Basic fluorescence toolkit

HW 4 posted

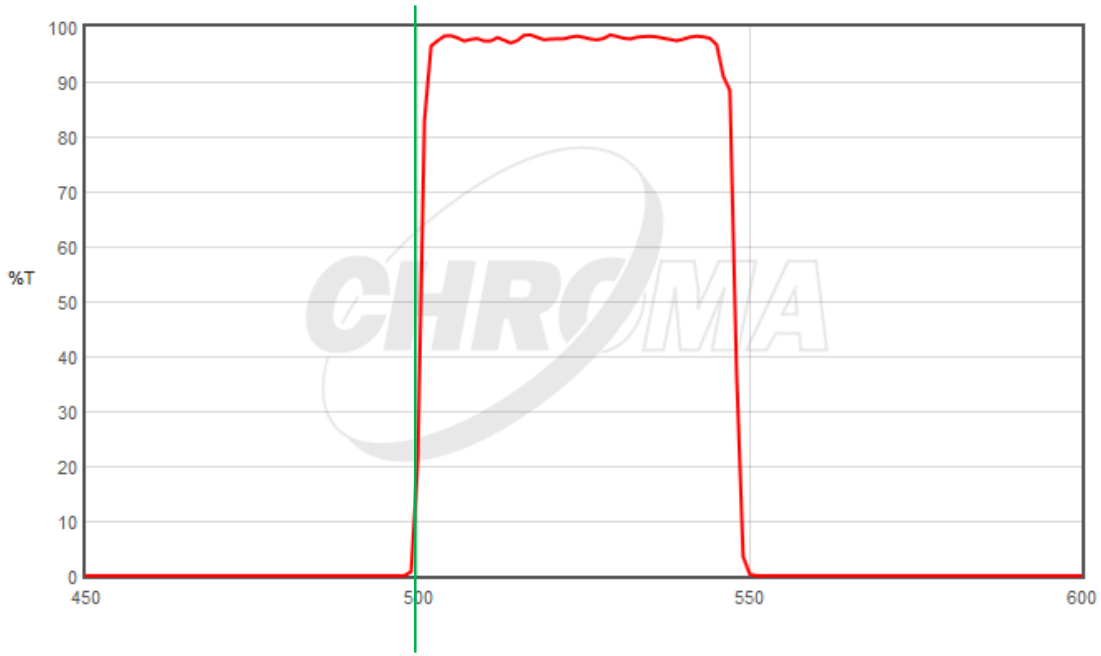
- Due next Fri (10/13)

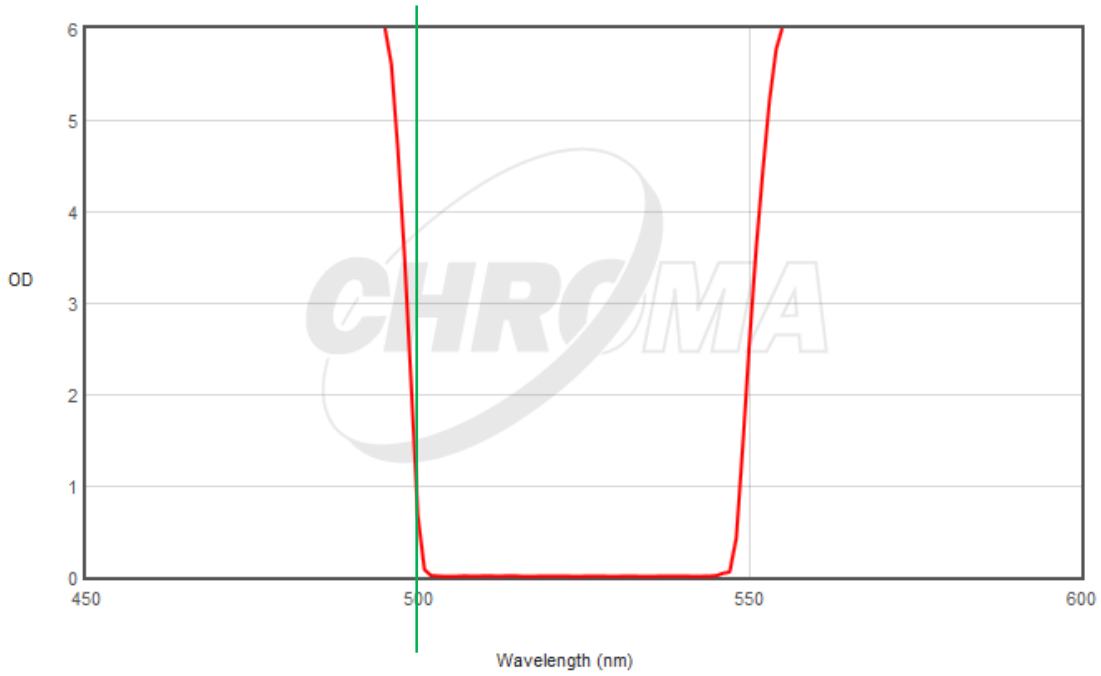
- Last class
  - FPs
  - GFP/mCherry
  - Exotic fluorophores
  - Filters
- This class
  - Basic applications of fluorescence
  - Limitations
  - Quantitation

OD vs % transmission

$OD = -\log_{10}(Transmission)$

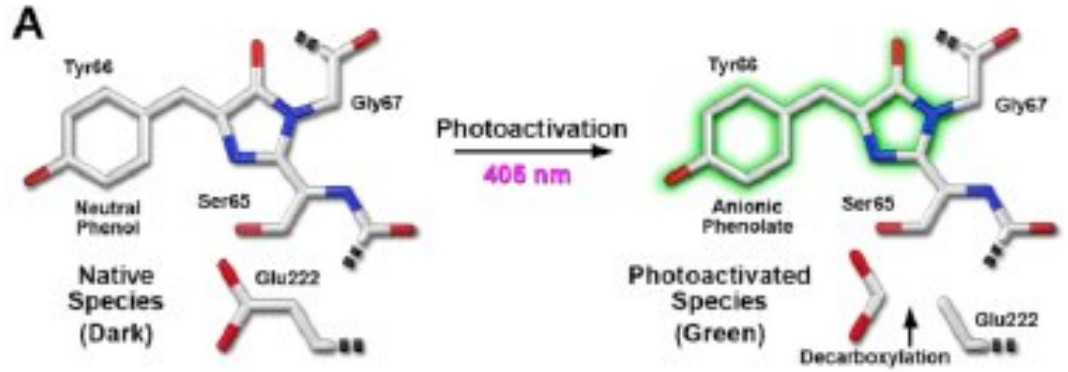
1 OD = 10% transmission, 2OD = 1% transmission, 3 OD = .1% transmission





Photoactivatable and photoconvertible GFPs

- Initially dark – can be activated by fluorescence with UV light
- Initially green, can be converted to red with UV light
- Some will also photoconvert with time (Timers)



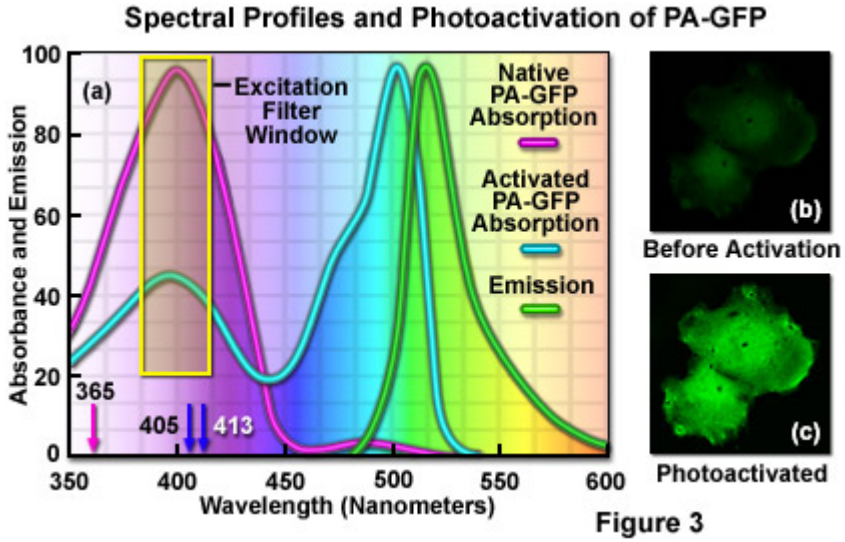


Figure 3

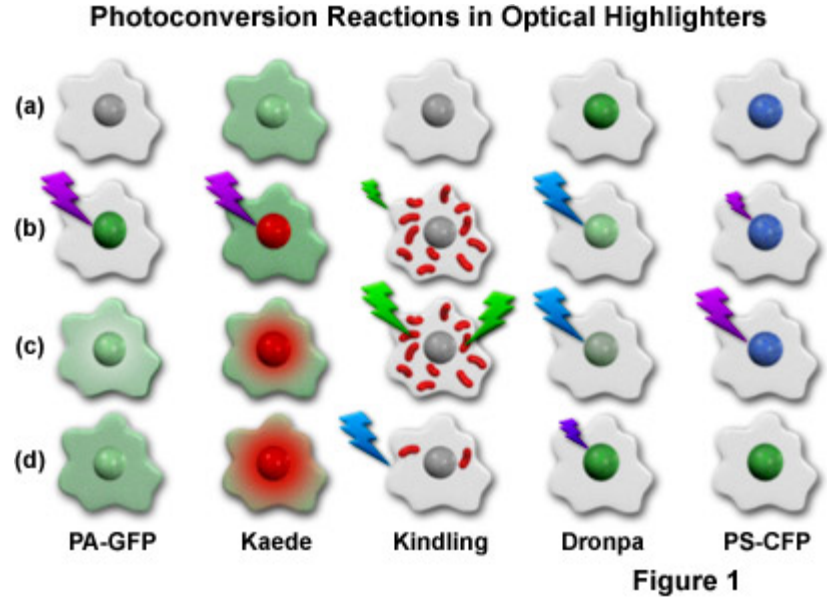


Figure 1

Basic Applications

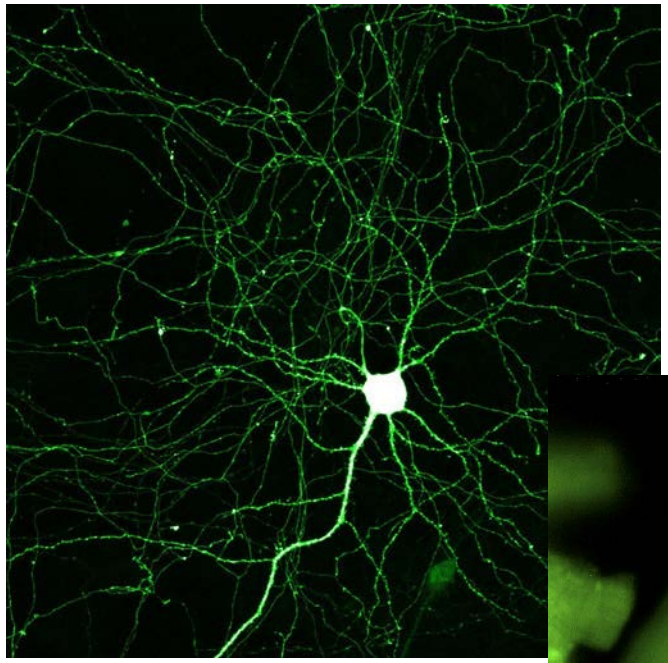
- Cell labeling
- Molecule labeling
- Molecule interactions

