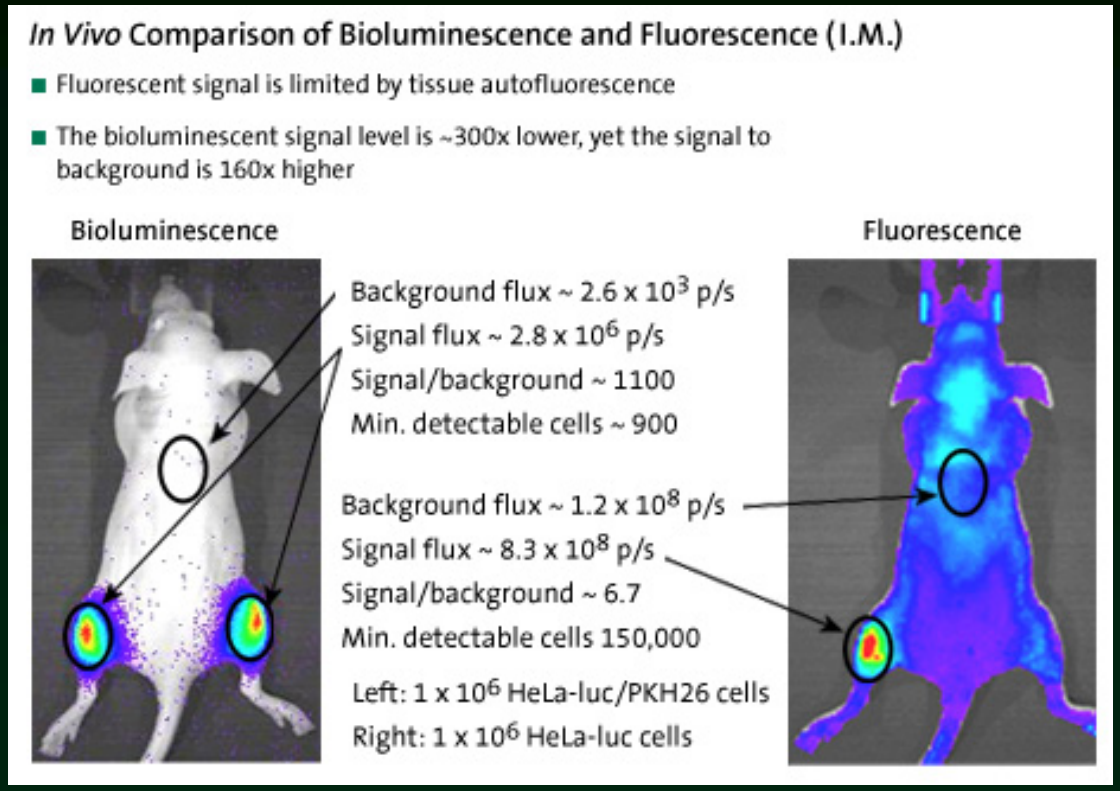
- LacZ, GUS
- Luciferase
- GFP

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; free GFP sometimes moves

# Luminiscent mouse better than phluorescent mouse



# Promoter activity monitoring

**Pros:**

- 

**Cons:**

- 

# Promoter activity monitoring

**Pros:**

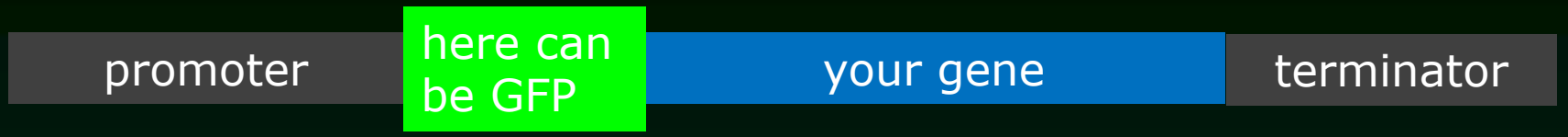
- easy to clone, easy to visualize
- usually some signal seen – cheers you up!
- can be used in less accessible organs

**Cons:**

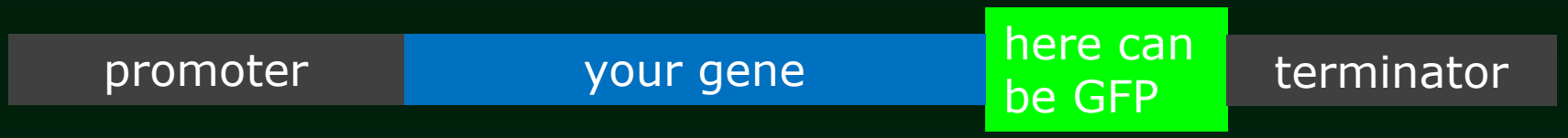
- limited information about gene product (mRNA, protein etc)
- needs cloning and transformation
- neglects regulatory elements (introns, UTRs etc.)
- length of promoter given arbitrarily

# Translational GFP fusions

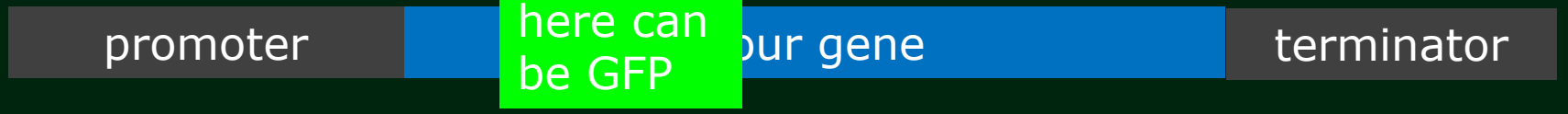
N-terminal fusion



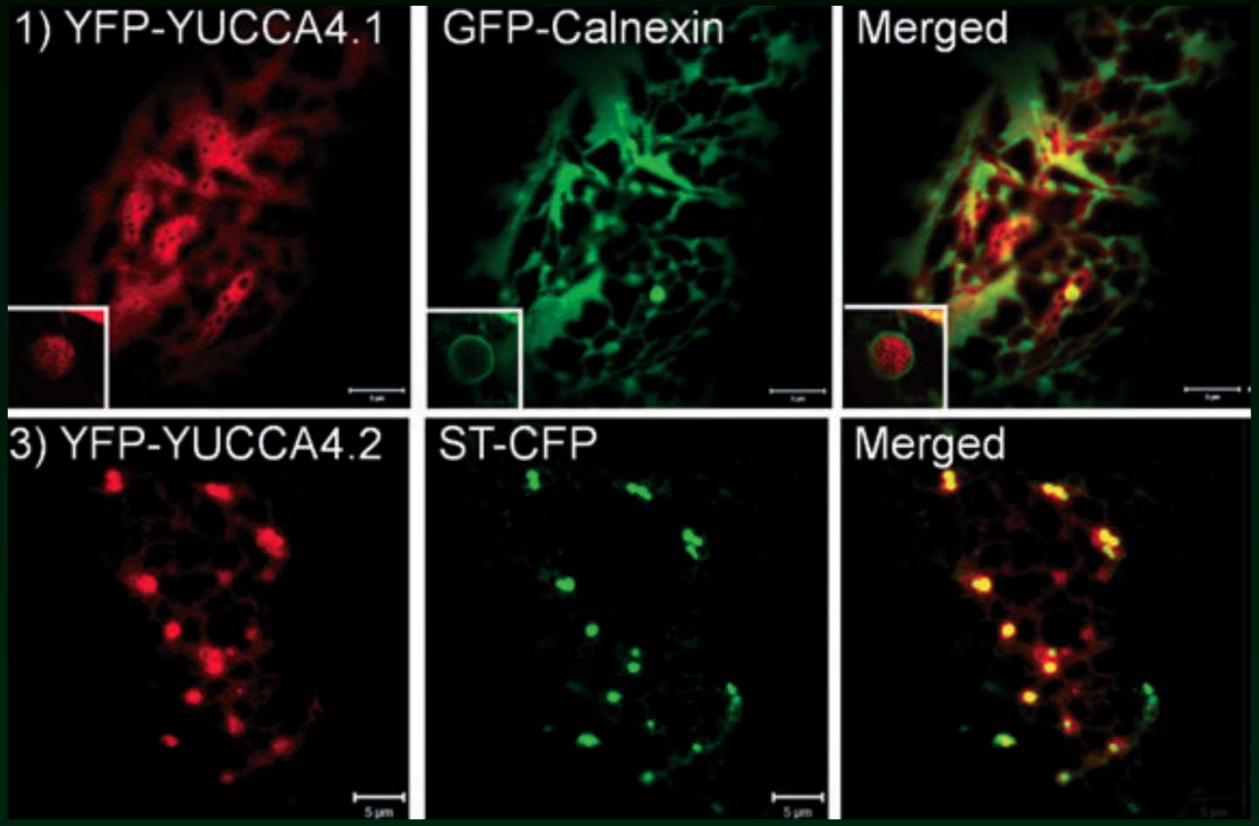

C-terminal fusion




fusion inside the coding sequence

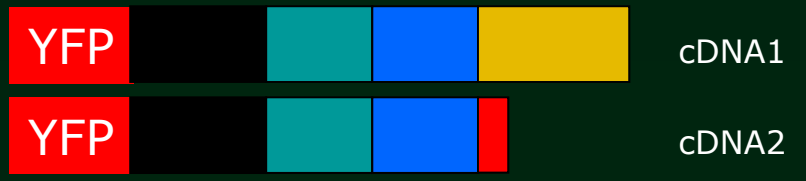


# Expression of isoforms




endoplasmic reticulum

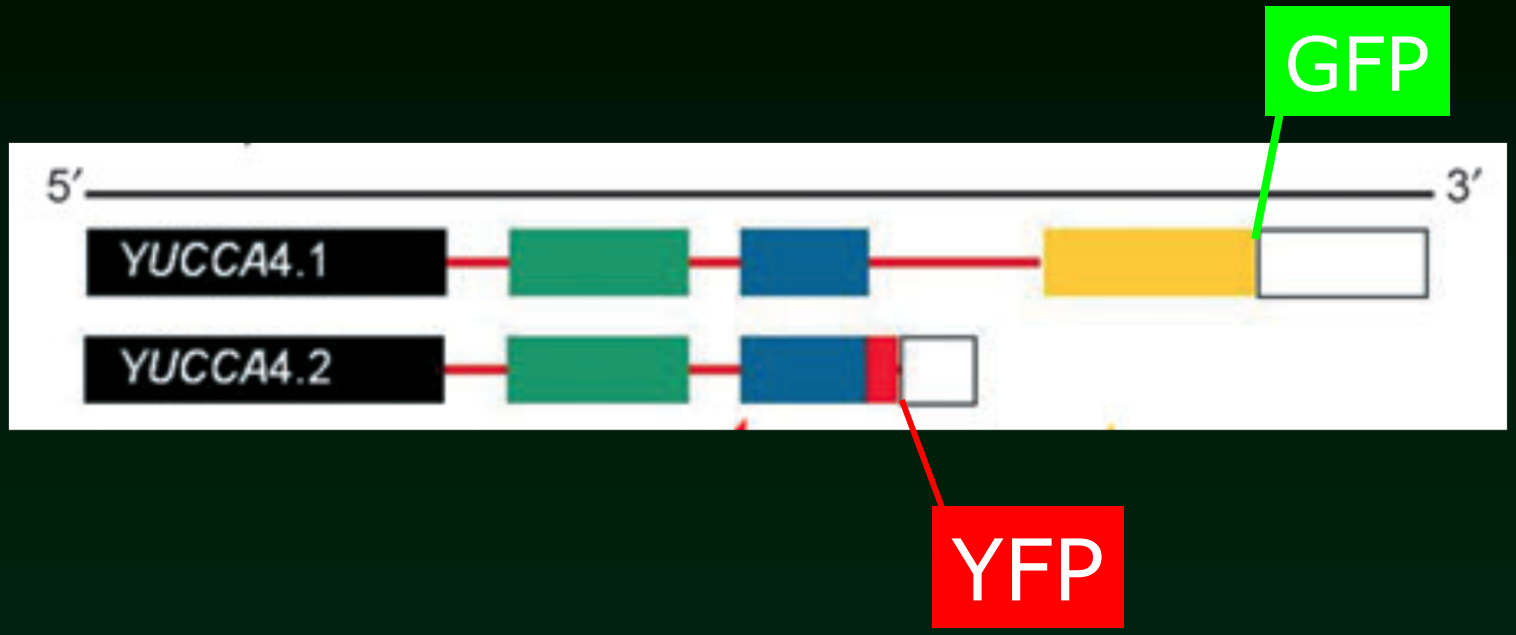
cytosol

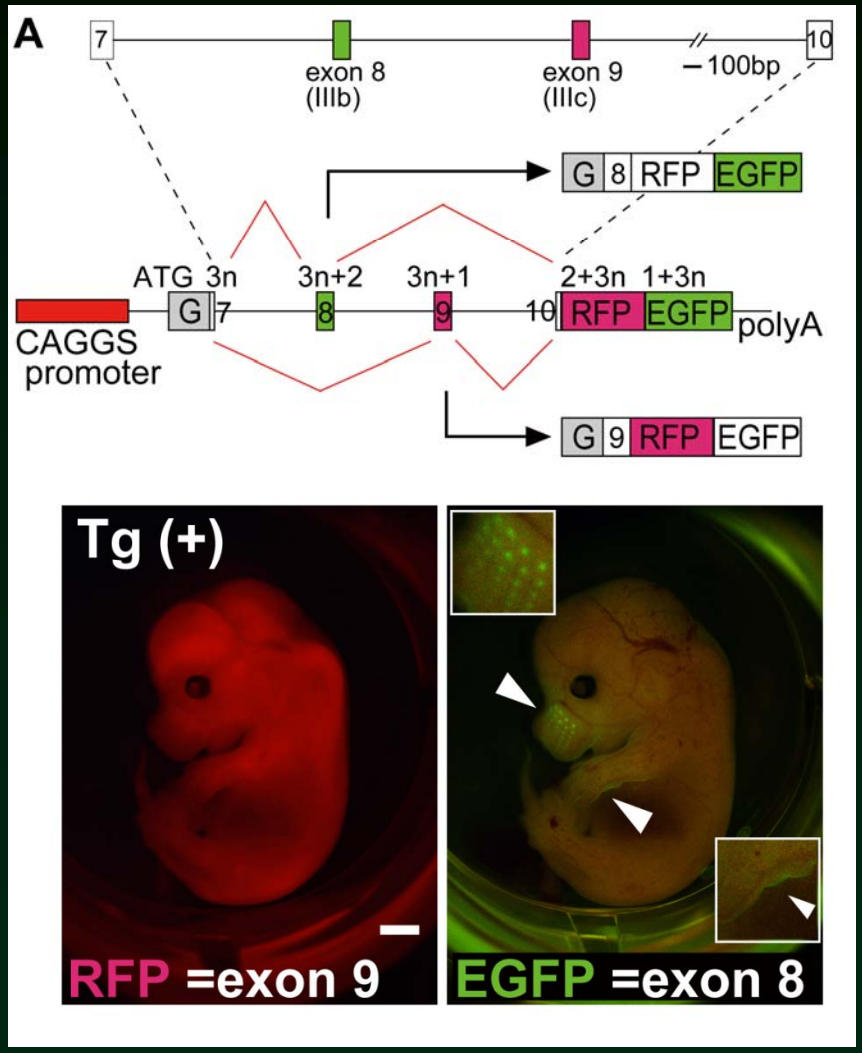

Not the best option available – can you guess?

