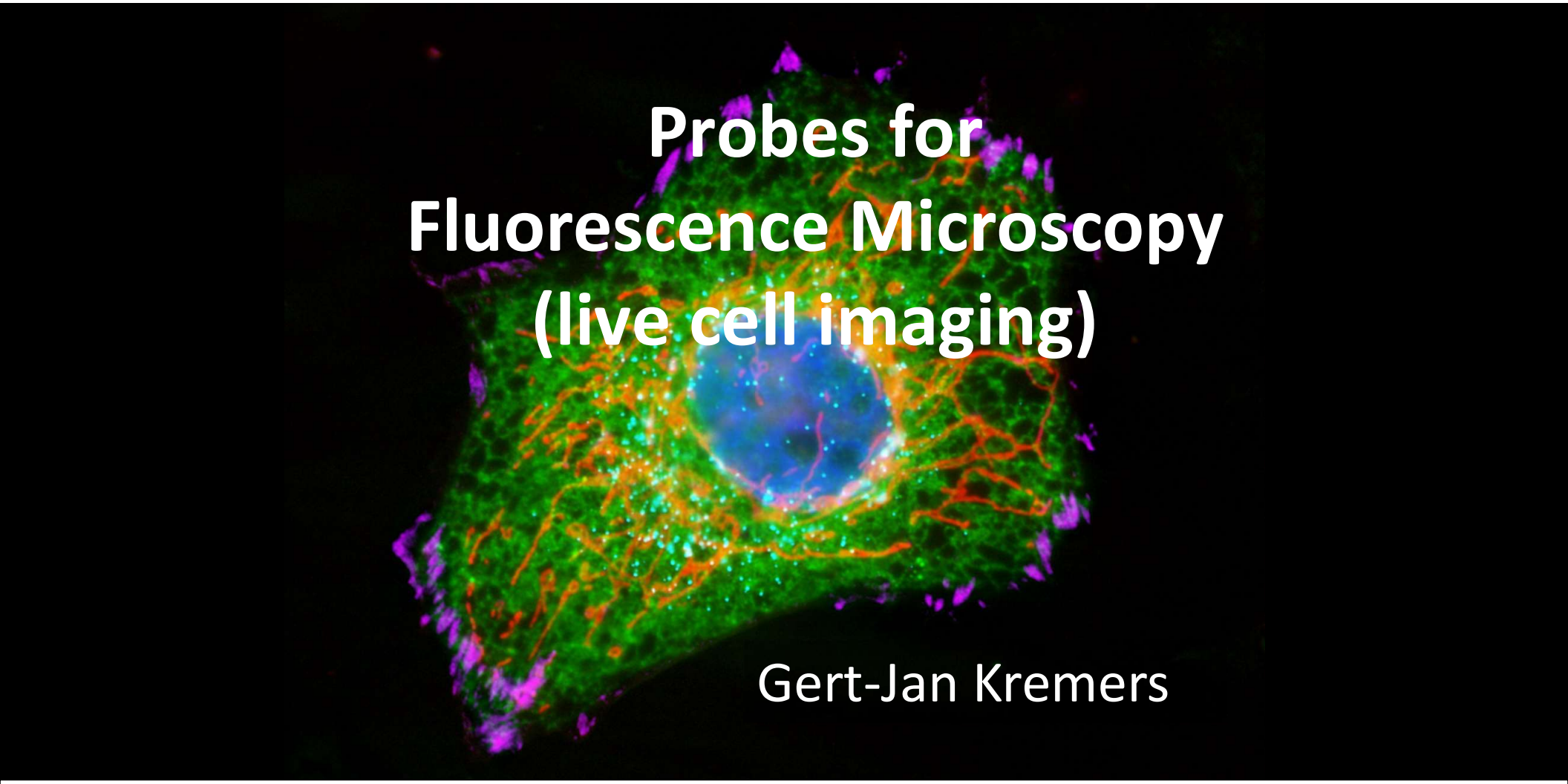


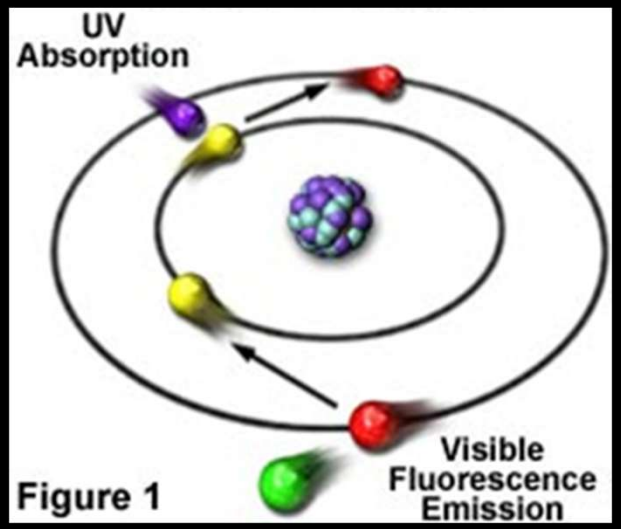
Probes for Fluorescence Microscopy (live cell imaging)

