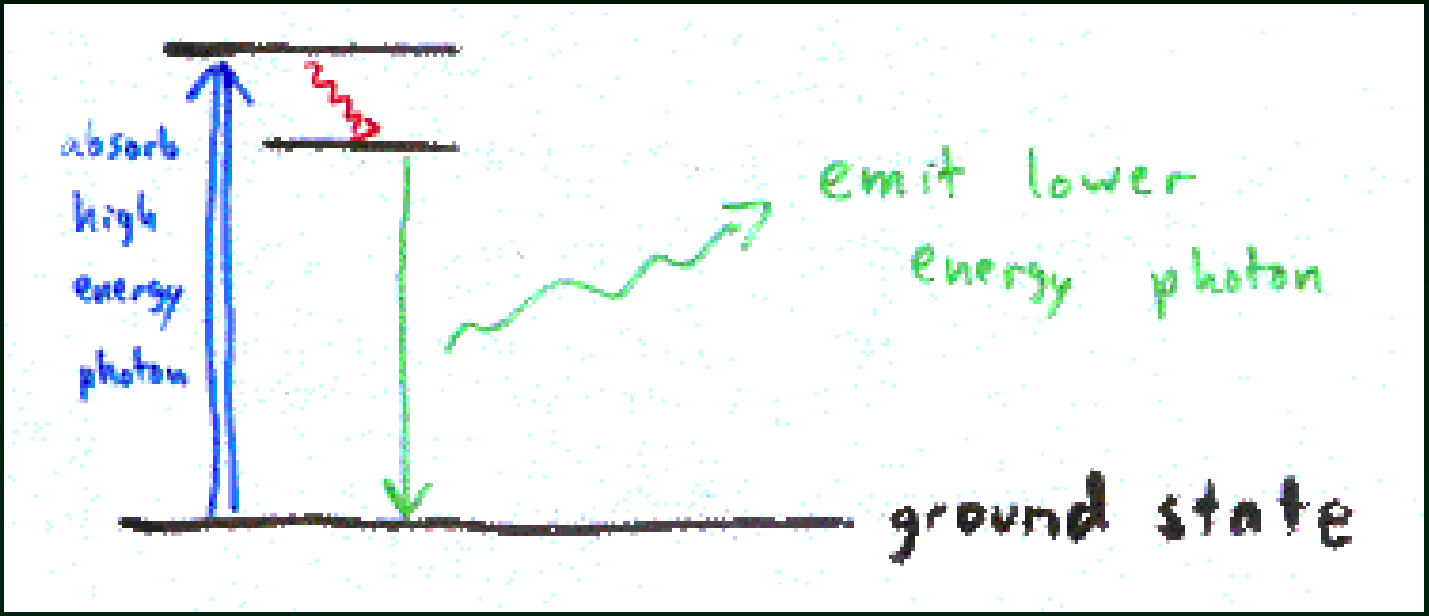
Cellular Communication

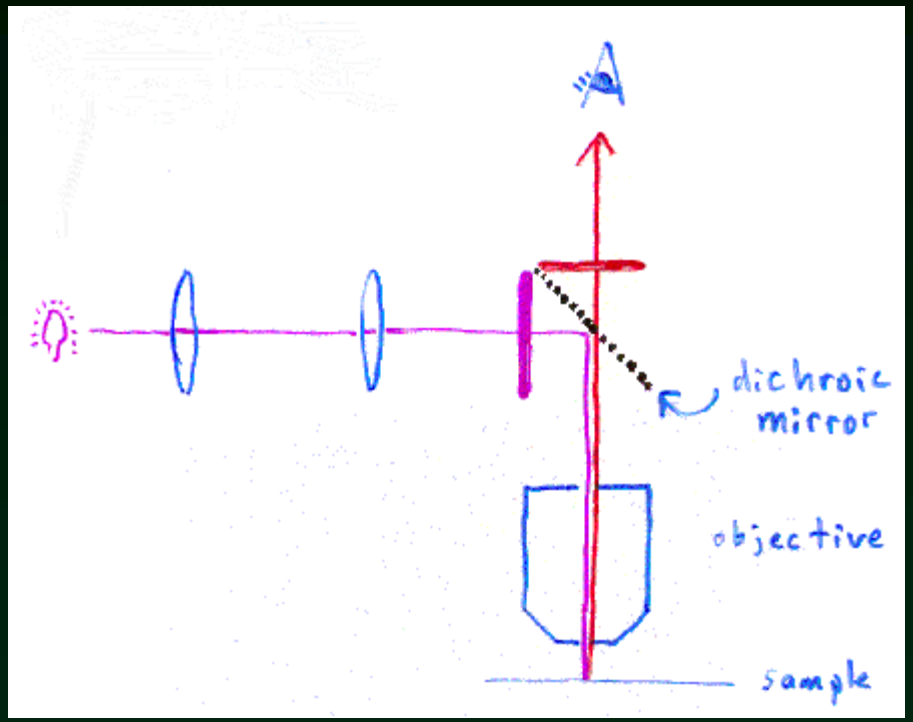
Genomics Lectures

Kamil Růžička
FGP CEITEC MU

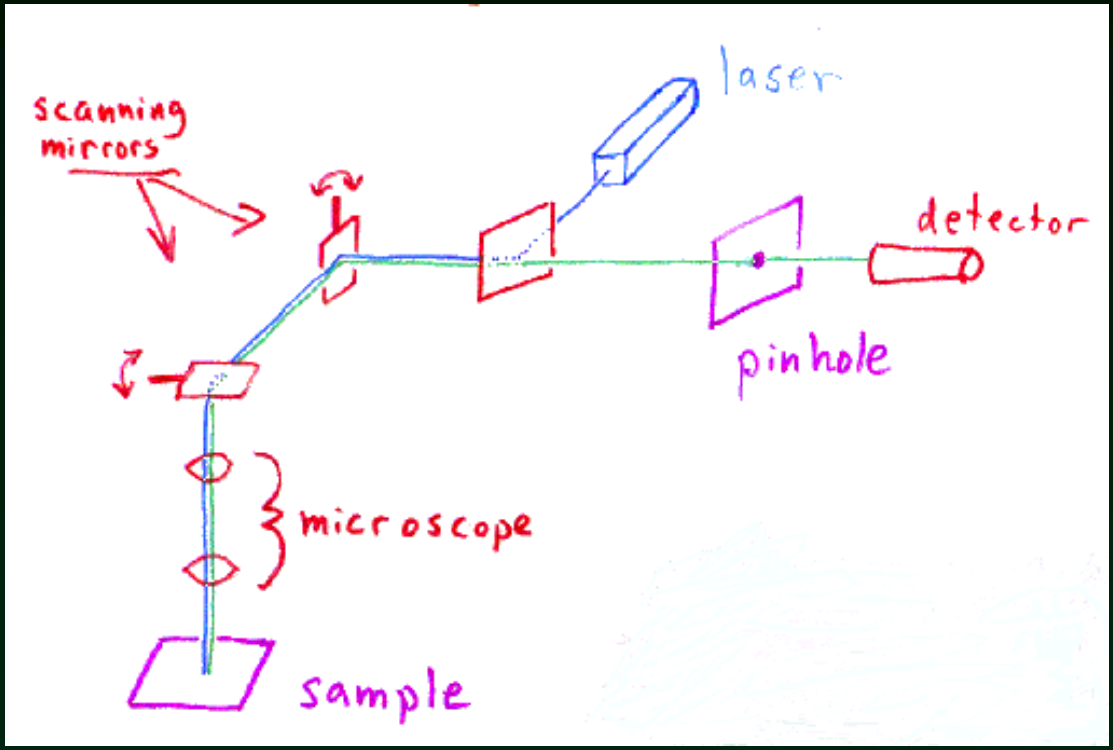
How does fluorescence work?



How does a fluorescence microscope work?



How does a confocal microscope work?



Protein localization

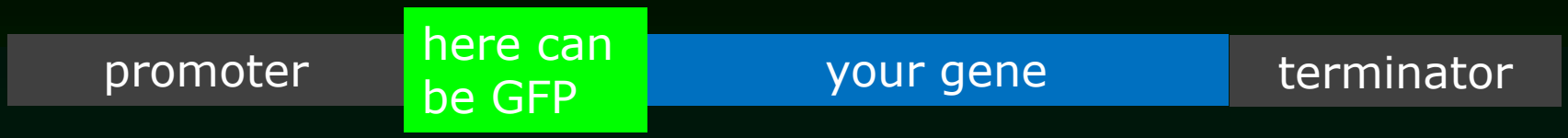
live imaging

GFP discovery - Nobel Prize 2008

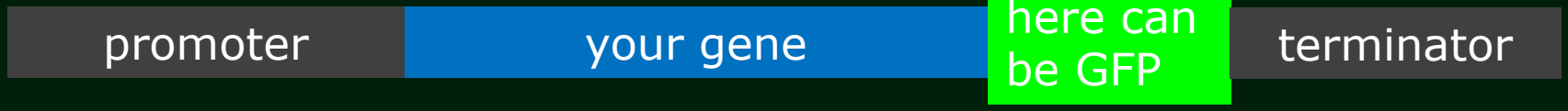
Osamu Shimomura Martin Chalfie Roger Tsien

