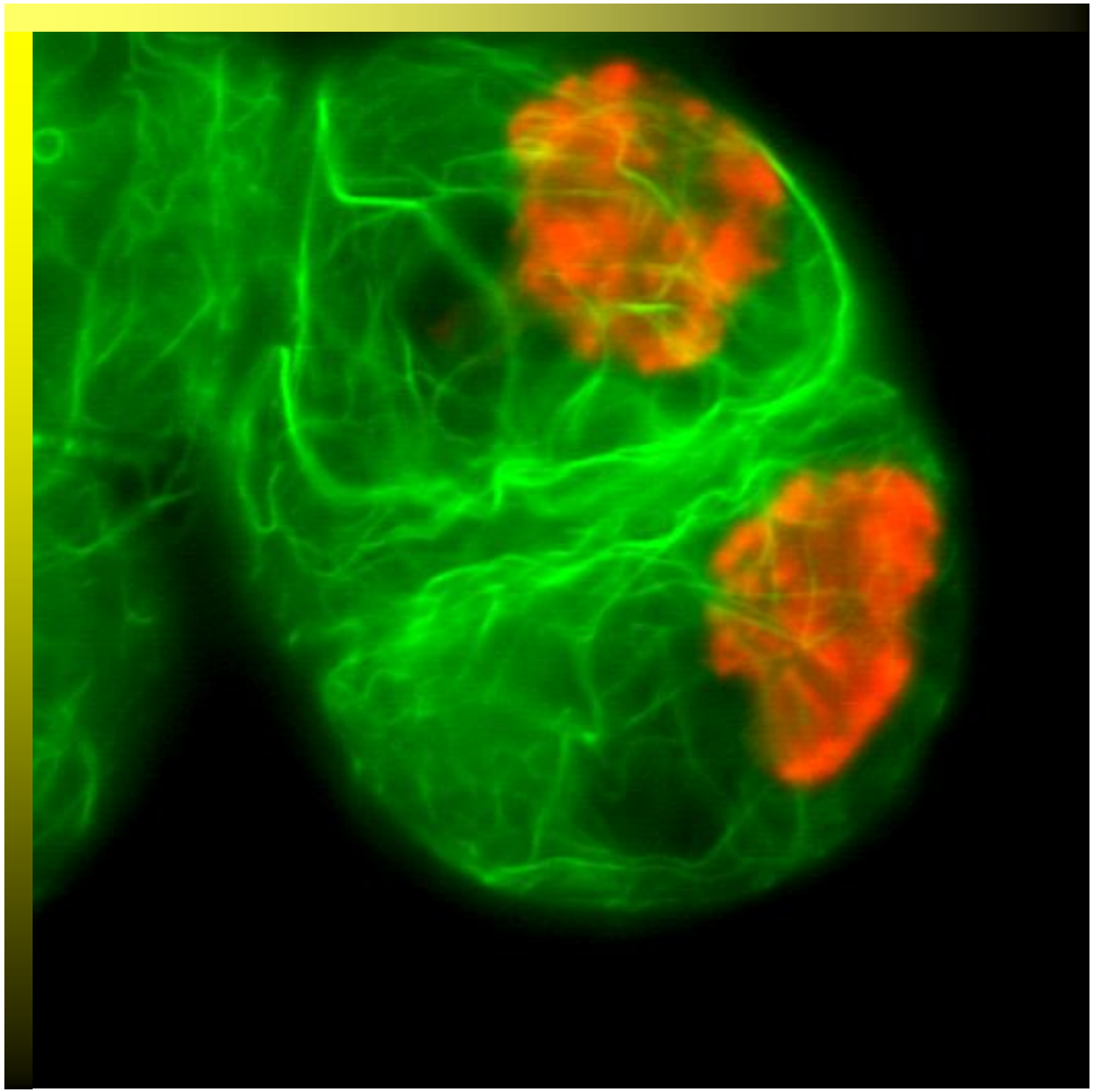
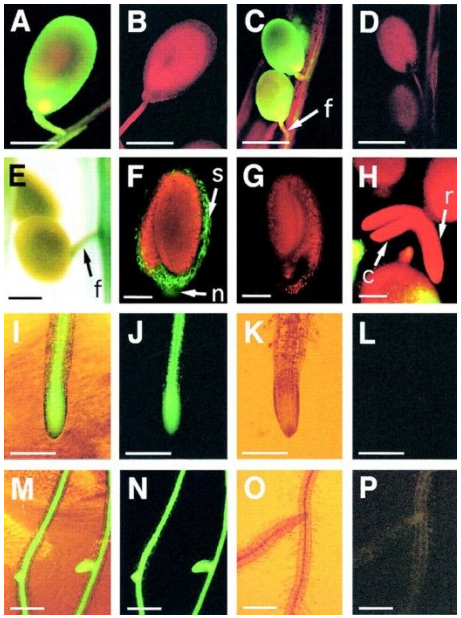
Fluorescence, fluorochromes and confocal microscopy



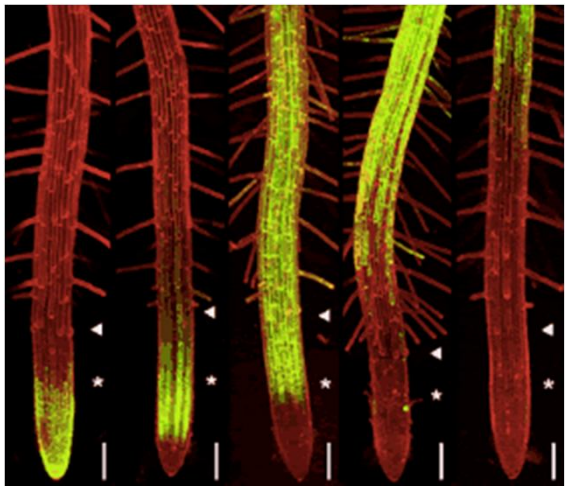
Outline

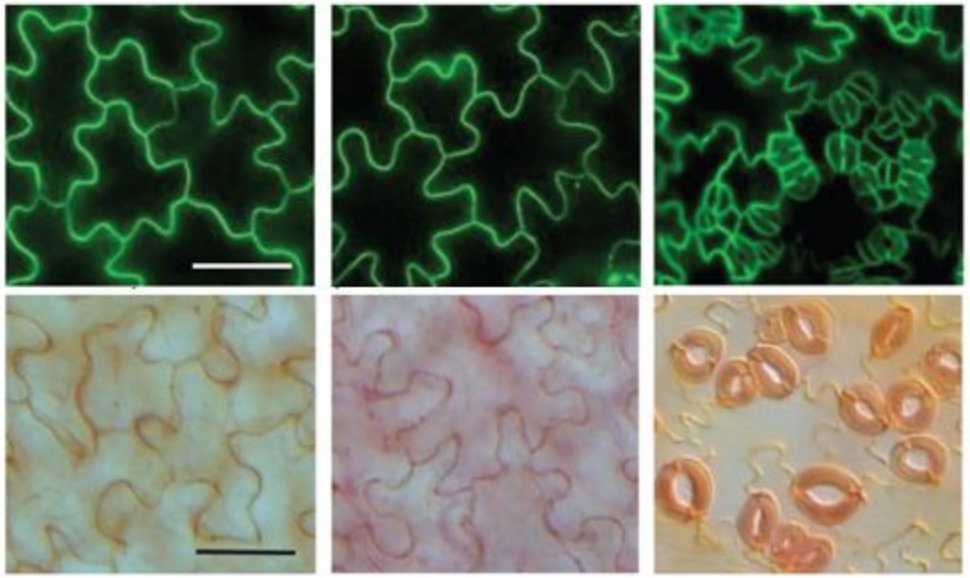
Phenomics and microscopy
History of microscopes
Fluorescence and Fluorophores
Fluorescence Microscope
Confocal Microscope
Advanced Applications of Confocal Microscope

Plant phenomics, fluorescence and confocal microscopy

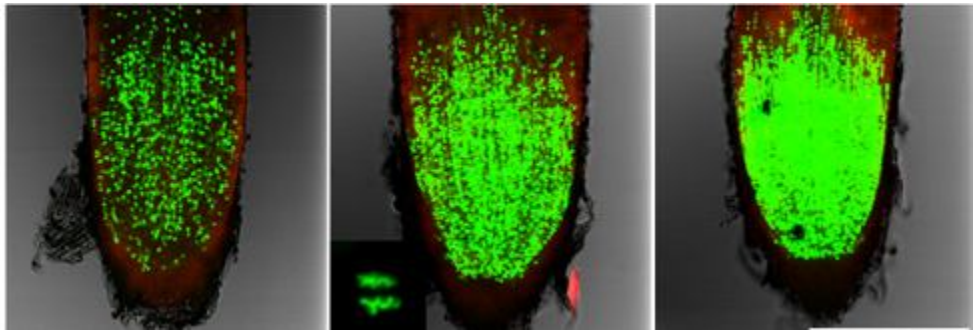


Location and expression level of a “gene of interest”



