How to visualize genes and their products
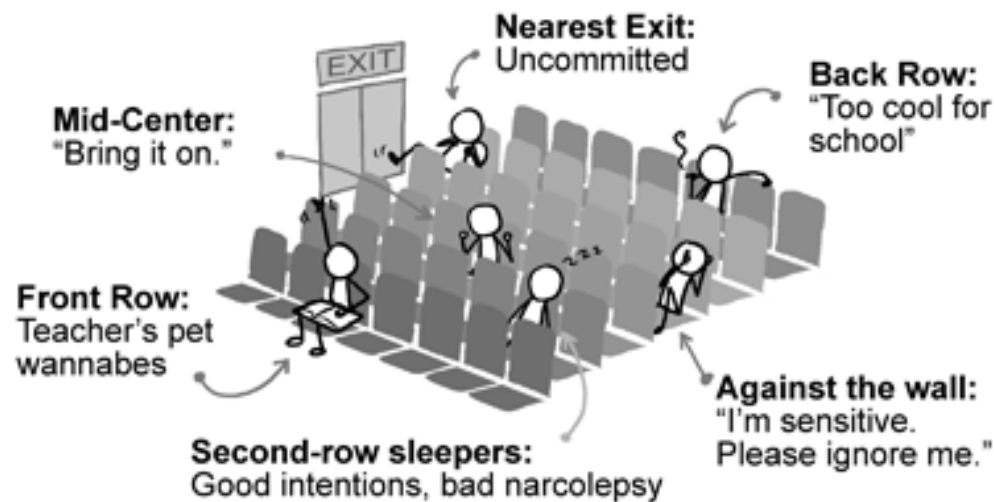
CG920 Genomics
Lecture 9

Kamil Růžička
FGP CEITEC MU

Outline

- reporter genes

- promoter fusions

- visualizing proteins

- visualizing RNA

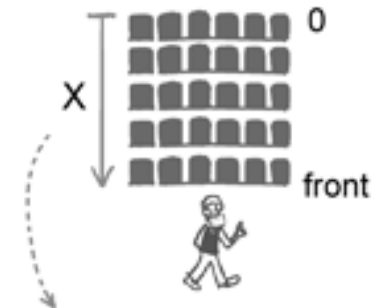- dynamics of protein imaging: FRAP, photoactivable proteins, FLIM, FCS

3

Promoter activity monitoring

promoter	here can be reporter	terminator

1-10 kb prior to ATG

LacZ, GUS

Luciferase

GFP

Reporter genes

- LacZ, GUS

- Luciferase

- GFP

some need external substrate, some do not
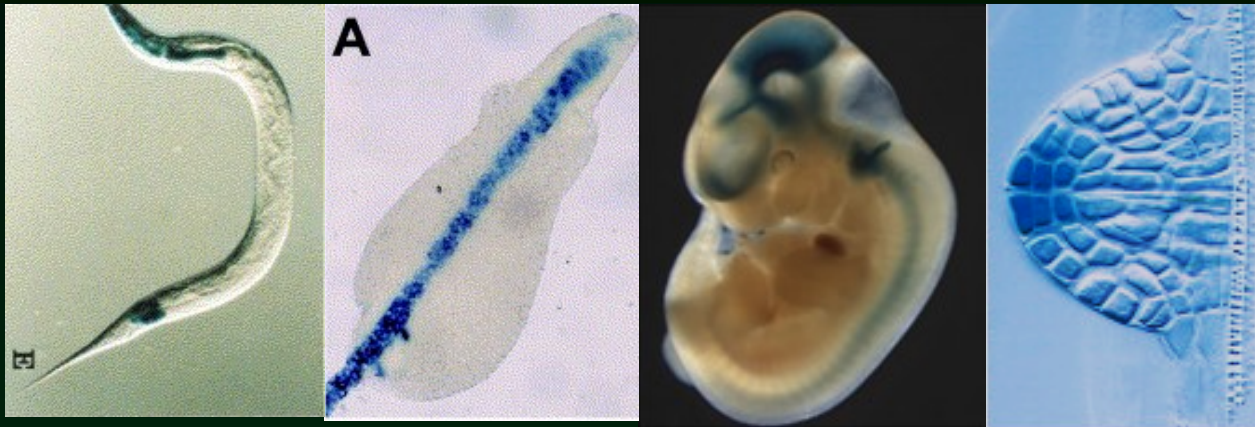
LacZ, GUS – rhapsody in blue

promoter	LacZ	terminator

soluble

insoluble
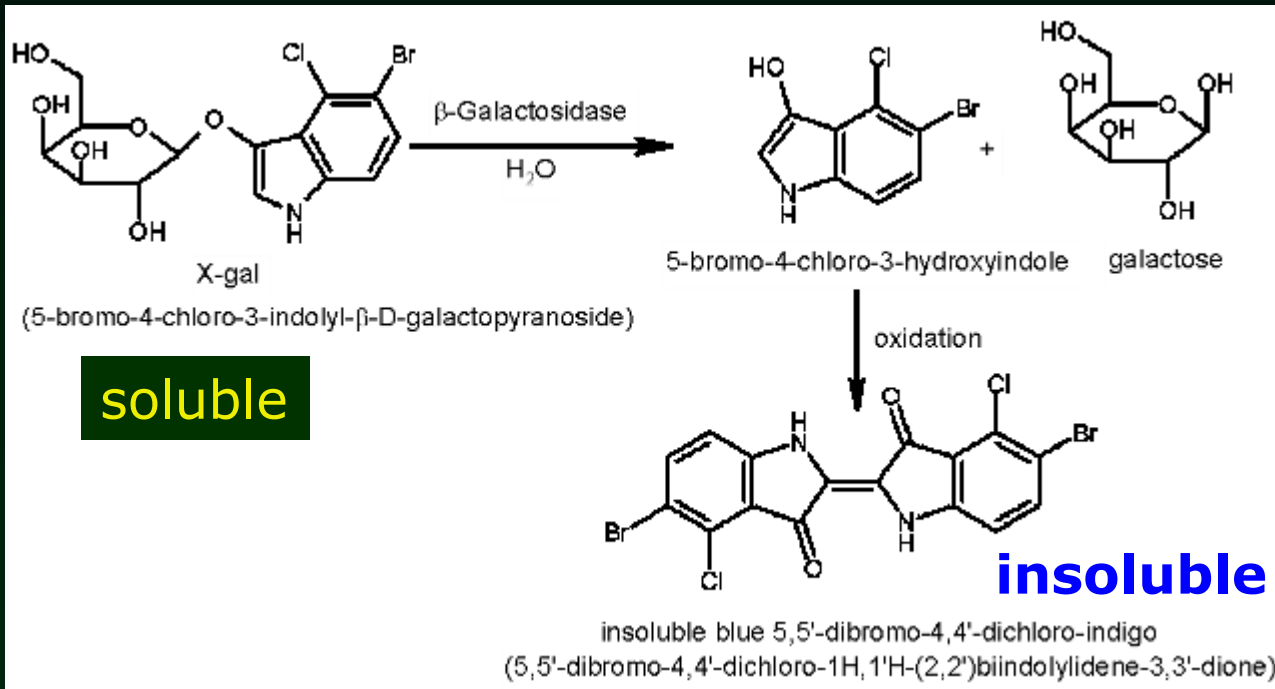
(in case of GUS – X-Gluc)

LacZ, GUS

LacZ/ GUS:

worm, mouse – LacZ, plants - GUS

Luciferase

promoter	luciferase	terminator

Bioluminescence

D-Luciferin

Firefly Luciferase + Mg^{2+}

Light

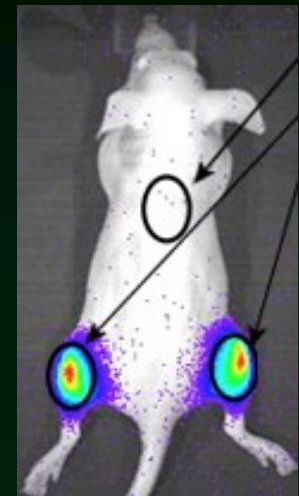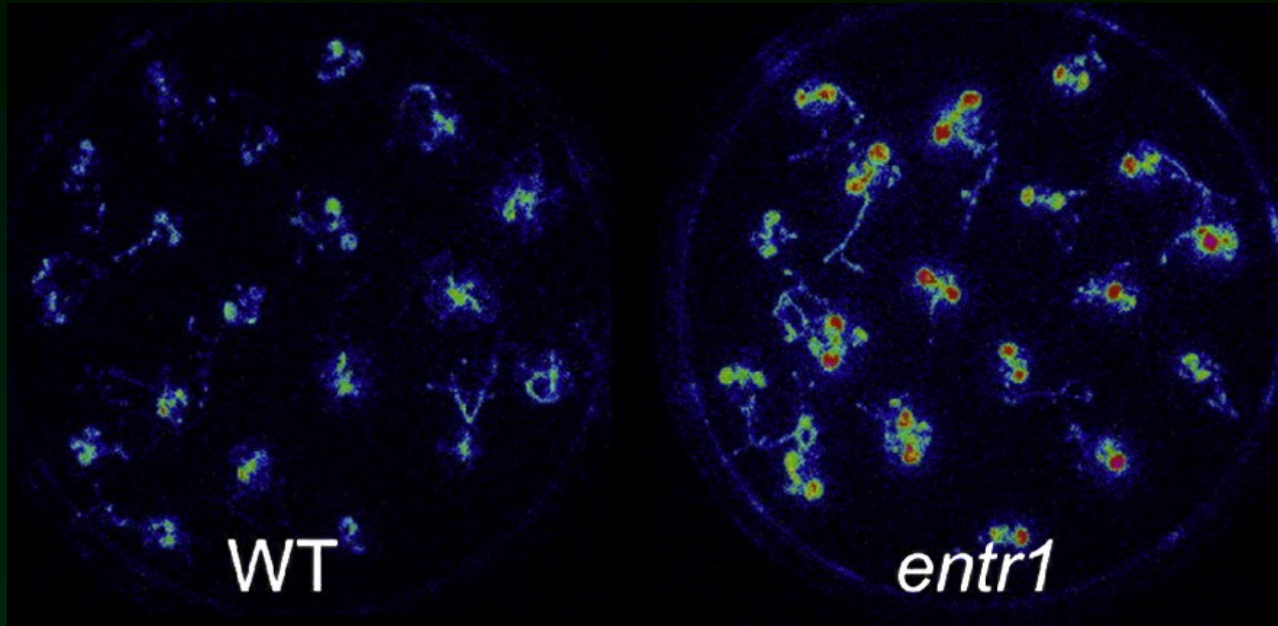Oxyluciferin

+ ATP + O$_2$

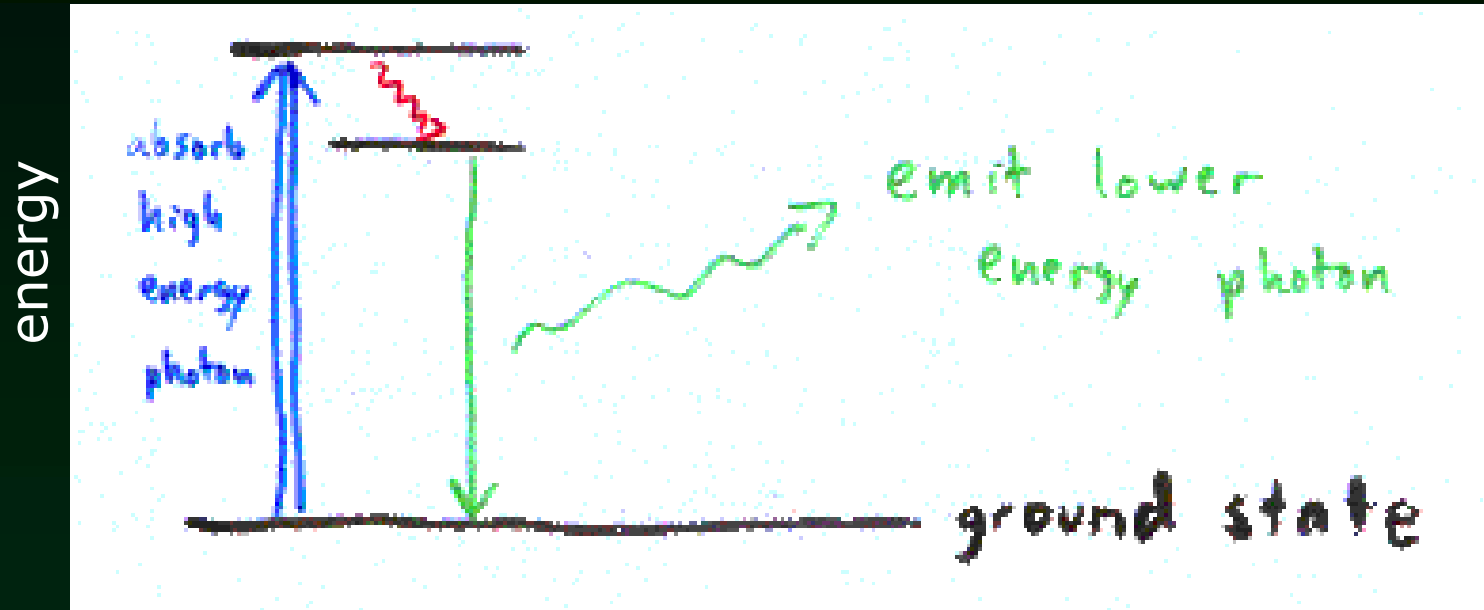+ PPi + AMP + CO$_2$
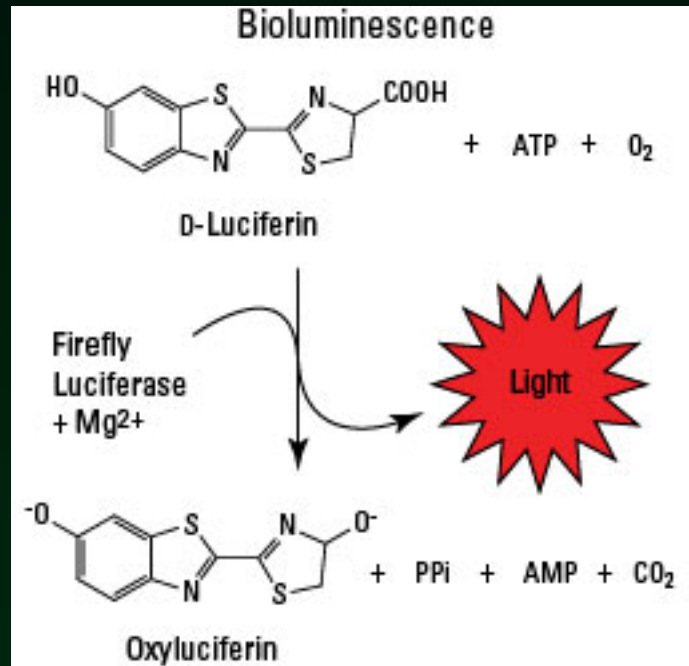
(similar to chemiluminiscence)

What's the difference between fluorescence and luminiscence?

Luciferase

WT

entr1
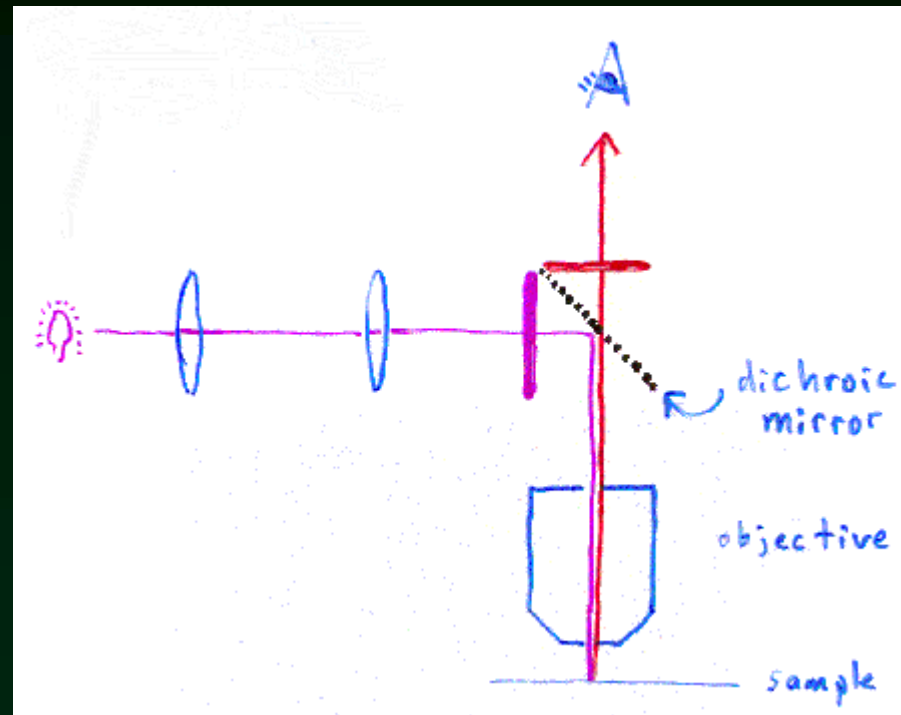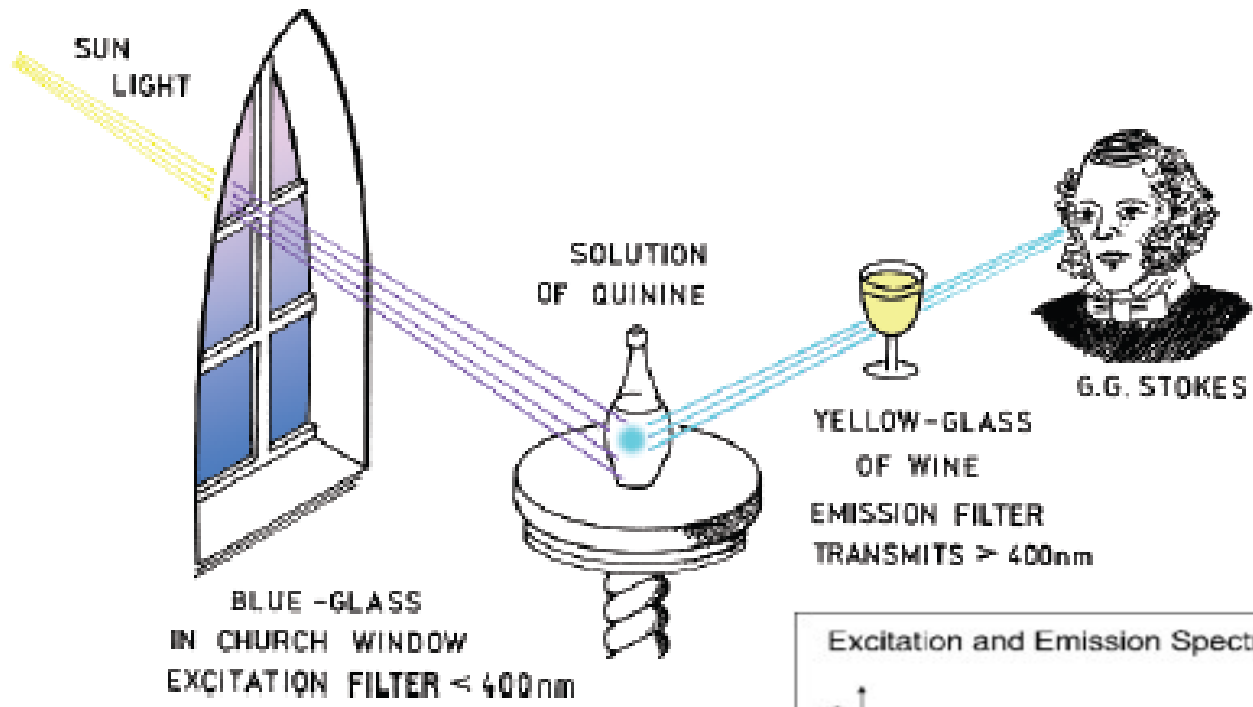
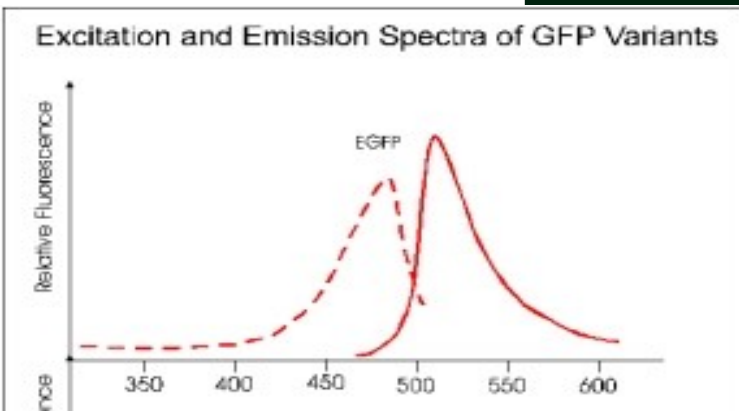# How does fluorescence work?

