How to visualize genes and their products

Genomics Lecture Series

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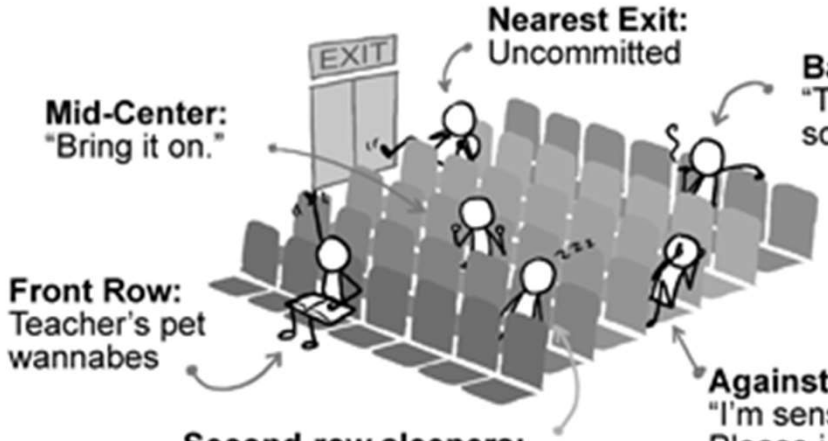
Outline

- 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:



Nearest Exit:
Uncommitted

Back Row:
"Too cool for school"

Mid-Center:
"Bring it on."

Front Row:
Teacher's pet wannabes

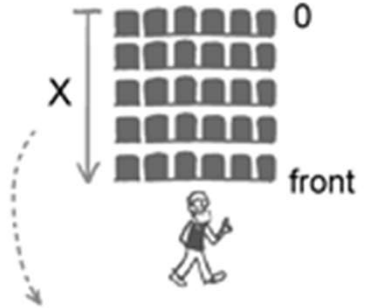
Against the wall:
"I'm sensitive. Please ignore me."

Second-row sleepers:
Good intentions, bad narcolepsy

WWW.PHDCOMICS.COM

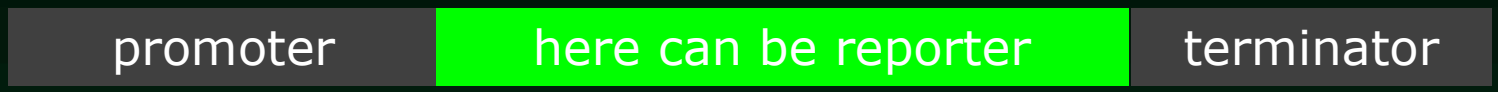
JORGE CHAM © 2008

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

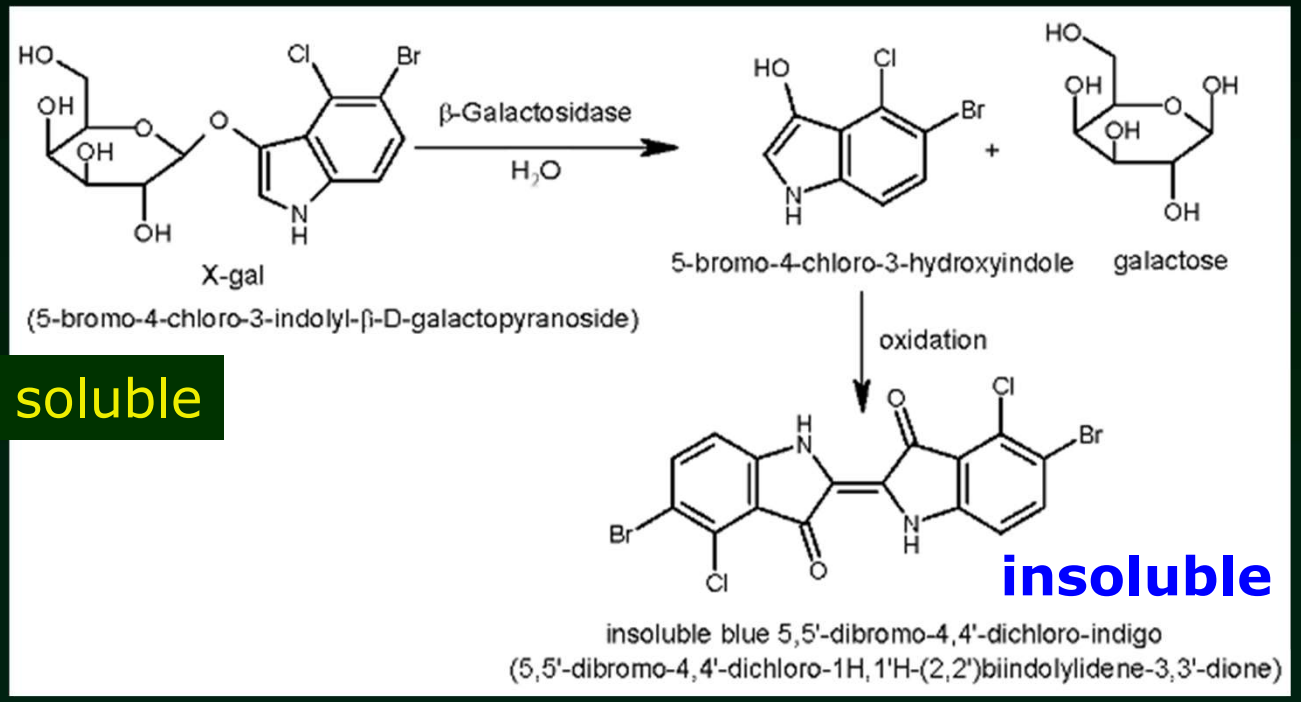
Reporter genes

- LacZ, GUS
- Luciferase
- GFP

some need external substrate, some not

LacZ, GUS – rhapsody in blue

promoter	LacZ	terminator



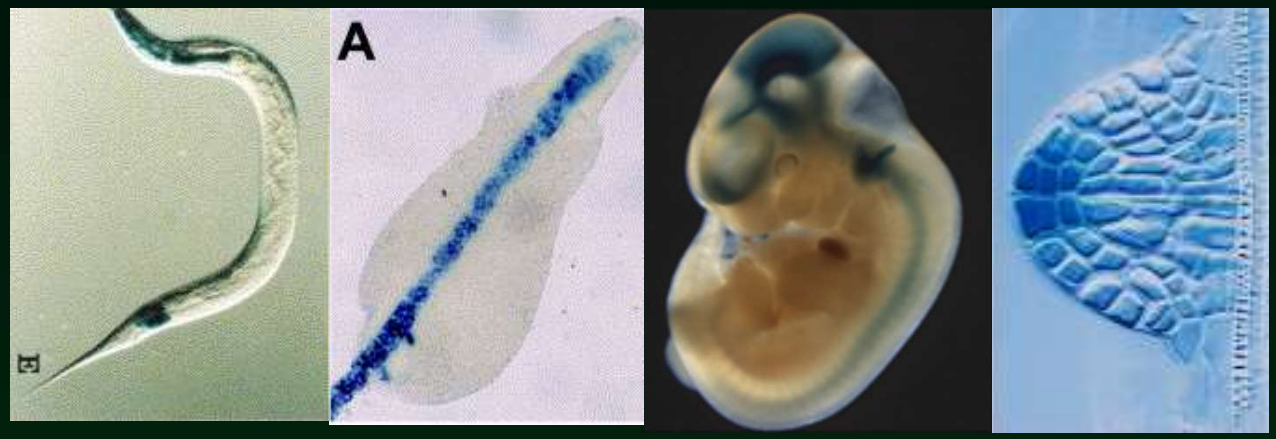
soluble

insoluble

(in case of GUS – X-Gluc)

LacZ, GUS

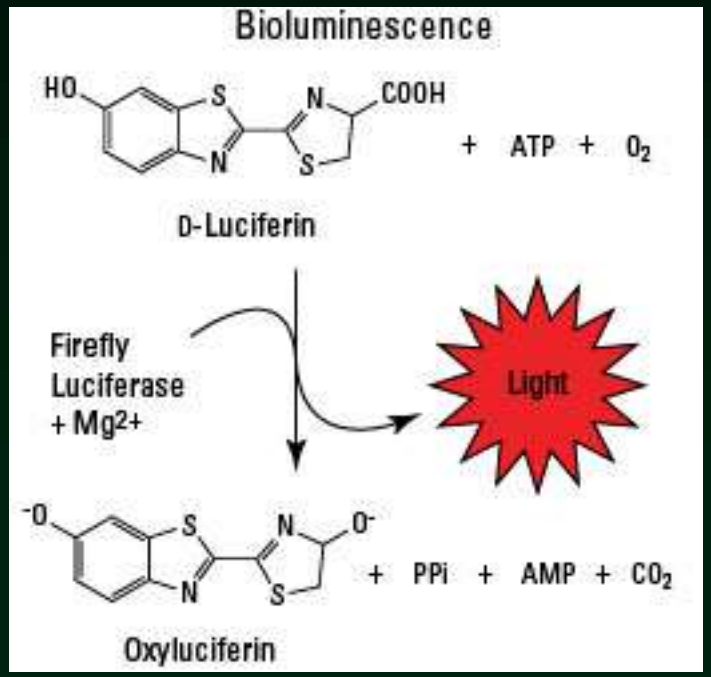
LacZ/ GUS:



worm, mouse – LacZ, plants - GUS

Luciferase

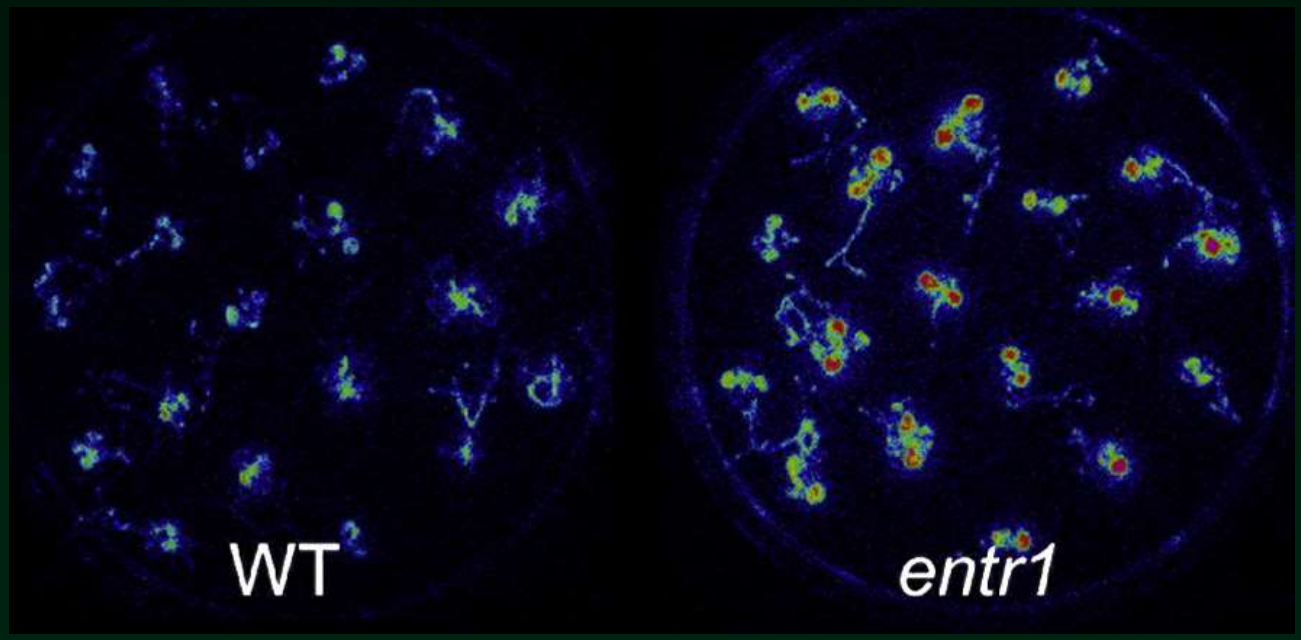
promoter	luciferase	terminator

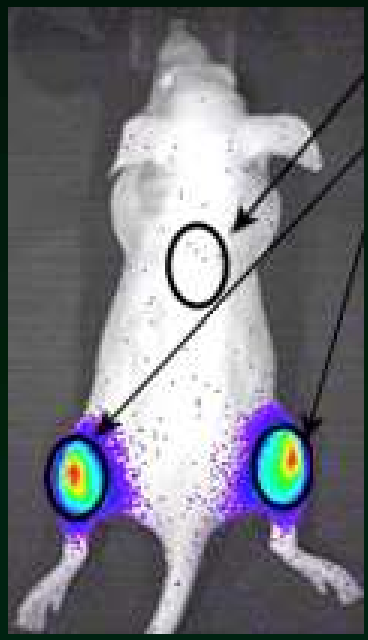


(principle of chemiluminiscence)

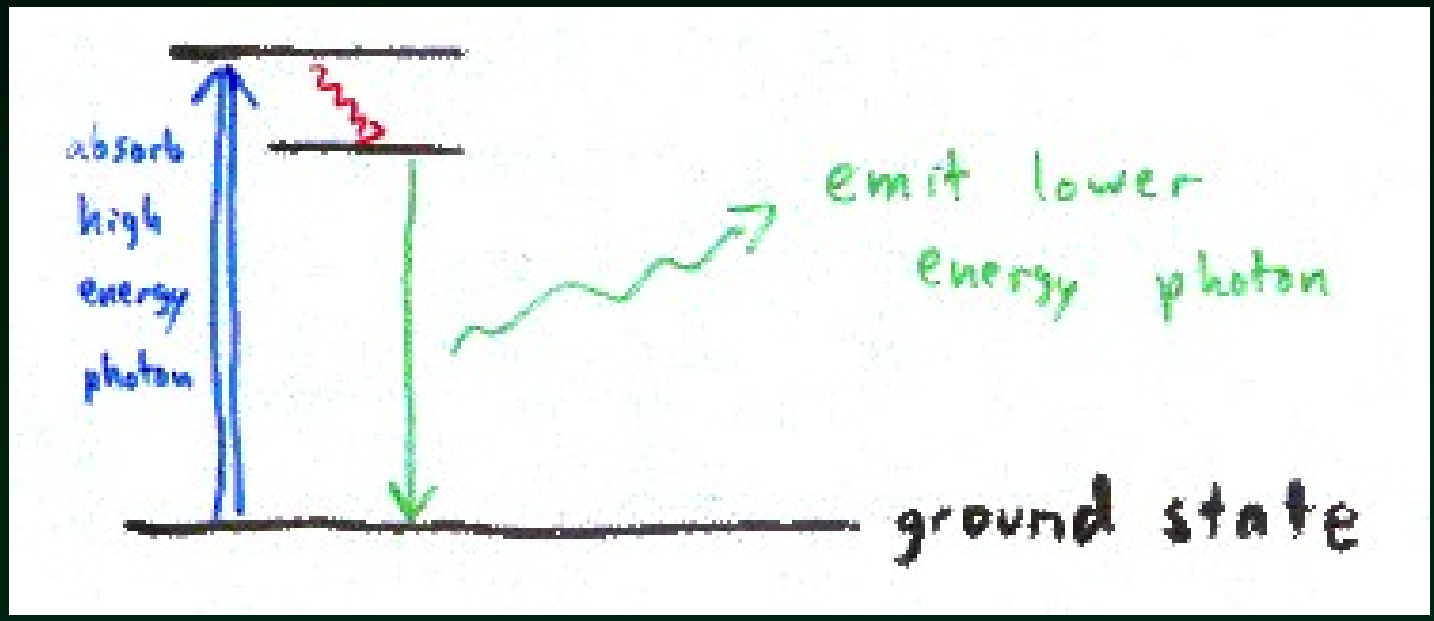
What's difference between flurescence and luminiscence?

Luciferase

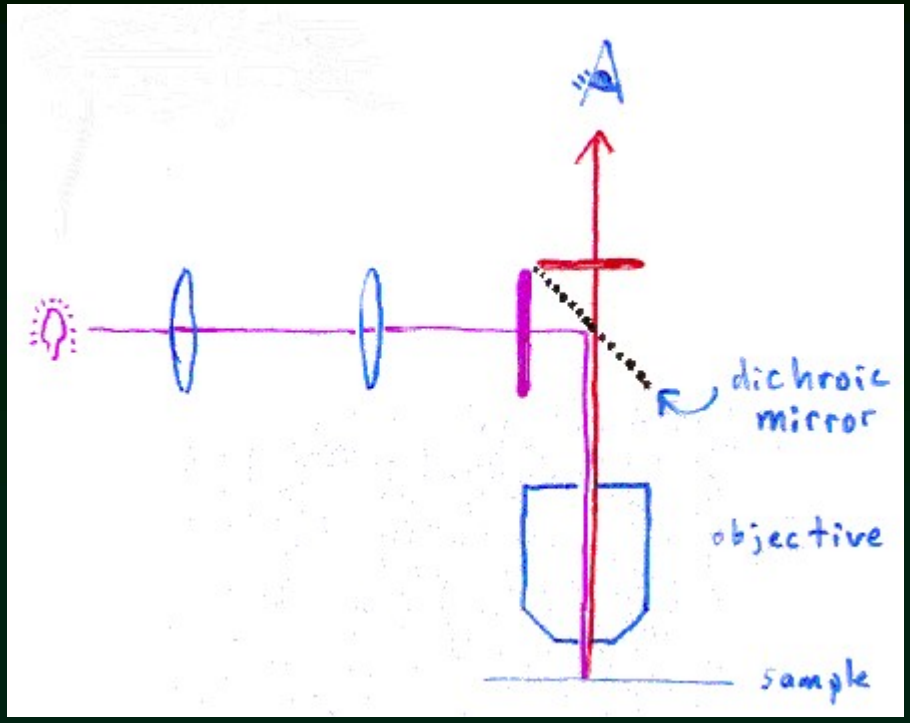




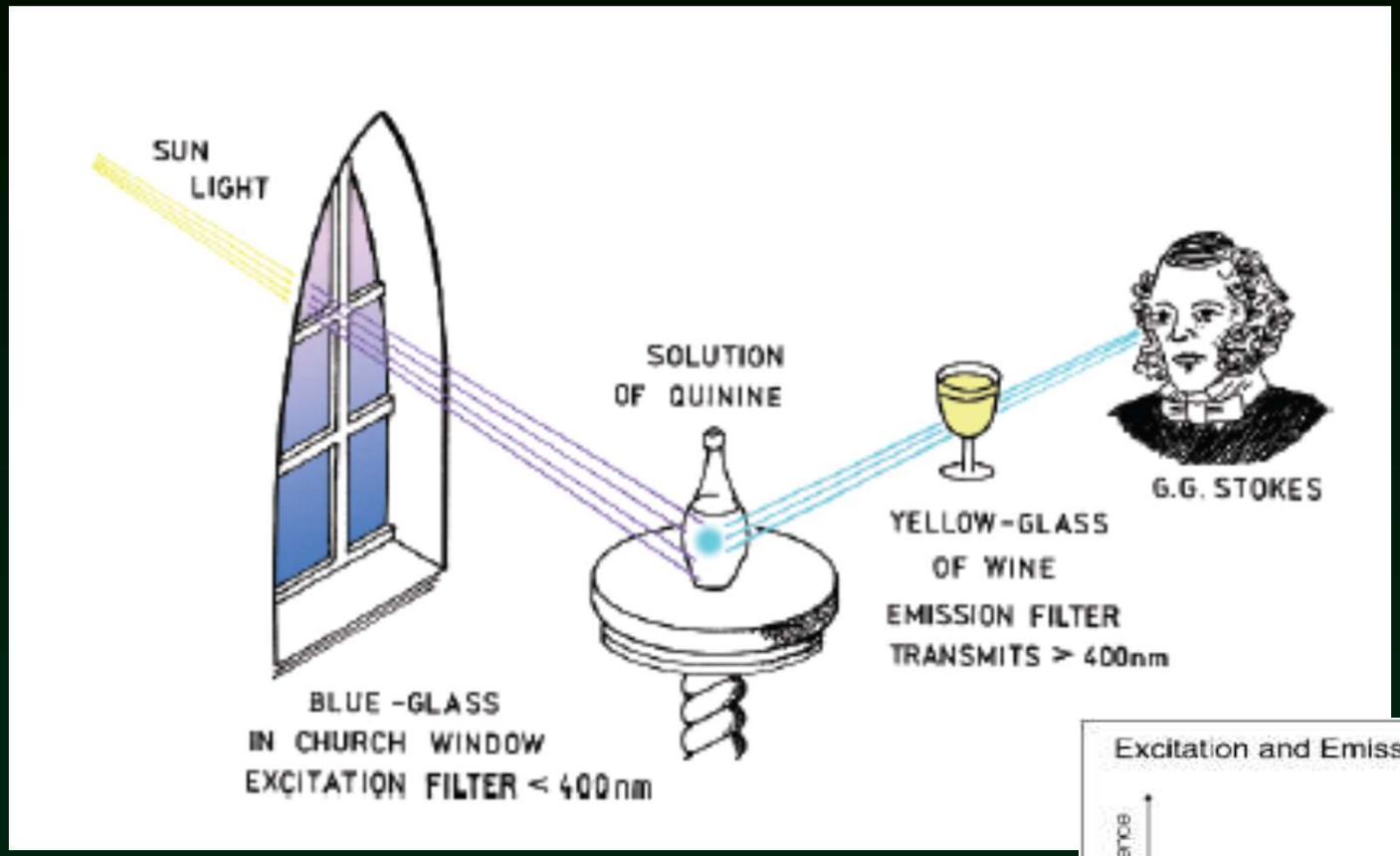
How does fluorescence work?