GFP fusions

N-terminal fusion

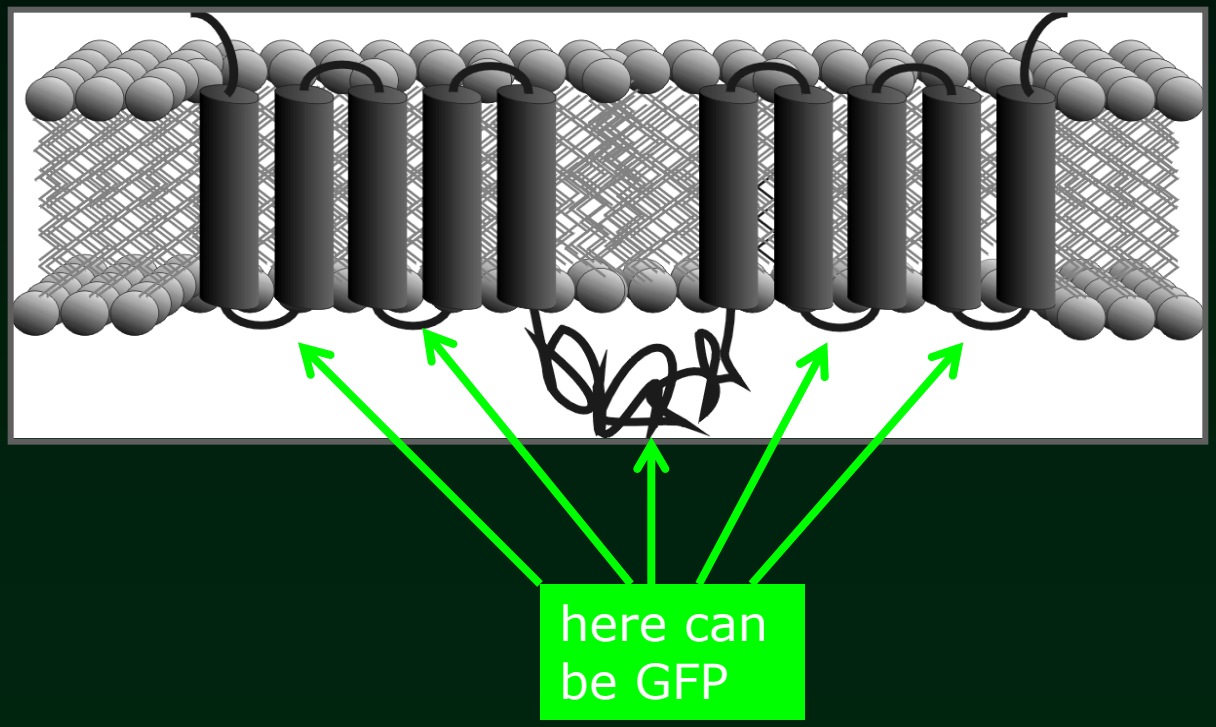


C-terminal fusion



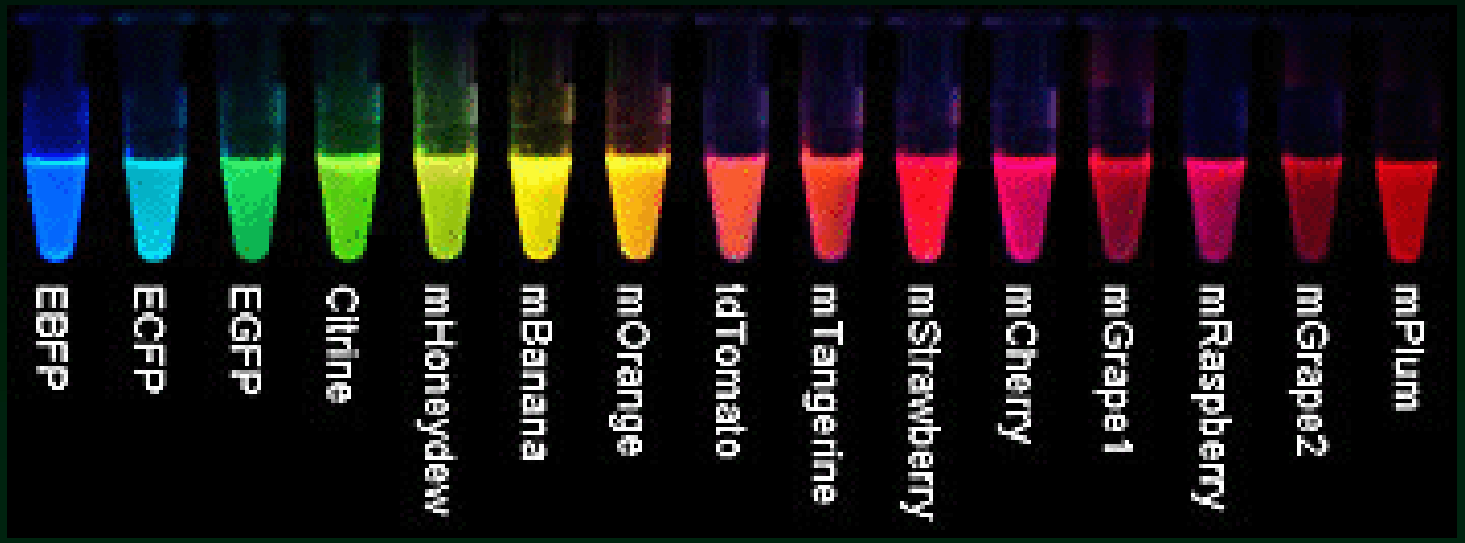
fusion inside the coding sequence

GFP and membrane proteins

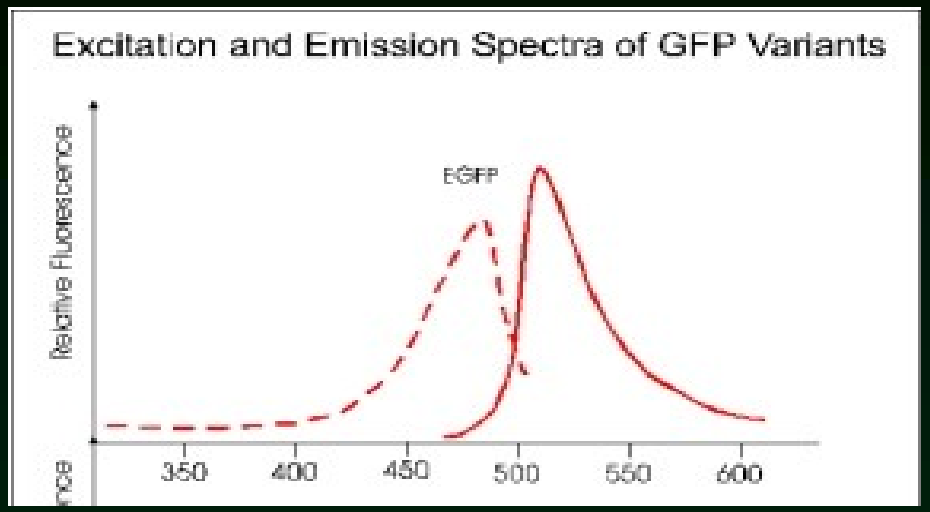


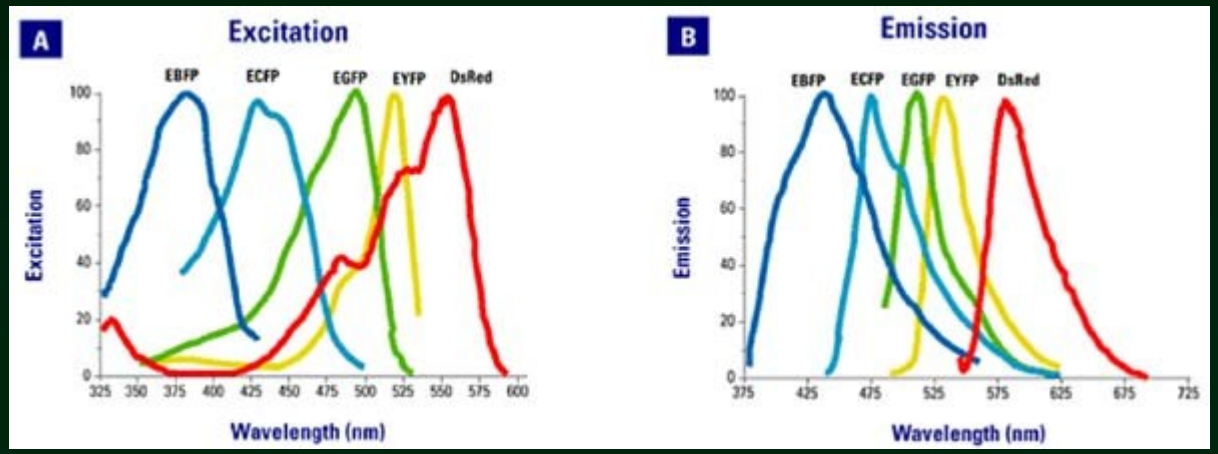
It is good to have GFP tag localized inside the cell

Fluorescent proteins on the market

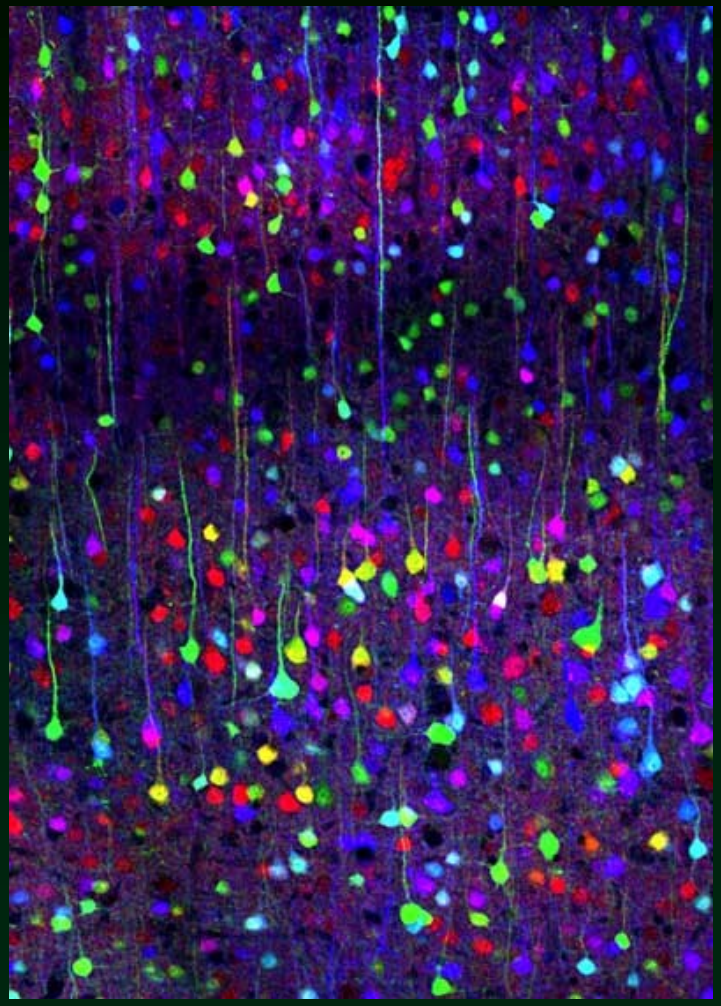


Excitation and emission

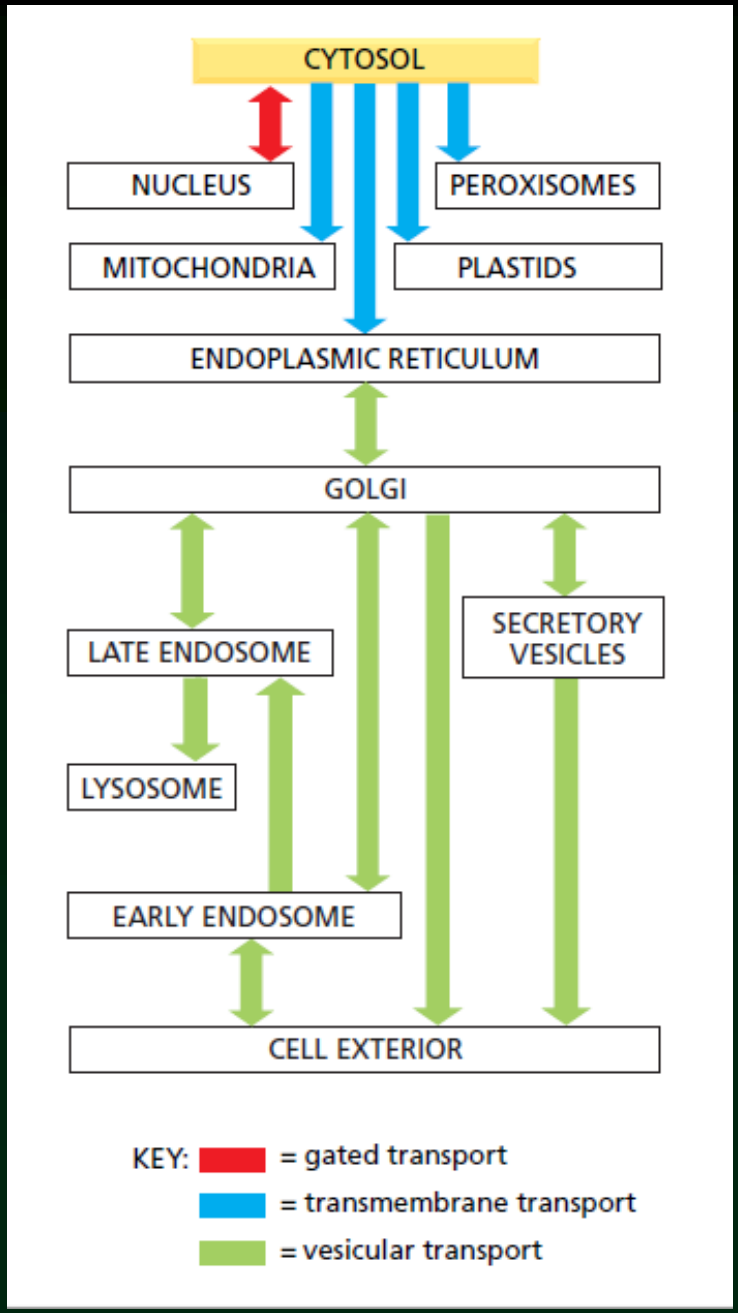




Multicolored fluorescent protein (neurones)

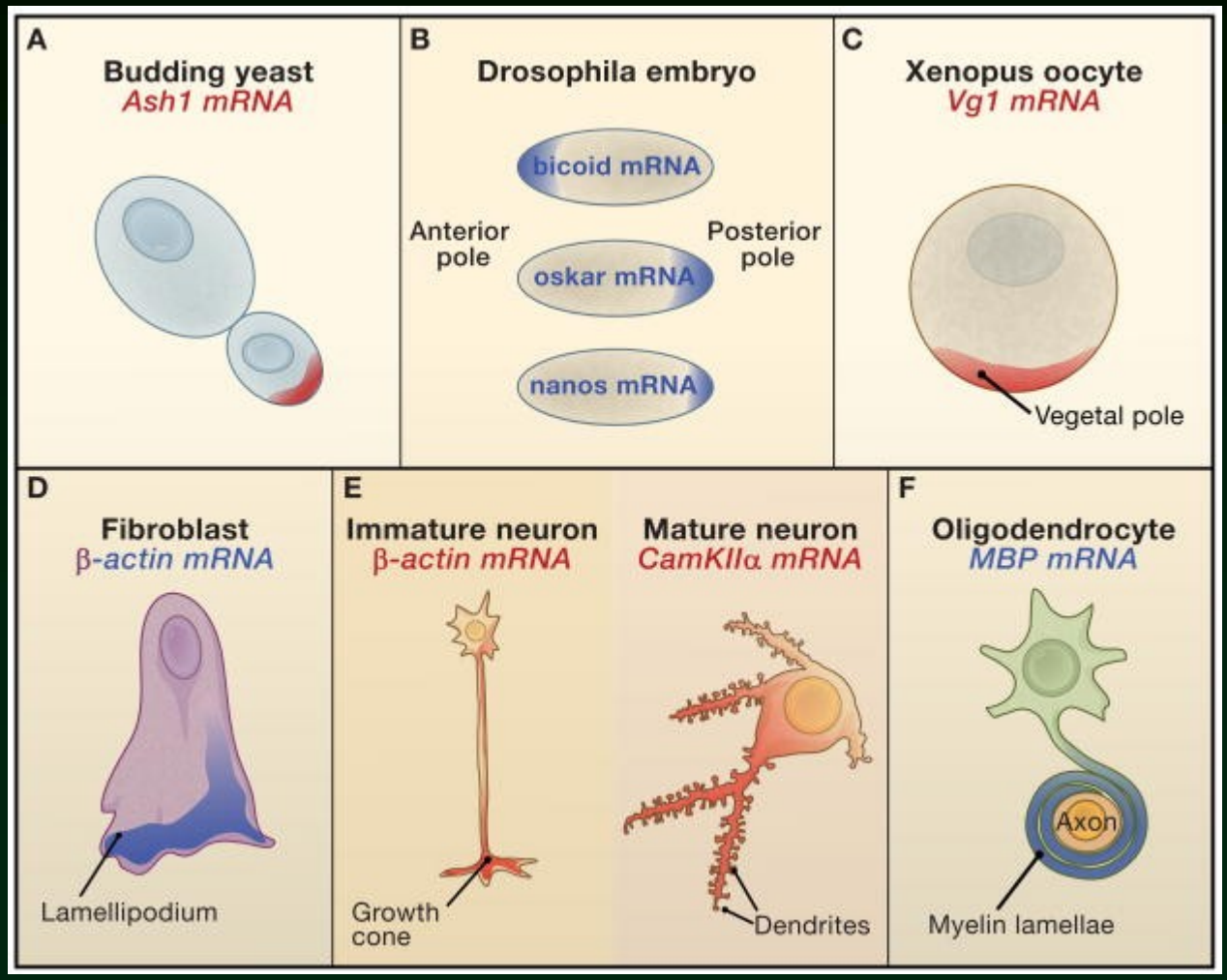


Transport among compartments

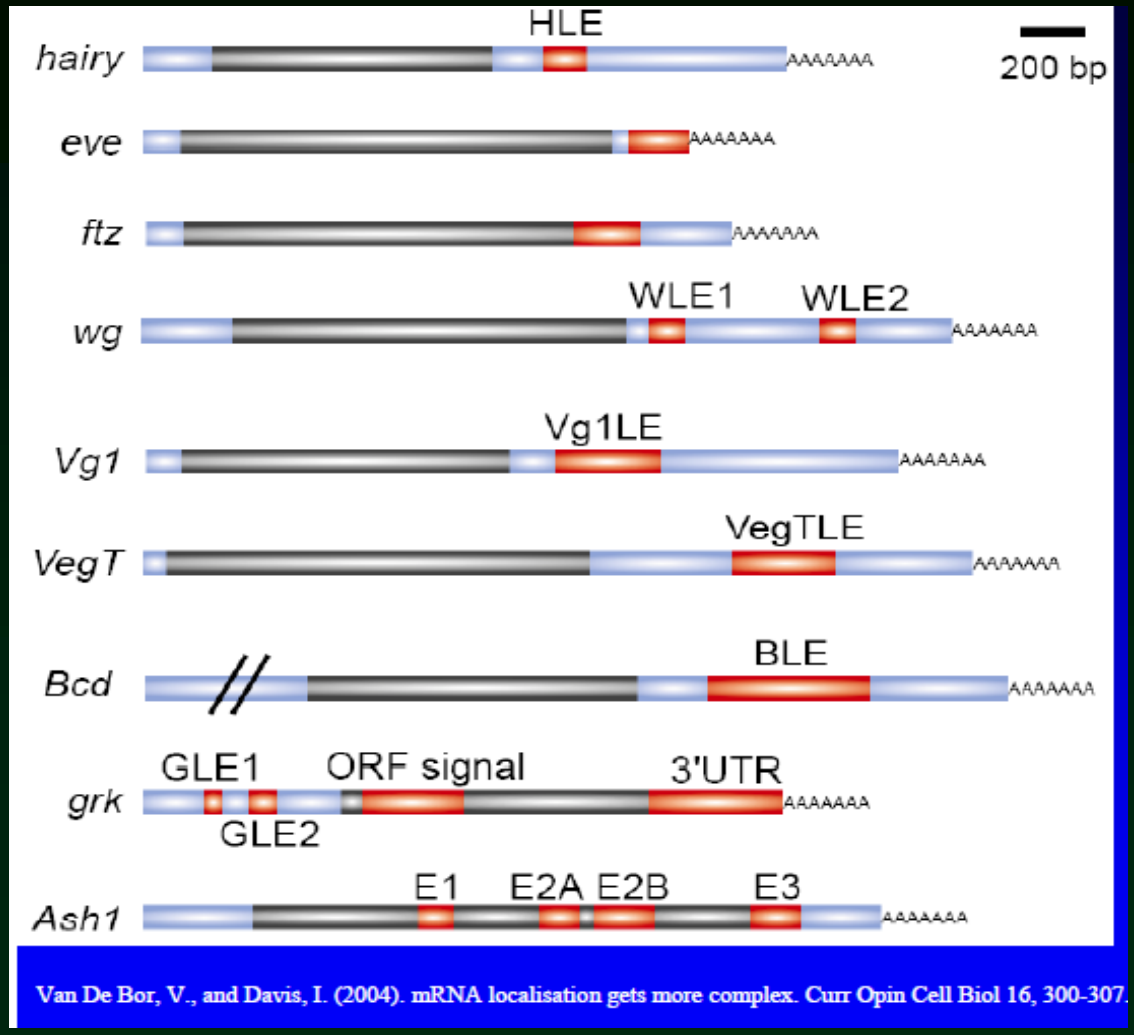


Alberts et al. 2008

Also RNA can be differentially localized



RNA ZIP codes for localization



Van De Bor, V., and Davis, I. (2004). mRNA localisation gets more complex. Curr Opin Cell Biol 16, 300-307.