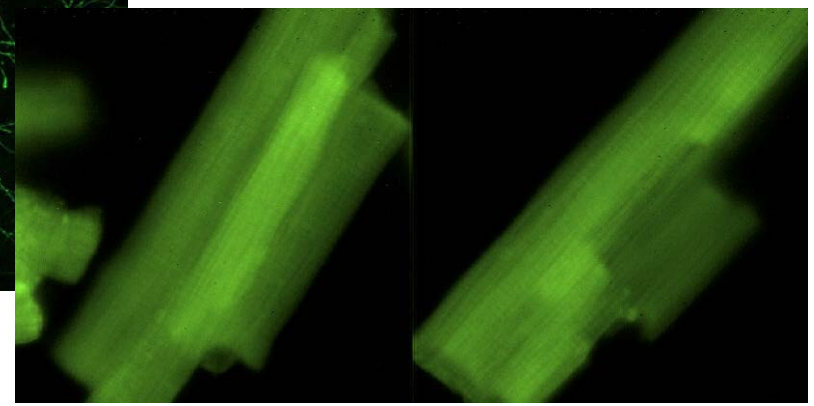
Cell labeling

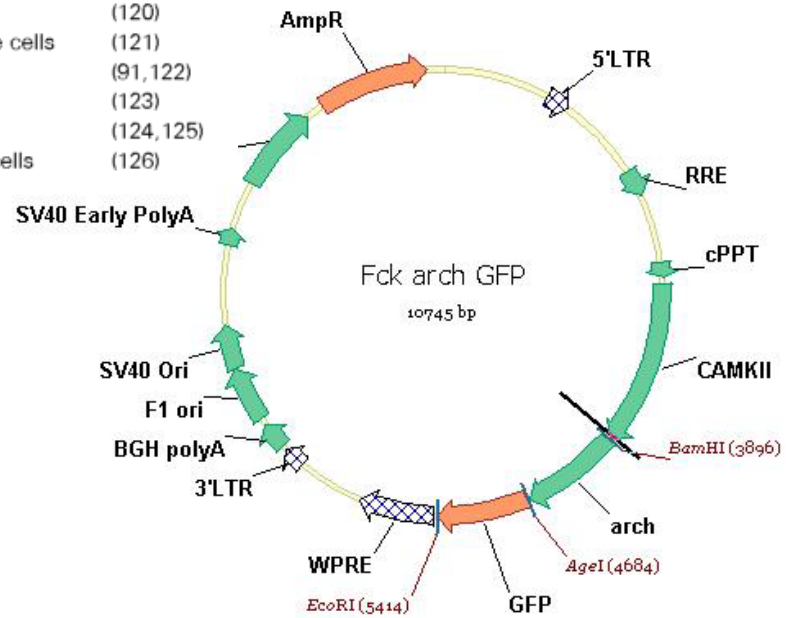
- Put FP downstream of cell specific promoter

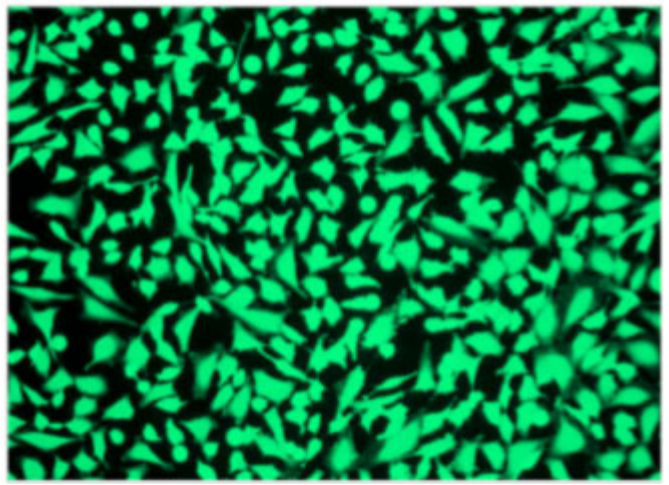
Table 1 - Promoters of tissue-specific expression of transgenes.

Promoter	Abbreviation	Cell type of highest activity	Reference
Neuron-specific enolase	NSE	Neurones	(112)
Tubulin α1	T-α1	Neurones	(113)
Glial-fibrillary acidic protein	GFAP	Astrocytes	(25,114,115)
Myosin light chain-2	MLC2	Cardiomyocytes	(116-118)
Preproendothelin-1	ET-1	Endothelial cells	(119)
Tie	tie	Endothelial cells	(120)
SM22α	SM22α	Vascular smooth muscle cells	(121)
α1-Antitrypsin	α1-AT	Hepatocytes	(91,122)
Albumin	ALB	Hepatocytes	(123)
Side-chain-cleavage enzyme	SCC	Steroidogenic cells	(124,125)
Kidney-androgen responsive protein	KAP	Renal proximal tubular cells	(126)



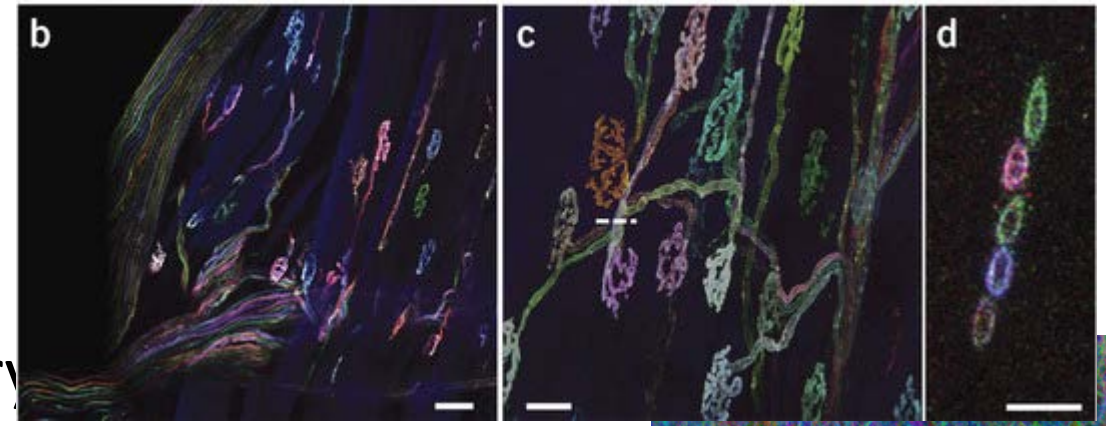


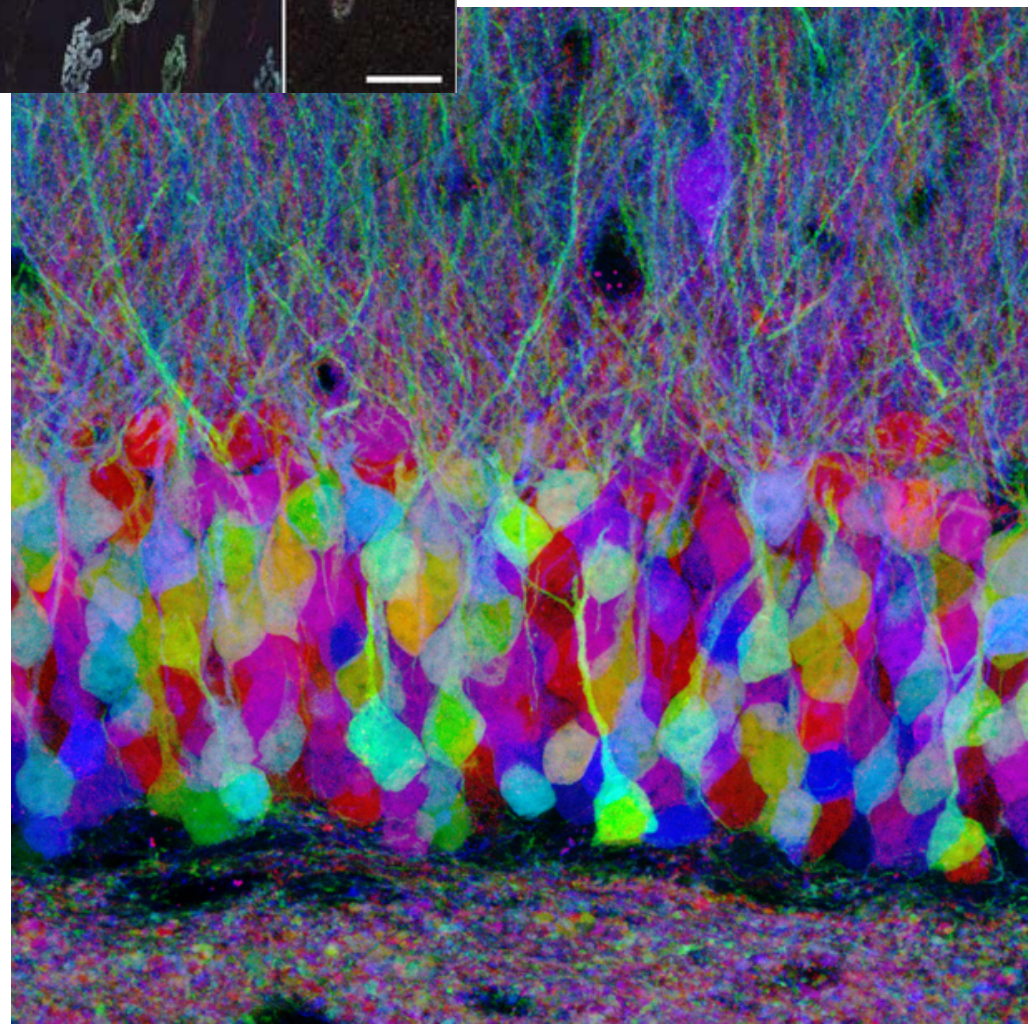


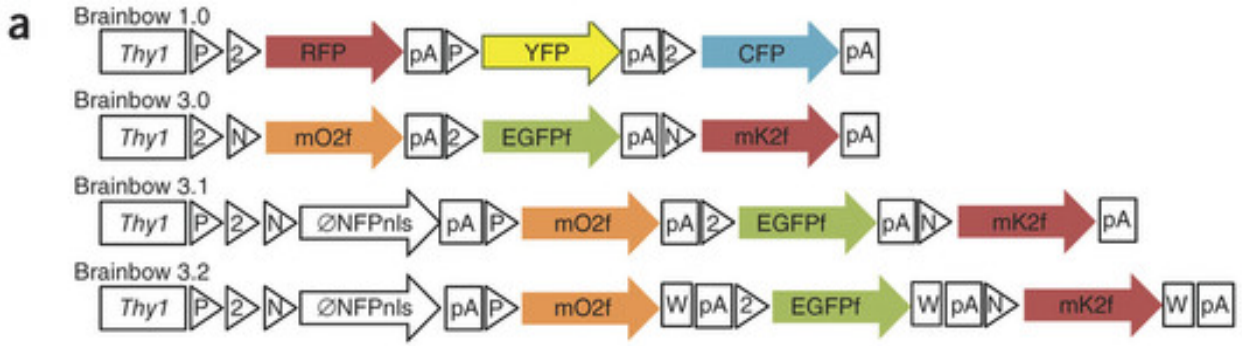


Brainbow

- Used to resolve individual cells in very dense samples
- Use stochastic Cre recombination