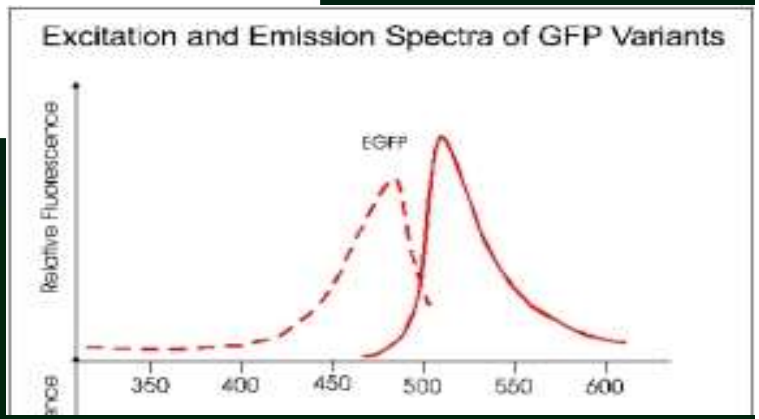


How does a fluorescence microscope work?



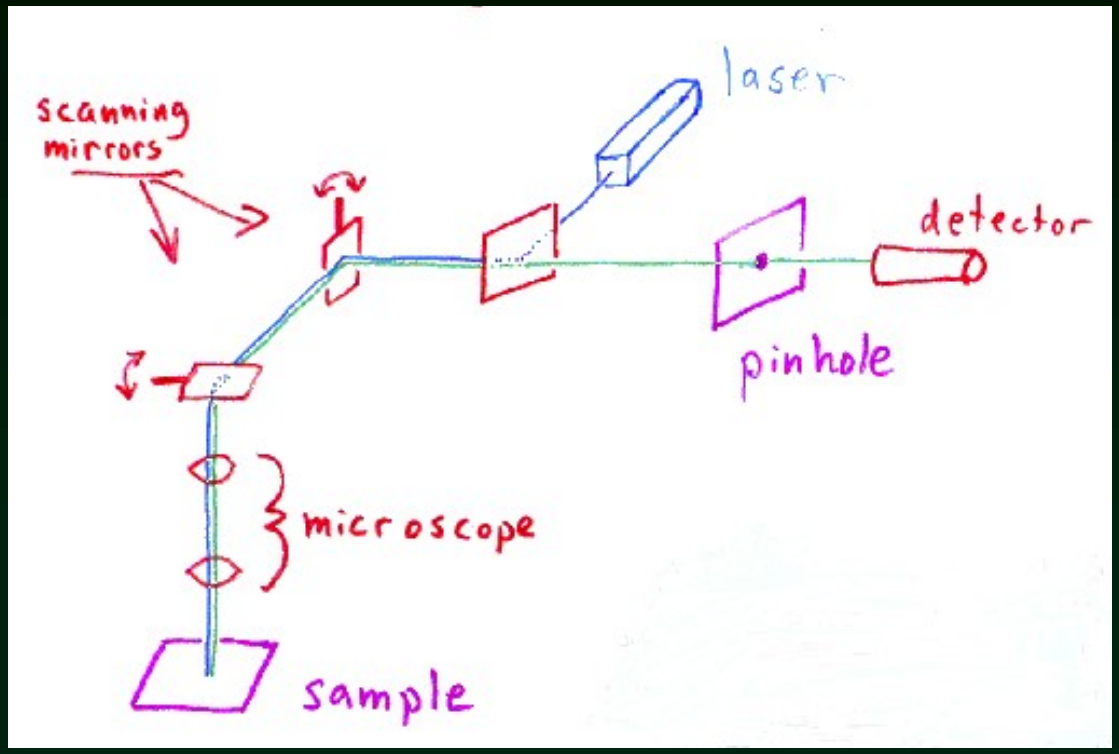
Stokes shift





George G. Stokes

How does a confocal microscope work?



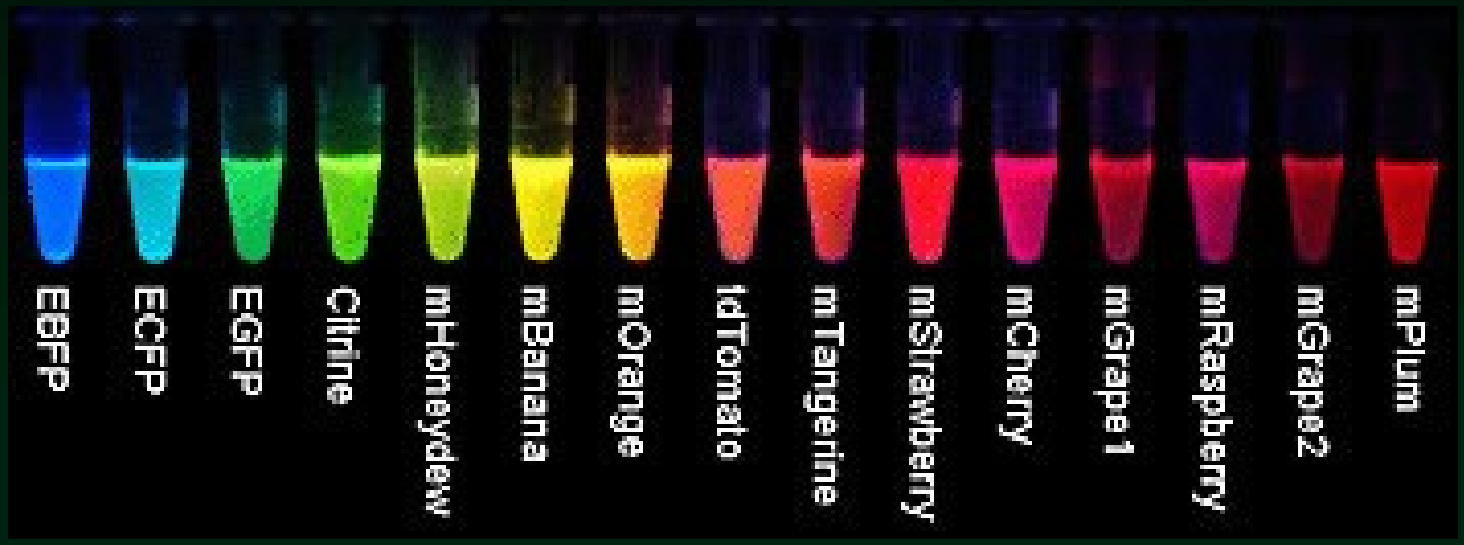
What are advantages of confocal microscopy?

Live imaging

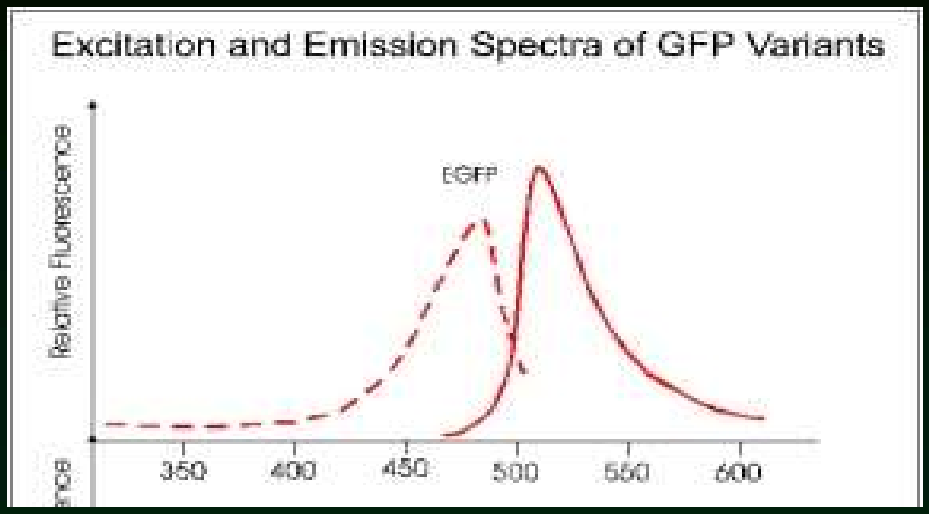
GFP discovery - Nobel Prize 2008

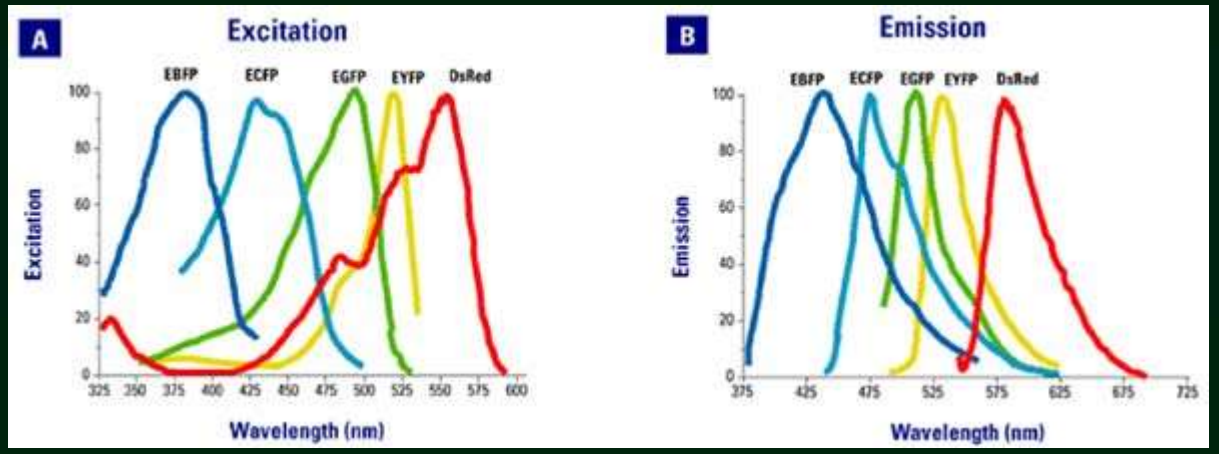
Osamu Shimomura Martin Chalfie Roger Tsien

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

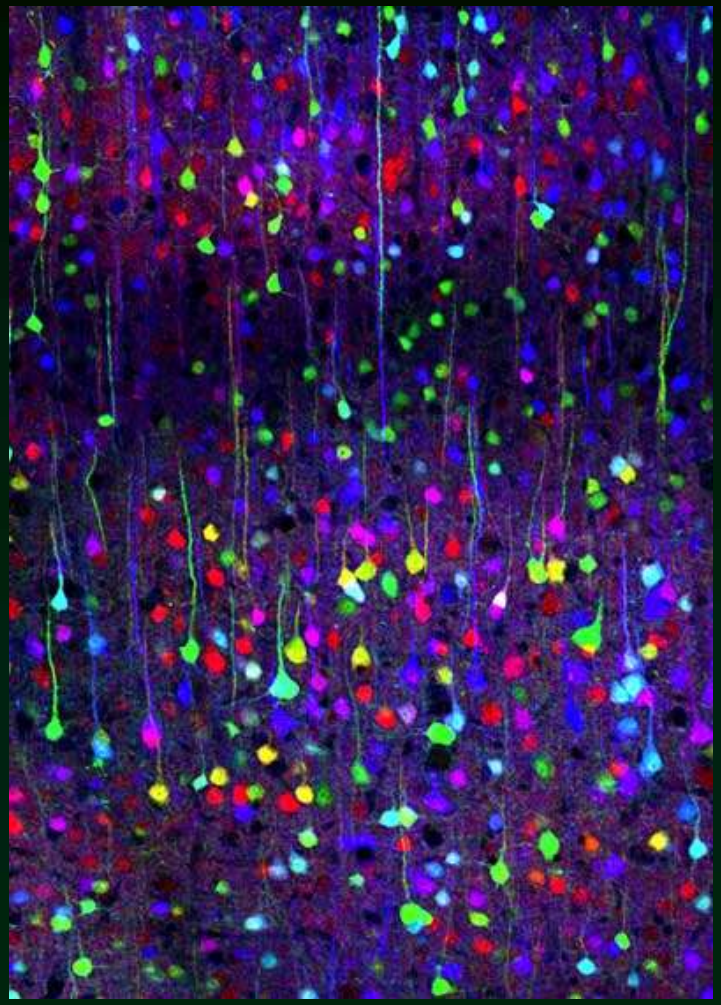


Excitation and emission



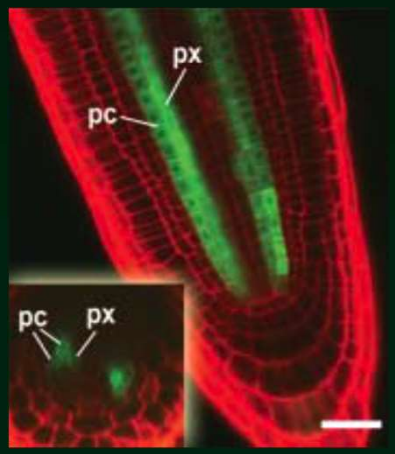


Multicolored fluorescent protein image (neurones)



Promoter-GFP

promoter	GFP	terminator



Promoter activity monitoring

choice of suitable reporter

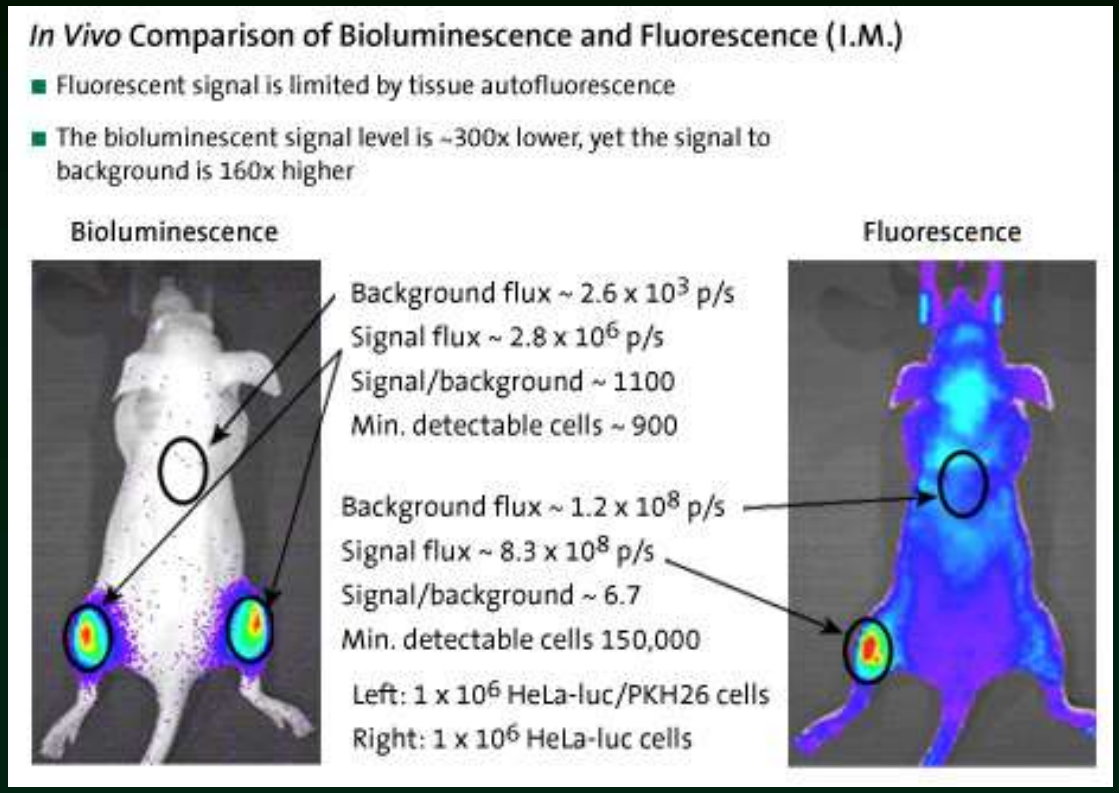
- LacZ, GUS
- Luciferase
- GFP

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; 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

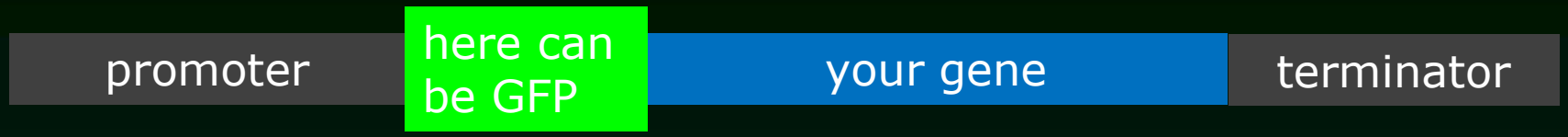
Cons:

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

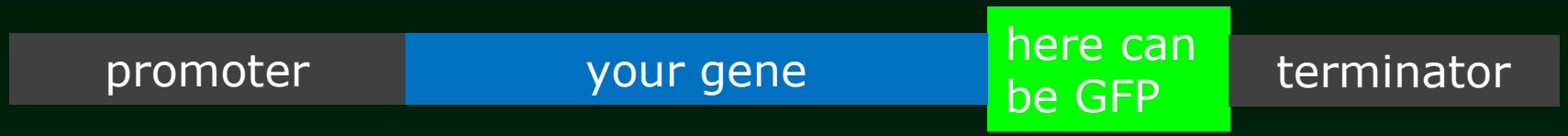
"We received him from a lab in U.S."