Mikko Frilander

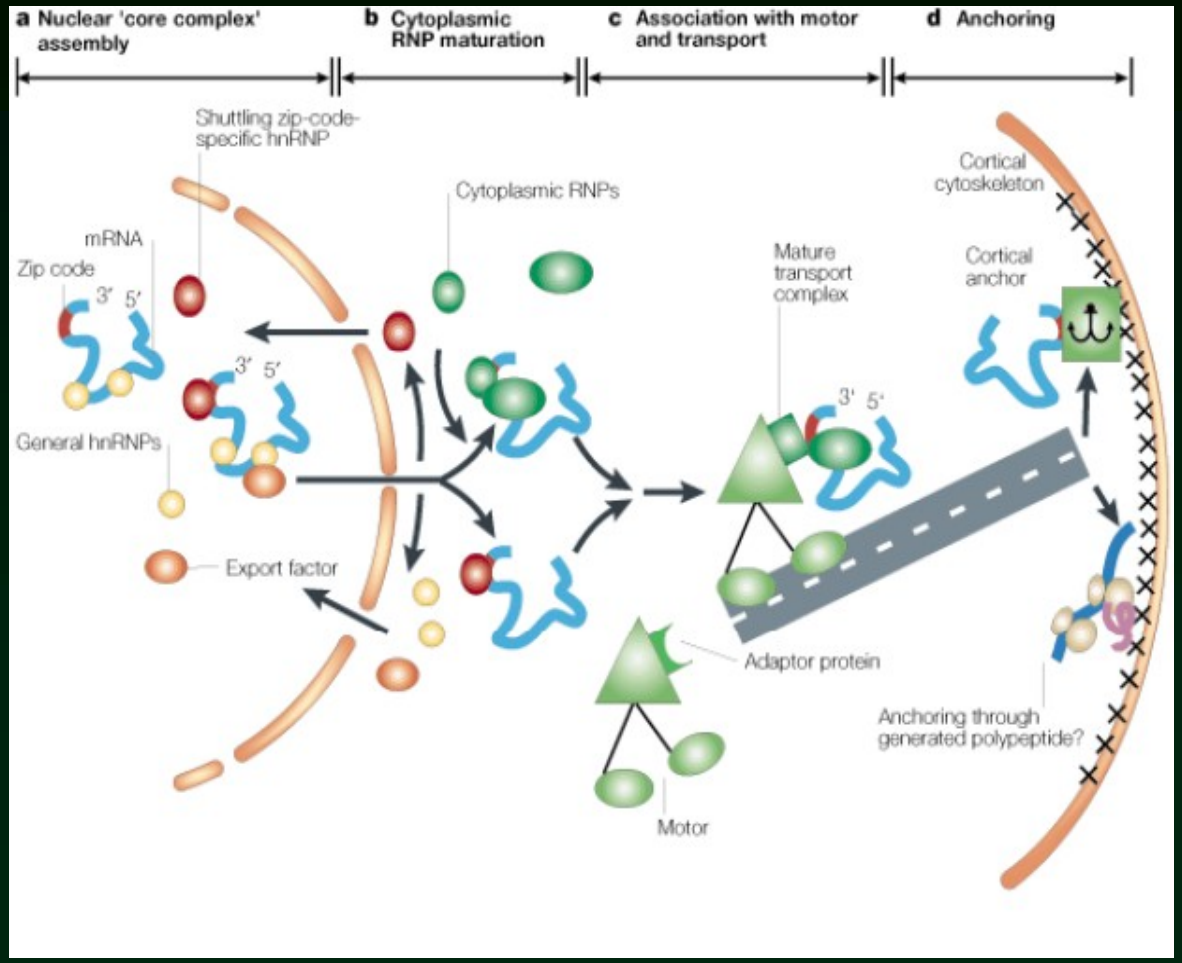
ZIP codes often motor protein bound

Table 1 | **Cross-species comparison of proteins involved in cytoplasmic mRNA localization**

Function/feature	Yeast	*Drosophila melanogaster*	Vertebrates
Zip-code-binding hnRNP protein (located in nucleus and cytoplasm)	Not yet identified	Squid (*grk*)[20]	hnRNP A2 (*MBP*)[21] VgRBP60* (*Vg1*)[62] Vera/VgRBP** (*Vg1*)[59,60] ZBP-1* (*β-actin*)[22]
Cytoplasmic zip-code-binding RNP	She2 (*ASH1*, *IST2?*)[6,63,64]	Staufen (*osk*, *pros*)[65] Swallow* (*bcd*)[82] Ypsilon-Schachtel* (*osk*)[83]	mStaufen* (?)[68,79,80] VILIP* (*trk*)[69] TB-RBP (*CaMKII?*)[98,99]
Motor protein for RNP	Myo4 (*ASH1*, *IST2?*)[4,6]	Kinesin I (*osk*)[88] Dynein* (*bcd*)[82]	Kinesin* (*MBP*, *CaMKII*)[75,99]
RNP motor adaptor	She3 (*ASH1*, *IST2?*)[6,63]	Dynein light chain (*bcd*)[82]	Not yet identified
mRNA/RNP anchor	Bni1*, Bud6* (*ASH1*)[32]	Staufen (*bcd*)[30] Oskar (*osk*)[37]	*Xlsirt* mRNAs* (*Vg1*)[38]

Mikko Frilander

Delivering at the correct address



1. Localisation complex assembly starts already in the nucleus

2. Cytoplasmic mRNP is "matured", nuclear export proteins removed, additional proteins attached.

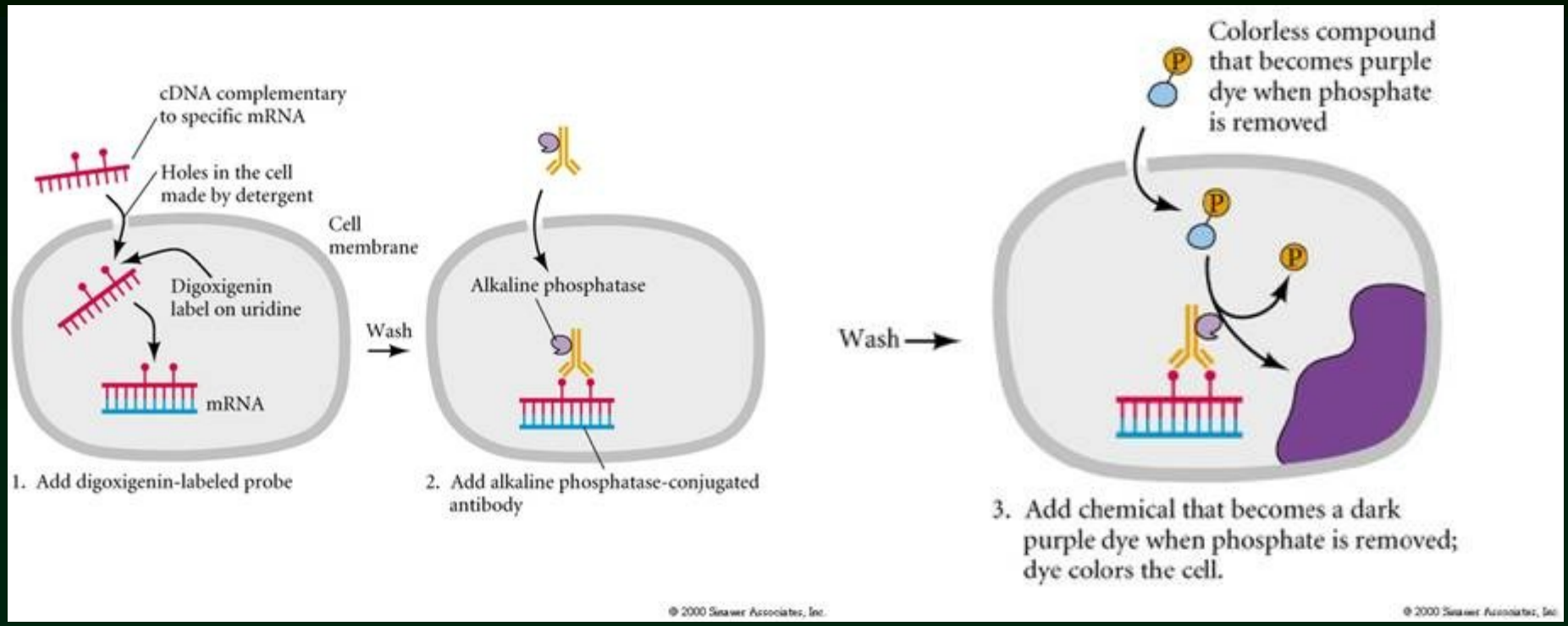
3. mRNP is associated with motor and transport system and delivered to the destination

4. Delivered mRNP is anchored to the destination (for storage) or translated directly.

Mikko Frilander

Localization of mRNA

RNA hybridization *in situ*



Localization of mRNA

RNA hybridization *in situ*

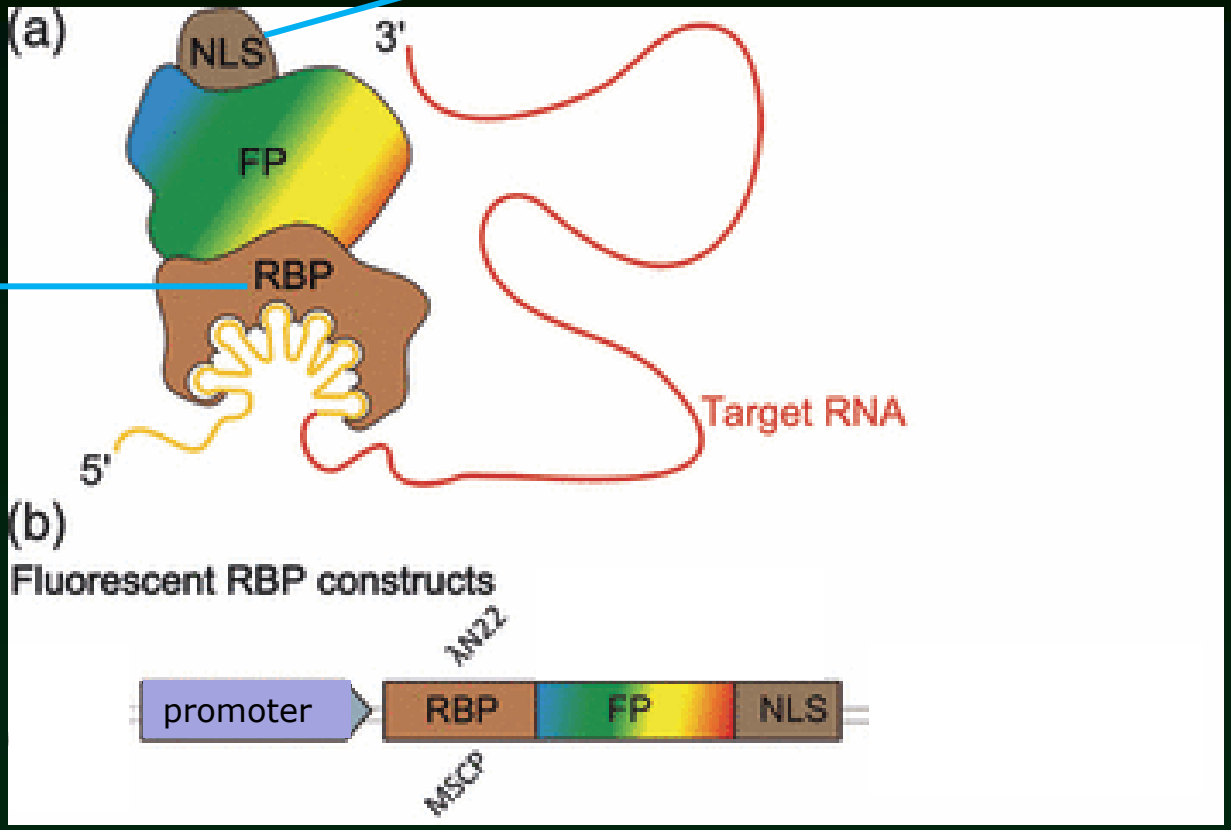
- classical technique, no alternative in developmental biology
- results often clear
- can be done without generating transgenic lines