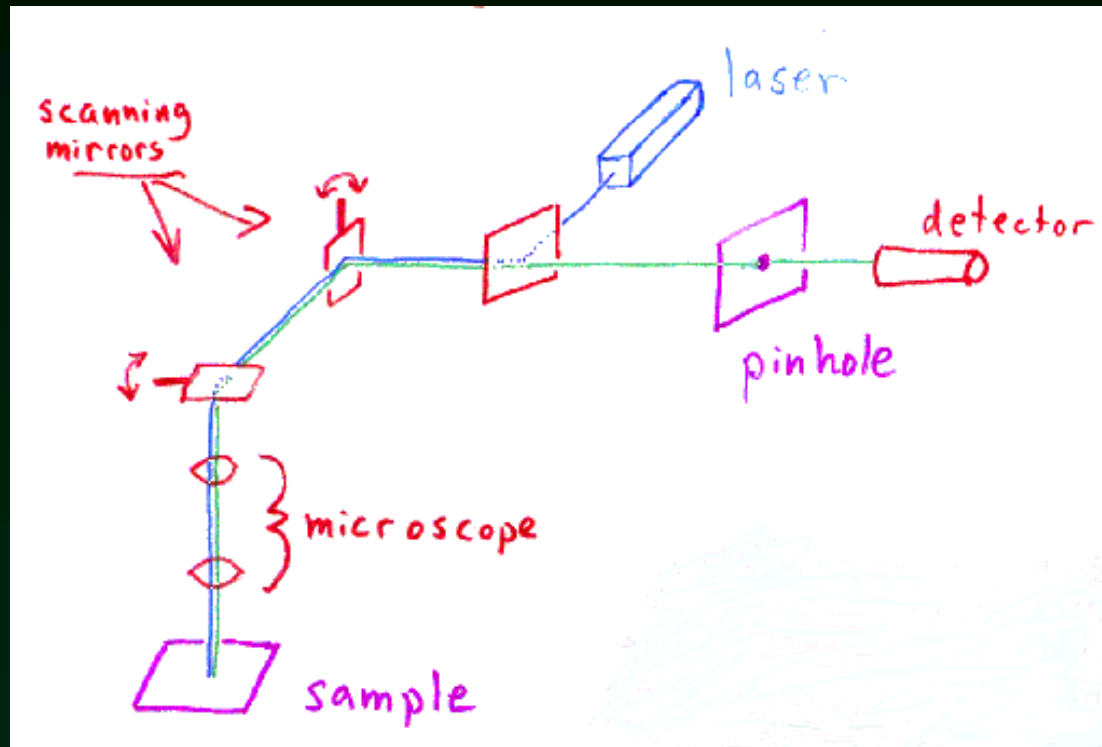# How does a fluorescence microscope work?

Stokes shift

George G. Stokes

How does a confocal microscope work?

What are advantages of confocal microscopy?

Live imaging

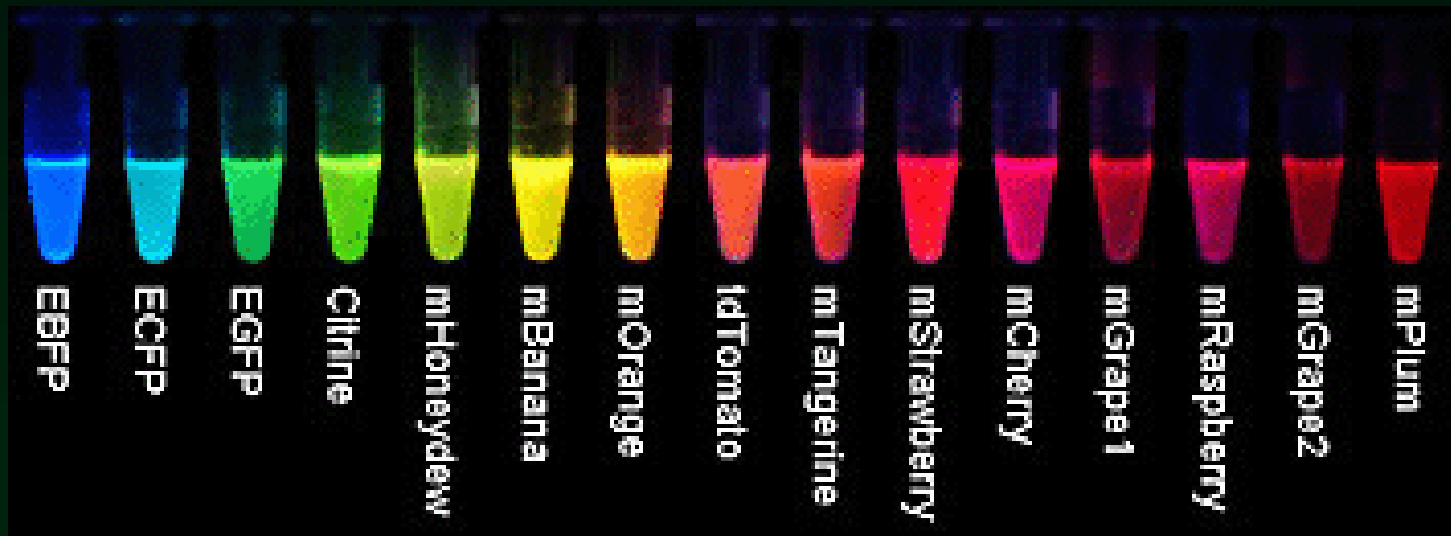GFP discovery - Nobel Prize 2008
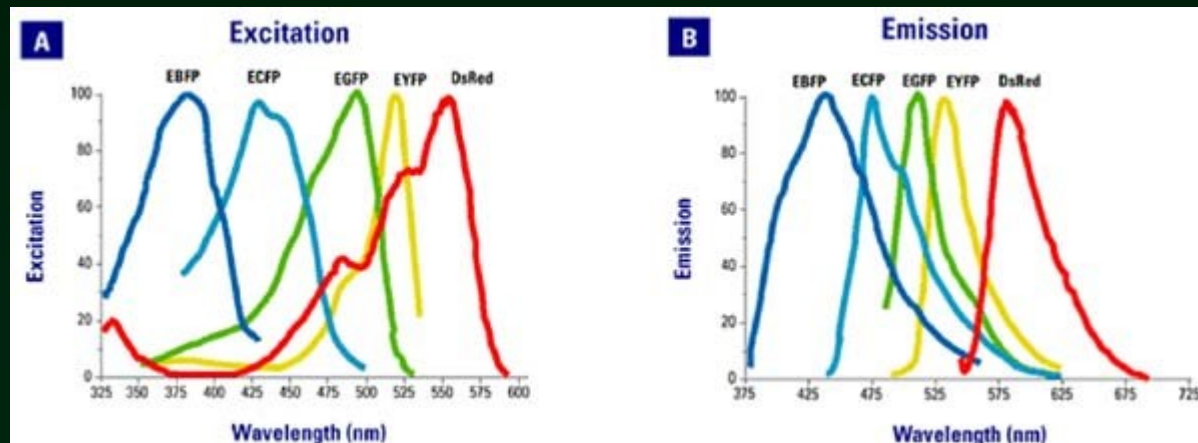
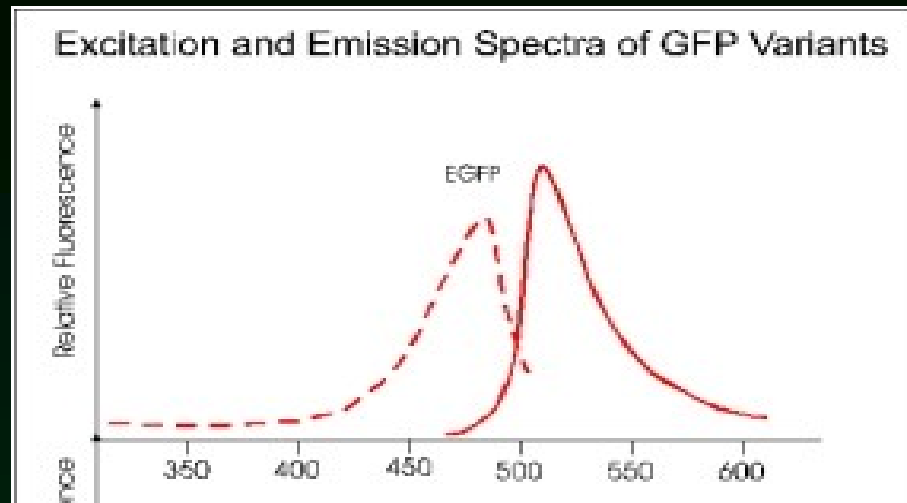Osamu Shimomura   Martin Chalfie   Roger Tsien

Fluorescent proteins on the market (Tsien's fruits)

Excitation and emission

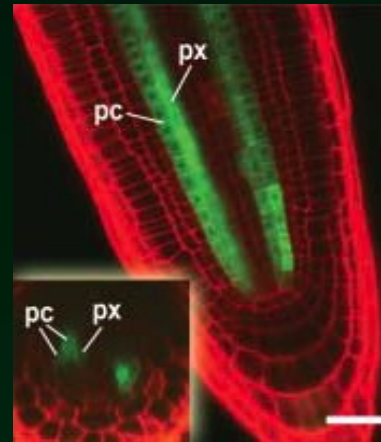# Multicolored fluorescent protein image (neurones)
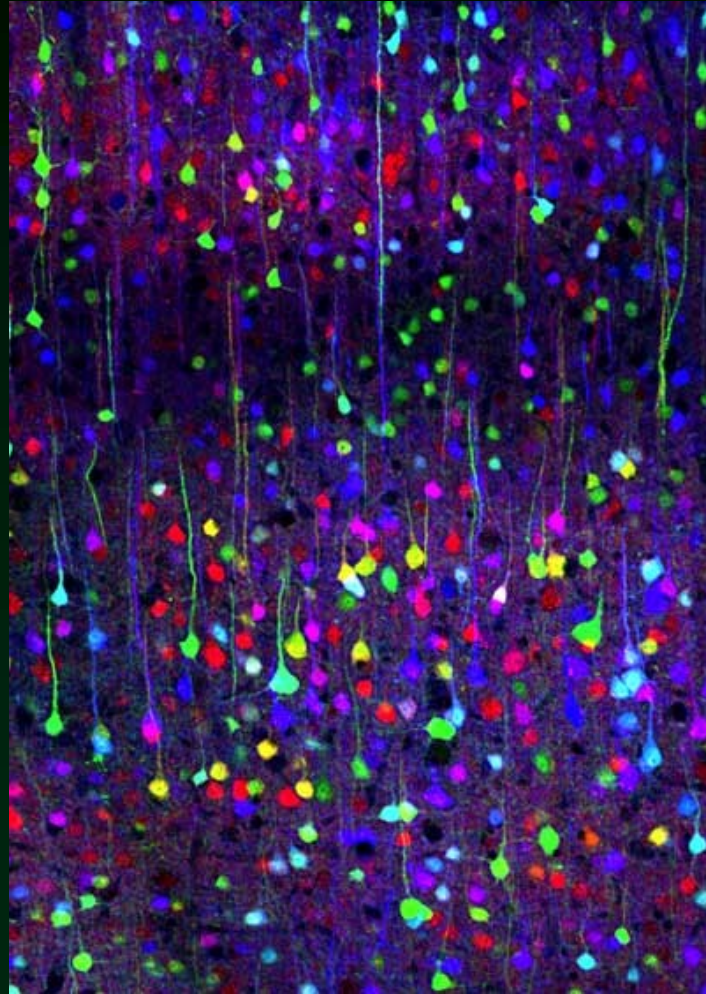
Promoter-GFP

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 autofluorescence
  - substrate must diffuse, special machine, dark
- GFP
  - good sensitivity, colocalization with other dyes/promoters possible, no substrate needed
  - only in vivo, autofluorescence, thin transparent sample; it should be ER localized in plants

Luminiscent mouse better than phluorescent mouse

In Vivo Comparison of Bioluminescence and Fluorescence (I.M.)

- Fluorescent signal is limited by tissue autofluorescence
- The bioluminescent signal level is ~300x lower, yet the signal to background is 160x higher
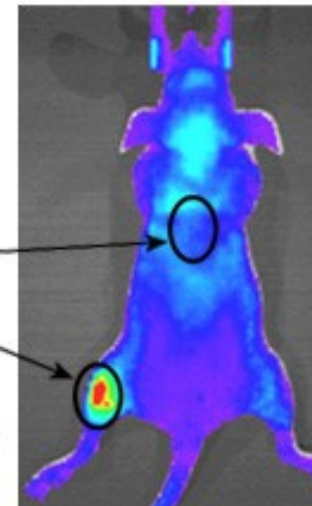
Bioluminescence
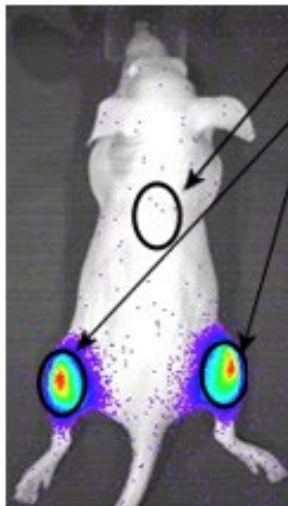
Background flux ~ 2.6×10^3 p/s
Signal flux ~ 2.8×10^6 p/s
Signal/background ~ 1100
Min. detectable cells ~ 900

Fluorescence

Background flux ~ 1.2×10^8 p/s
Signal flux ~ 8.3×10^8 p/s
Signal/background ~ 6.7
Min. detectable cells 150,000

Left: 1×10^6 HeLa-luc/PKH26 cells
Right: 1×10^6 HeLa-luc cells

Promoter activity monitoring
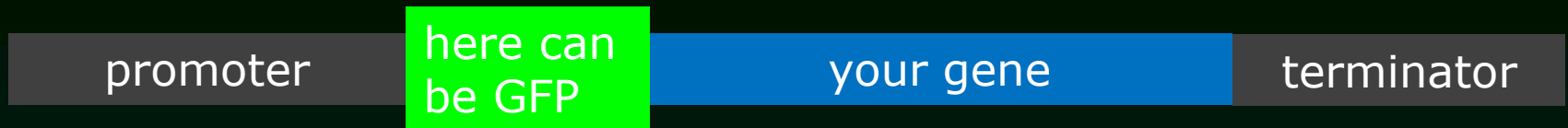
<u>Pros:</u>
- easy to clone, easy to visualize
- usually some signal seen – cheers you up!
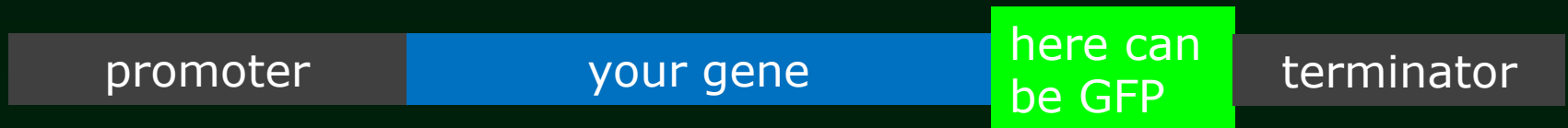- can be used in less accessible organs

<u>Cons:</u>
- limited information about gene product (mRNA, protein etc.)
- needs cloning and transformation
- neglects regulatory elements (introns, UTRs etc.)
- length of promoter given arbitrarily
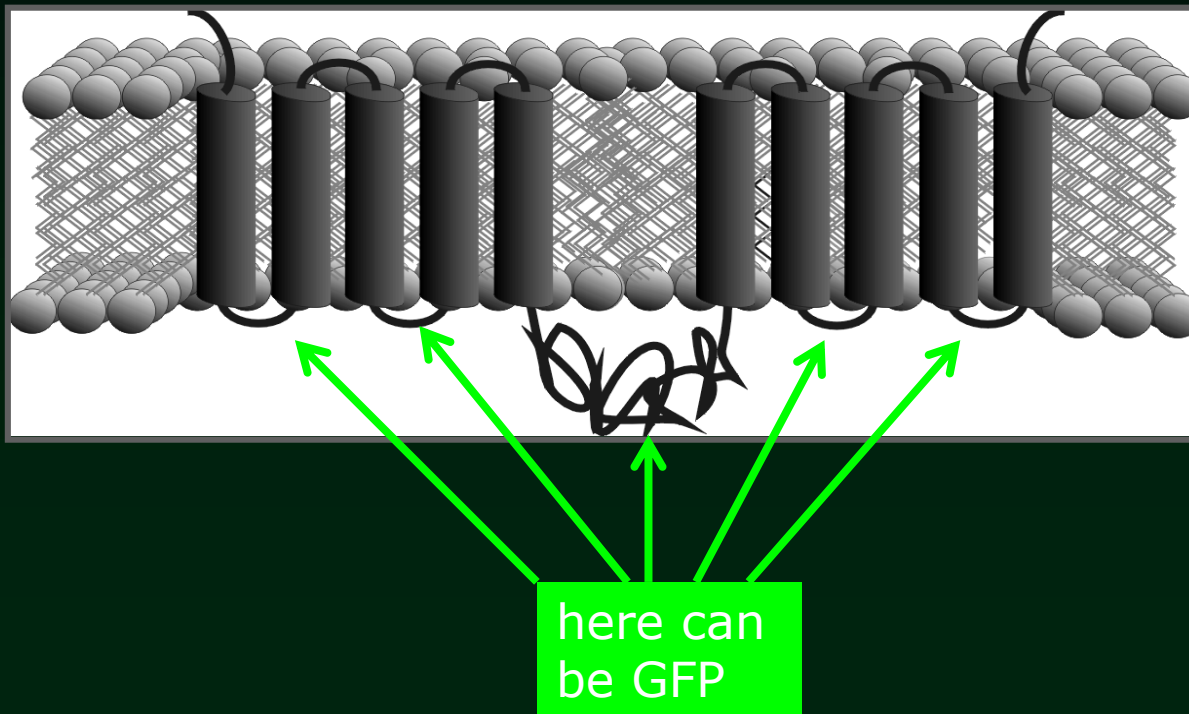
Translational GFP fusions

N-terminal fusion

promoter	here can be GFP	your gene	terminator

C-terminal fusion

promoter	your gene	here can be GFP	terminator

fusion inside the coding sequence

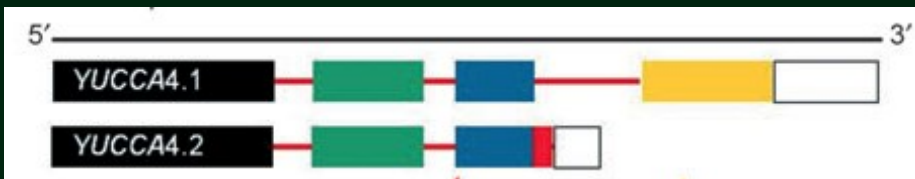| promoter	here can be GFP / your gene	terminator

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GFP and membrane proteins

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

here can
be GFP

Expression of isoforms

endoplasmic reticulum

cytosol

Not the best option available – can you guess?