Kriechenbaumer et al 2011

# Isn’t this better?



# Expression of isoforms



# Fluorescent protein fusion

Pros:

•

Cons:

# Fluorescent protein fusion

Pros:

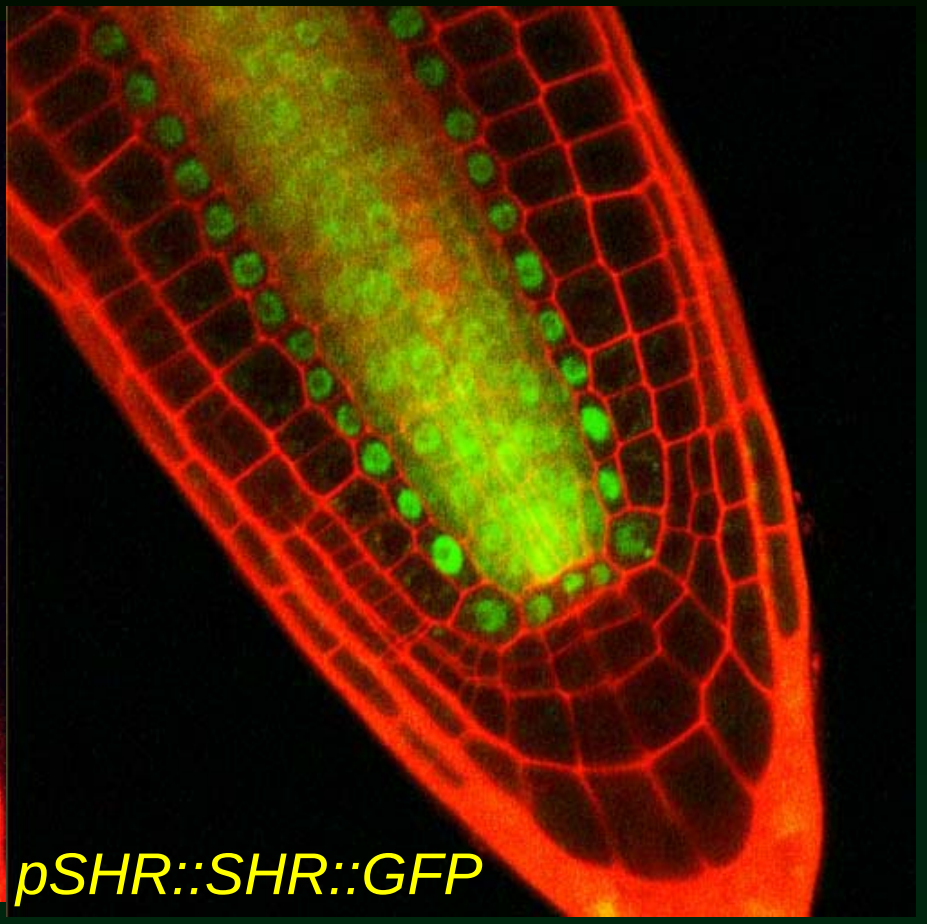
- in vivo imaging

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

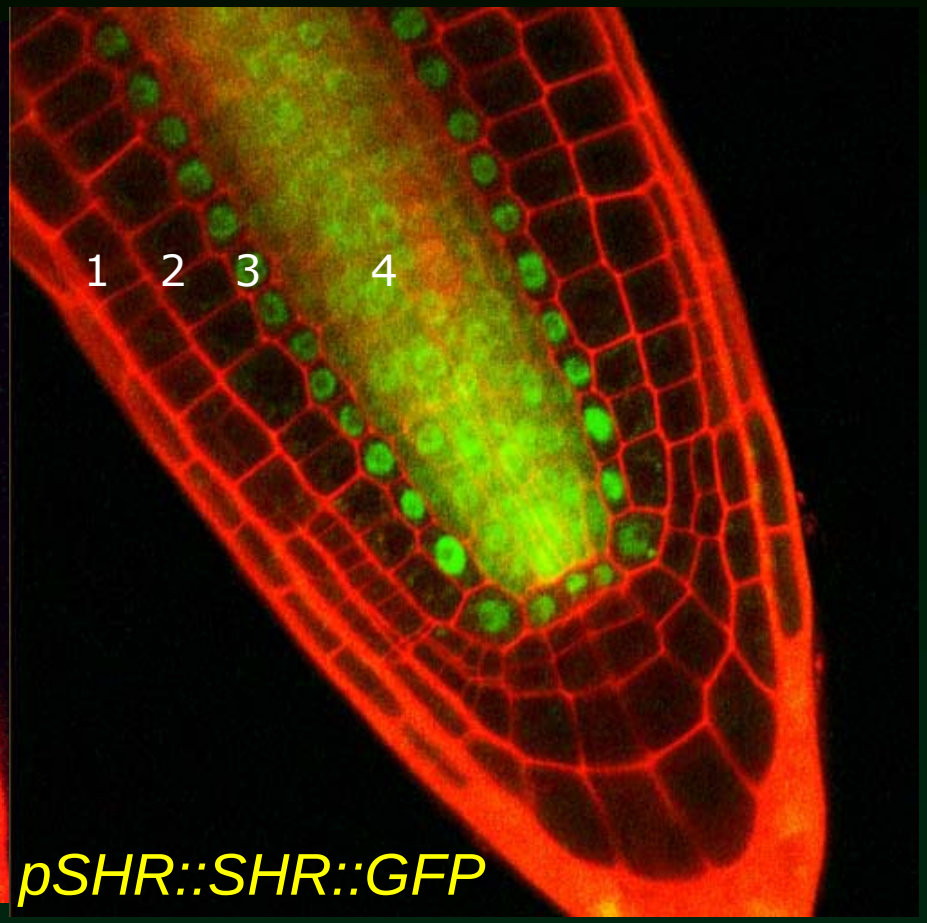



translational

Nakajima et al, Nature 2001

# Why to visualize all this stuff

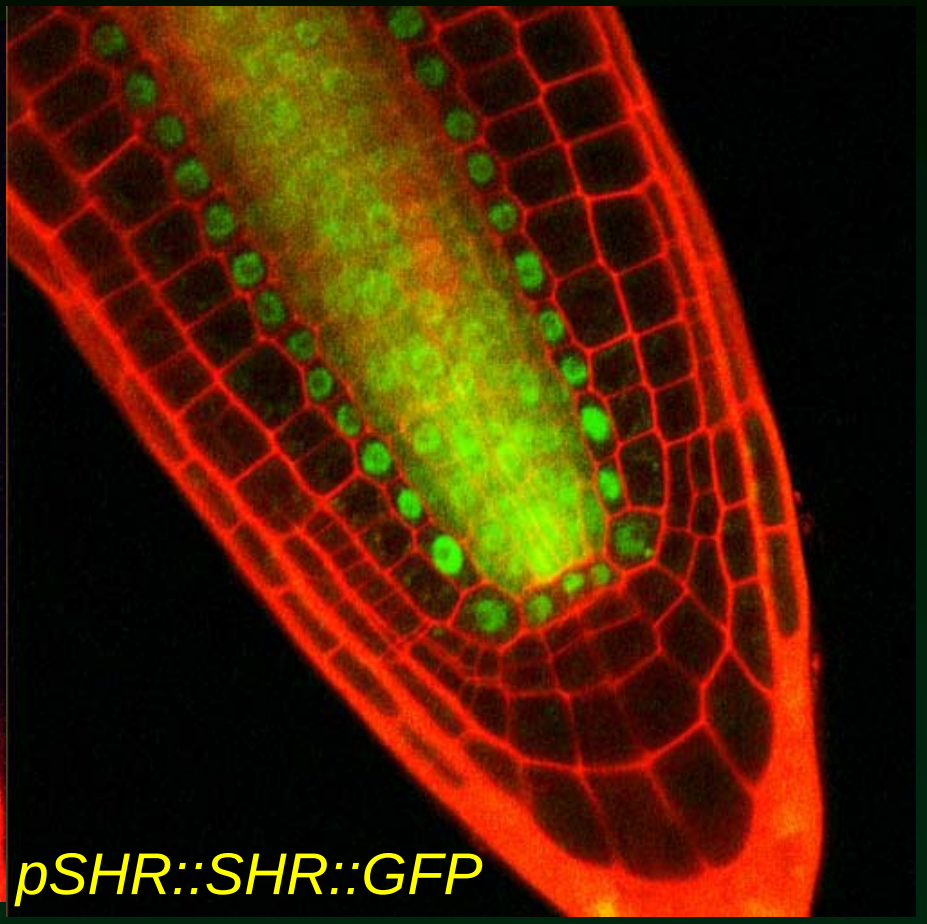

promoter




translational

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

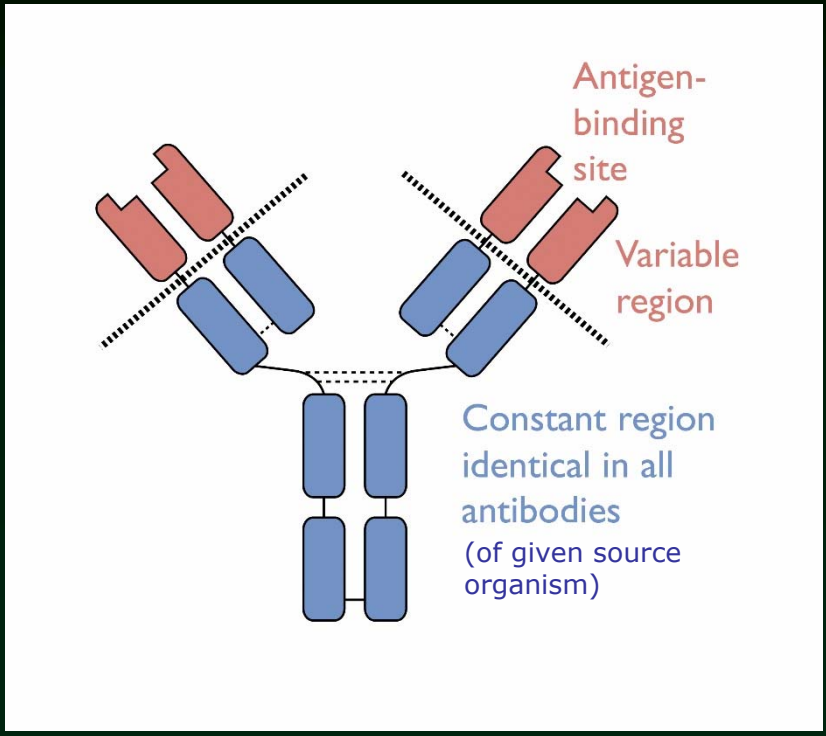
# Why to visualize all this stuff


BANG! SHR moves from stele to endodermis

Nakajima et al, Nature 2001

# Protein immunolocalization

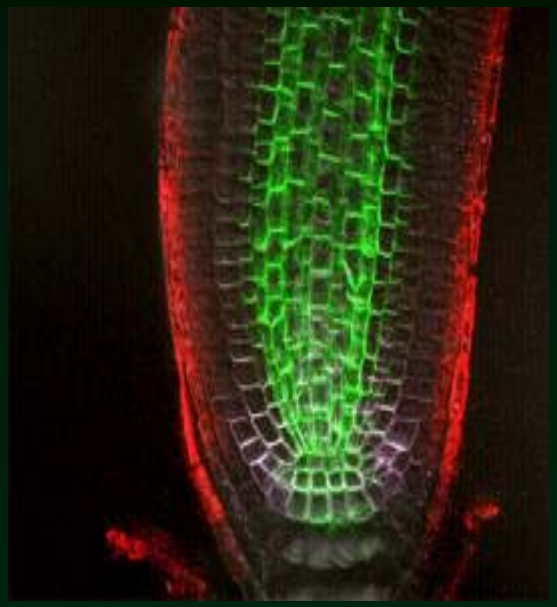


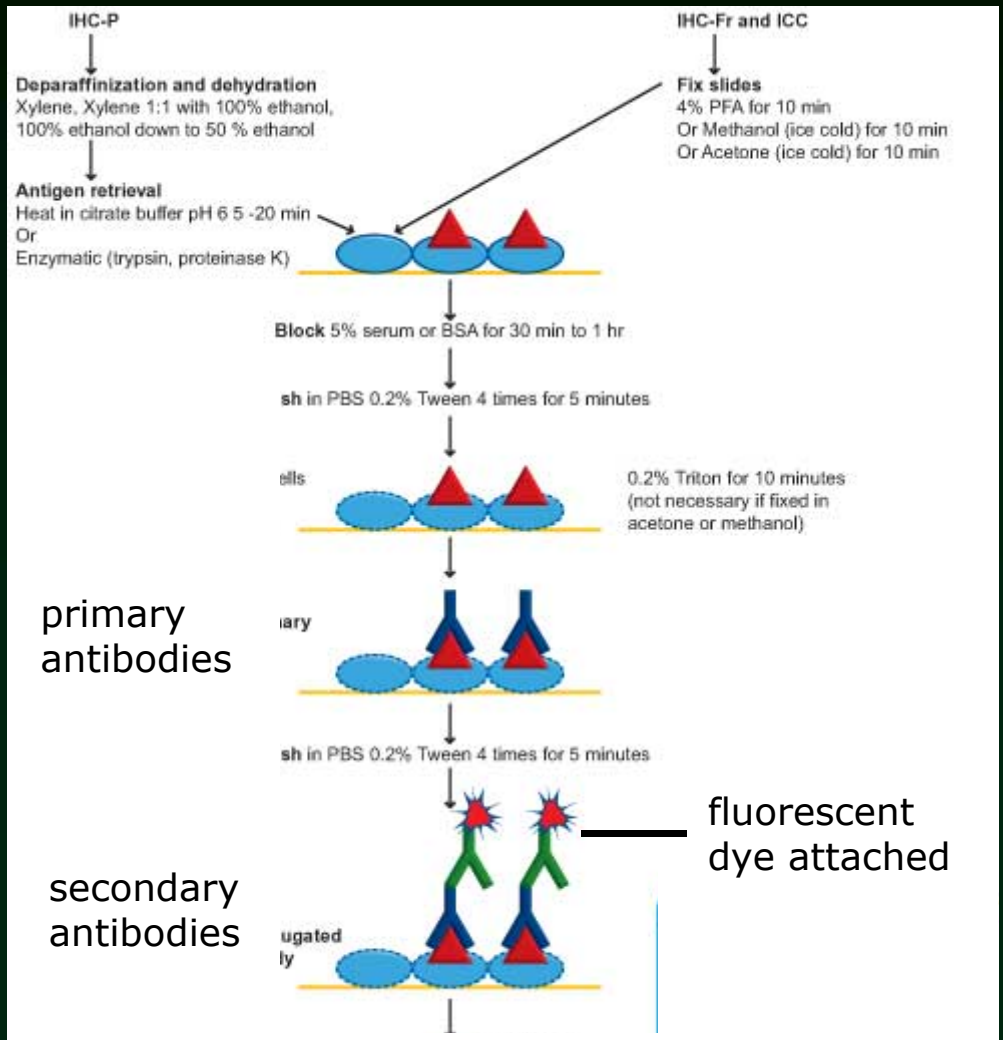

Most favorite animals:
-rabbit (too many rabbits)
-mouse (low volume)
-goat
-chicken
-rat
-sheep
-donkey
-guinea pig

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

# Protein immunolocalization

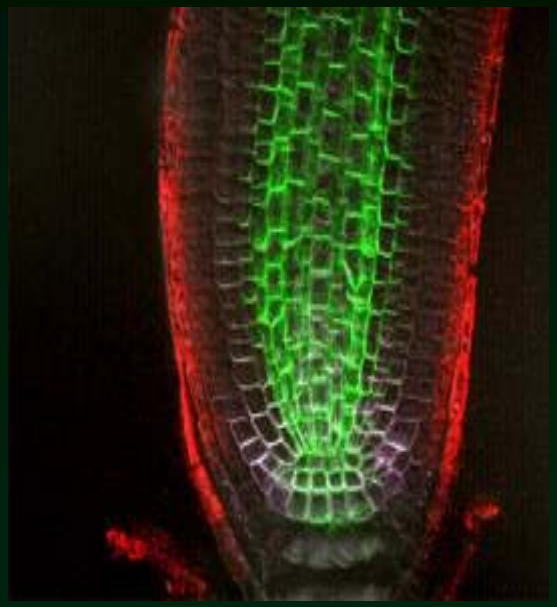
## immunolocalization - fluorescently





# Protein immunolocalization

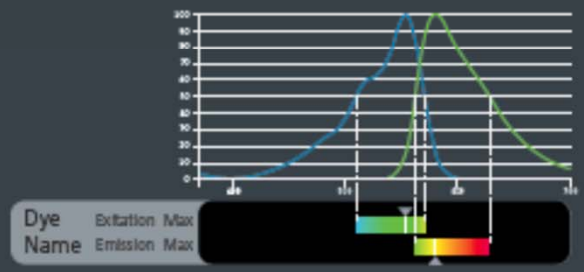
immunolocalization



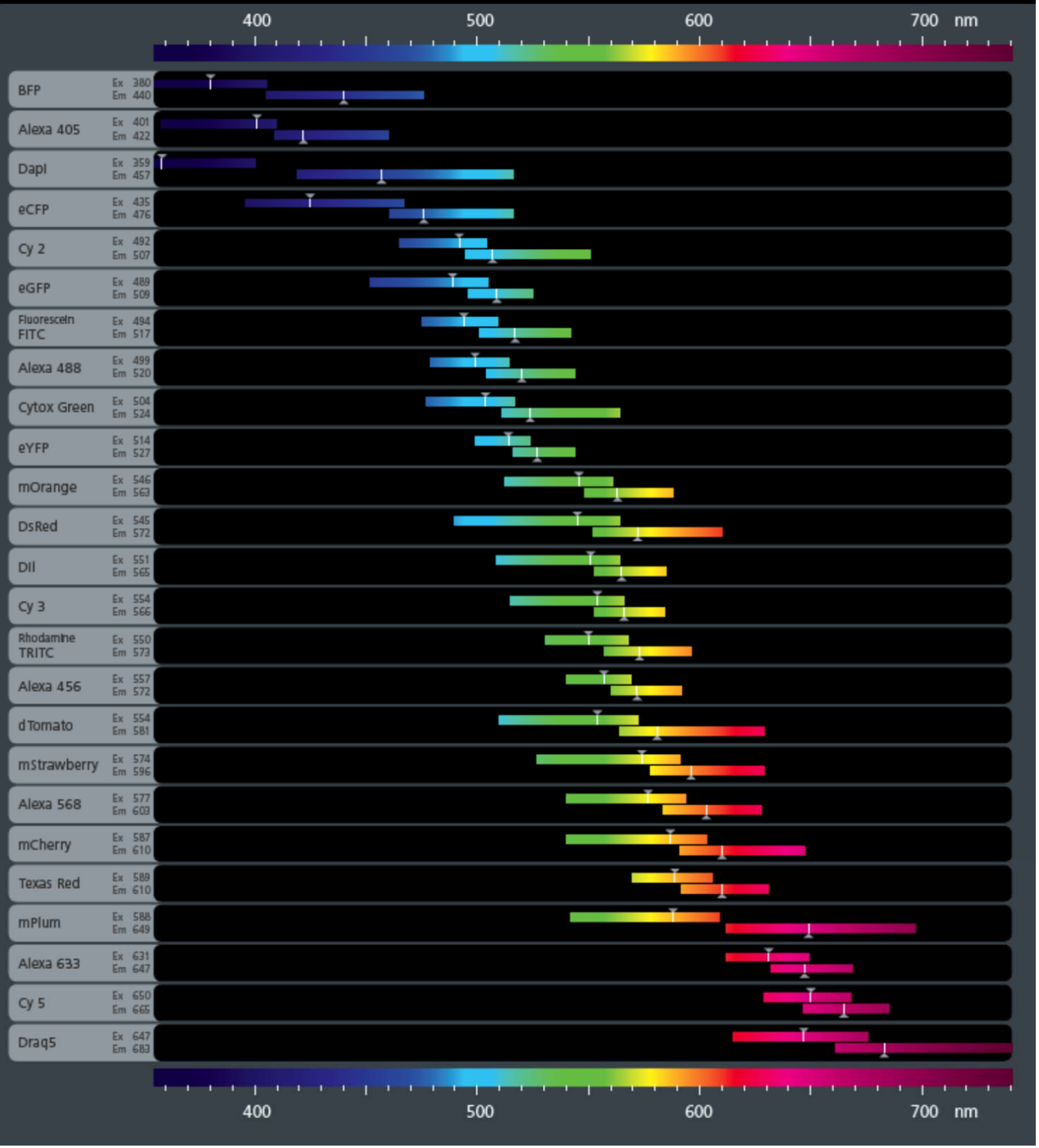
Fluorescent dyes conjugated to 2ndary (examples):