- tedious
- only on “dead” samples

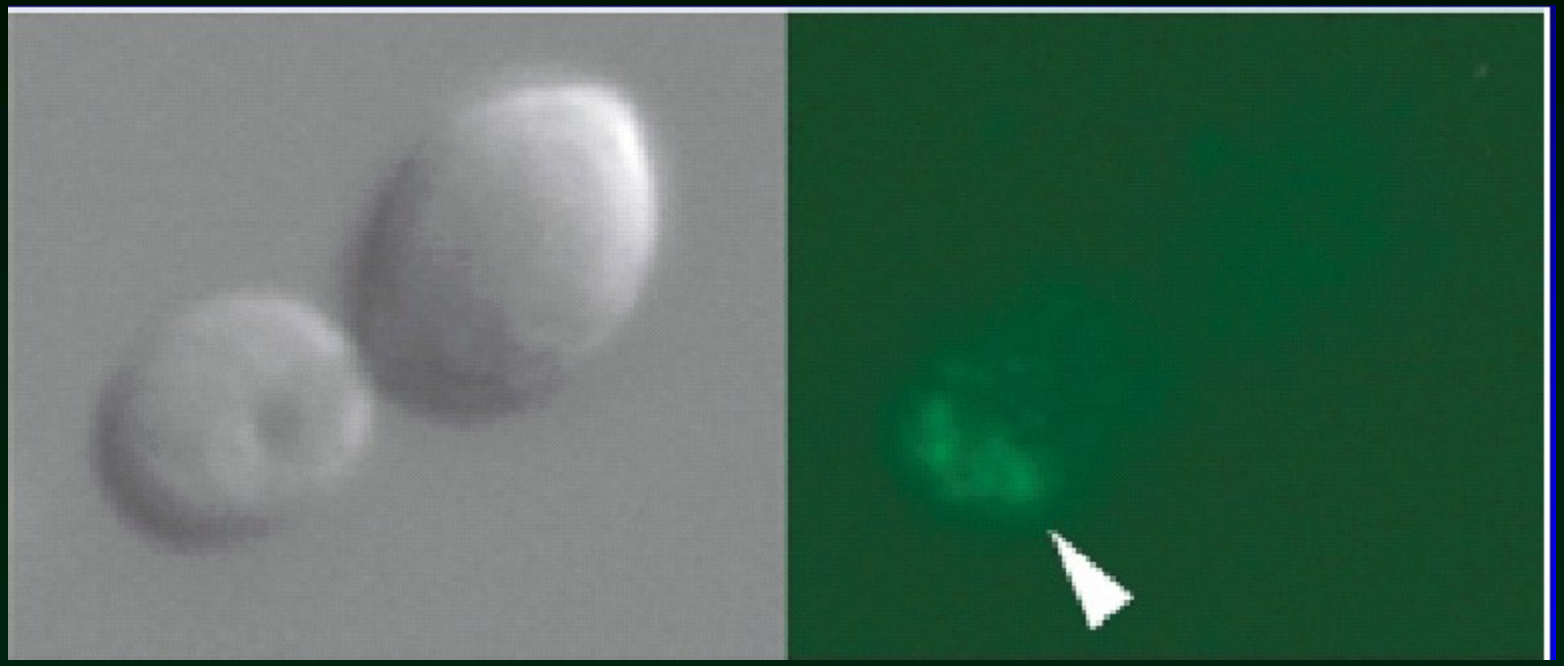
λN_{22} system

nuclear localization signal

viral RNA binding protein

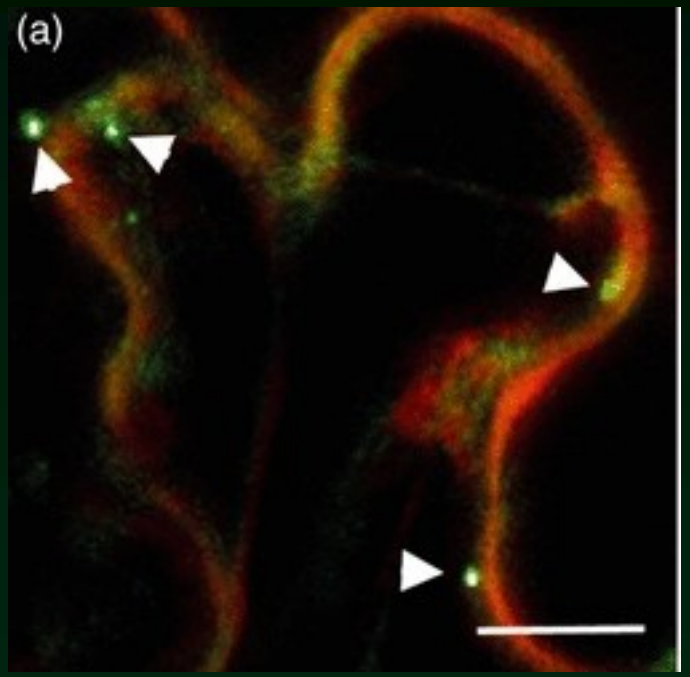


Also mRNA can be differentially localized

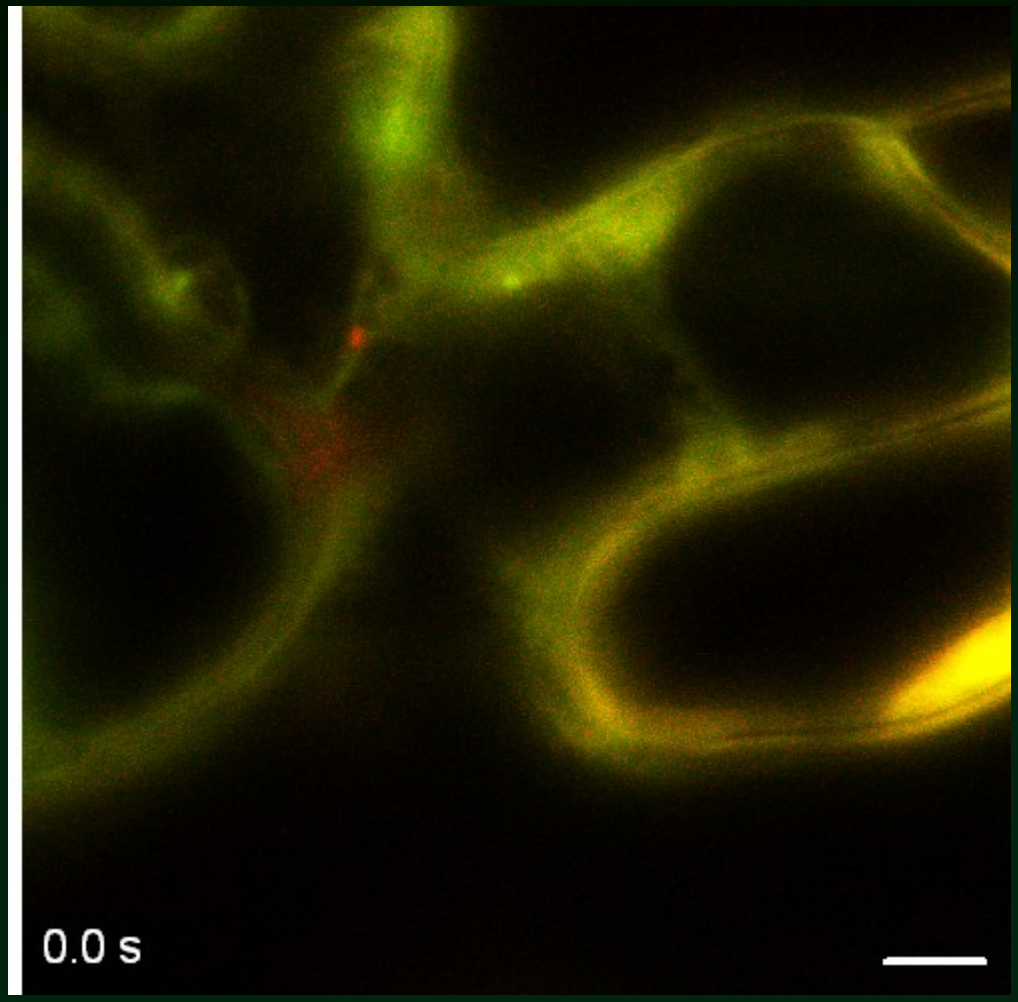


Ash1 mRNA localized to the tip of the daughter cell

Also mRNA can be differentially localized

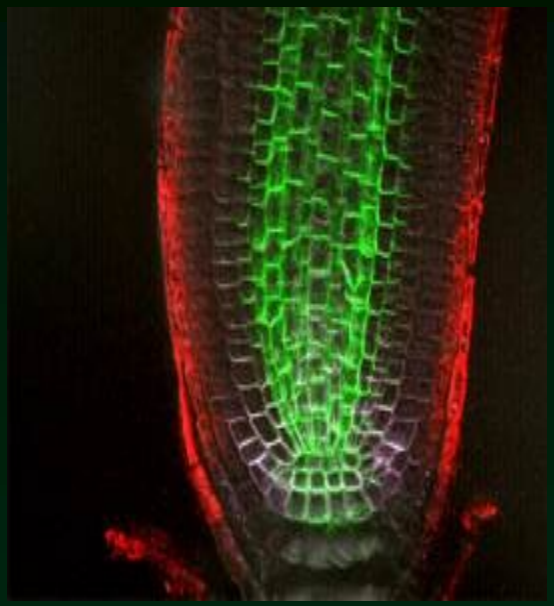


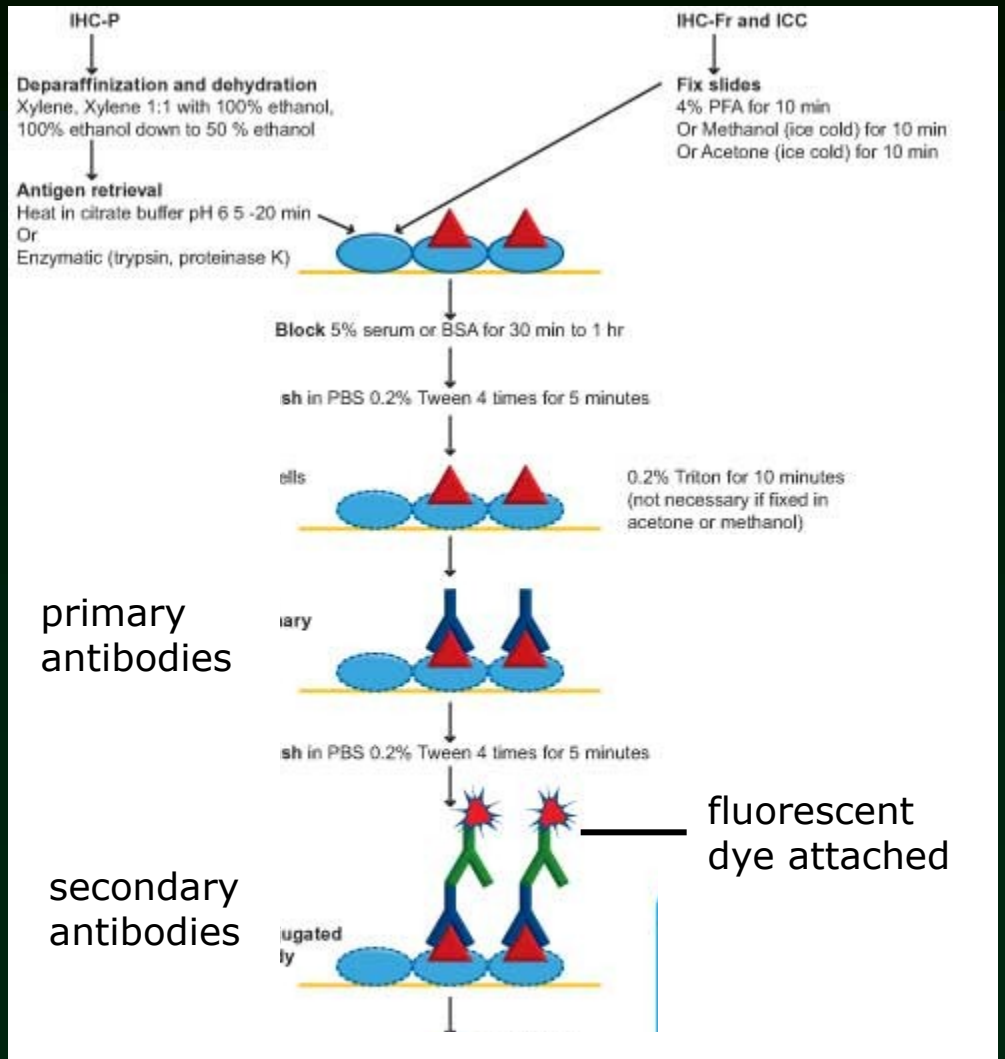
Also mRNA can be differentially localized