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Kriechenbaumer et al 2011
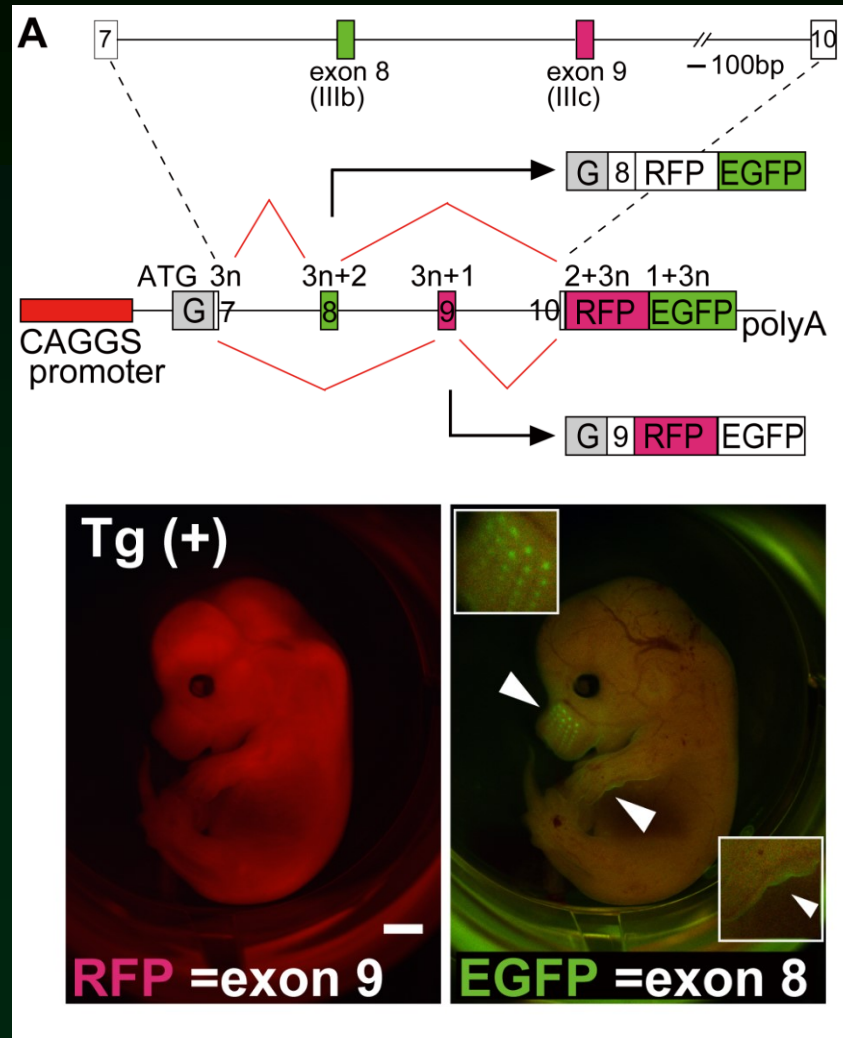
Isn't this better?

26
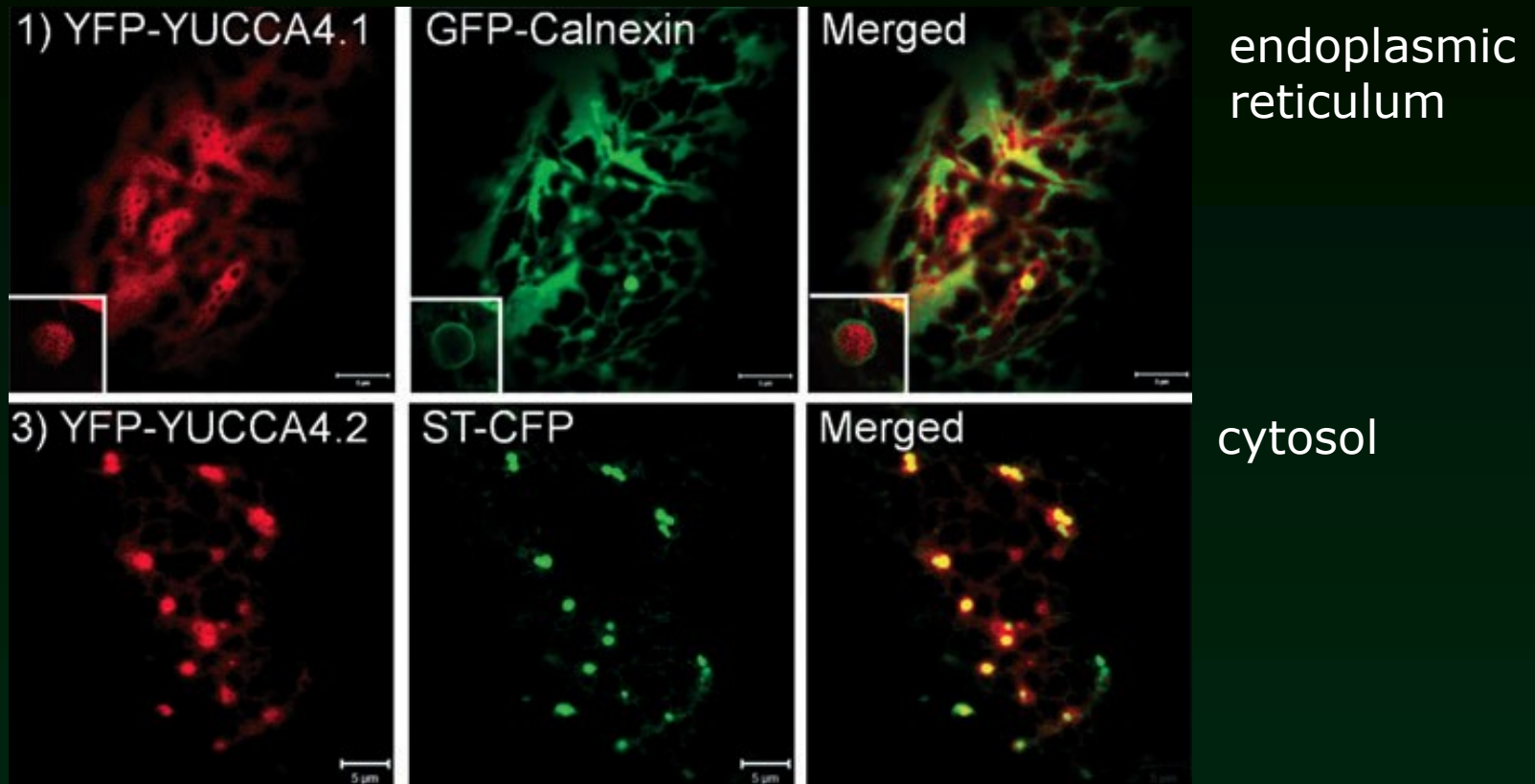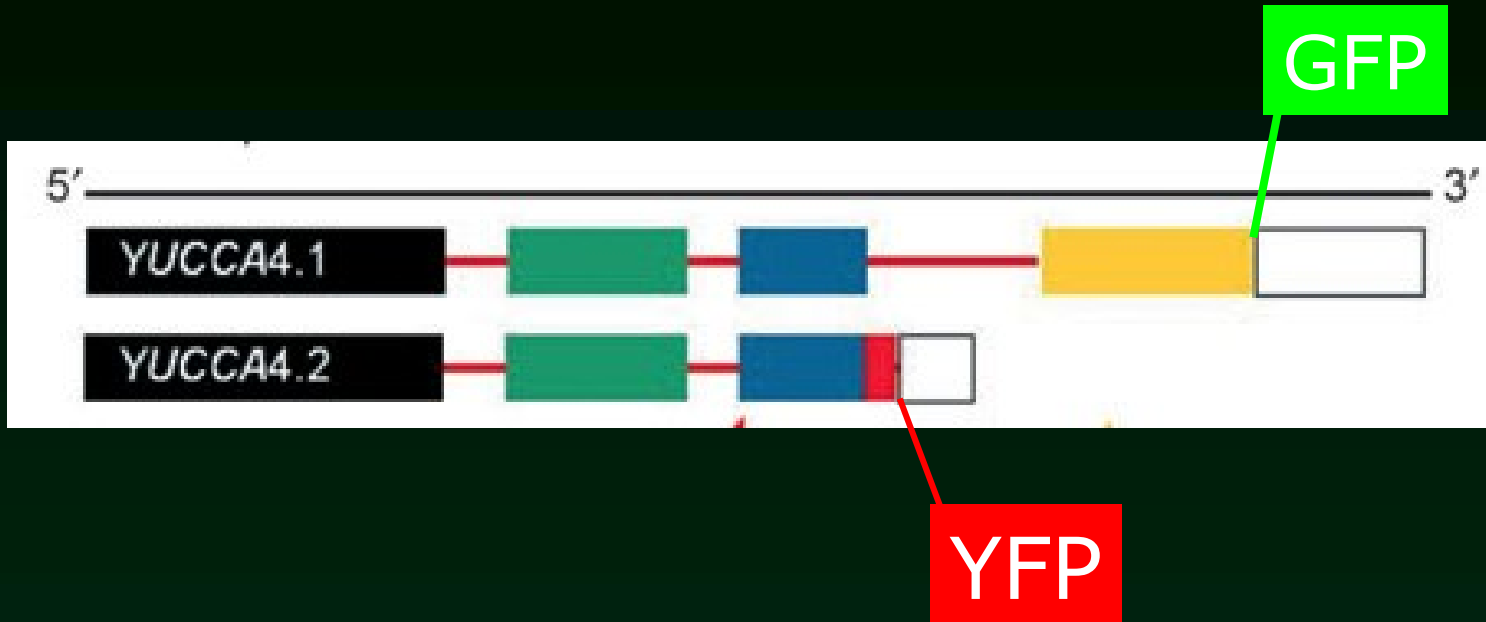
Expression of isoforms
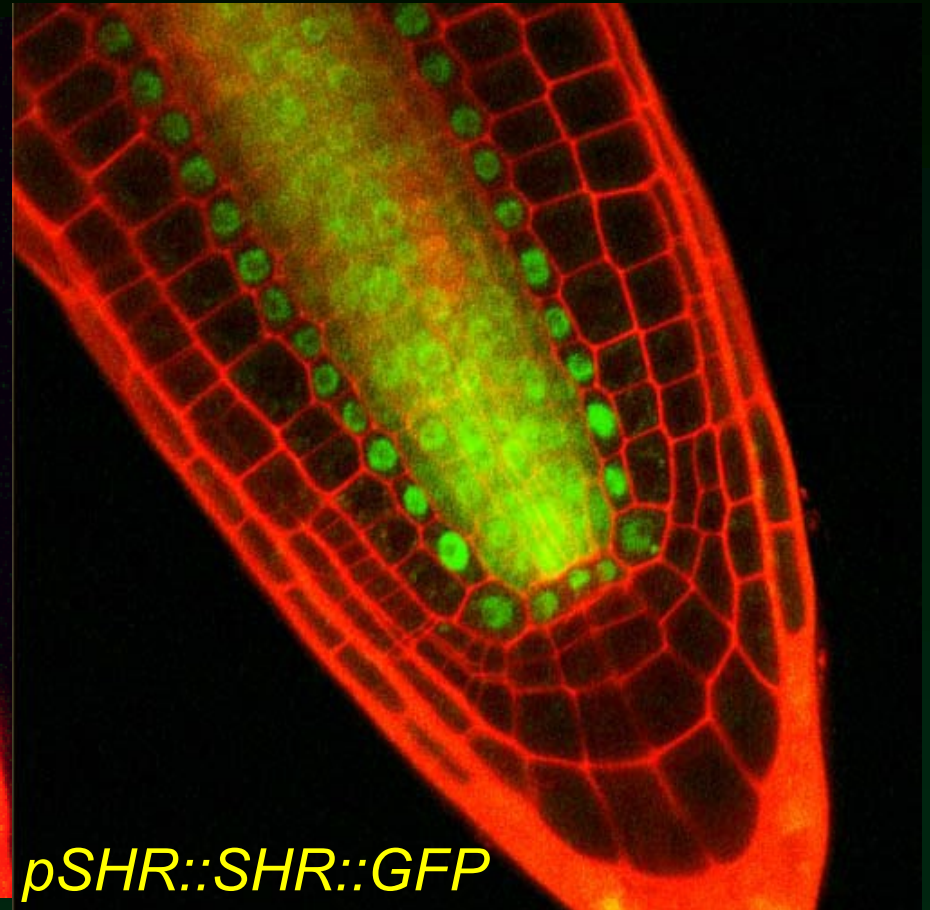
Fluorescent protein fusion

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
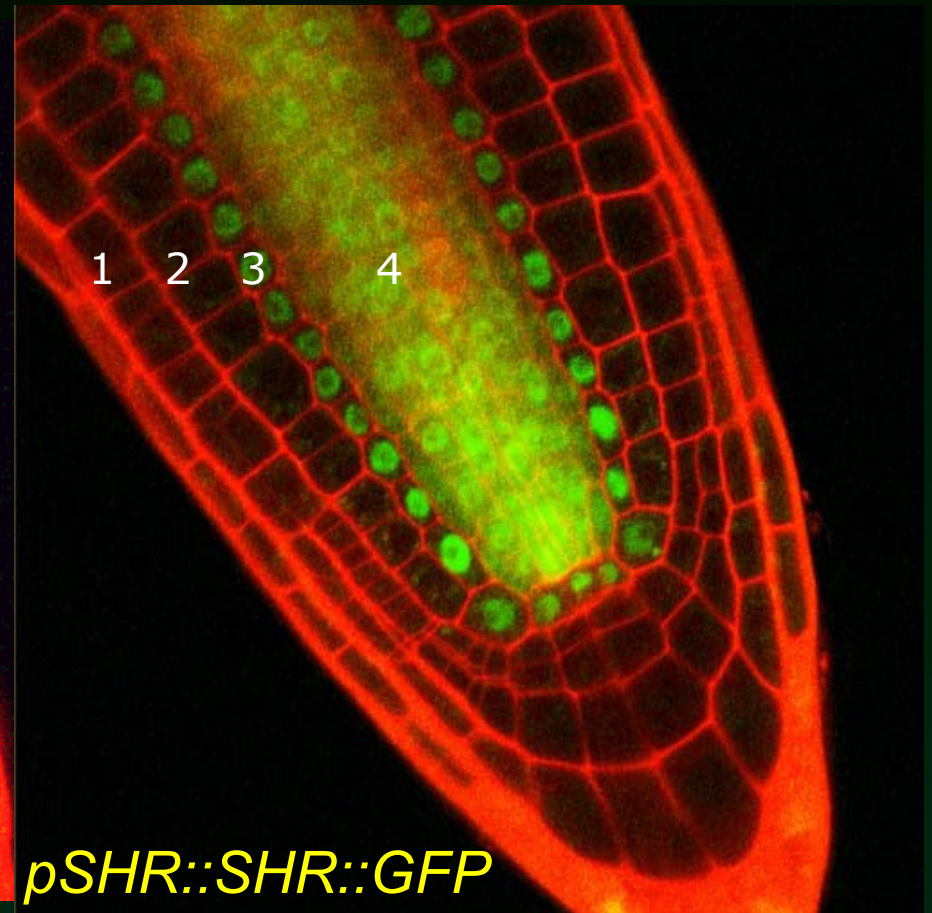

pSHR :: GFP

promoter

pSHR::SHR::GFP

translational

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Nakajima et al, Nature 2001

Why to visualize all this stuff

pSHR :: GFP

promoter

pSHR::SHR::GFP

translational

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

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Why to visualize all this stuff

pSHR :: GFP

pSHR::SHR::GFP
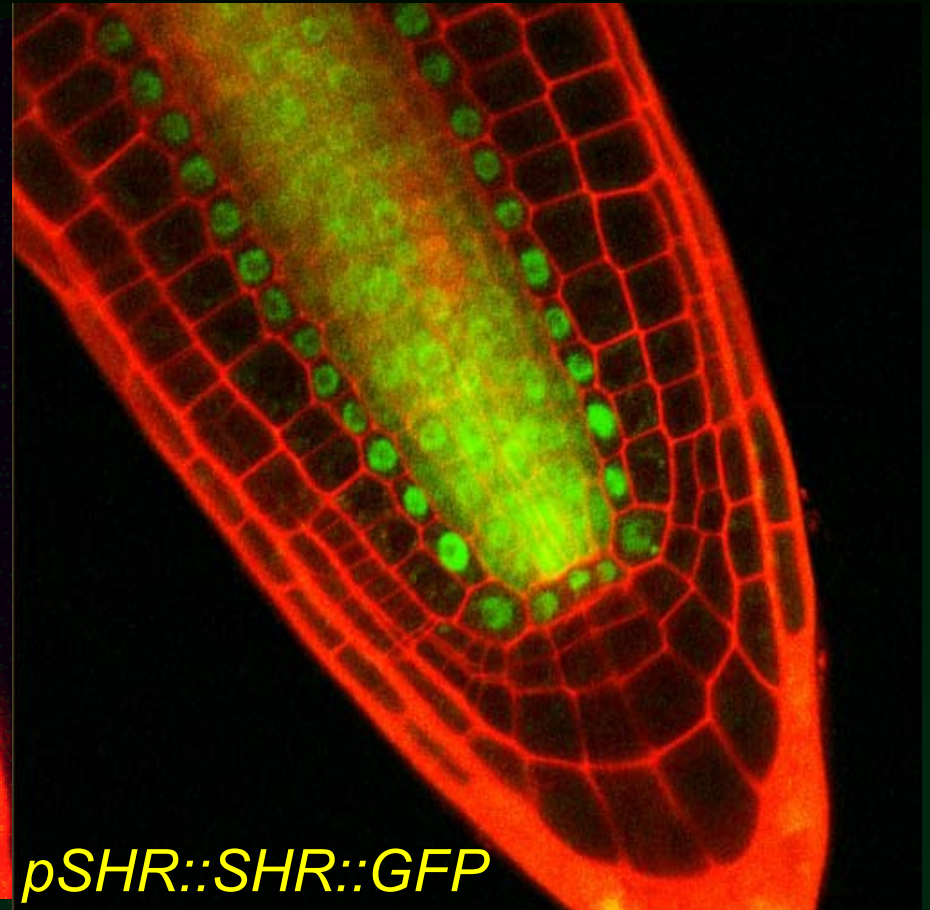
BANG! SHR moves from stele to endodermis

Nakajima et al, Nature 2001

Protein immunolocalization

Antigen-binding site

Variable region

Constant region identical in all antibodies
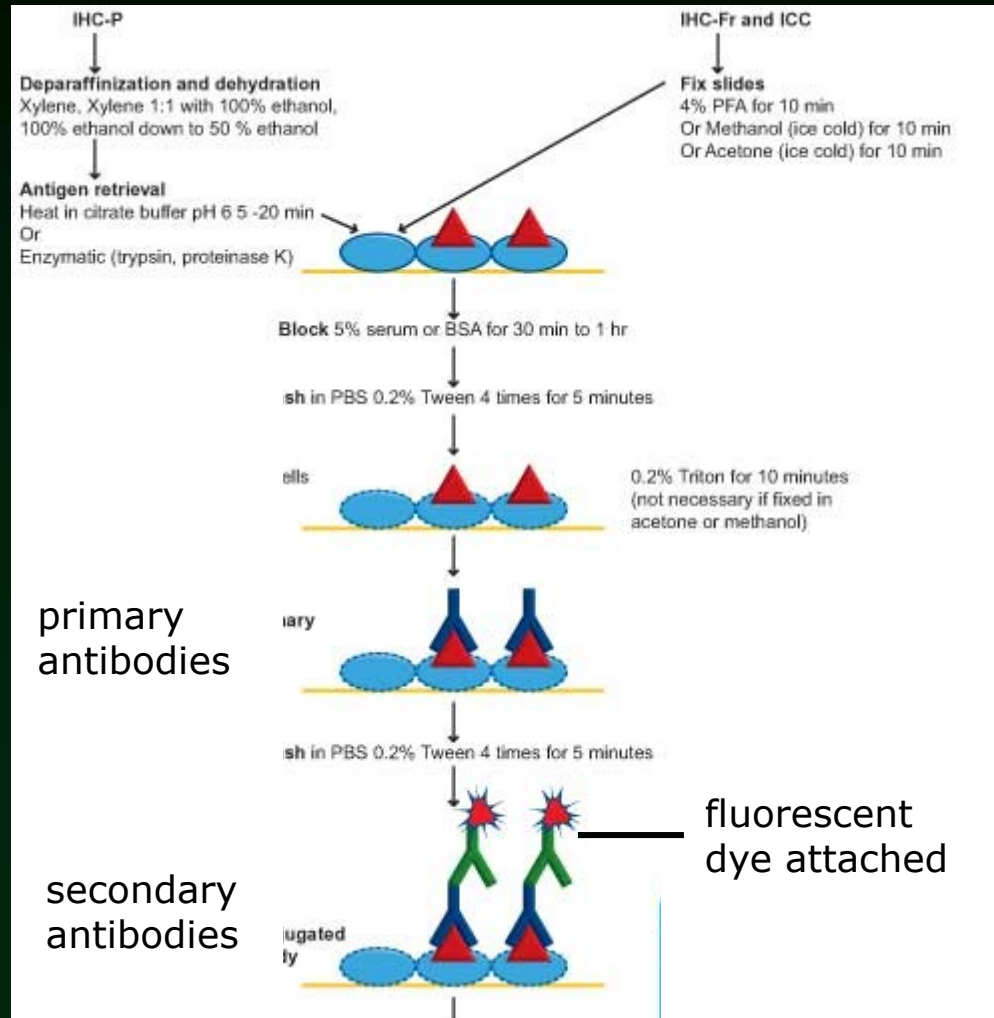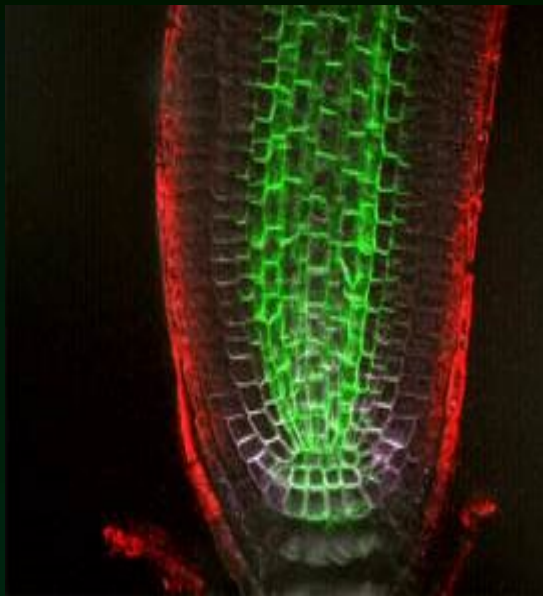(of given source organism)
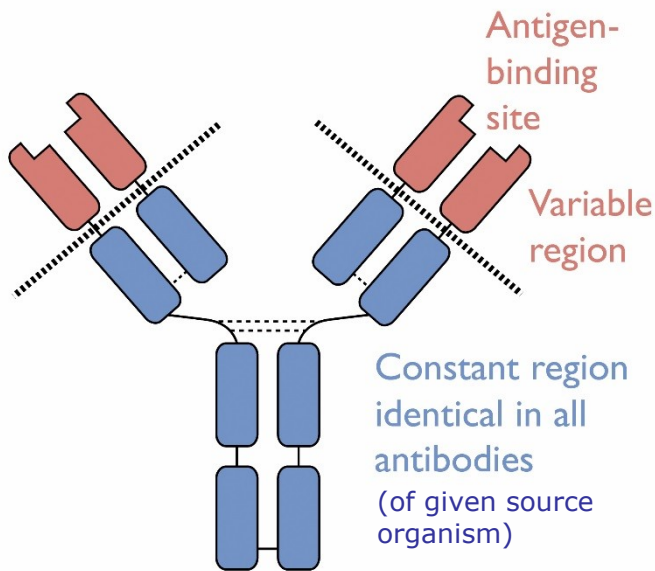
Most favorite animals:
- rabbit (too many rabbits)
- mouse (low volume)
- goat
- chicken
- rat
- sheep
- donkey
- guinea pig