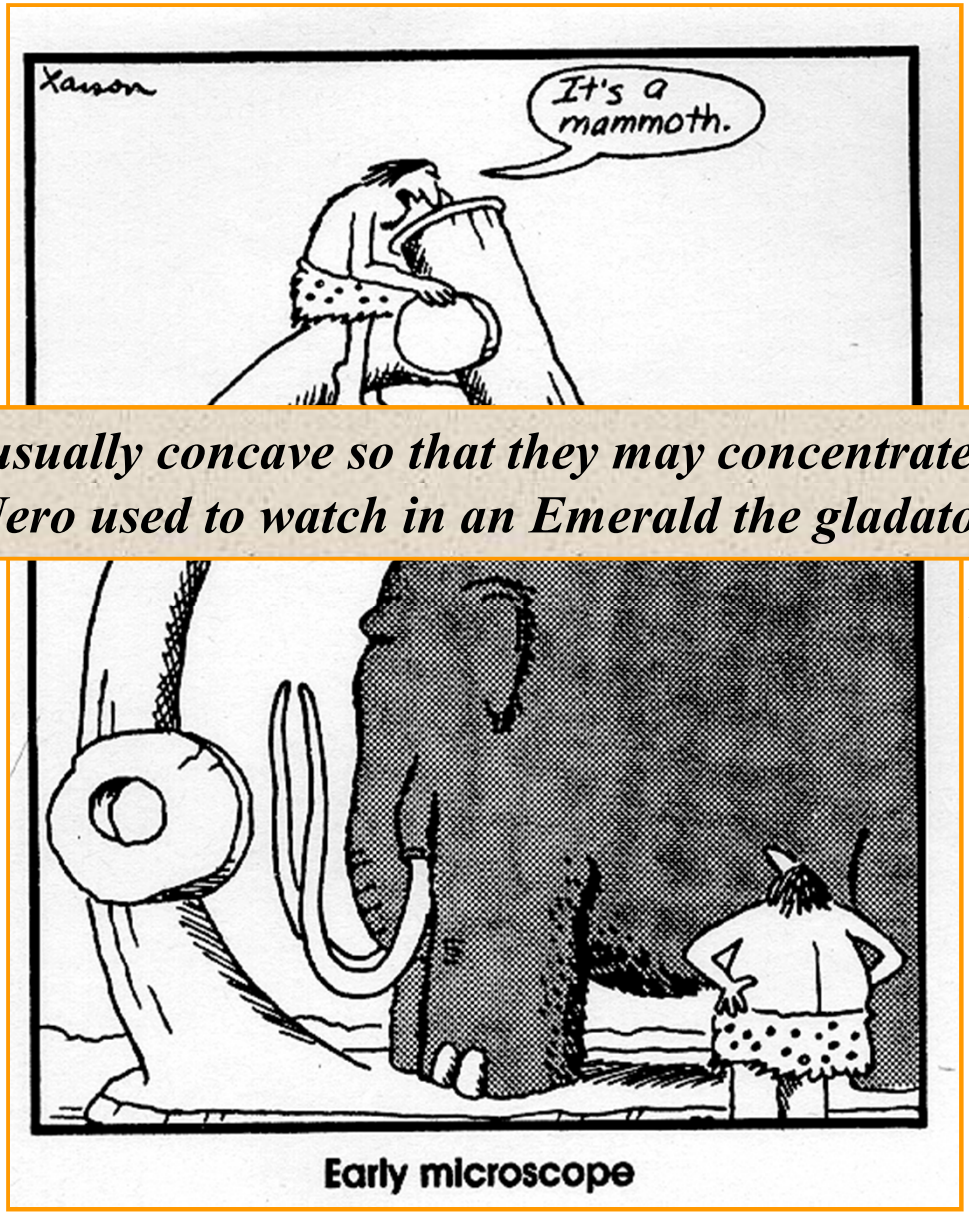


Leaf surface, epidermis morphology, stomata density



Cell division / cell elongation / cell differentiation

History of Microscopy



"Emeralds are usually concave so that they may concentrate the visual rays. The Emperor Nero used to watch in an Emerald the gladatorial combats."

Pliny the Elder 23-79 A.D

History of Microscopy

The First Compound Microscope (circa 1595)

The first known compound microscope, made by Zacharias and Hans Janssen in the 1590's.

Leeuwenhoek Microscope (circa late 1600s)

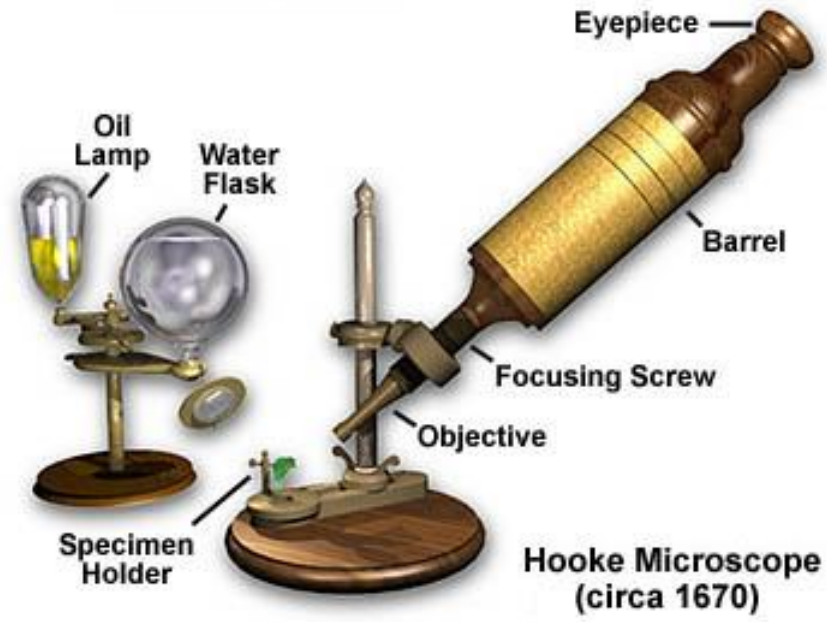
Antoni van Leeuwenhoek was an amateur Dutch scientist who was granted for his discoveries in microscopy and high quality, but crude optical microscopes.

History of Microscopy

Simple Sliding Rod Microscope (circa 1640s)

This middle seventeenth century version of the simple one-lens microscope uses a sliding rod to focus the specimen.

The Hooke design was a functional improvement over the traditional motif, and even included a lighting apparatus to aid in specimen illumination



Hooke Microscope (circa 1670)

History of Microscopy

Cuff's Microscope
(circa mid 1700s)

Jones' Most
Improved Compound
Microscope
(circa late 1700s)