Protein localization

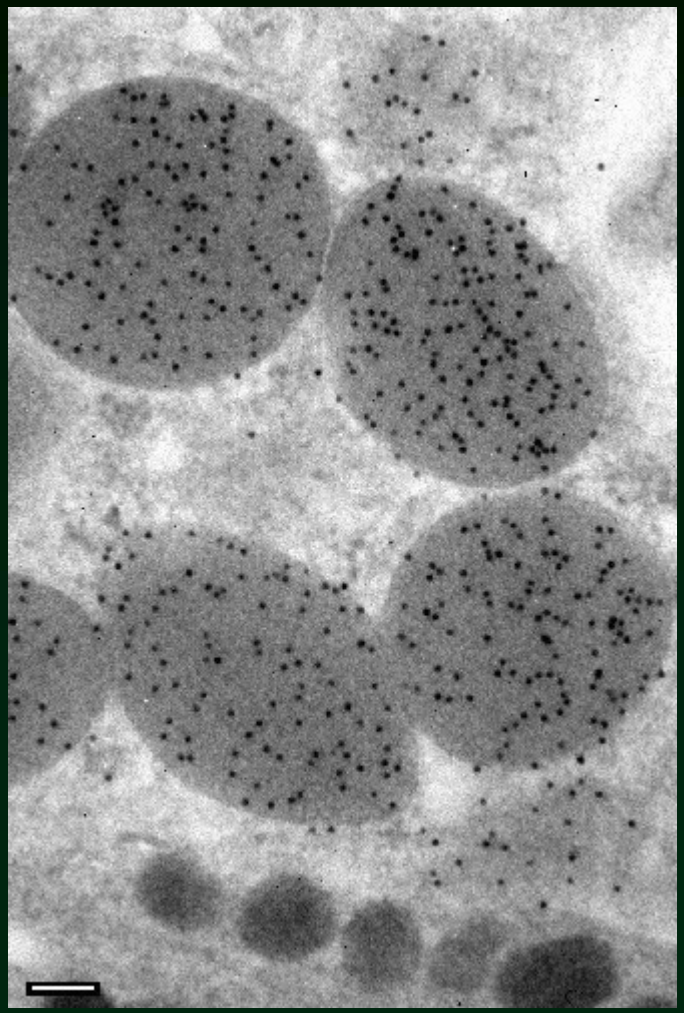
immunolocalization - fluorescently



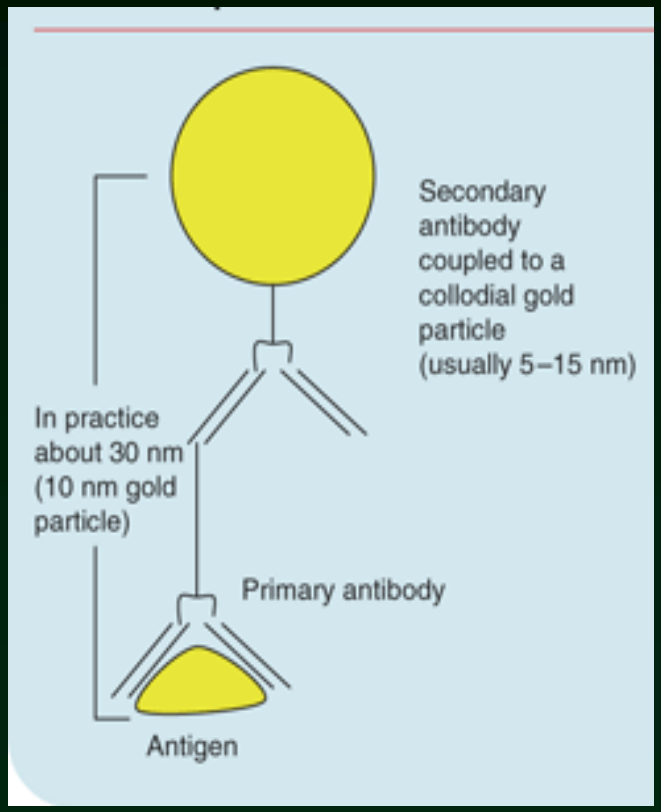


Protein localization

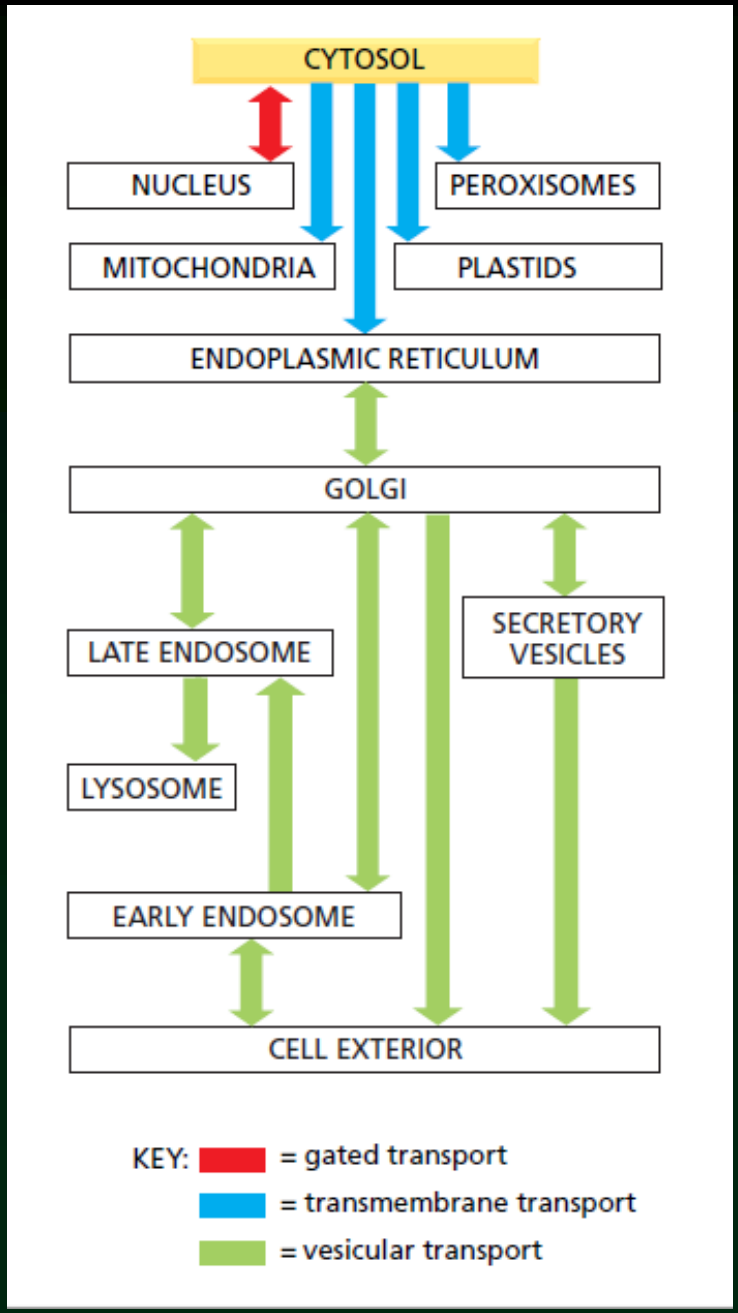
immunolocalization - immunogold



electron microscope



Transport among compartments

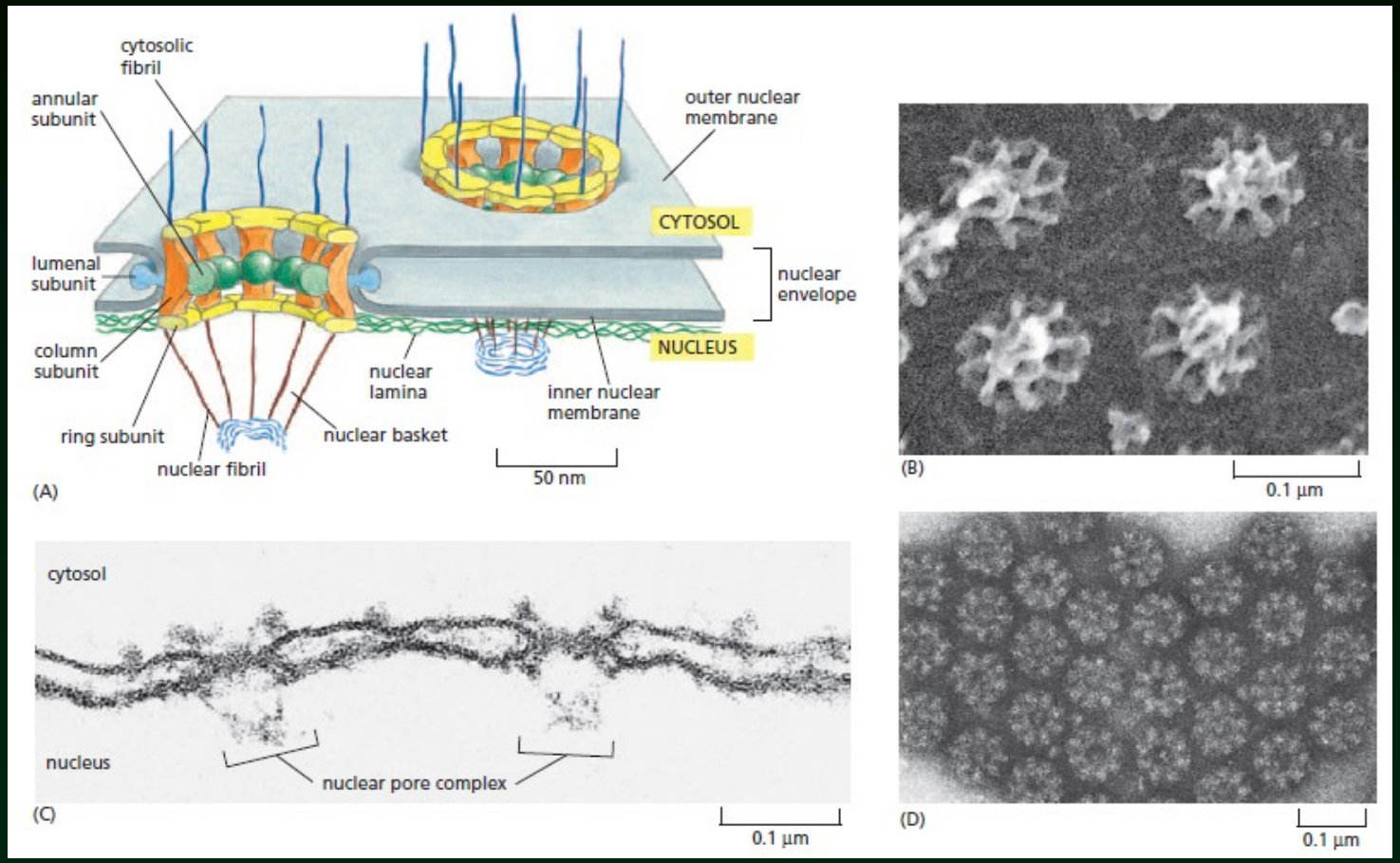


Alberts et al. 2008

Protein sorting – target peptides

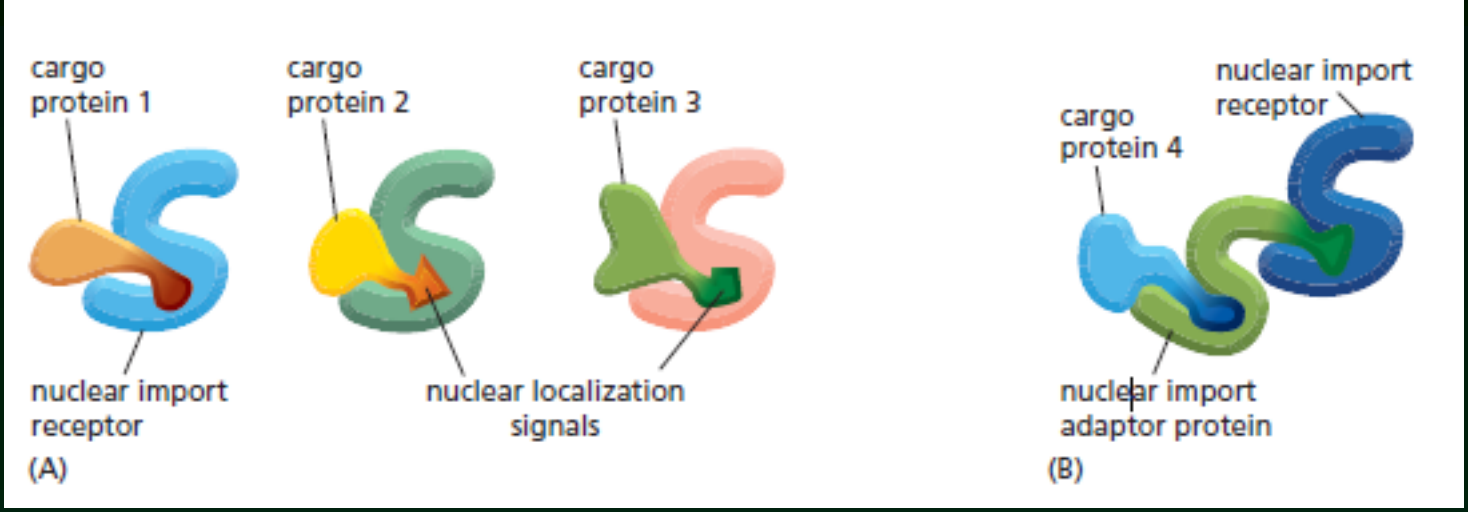
Location	Type of targeting signal	Properties
Nucleus	Nuclear localization signal (NLS)	Short clusters of basic amino acids
Endoplasmic reticulum	Signal peptide	Cleavable N-terminal presequence
	ER retention signal	C-termini, H/KDEL motif
Plastid	Transit peptide	Usually cleavable N-terminal presequence
Mitochondrion	Presequence/Transit peptide	Usually cleavable N-terminal presequence
Peroxisome	Peroxisome targeting sequence 1 (PTS1)	C-termini, a conserved short motif
	Peroxisome targeting sequence 1 (PTS2)	Cleavable N-terminal presequence
Tonoplast/vacuole	Signal peptide	Cleavable N-terminal presequence
	Vacuolar sorting signals	Internal short sequence at near N-terminal
		C-termini, targeting to protein storage vacuole
Apoplast	Signal peptide	Cleavable N-terminal presequence