Translational GFP fusions

N-terminal fusion

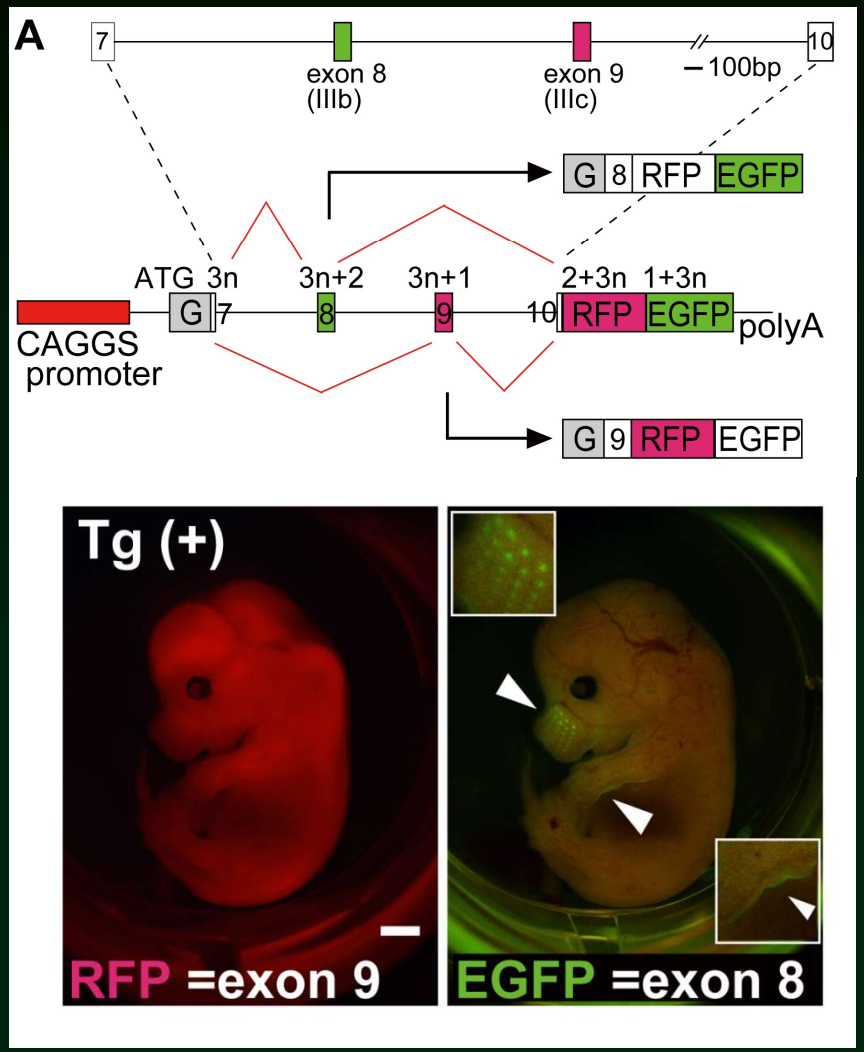


C-terminal fusion



fusion inside the coding sequence

Expression of isoforms



Fluorescent protein fusion

Pros:

•

Cons:

Fluorescent protein fusion

Pros:

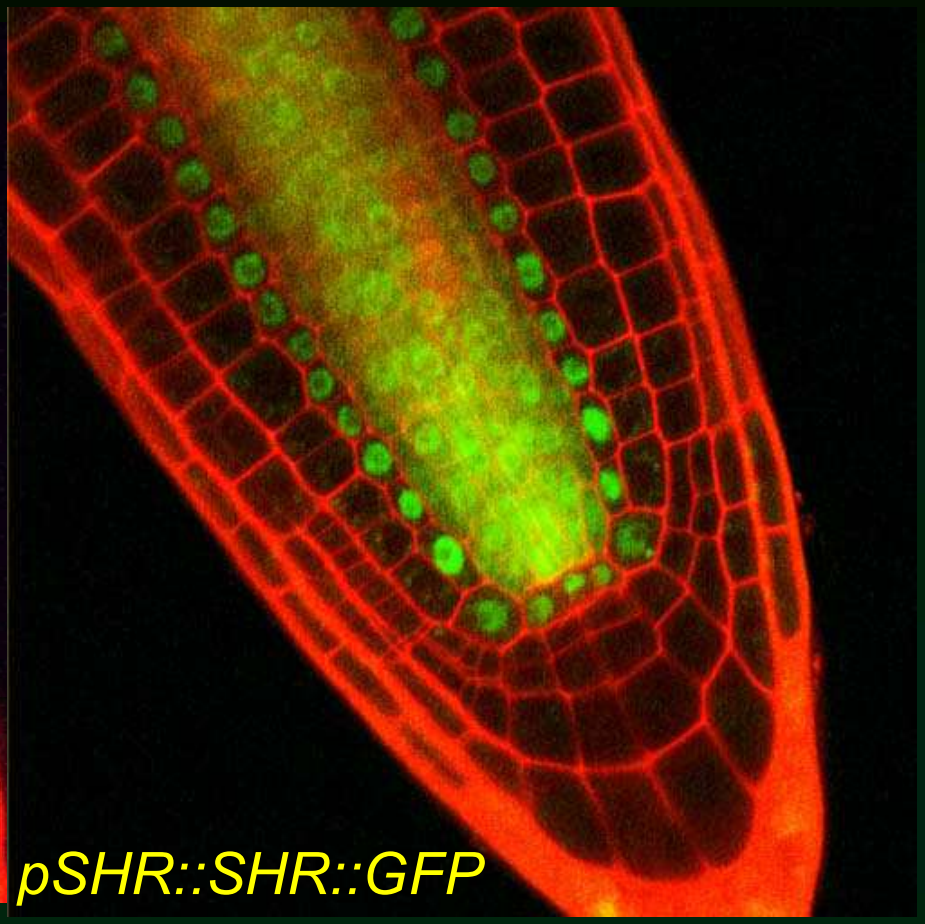
- in vivo imaging

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

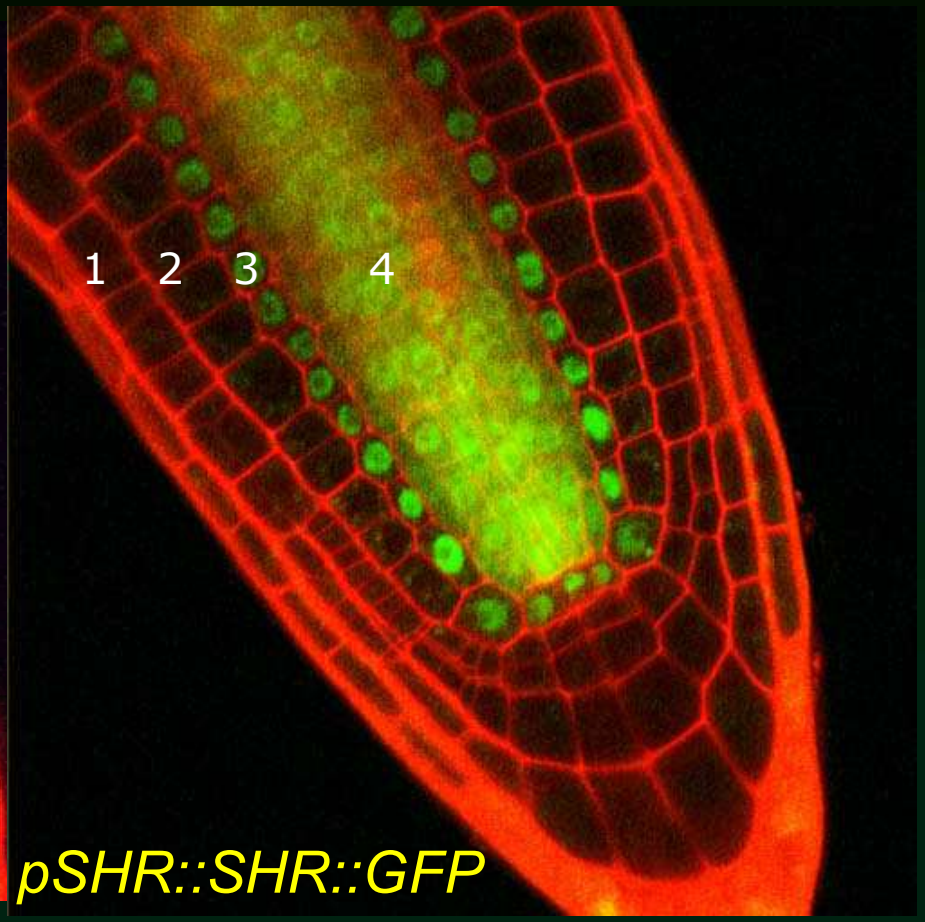


translational

Nakajima et al, Nature 2001

Why to visualize all this stuff

promoter



translational

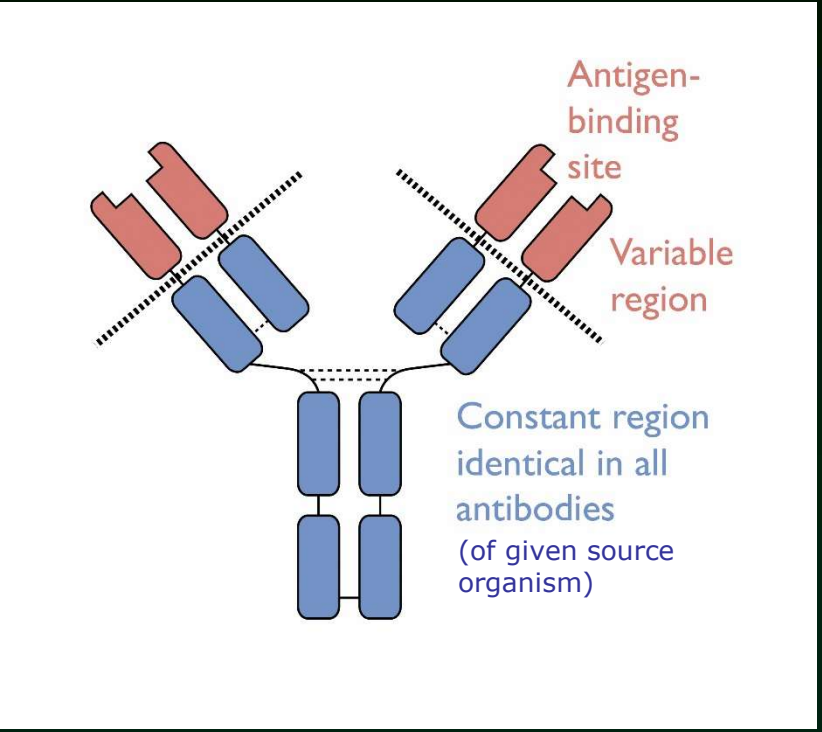
1 – epidermis
2 – cortex
3 – endodermis
4 – stele

Why to visualize all this stuff

BANG! SHR moves from stele to endodermis

Nakajima et al, Nature 2001

Protein immunolocalization

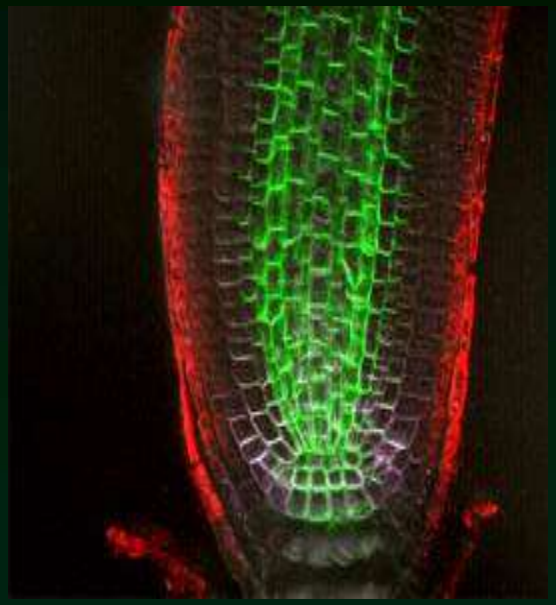


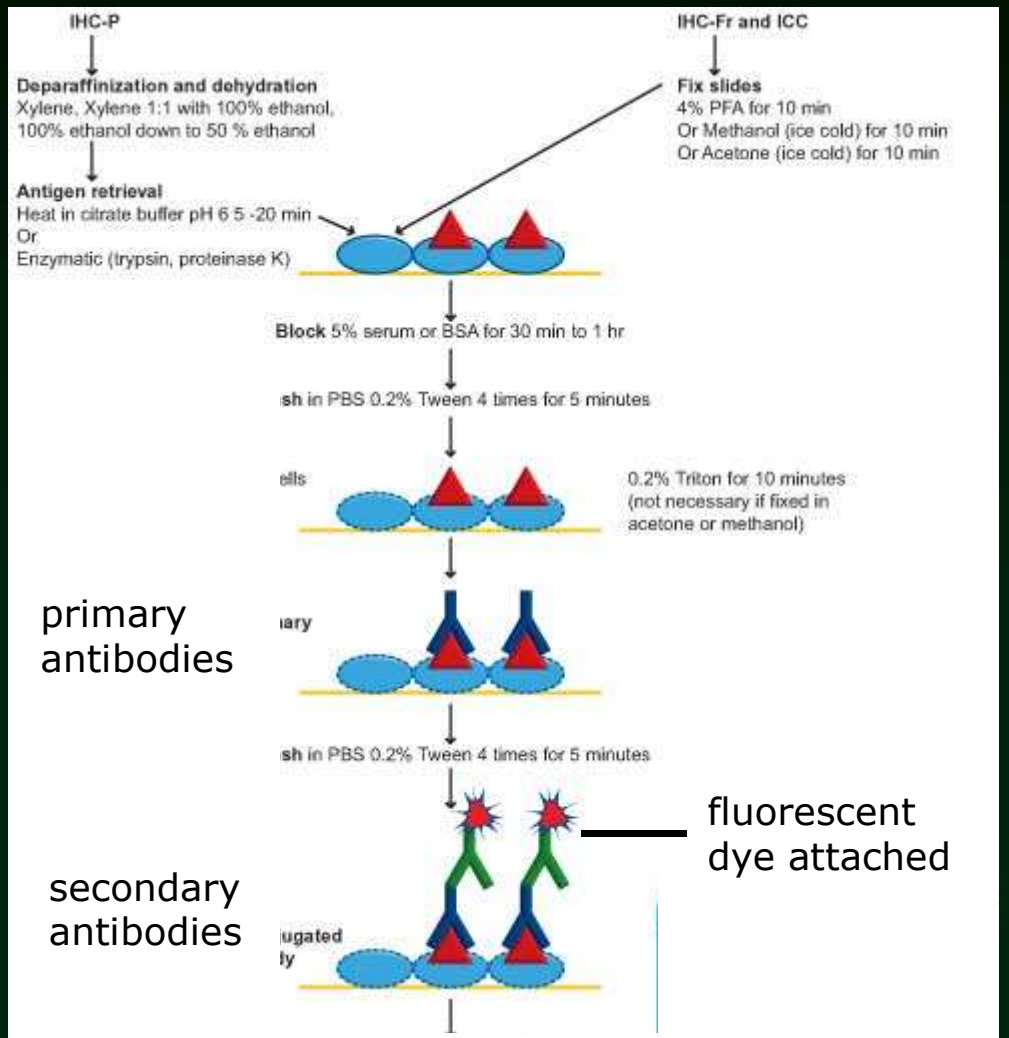
Most favorite animals:
-rabbit (too many rabbits)
-mouse (low volume)
-goat
-chicken
-rat
-sheep
-donkey
-guinea pig

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

Protein immunolocalization

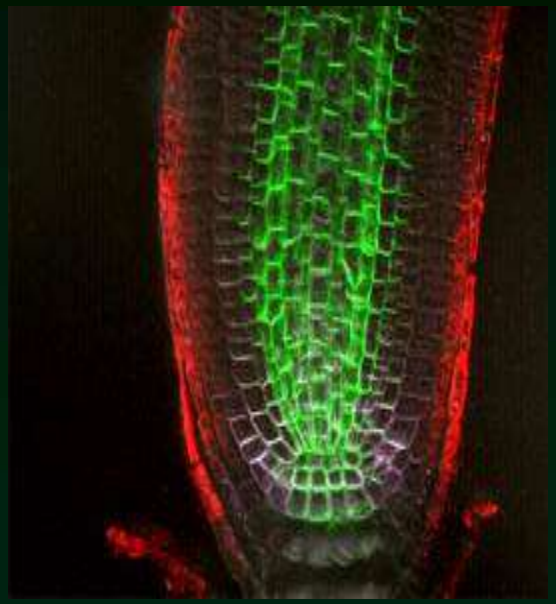
immunolocalization - fluorescently





Protein immunolocalization

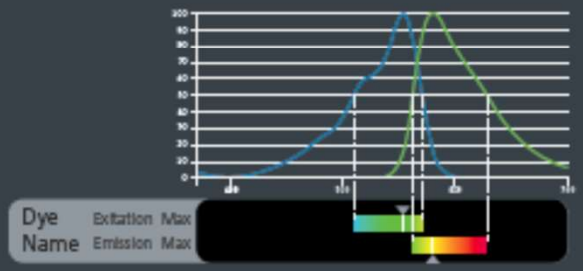
immunolocalization

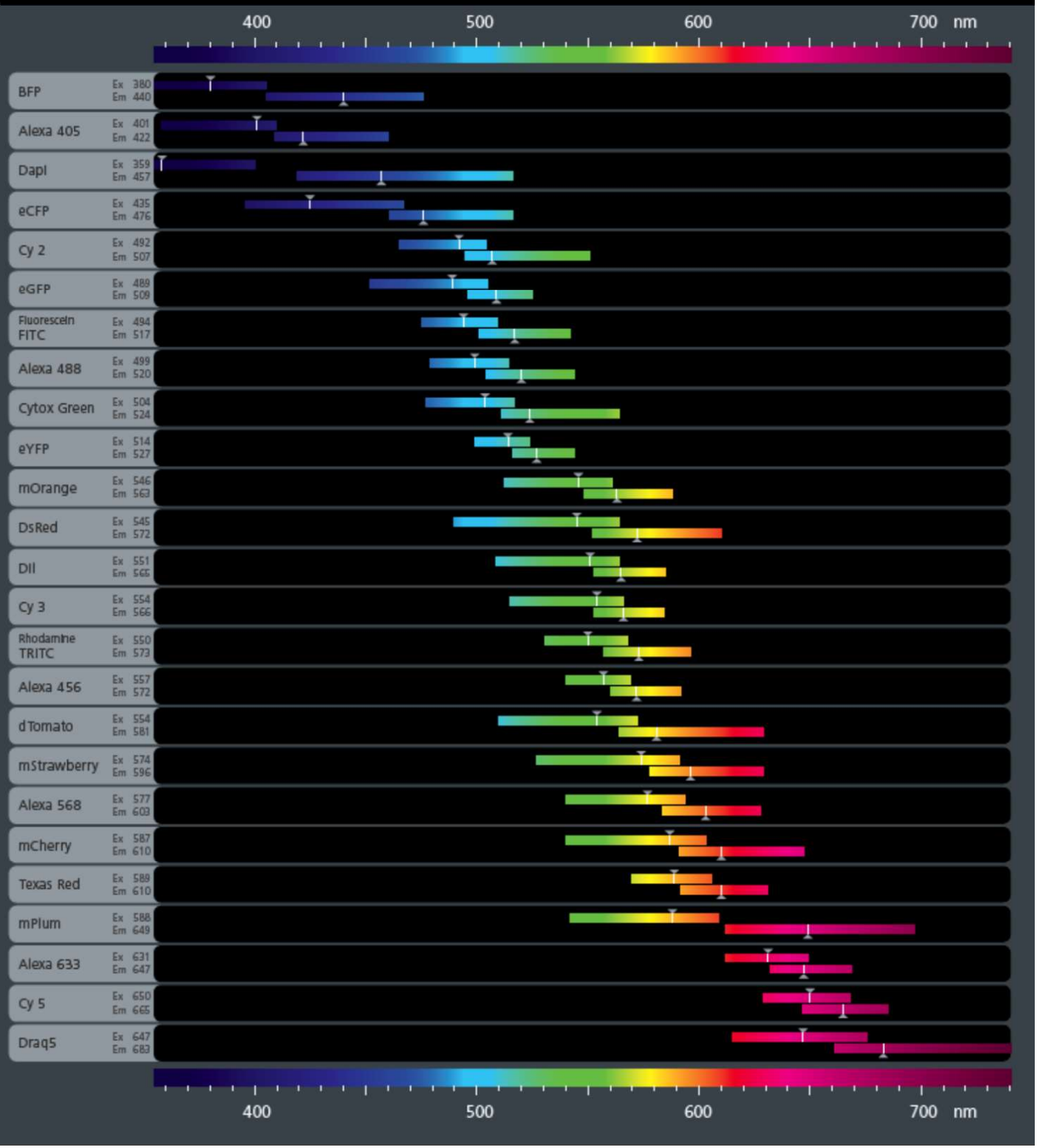


Fluorescent dyes conjugated to 2ndary (examples):