Gert-Jan Kremers

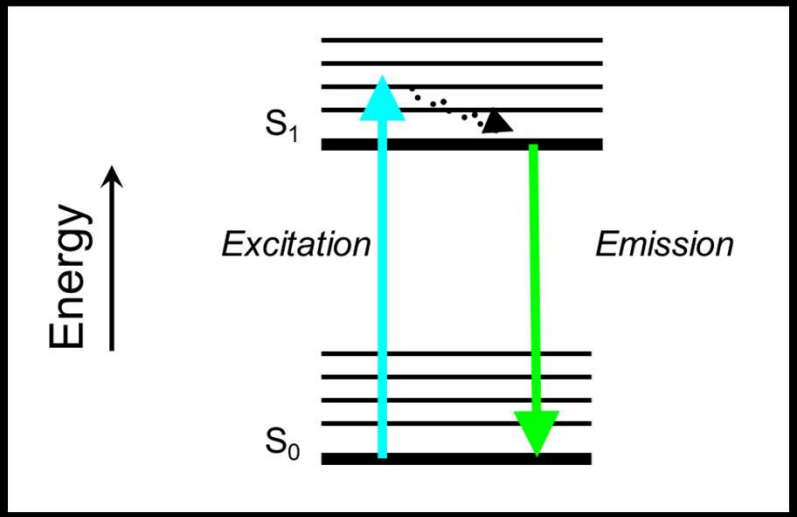
Erasmus Optical Imaging Centre

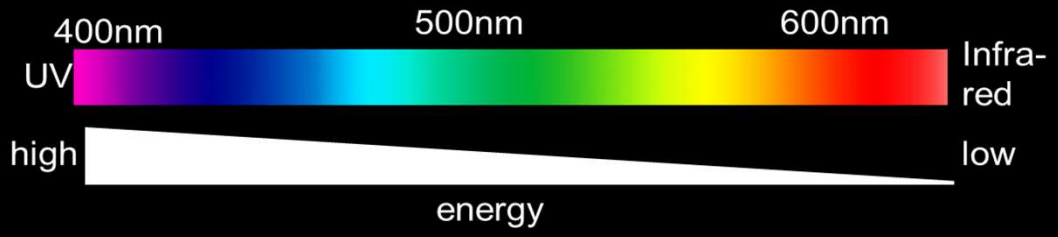
PhD imaging course, November 9th 2026

Fluorescence

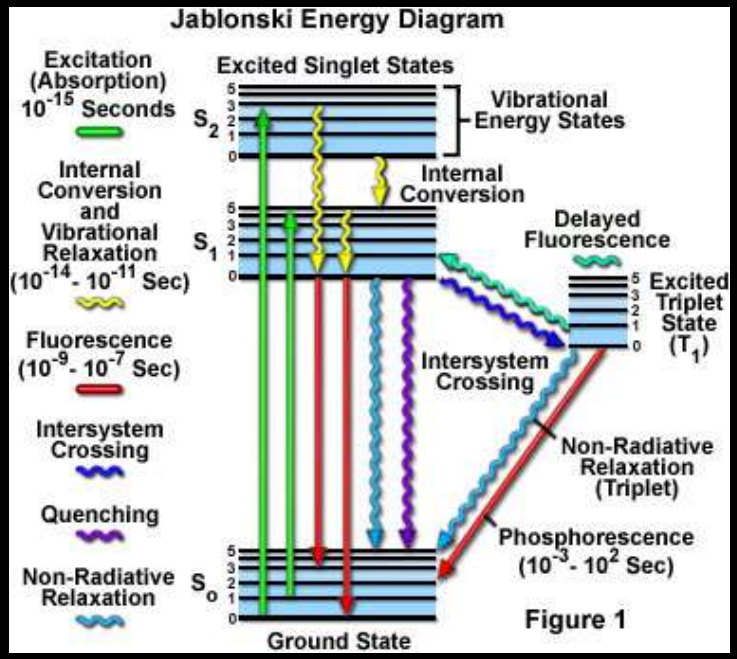


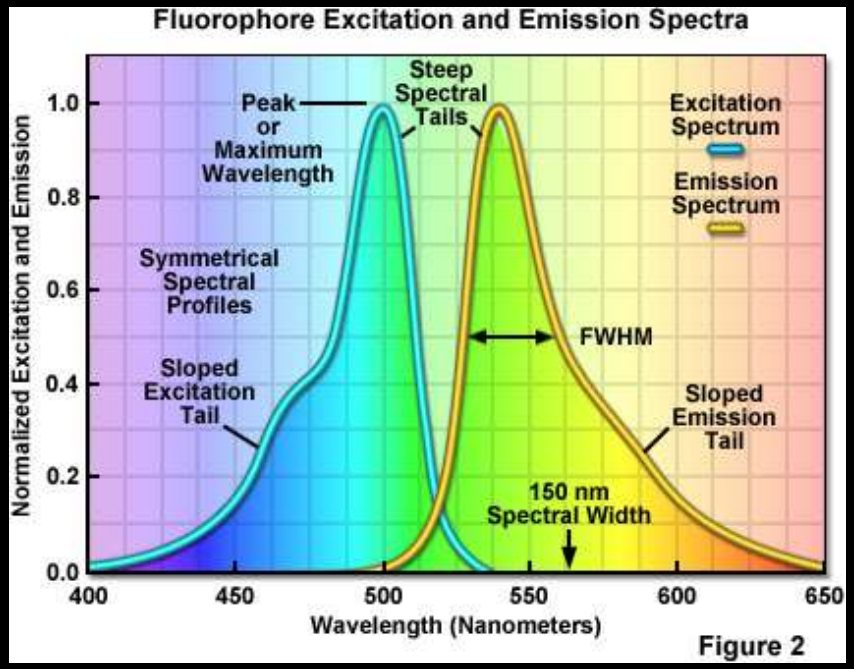
Jablonski diagram



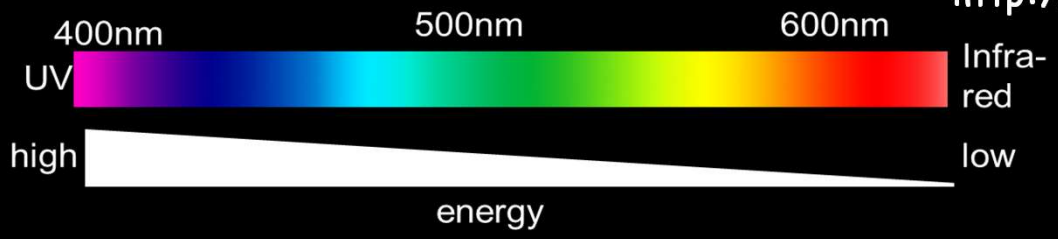


Fluorescence

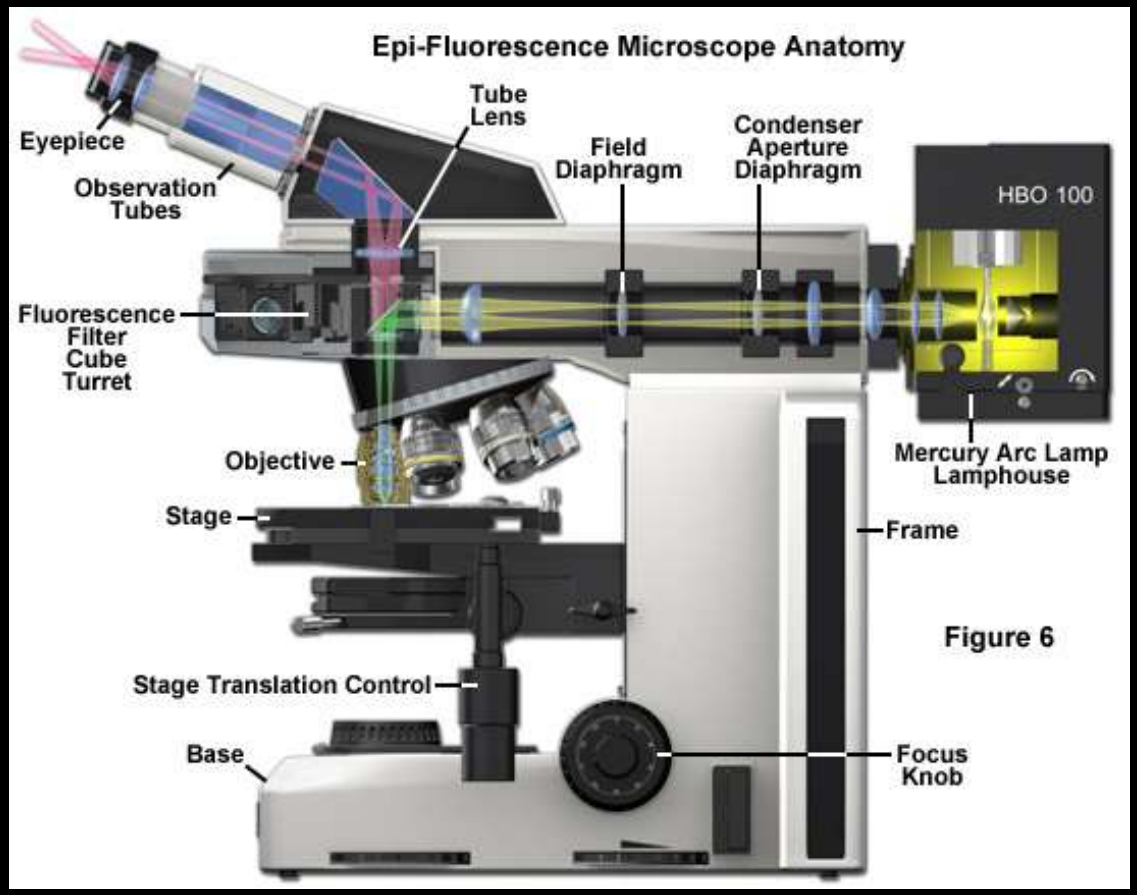




http://www.microscopyu.com/

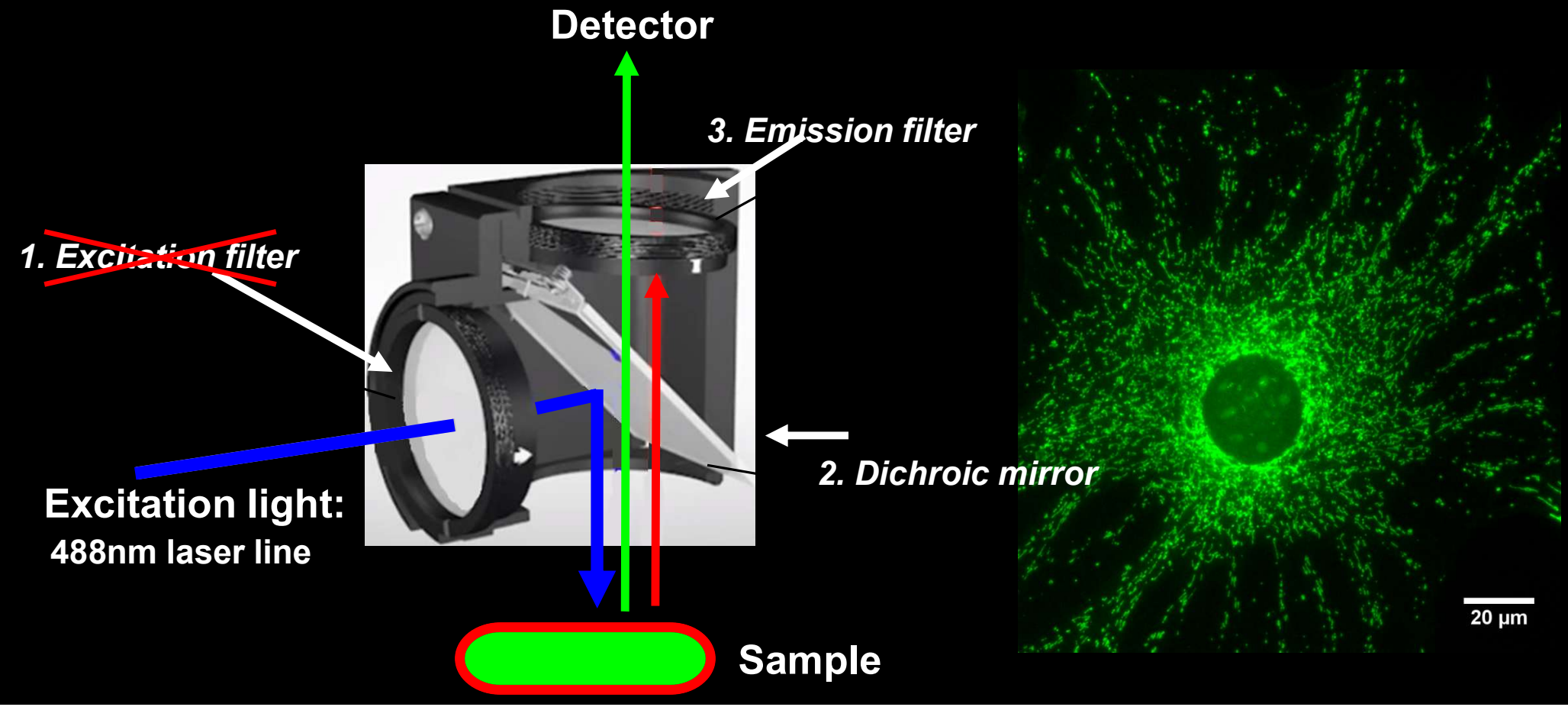


Fluorescence microscope

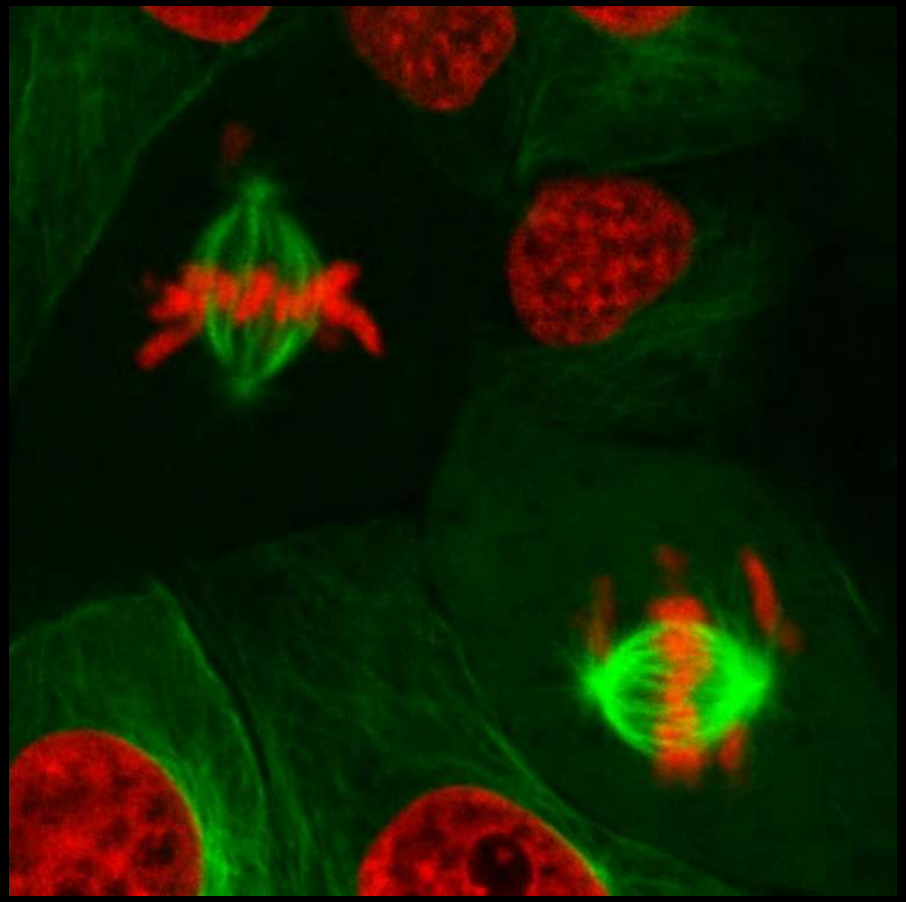


http://zeiss-campus.magnet.fsu.edu

Fluorescence lightpath



Live cell imaging

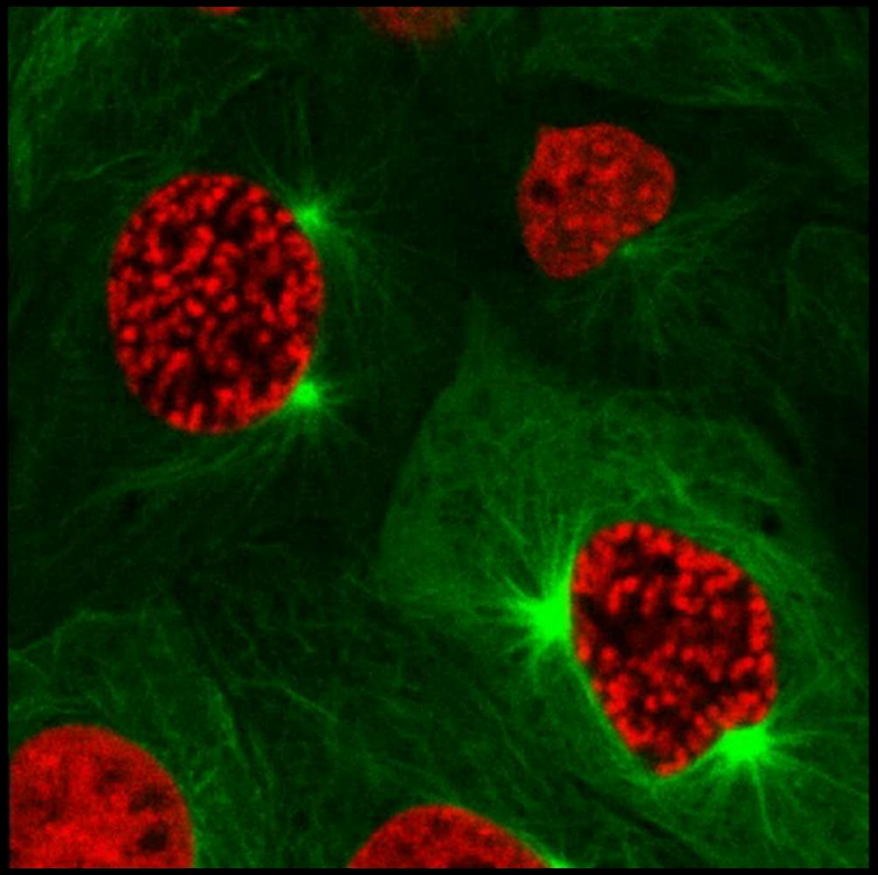


Mike Davidson, Florida State University

LLC-PK1 (Pig Kidney) Cells
EGFP-Tubulin
mCherry-H2B

Will these metaphase cells complete cell division ?