Nuclear transport

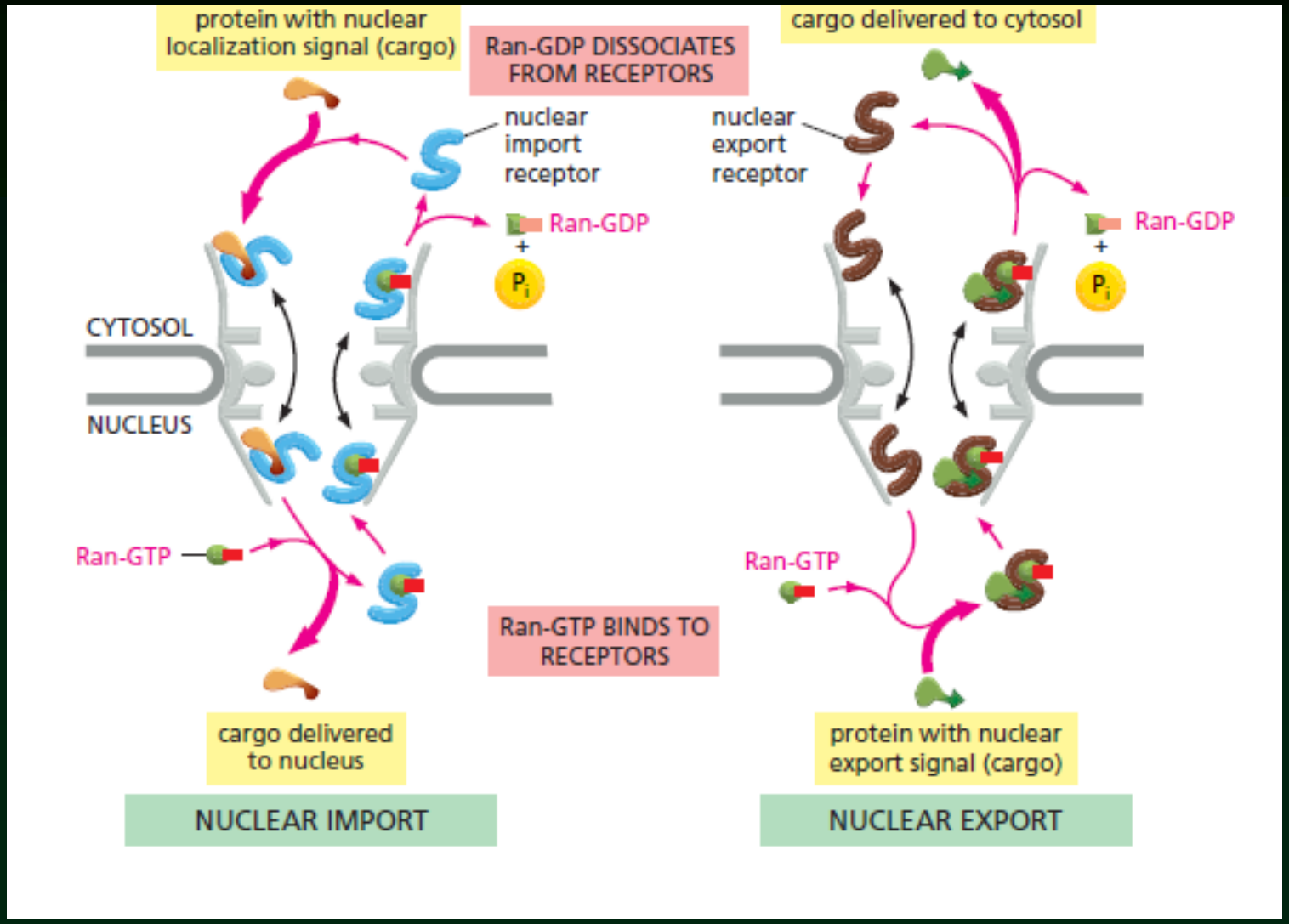


nucleoporins

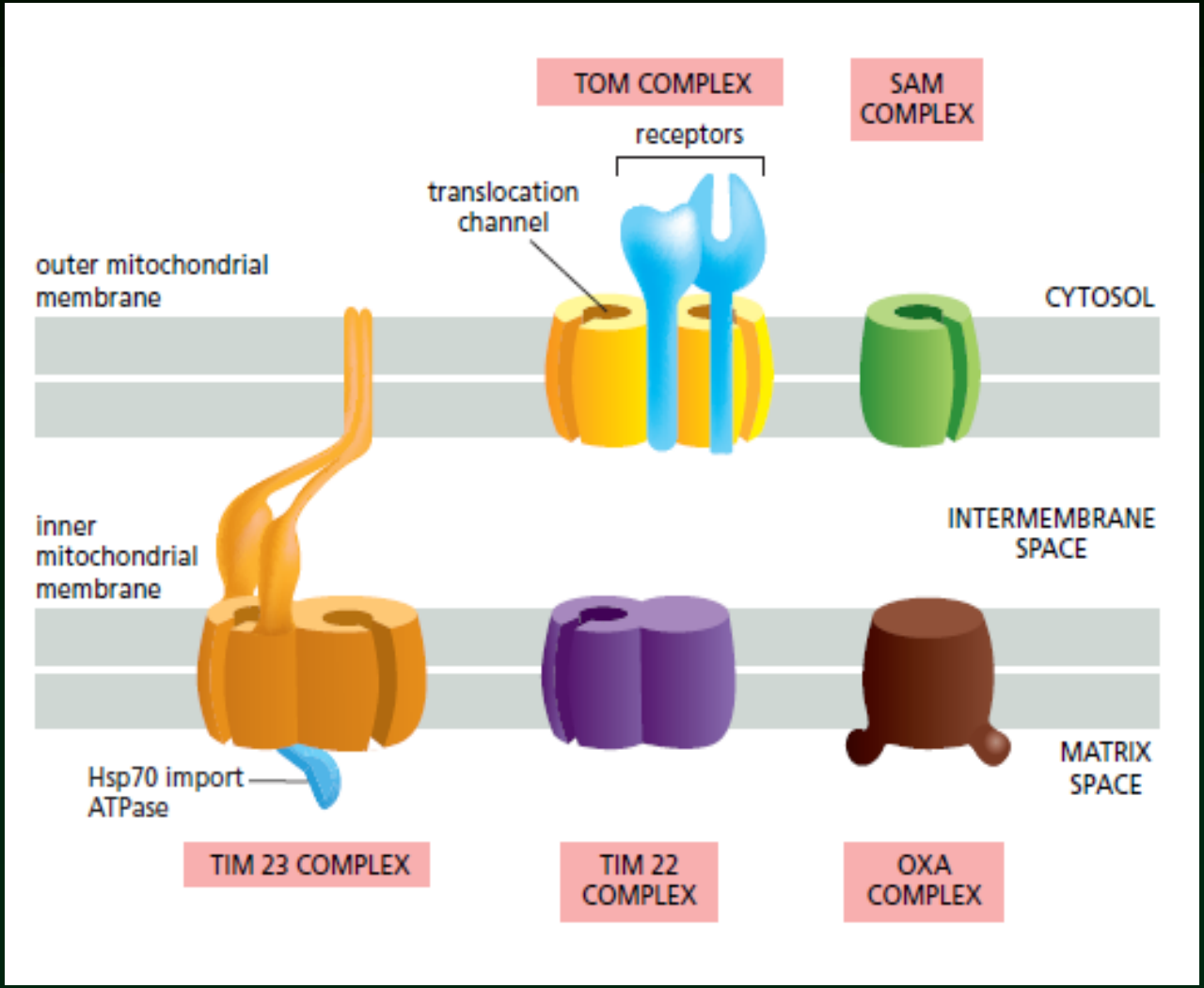
Nuclear import



Nuclear import



Mitochondrial transport



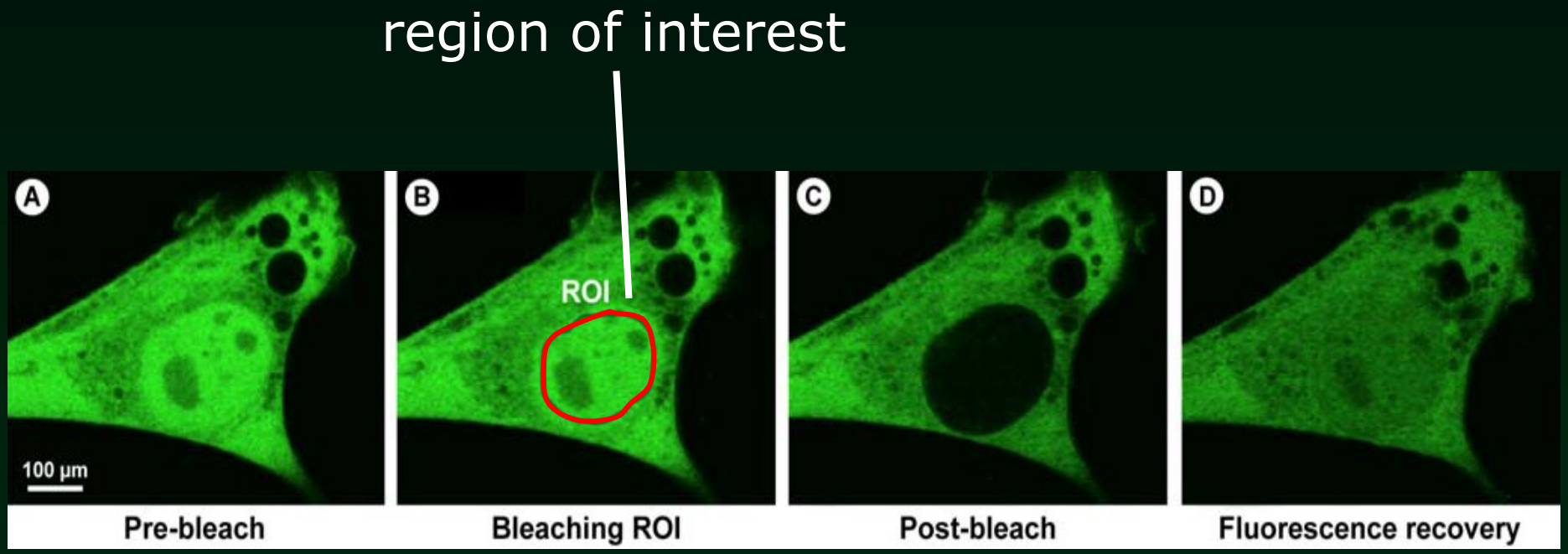
TIM and TOM complexes decide at which side of mitochondria the protein will be transported.

Advanced confocal techniques

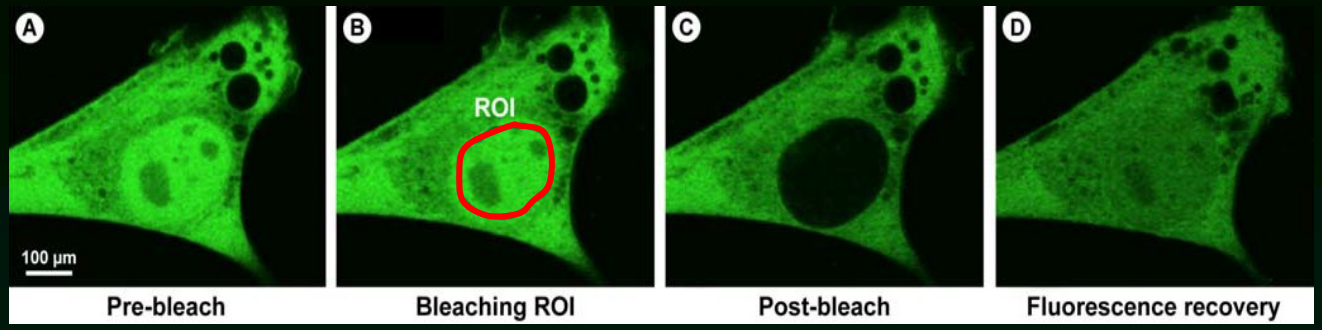
- FRAP
- photoactivatable FP
- FCS

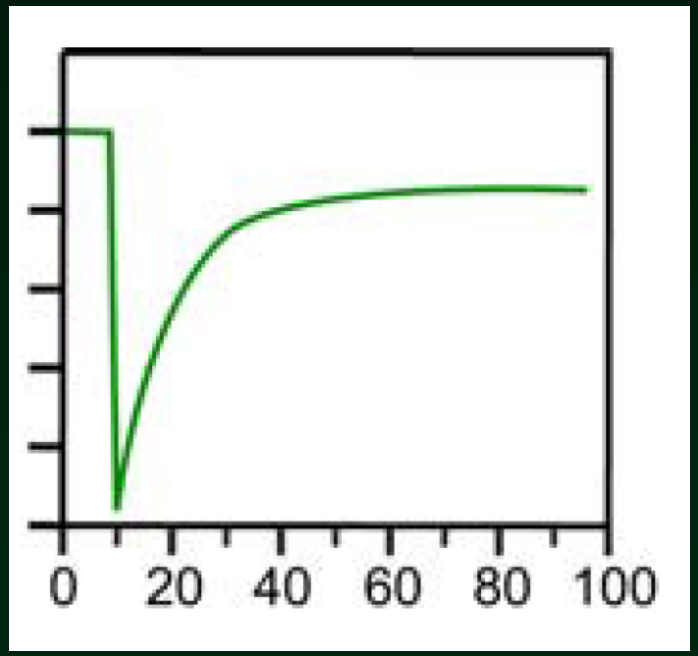
FRAP

Fluorescence Recovery After Photobleaching

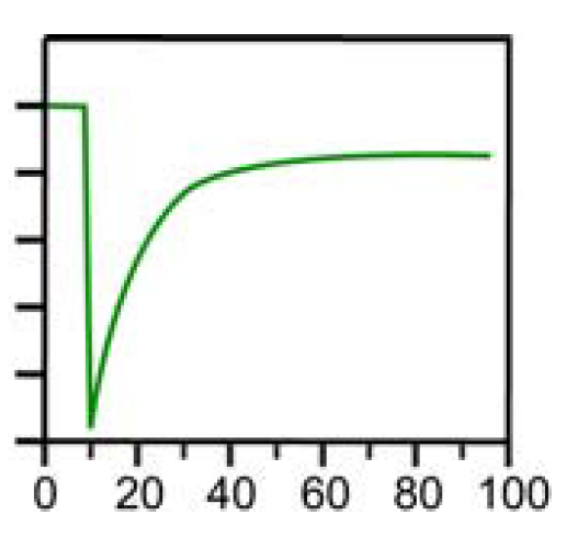


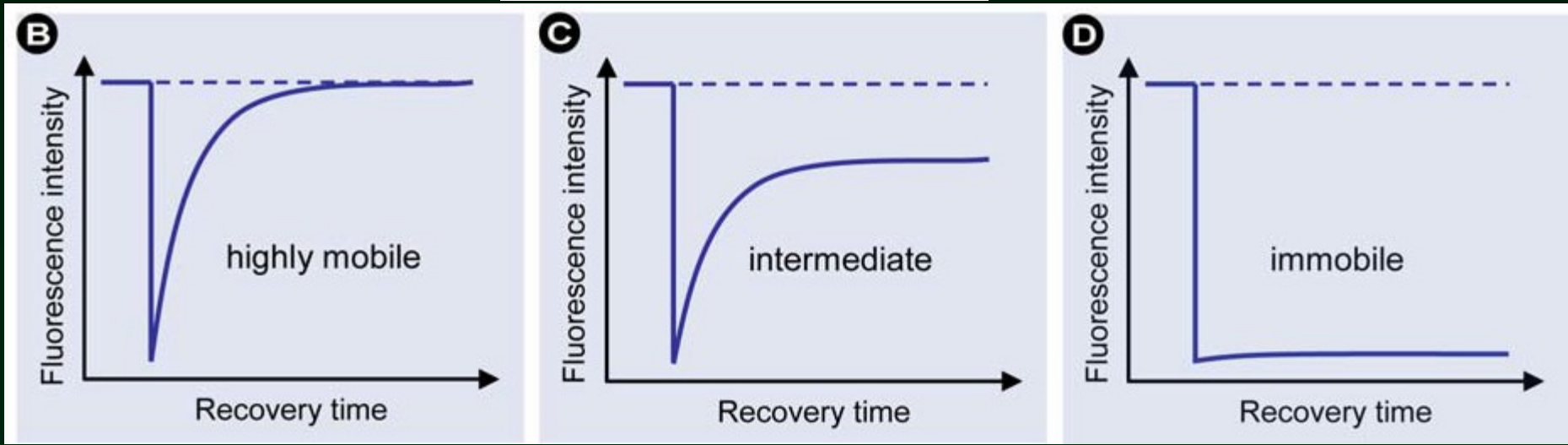
FRAP


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


0
20
40
60
80
100

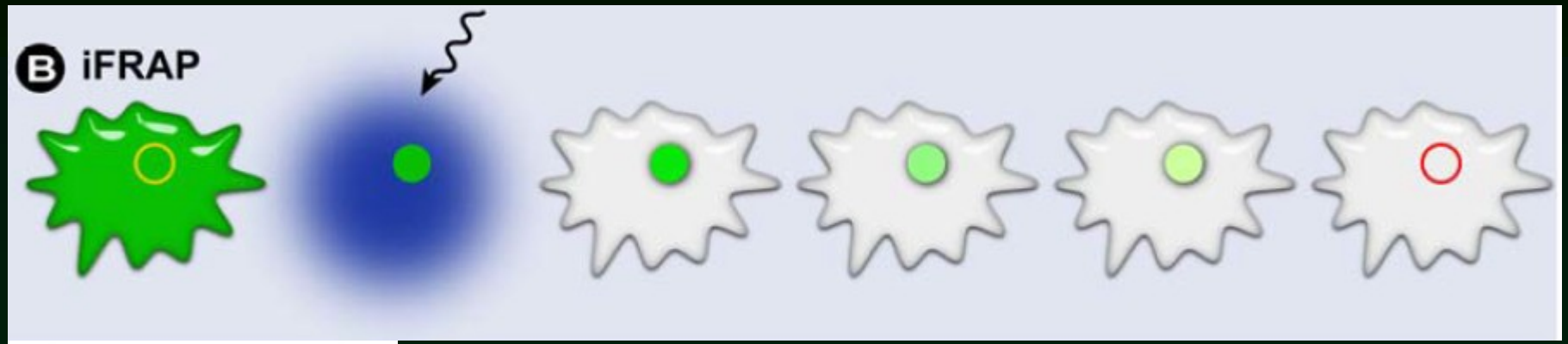
FRAP – bleaching curve

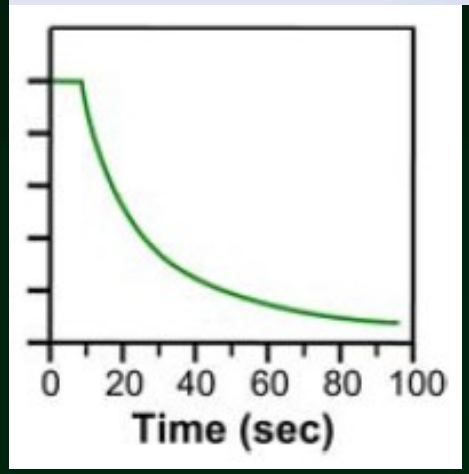




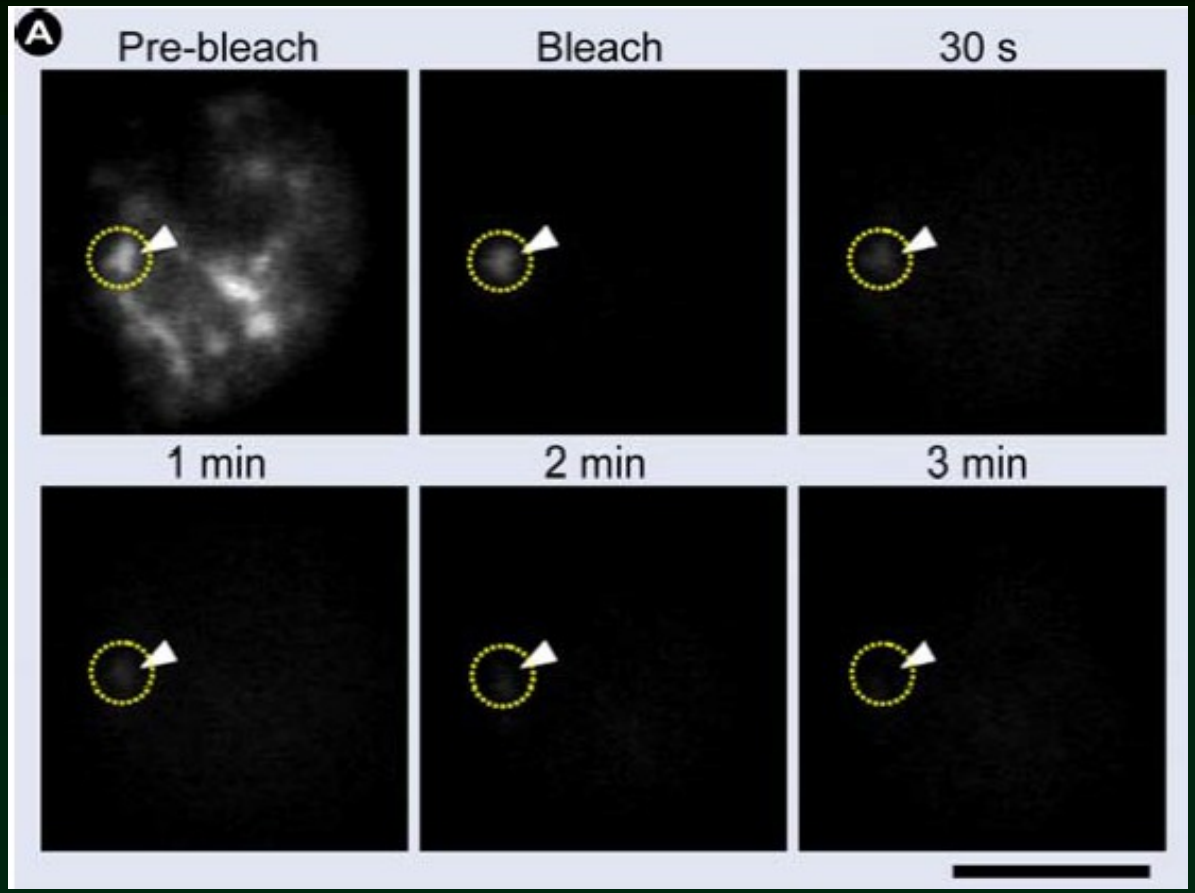
iFRAP

inverse FRAP





iFRAP – dissociation of premRNA from specles



FRAP - advantages

- not only proteins (also other dyes)