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

# Fluorescent Dyes and Proteins




www.zeiss.com/microscopy



# Protein immunolocalization

Pros:

- 
- 

Cons:

- 
- 

# Protein immunolocalization

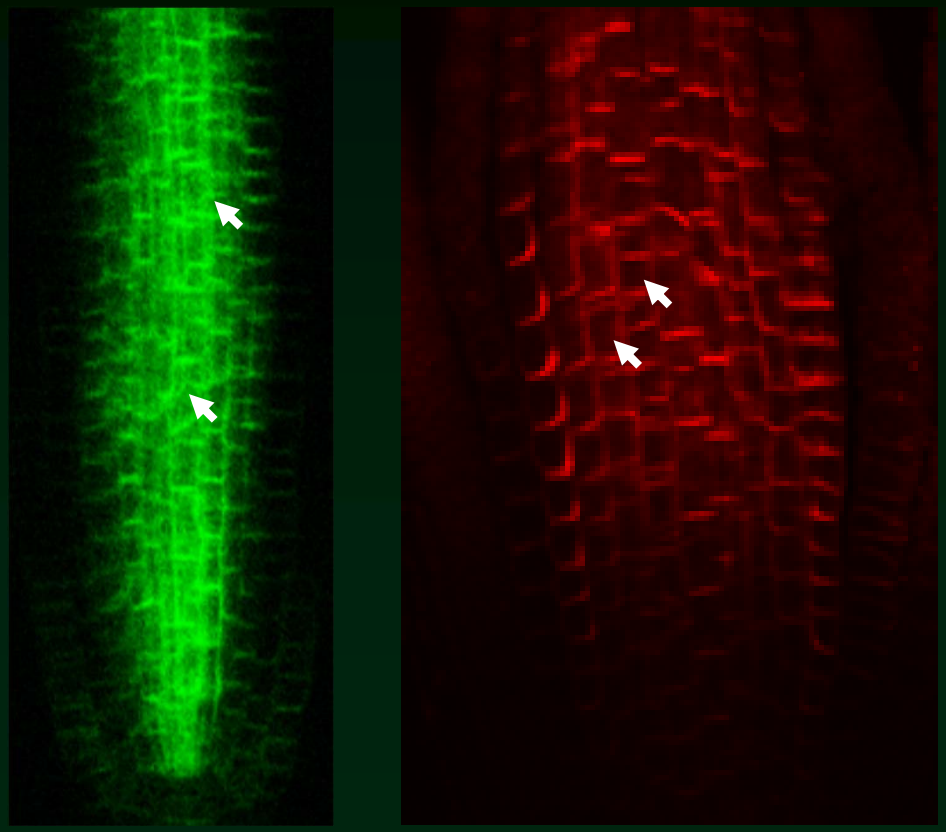
Pros:

- 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

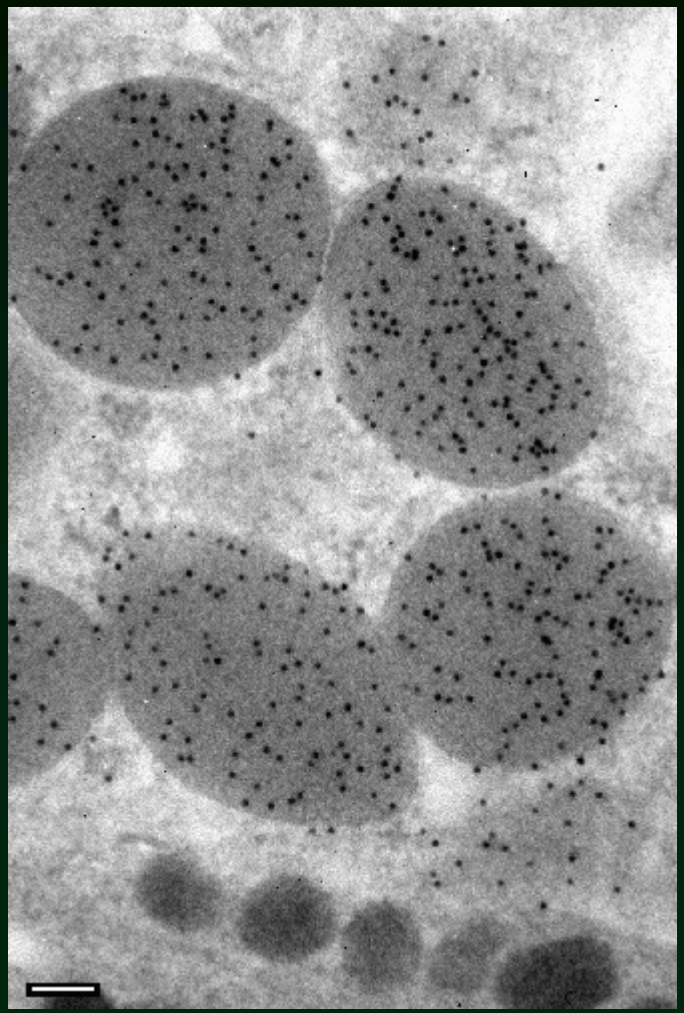
# GFP tag partially retains PIN1 in endoplasmic reticulum (-> artifact)



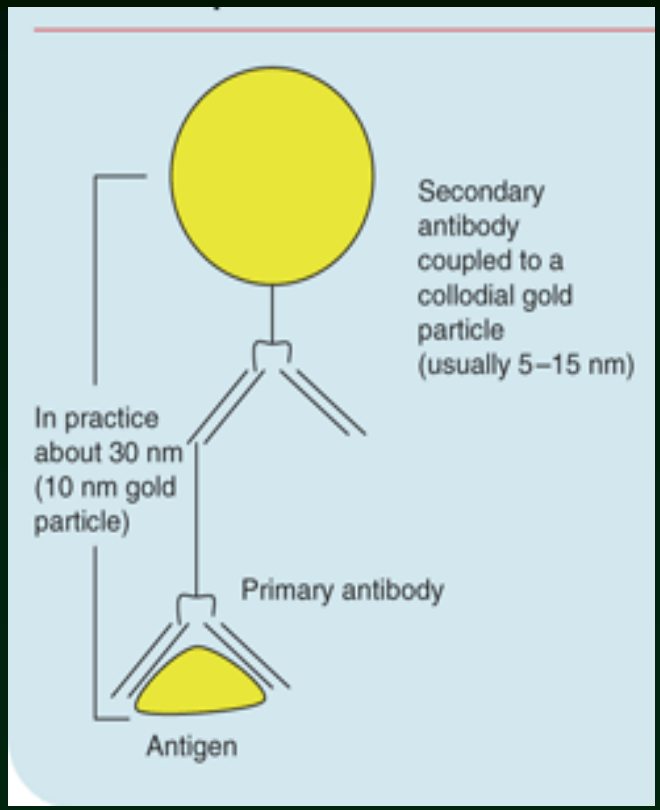
PIN1-GFP anti-PIN1

# Protein localization - immunogold

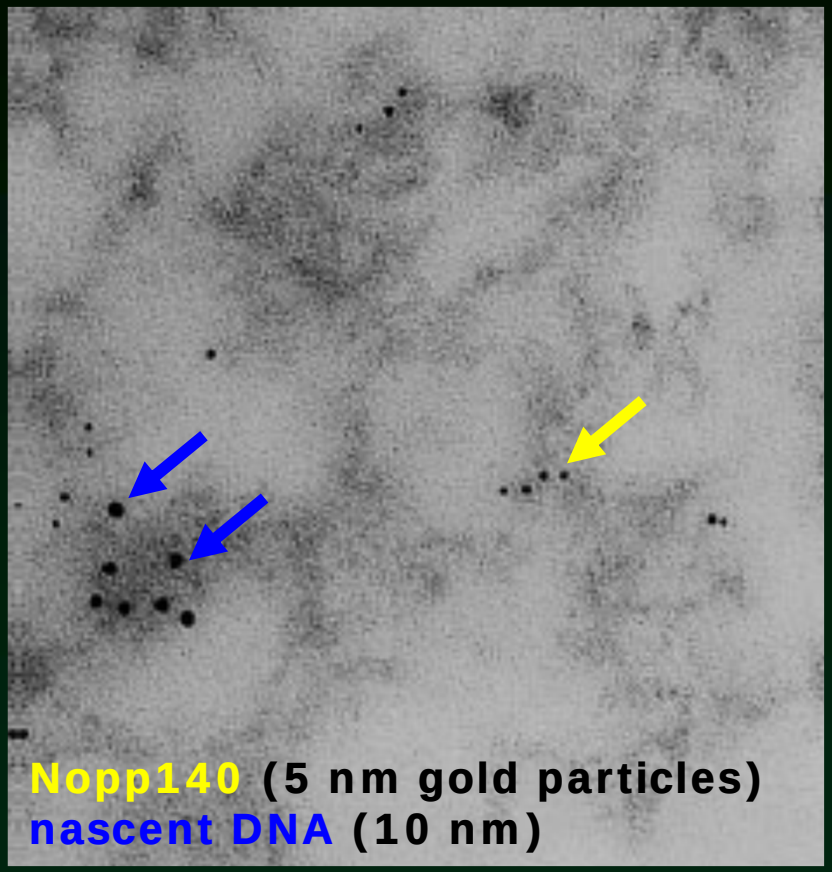
immunolocalization - immunogold

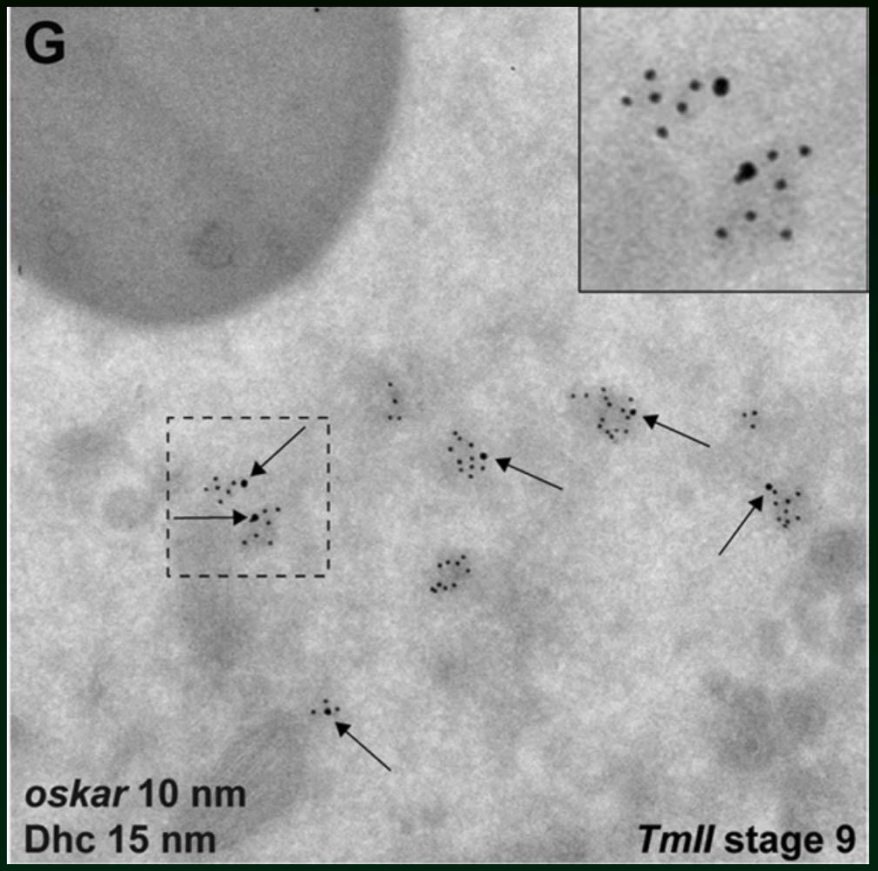


electron microscope



# Immunogold collocalization




Philimonenko et al 2000, and an unfortunate Cell paper

# Pros/cons

Pros:

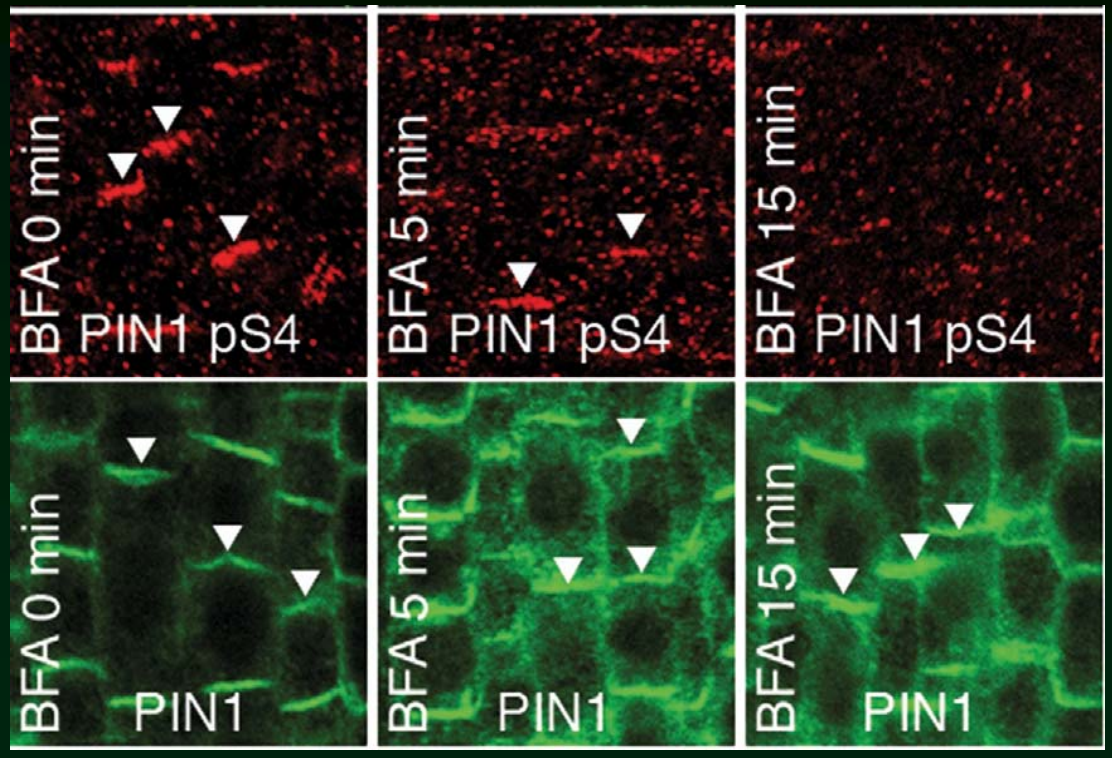
- direct
- nothing can beat the resolution

Cons:

- very tricky (needs rather expert)
- huge experience for interpretation needed

# Can we visualize postranslational modifications?

# Can we visualize postranslational modifications?




antibodies against phosphate pS4

-> phosphorylation is required for PIN1 to stay on the membrane

Yes, we can.