18th Century Microscopes

History of Microscopy

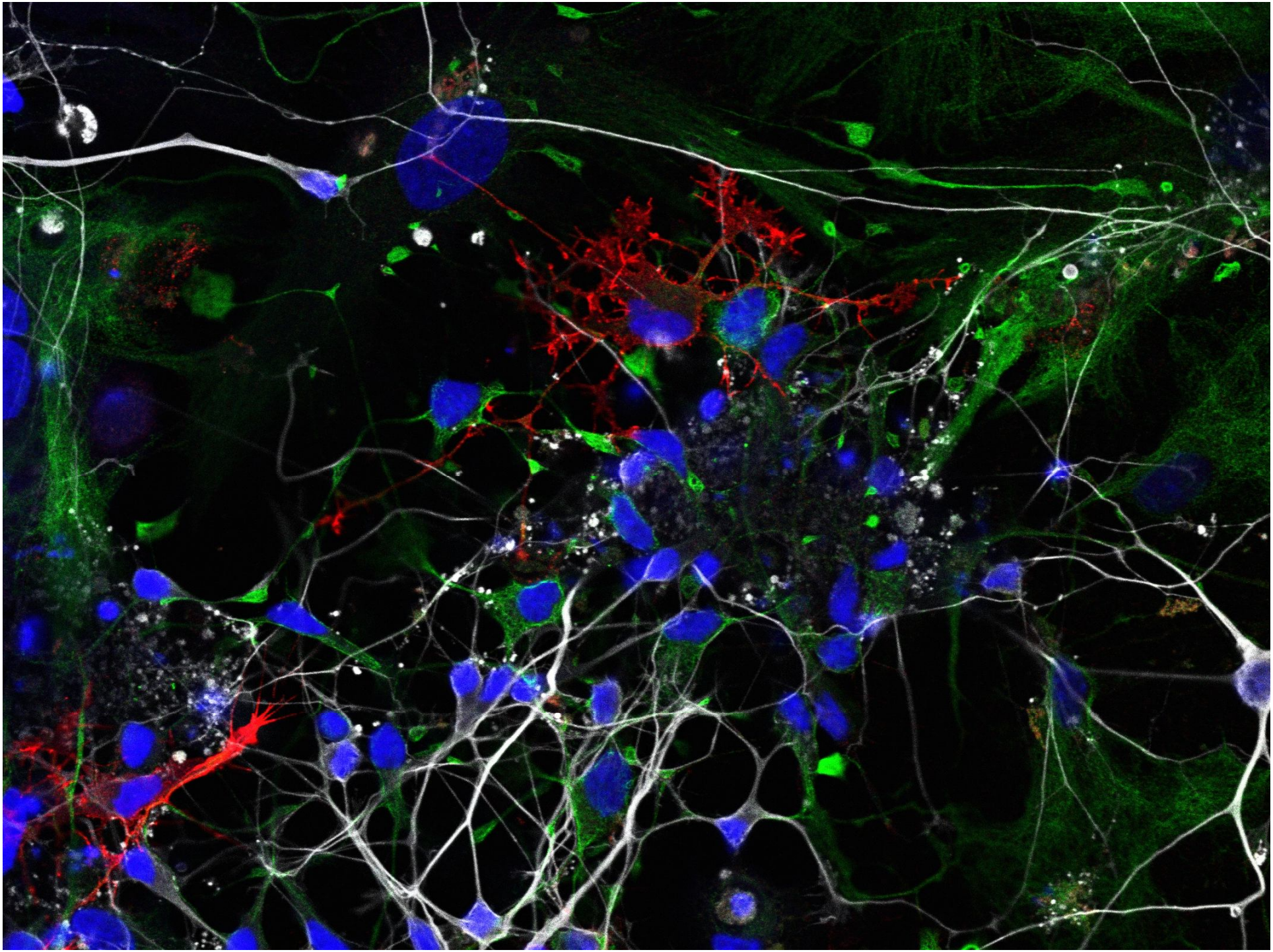
19th Century Microscopes

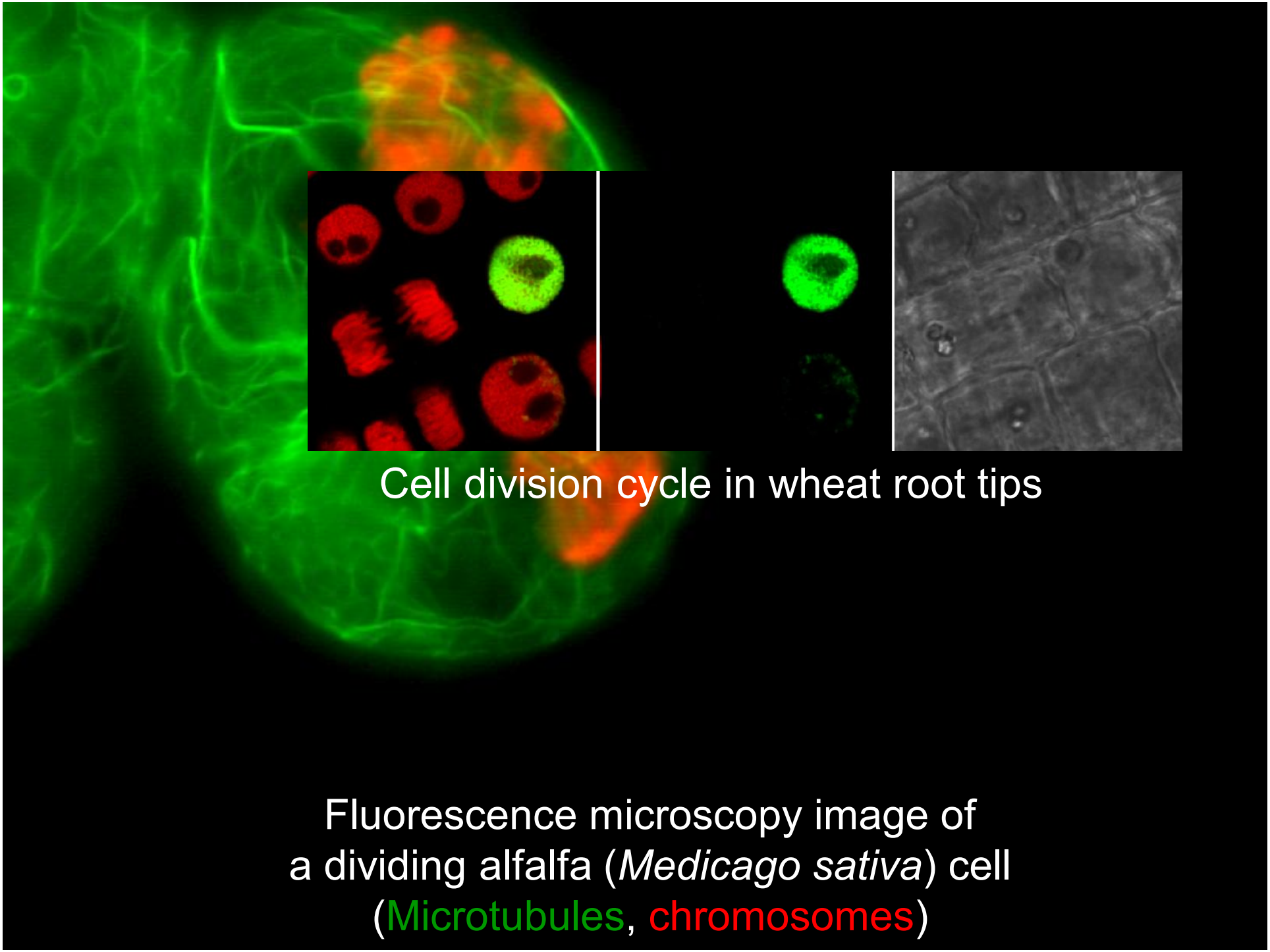
History of Microscopy

Olympus
Vanox
Microscope
(circa 1971)

Nikon Diaphot
Inverted Tissue
Culture Microscope
(circa 1985)

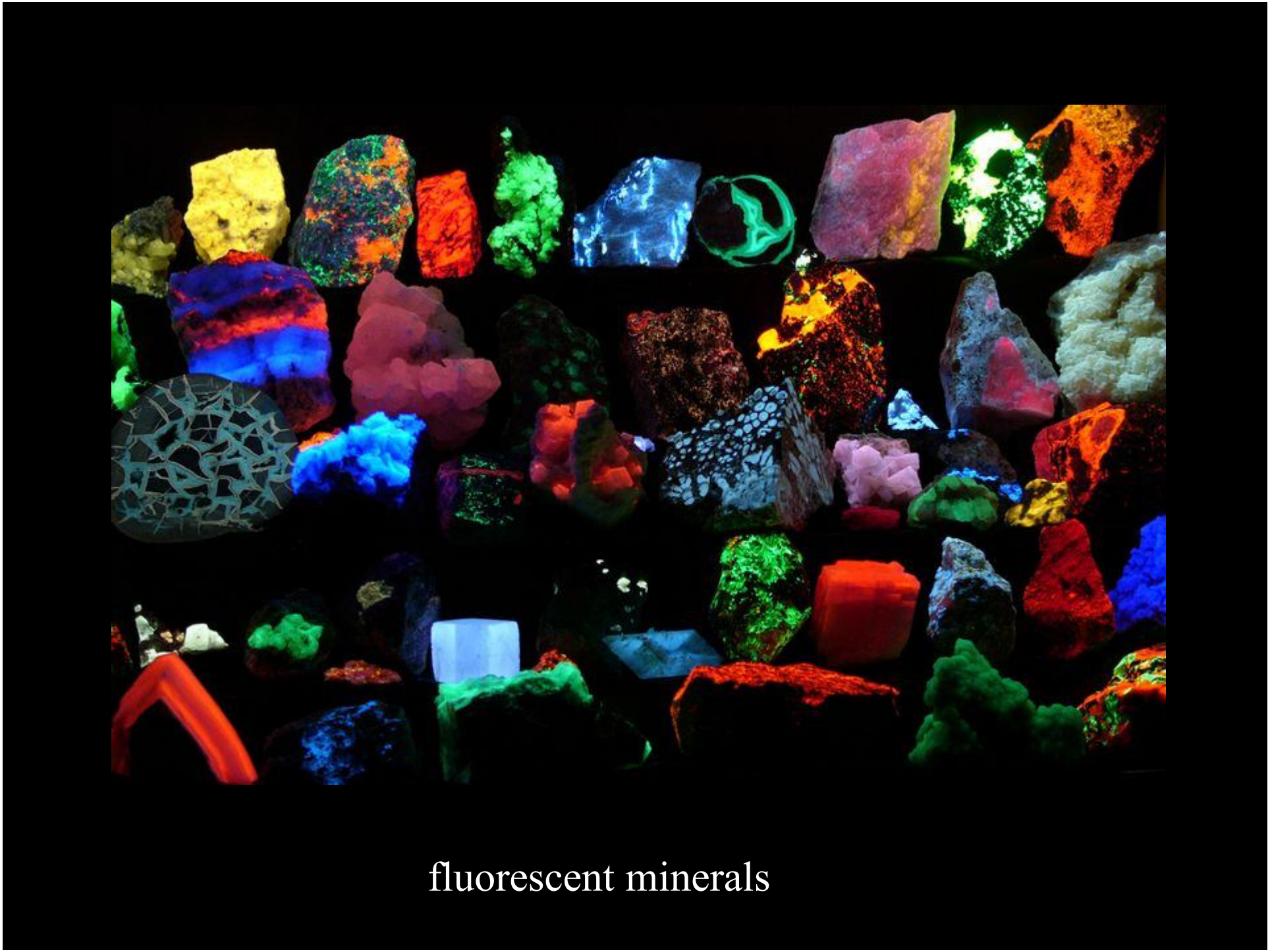
20th Century Microscopes





Cell division cycle in wheat root tips

Fluorescence microscopy image of
a dividing alfalfa (*Medicago sativa*) cell
(Microtubules, chromosomes)