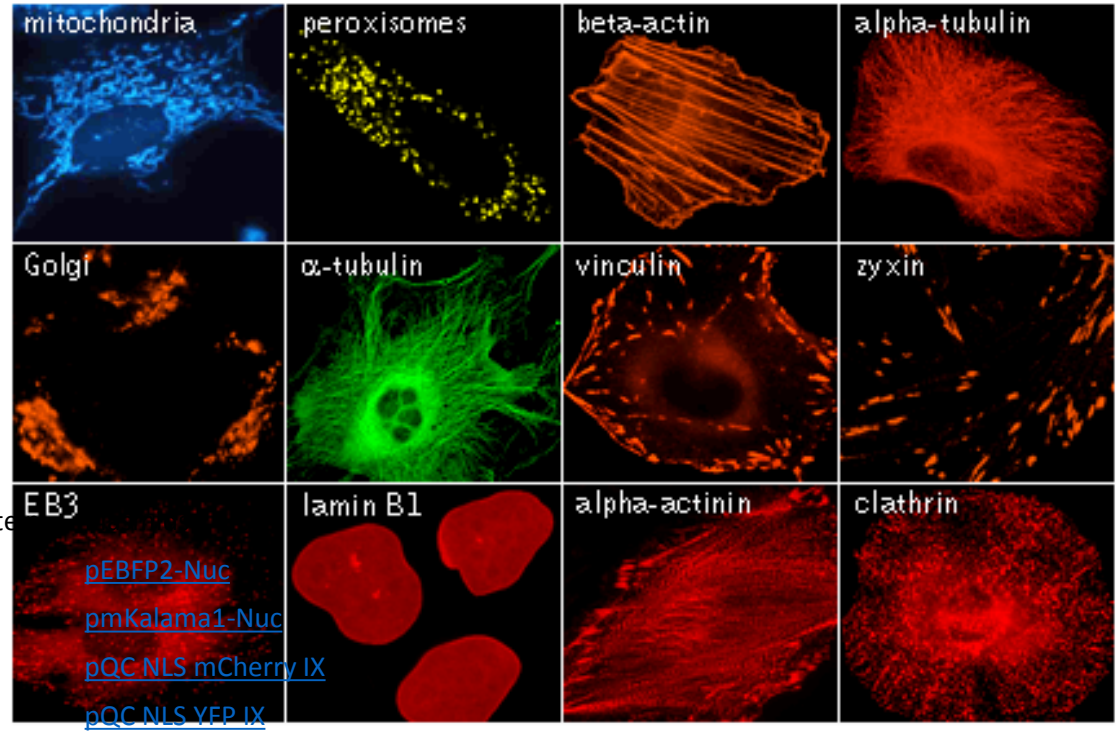






Organelle labeling

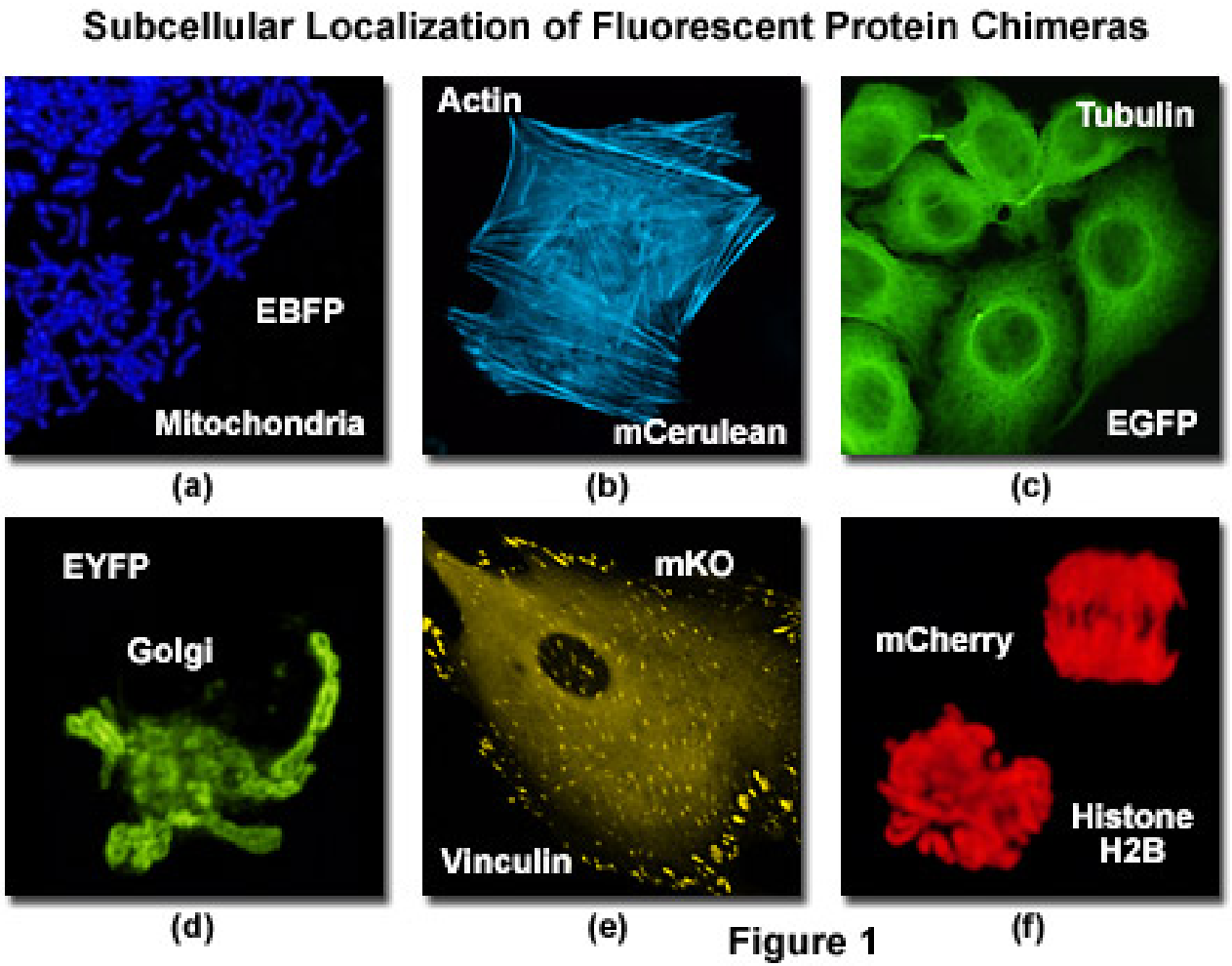
- Use targeting sequence
- Translated peptide before or after the FP



Structure	Targeting Gene/Tag	Fluorescent Prote	
Nucleus	NLS	EBFP2	pEBFP2-Nuc
Nucleus	NLS	mKalama1	pmKalama1-Nuc
Nucleus	NLS	mCherry	pQC NLS mCherry IX
Nucleus	NLS	YFP	pQC NLS YFP IX
Nucleus	H2A	mTurquoise2	pmTurquoise2-H2A
Non-nucleus	Nuclear Export Sequence	mTurquoise2	pmTurquoise2-NES
Mitochondria	ABCb10	GFP	pABCb10-GFP
Mitochondria	COX8A(1-29)	mTurquoise2	pmTurquoise2-Mito
Peroxisomes	Peroxisomal Targeting Sequence	mTurquoise2	pmTurquoise2-Peroxi
Extracellular milieu	cfSGFP2-N	EGFP	cfSGFP2-N
Membrane	palmitoylation sequence from GAP43	EGFP	pCAG-mGFP
Membrane	palmitoylation sequence from p63	mTurquoise2	pPalmitoyl-mTurquoise2
Periplasmic space	PelB signal sequence	YFP	pTDpelB-C_sfYFPTwinStrep

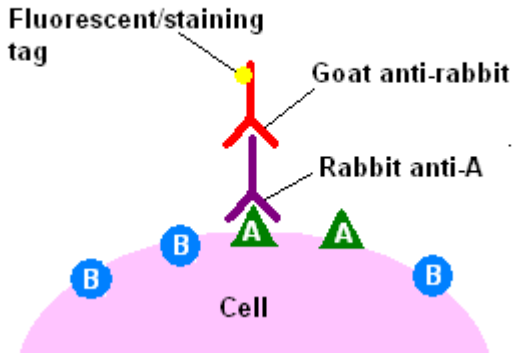
Protein labeling

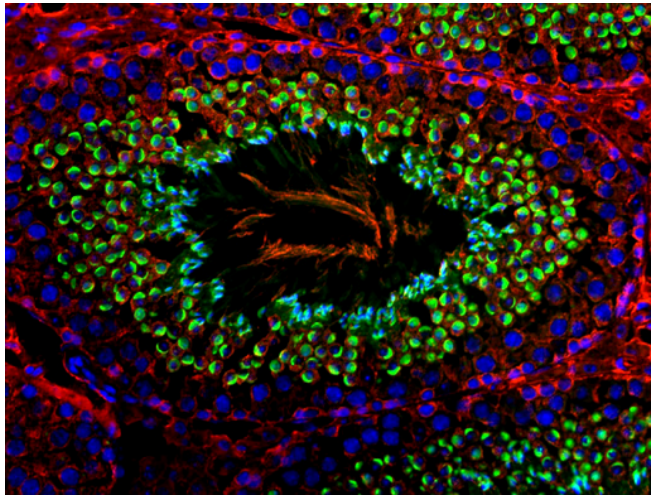
- Can add it at the end of a protein sequence – get rid of stop codon, and add on the extra 700 AA.
- Can add localization signals
- Can put under a promoter to look at gene expression

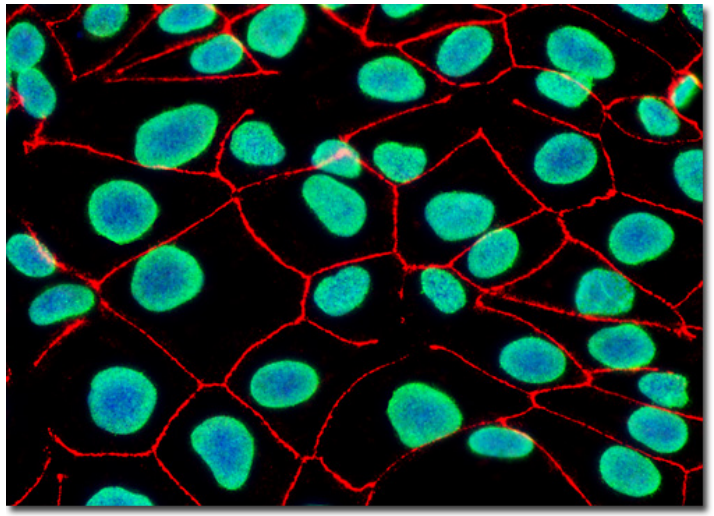


Immunohistochemistry

- Protein localization on fixed and permeabalized samples
- Can do membrane staining on live cells
- Necessary to buy antibodies with labels attached