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

Fluorescent Dyes and Proteins





www.zeiss.com/microscopy

Protein immunolocalization

Pros:

-
-

Cons:

-
-

Protein immunolocalization

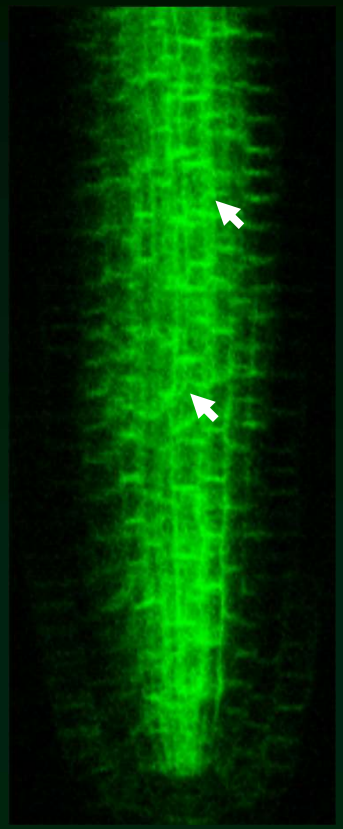
Pros:

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

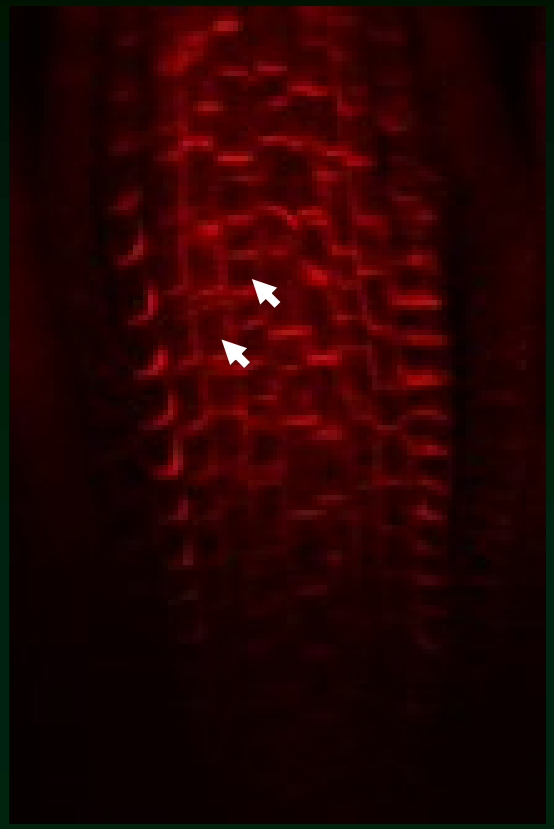
Cons:

- fixed material only
- excellent antibodies only, sometimes tricky

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



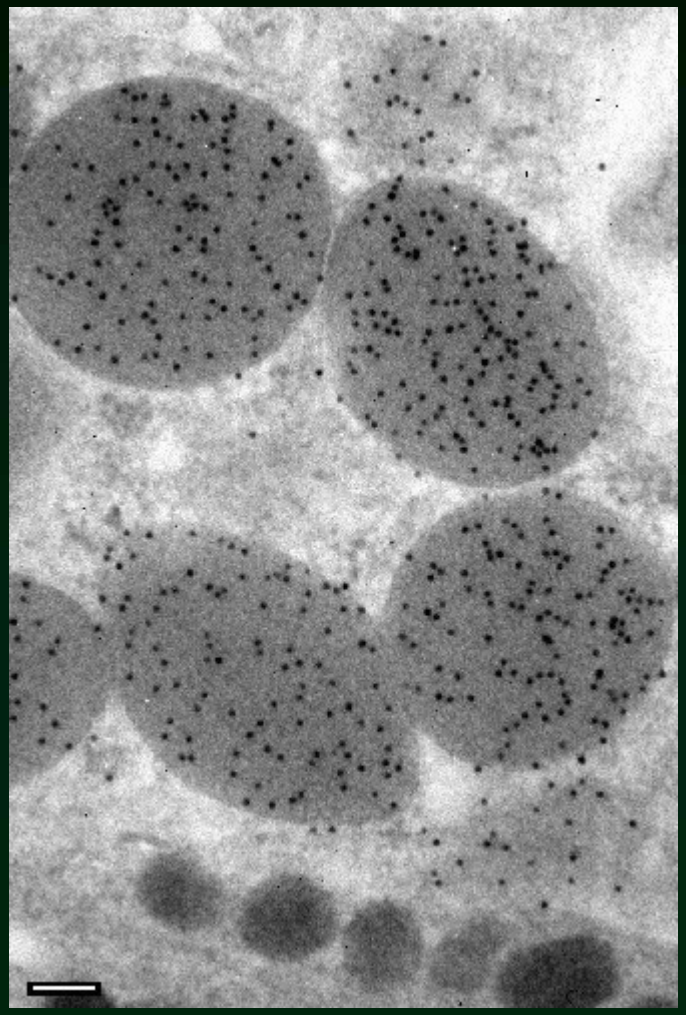
PIN1-GFP



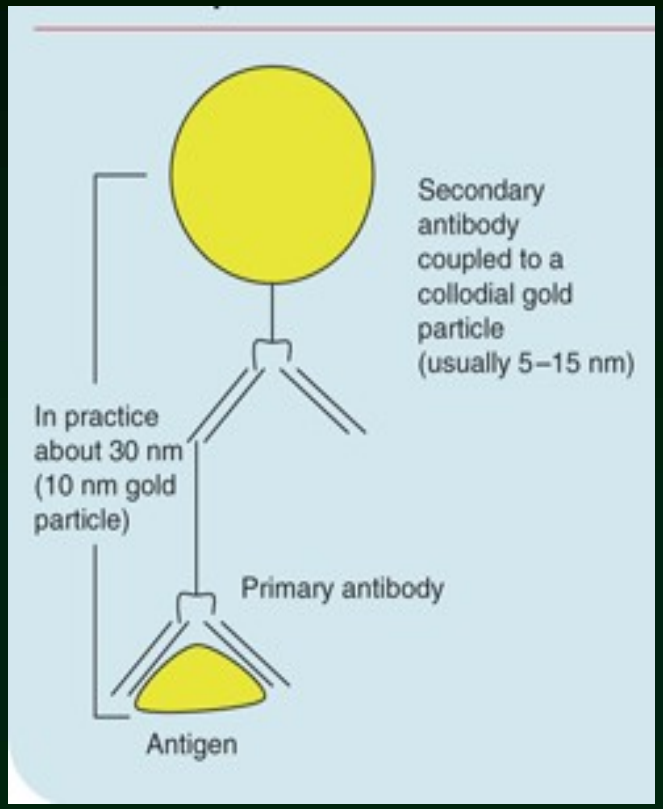
anti-PIN1

Protein localization - immunogold

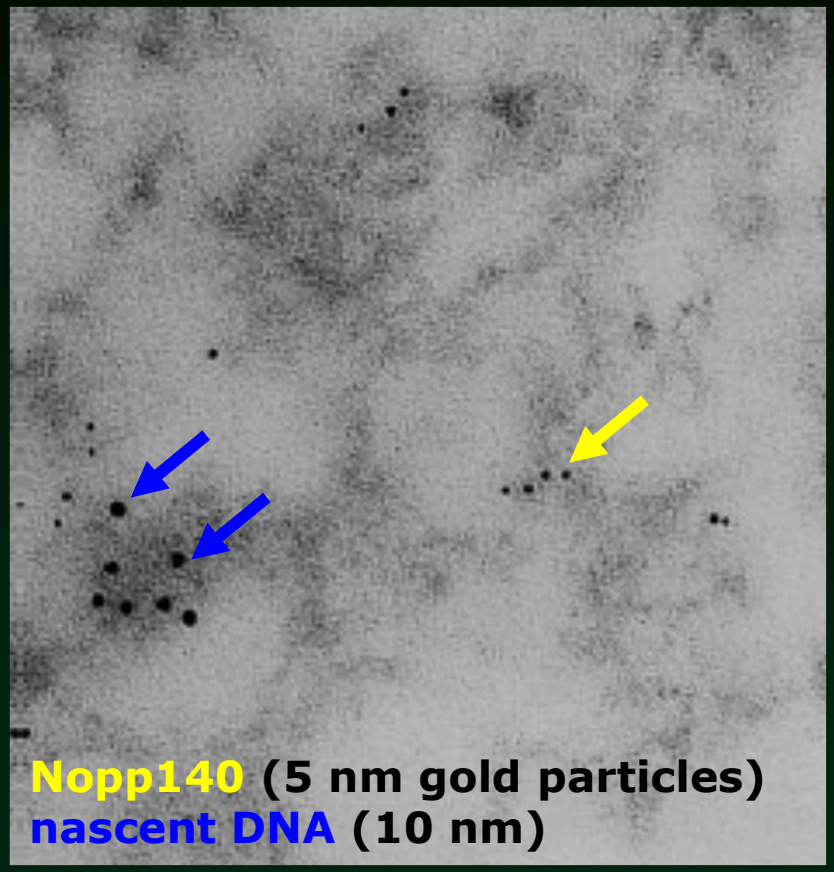
immunolocalization - immunogold

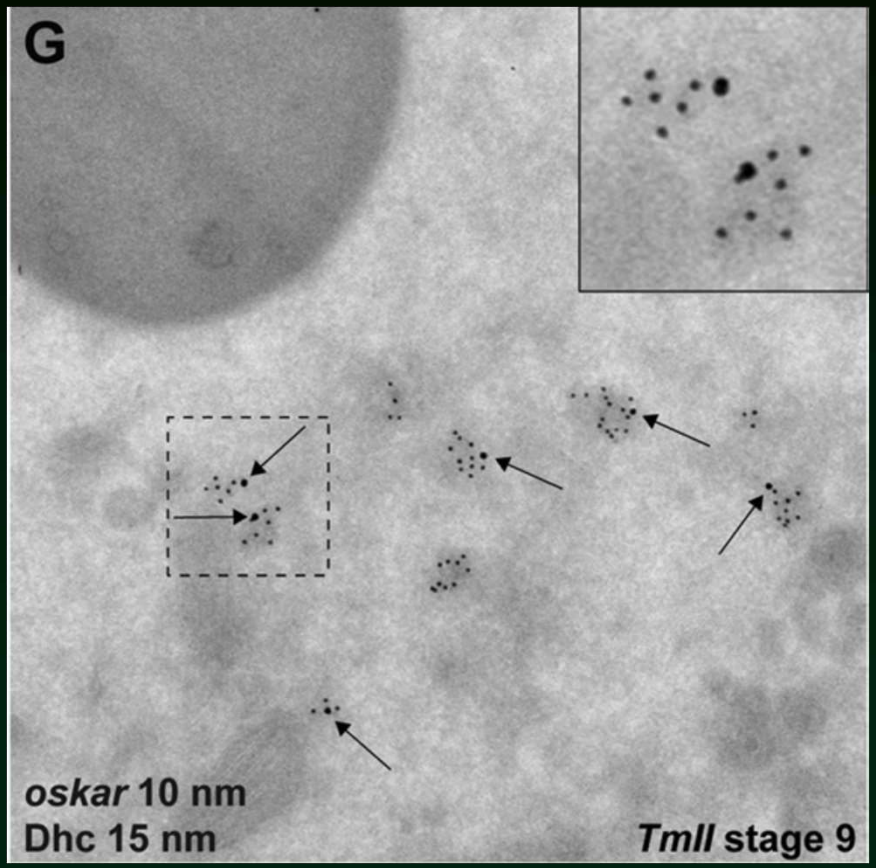


electron microscope



Immunogold collocalization





Philimonenko et al 2000, and an unfortunate Cell paper

Imunohistochemistry pros/cons

Pros:

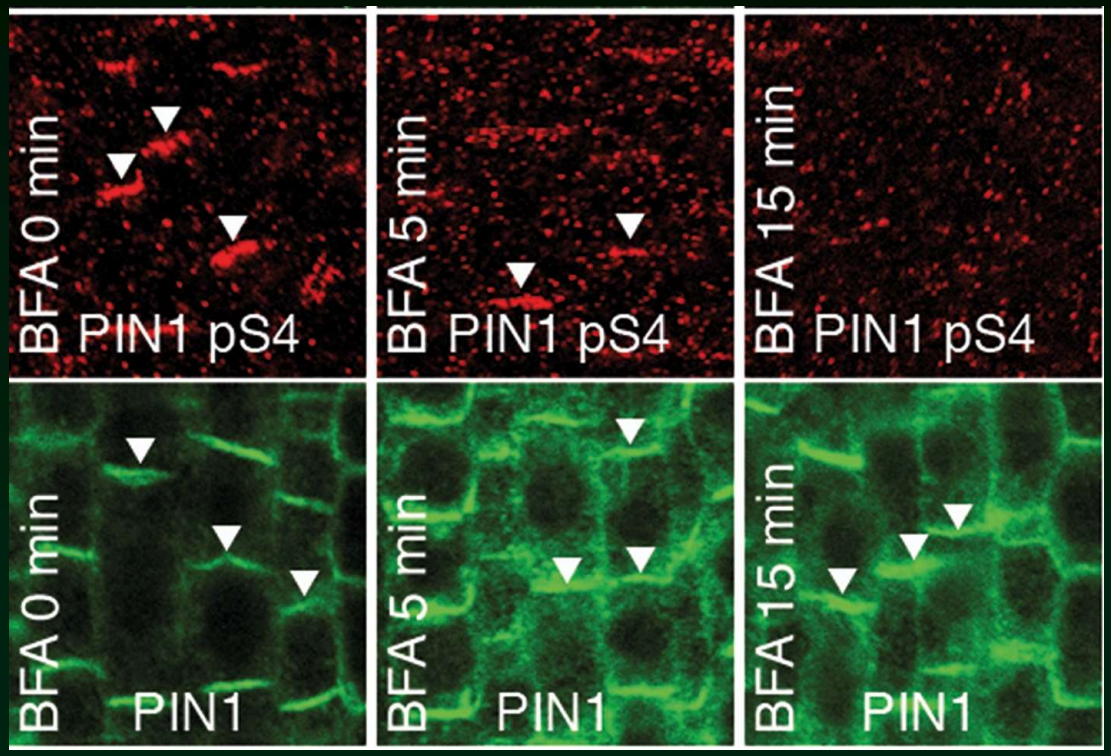
- direct
- nothing can beat the resolution

Cons:

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

Can we visualize postranslational modifications?

Can we visualize postranslational modifications?



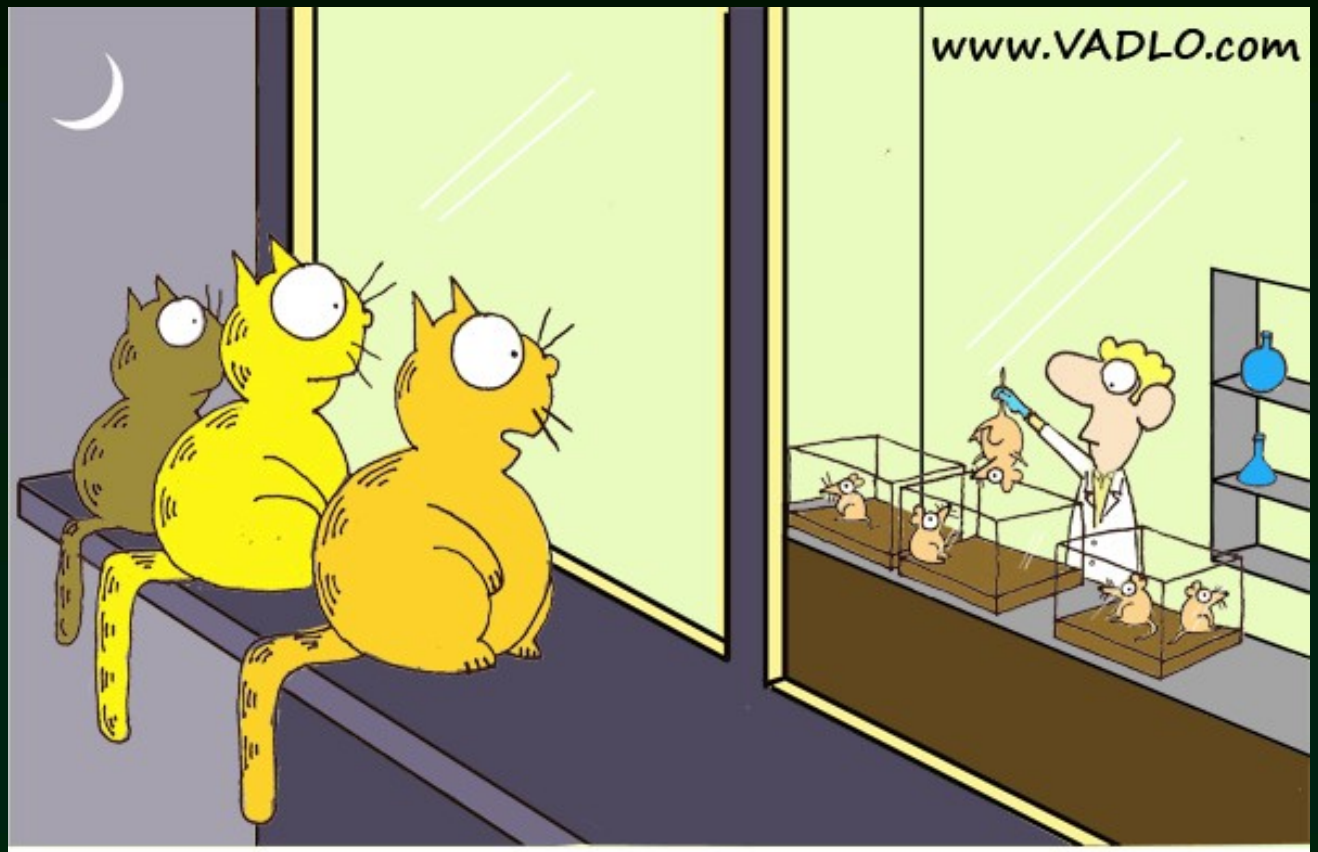
antibodies against phosphate pS4

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

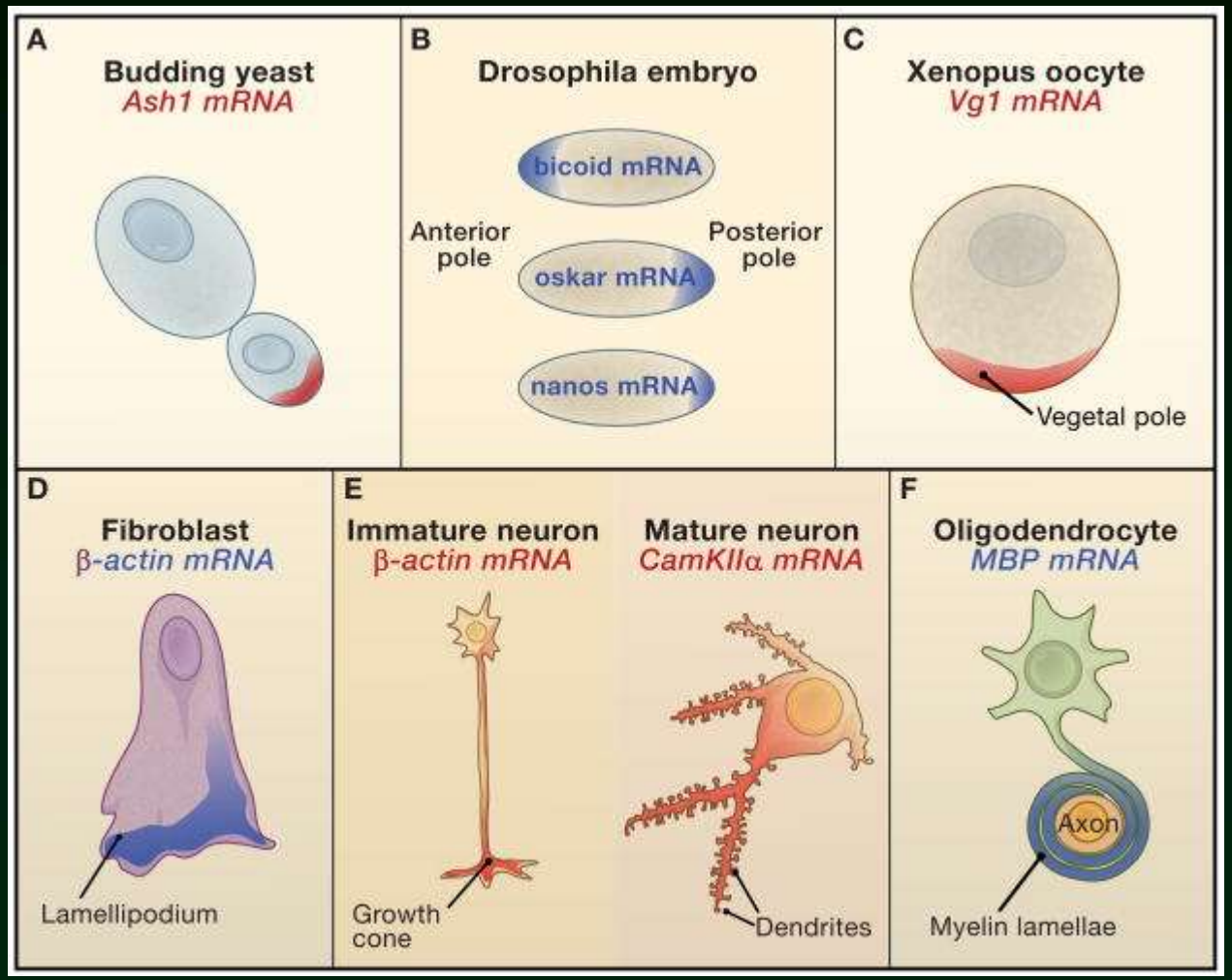
Yes, we can.