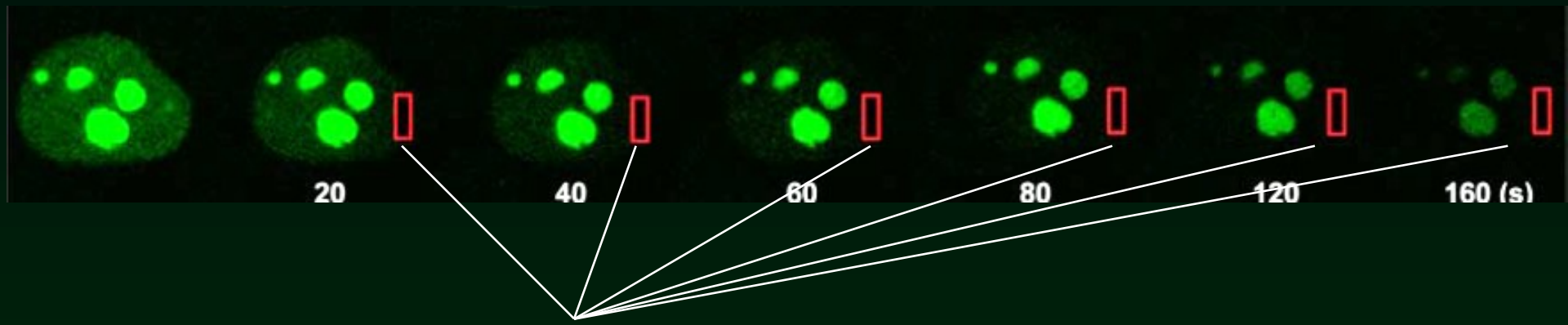
FRAP – disadvantages

- your cells are moving
- high energy needed to bleach the ROI
  - can damage your material
  - long time needed to bleach
- usually only one ROI can be observed – time consuming

FRAP derivatives

FLIP

Fluorescence Loss After Photobleaching



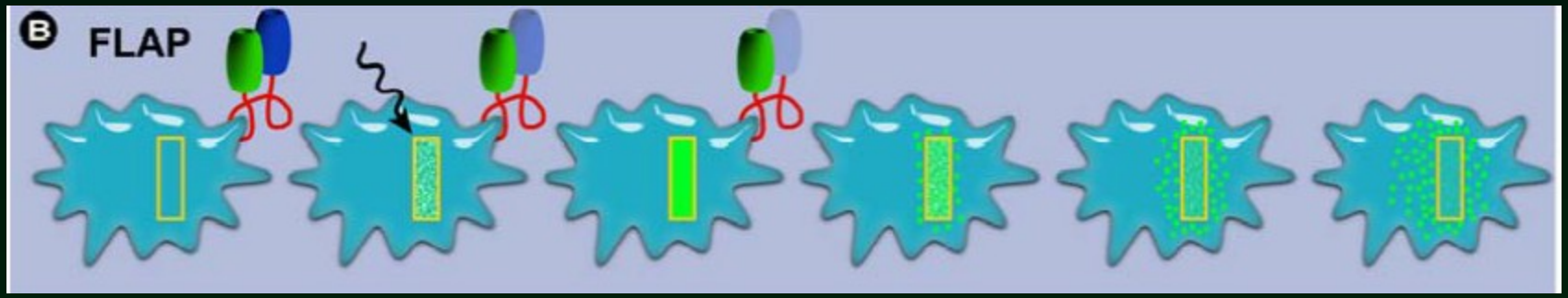
continuous bleaching here

- bleaching process is repeated during the experiment
- for studying general protein turnovers in compartments
- less often used

FRAP derivatives

FLAP

Fluorescence Localization after Photobleaching

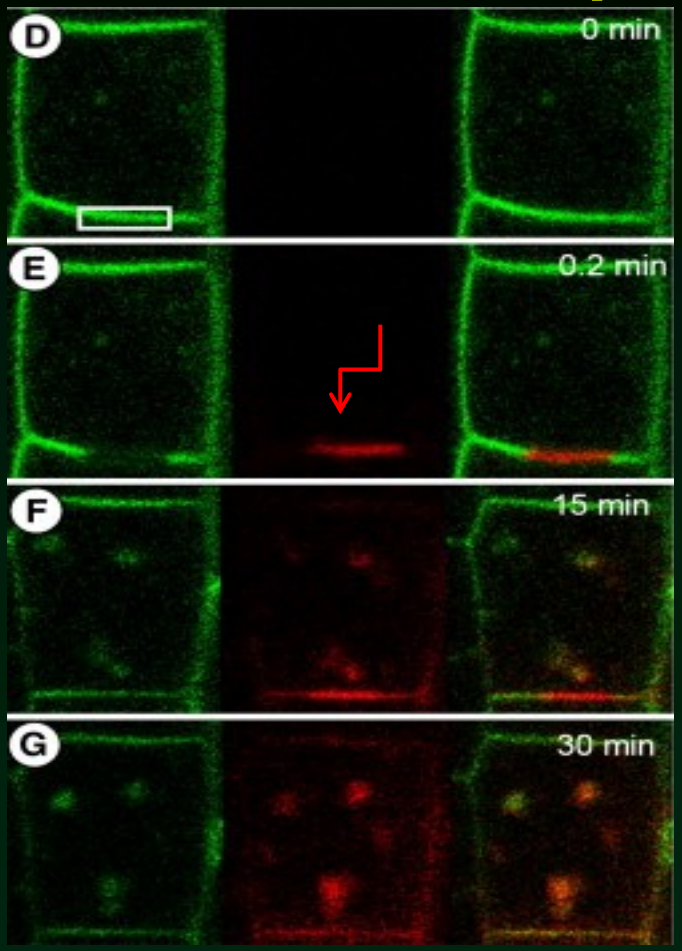


- two fluorochromes on one protein– one bleached, non bleached as control

Intermezzo: story from a conference

even top scientists can be wrong

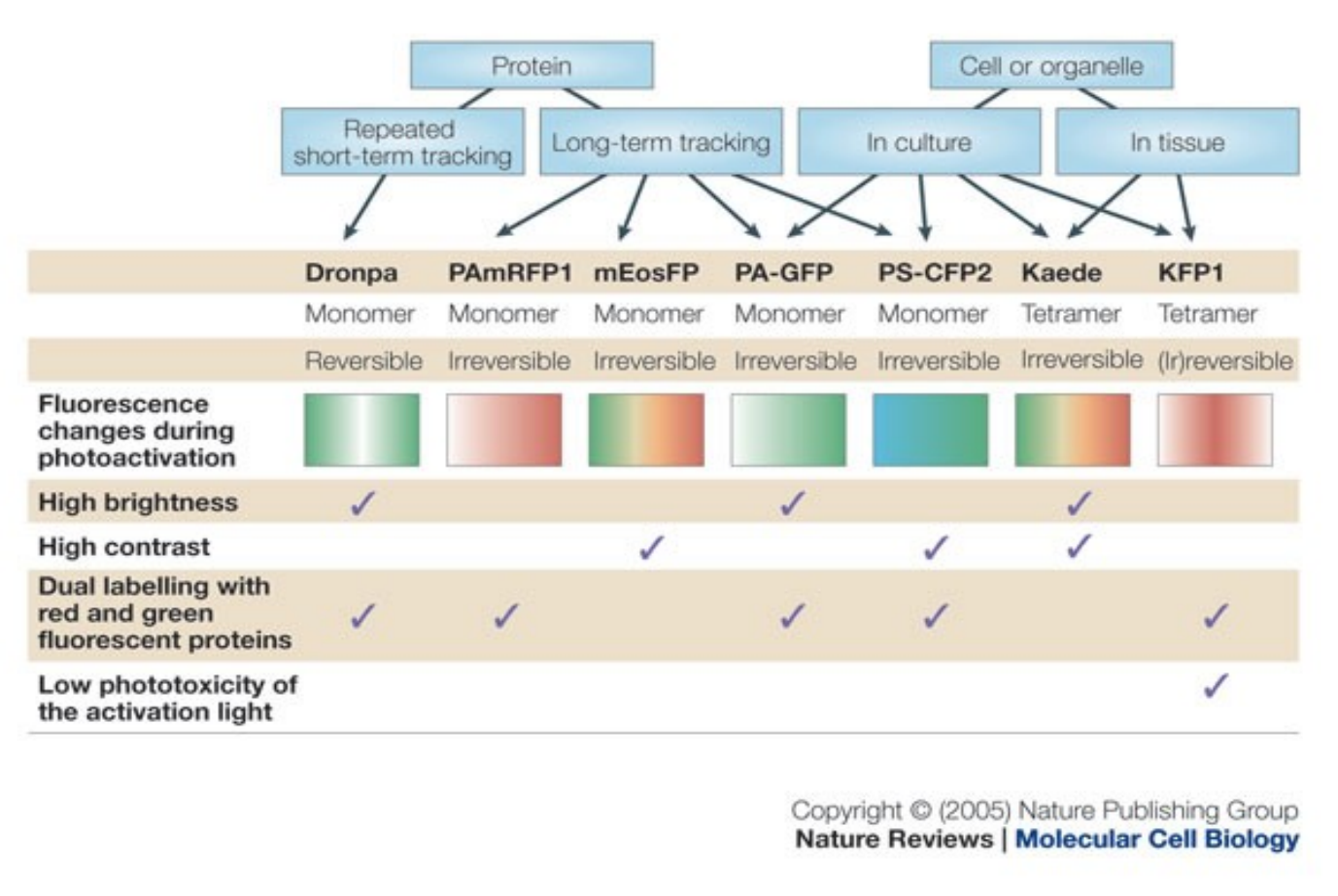
Photoactivable proteins



photoactivation (UV)

non activated | activated | overlay

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:
- most elegant, most 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

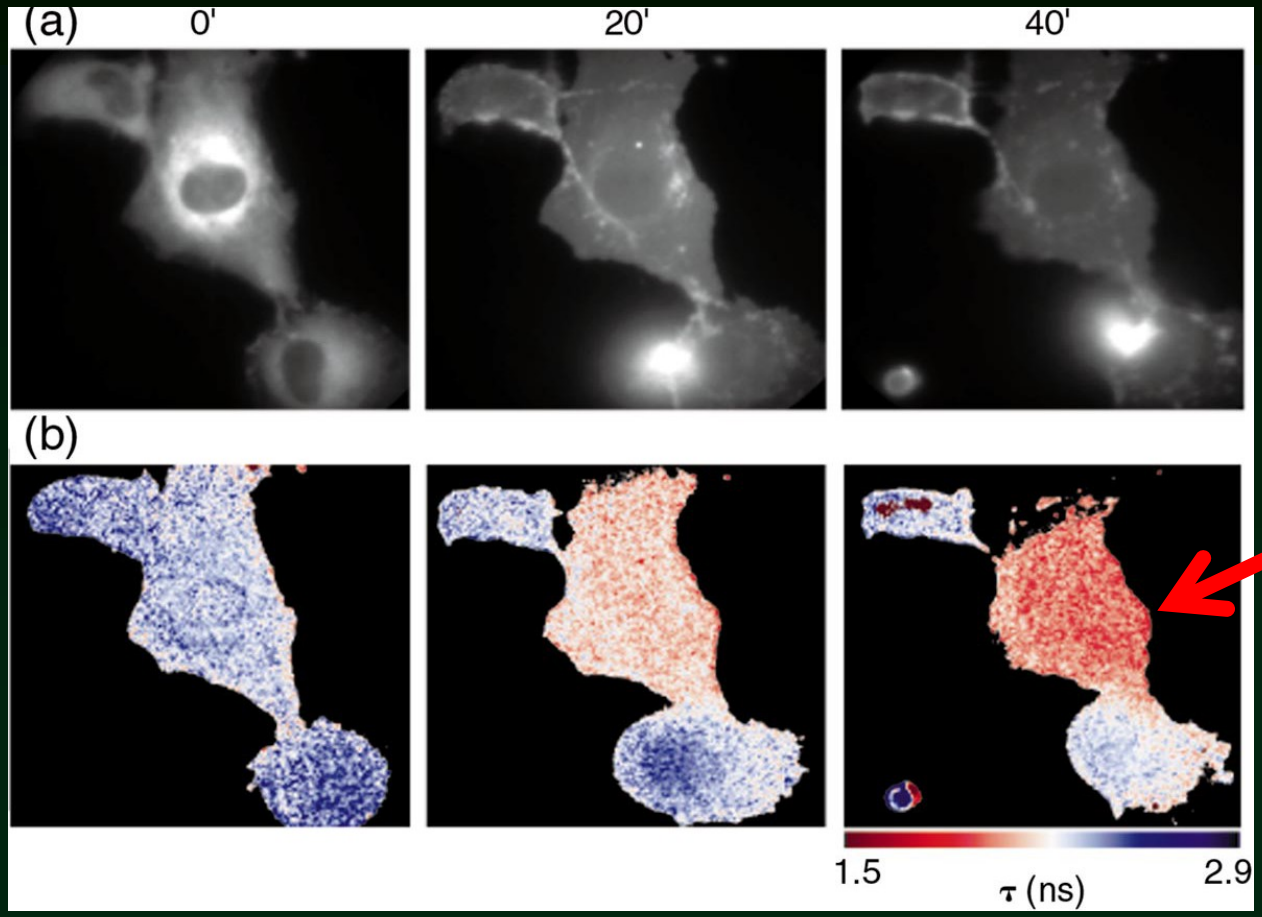
Lifetime sensitive to almost everything:
- pH
- ionic strength
- polarity
- other fluorochrome