fluorescent minerals

Fluorescence and Fluorophores

white light

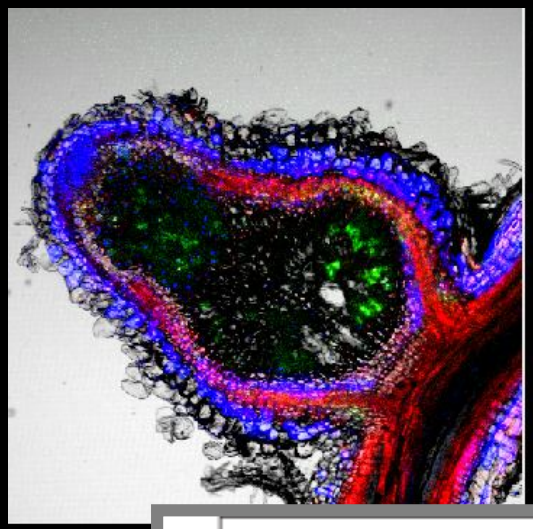
the term fluorescence comes from t

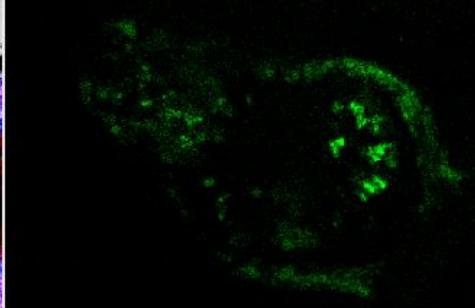
Fluorescence occurs when a molecule relaxes to it

Excitation: S0+hv→ S1 Emiss

Stoke's shift

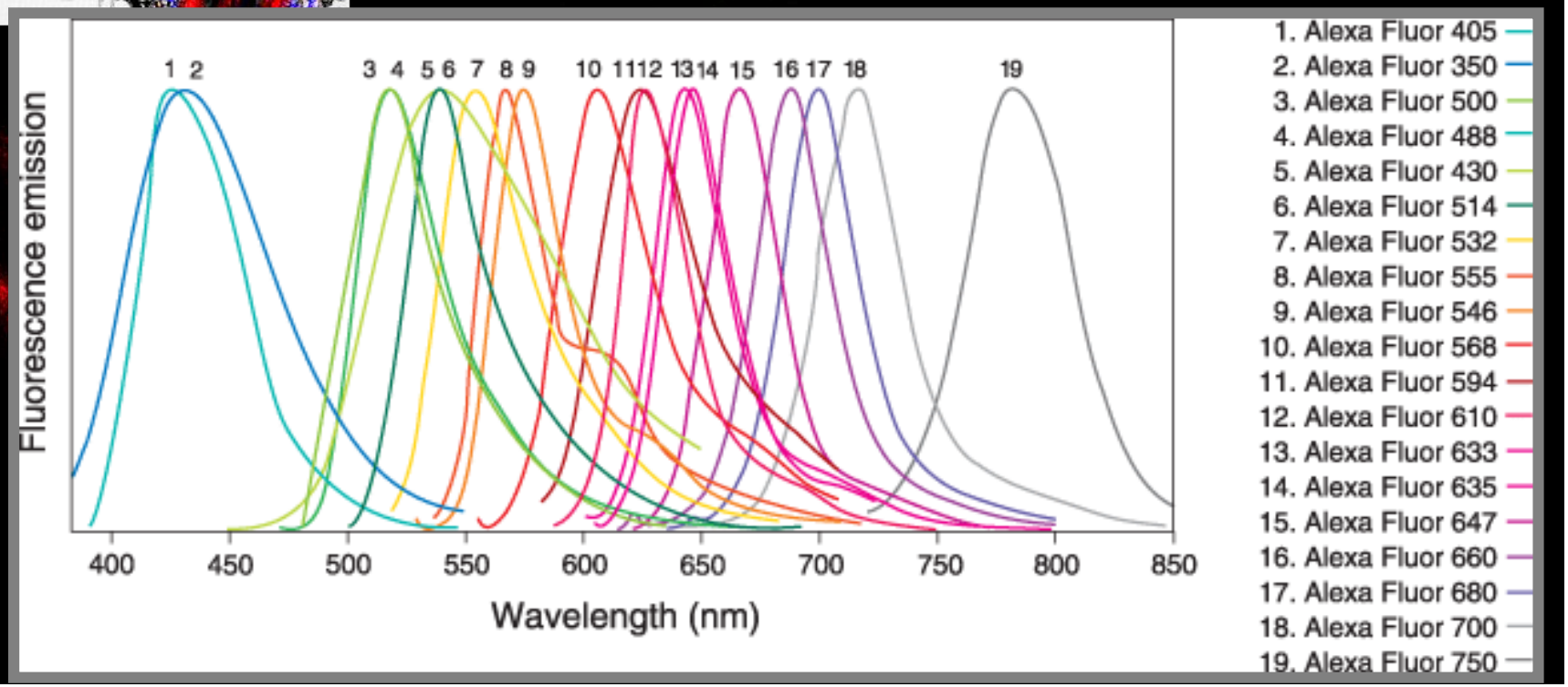
Detection of proteins by Immunofluorescence