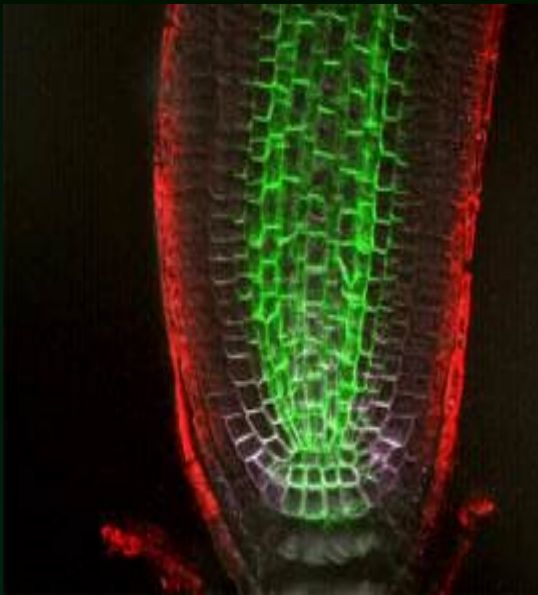secondary: anti-rabbit from no-rabbit, anti-mouse from no-mouse, etc.

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Protein immunolocalization

immunolocalization - fluorescently
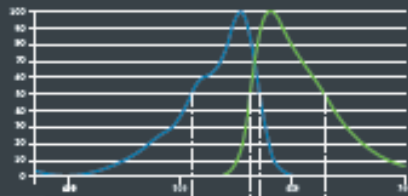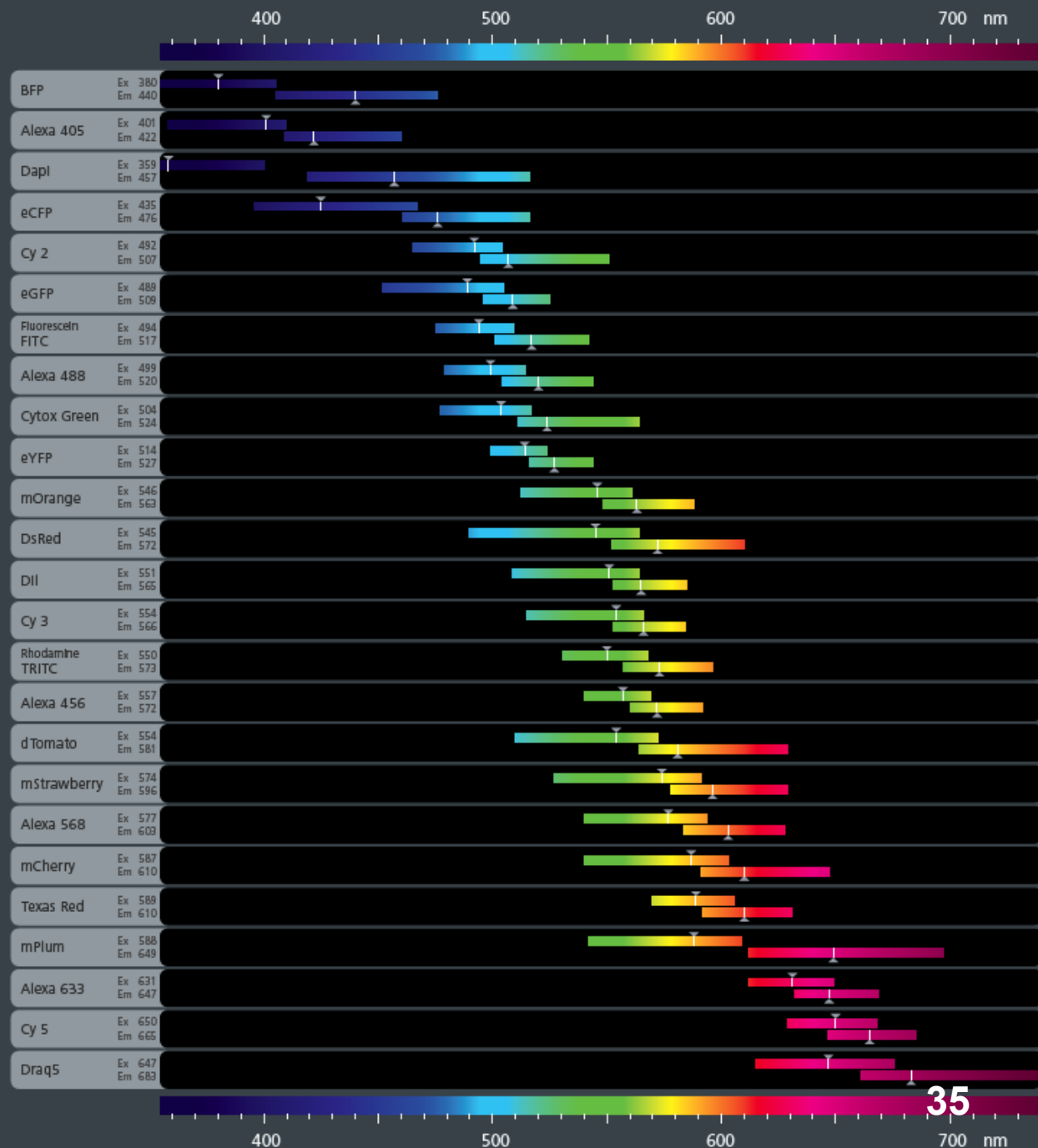

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Protein immunolocalization
immunolocalization

Fluorescent dyes conjugated to secondary (examples):

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

Fluorescent Dyes and Proteins

www.zeiss.com/microscopy

Dye Name	Ex	Em
BFP	380	440
Alexa 405	401	422
DapI	359	457
eCFP	435	476
Cy 2	492	507
eGFP	489	509
Fluorescein FITC	494	517
Alexa 488	499	520
Cytox Green	504	524
eYFP	514	527
mOrange	546	563
DsRed	545	572
DII	551	565
Cy 3	554	566
Rhodamine TRITC	550	573
Alexa 456	557	572
dTomato	554	581
mStrawberry	574	596
Alexa 568	577	603
mCherry	587	610
Texas Red	589	610
mPlum	588	649
Alexa 633	631	647
Cy 5	650	665
Draq5	647	683

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Protein immunolocalization

Pros:

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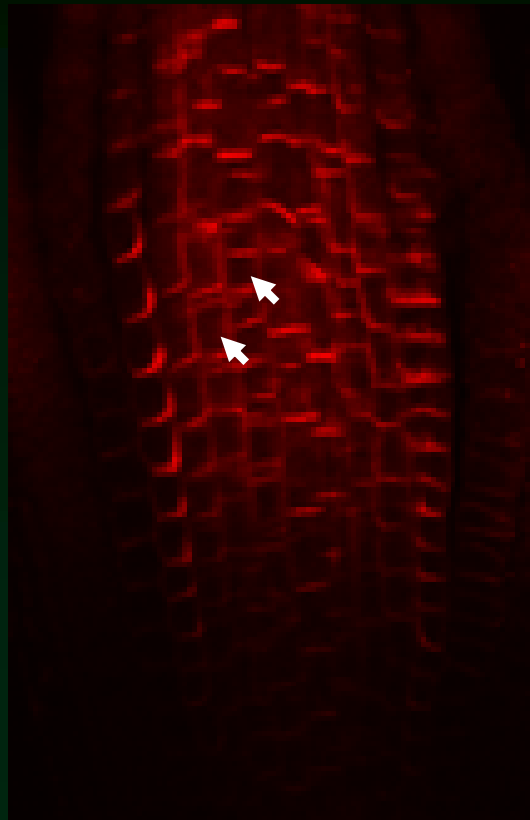- allows sectioning (less accessible tissues)

Cons:

- fixed material only
- excellent antibodies only, sometimes tricky

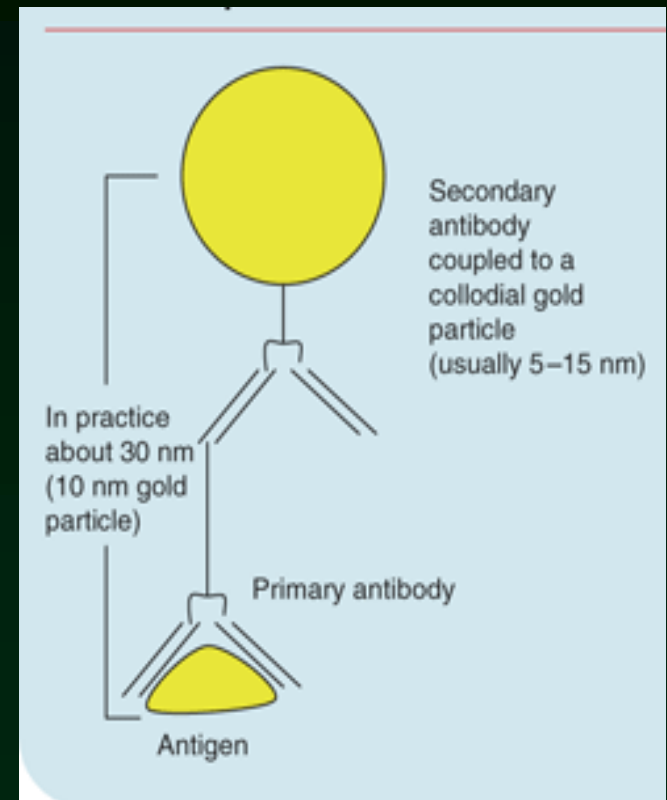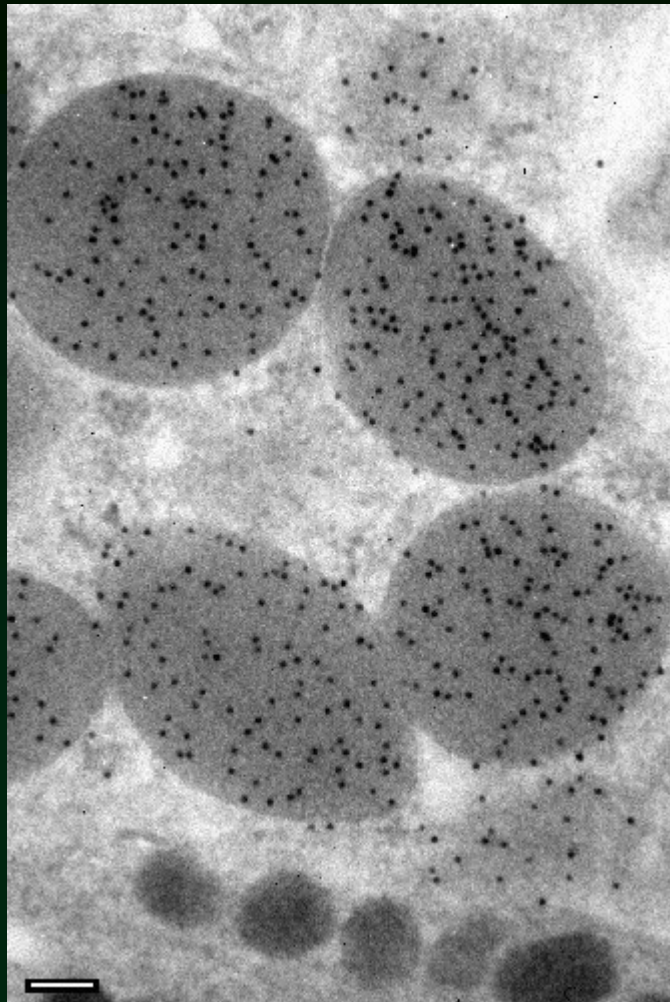# GFP tag partially retains PIN1 in endoplasmic reticulum (-> artifact)
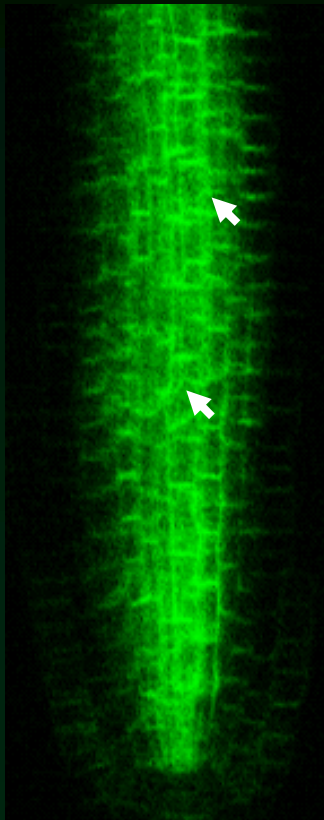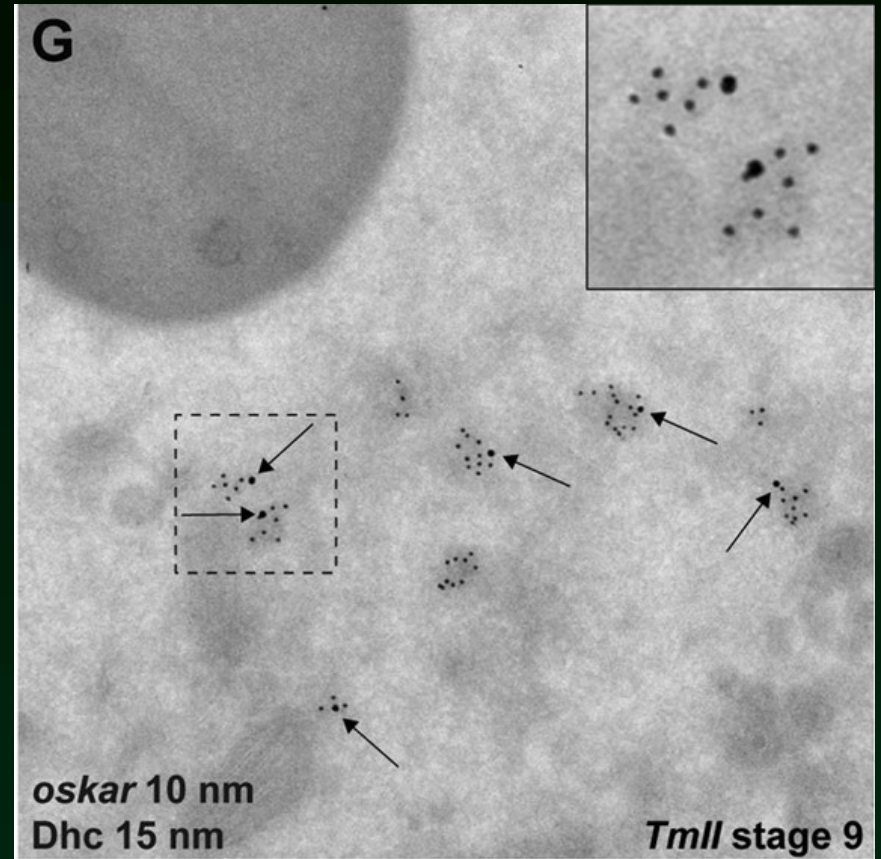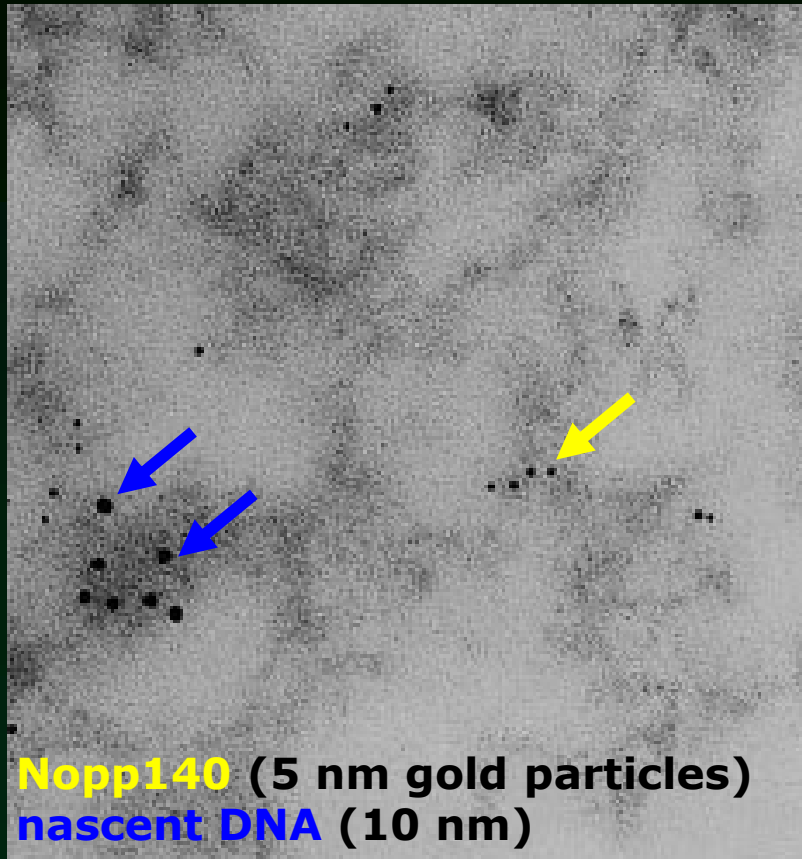

PIN1-GFP

anti-PIN1

Protein localization - immunogold

immunolocalization - immunogold

electron microscope

Secondary antibody coupled to a collodial gold particle (usually 5–15 nm)

In practice about 30 nm (10 nm gold particle)

Primary antibody

Antigen

Immunogold collocalization

Nopp140 (5 nm gold particles)
nascent DNA (10 nm)

G

oskar 10 nm
Dhc 15 nm

TmII stage 9

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 posttranslational modifications?