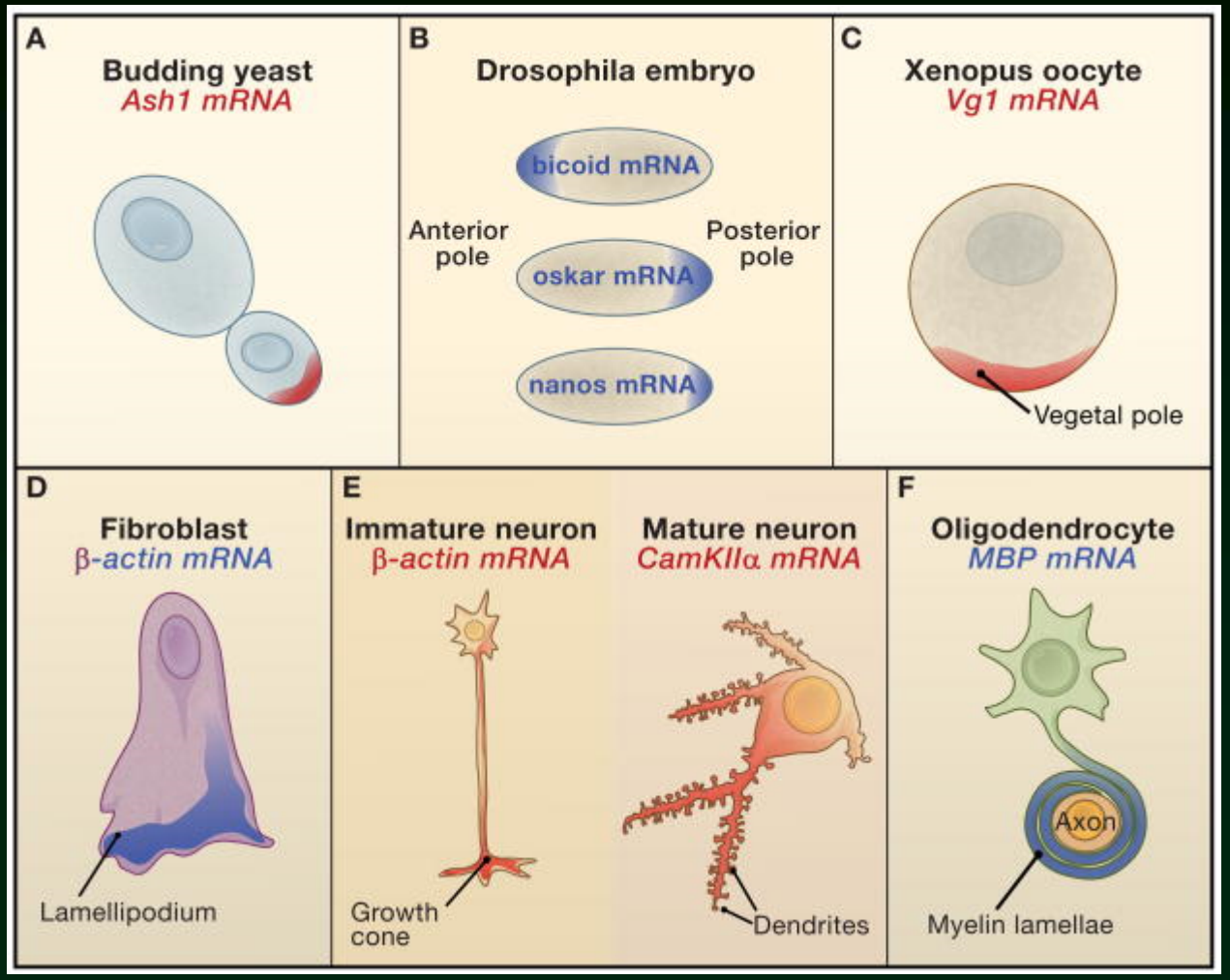
Stanislas et al. 2016

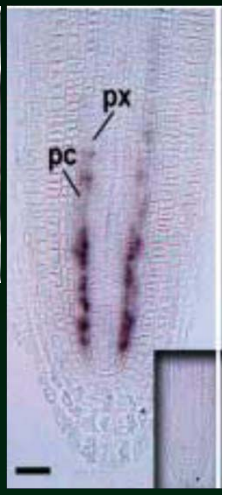
# Can we visualize postranslational modifications?

Yes, we can – make biology great again!

Stanislas et al. 2016

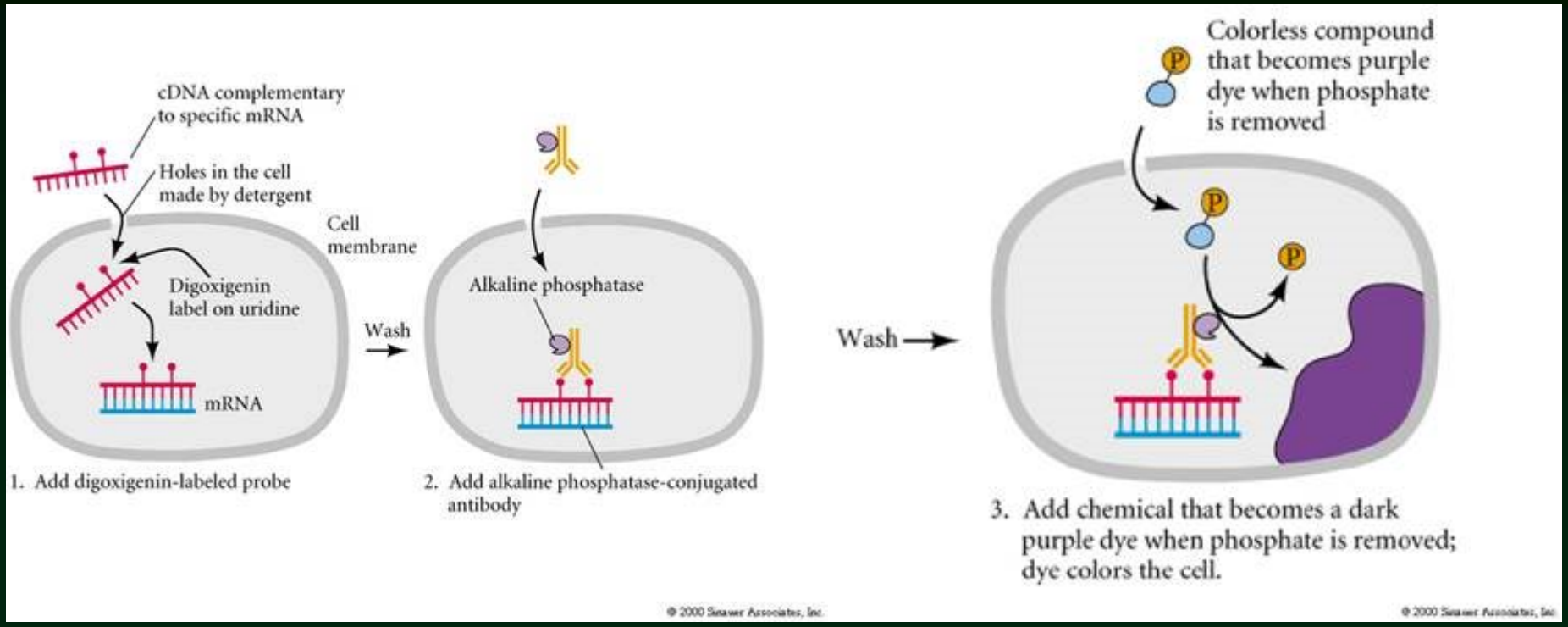
# Also RNA can be visualized



# Localization of mRNA

## RNA hybridization *in situ*



# Visualization of mRNA

## RNA hybridization *in situ*

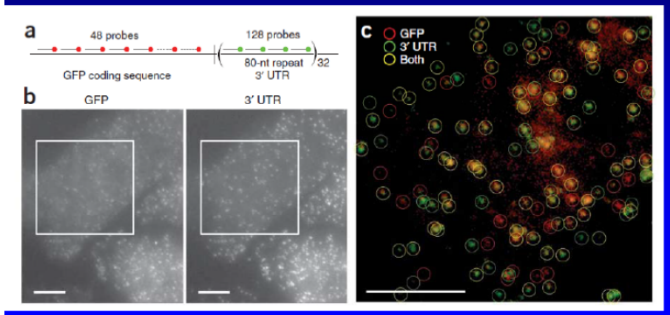
Pros
- classical technique in developmental biology
- no transgenes needed

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

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

## Single-molecule detection using multiple probes

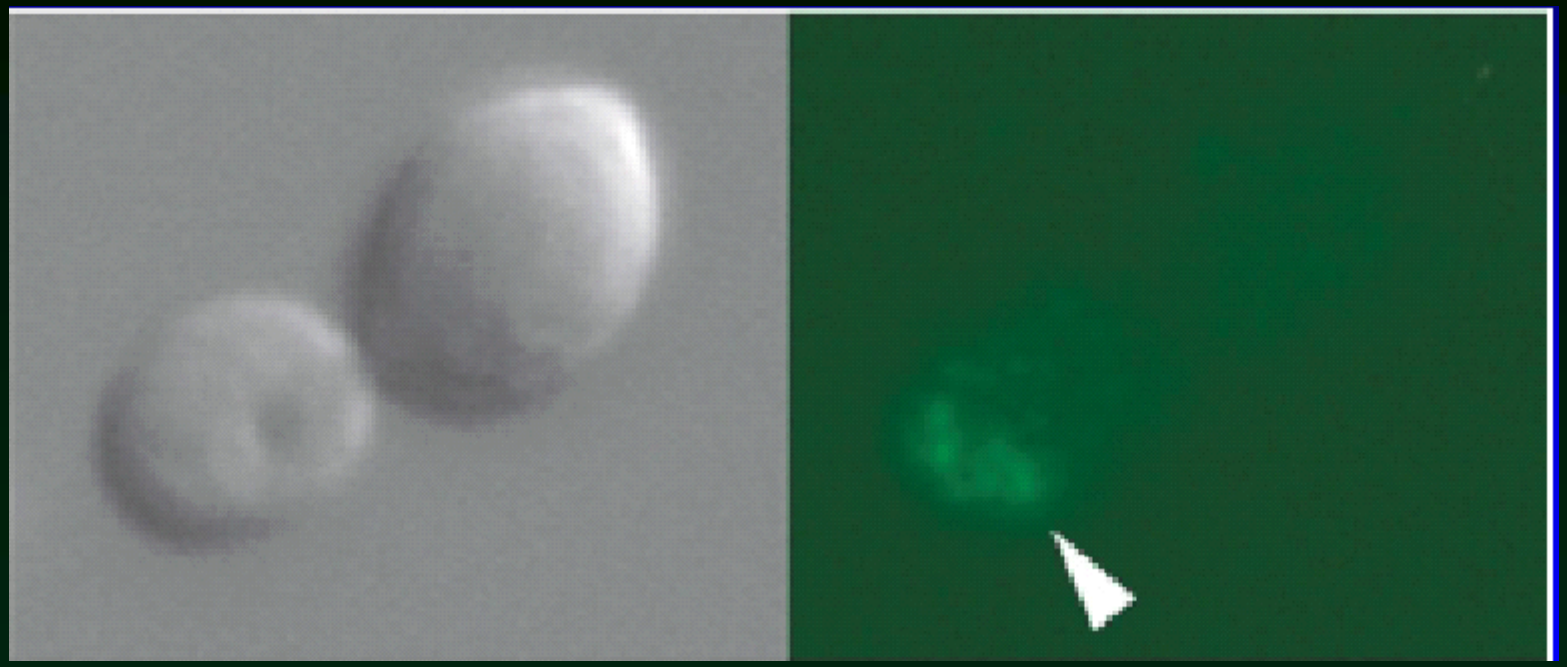
- ~ 48 oligonucleotide probes provide sufficien signal to detect a single mRNA molecule




Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. Nature Methods *5*, 877-879.

(slide by Mikko Frilander)

# Also mRNA can be visualized *in vivo*

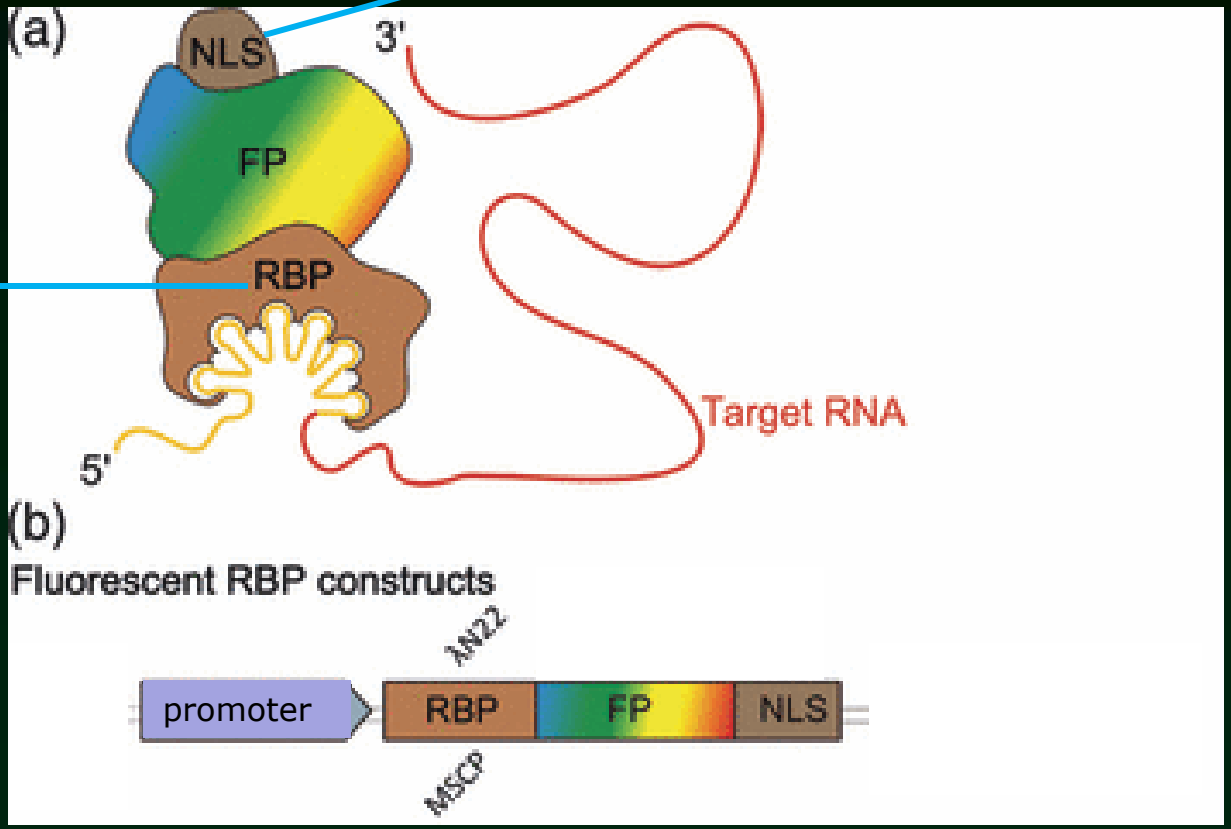


Ash1 mRNA localized to the tip of the daughter cell

# $\lambda N_{22}$ system – RNA imaging *in vivo*

nuclear localization signal

viral RNA binding protein

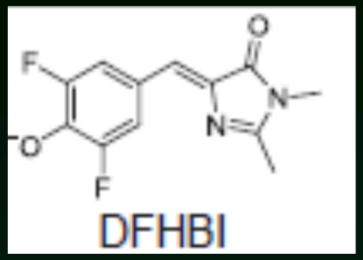


# Drawbacks of $\lambda N_{22}$ system - we have SPINACH

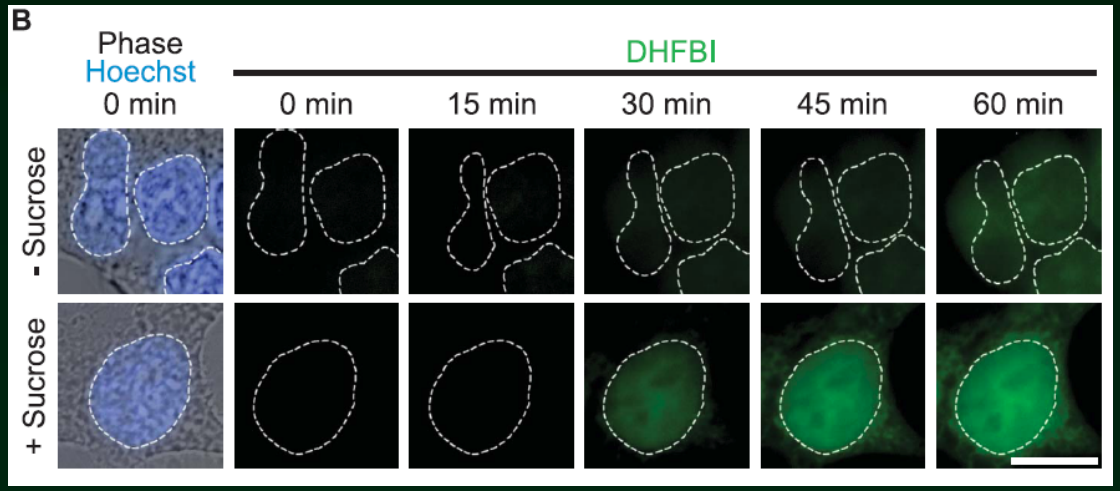
GACGCAACUGAAUGAAA
UGGUGAAGGACGGGUCC
AGGUGUGGCUGCUUCGG
CAGUGCAGCUUGUUGAG
UAGAGUGUGAGCUCCGU
AACUAGUCGCGUC