Live cell imaging



Mike Davidson, Florida State University

LLC-PK1 (Pig Kidney) Cells
EGFP-Tubulin
mCherry-H2B

Live cell imaging

- Spatial information (Where?)
- Temporal information (When?)
- Functional imaging

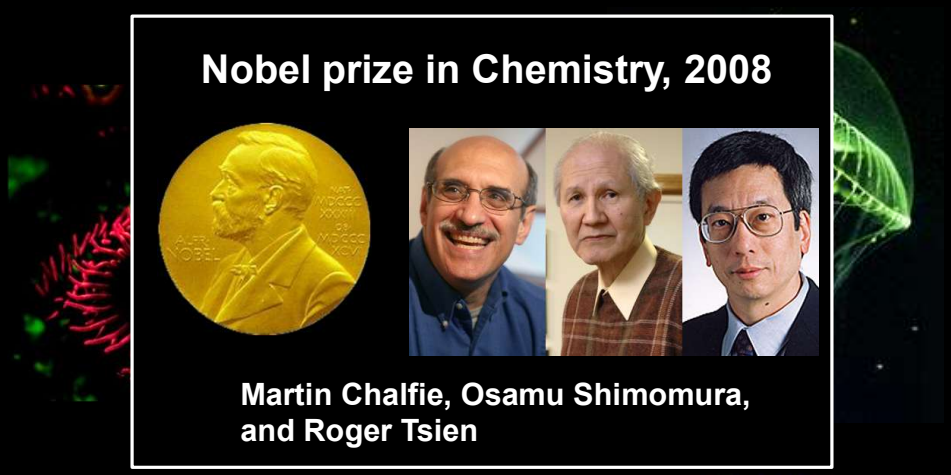
Probes for live cell imaging

- Fluorescent proteins
- Cell permeable organic dyes
- Chimeric approaches (part genetic / part organic)

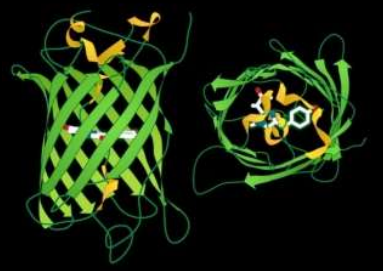
Fluorescent Proteins (FPs)

- **Fluorescent proteins isolated from jellyfish, corals and many other (marine) animals**

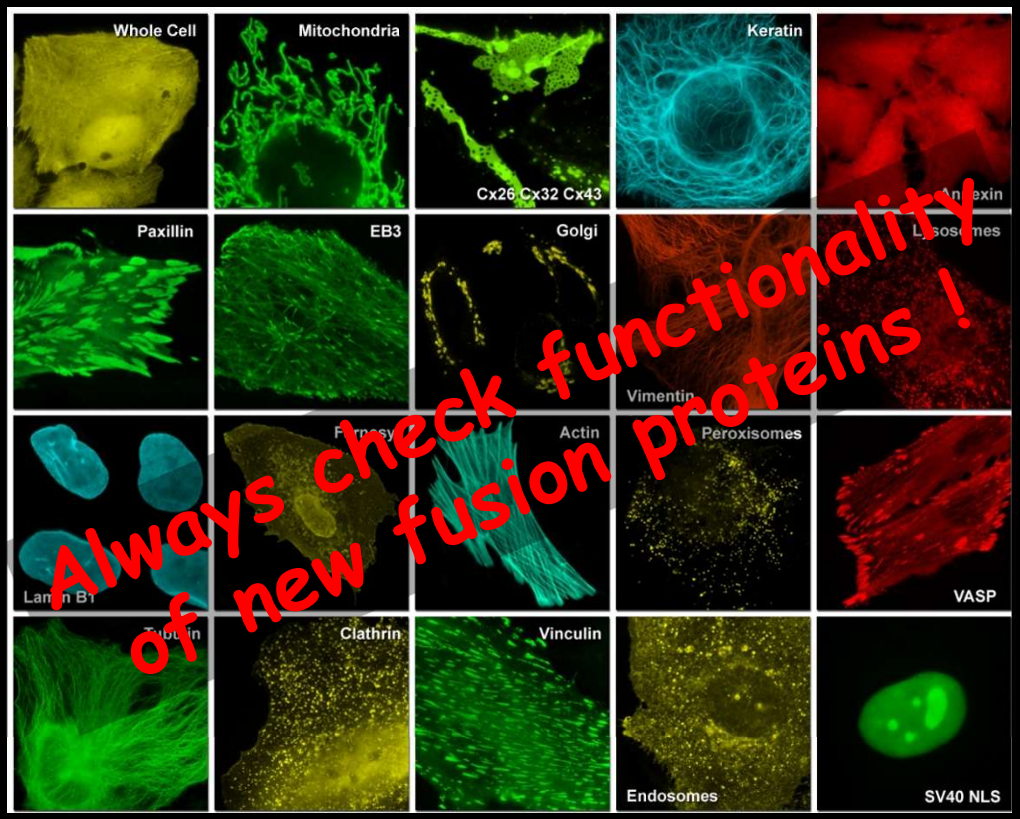
- **Spectral variants:**
  - EGFP (Green)
  - TagBFP2 (Blue)
  - mTurquoise3 (Cyan)
  - mVenus (Yellow)
  - mScarlet3 (Red)