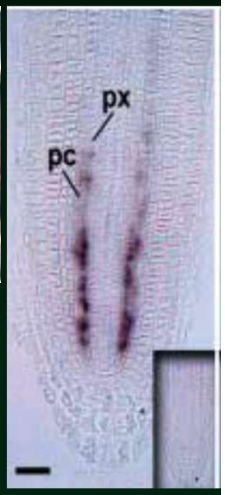
Stanislas et al. 2016

If missing other model organism than *Arabidopsis*



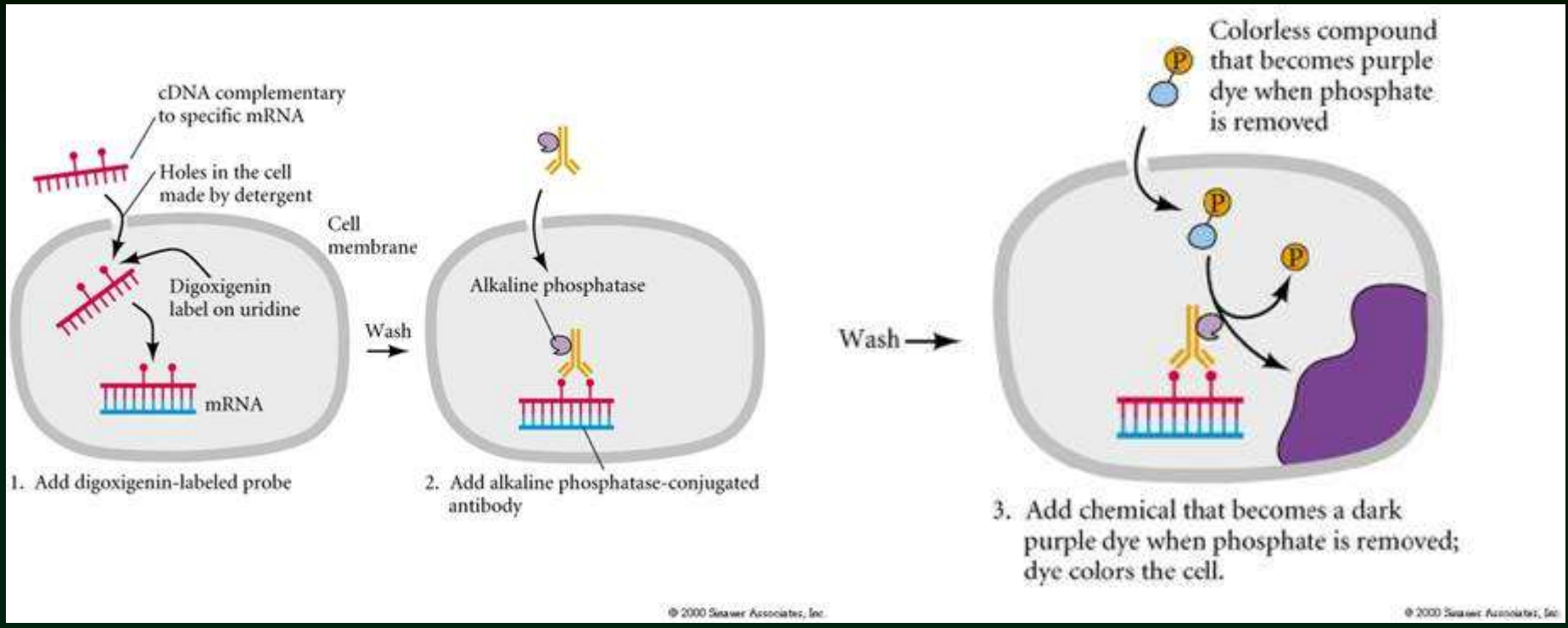
Also RNA can be visualized





Localization of mRNA

RNA hybridization *in situ*



Visualization of mRNA

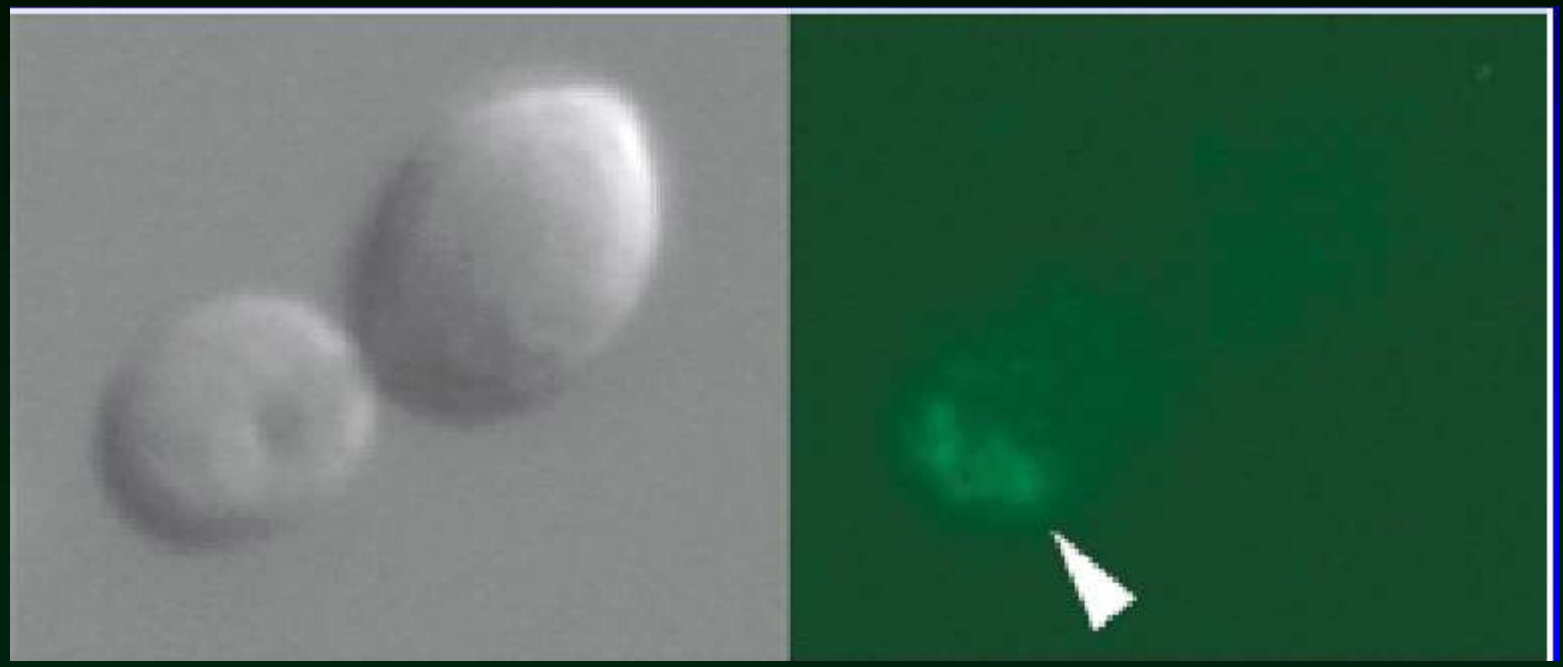
RNA hybridization *in situ*

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*

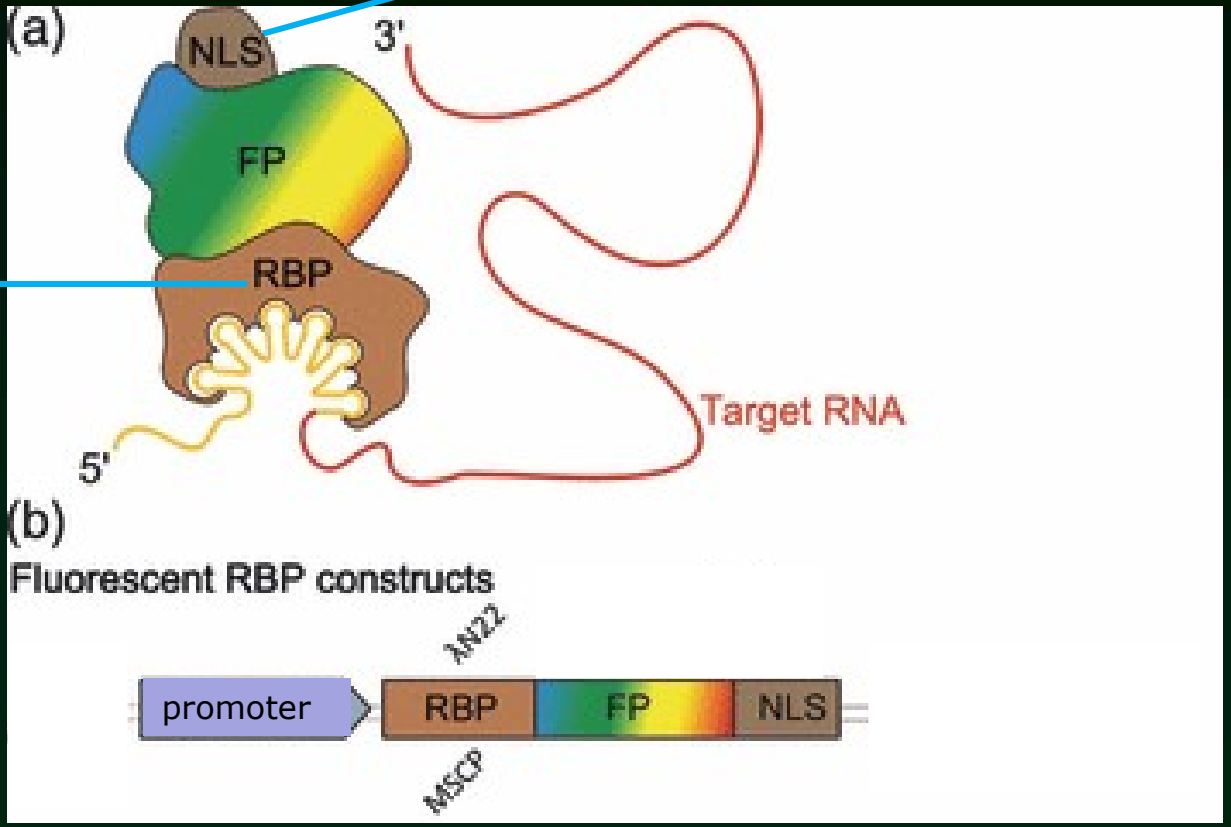


Ash1 mRNA localized to the tip of the daughter cell

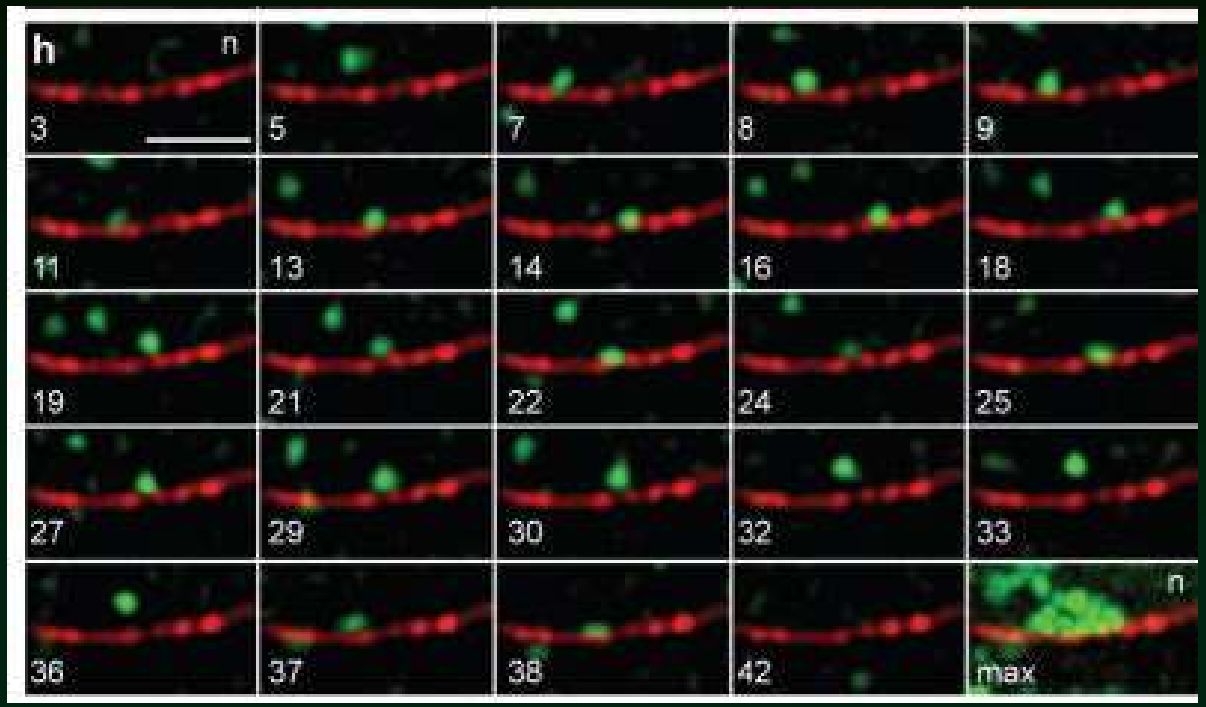
λN_{22} system – RNA imaging *in vivo*

nuclear localization signal

viral RNA binding protein



Similar system in single molecule resolution

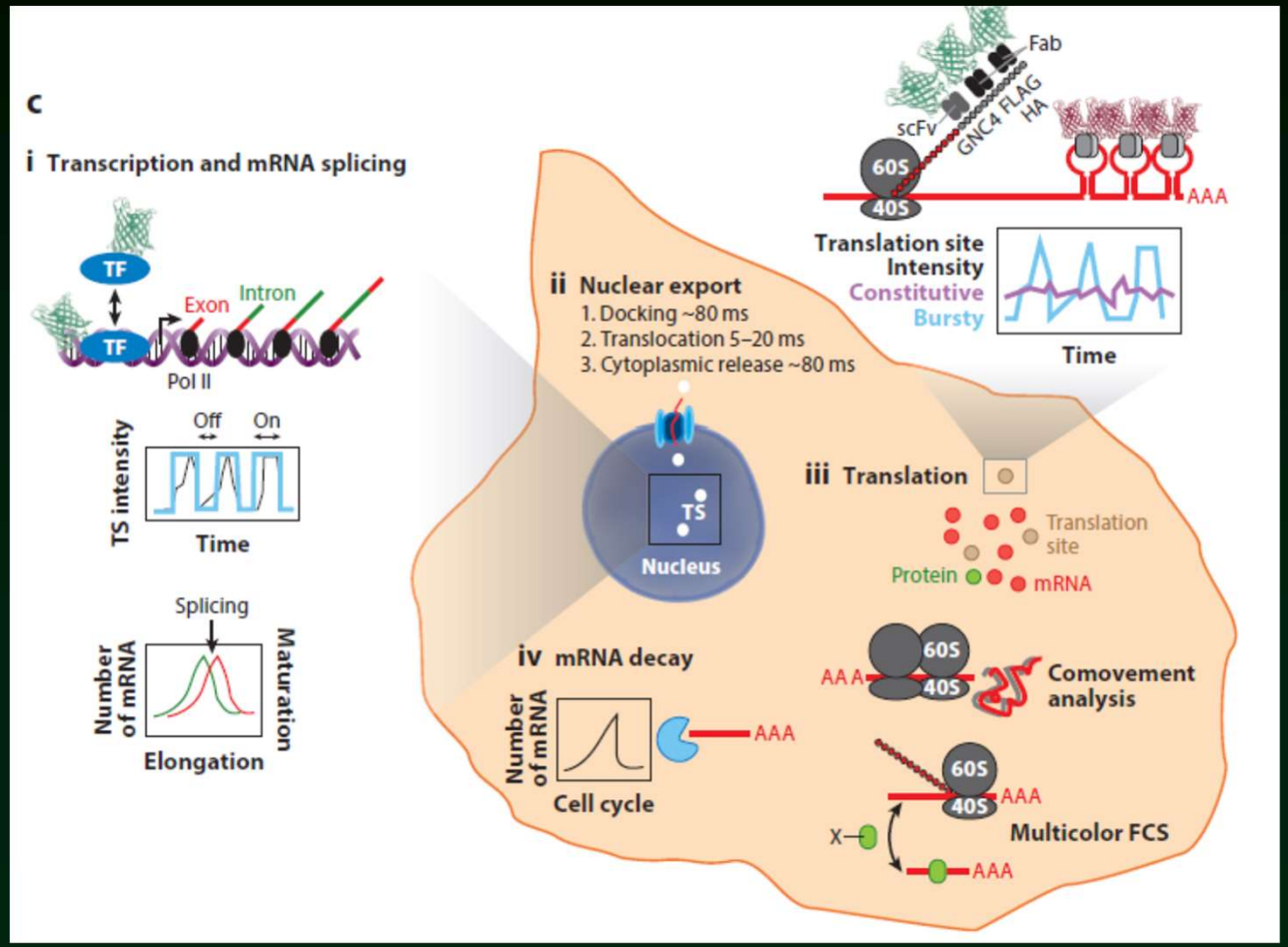


export of β-actin mRNA from nucleus

(smFISH and stem loops)