RNA fusion

+




aptamer

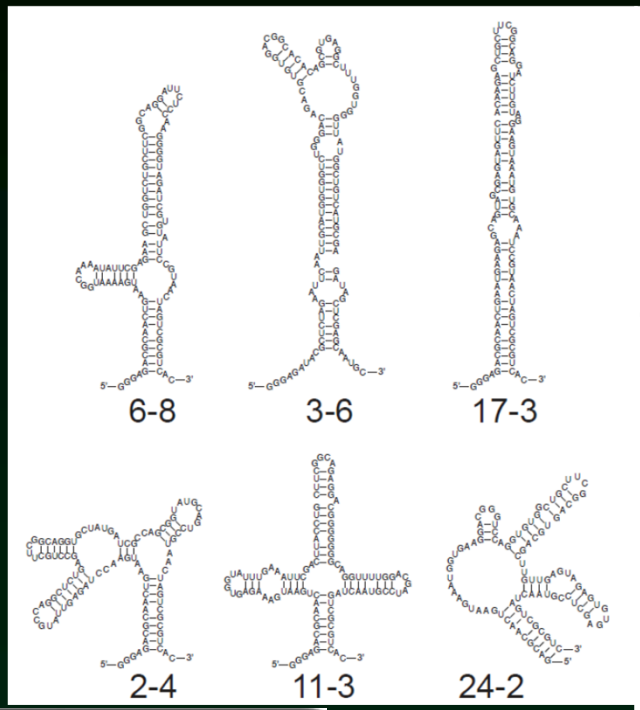


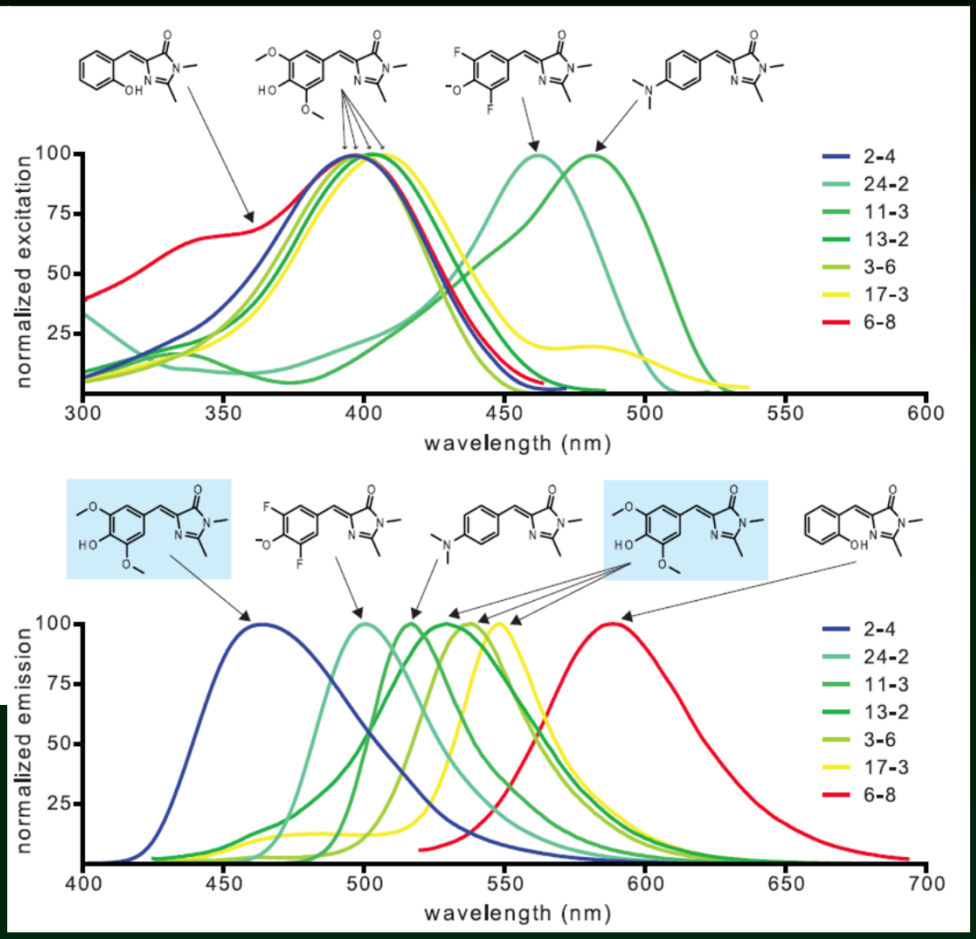

blue-DNA

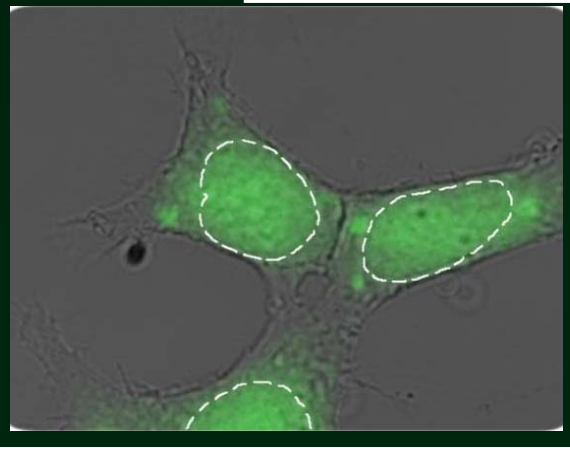
green-RNA

Paige et al. 2012

# Other vegetables than SPINACH

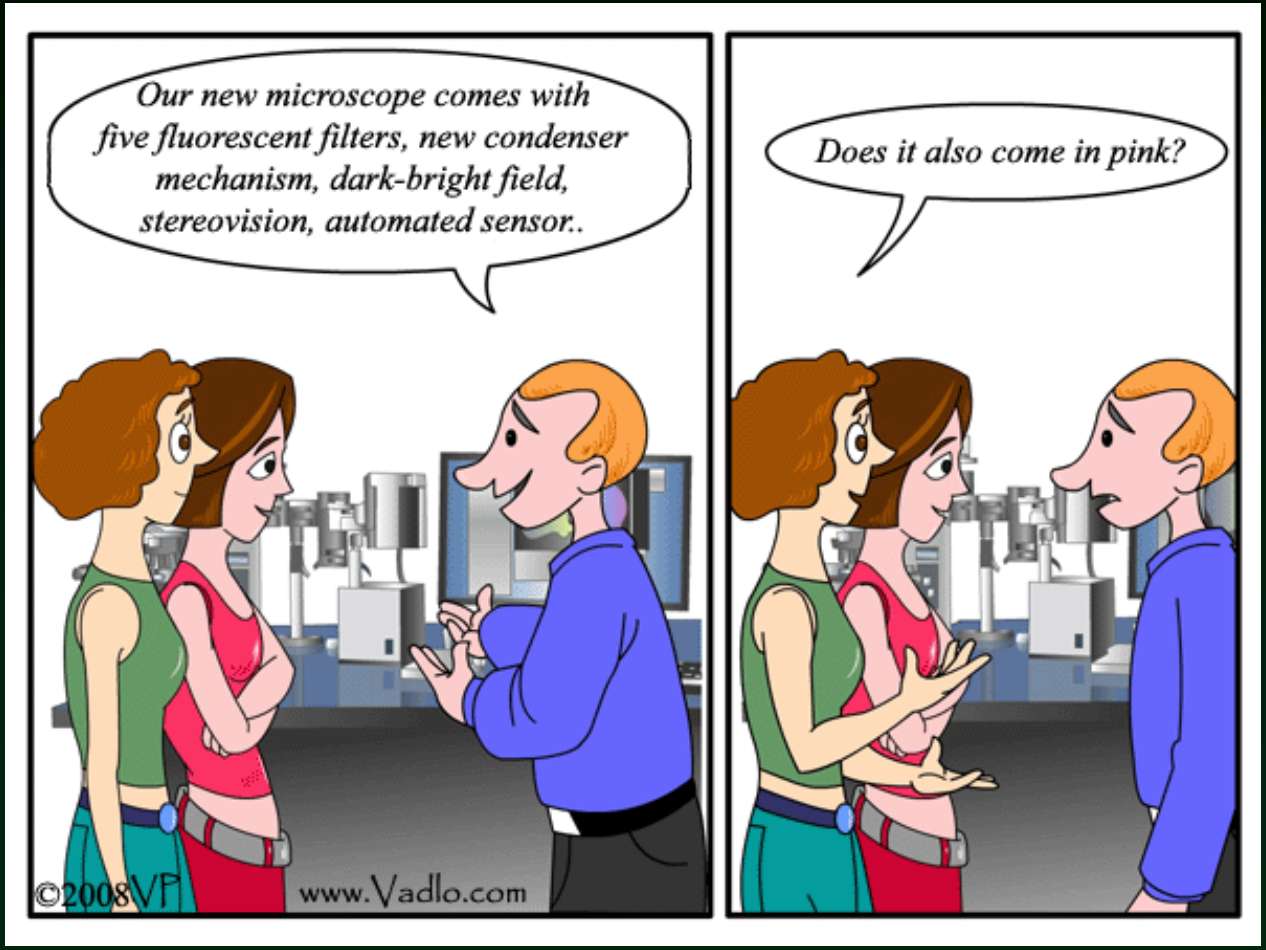


Paige et al. 2012; Song et al. 2014

# Advanced confocal techniques

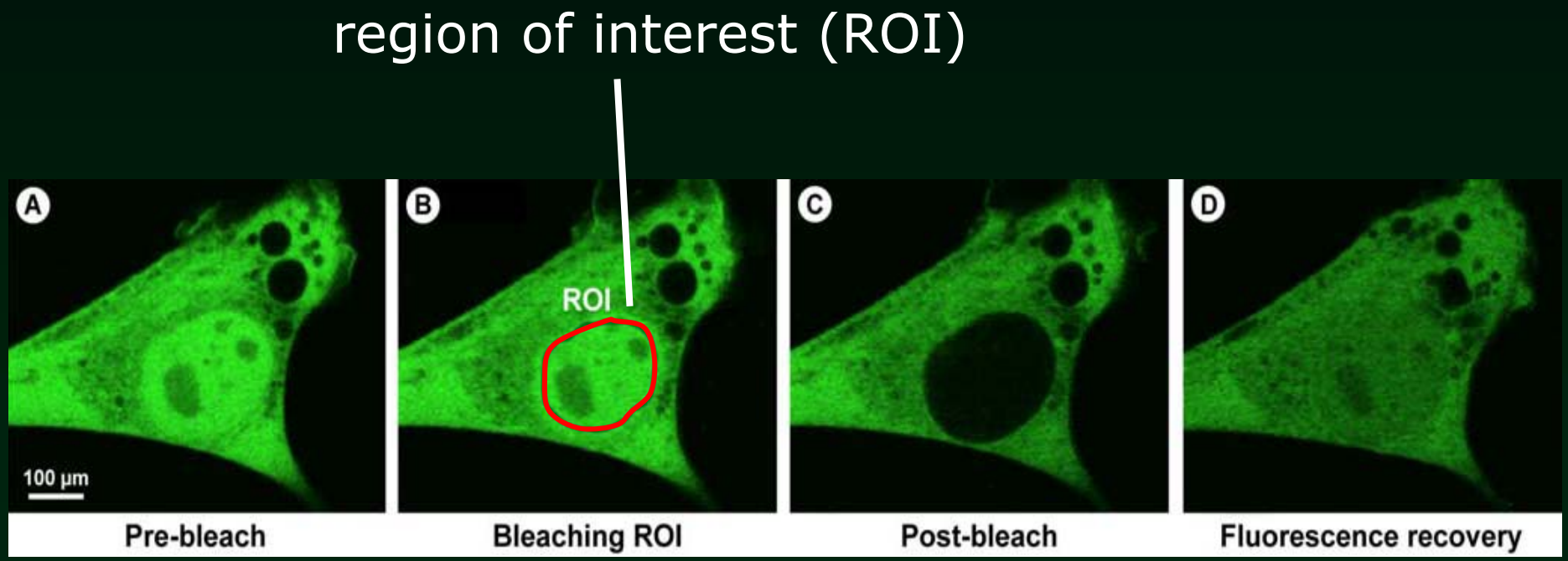


# (slightly) Advanced confocal techniques

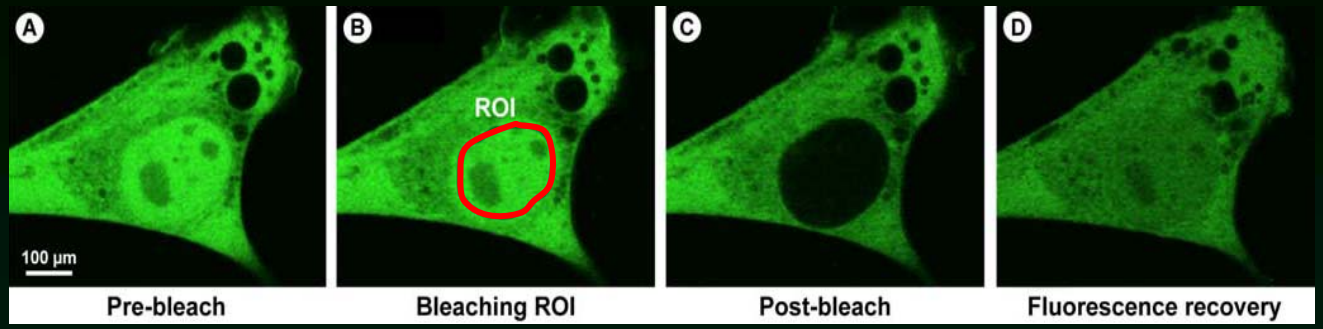
- FRAP
- photoactivatable FP
- FCS

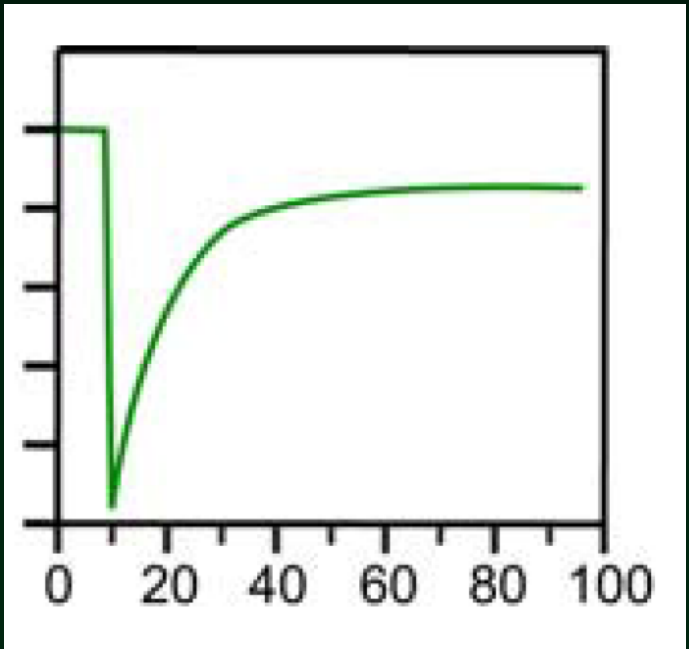
# FRAP

## Fluorescence Recovery After Photobleaching

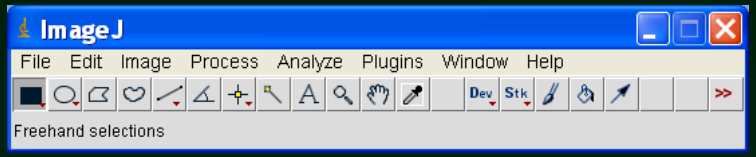


# FRAP



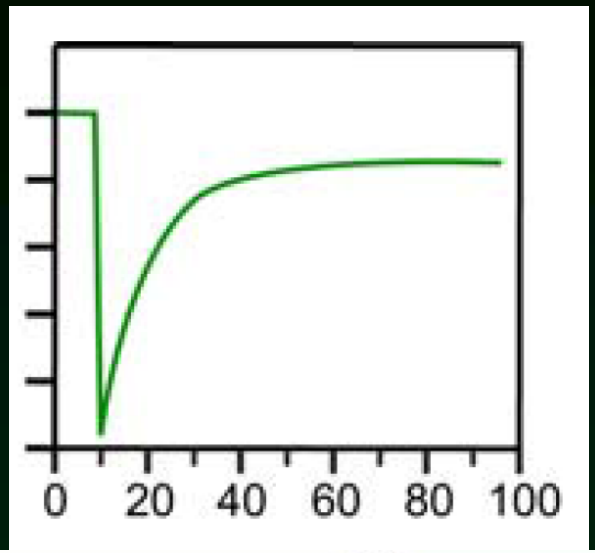




you can quantify fluorescence..
(ImageJ is our friend)