Also RNA can be visualized

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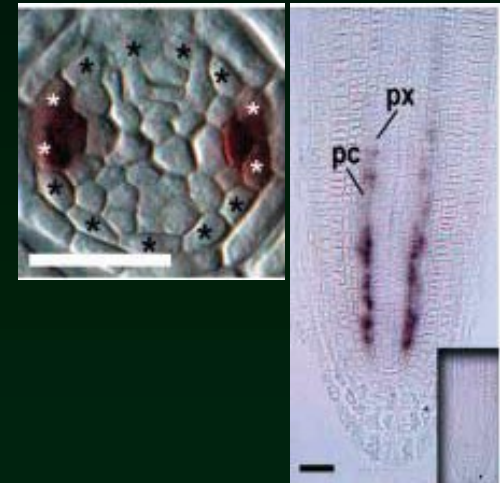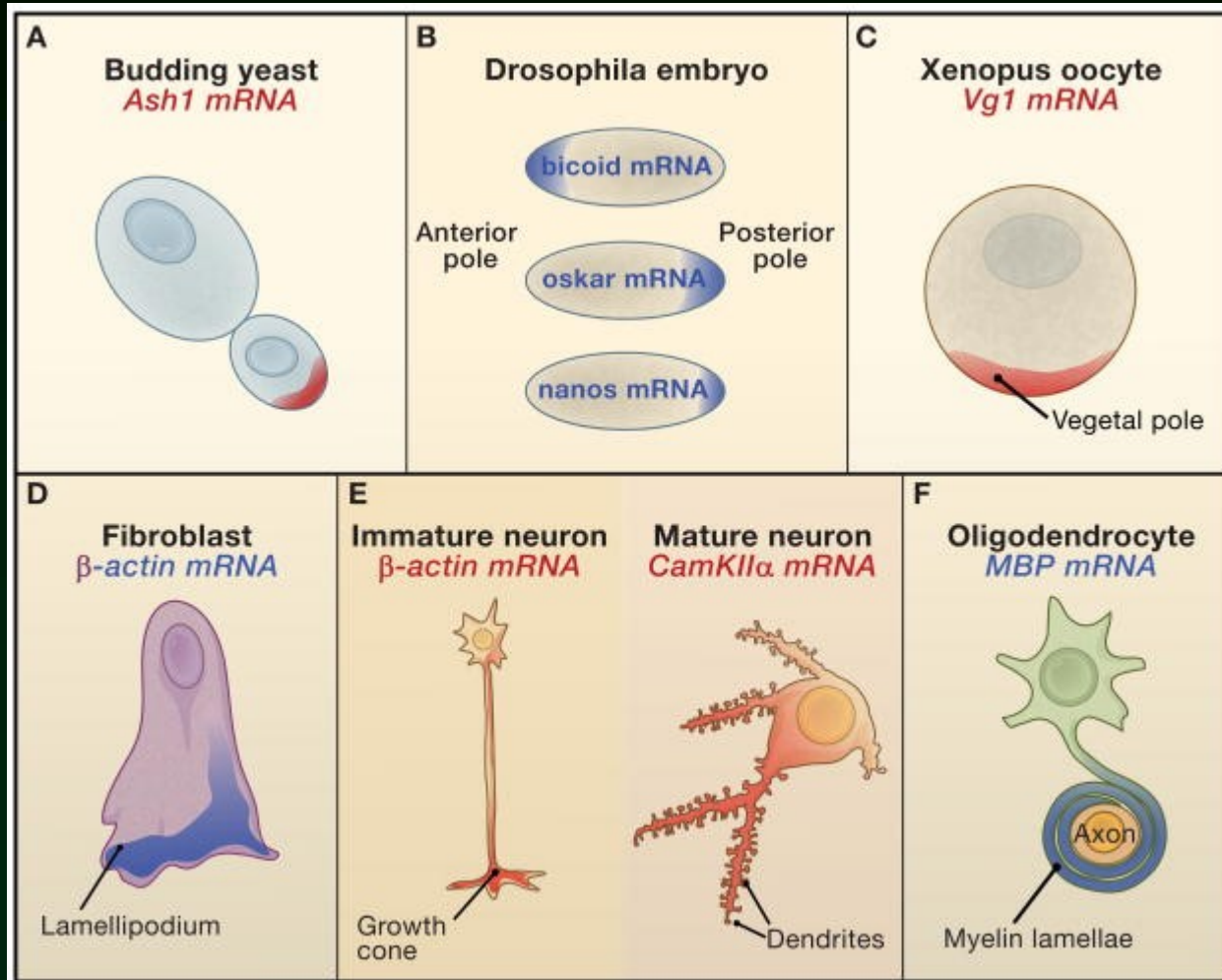
Localization of mRNA
RNA hybridization *in situ*
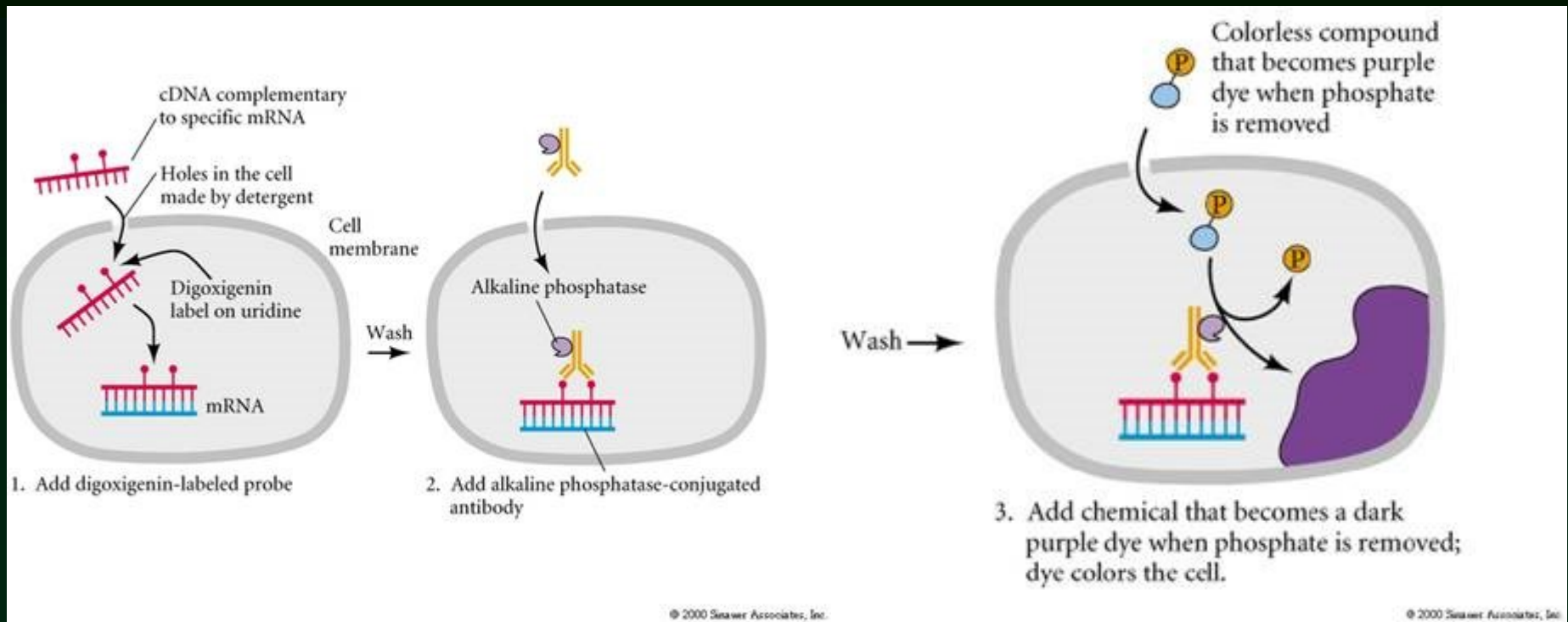
Visualization of mRNA
RNA hybridization *in situ*

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.
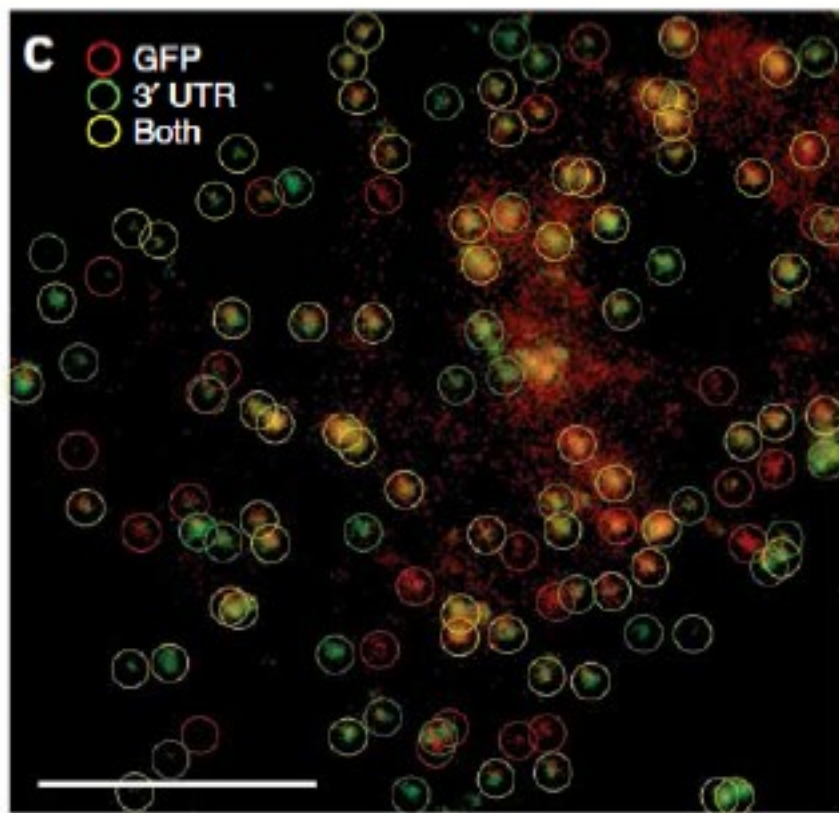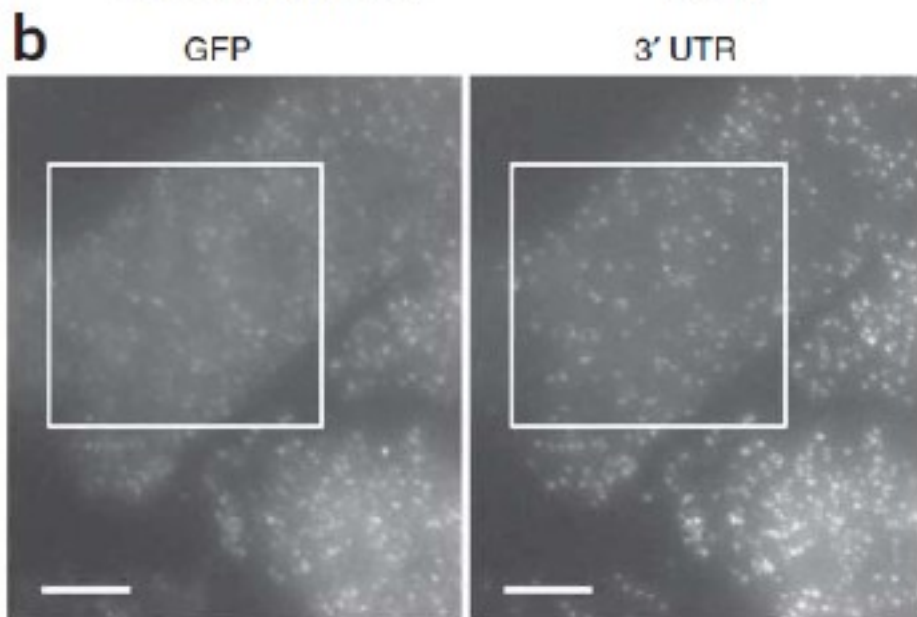
Also mRNA can be visualized *in vivo*
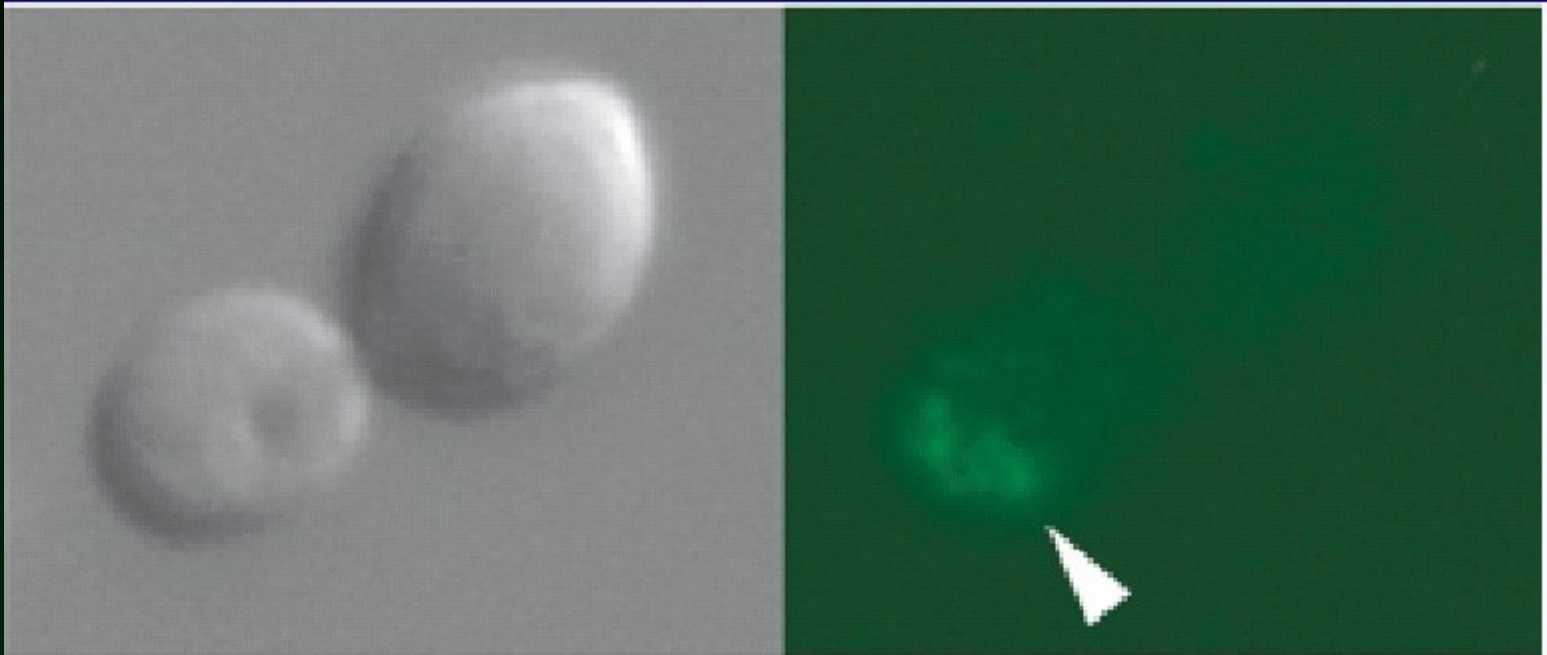
Ash1 mRNA localized to the tip of the daughter cell

λN$_{22}$ system – RNA imaging
in vivo

nuclear localization signal

viral RNA binding protein

(a) NLS 3′
FP
RBP
5′

(b)
Fluorescent RBP constructs

λN22
MSCP

promoter | RBP | FP | NLS

Target RNA
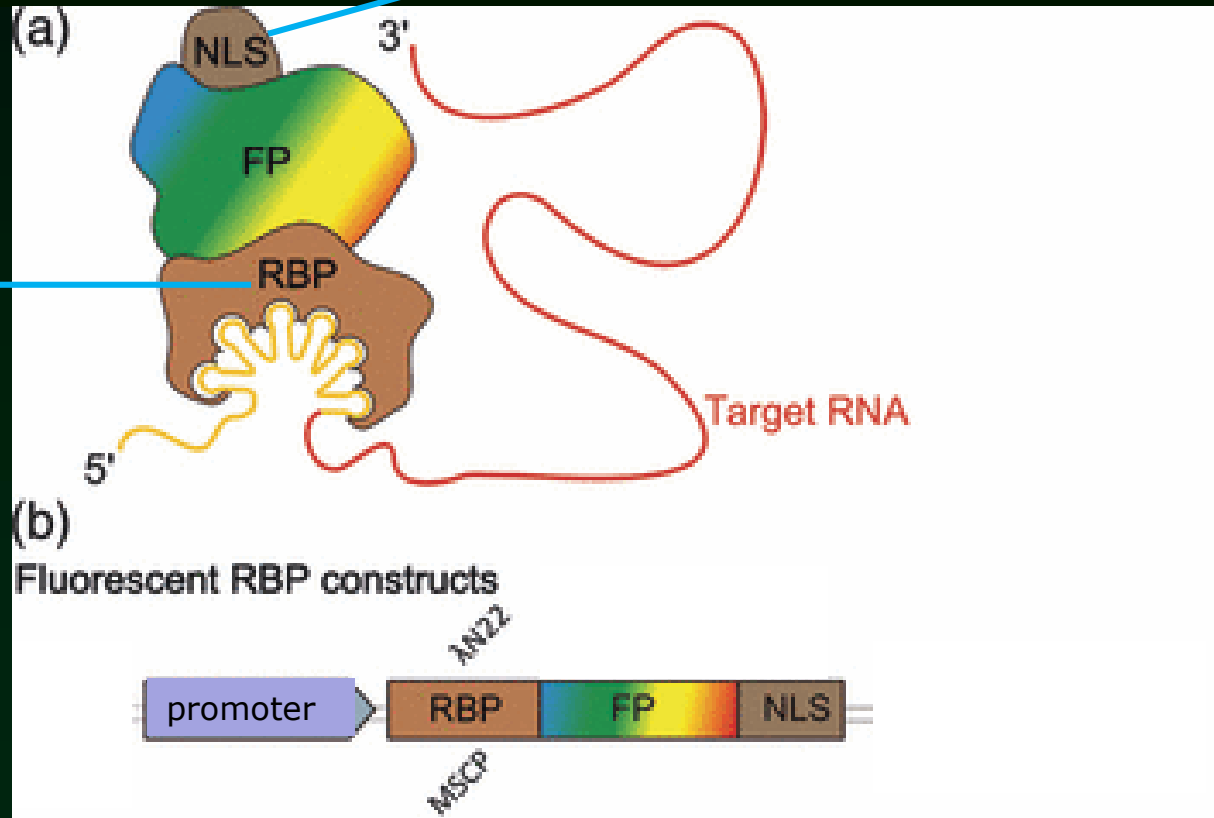
Drawbacks of λN$_{22}$ system - we have SPINACH

GACGCAACUGAAUGAAA
UGGUGAAGGACGGGUCC
AGGUGUGGCUGCUUCGG
CAGUGCAGCUUGUUGAG
UAGAGUGUGAGCUCCGU
AACUAGUCGCGUC

RNA fusion

$+$

DFHBI

aptamer

B Phase Hoechst 0 min | DHFBI 0 min | 15 min | 30 min | 45 min | 60 min

− Sucrose

+ Sucrose

blue-DNA   green-RNA

48
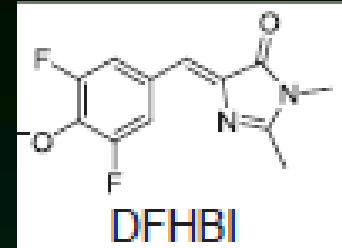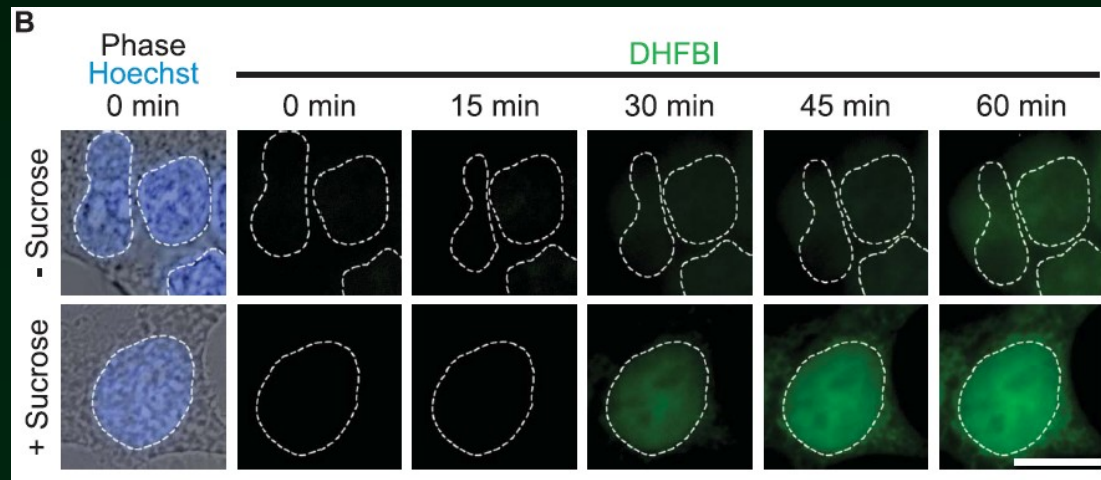
Other vegetables than SPINACH