Antibody production

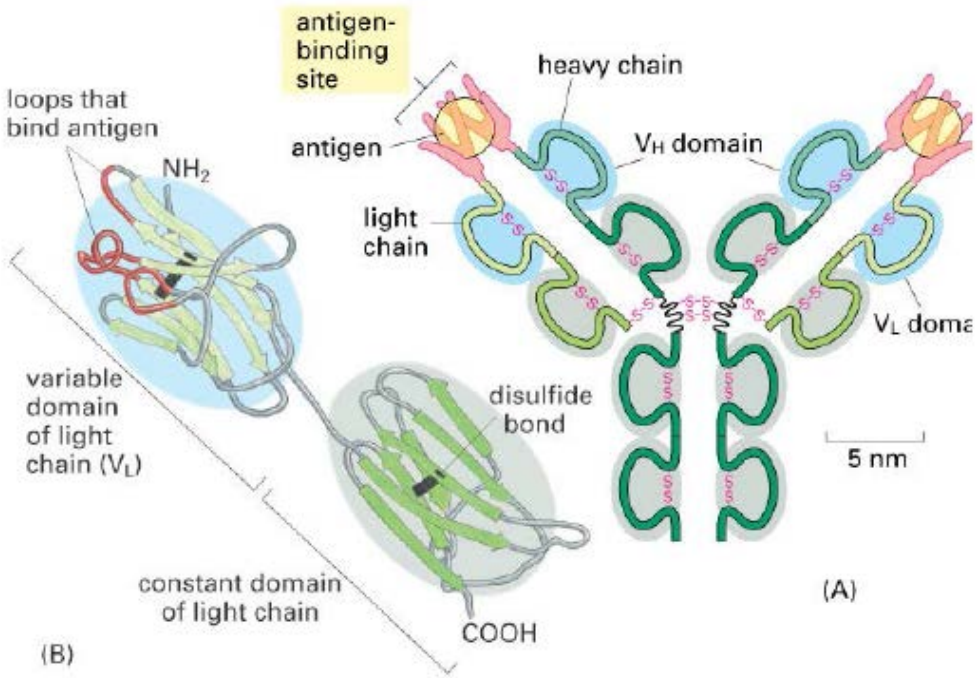
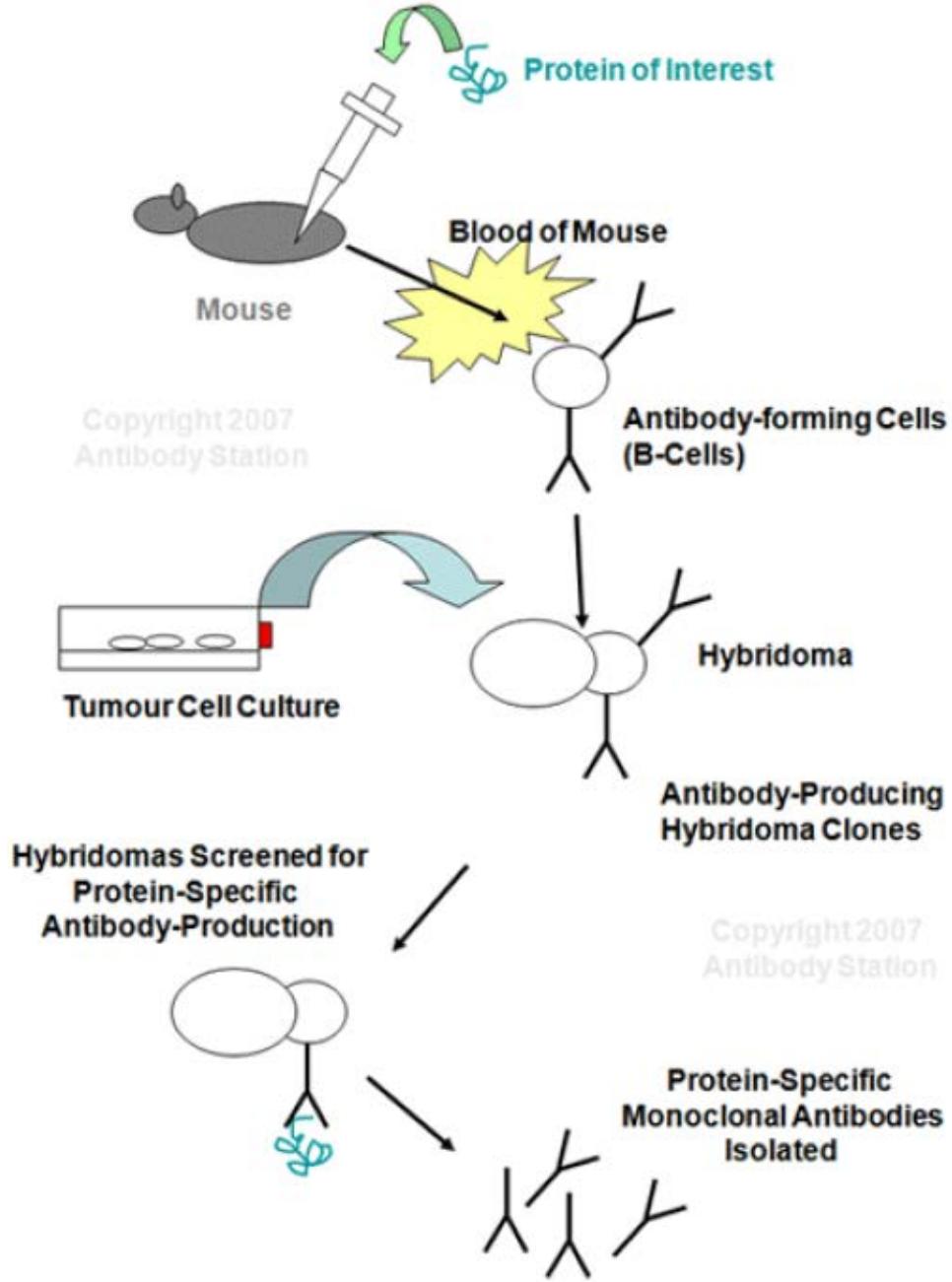


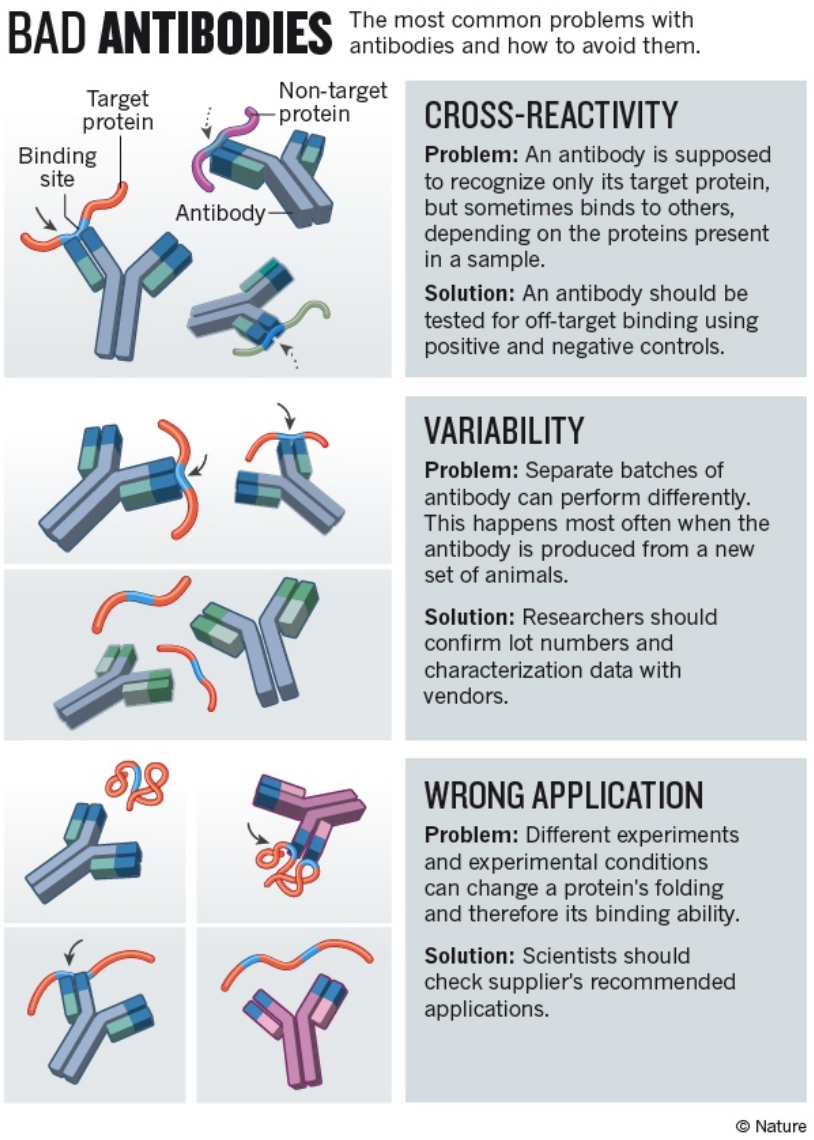
Figure 4-32 Essential Cell Biology, 2/e. (© 2004 Garland Science)



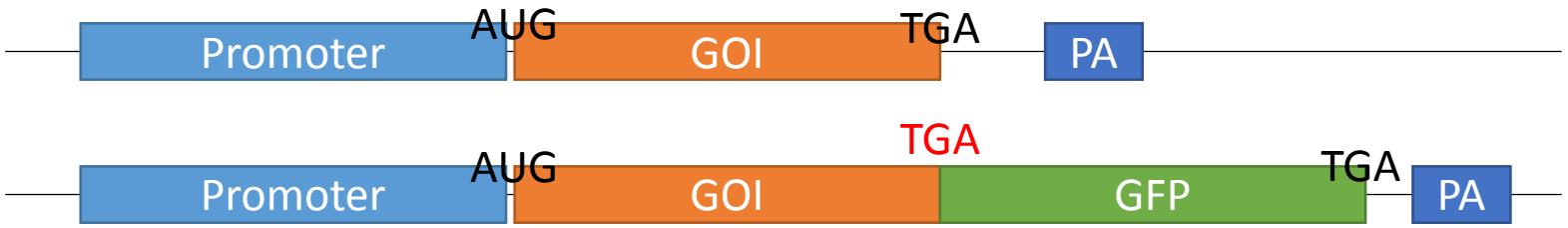
Issues with immunohistochemistry

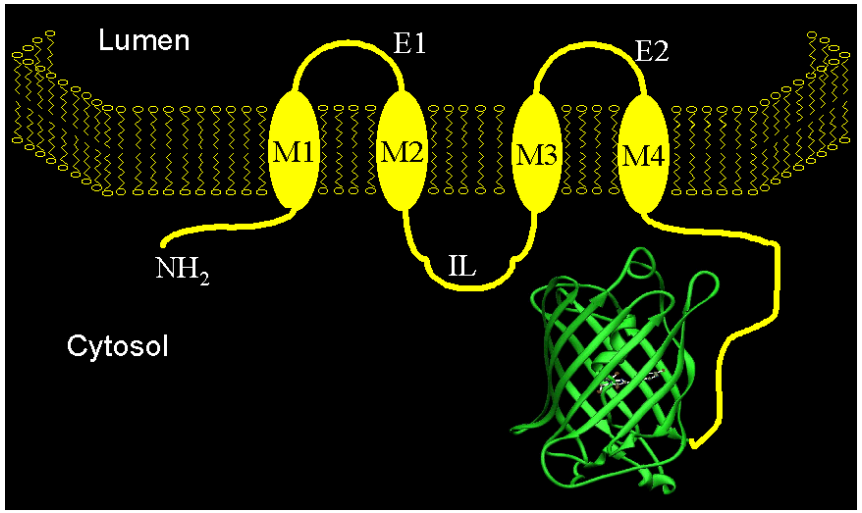
- Antibodies don’t always do what the label says
- If you’re basing a research project on antibody imaging, make sure it’s targeting your protein of interest
- Non-specific targeting, incorrect targeting, batch to batch variability

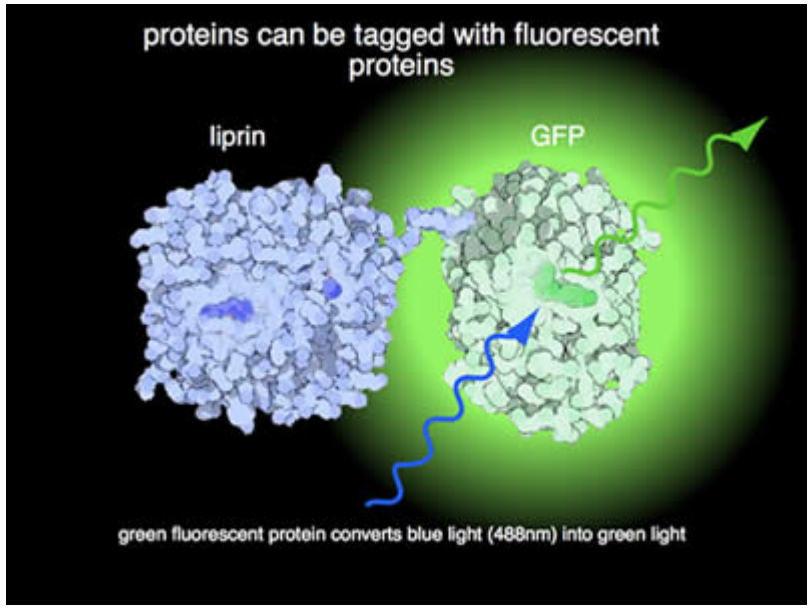
Poorly characterized antibodies probably contribute more to the problem than any other laboratory tool, says Glenn Begley, chief scientific officer at TetraLogic Pharmaceuticals in Malvern, Pennsylvania, and author of a controversial analysis[1] showing that results in 47 of 53 landmark cancer research papers could not be reproduced.