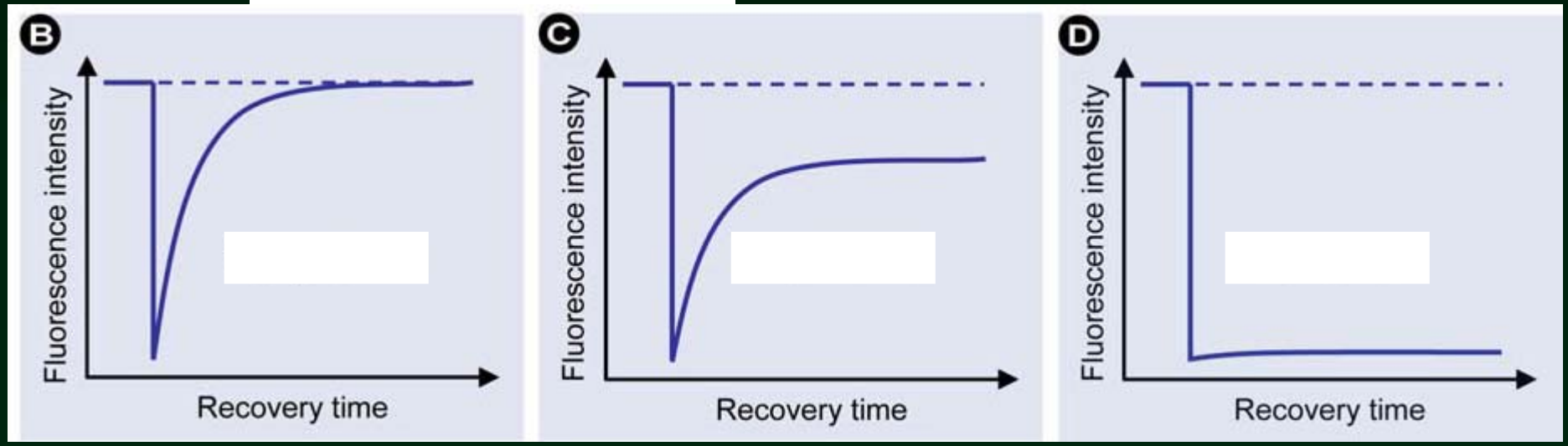



| | mean | min | max |
|---|---|---|---|
| A | 90.404 | 49 | 113 |
| C | 8.556 | 3 | 8 |
| D | 39.934 | 19 | 63 |

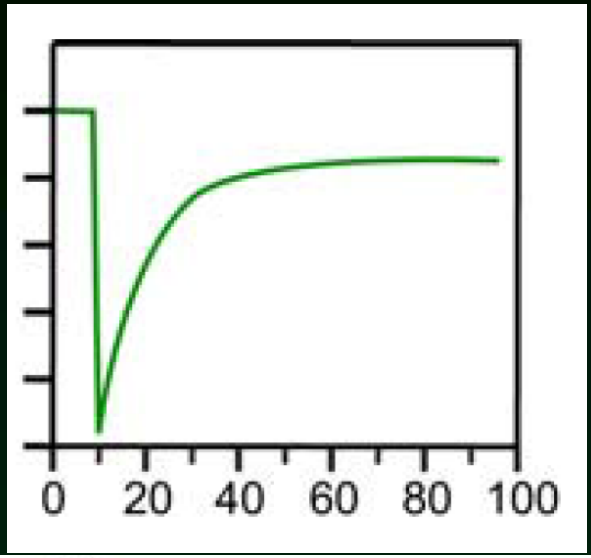
# FRAP – bleaching curve

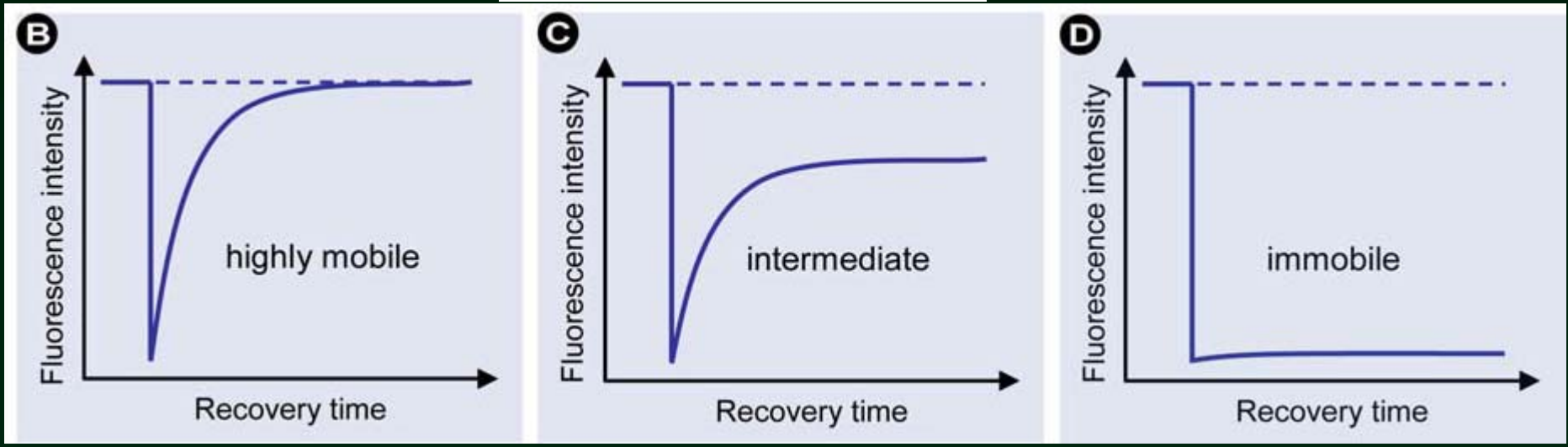



What does the curve tell?



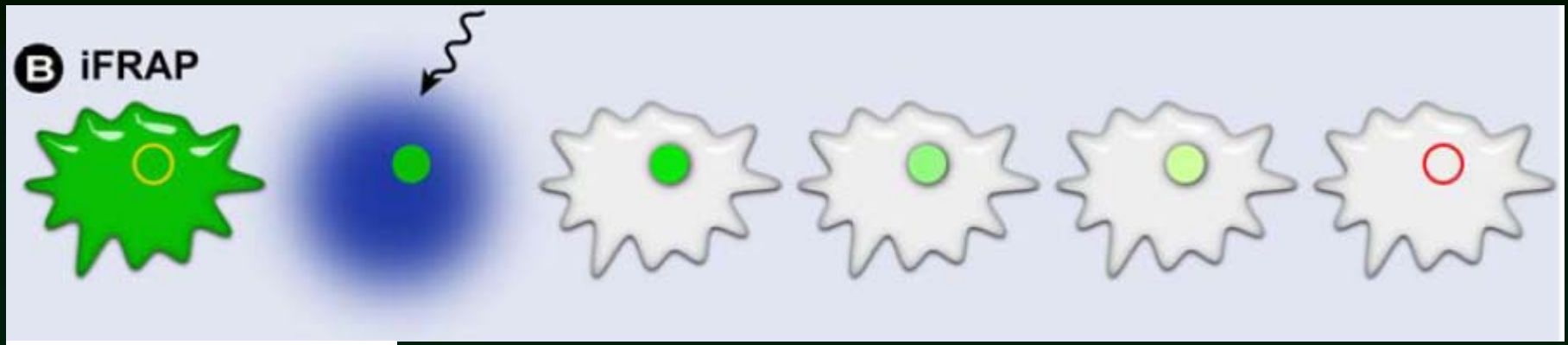
# FRAP – bleaching curve

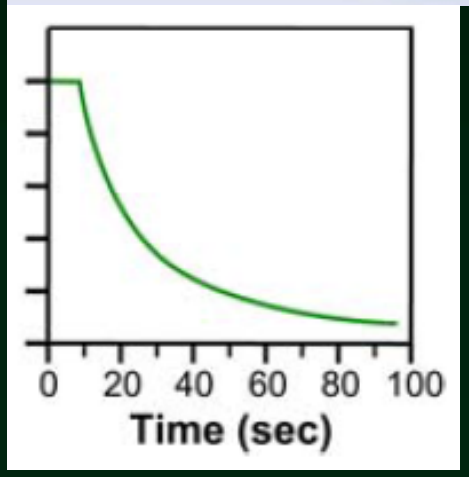


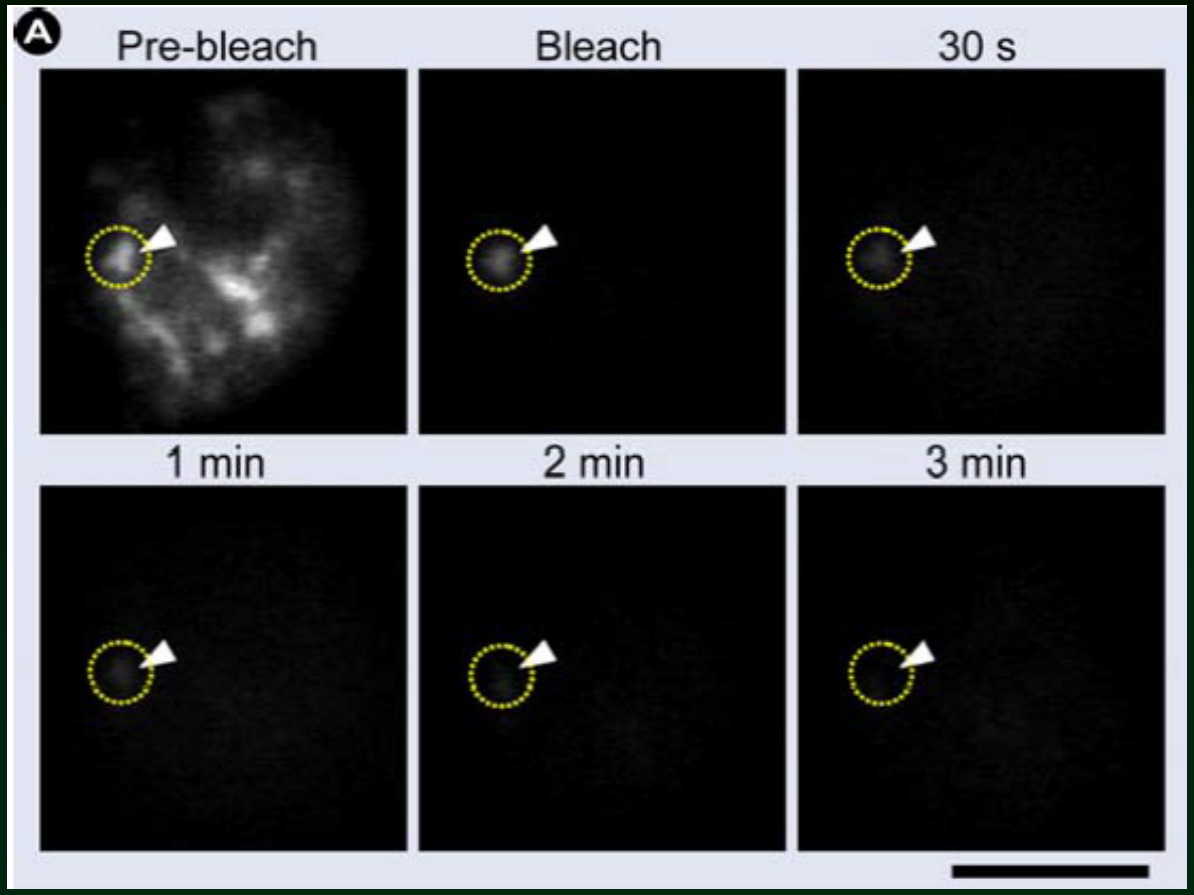
# iFRAP

inverse FRAP



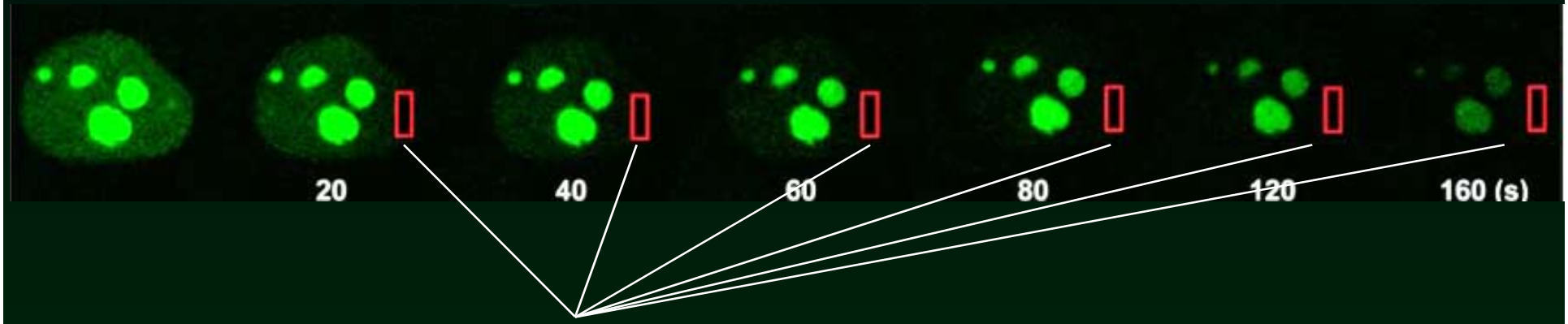
# iFRAP – dissociation of premRNA from specles