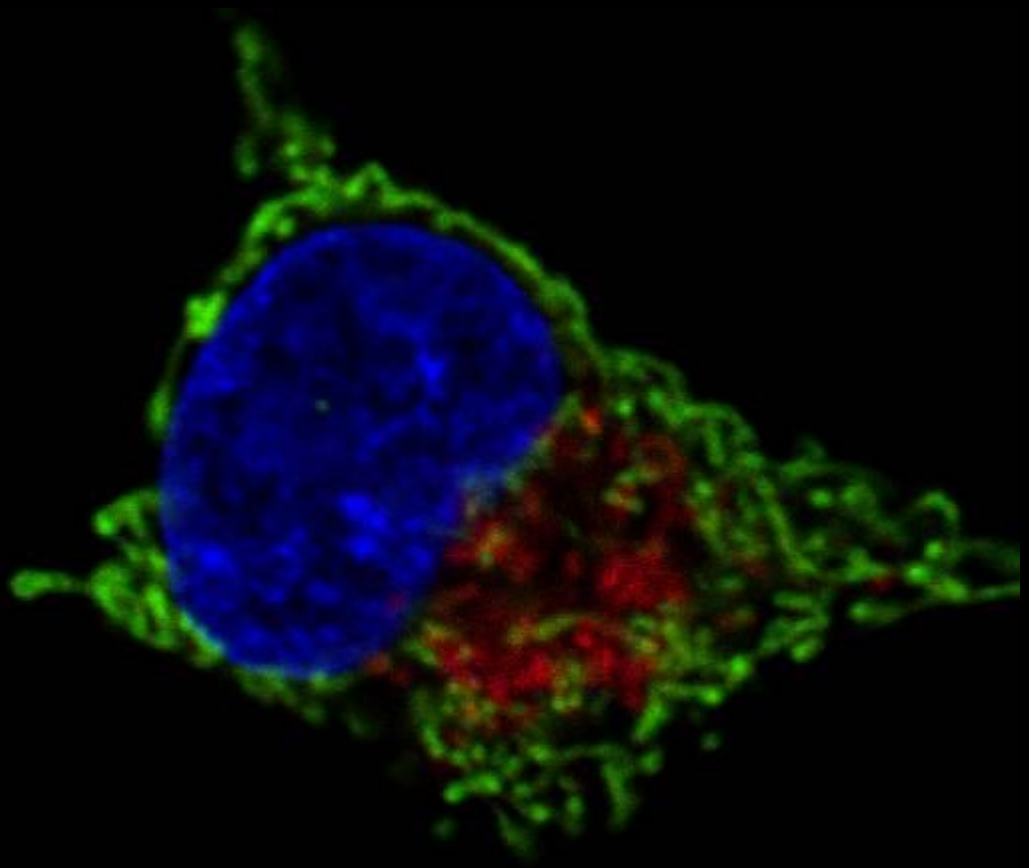


Common Fluorochromes
FITC
Rhodamine
Texas Red
Cyanine dyes

AlexaFluor dyes
wide spectrum, stable
brighter and bleach resistant



Staining Organelles with Fluorochromes



Nucleus
DAPI
Hoechst dyes
Ethidium Bromide
Propidium Iodide
Acridine Orange

Mitochondria
Mitotracker
Mitofluor dyes
Nonyl acridine orange

Golgi/ER
ER tracker
fluorescent Ceramide
fluorescent Sphingomyosin

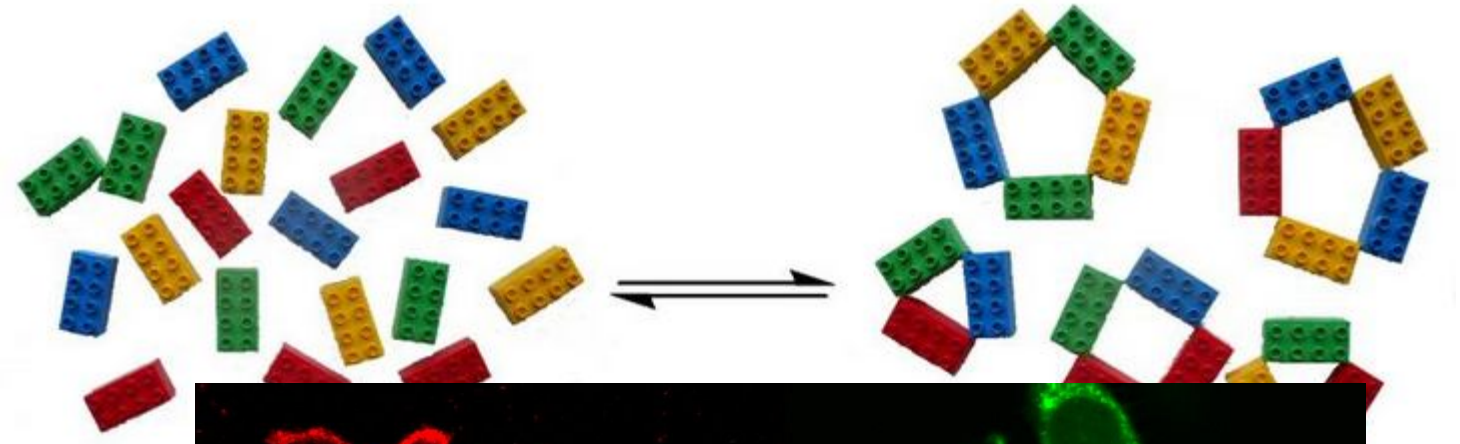
Lysozme
Lysotracker

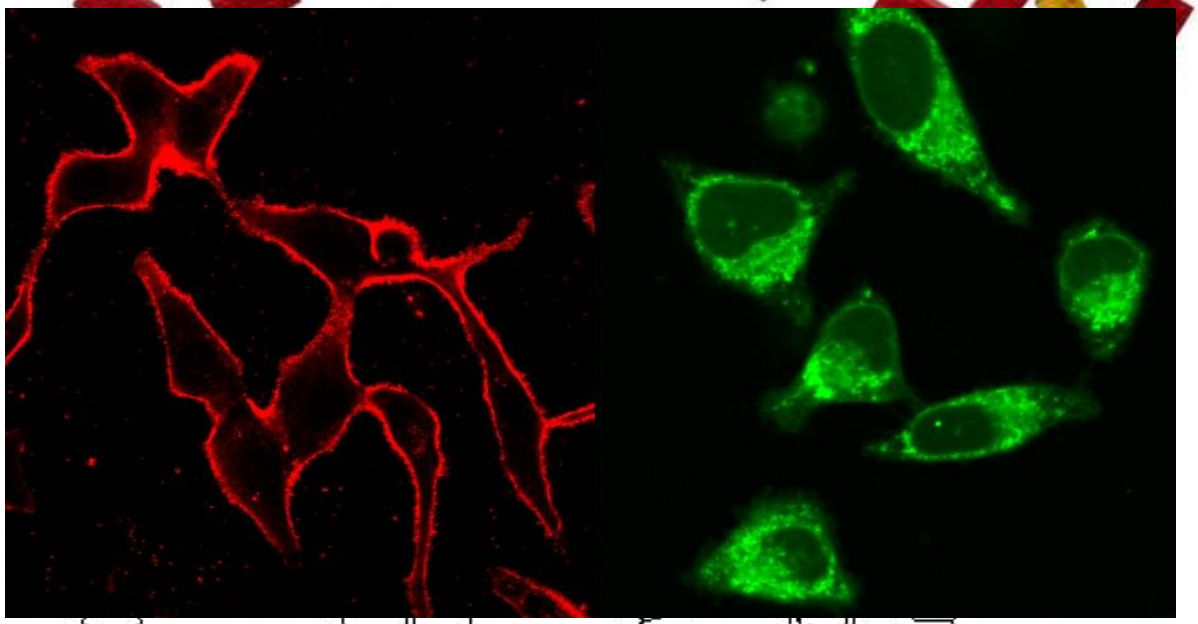
My phenomics project requires a fluorescent dye that….

… a fluorescent dye that specifically stains the root hairs of Arabidopsis

… a fluorescent dye that specifically stains leaf oils of Cannabis

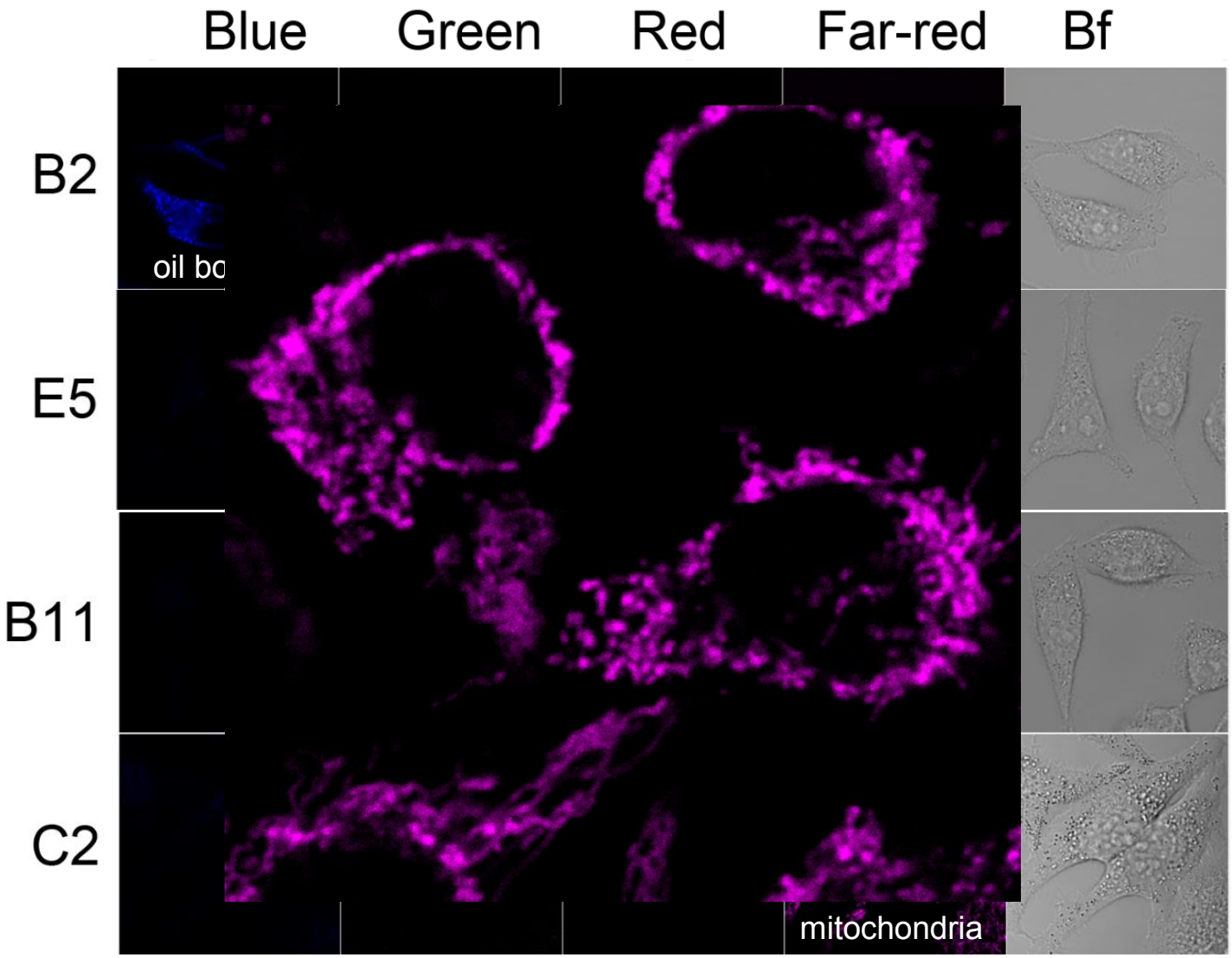
Novel fluorescent chemical discovery through combinatorial chemistry