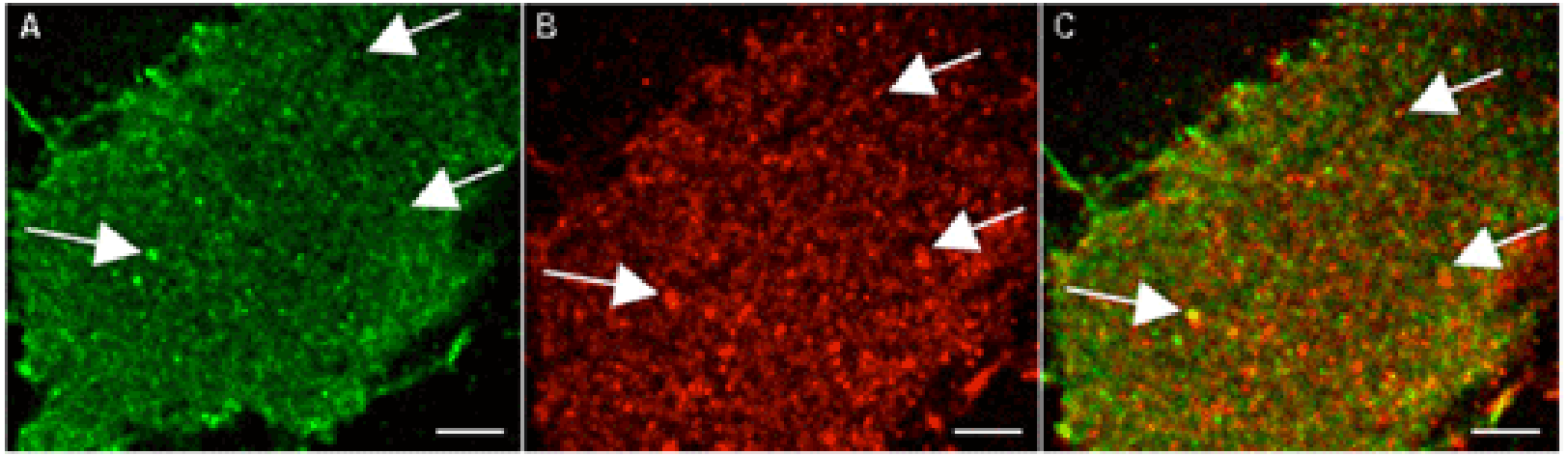


Molecule tracking

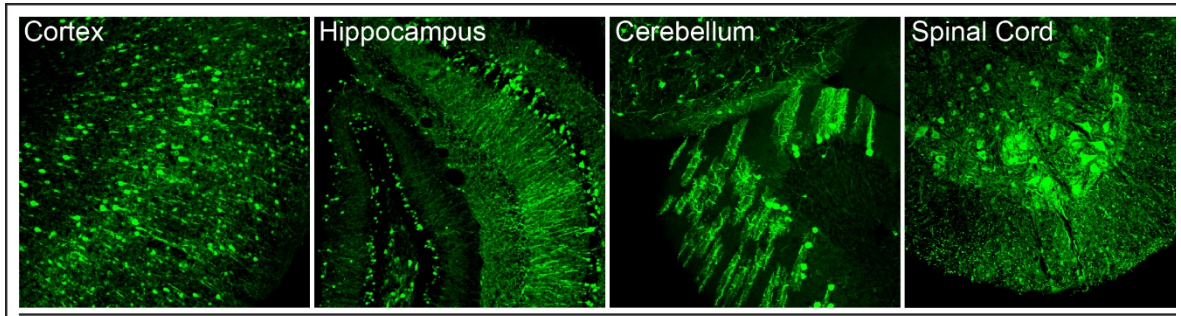




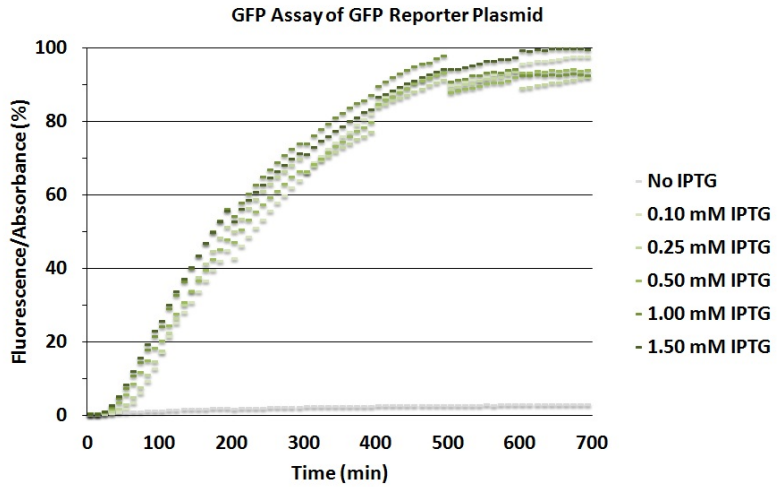


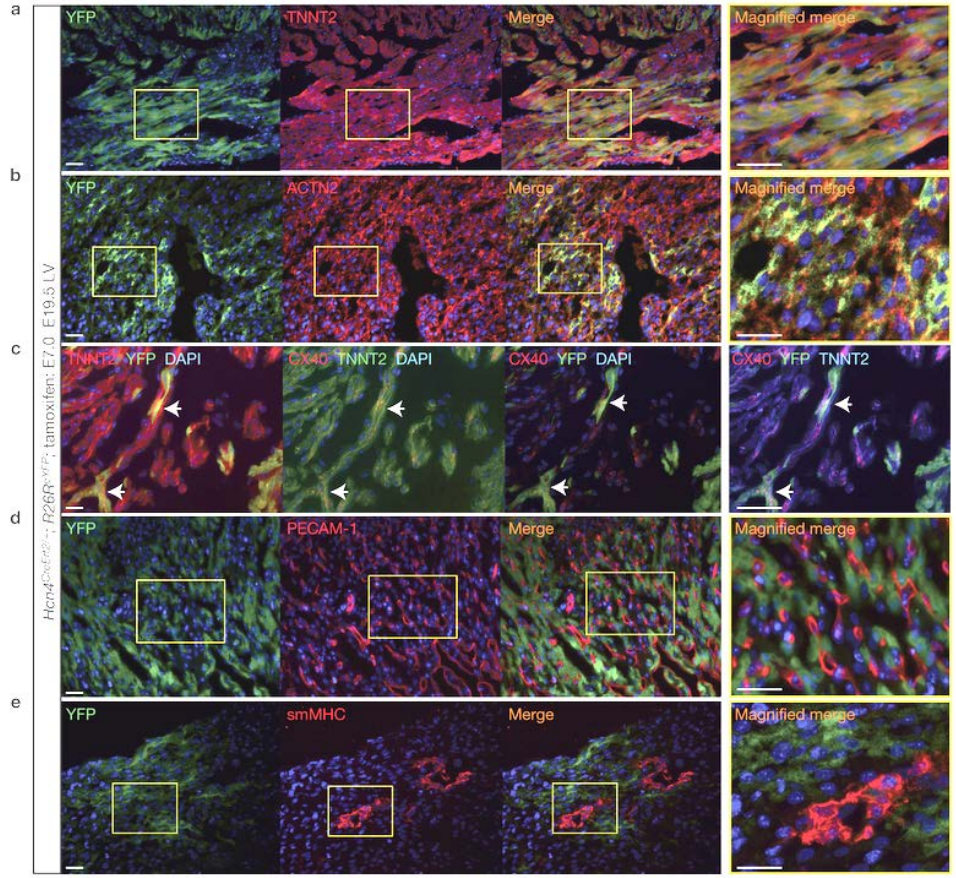


Gene expression



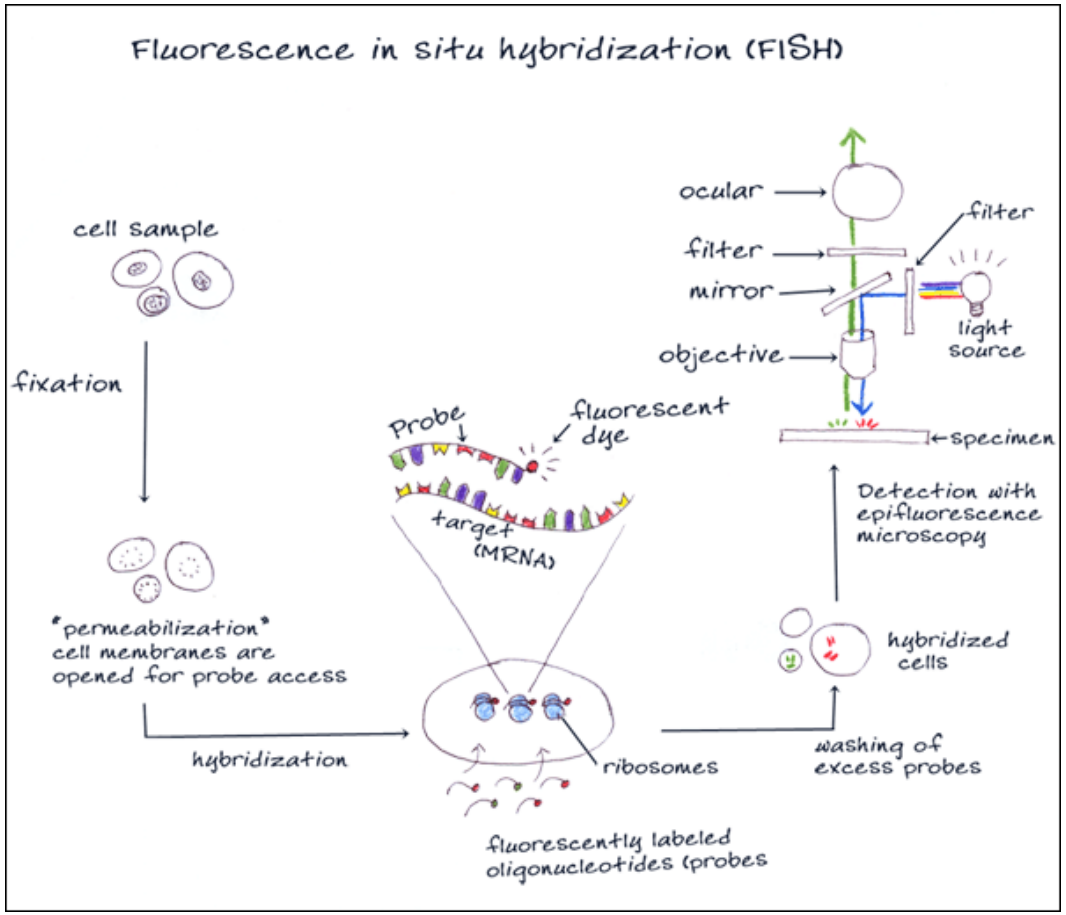
Representative images (10x) of GFP expression (green) in cortex, hippocampus, cerebellum, and cervical spinal cord from 6-week-old mouse which received a bilateral i.c.v. injection of AAV9 encoding the GFP transgene under the hSYN promoter c post-natal day 2. We observed that transgene expression was restricted to neurons throughout the CNS and PNS.