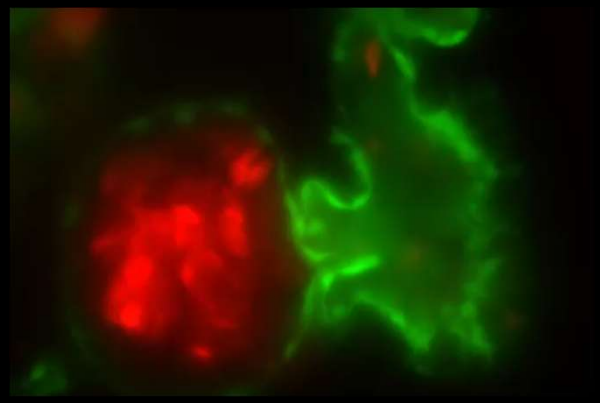




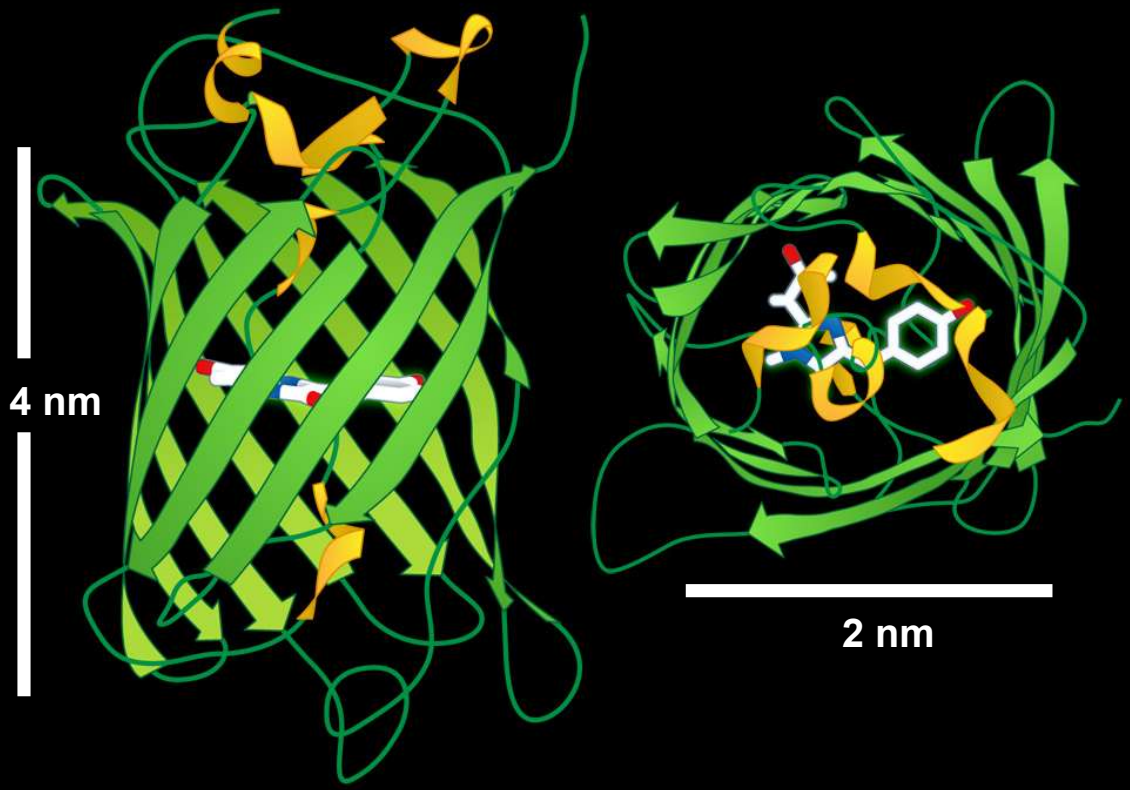
Genetic labeling







EGFP structure

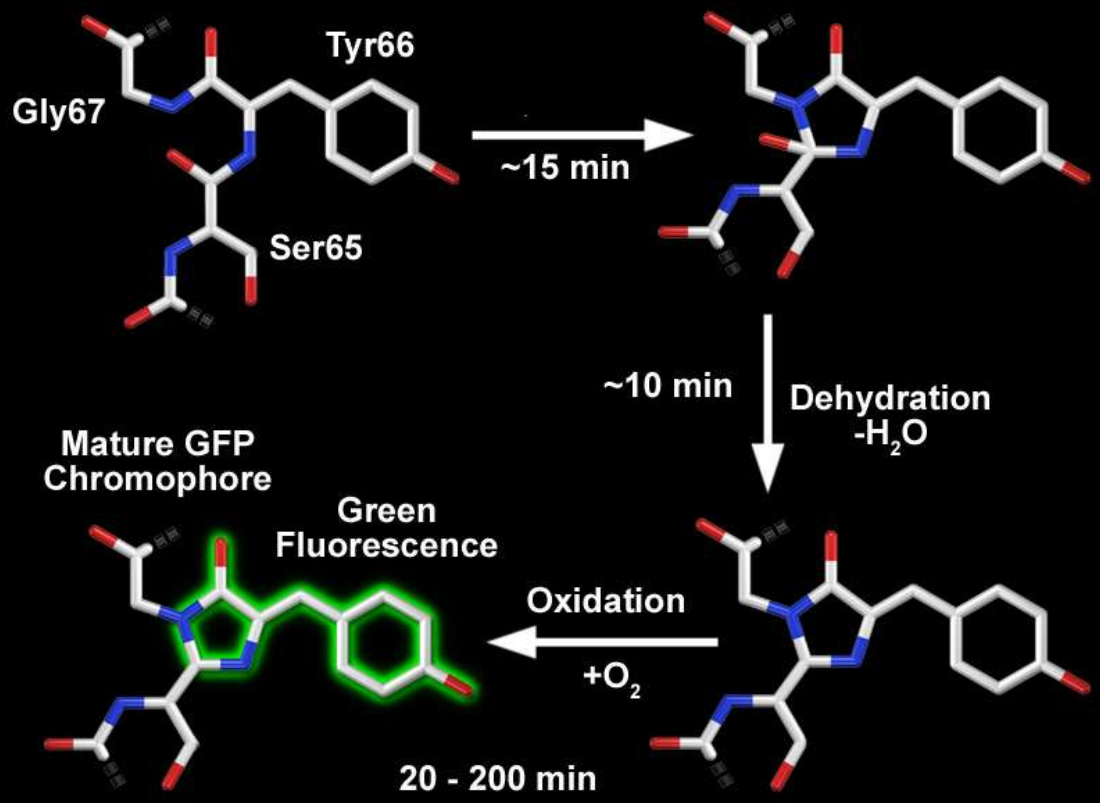




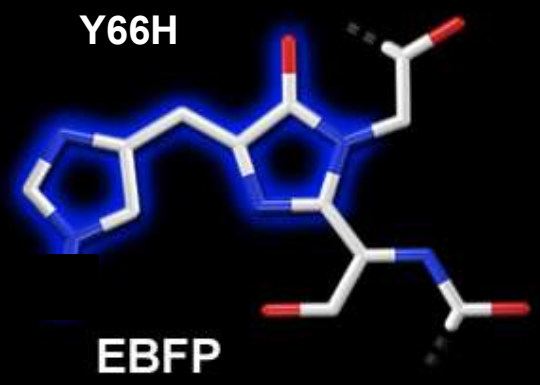
Aquoria victoria

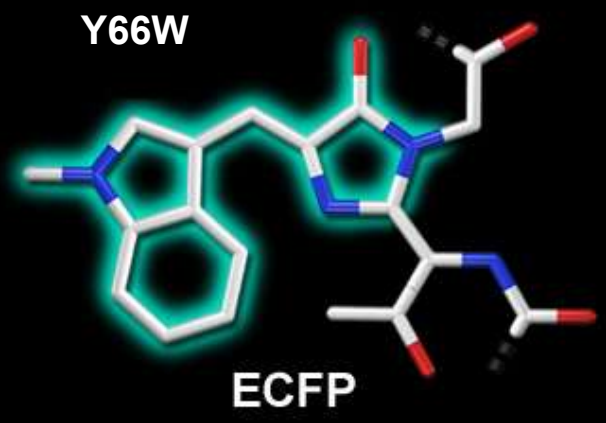
717 bp
238 aa
27 kDa

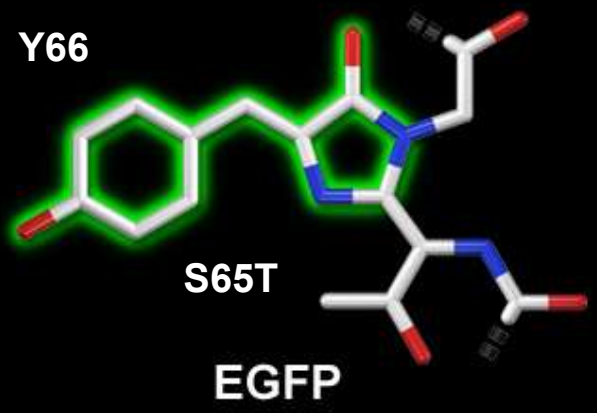
Chromophore formation

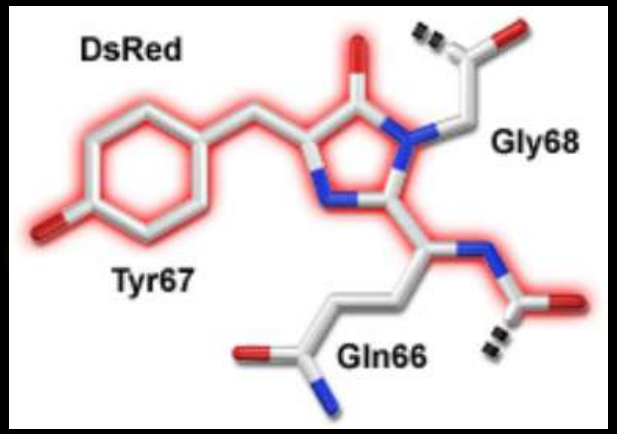


GFP color variants



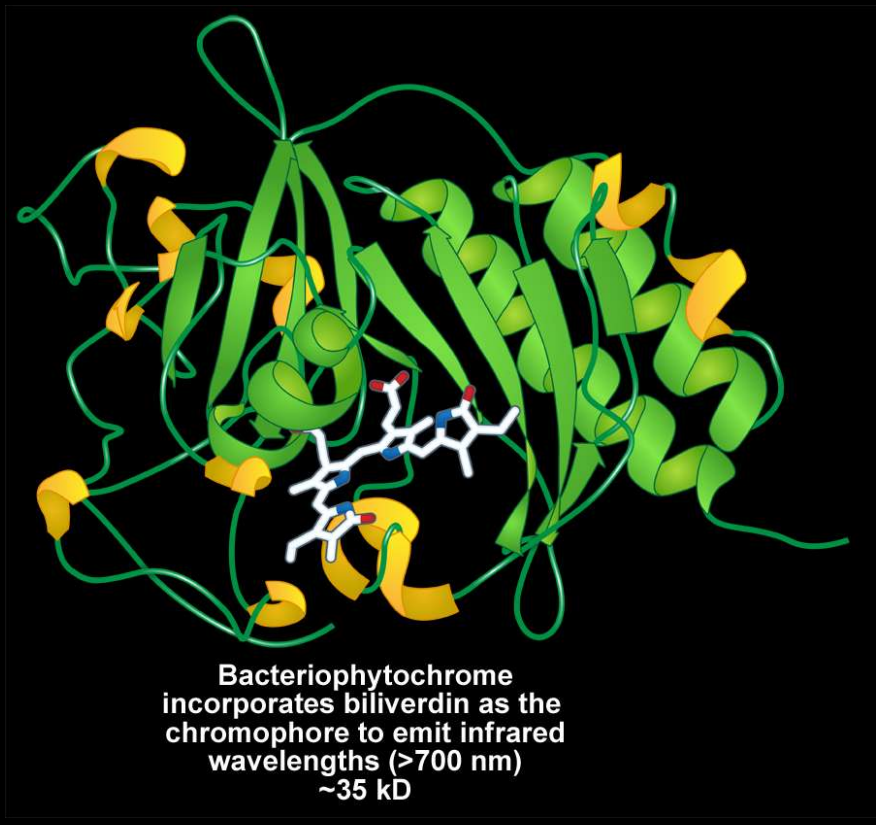






near-infrared FPs

- **iRFPs**
  - Emit fluorescence >700nm
  - Derived from bacteriophytochromes
  - Biliverdin co-factor required
  - Is endogenously present in mammalian cells
  - ~35kD

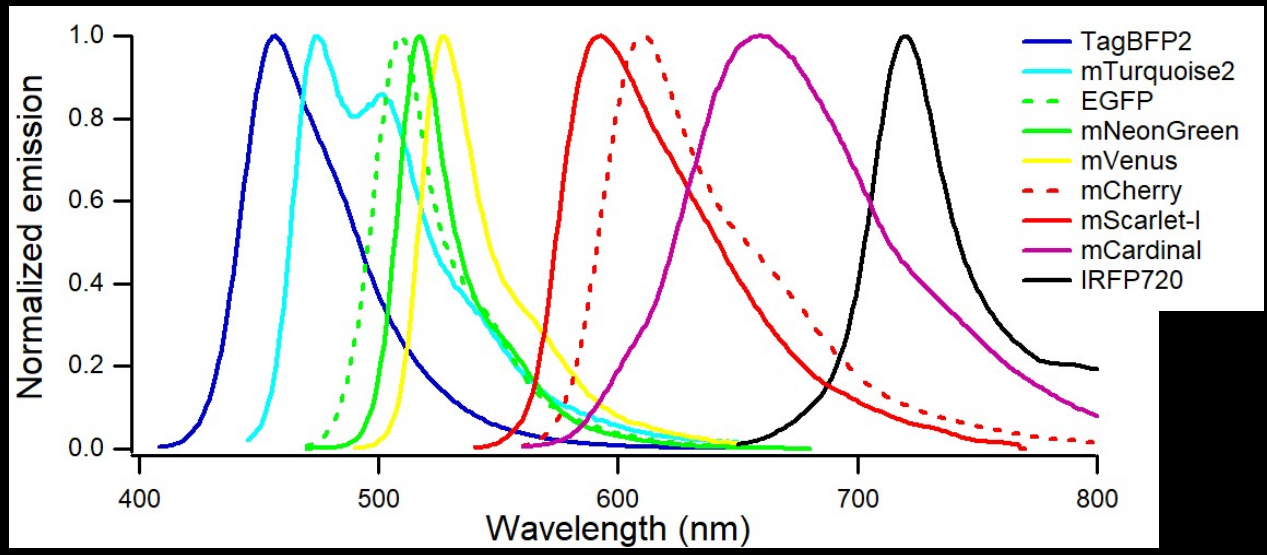


Bacteriophytochrome incorporates biliverdin as the chromophore to emit infrared wavelengths (>700 nm) ~35 kD

How to choose the “best” FP?