Discovery of novel live cell permeable fluorescent chemicals

14585 compounds > microarray scanner > confocal microscopy

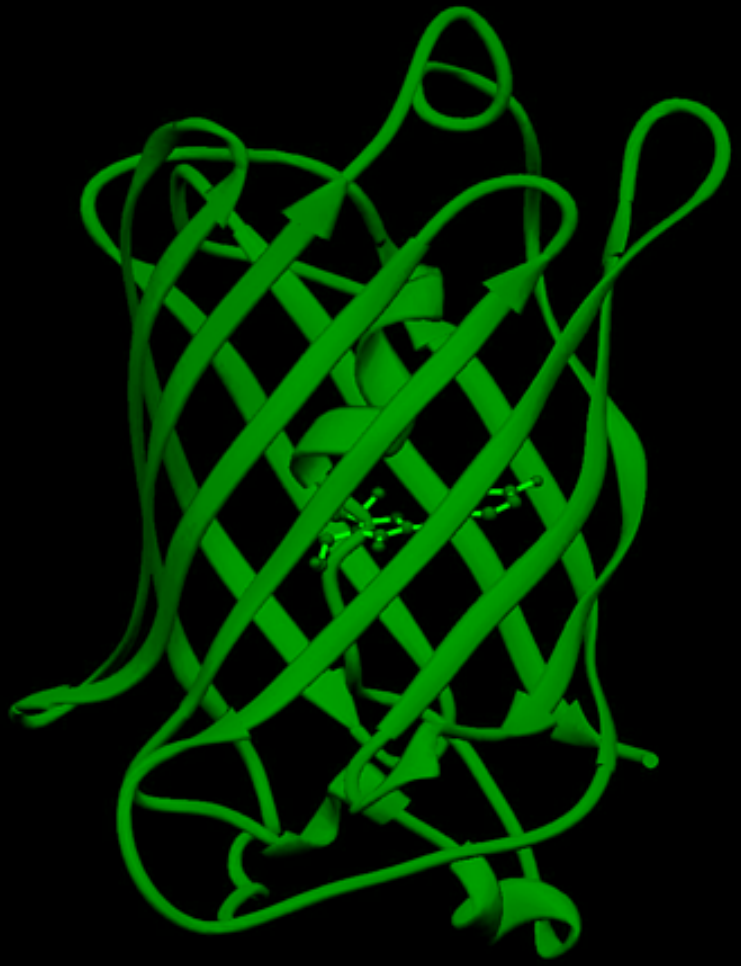


Novel dyes to stain plant oil bodies in live cells

B2

C6

Green Fluorescent Protein :GFP

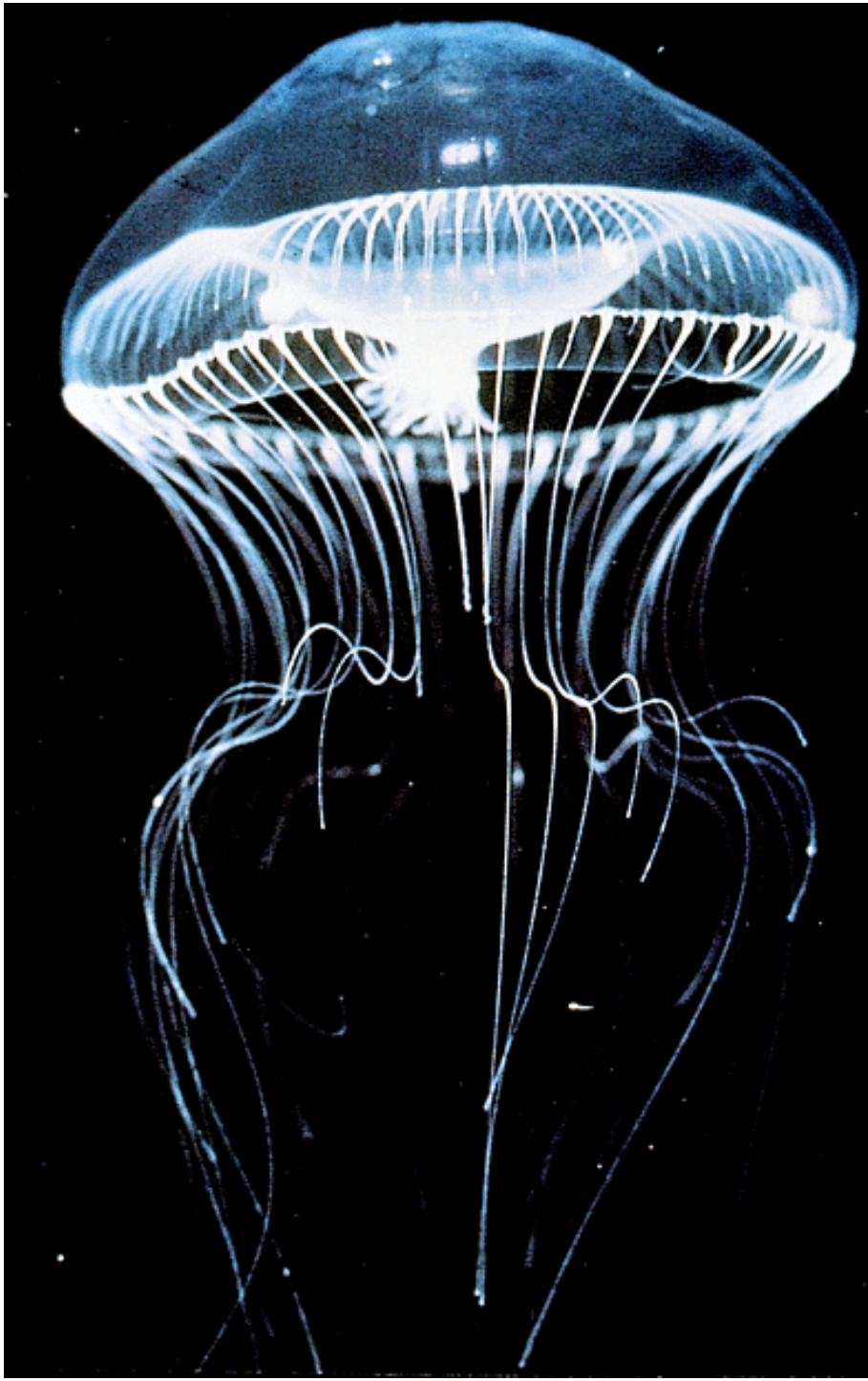


GFP is a small protein (27 kD) and the DNA sequences coding for GFP can be manipulated by recombinant DNA technology to create gene fusion

promoter | GFP | your favorite protein

promoter | your favorite protein | GFP

Aequorea victoria



The Mechanism of Glow

- The GFP chromophore consists of a cyclic tripeptide derived from Ser-Tyr-Gly at positions 65–67 in the protein and is only fluorescent when embedded within the fully folded, complete GFP molecule.

- EGFP: Ser65 to Thr mutation (near-UV to blue excitation)

- Nascent GFP is not fluorescent, since chromophore formation occurs post-translationally. The chromophore is formed by a cyclization reaction and an oxidation step at Tyr66 that requires molecular oxygen

Fluorescent Protein Color Variants

YELLOW Fluorescent Protein (YFP)
(Thr 203 to Tyr)