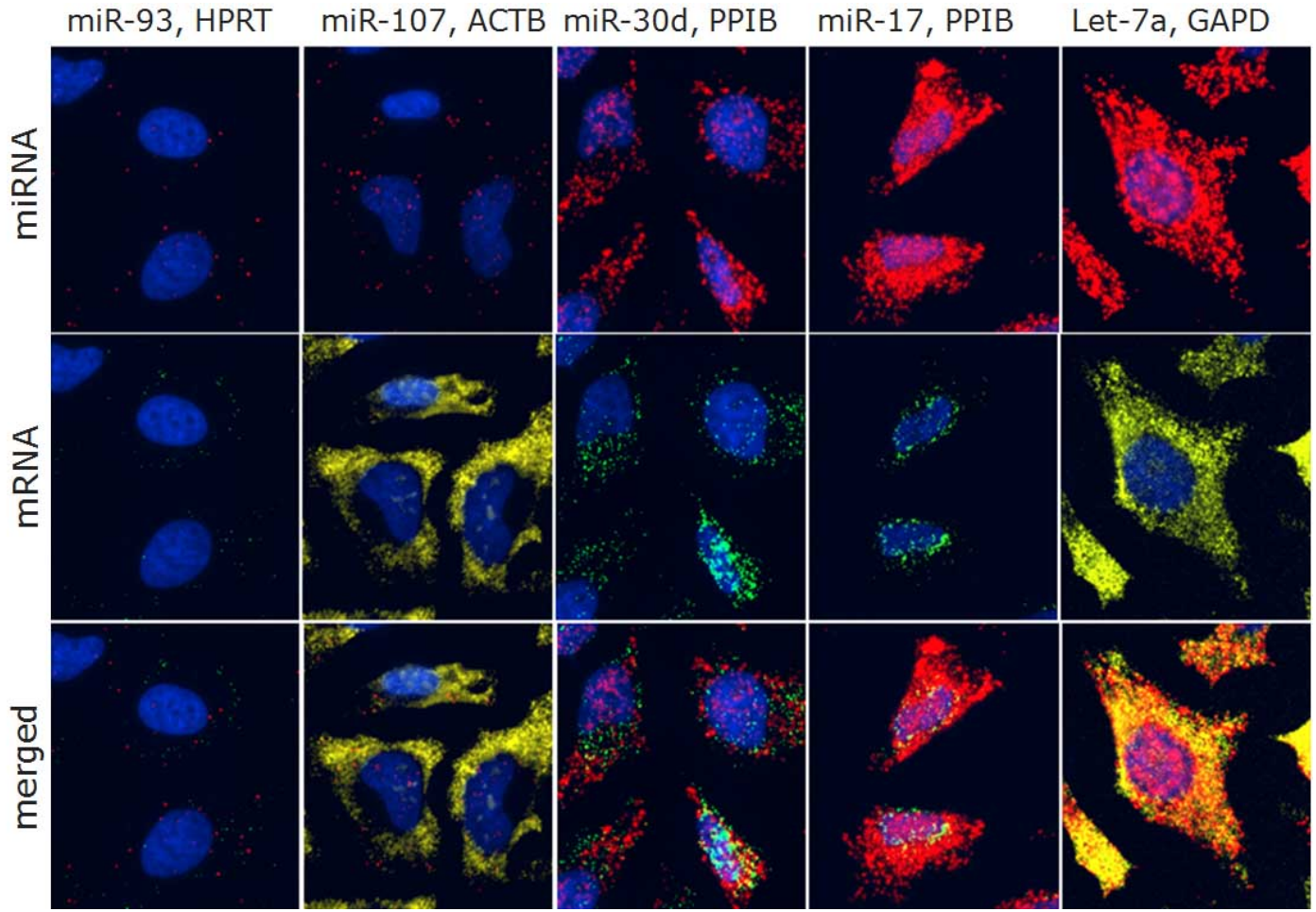




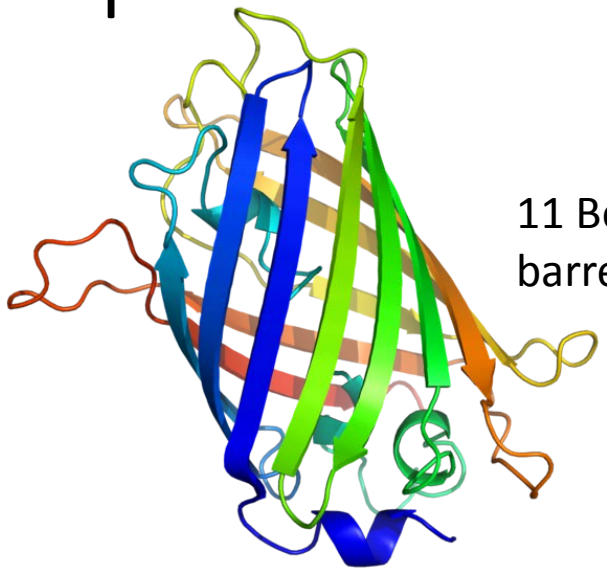
FISH (Fluorescence *in situ* hybridization)

- Track nucleic acids in fixed cells

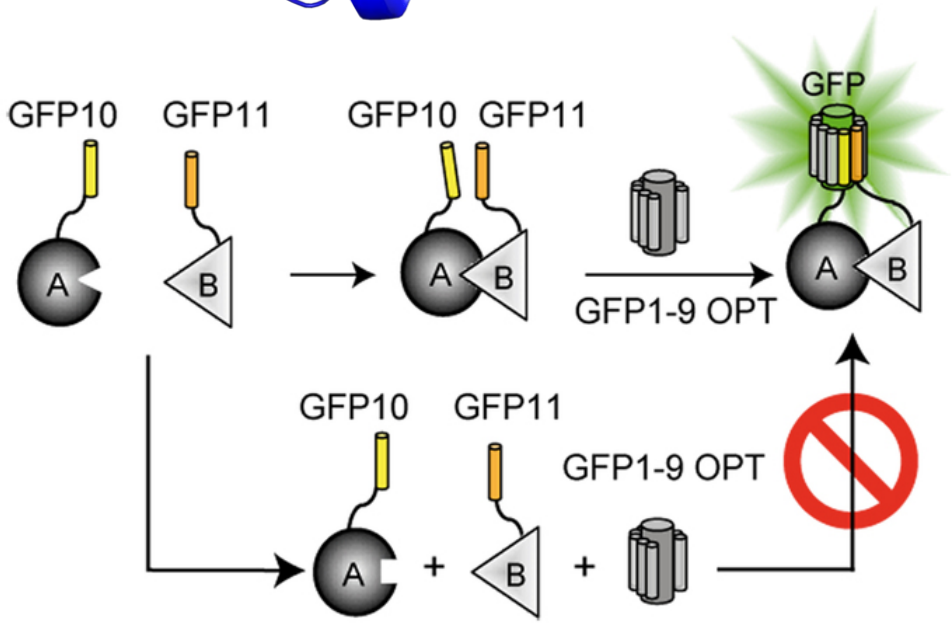


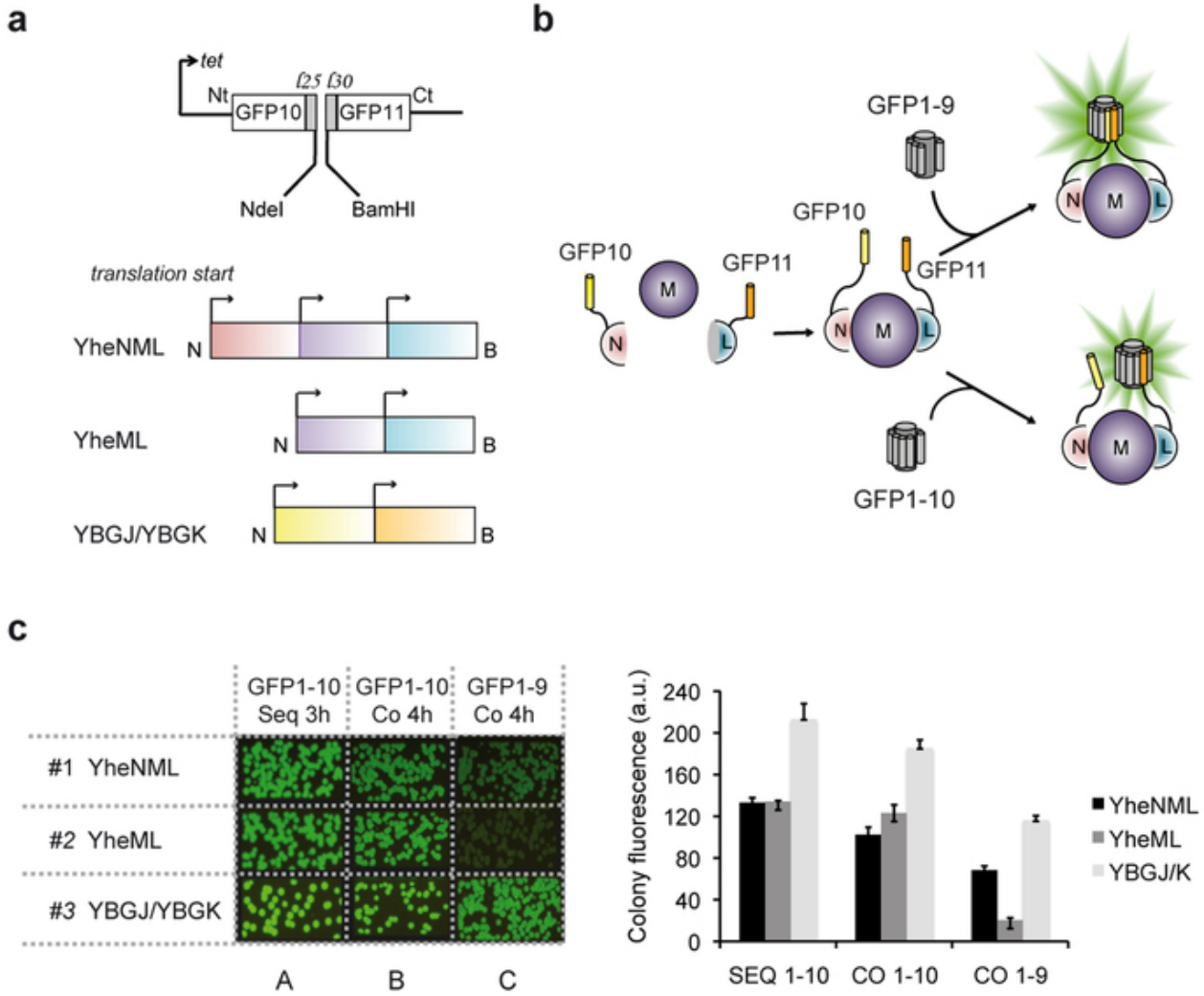


Split GFP



11 Beta-barrel strands



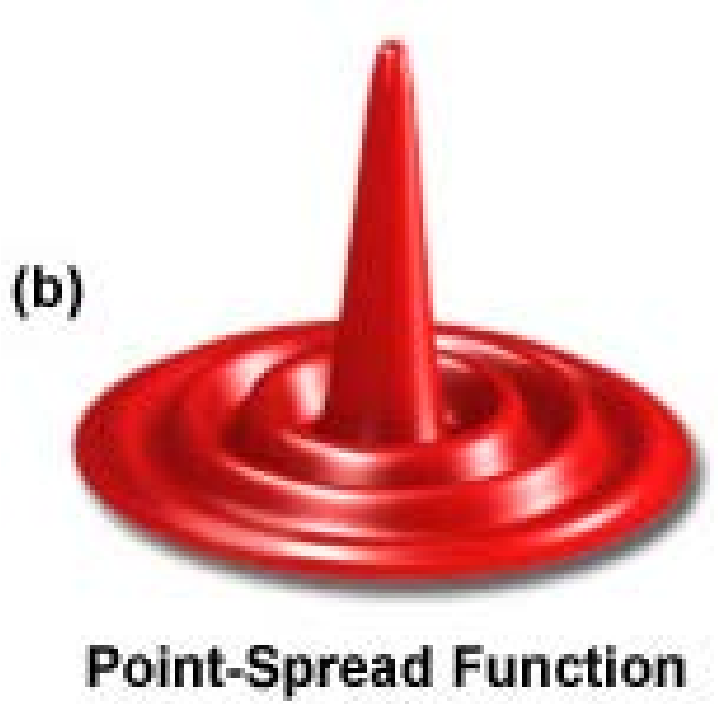


Limitations of fluorescent microscopy in biology

- Bleedthrough
- Autofluorescence
- Photobleaching\Phototoxicity
- Labeling
- Quantitation