FLIM - applications

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

FLIM - applications

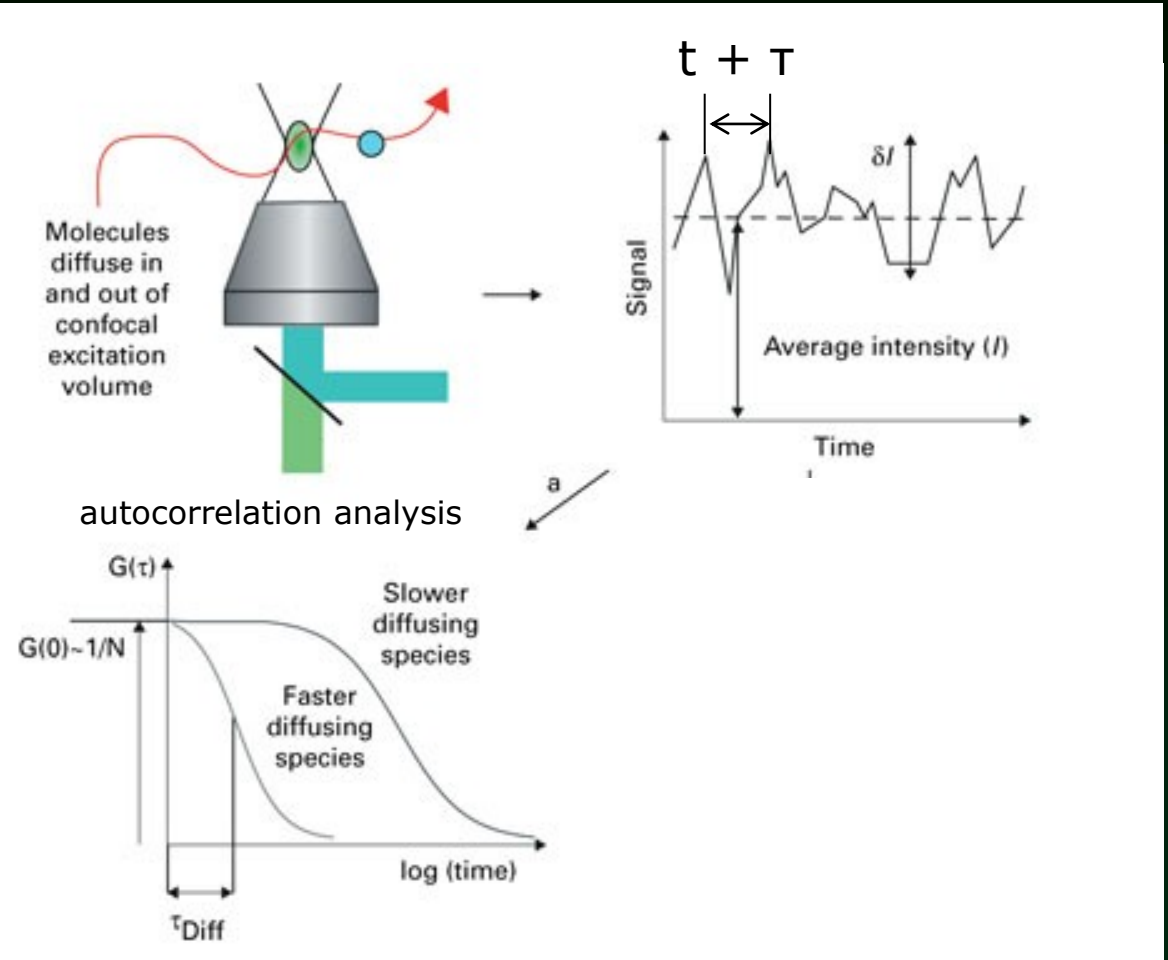
phosphorylation assay



lifetime decreased by site specific IgG injection

FCS

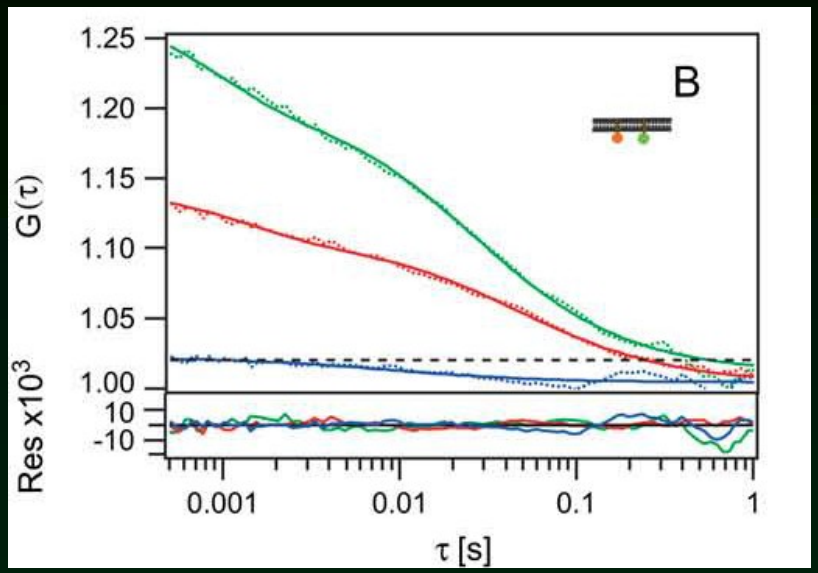
Fluorescence Correlation Spectroscopy



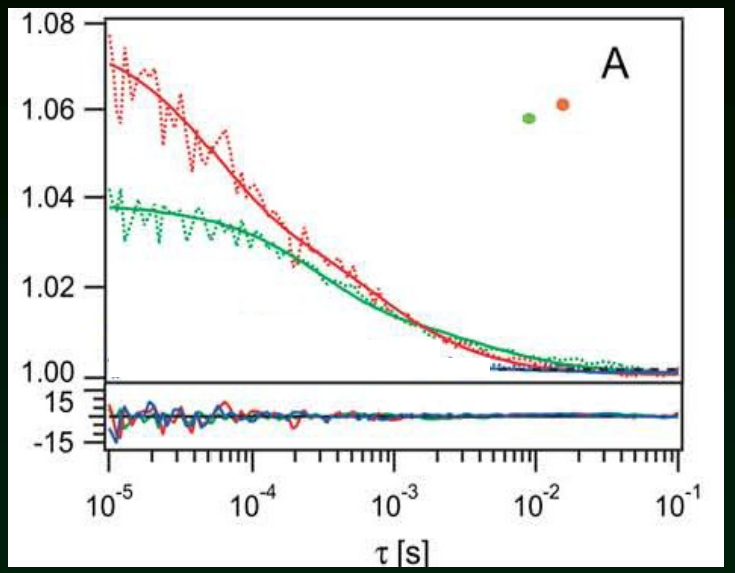
It is counted, how many times the fluorescent molecule comes through the focal plane.

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

FCS

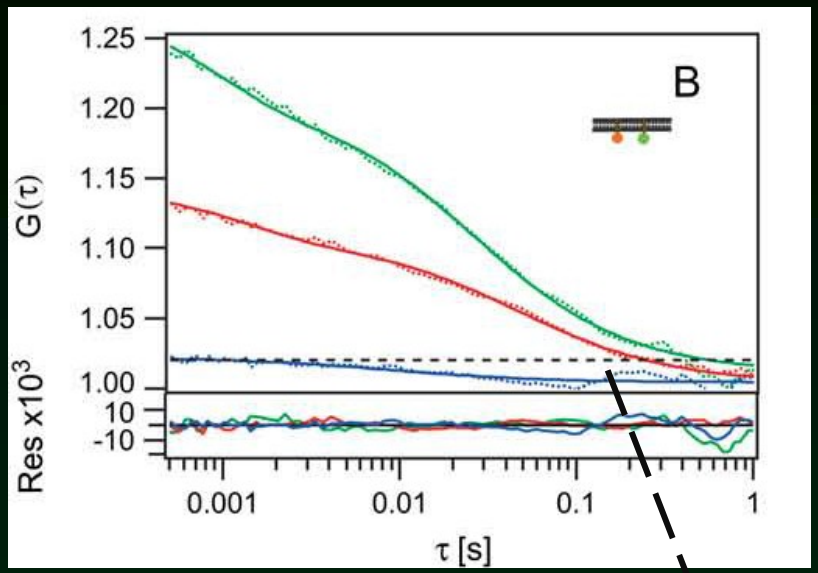


control membrane bound GFP and RFP (crosscorrelation curve)



free GFP and RFP

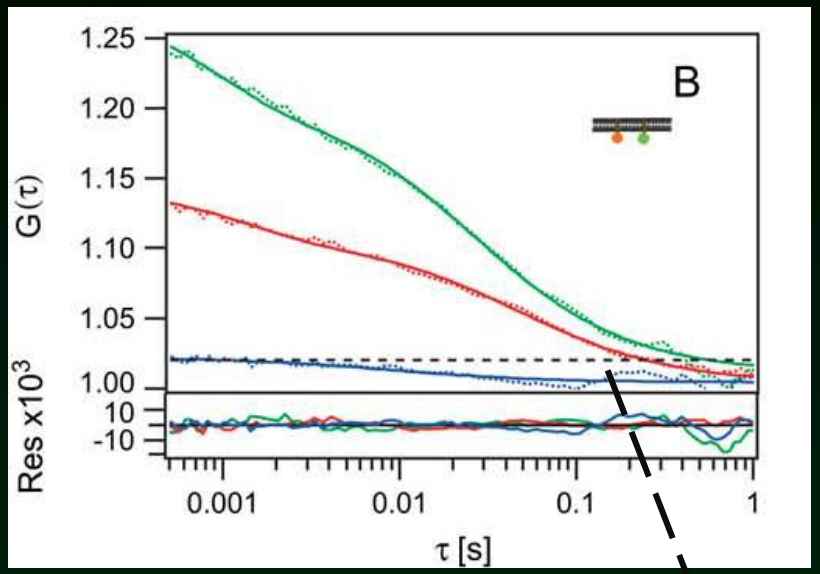
FCS



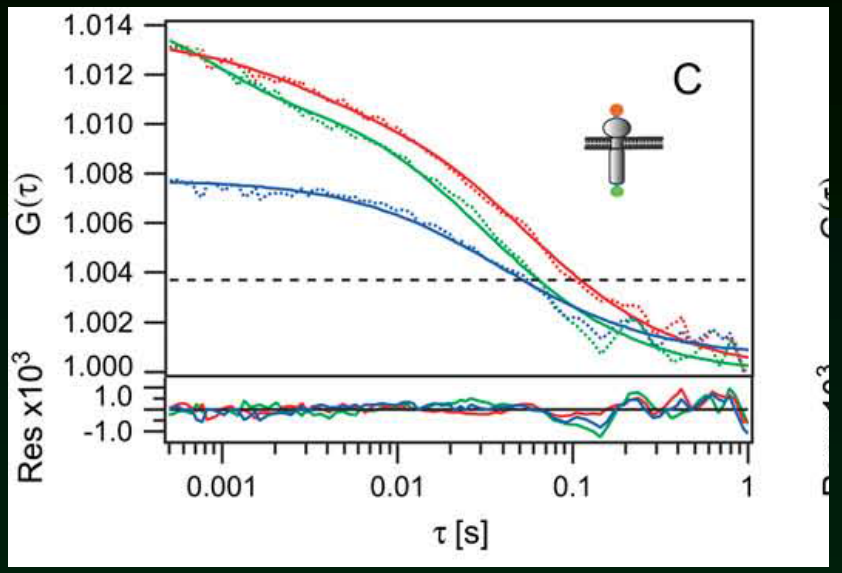
control membrane bound
GFP and RFP
(crosscorrelation curve)

channel crosstalk threshold

FCS



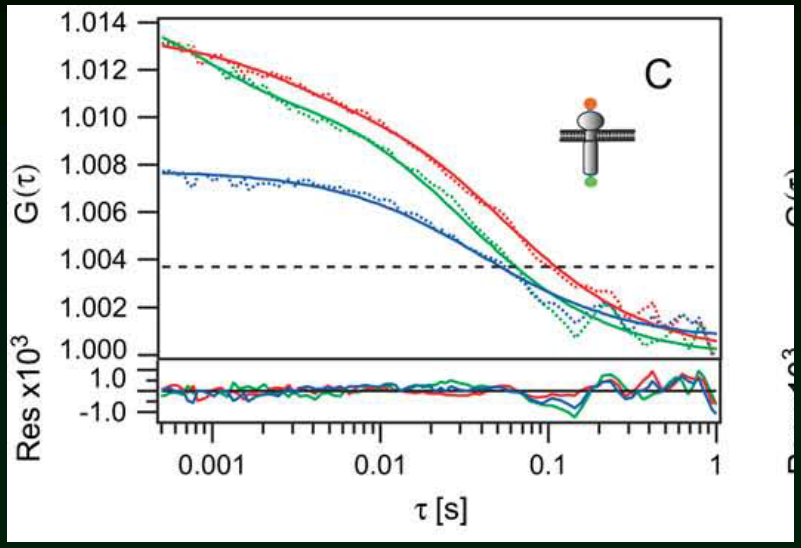
control membrane bound GFP and RFP (crosscorrelation curve)



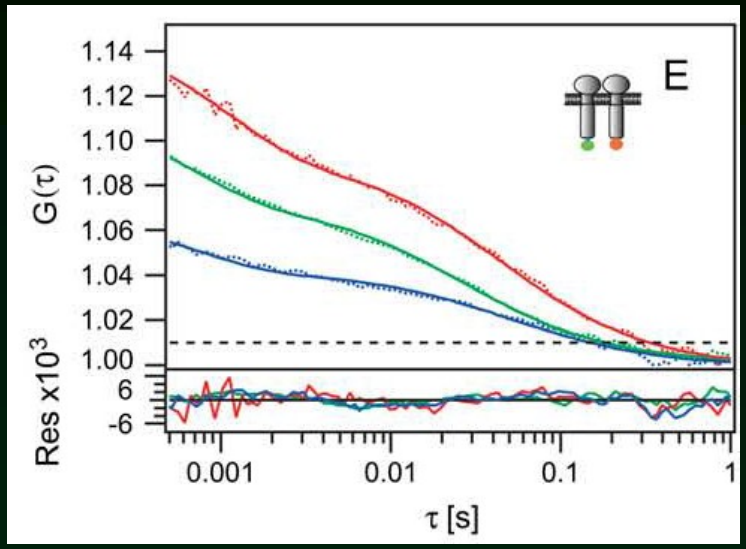
receptor with two labels

channel crosstalk threshold

FCS



receptor with two labels



the crosscorelation curve is above threshold -> EGFR protein dimerizes

Liu et al., 2007