- **Color**
- **Brightness (Ext Coeff x QY)**
- **Photostability**
- **Oligomerization**

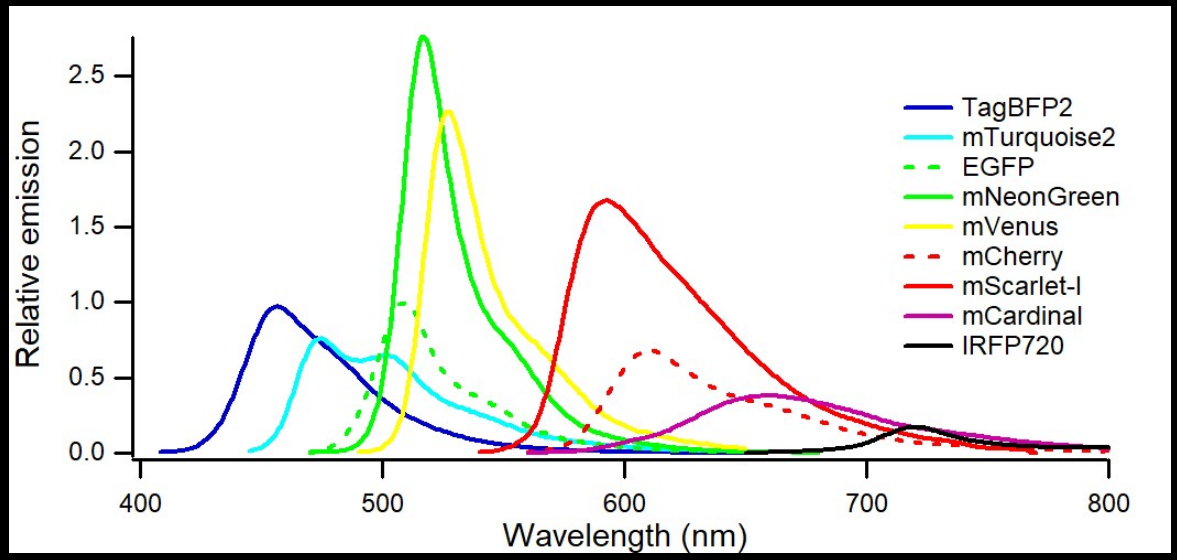
Application and microscope!!!



Adapted from Cranfill et al Nat Methods 2016

How to choose the “best” FP?

- **Color**
- **Brightness (Ext Coeff x QY)**
- **Photostability**
- **Oligomerization**



Adapted from Cranfill et al Nat Methods 2016

How to choose the “best” FP?

Fluorescent protein properties

Name	Excitation maximum (nm)	Emission maximum (nm)	Extinction Coefficient x10^3 ($M^{-1}cm^{-1}$)	Quantum yield	Relative brightness (% of EGFP)
mTagBFP2	400	454	76	0.48	97
mTurquoise2	434	473	31	0.93	77
EGFP	488	508	56	0.67	100
mNeonGreen	506	517	116	0.80	247
mStayGold	499	510	164	0.83	363
mVenus	515	528	127	0.67	227
mKO2	551	565	105	0.57	160
~~mCherry~~	~~586~~	~~610~~	~~85~~	~~0.30~~	~~68~~
mScarlet3	569	592	104	0.75	208
mCardinal	603	651	79	0.18	38
miRFP720	702	720	98	0.06	15.7

How to choose the “best” FP?



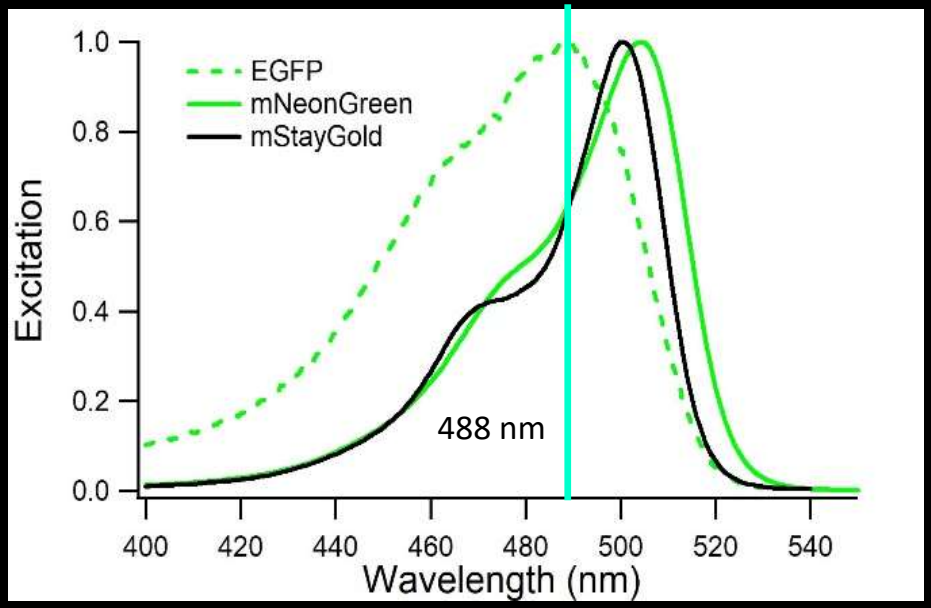
fpbase.org

Brightness

Fluorescent protein properties

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mCardinal	603	651	79	0.18	38
miRFP720	702	720	98	0.06	15.7

Brightness



EGFP: 100% x 1.00 = 100%
mNeon: 247% x 0.61 = 151%
mStayGold: 363% x 0.56 = 203%

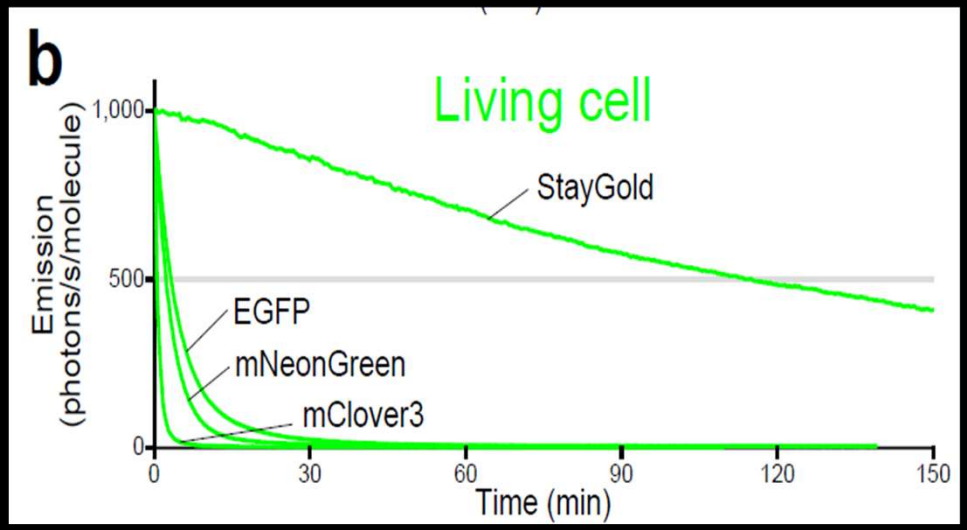
mNeon "only" 1.5x brighter than EGFP
mStayGold "only" 2x brighter than EGFP

Photostability



https://www.nature.com/articles/s41592-023-02085-6#Abs1

Photostability

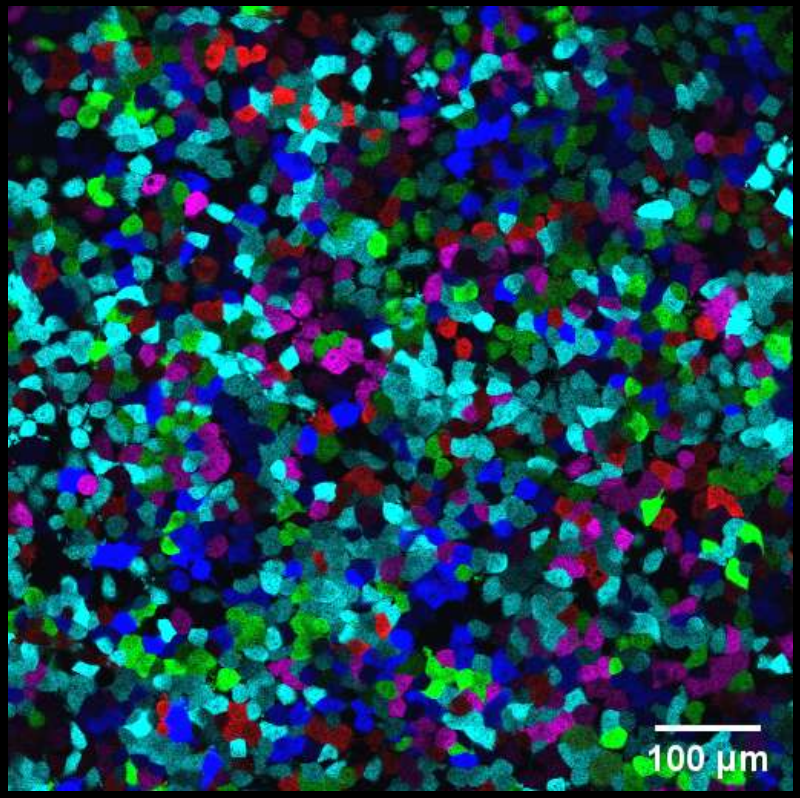


Widefield illumination

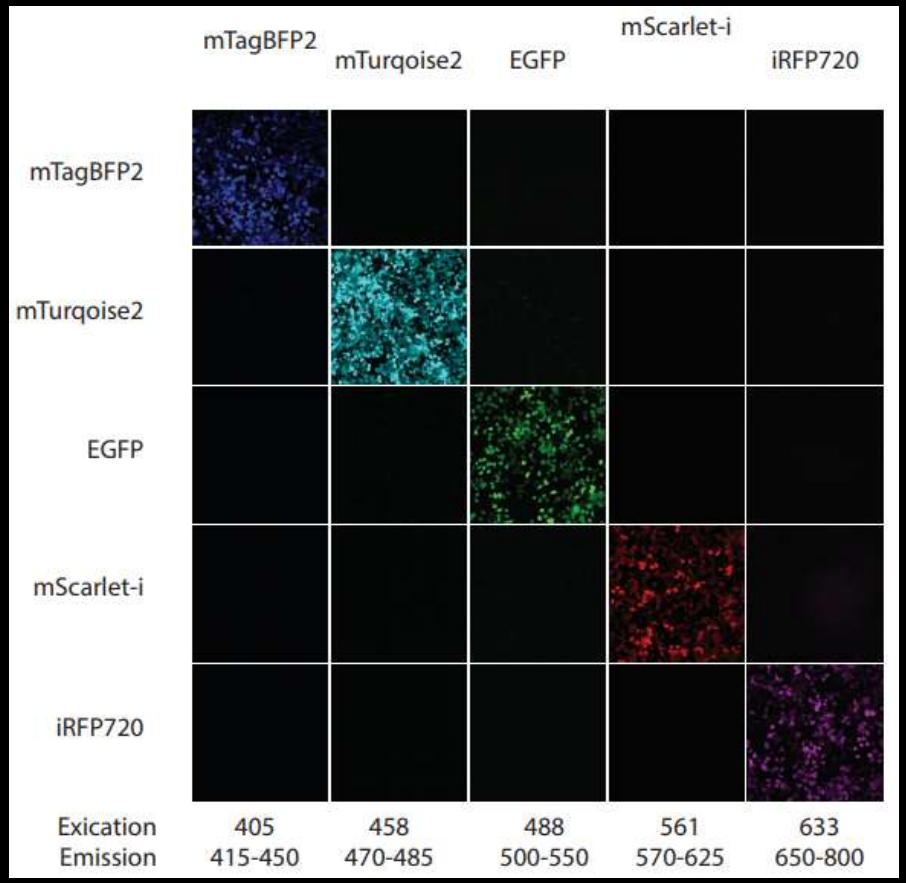
	WF	SpinD	LSCM
StayGold	34.6	5.8	2.2
EGFP	1.0	1.0	1.0
mNeonGreen	0.7	0.6	0.7