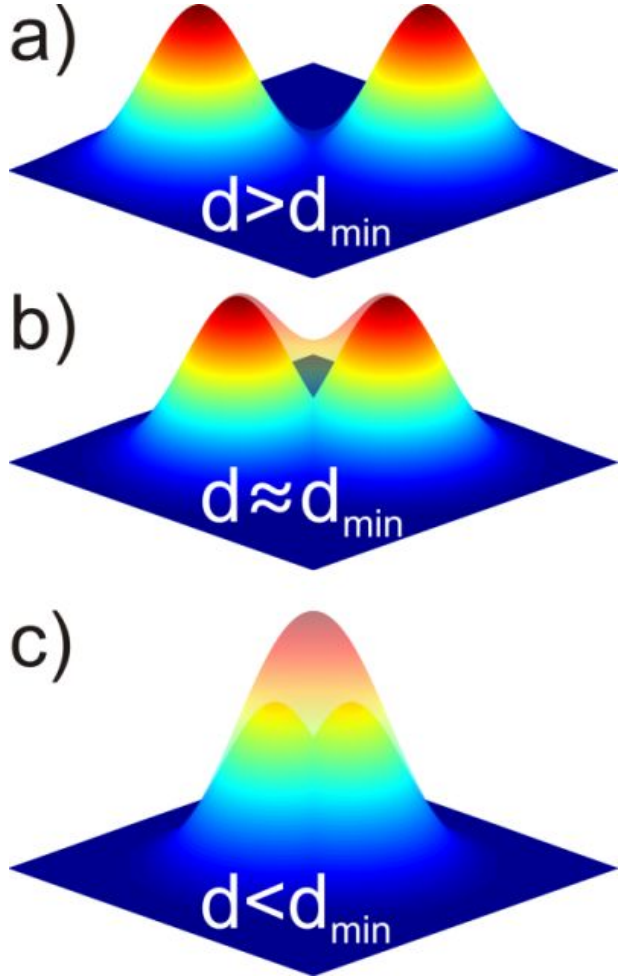
Resolution

- Always have to deal with the diffraction limit (though there are a few tricks)
- Typically on the order of 200-300 nm



Diffraction limited spot

$$d = \frac{\lambda}{2NA}$$



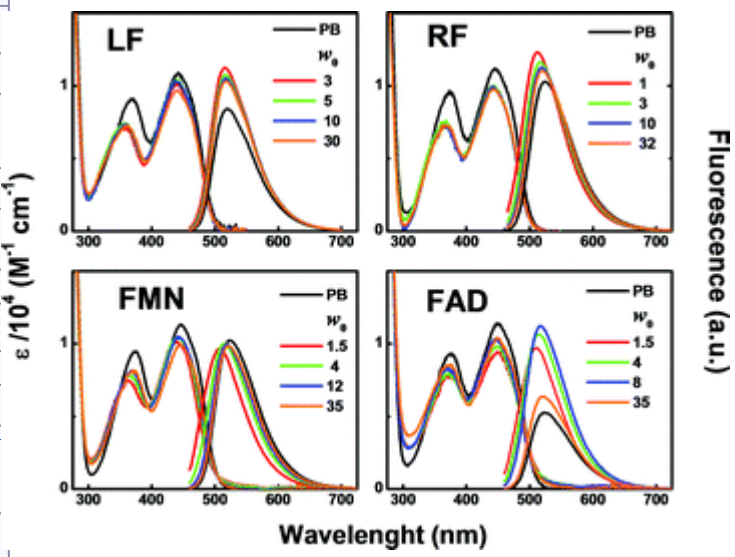
Resolution limit

$$R = \frac{0.61\lambda}{NA}$$

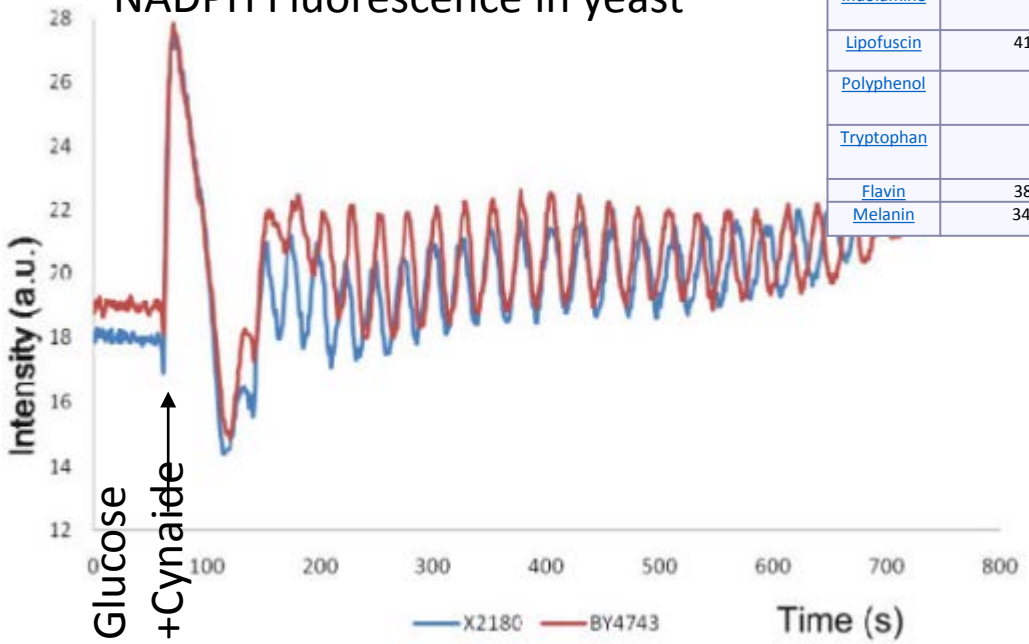
Autofluorescence

- NADPH, flavins
- Amino acids
- Chlorophyll!

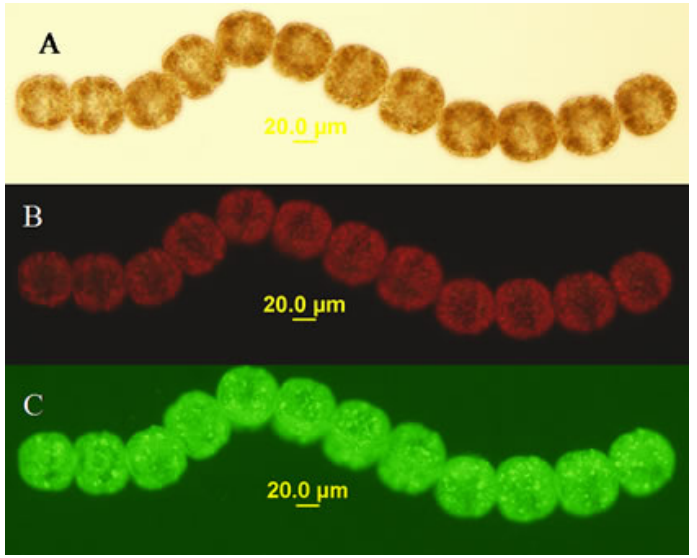
Molecule	Excitation (nm)	Emission (nm)	Organisms	Reference
NAD(P)H	340	450	All	[8]
Chlorophyll	465, 665	673, 726	Plants	
Collagen	270-370	305-450	Animals	[8]
Retinol		500	Animals & bacteria	[9]
Riboflavin		550	All	[9]
Cholecalciferol		380-460	Animals	[9]
Folic acid		450	All	[9]
Pyridoxine		400	All	[9]
Tyrosine	270	305	All	[2]
Dityrosine	325	400	Animals	[2]
Excimer-like aggregate	270	360	Animals	collagen[2]
Glycation adduct	370	450	Animals	[2]
Indolamine			Animals	
Lipofuscin	410-470	500-695	Eukaryotes	[10]
Polyphenol			Plants	
Tryptophan	280	300-350	All	
Flavin	380-490	520-560	All	
Melanin	340–400	360–560	Animals	[11]



NADPH Fluorescence in yeast



Dinoflagellates



Fluorophore bleedthrough

- Fluorescence excitation and emission are broad
- Broad excitation tails will likely hit a bit of each fluorophore
- Can try to get around it with clever choice of excitation and emission filters