Paige et al. 2012; Song et al. 2014

49
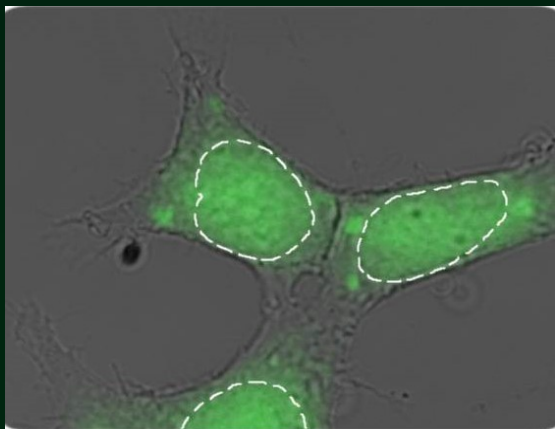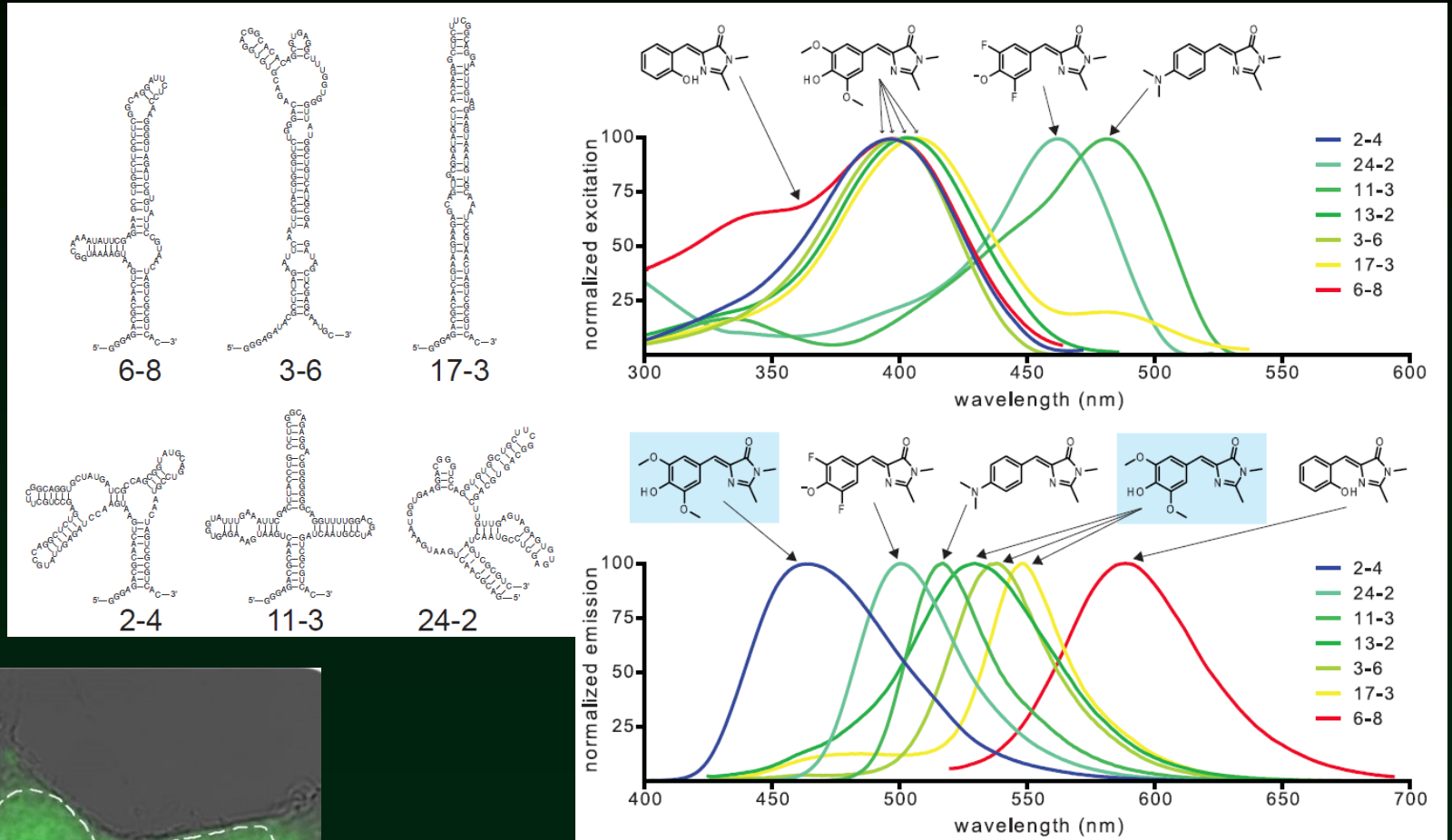
Advanced confocal techniques
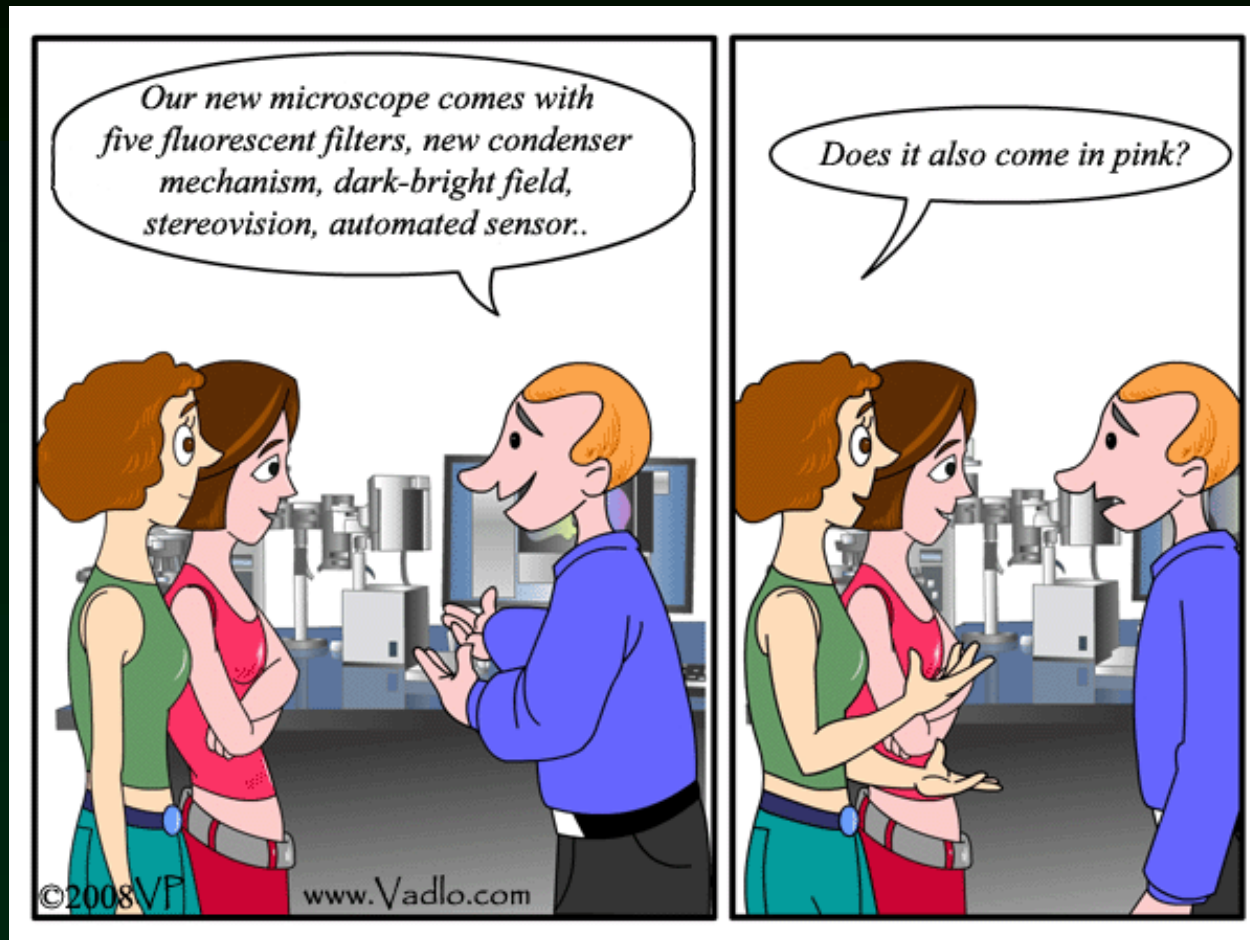
(slightly) Advanced confocal techniques

- FRAP

- photoactivatable FP

- FCS

FRAP

Fluorescence Recovery After Photobleaching

region of interest (ROI)

| A Pre-bleach | B Bleaching ROI | C Post-bleach | D Fluorescence recovery |

100 μm

FRAP

A. Pre-bleach B. Bleaching ROI C. Post-bleach D. Fluorescence recovery
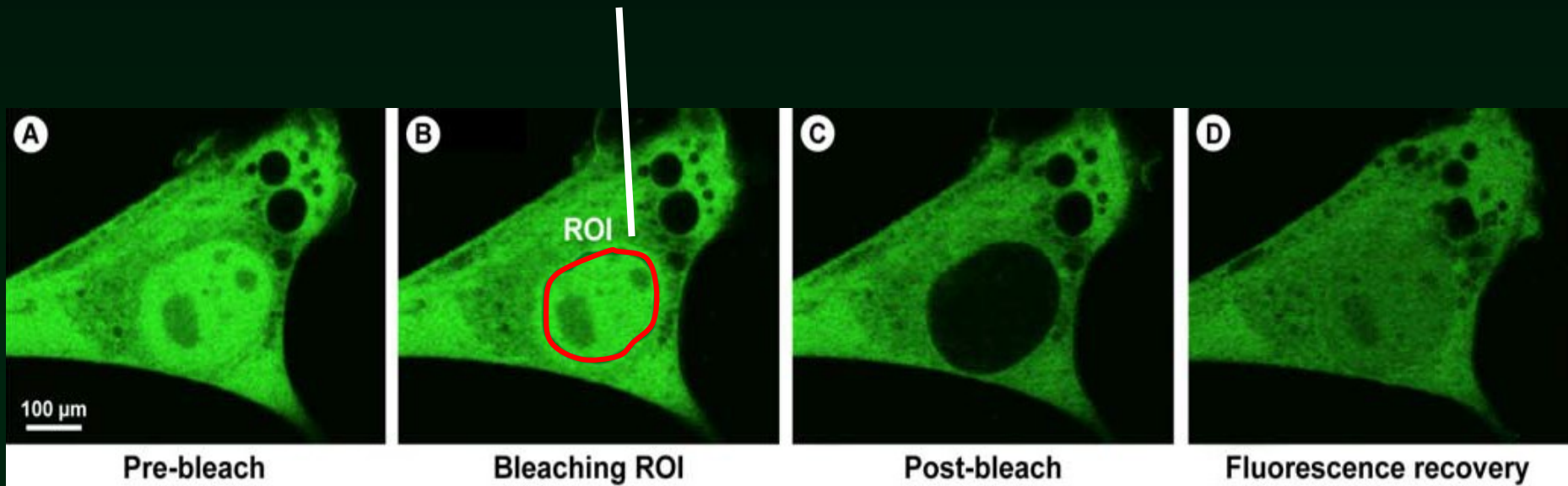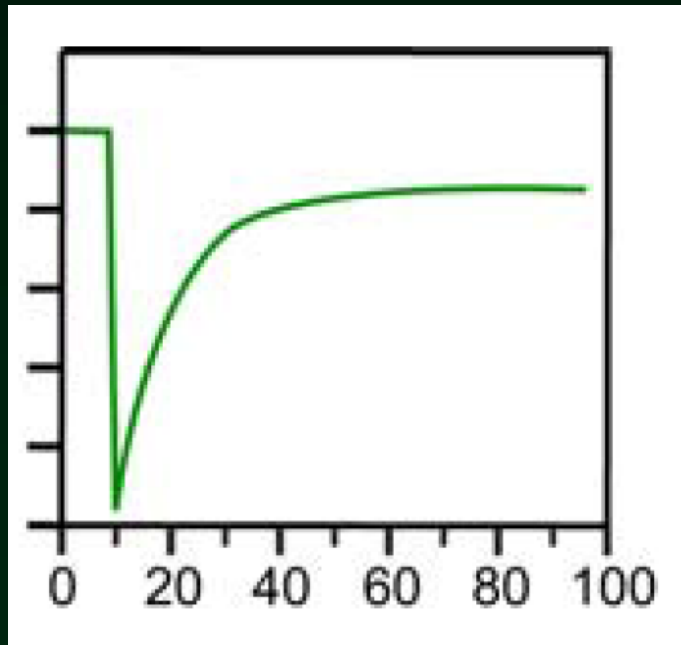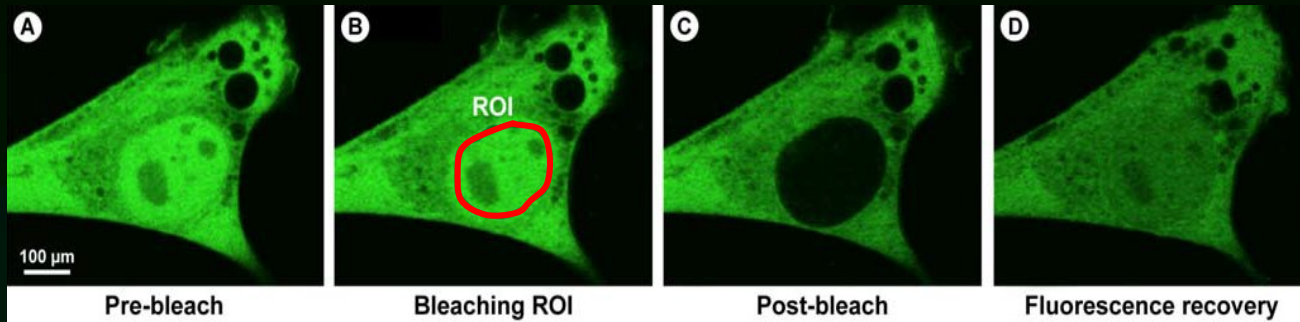
you can quantify fluorescence..
(ImageJ is our friend)
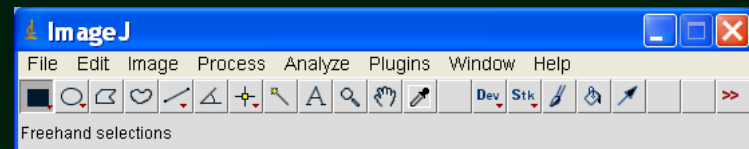
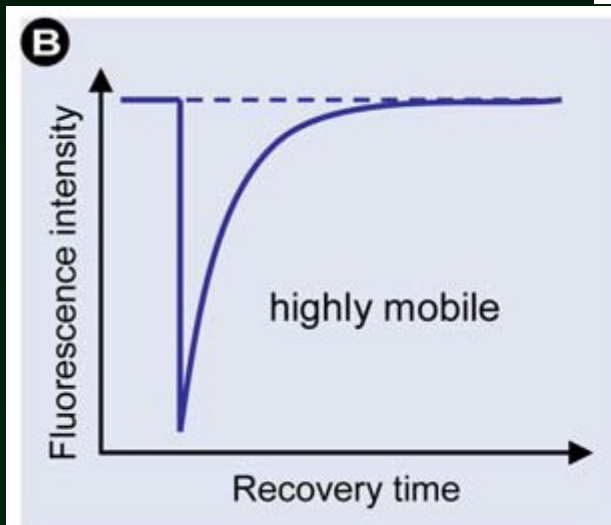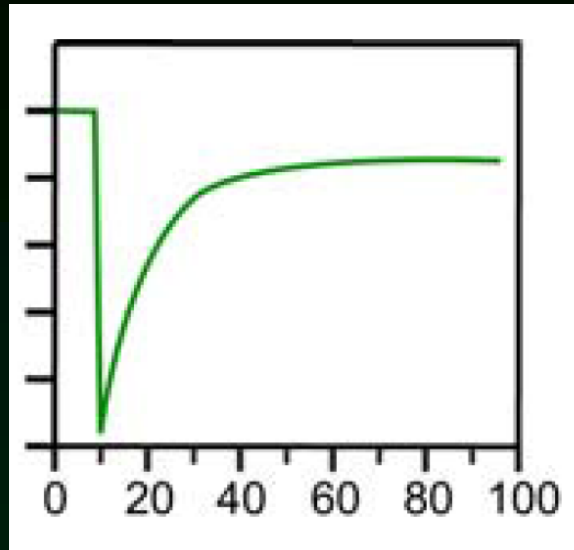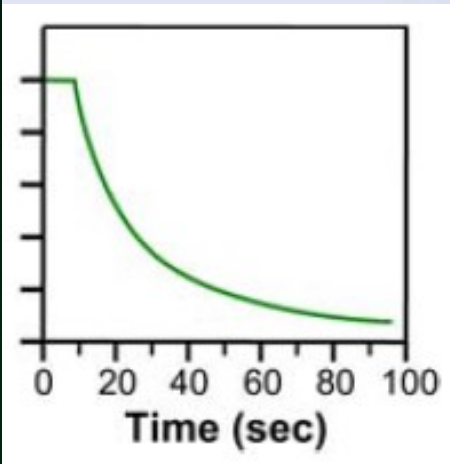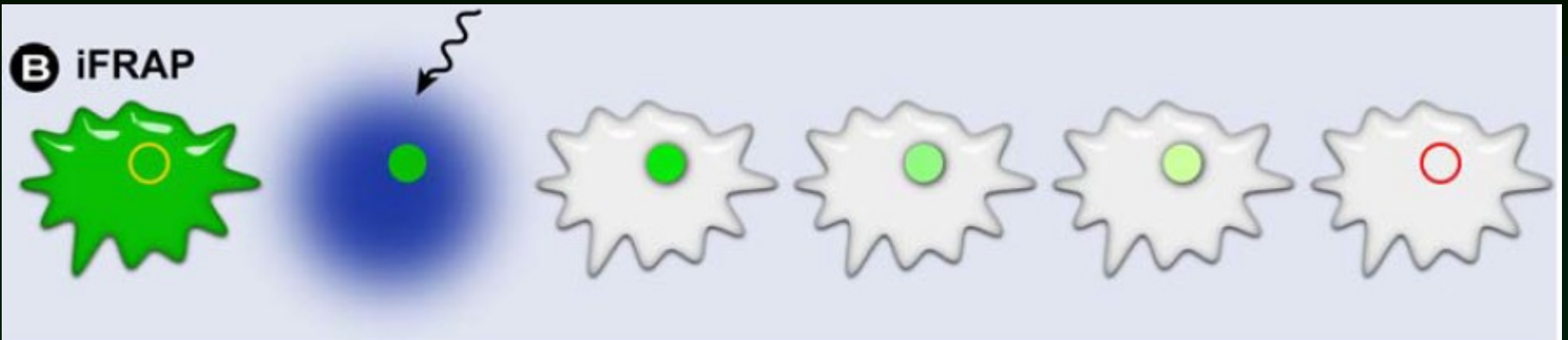|	mean	min	max
A	90.404	49	113
C	8.556	3	8
D	39.934	19	63