https://www.biorxiv.org/content/10.1101/2023.11.23.568490v1

5 color cell lines



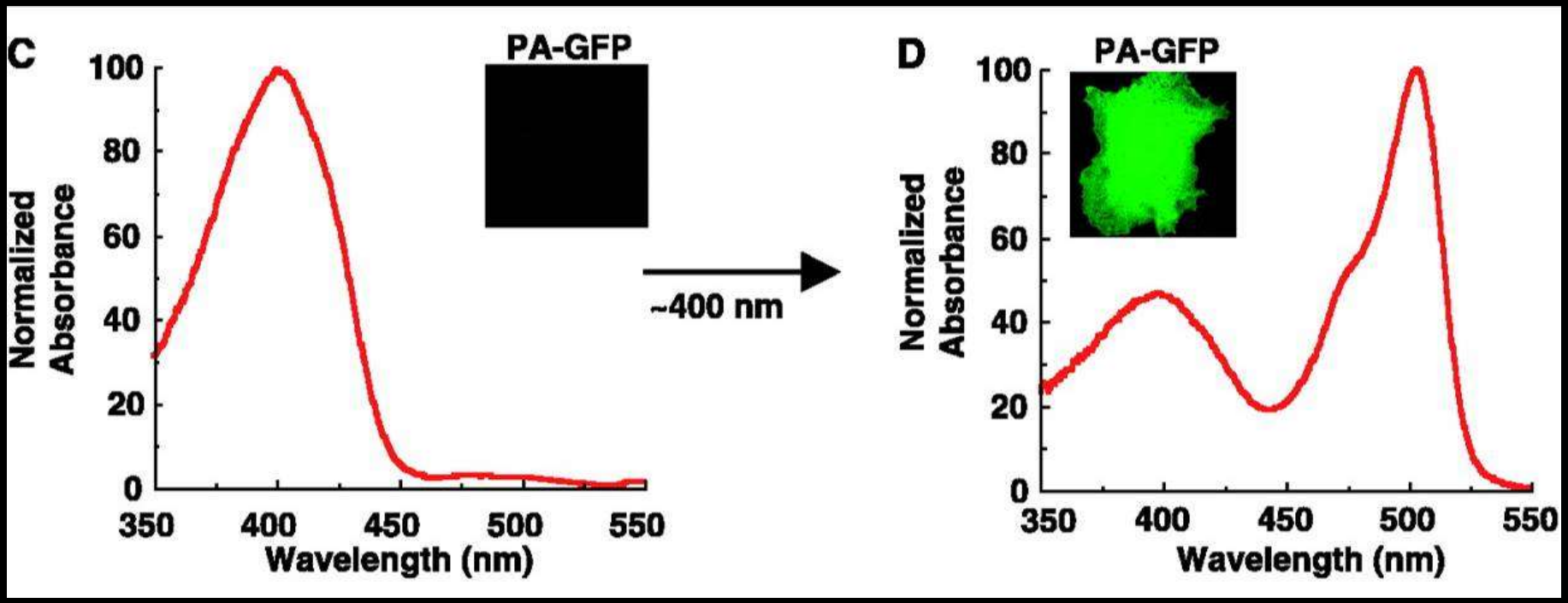
Maayke Kuijten, Mol. Genetics



Optical highlighter FPs

- **Optical highlighter FPs**
  - Change in fluorescence upon (typically) 405nm illumination
  - Photoactivatable FPs: to green (PA-GFP)
  - Photoconvertible FPs: green to red (mEos3.2, mMaple3)

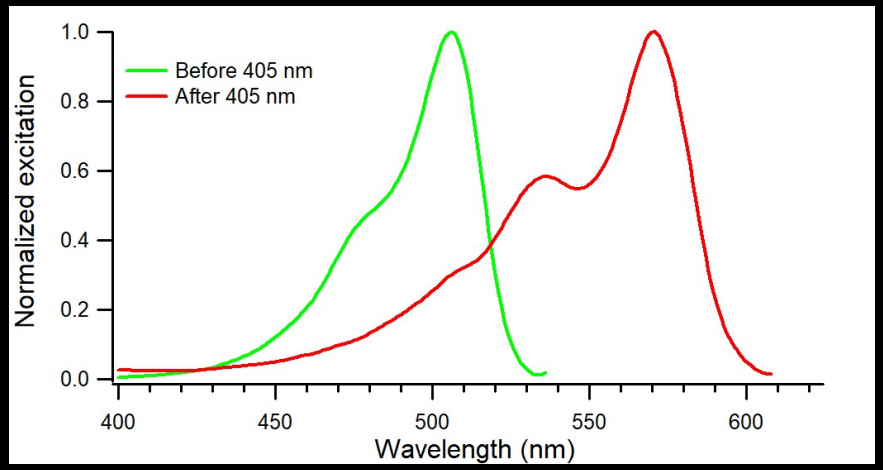
Photoactivation



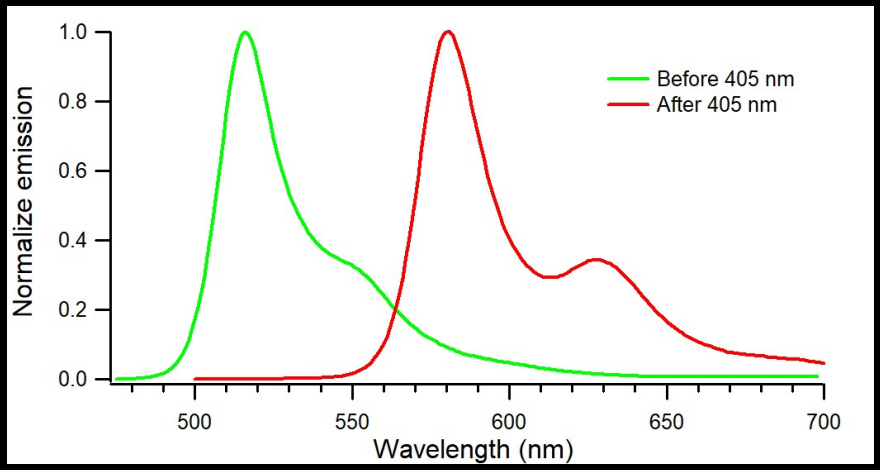
Patterson and, Lippincott-Schwartz 2002 Science. 297