Grünwald and Singer 2010

Big science of single molecule microscopy



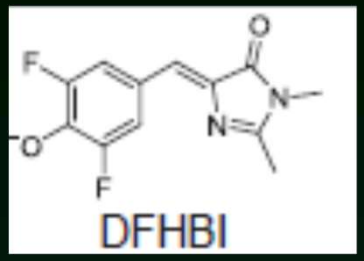
Vera et al. 2016
(Singer lab)

Alternative to λN_{22} system etc. - we have SPINACH

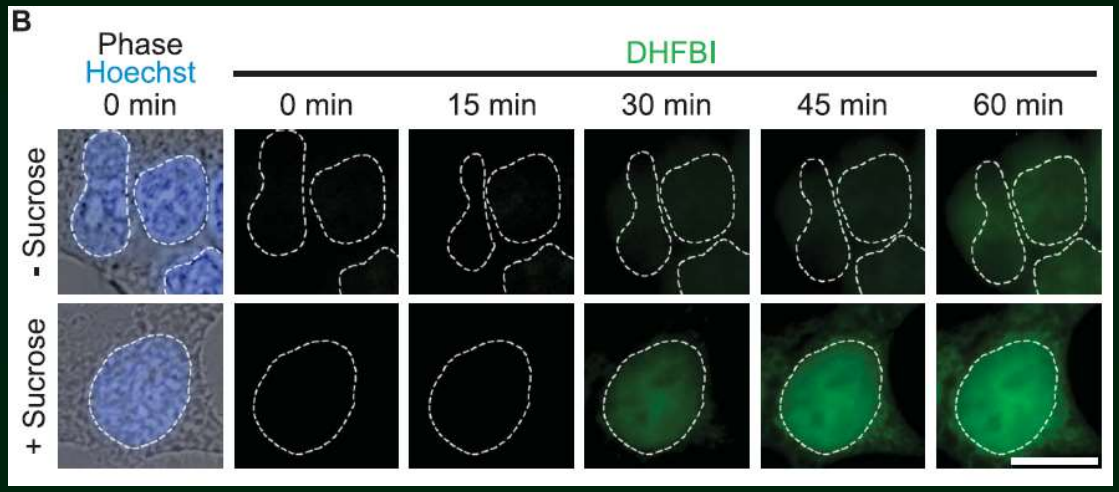
GACGCAACUGAAUGAAA
UGGUGAAGGACGGGUCC
AGGUGUGGCUGCUUCGG
CAGUGCAGCUUGUUGAG
UAGAGUGUGAGCUCCGU
AACUAGUCGCGUC

RNA fusion

+



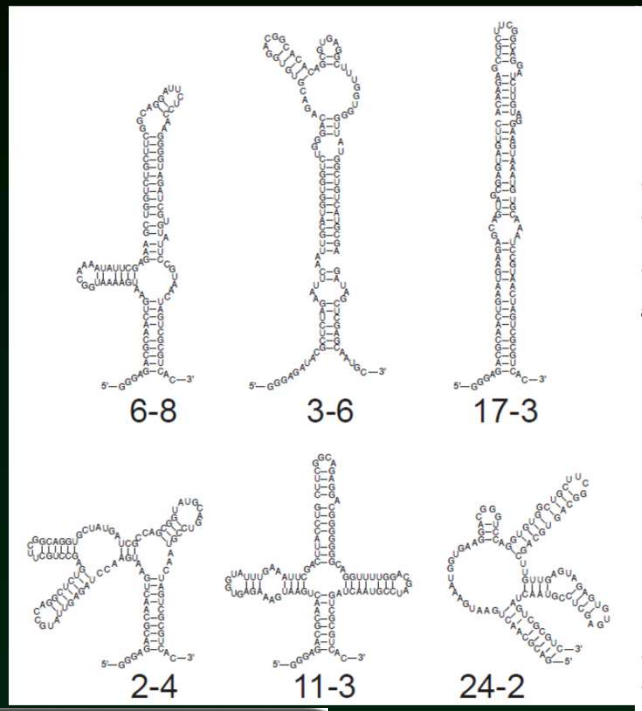
aptamer

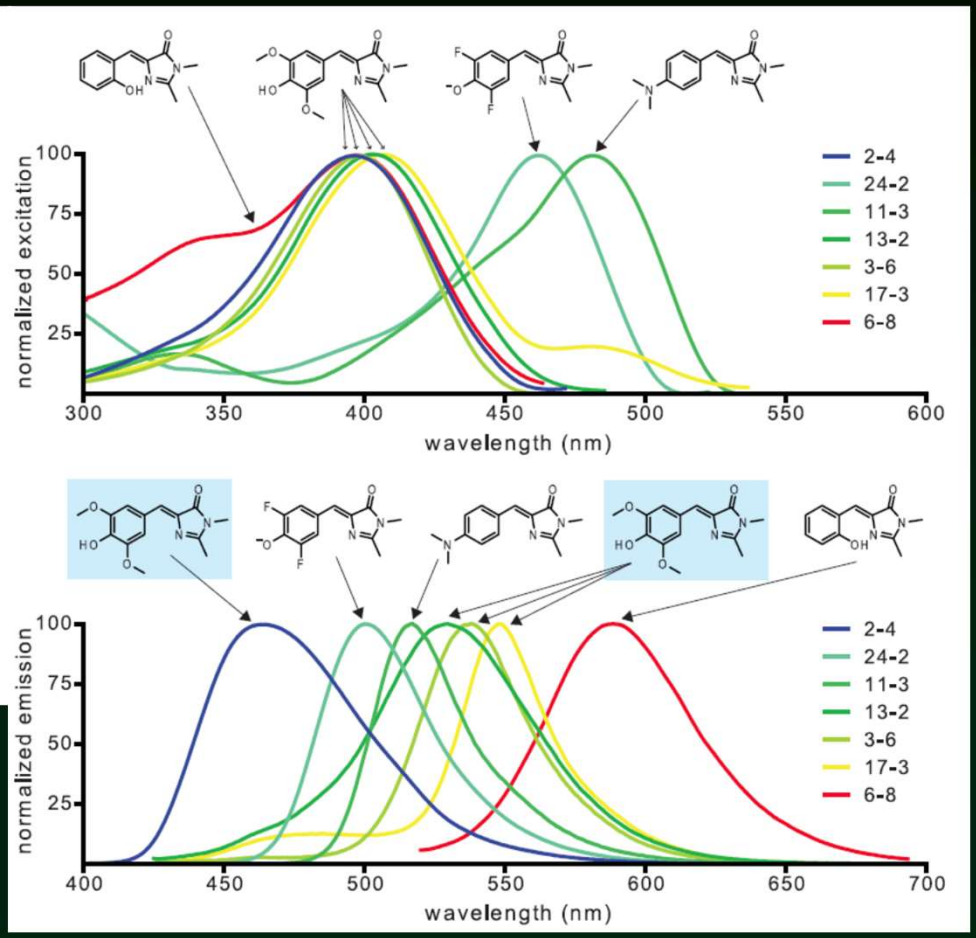


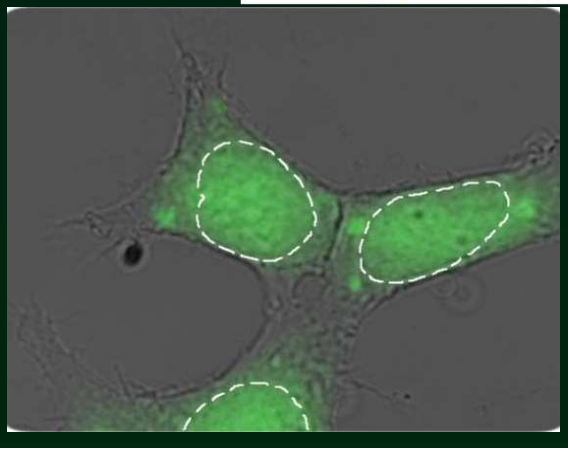
blue-DNA green-RNA

Paige et al. 2012

Other vegetables than SPINACH

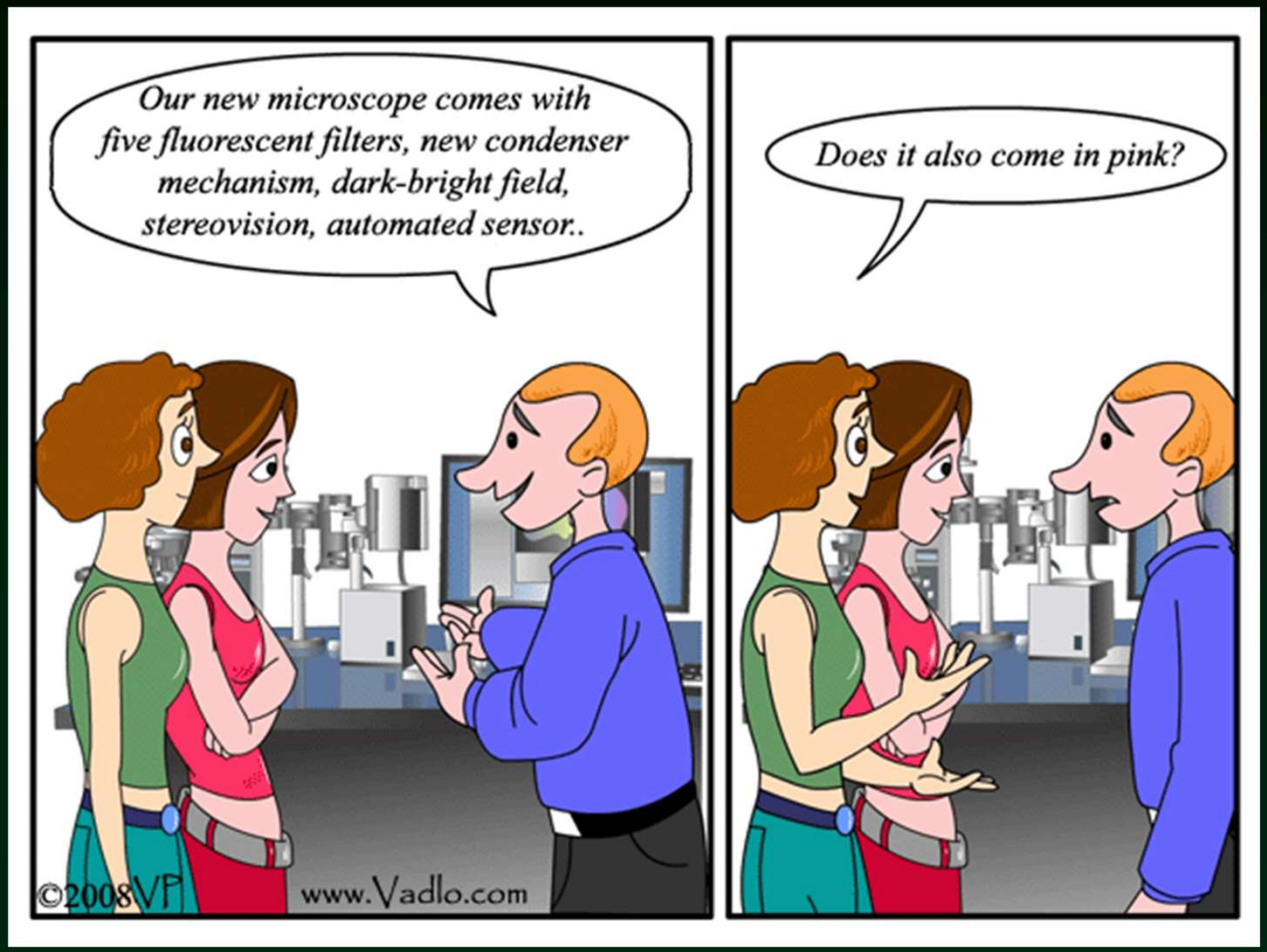






Paige et al. 2012; Song et al. 2014

Advanced confocal techniques



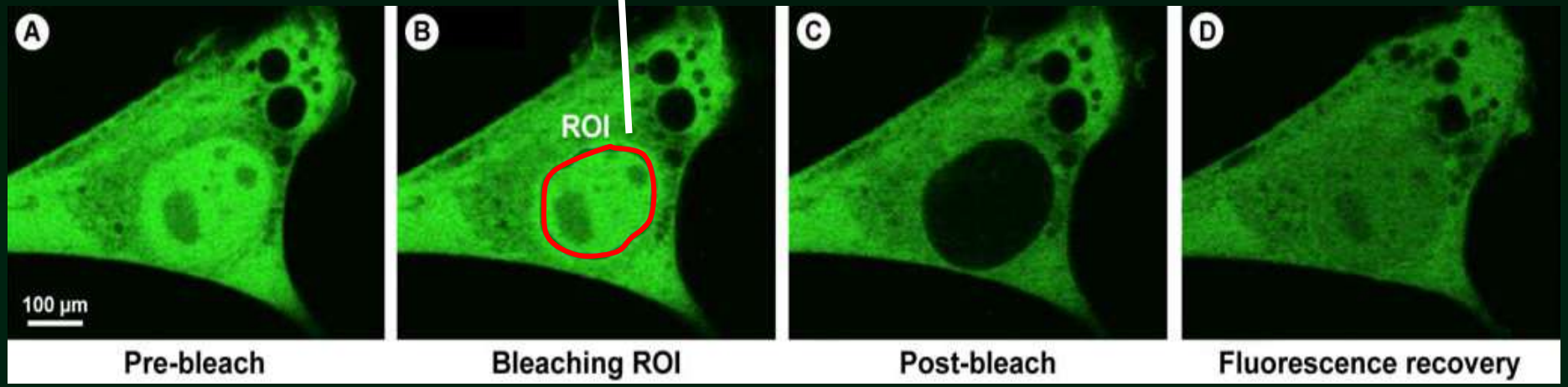
(slightly) Advanced confocal techniques

- FRAP
- photoactivatable FP
- FCS

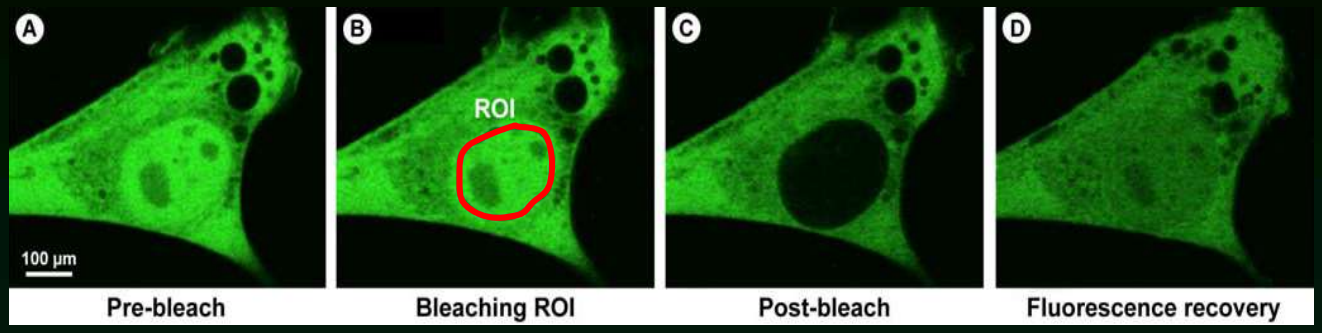
FRAP

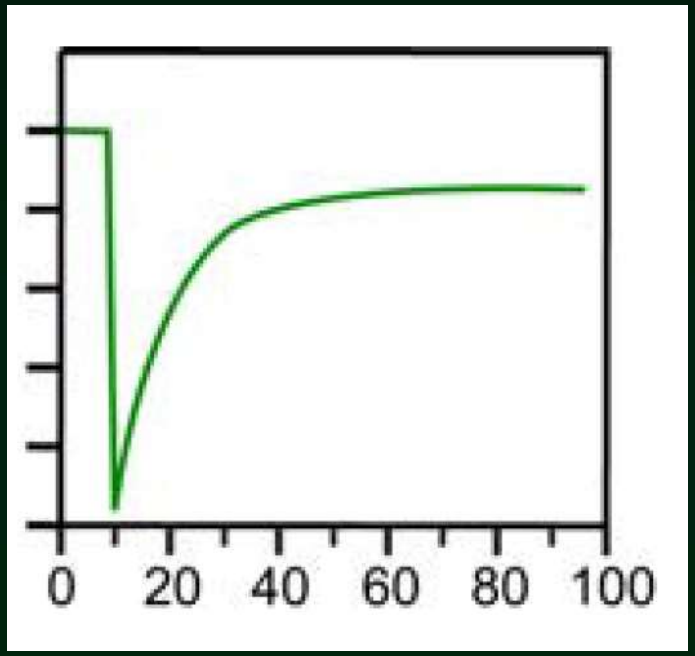
Fluorescence Recovery After Photobleaching

region of interest (ROI)

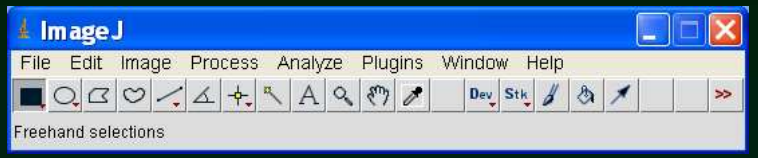


FRAP



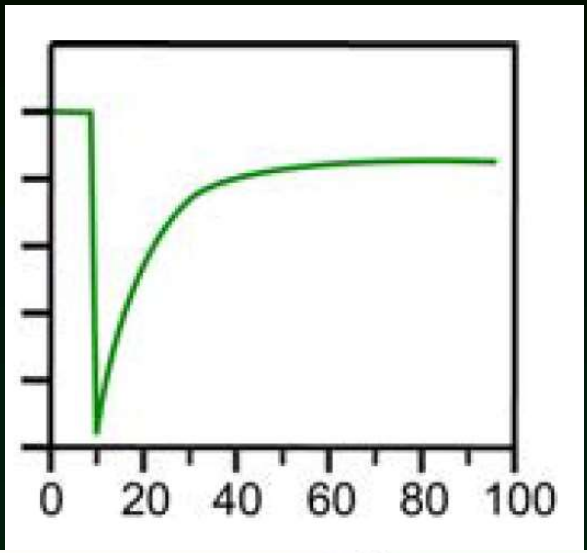


you can quantify fluorescence..
(ImageJ is our friend)