FRAP – bleaching curve
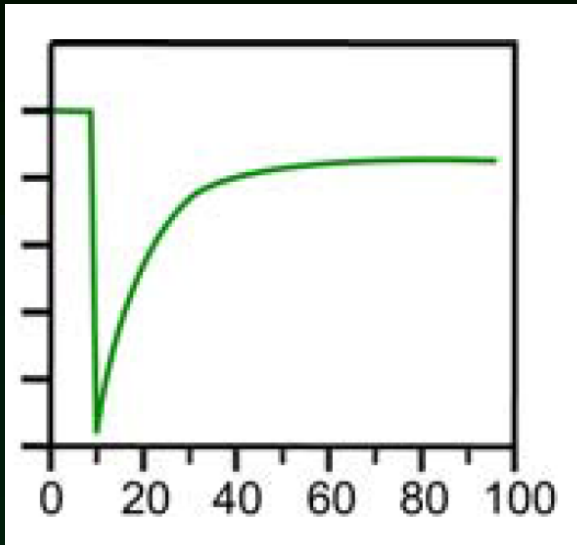
What does the curve tell?
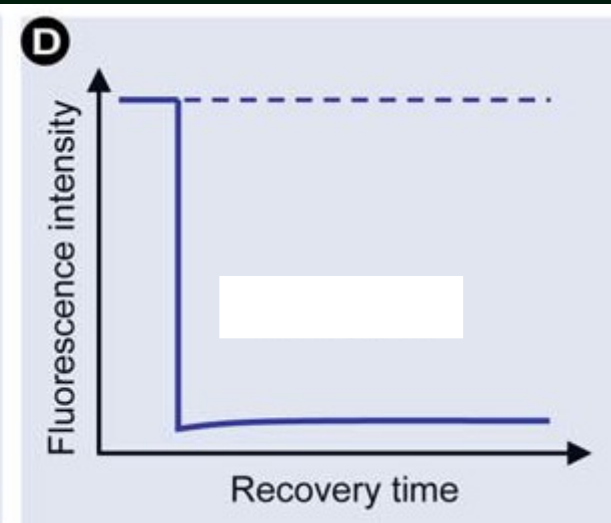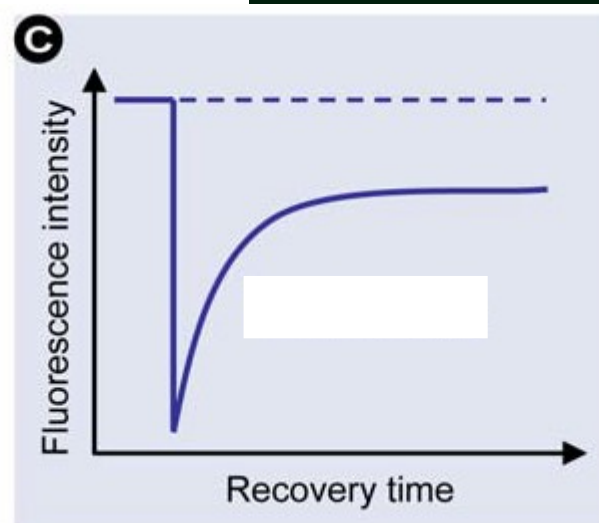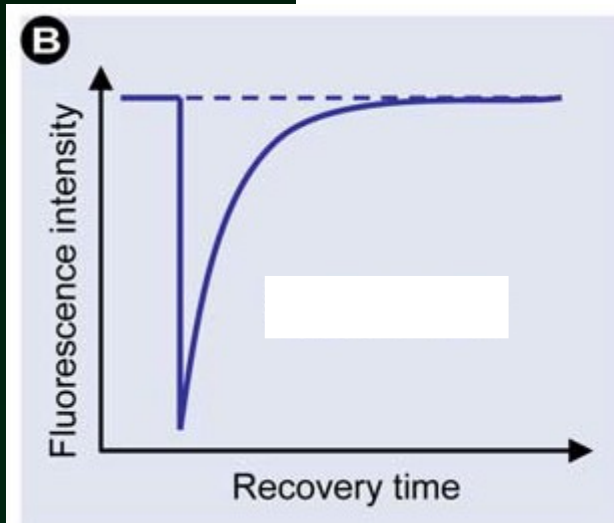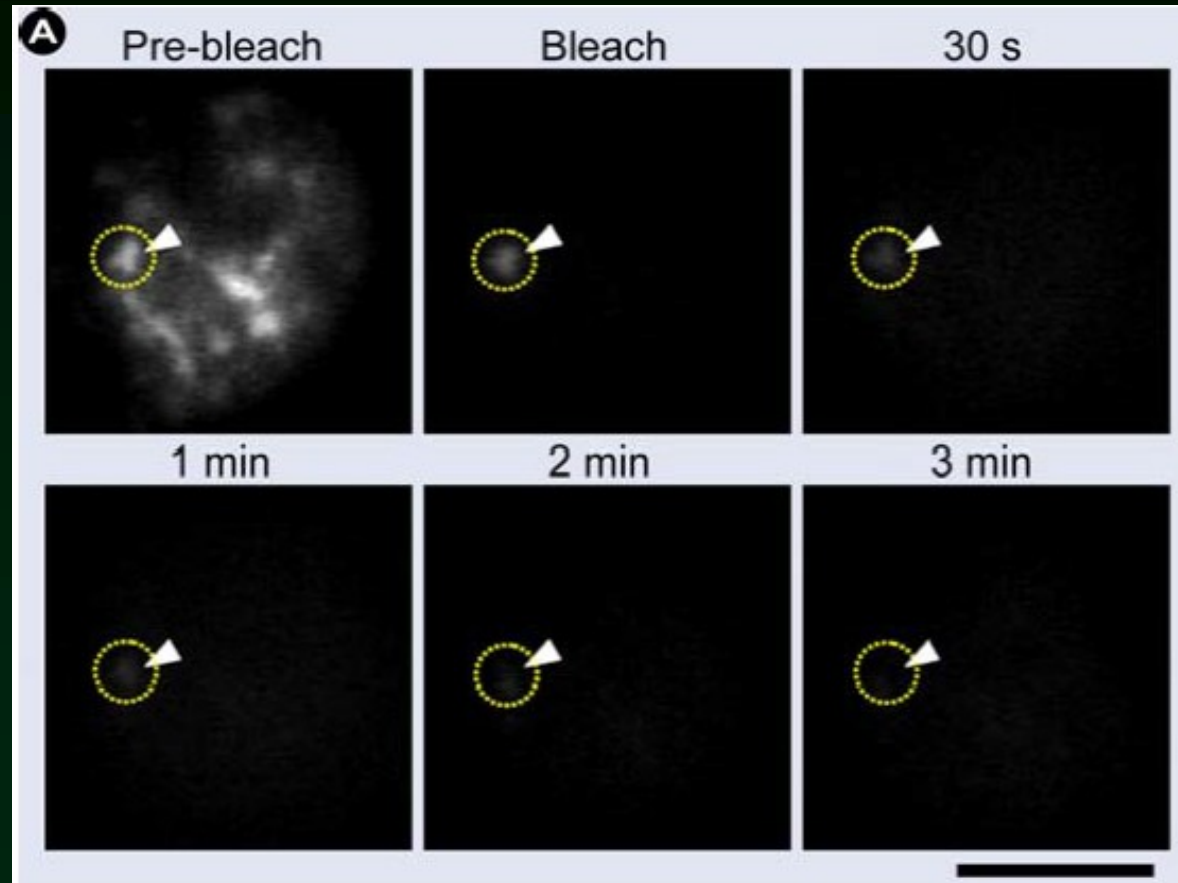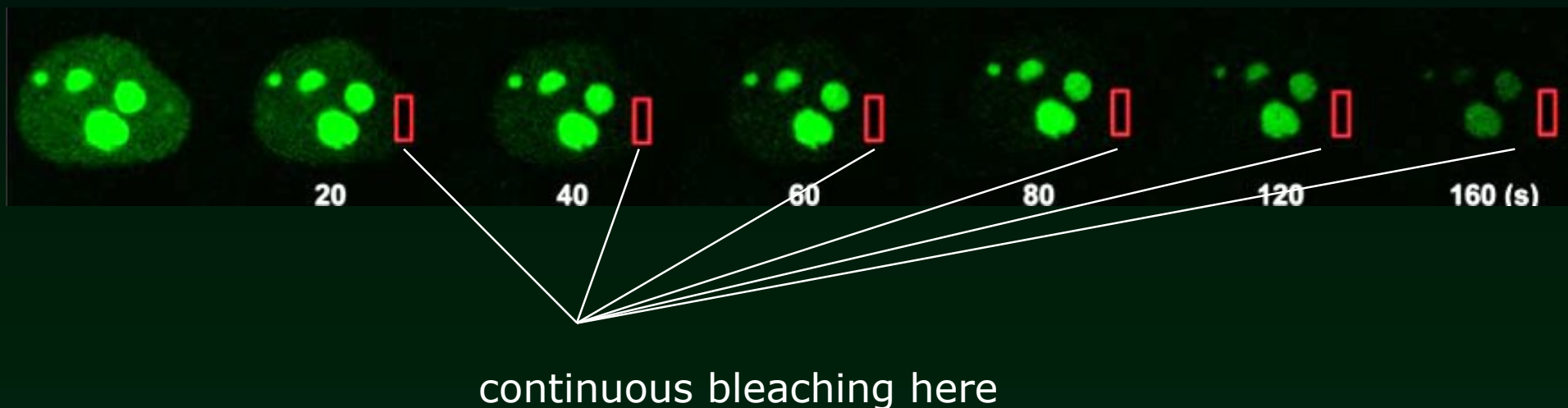
FRAP – bleaching curve
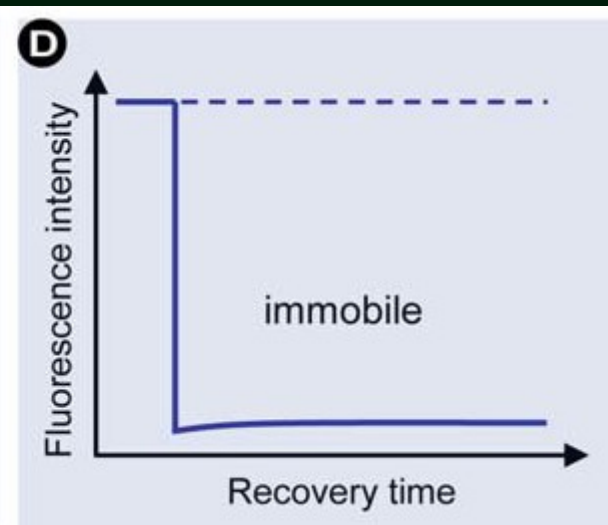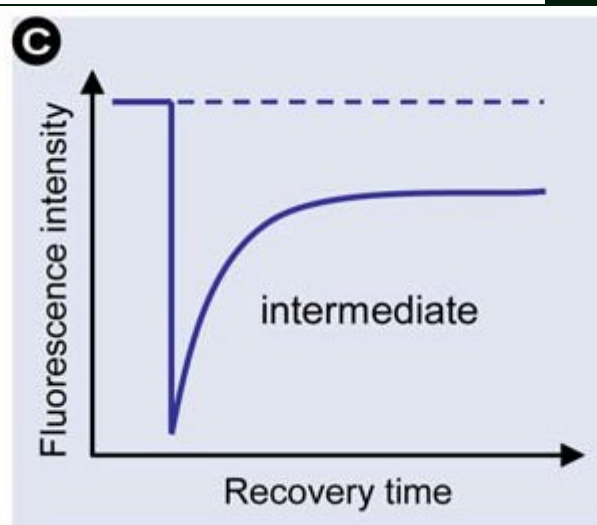
iFRAP

inverse FRAP

iFRAP – dissociation of premRNA from specles

FRAP derivatives
FLIP
Fluorescence Loss After Photobleaching
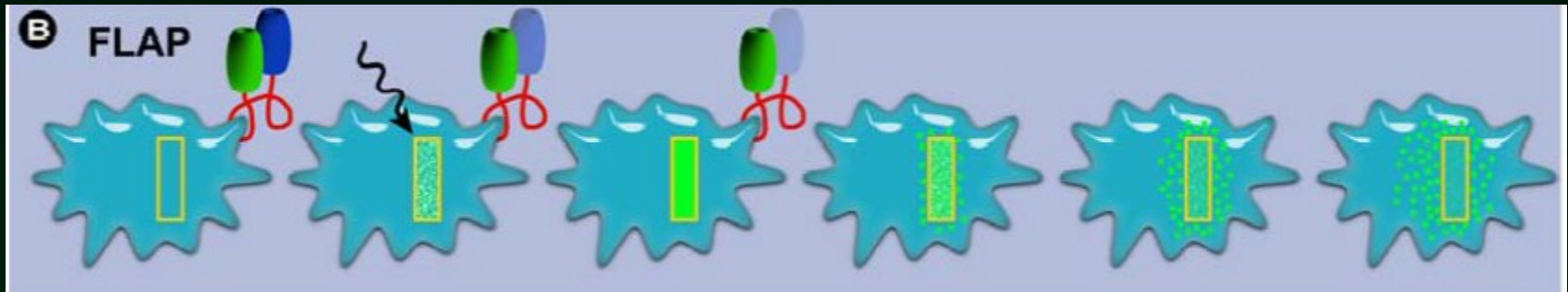

continuous bleaching here

- 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
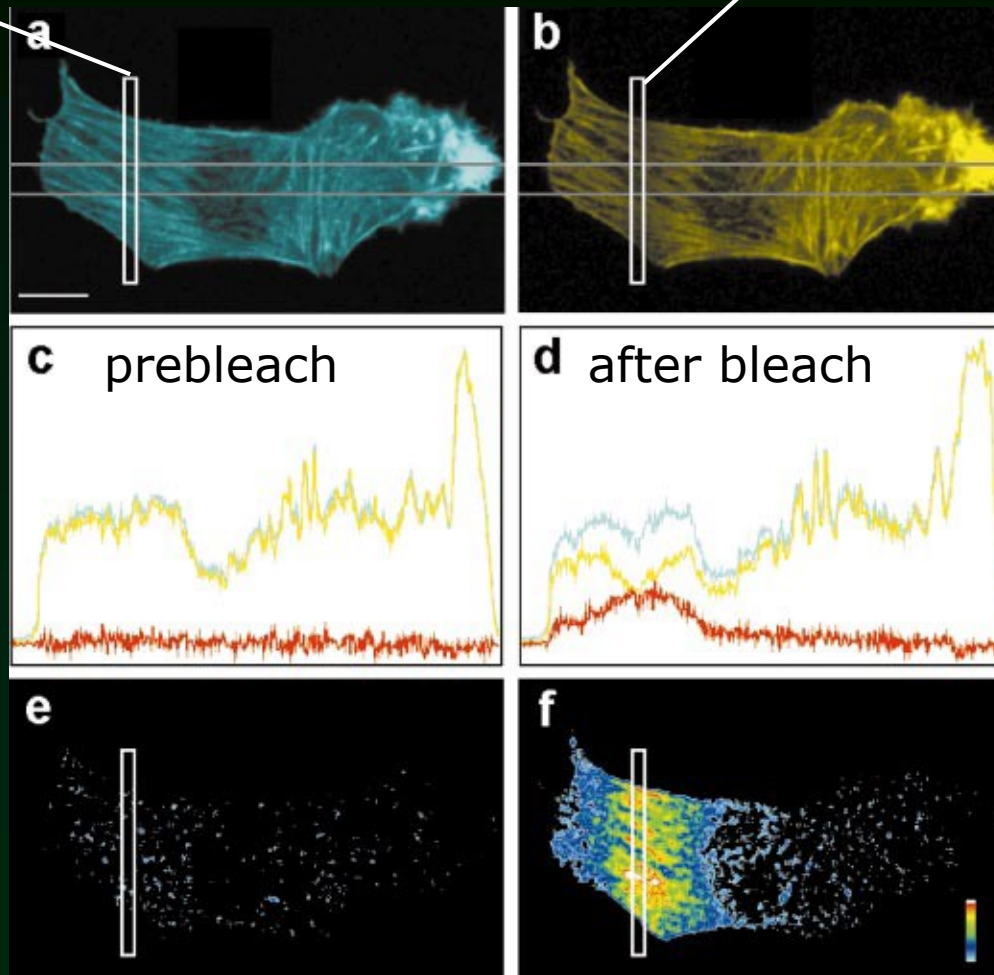## Fluorescence Localization after Photobleaching

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

Perhaps better scheme than previous

CFP not bleached

YFP bleached

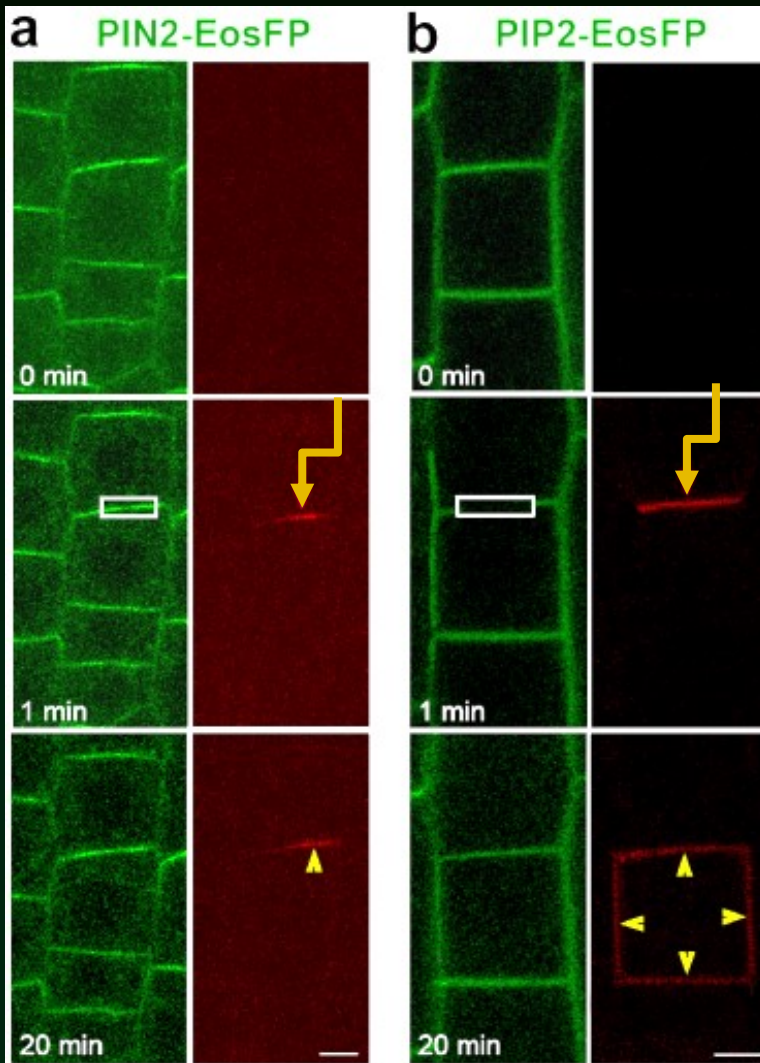RED=CFP-YFP

Dunn et al. 2002

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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 diffusion

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Photoactivable proteins

Dronpa, Kaede, Eos – probably the most popular

Photoactivable proteins

Advantages:
- elegant, can be convincing

Disadvantages:
- very weak signal
- each material needs optimization

Remarks

- your material is 3D

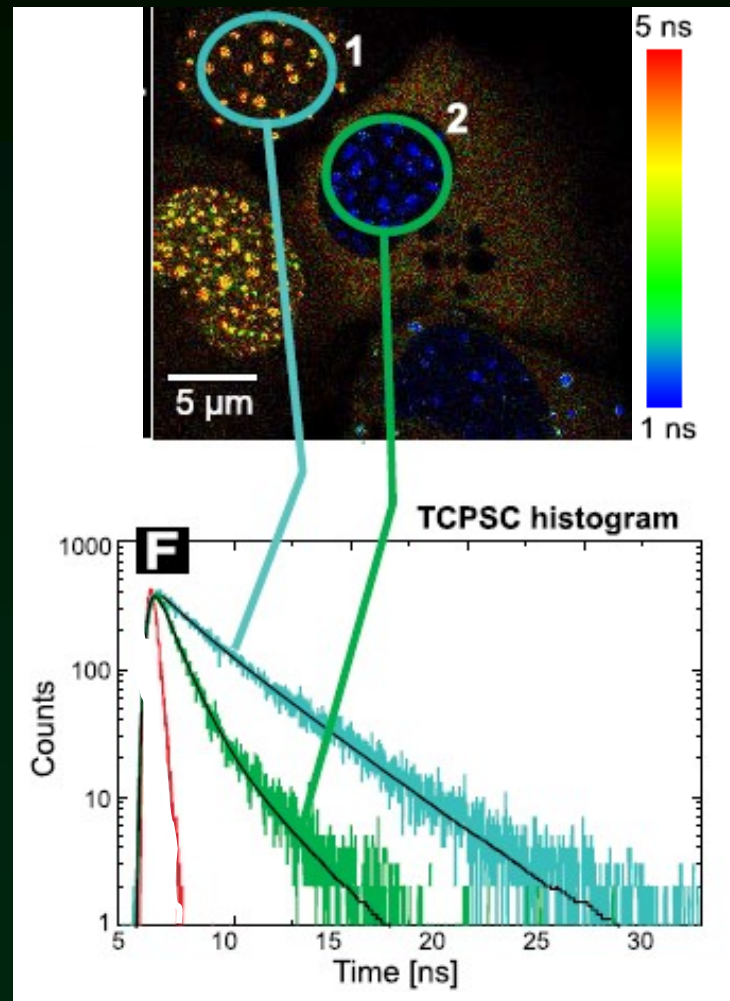- protein *de novo* synthesis in some experiments (e.g. cycloheximide stops translation)