# FRAP derivatives

# FLIP

## Fluorescence Loss After Photobleaching



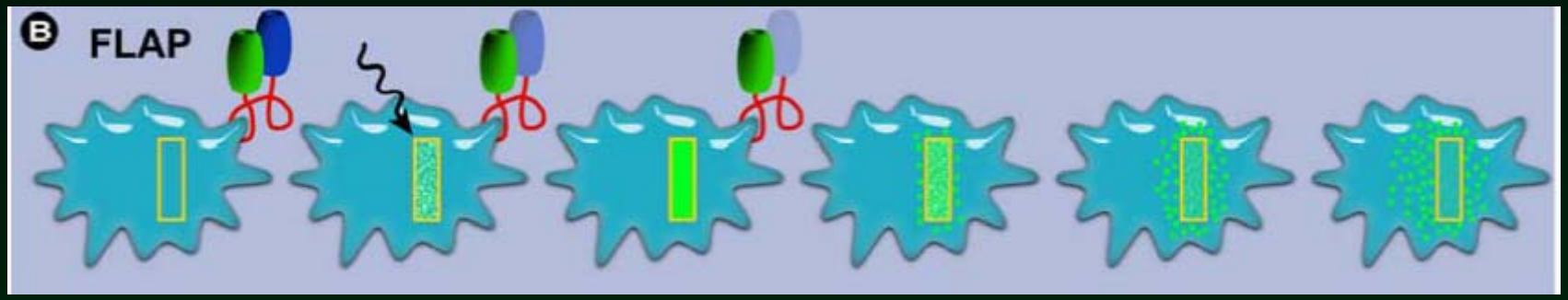

continuous bleaching here

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

# FRAP derivatives

## FLAP

Fluorescence Localization after Photobleaching

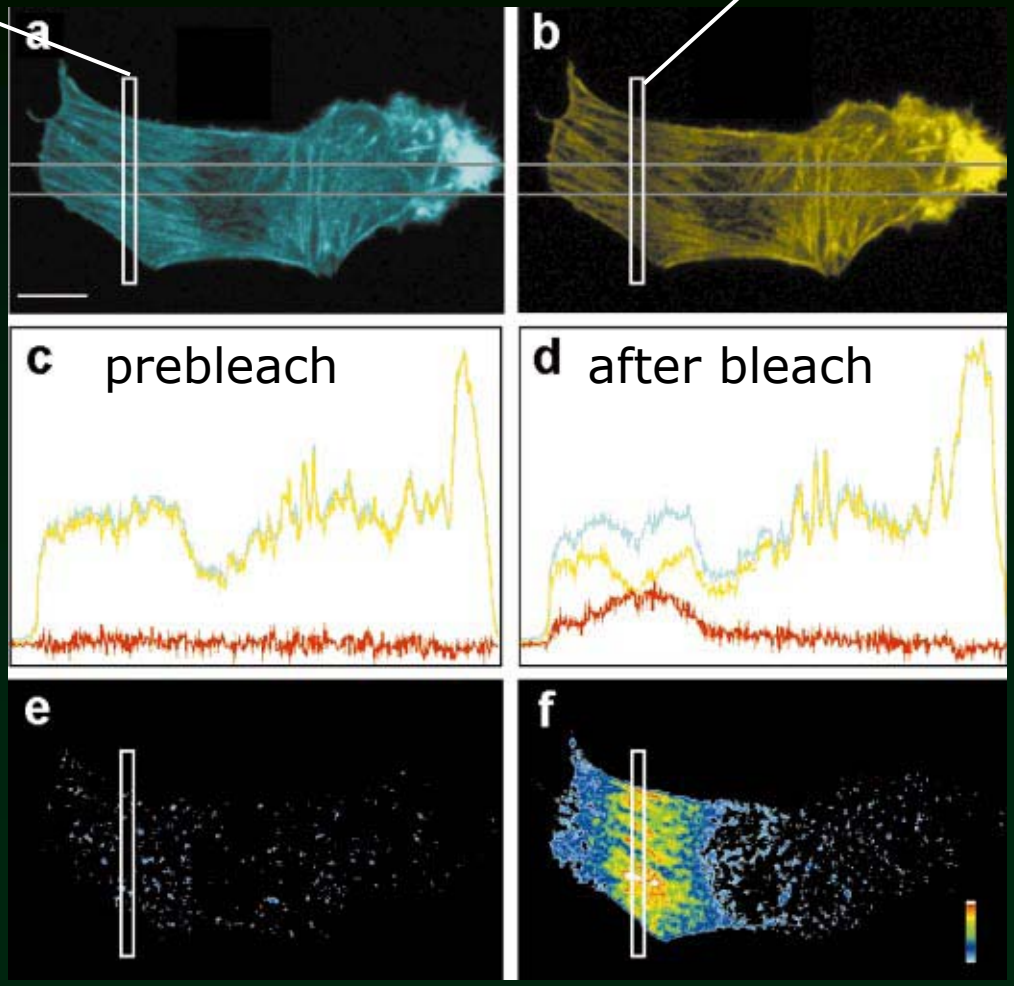



- two fluorochromes on one protein– one bleached, non bleached as control

# Perhaps better scheme than previous

CFP not bleached

YFP bleached




RED=CFP-YFP

Dunn et al. 2002

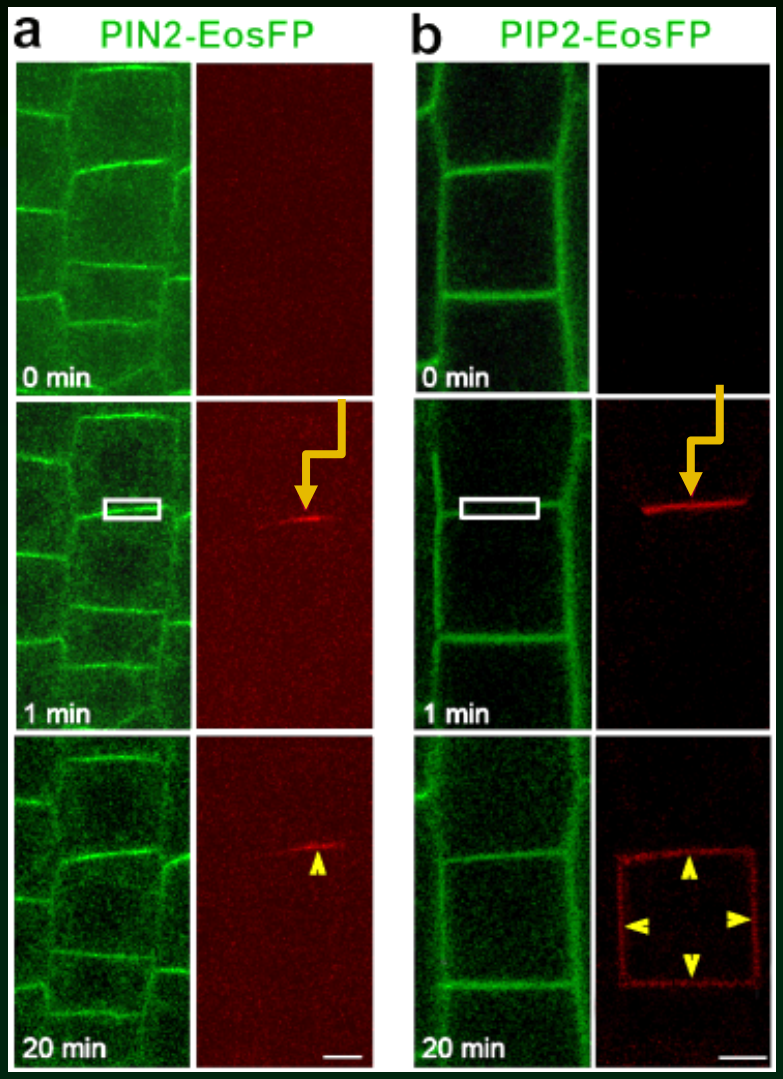
# FRAP - advantages

- 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)

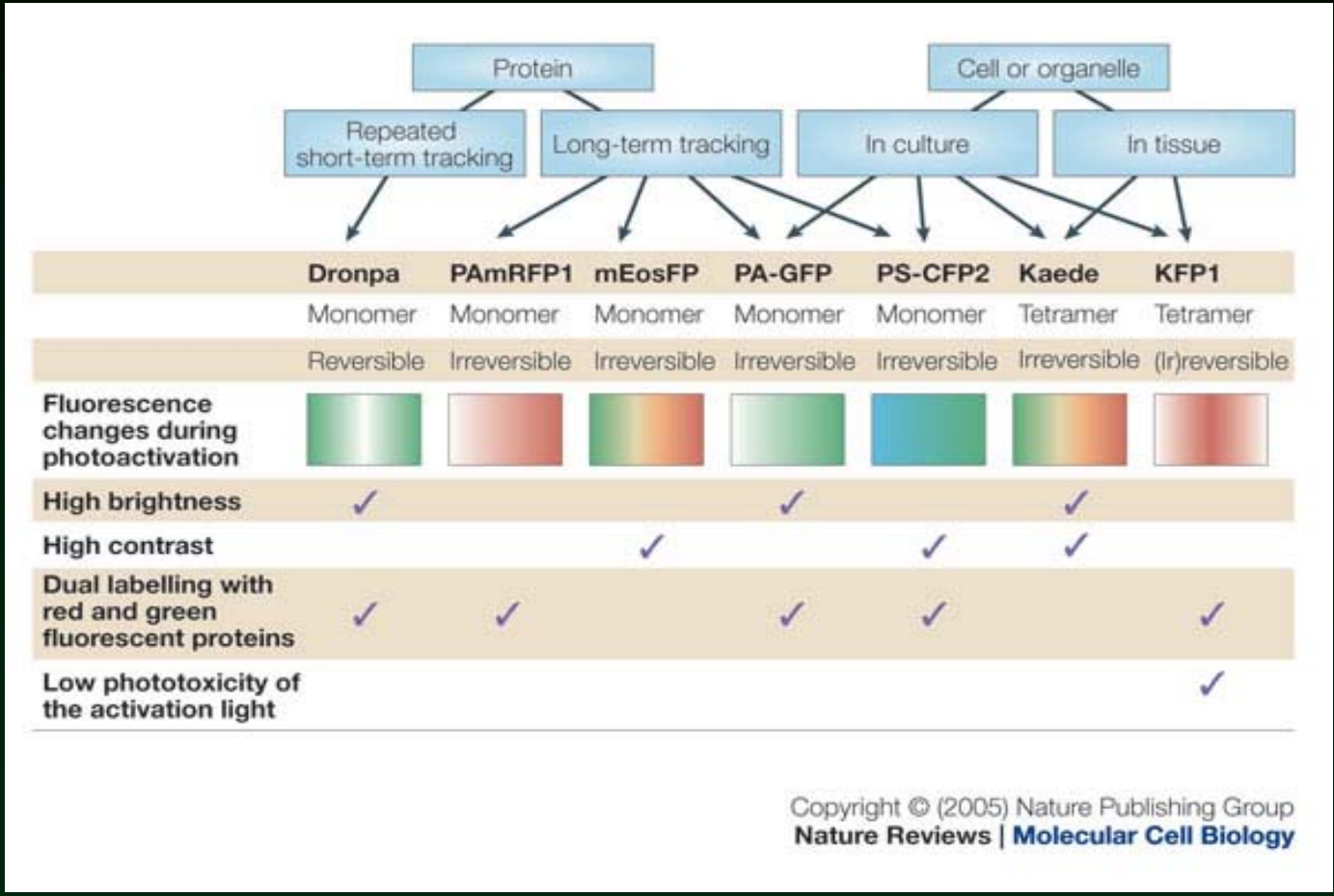
# Photoactivable fluorescent proteins




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)

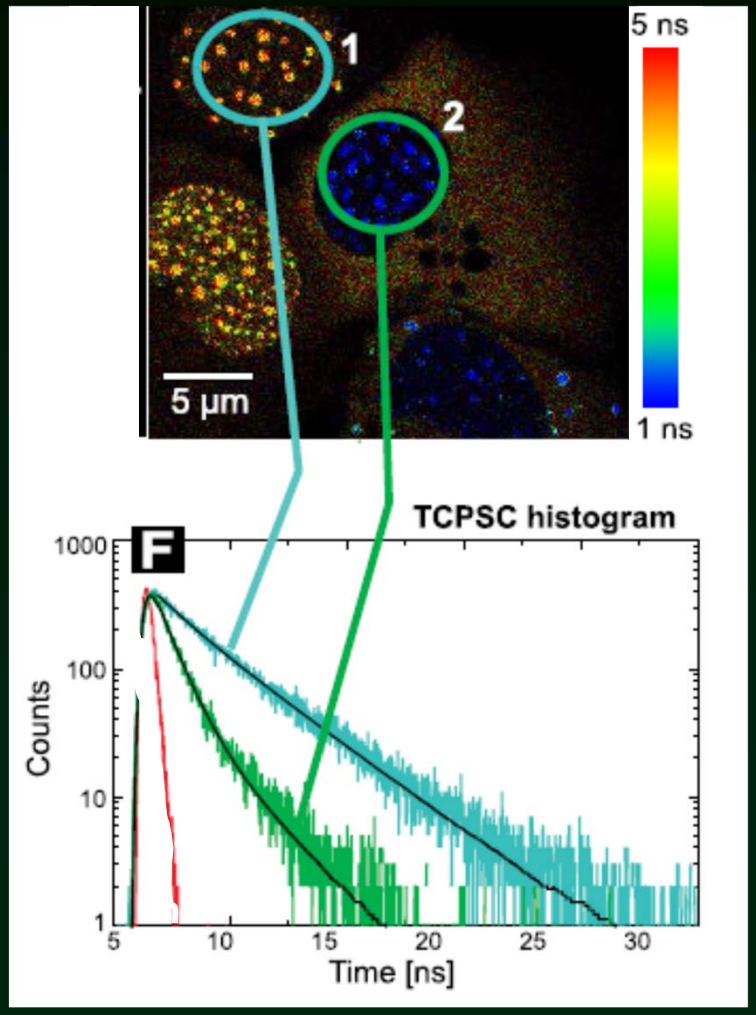
# FLIM

Fluorescence Life Time Imaging Microscopy

Fluorochromes

- excitation spectra
- emission spectra
- unique lifetime

# FLIM - applications



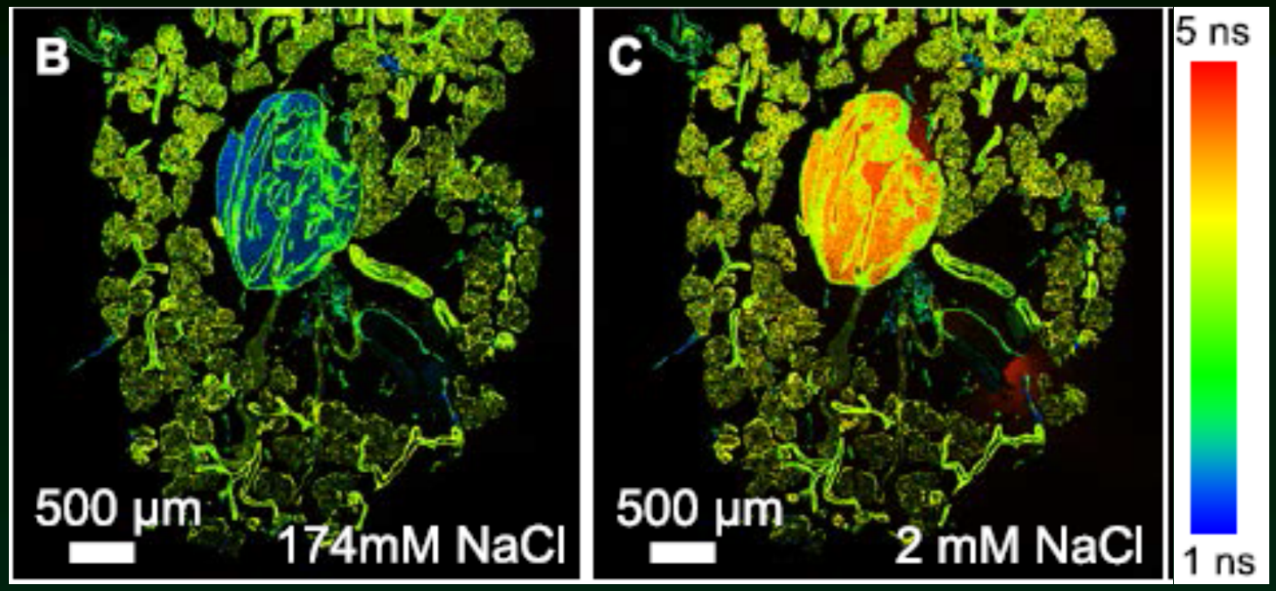
# FLIM - applications

Lifetime sensitive to almost everything:

- pH
- ionic strength
- solution polarity
- other fluorochrome

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

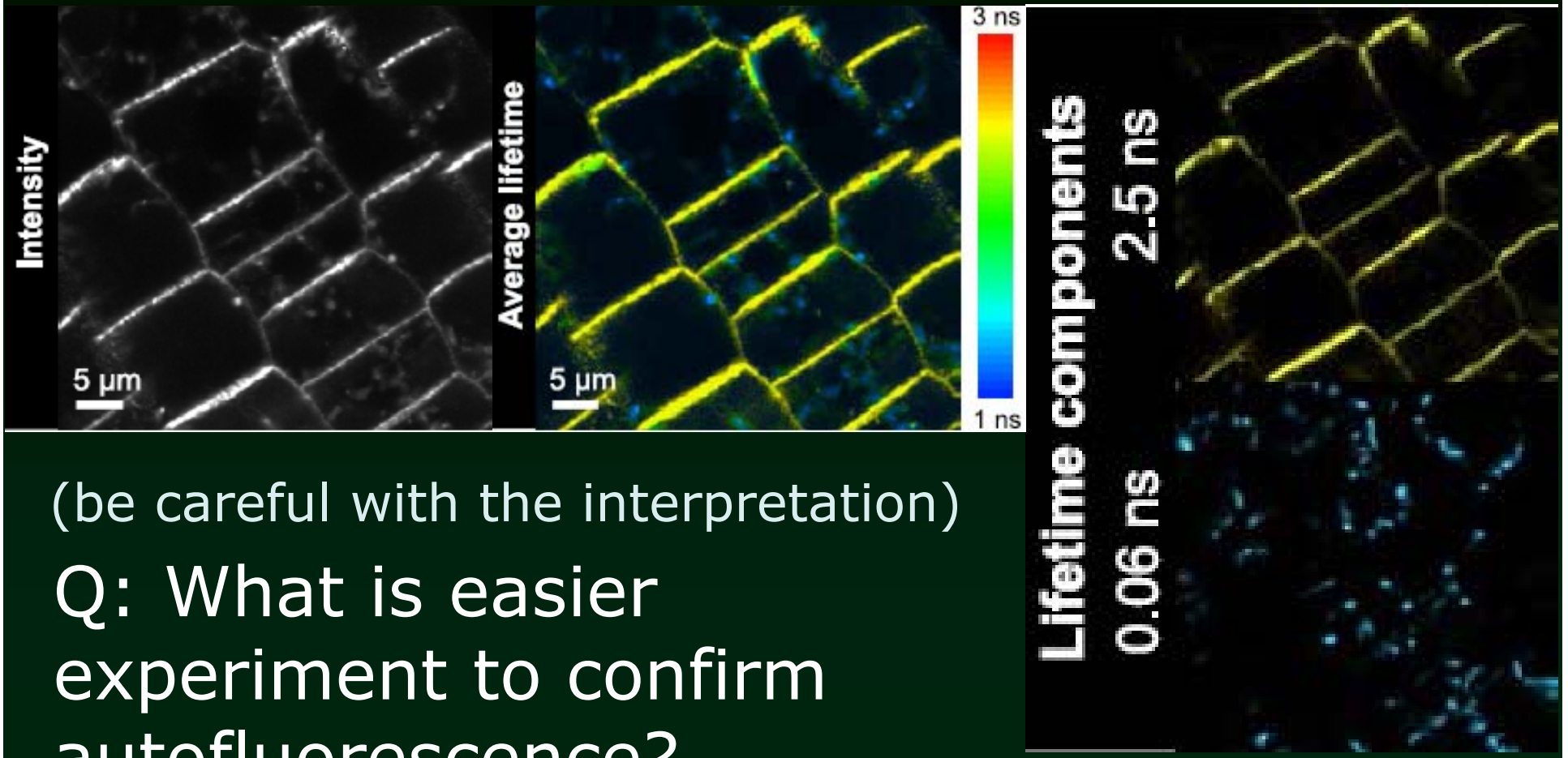
# FLIM




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)

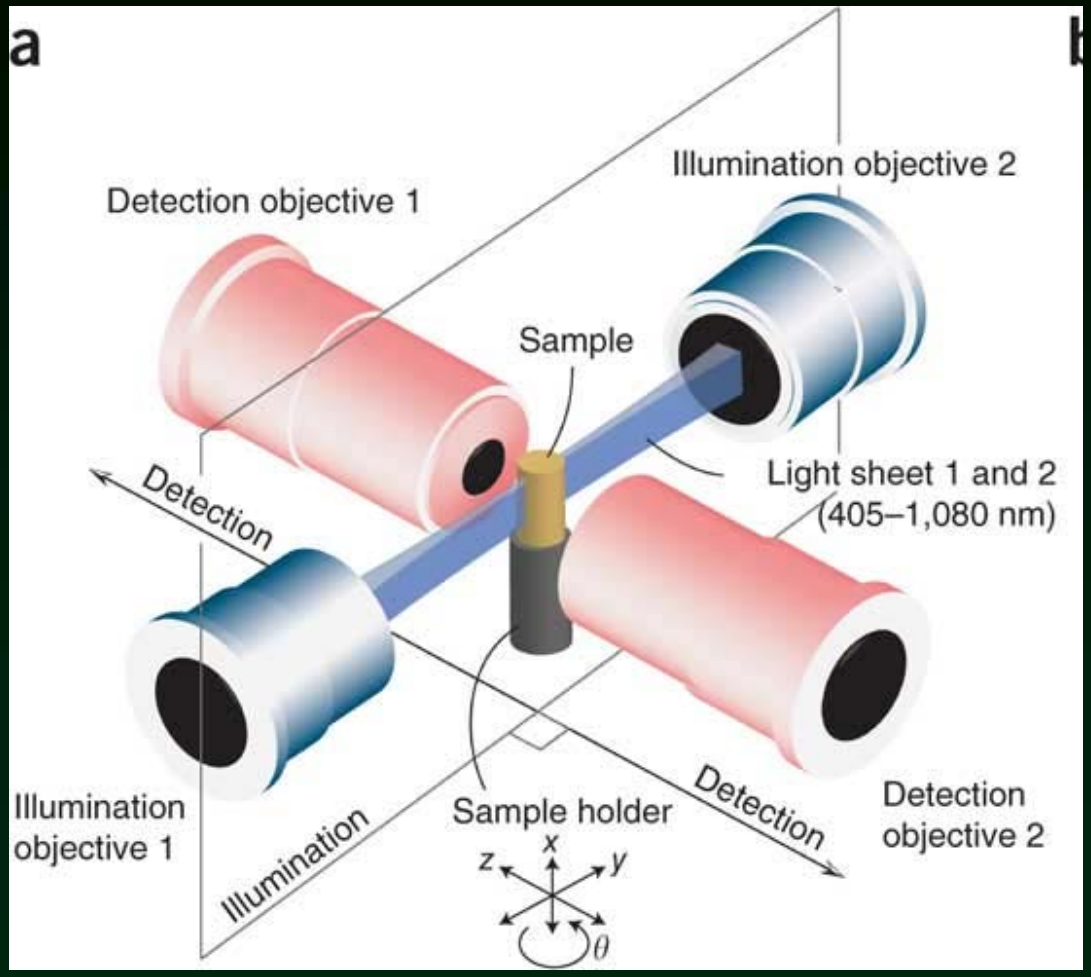
Q: What is easier experiment to confirm autofluorescence?