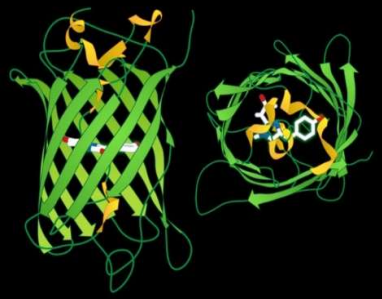
Photoconversion

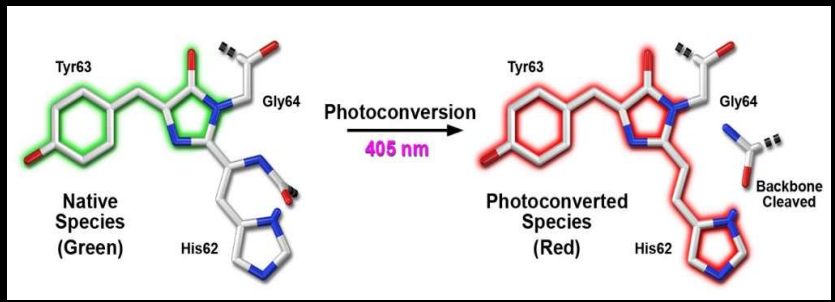
Excitation



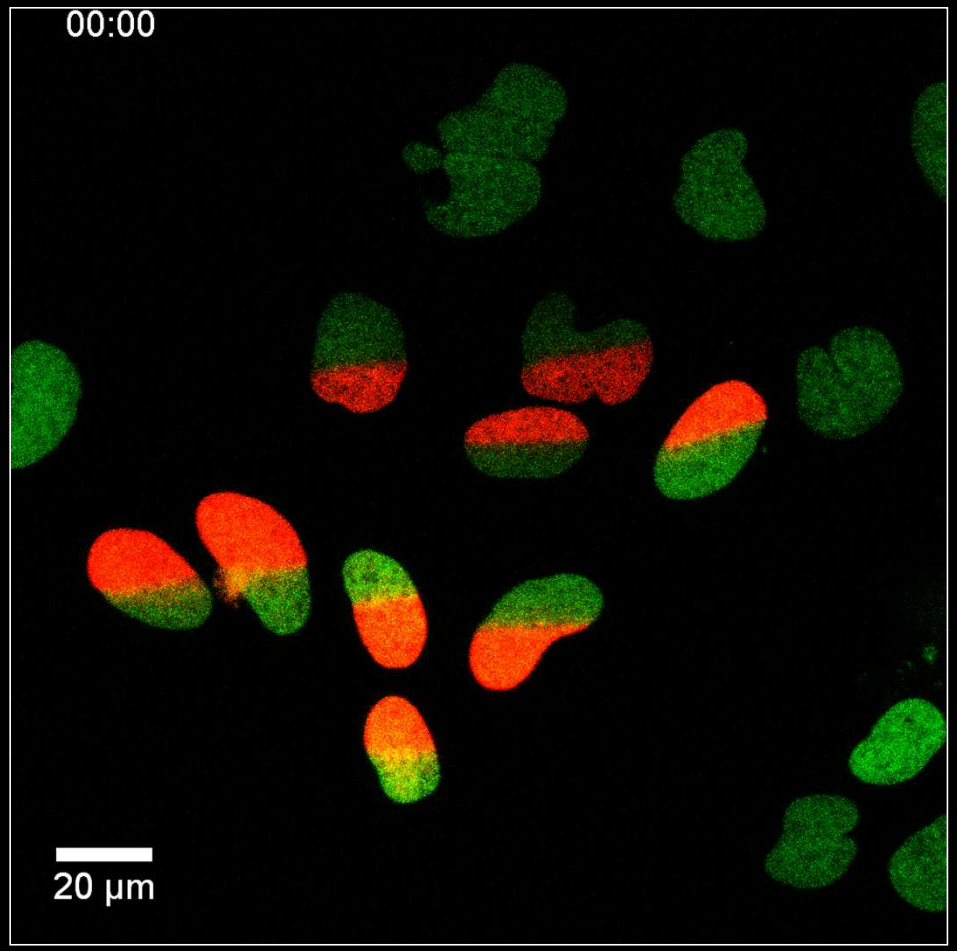
Emission







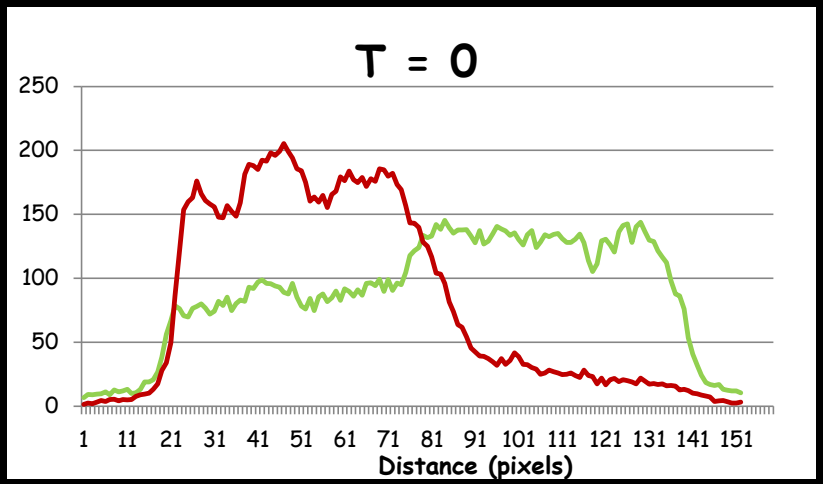
Optical highlighter FPs

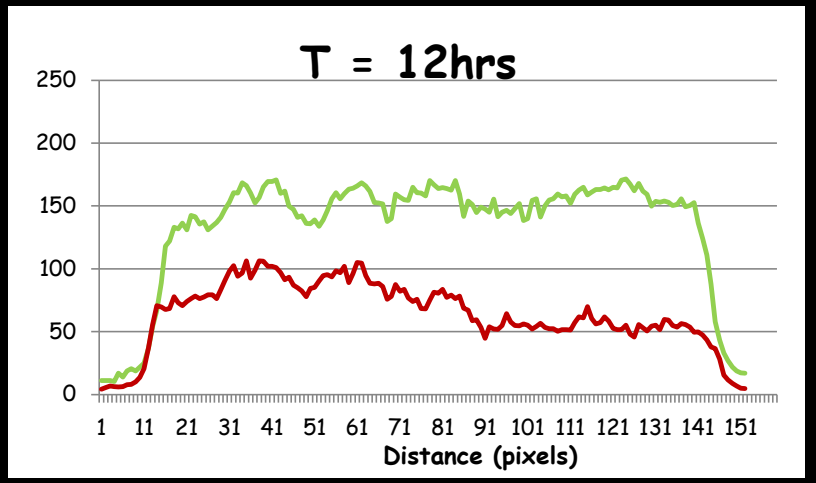


U2OS H2B-mMaple3

Pulse-chase labeling

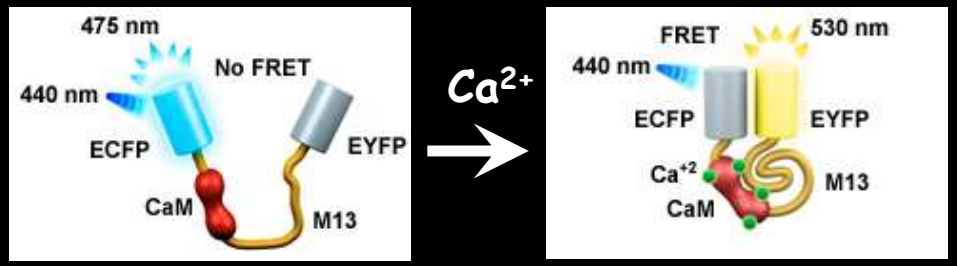






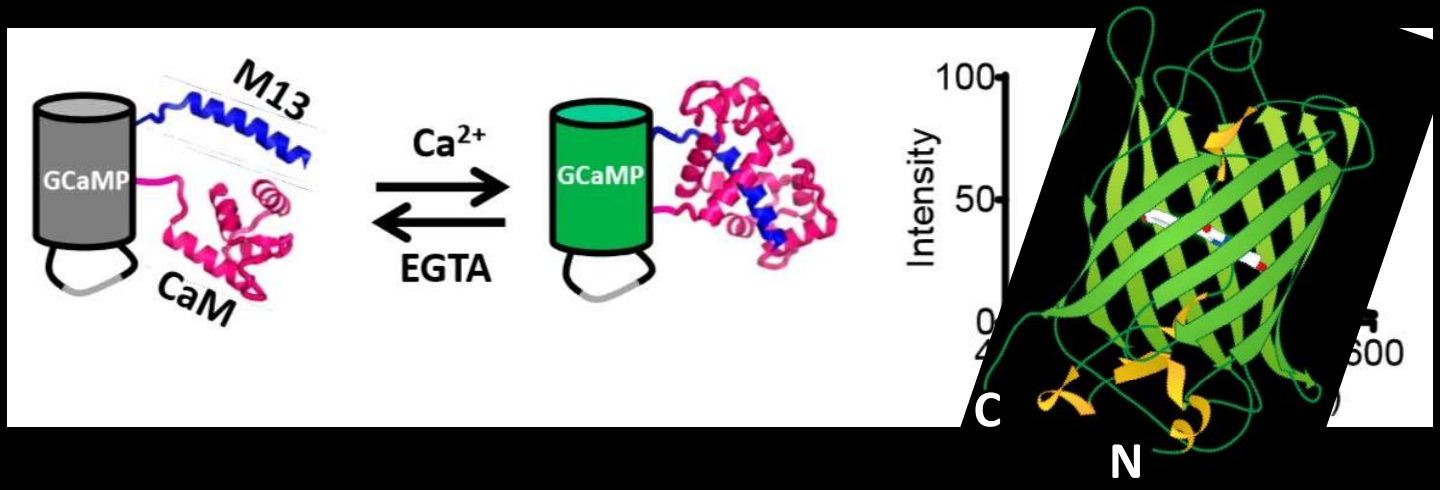
Biosensors

- **Engineered fusion protein consisting of 1 or 2 fluorescent proteins and protein domains**
- **Enable functional imaging of protein dynamics and other non-protein molecules**