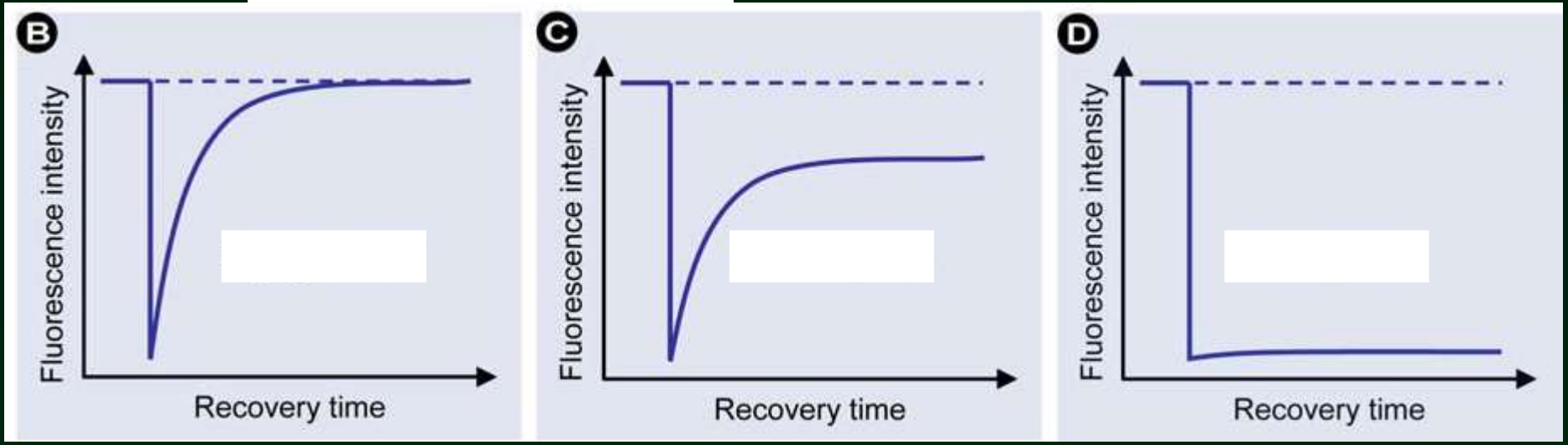


	mean	min	max
A	90.404	49	113
C	8.556	3	8
D	39.934	19	63

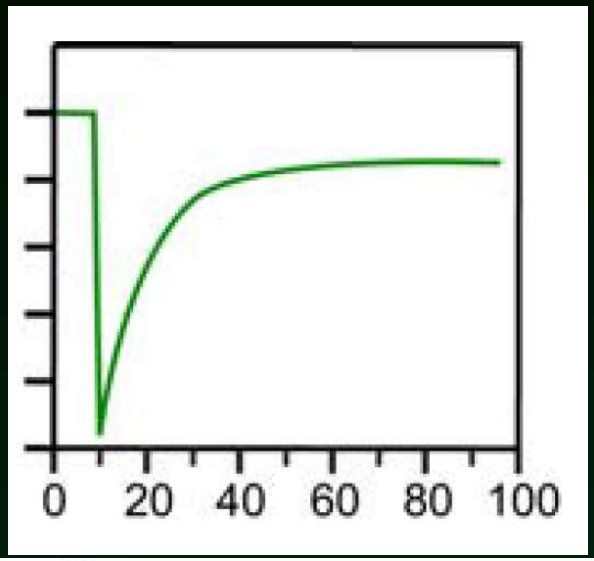
FRAP – bleaching curve

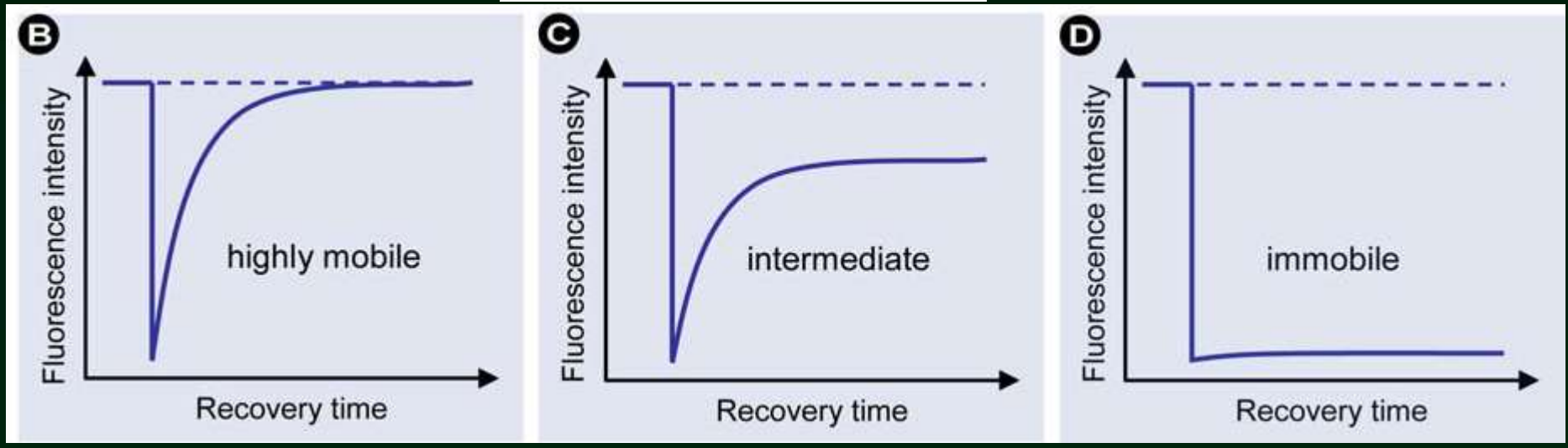


What does the curve tell?



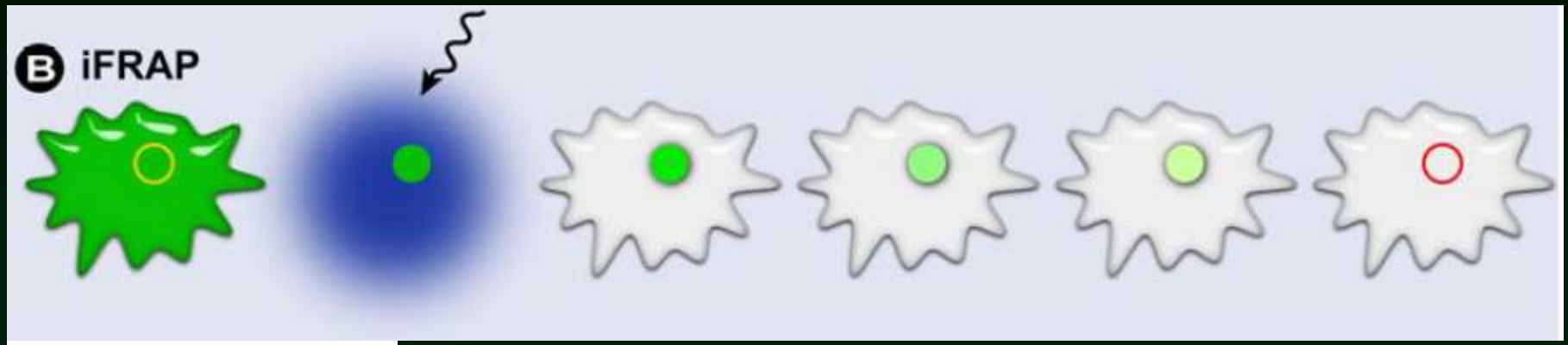
FRAP – bleaching curve

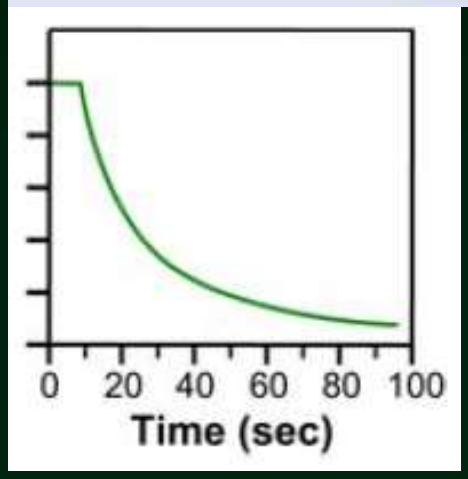




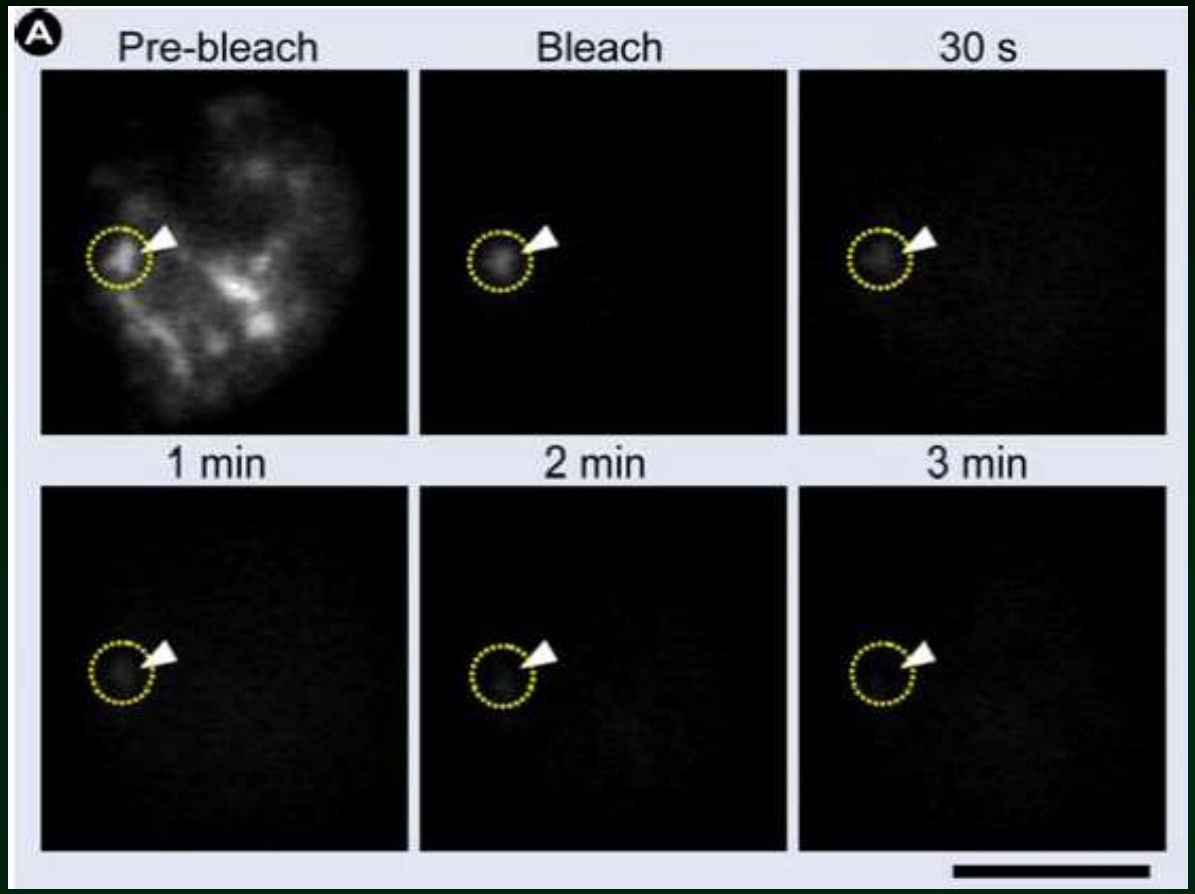
iFRAP

inverse FRAP





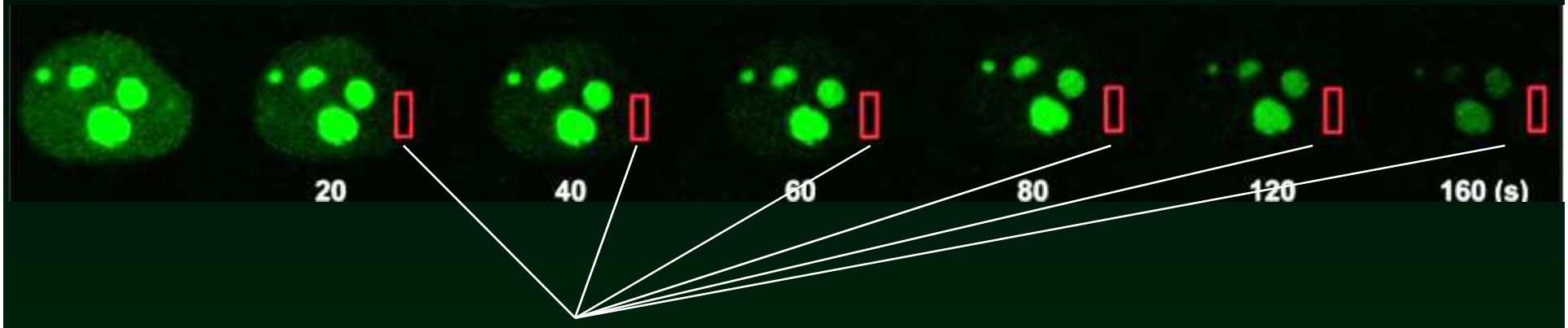
iFRAP – dissociation of premRNA from specles



FRAP derivatives

FLIP

Fluorescence Loss After Photobleaching



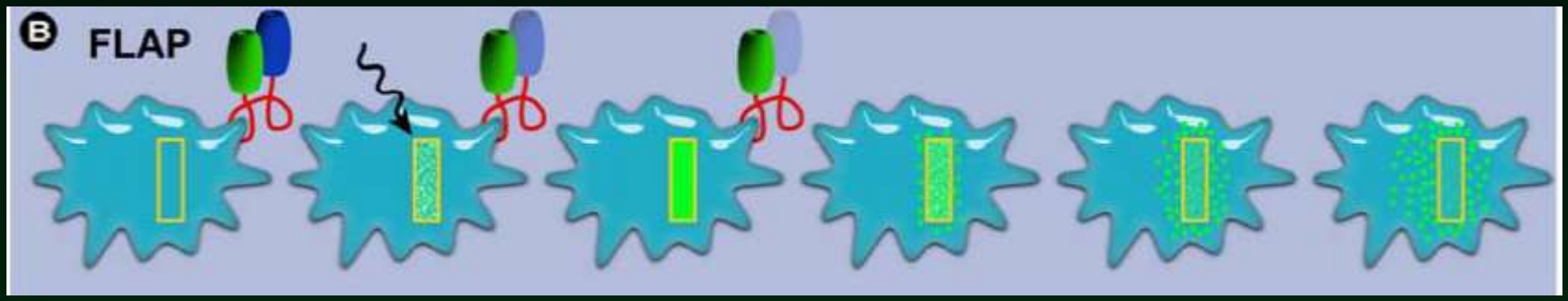
continuous bleaching here

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

FRAP derivatives

FLAP

Fluorescence Localization after Photobleaching

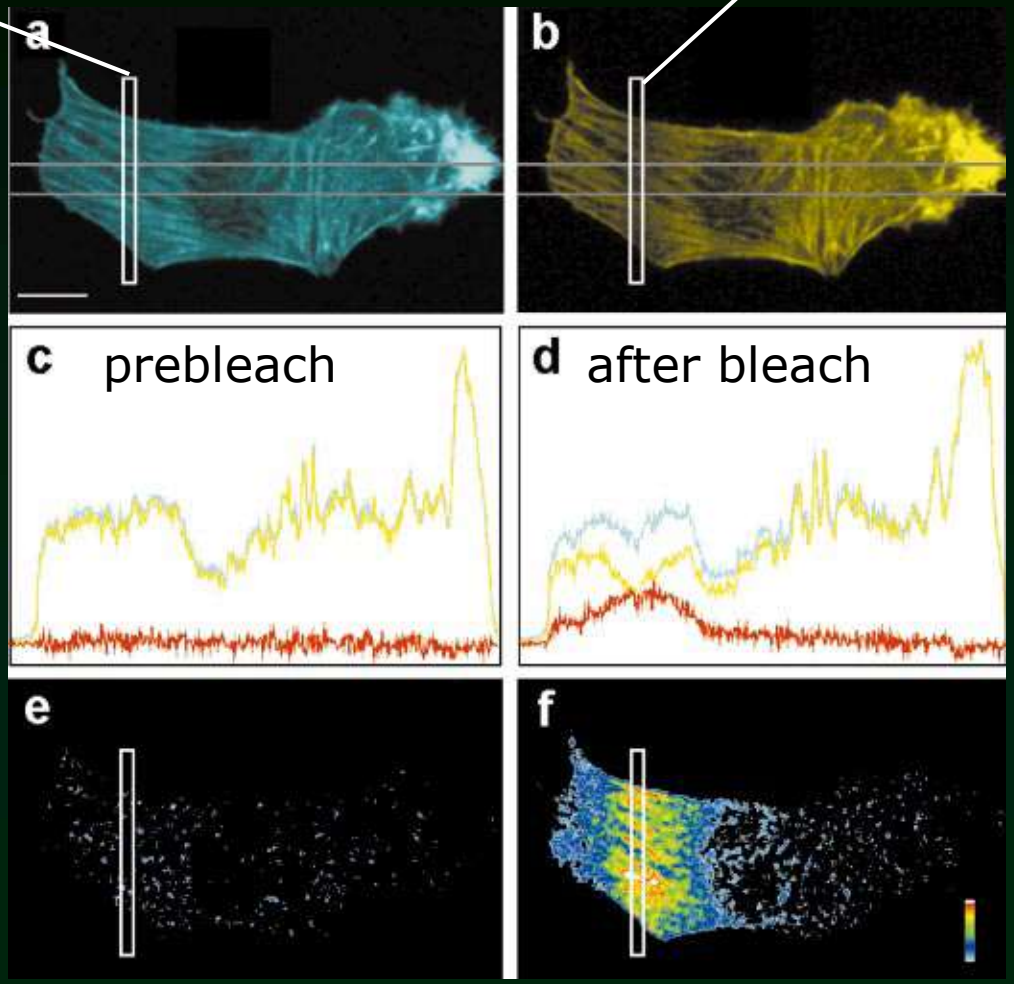


- two fluorochromes on one protein– one bleached, non bleached as control

Perhaps better scheme than previous

CFP not bleached

YFP bleached



RED=CFP-YFP

Dunn et al. 2002

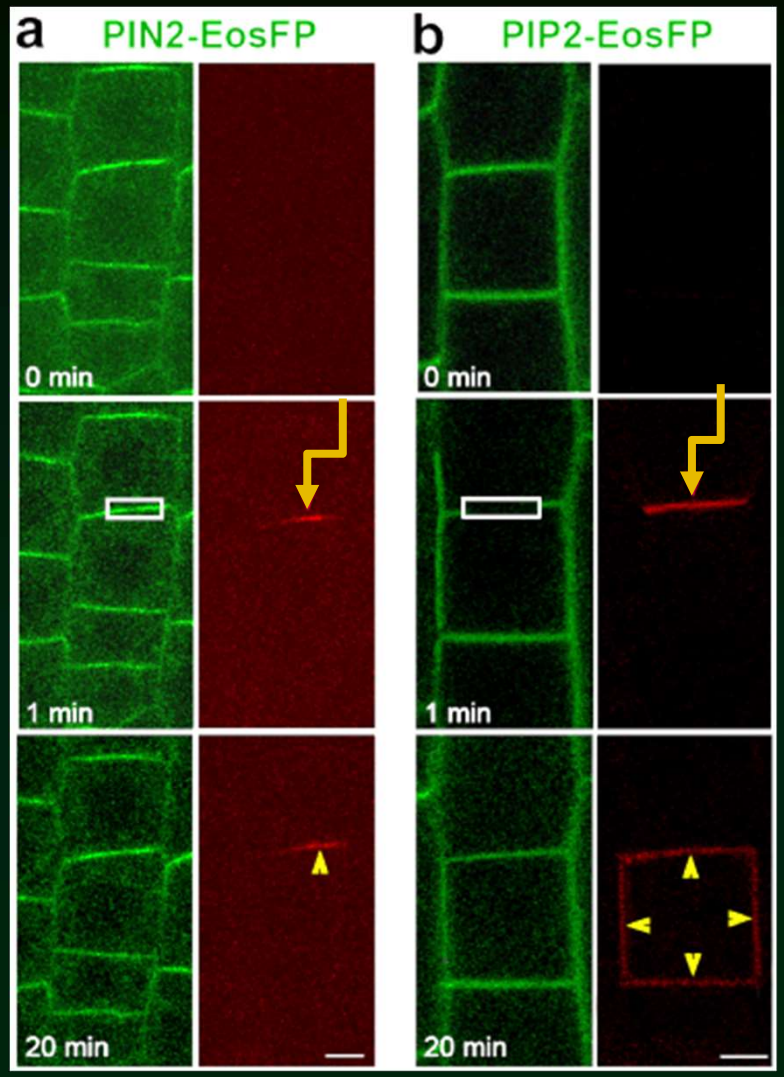
FRAP - advantages

- 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)