FLIM

Fluorescence Life Time Imaging Microscopy

Fluorochromes
- excitation spectra
- emission spectra
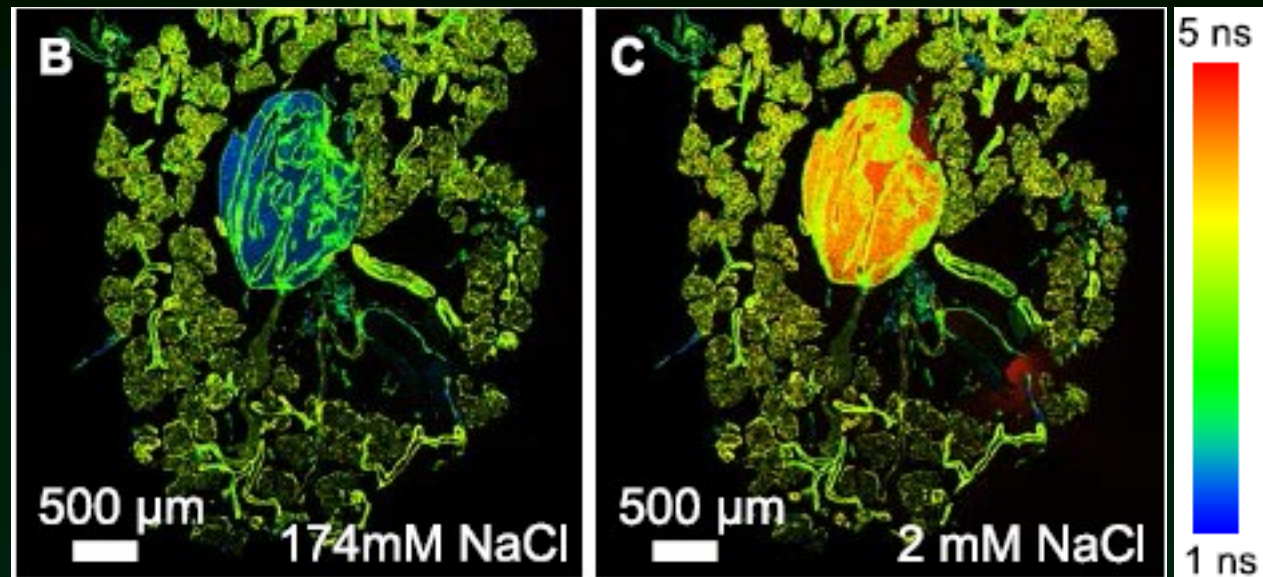- unique lifetime

FLIM - applications

FLIM - applications

Lifetime sensitive to almost everything:
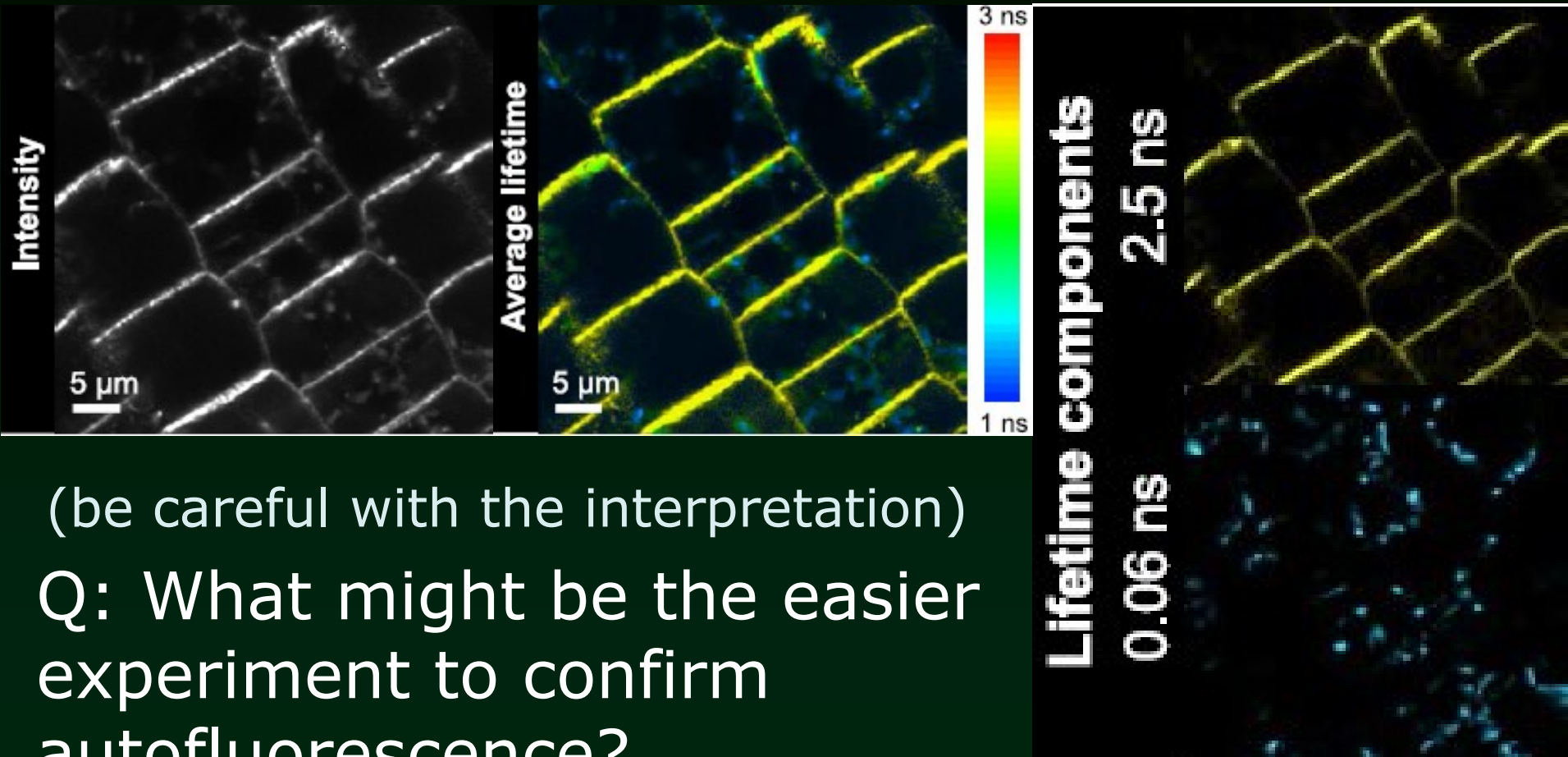- pH
- ionic strength
- solution polarity
- other fluorochrome

Protein-protein interactions
(FRET-FLIM)

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)

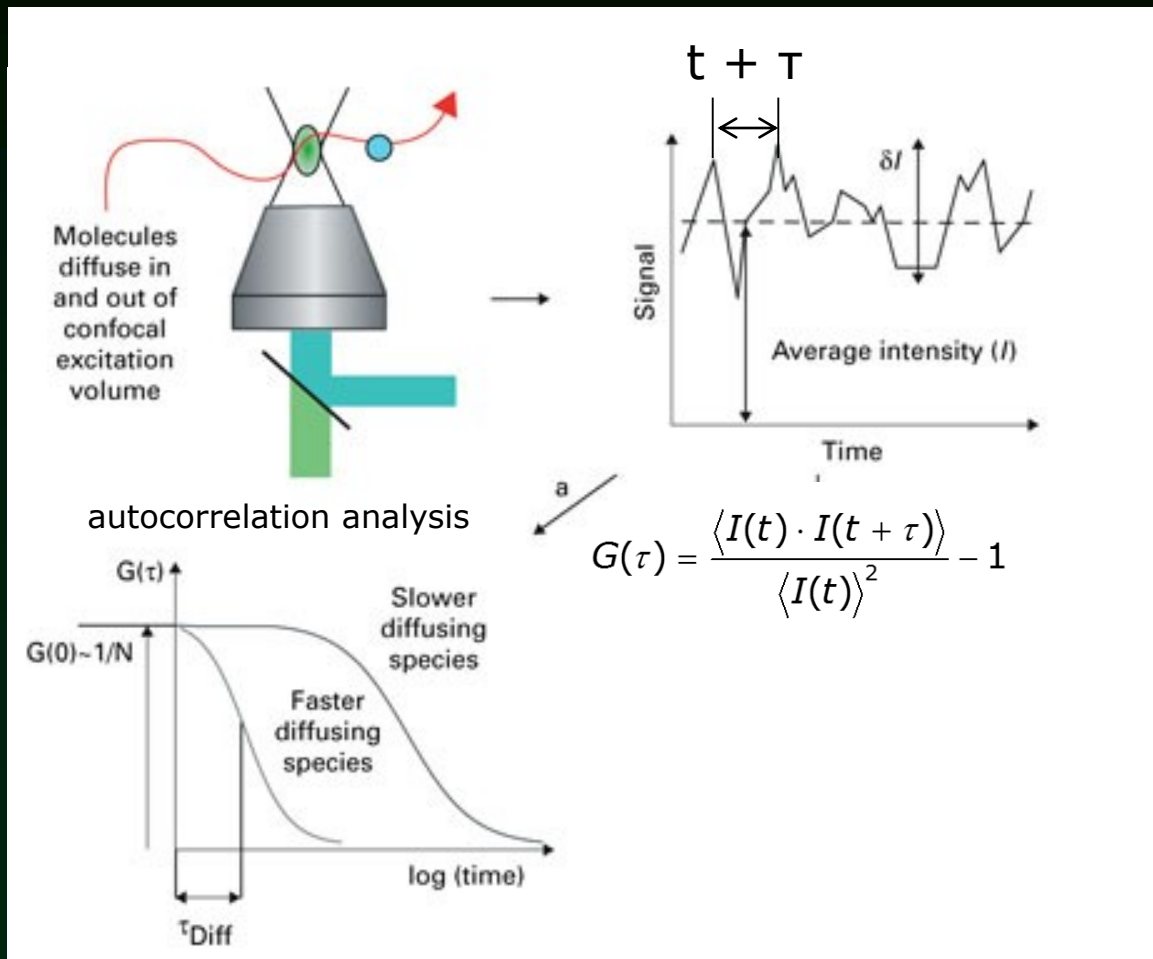Q: What might be the easier experiment to confirm autofluorescence?

FLIM

- experience needed
- special module on your confocal needed

FCS
Fluorescence Correlation Spectroscopy

$$G(\tau) = \frac{\langle I(t) \cdot I(t + \tau)\rangle}{\langle I(t)\rangle^2} - 1$$

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.
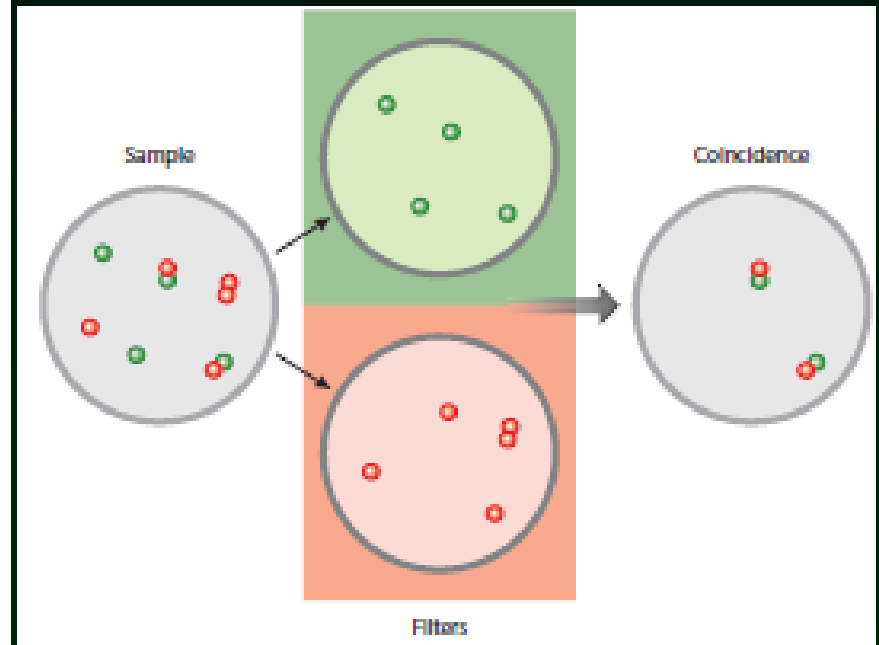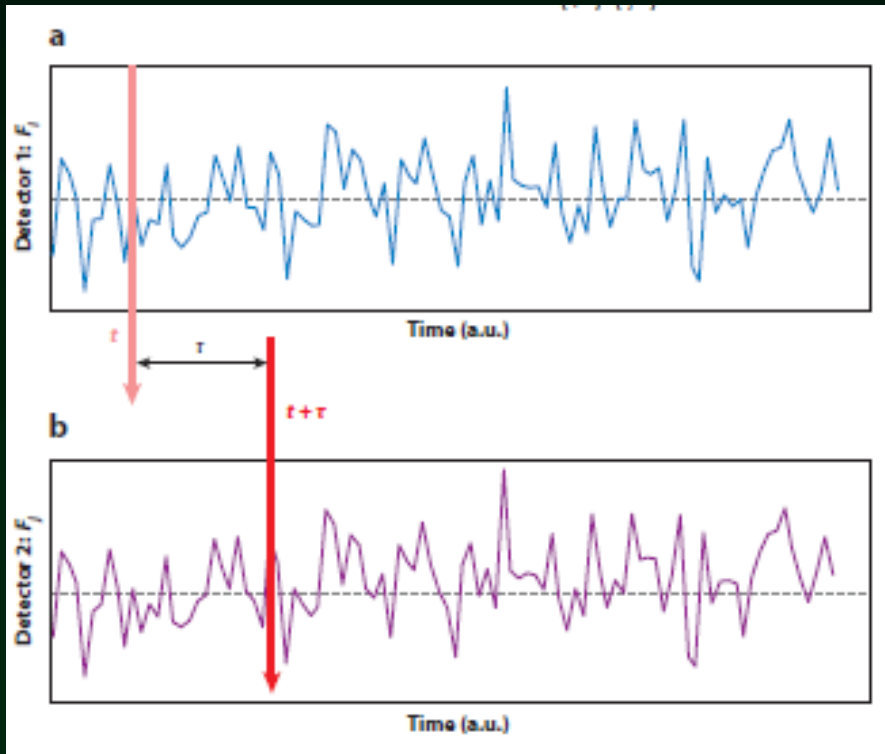
FCS
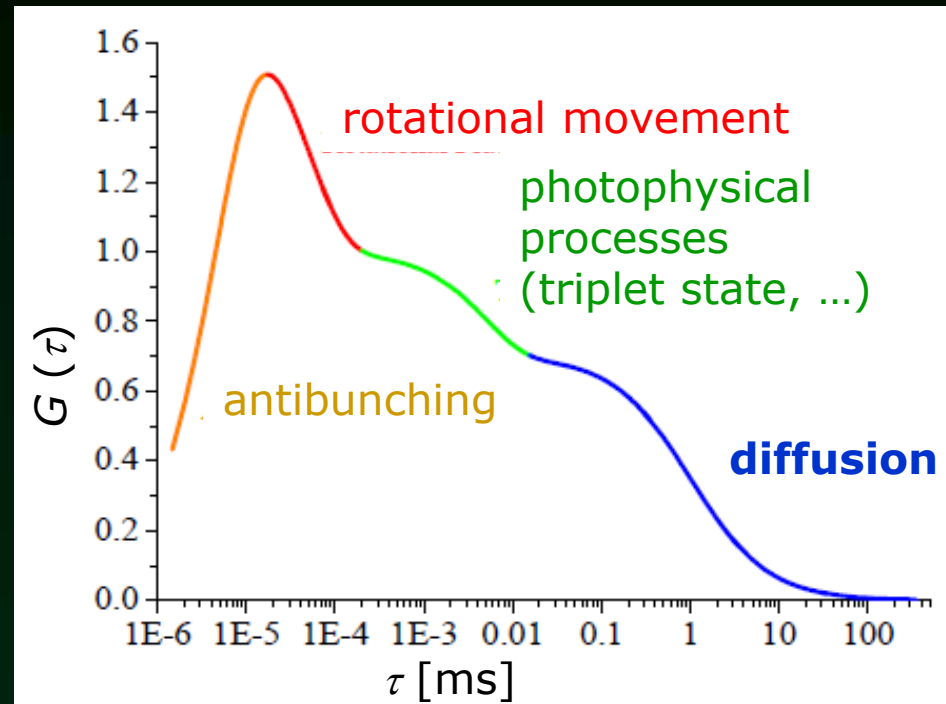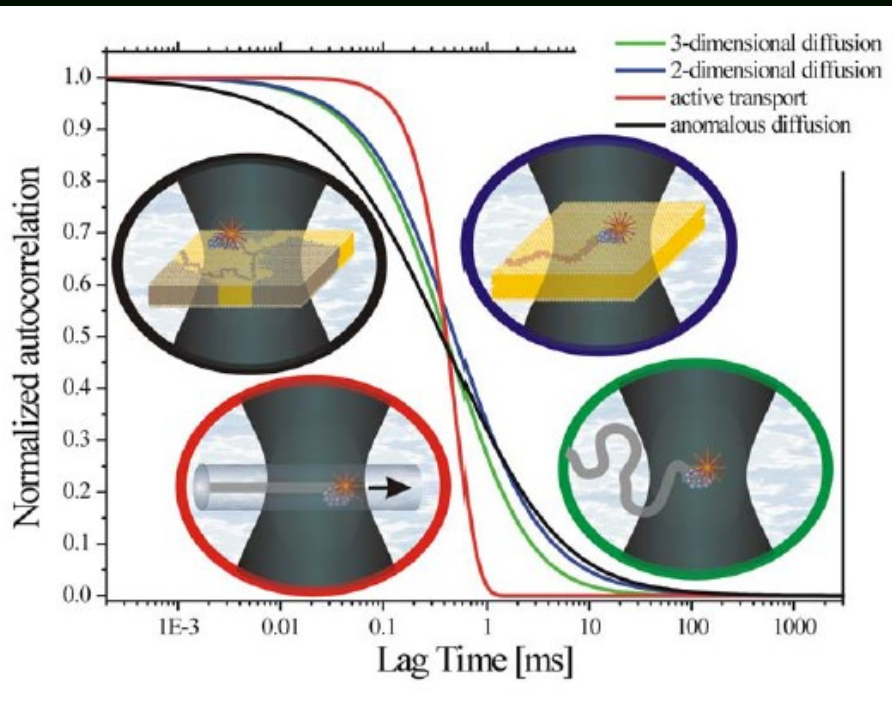
FCS (FCCS)

## 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

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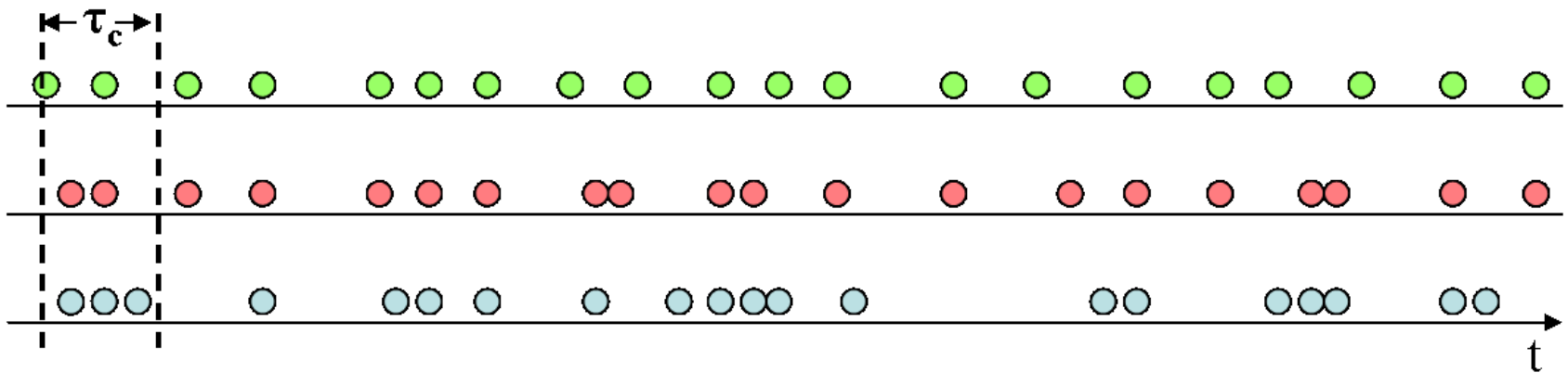
Photon detections as a function of time for a) antibunched, b) random, and c) bunched light