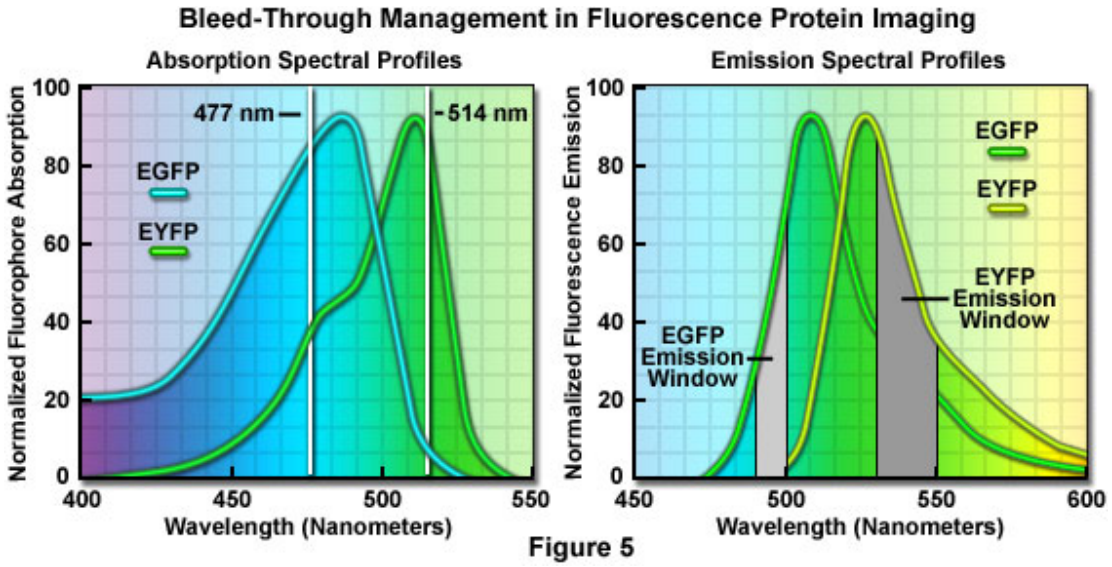


Figure 5

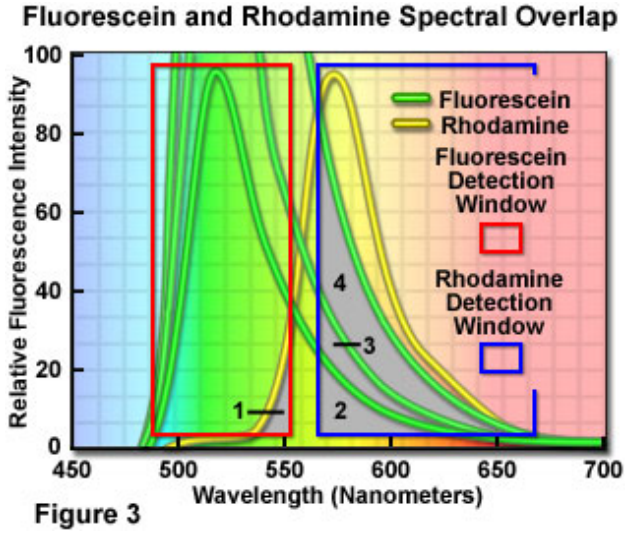


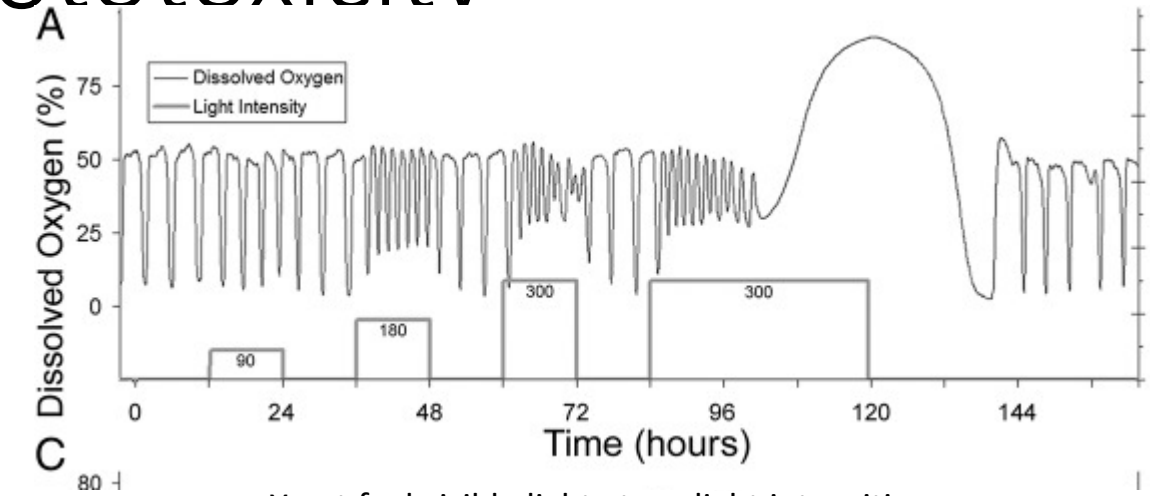
Figure 3

Allowable sets

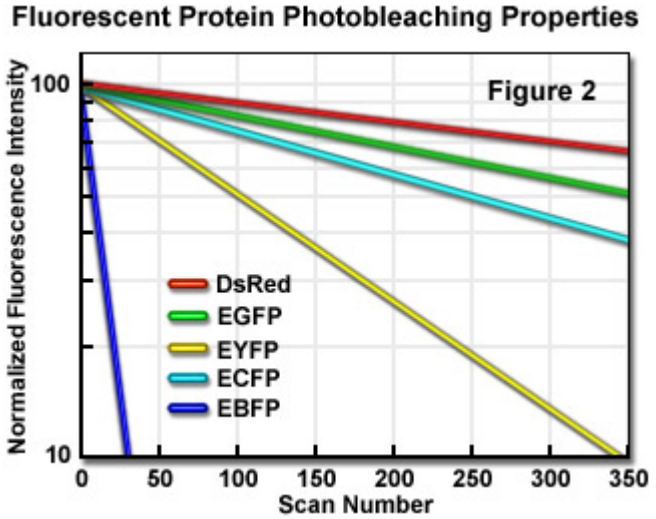
CFP/YFP
GFP/mCherry
Cy3/Cy5
FITC/TRITC

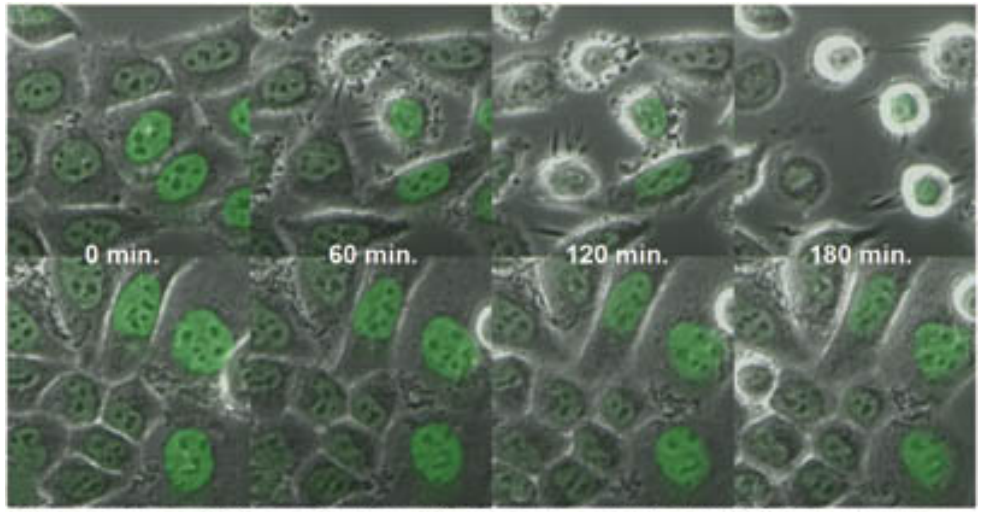
Photostability and phototoxicitv

- All fluorophores photobleach
- Autofluorescent molecules photobleach too
- Often generate free radicals
- Toxic to cells!
- “Dim” exposure to light can trigger



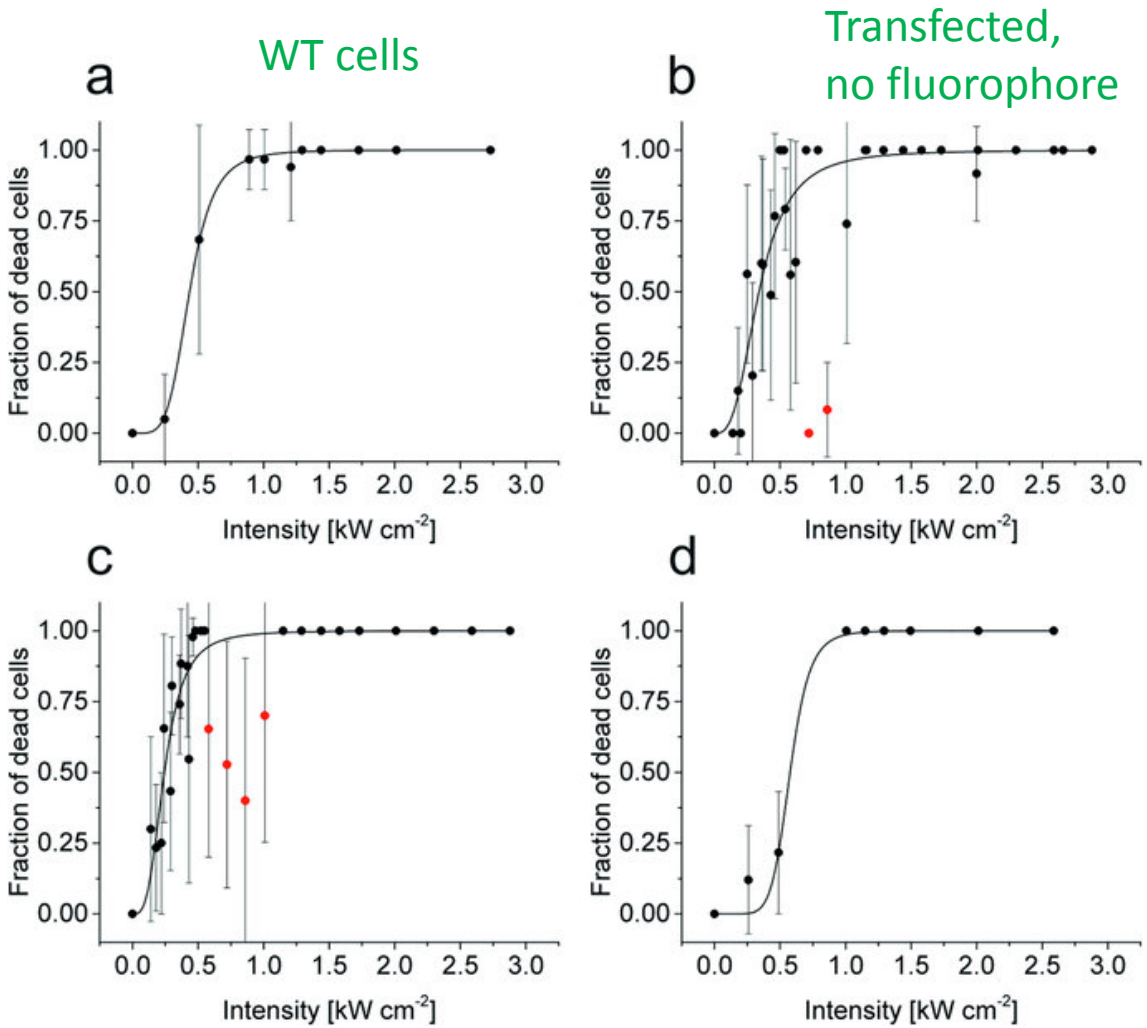
Yeast feel visible light at sunlight intensities

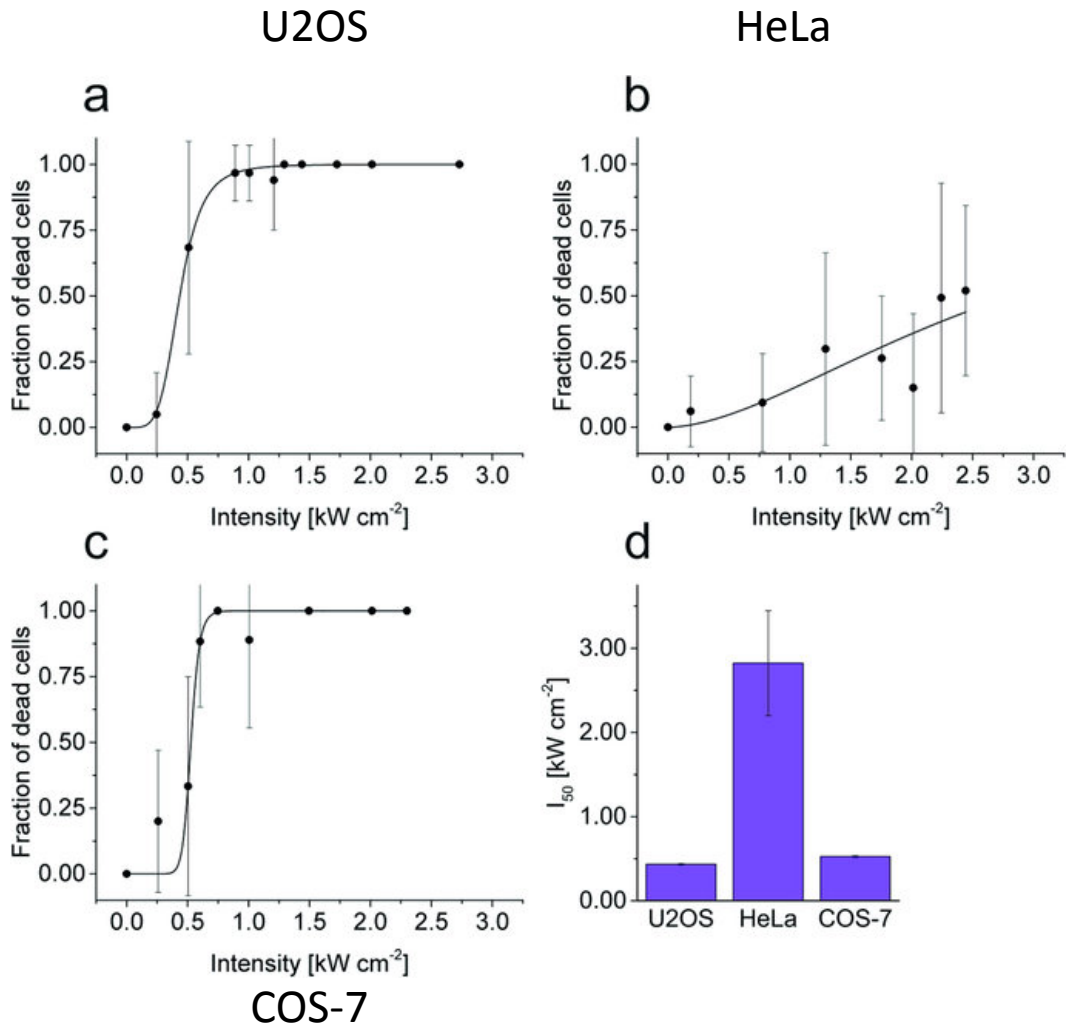




Time-lapse acquisition of HeLa cells expressing GFP tagged histone-2B. The transmitted light and fluorescence images were simultaneously acquired in the absence (A) or presence (B) of CLEM.

Quantitative phototoxicity





And on to Matlab…