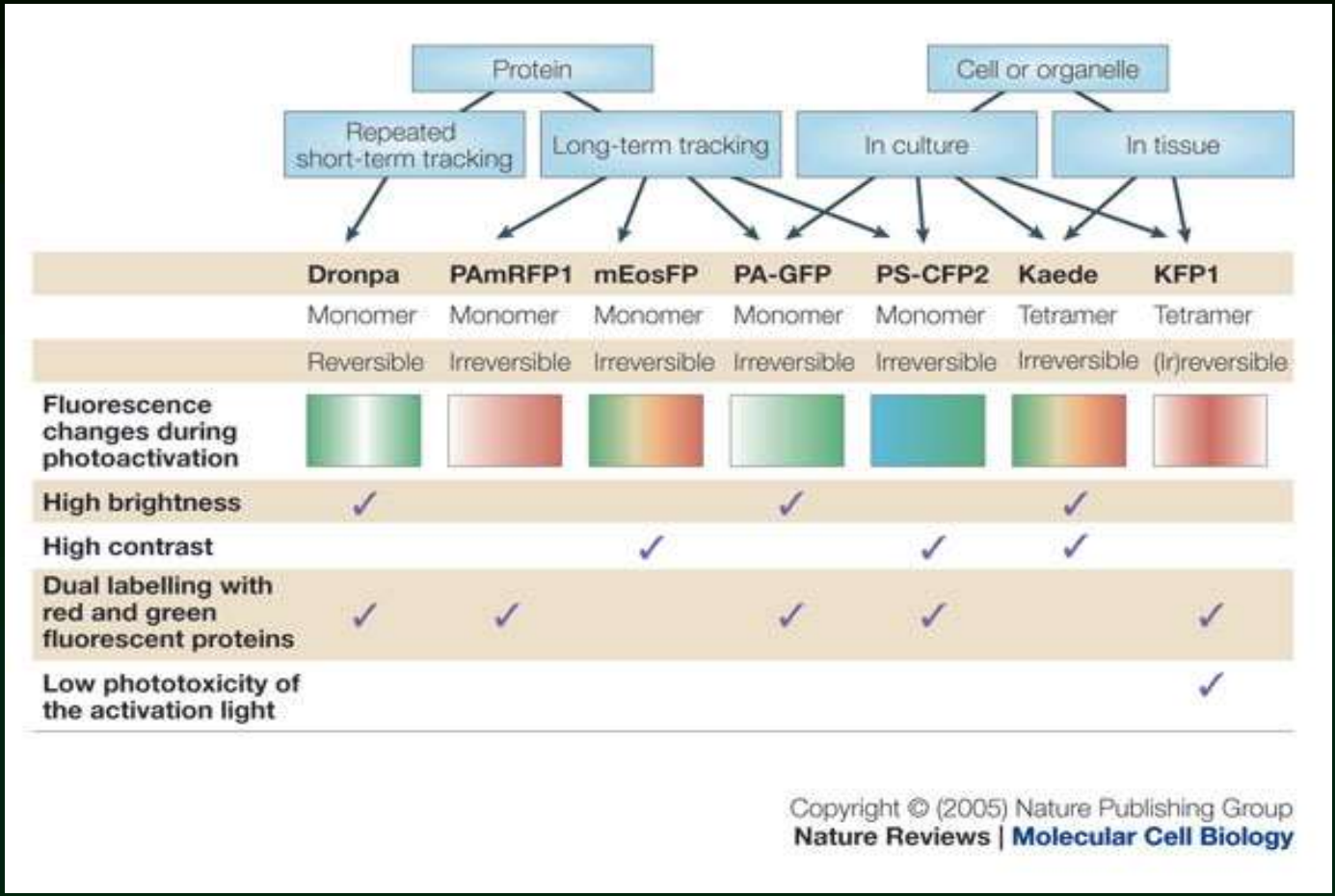
Photoactivable (photoswitchable) fluorescent proteins



photoactivation (UV)

aquaporin PIP2 undergoes lateral difussion

Photoactivable proteins



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)

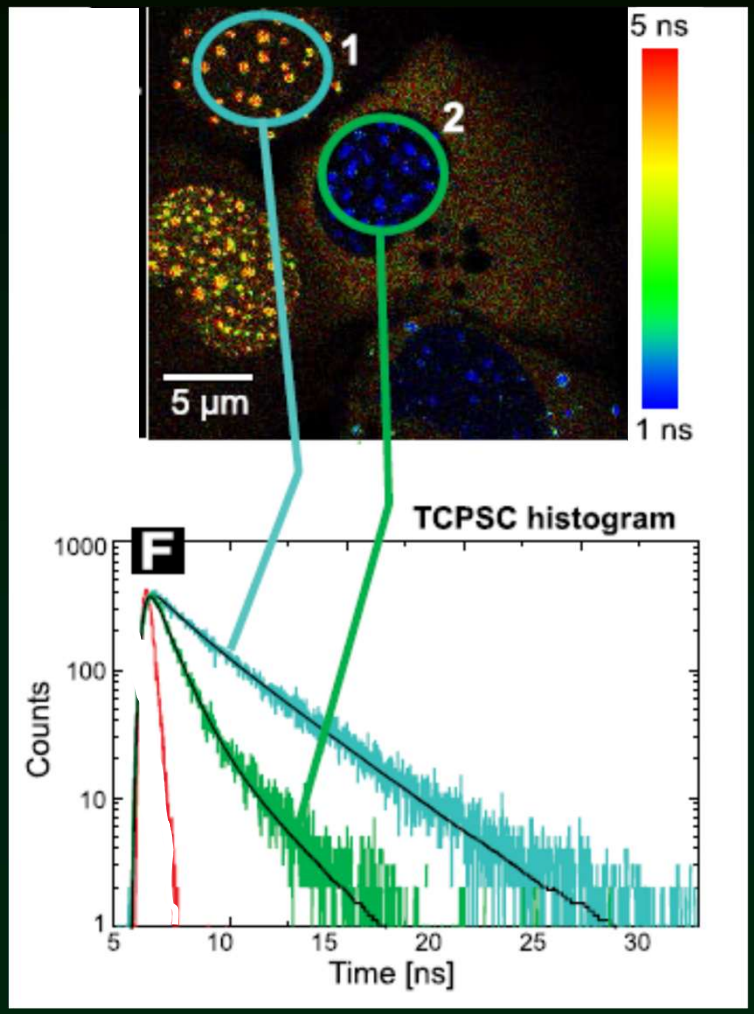
FLIM

Fluorescence Life Time Imaging Microscopy

Fluorochromes

- excitation spectra
- emission spectra
- unique lifetime

FLIM - applications



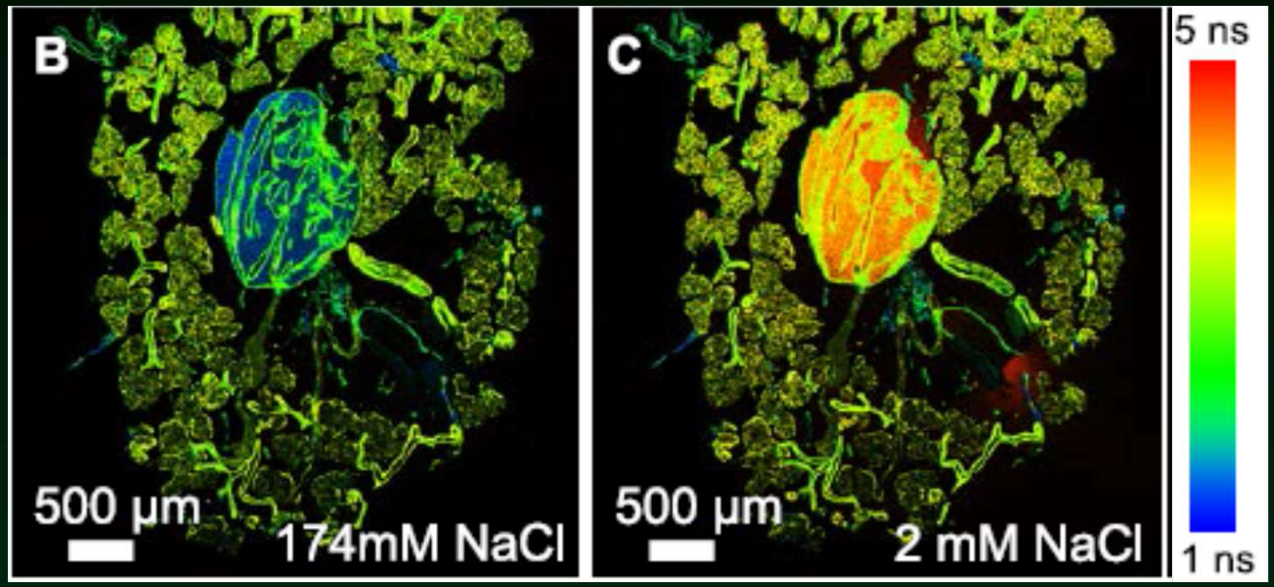
FLIM - applications

Lifetime sensitive to almost everything:

- pH
- ionic strength
- solution polarity
- other fluorochrome

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

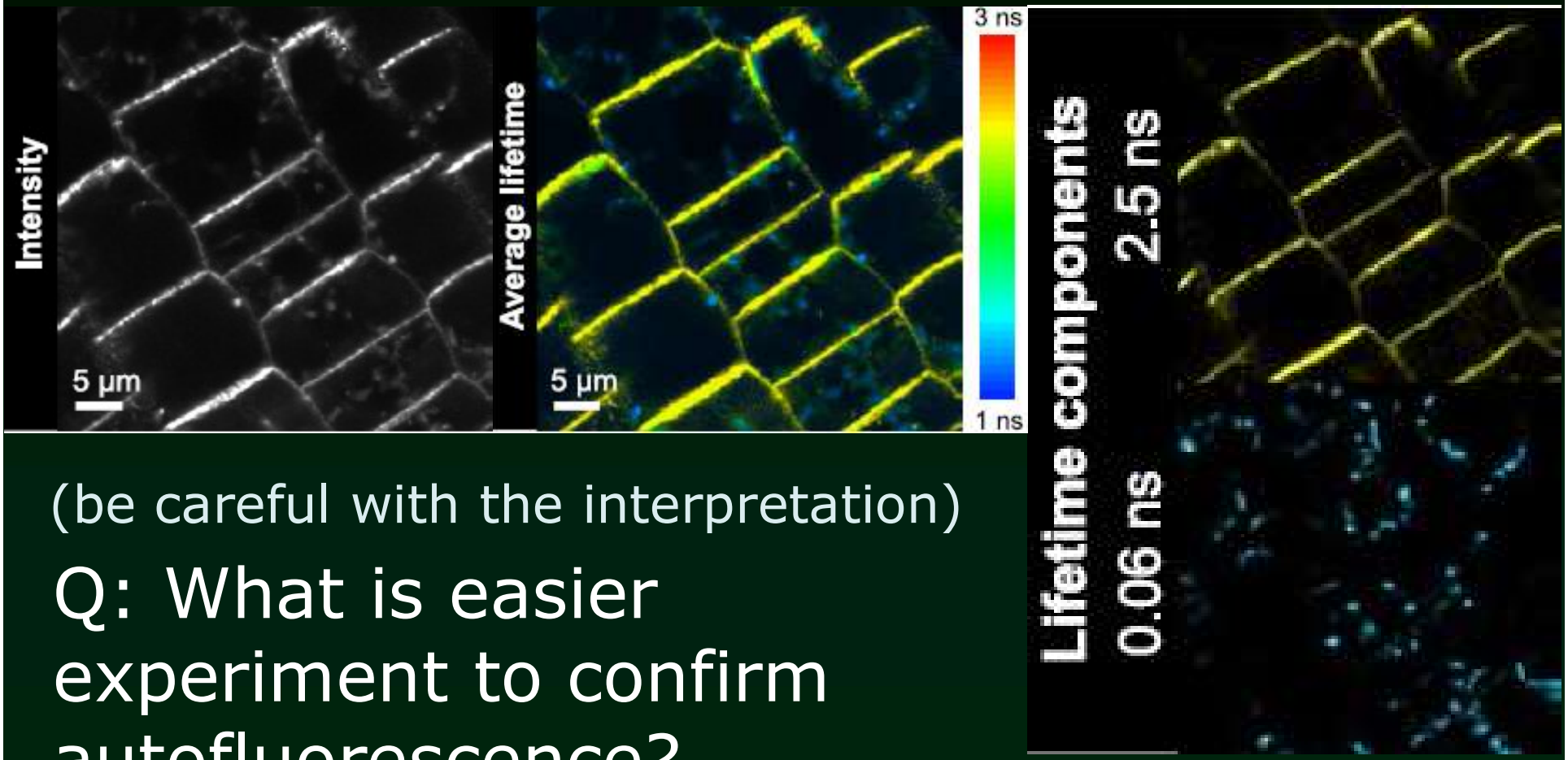
FLIM



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)

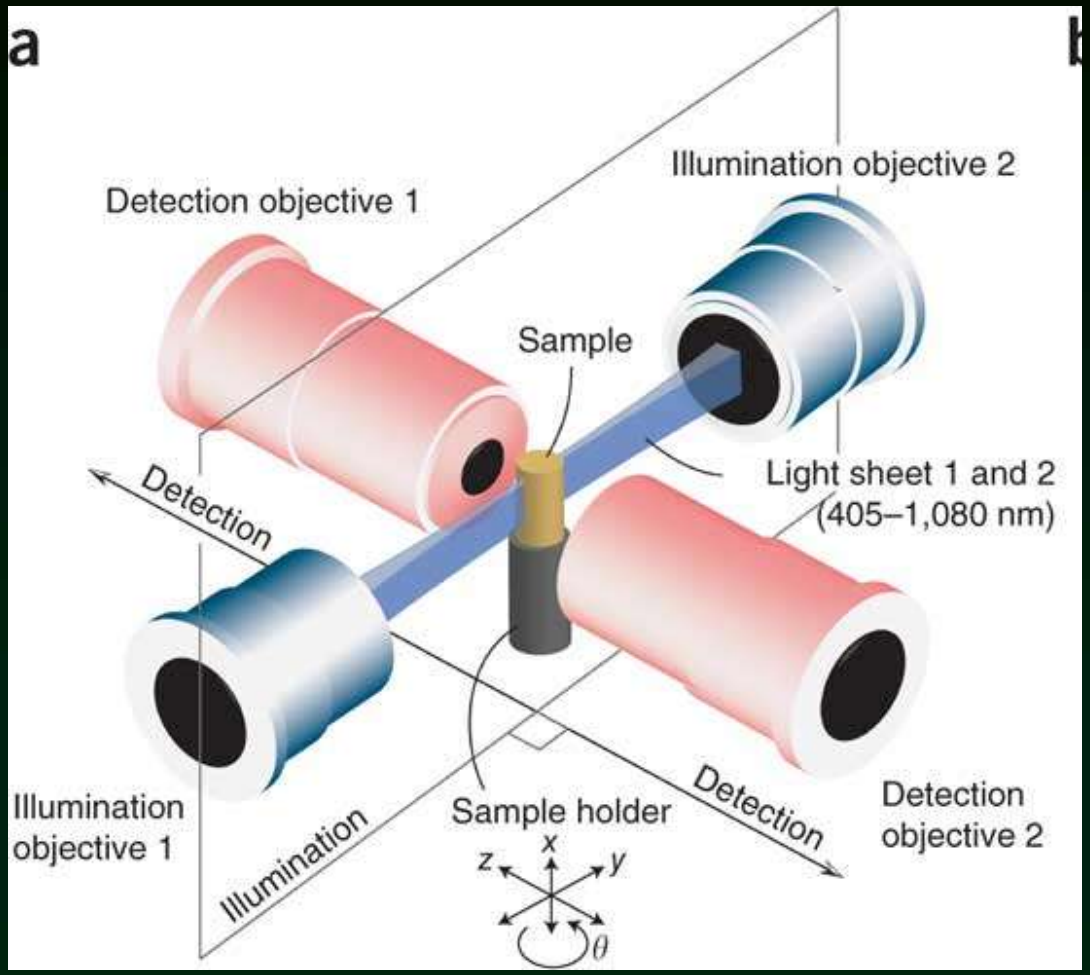
Q: What is easier experiment to confirm autofluorescence?

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:

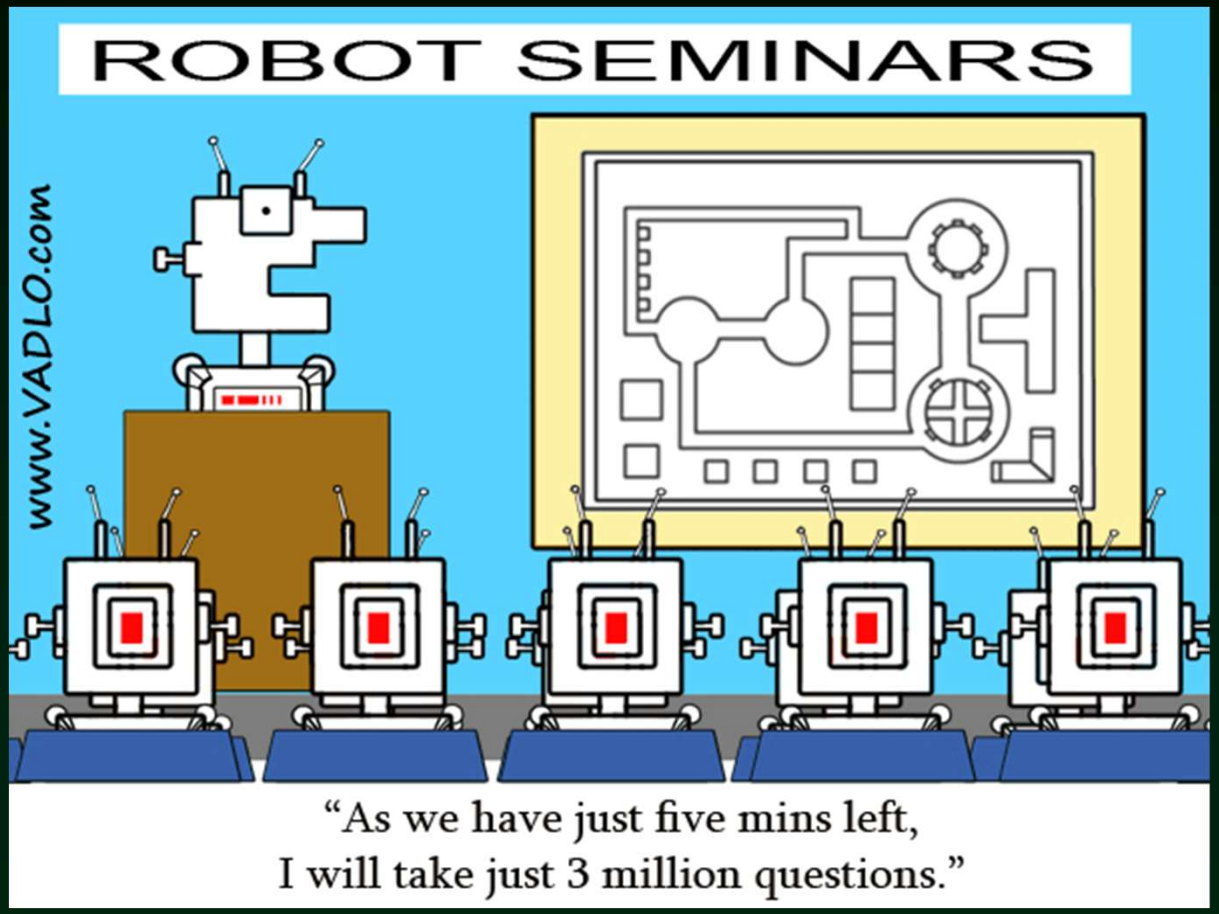
- less bleaching: better tissue penetrance, better resolution and sensitivity
- 3D structures fast

Cons:

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

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
- Single molecule analysis of gene expression (Vera et al. 2016): https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5149423/
- 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)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"As we have just five mins left,
I will take just 3 million questions."