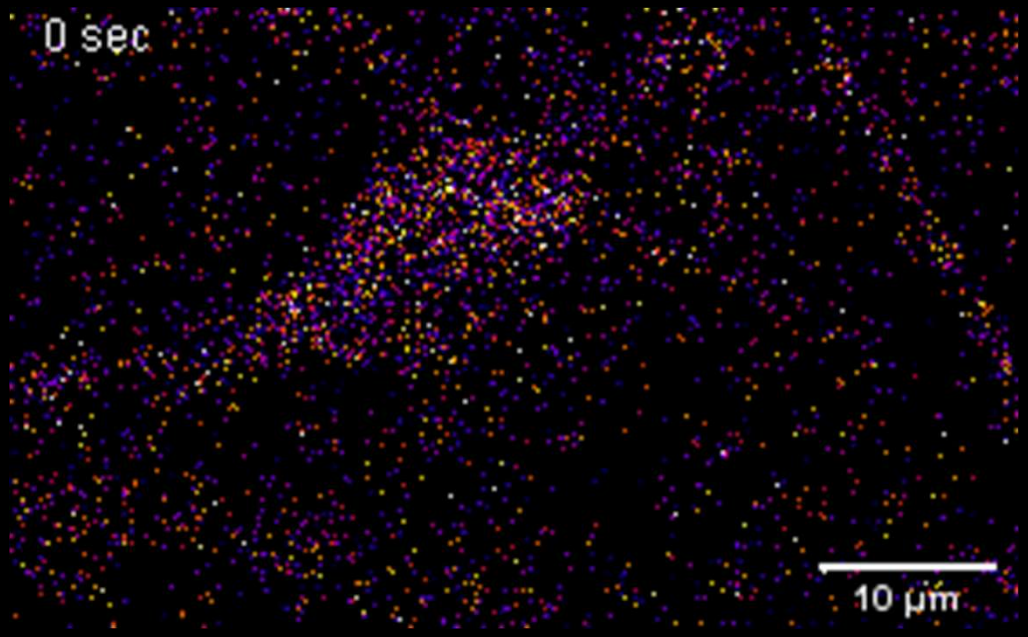


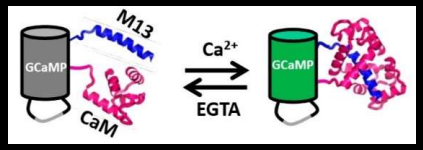
http://zeiss.magnet.fsu.edu

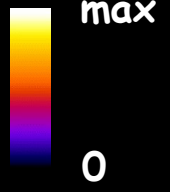
GCaMP Ca^{2+} biosensors



Neuronal activity

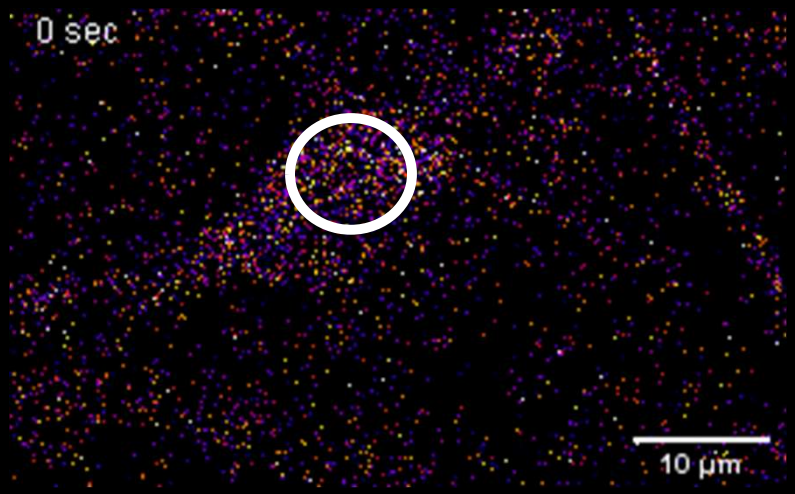


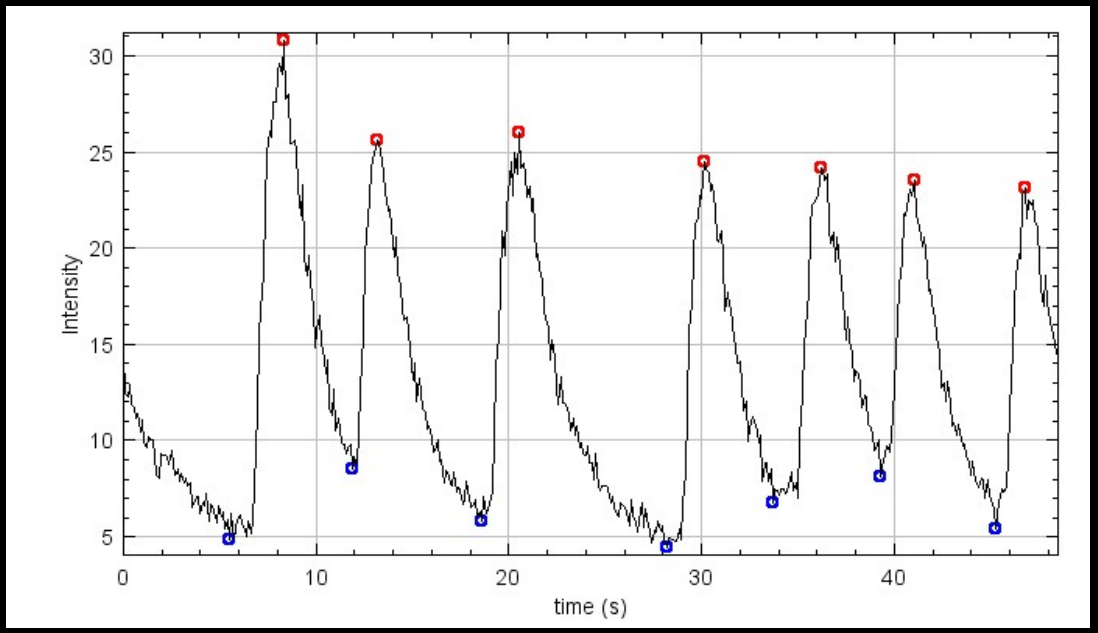




Nilhan Gunalar, Internal Medicine

Neuronal activity





Summary FPs

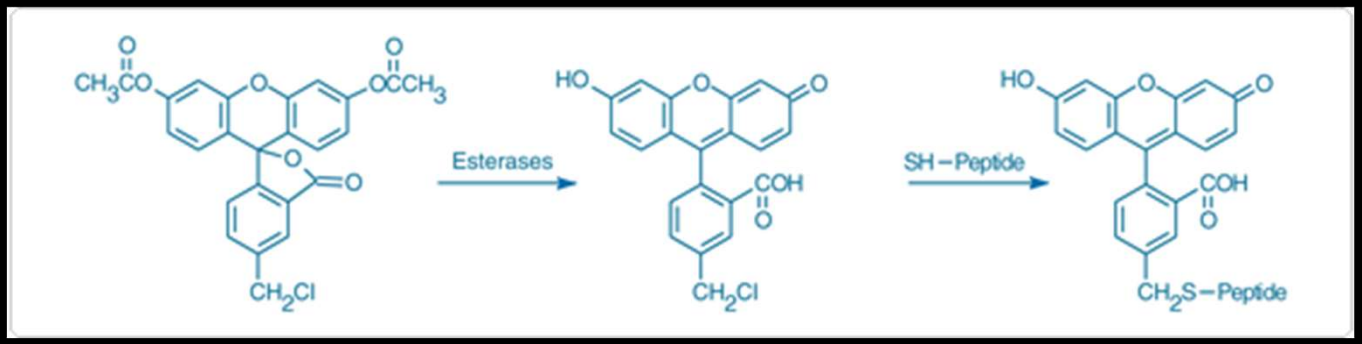
- **Fluorescent proteins**
  - Ideal for live cell multi-color imaging
  - Labeling of organism > tissue > cells > organelles > molecules
  - Optical highlighters for labeling sub-populations of molecules and super-resolution imaging
- **Biosensors**
  - Engineered fusion proteins
  - Functional imaging of non-protein dynamics
    - Phospholipids, Ca^{2+}, Glucose, cAMP, membrane potential, etc.
- **FP-fusion and overexpression artefacts**
- **Need for gene delivery**

Alternatives for FP labeling

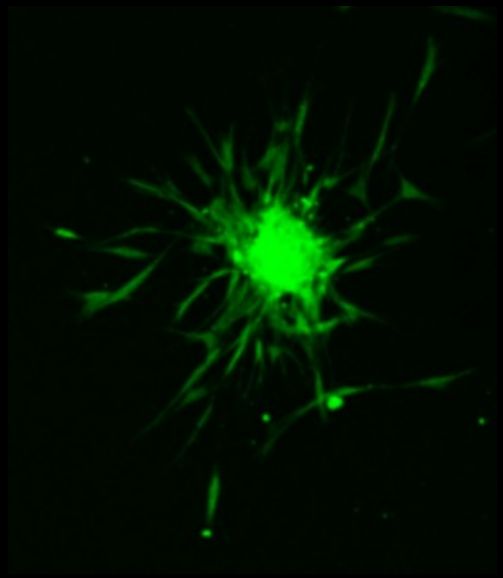
- ***Cell permeable organic dyes***
  - Cell / organelle tracker dyes, Hoechst, Fluo4, etc
  - SiR and SPY dyes
  - MemGlow dyes
- ***Chimeric approach: partly genetic / partly organic***
  - Halo-Tag
  - SNAP-Tag, Click-Tag, LigandLink

Cell permeable organic dyes

- **Cell-Trackers**
  - Fluorescent only upon cell entry
  - Short and long term labeling (several cell generations)
  - For example: Calcein Green and Carboxyfluorescein diacetate succinimidyl ester (CFSE)



www.thermofisher.com

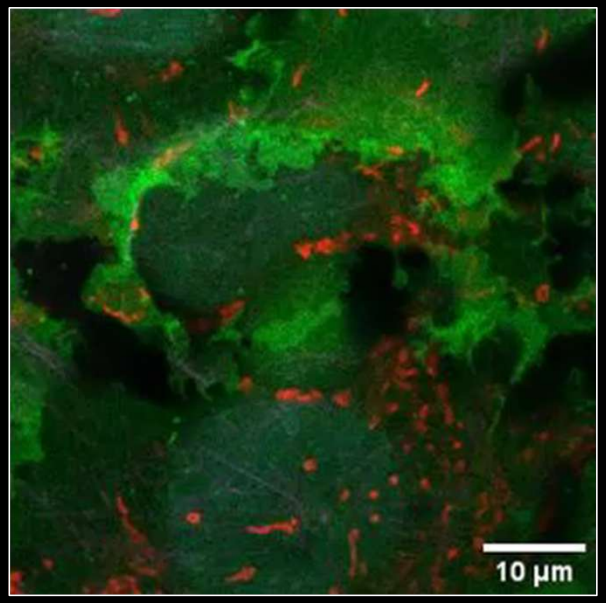


Cell migration in organoids cultured in Matrigel (4 days – 1 week)

Kavitha Sivasubramaniyan, Dept. Orthopedics

Small organic molecules

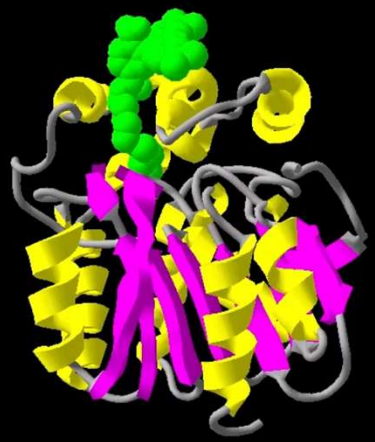
- **Most are cell impermeable and often chemically linked to other molecules**
  - Antibodies (immunofluorescence), receptor ligands, toxins
  - DAPI = cell impermeable DNA stain
- **Some are cell permeable and bind to specific targets**
  - DNA: Hoechst
  - Membranes: DiI, DiO, PKH, MemGlow
  - CellTracker, MitoTracker, LysoTracker
- **Source of autofluorescence**
  - NADH, Flavines, hemoglobin, elastin



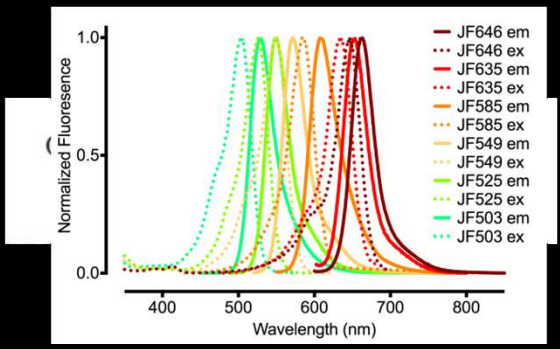
Hoechst, MemGlow488,
PKmito-Red, SPY640 tubulin

Chimeric approach: HaloTag

- **HaloTag**
  - 33 kDa protein
  - *Rhodococcus dehalogenase* DhaA
  - Catalyzes the conversion of haloalkanes to hydroxyalkanes
  - Modified to covalently bind ligands

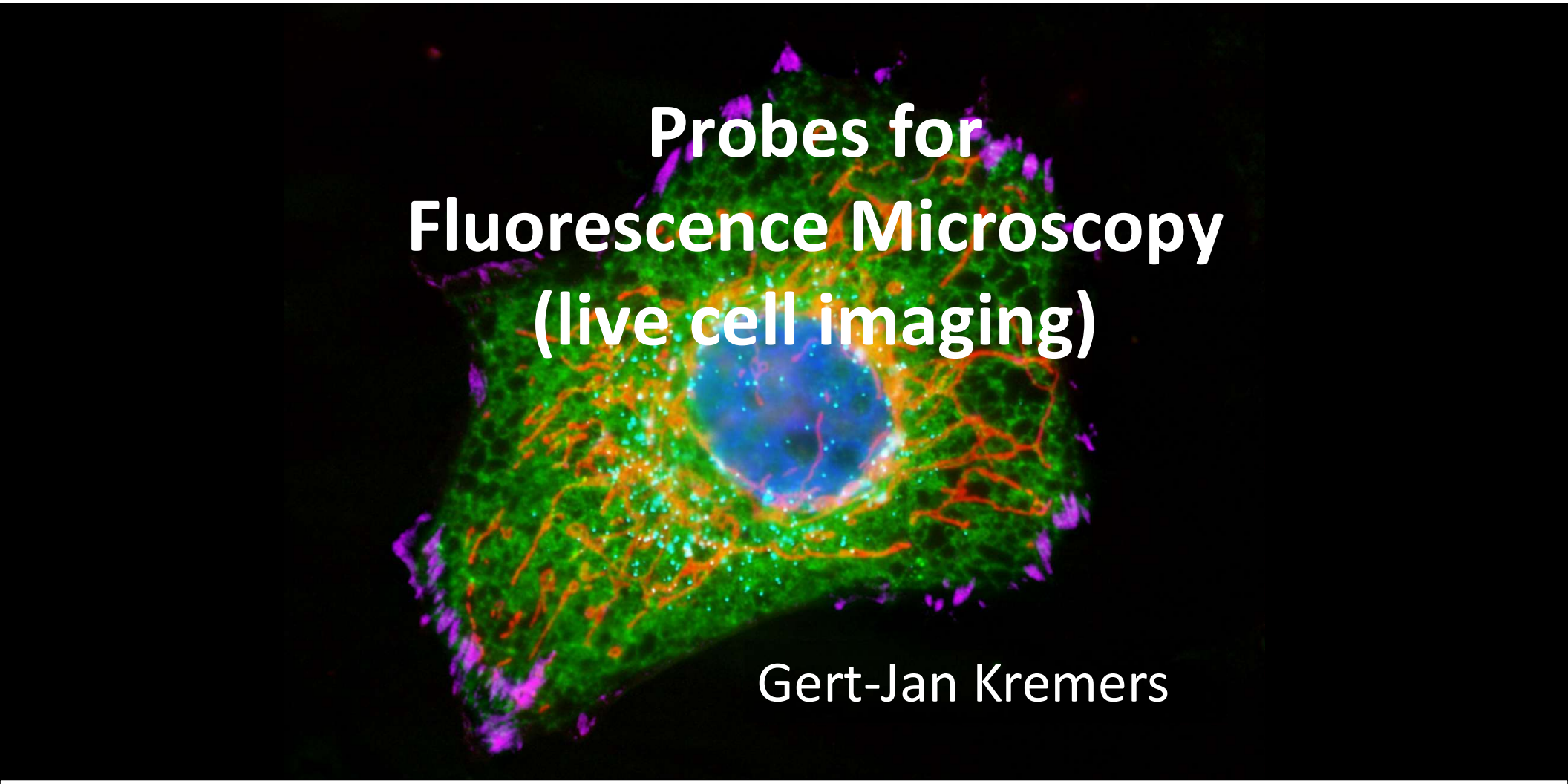


- **HaloTag ligand**
  - Chlorohexane derivative
  - Easy synthetic attachment to fluorophores
  - Biotin, Magnetic beads, etc



Conclusions

	FPs	Organic Dyes	Halo-tag
Cell permeability	---	+++	+/-
Probe retention	+++	+/-	+
Labeling targets	+++	+	+++
Functionality (probe size)	++	+++	++
(Non)toxicity	+++	+	+++
Protein purification	++		+++



Probes for Fluorescence Microscopy (live cell imaging)

Gert-Jan Kremers

Erasmus Optical Imaging Centre

PhD imaging course, November 9th 2026