Dovzhenko, TrautmannPicoQuant Application note 2013

# FLIM

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

# Light sheet microscopy




Tomer et al. Nat Methods 2012

# Light sheet microscopy

Pros:

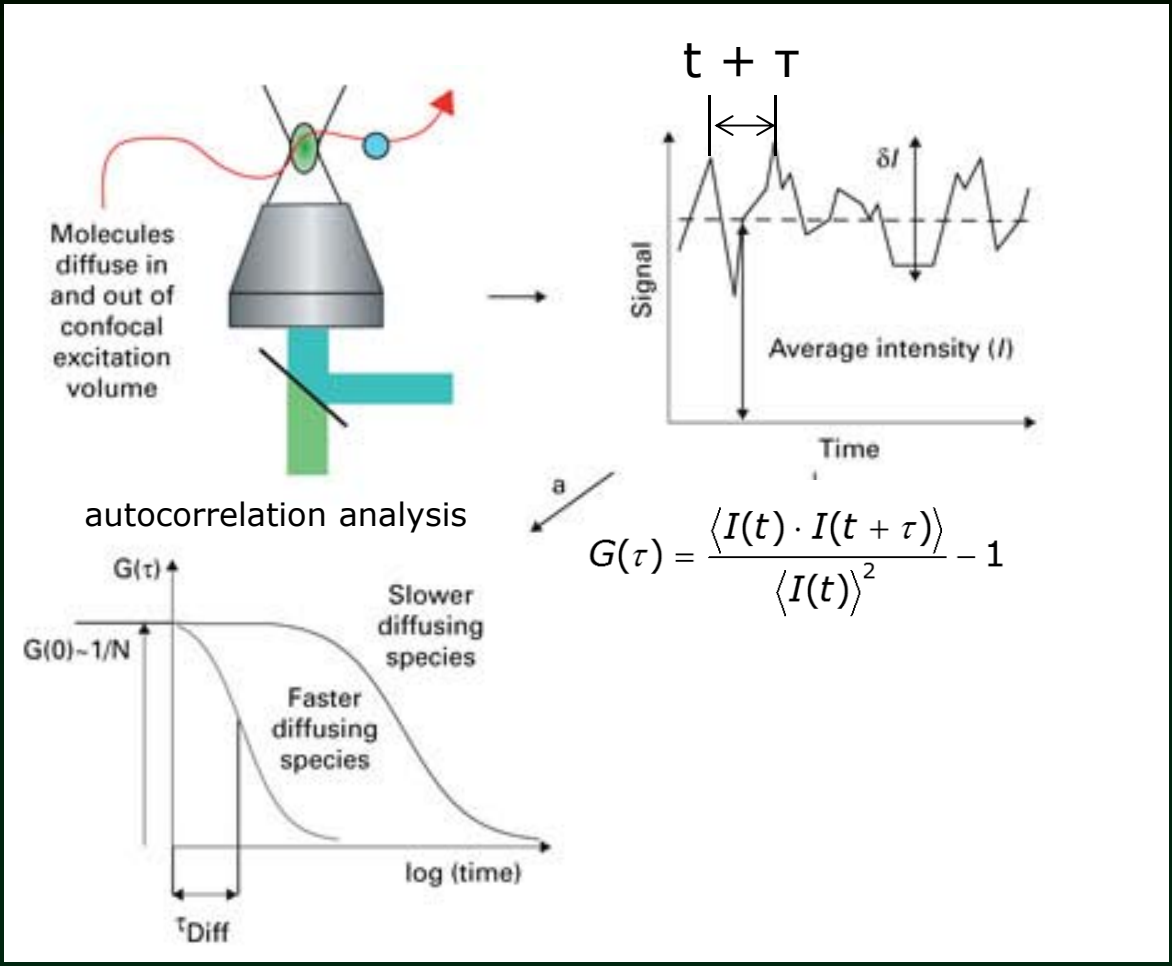
- less bleaching: better tissue penetrance, better resolution and sensitivity
- 3D structures fast

Cons:

- equipment price, availability
- sample preparation could be slower
- data handling

# FCS

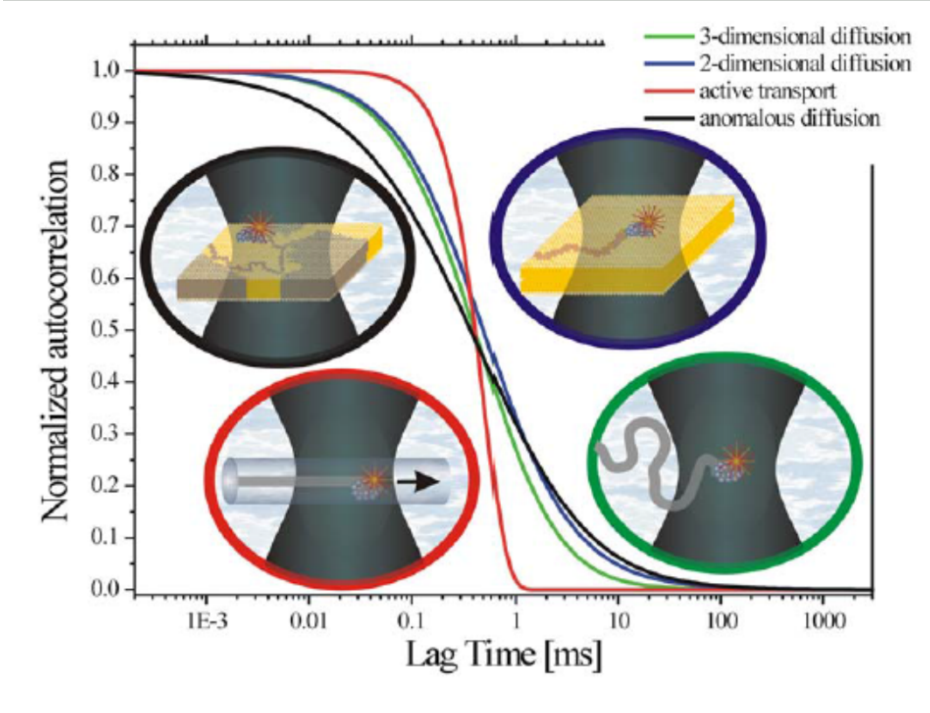
## Fluorescence Correlation Spectroscopy

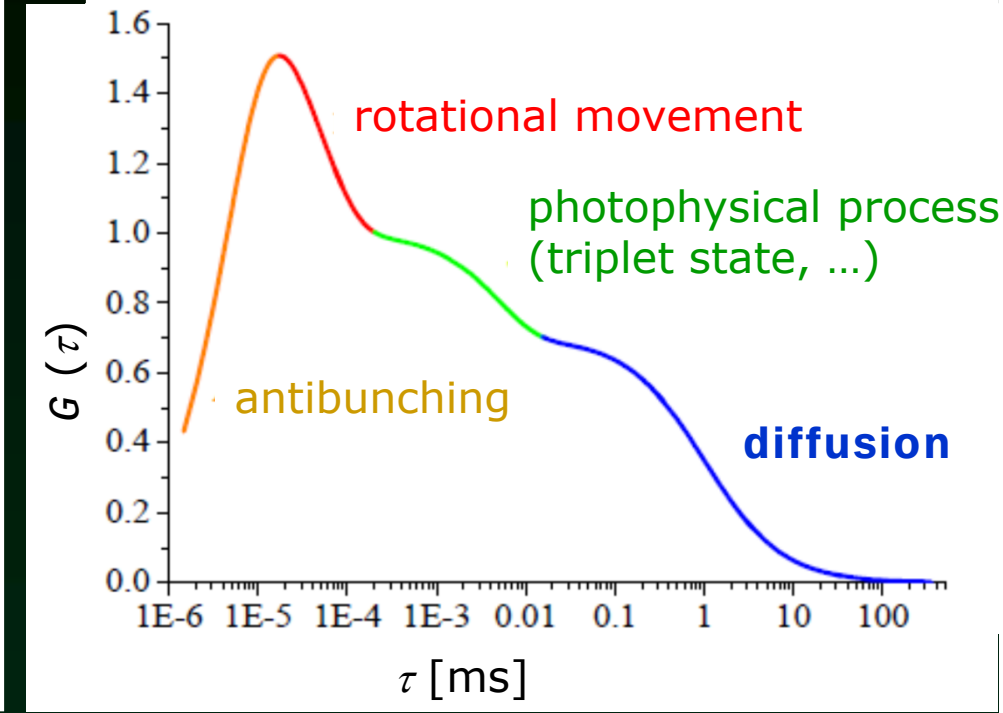



It is counted, how many times the fluorescent molecule comes through the focal plane.

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

# FCS



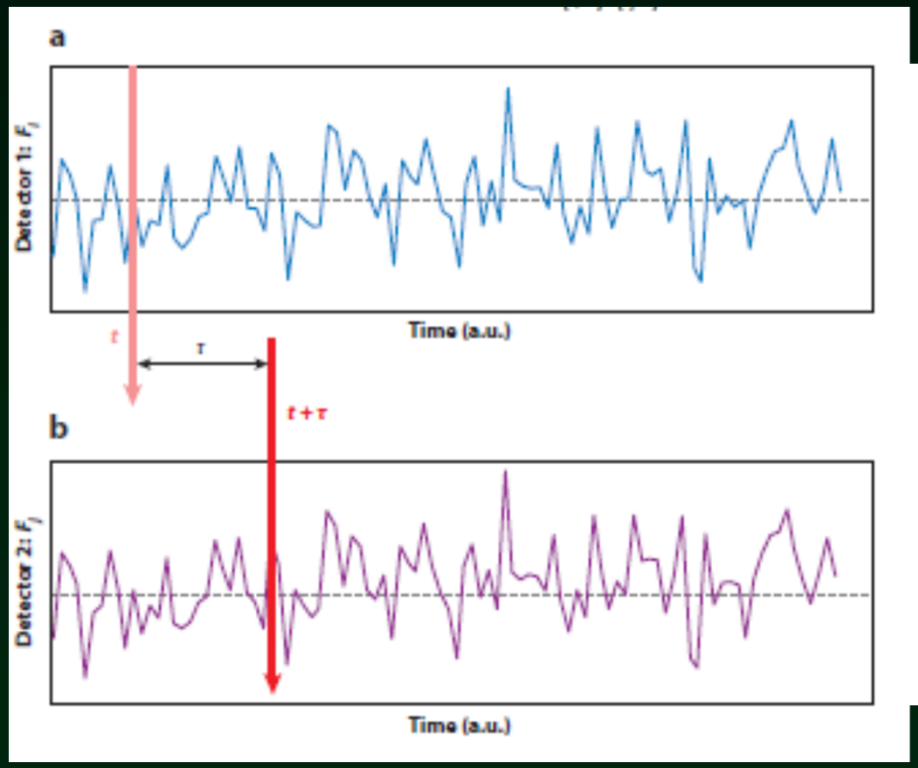



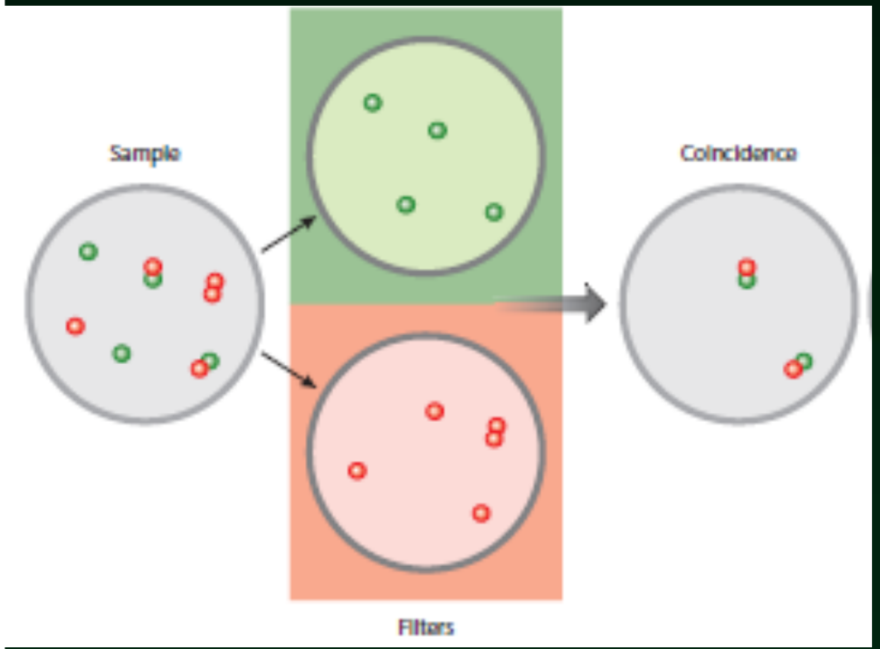

Schwille und Haustein

# FCS (FCCS)

## fluorescence cross-correlation spectroscopy

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



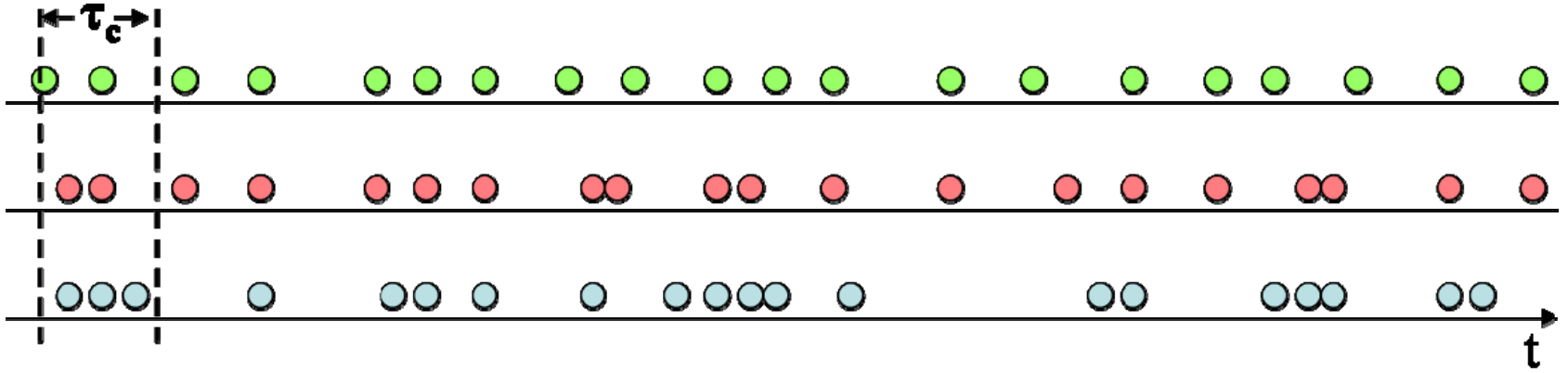




Digman and Gratton 2011

# 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)

# Photon bunching, if someone would ask




Photon detections as a function of time for a) antibunched, b) random, and c) bunched light