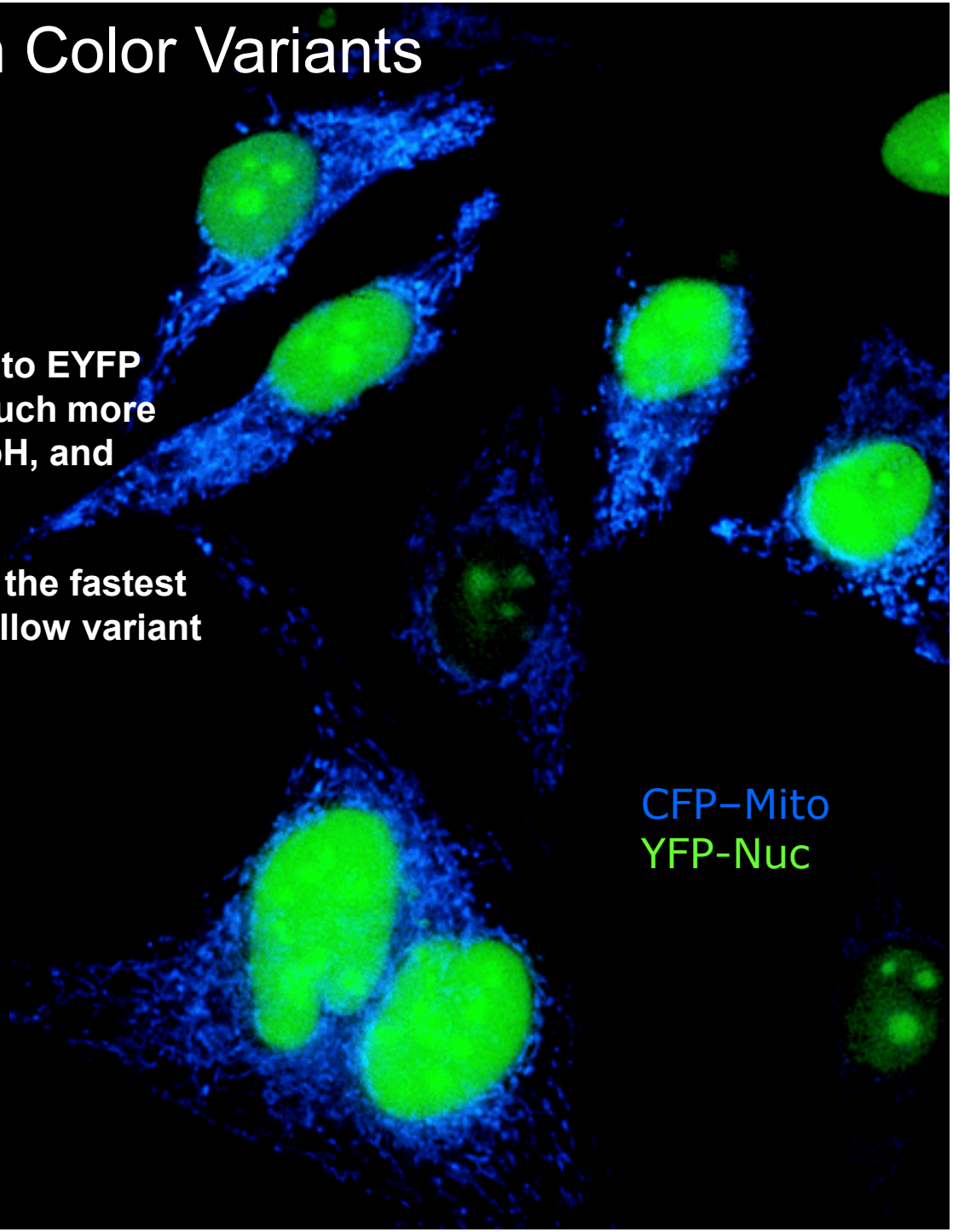
Citrine variant is very bright relative to EYFP and has been demonstrated to be much more resistant to photobleaching, acidic pH, and other environmental effects

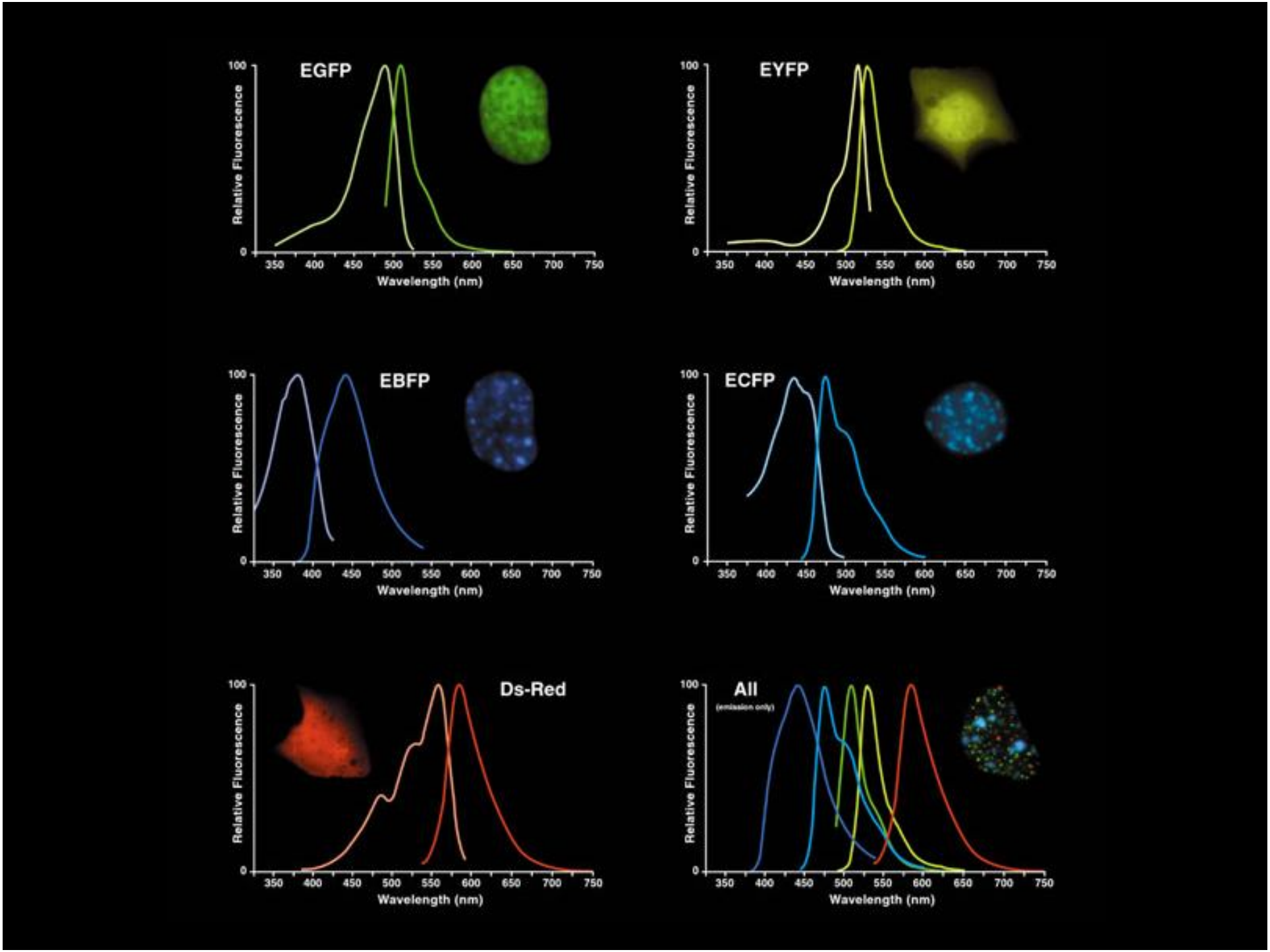
Another derivative, named Venus, is the fastest maturing and one of the brightest yellow variant

CYAN Fluorescent Protein (CFP)
(Tyr66 to Tryptophan)

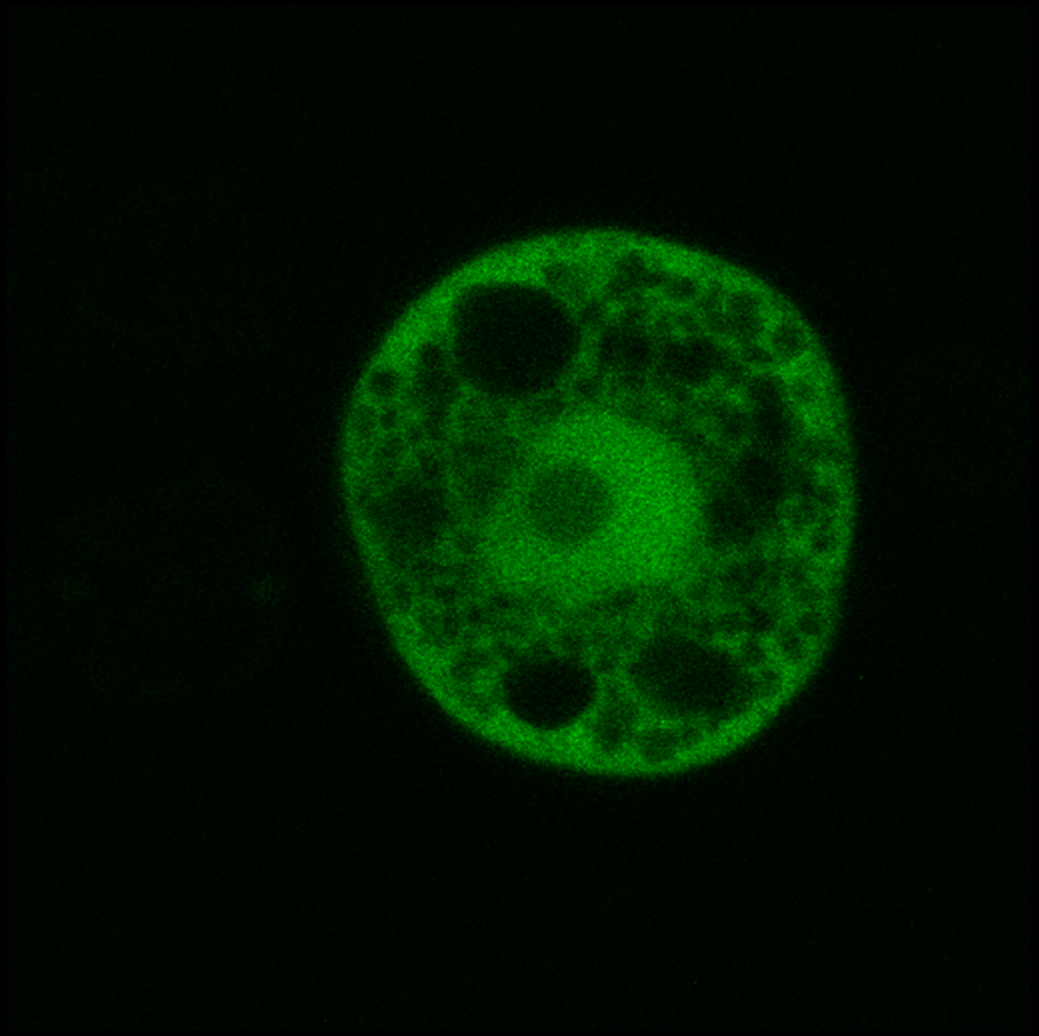
BLUE Fluorescent Protein (BFP)
(Tyr66 to His)

RED Fluorescent Protein (RFP)




EGFP
EYFP
EBFP
ECFP
Ds-Red
All
(emission only)
Relative Fluorescence
Wavelength (nm)
100
0
350
400
450
500
550
600
650
700
750

The biggest advantage of using GFP is...

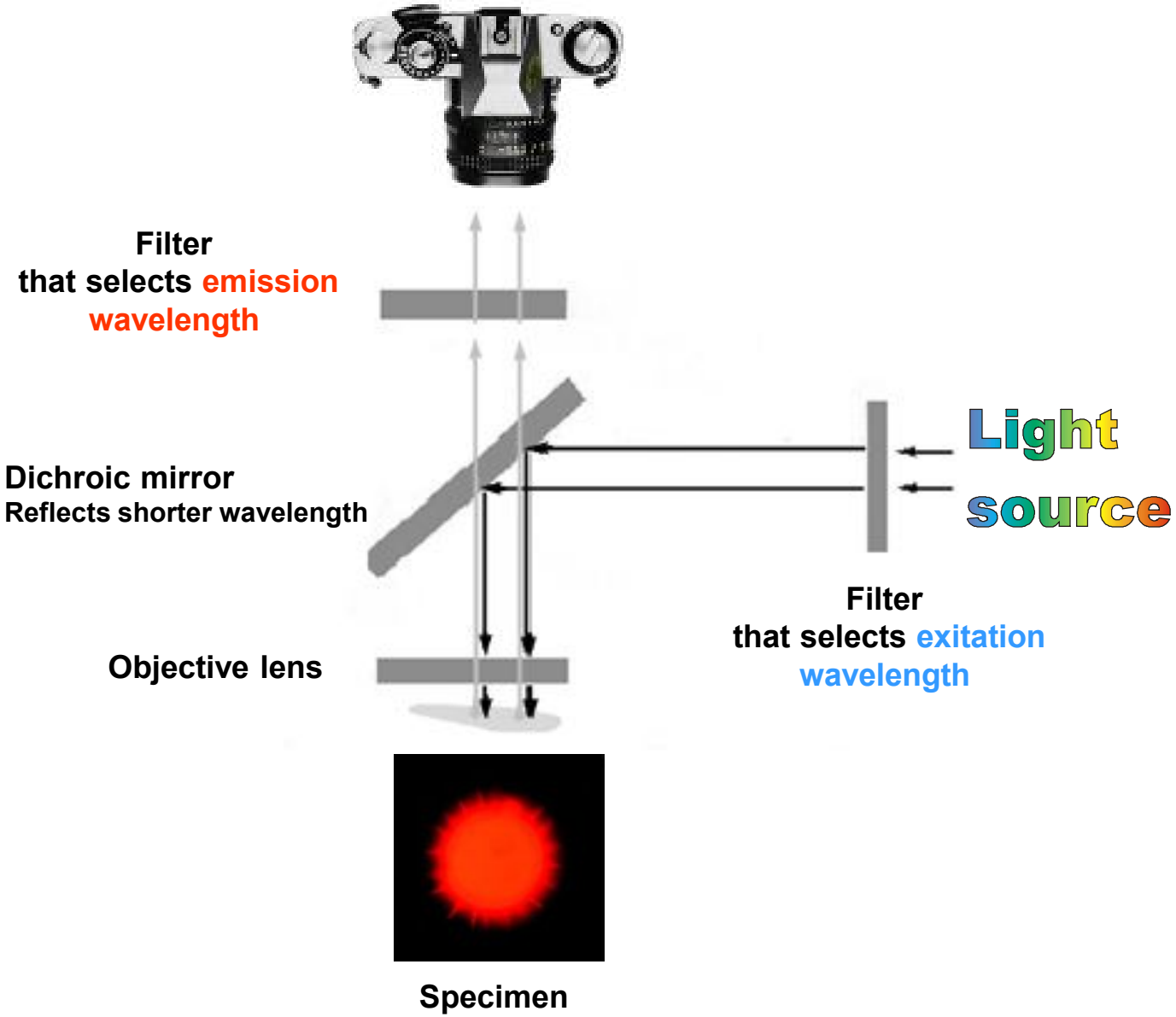


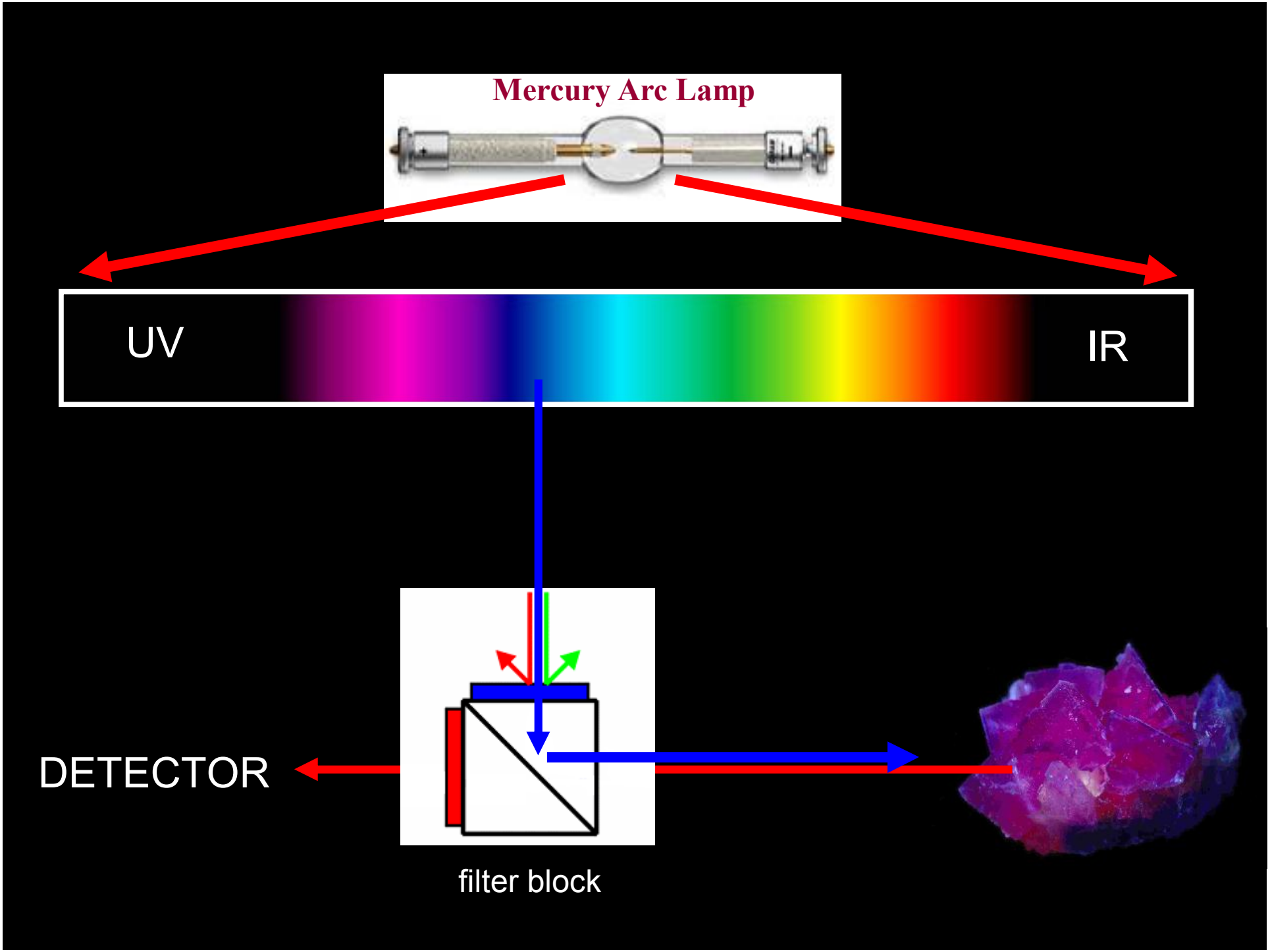
Fluorescence Microscope

upright

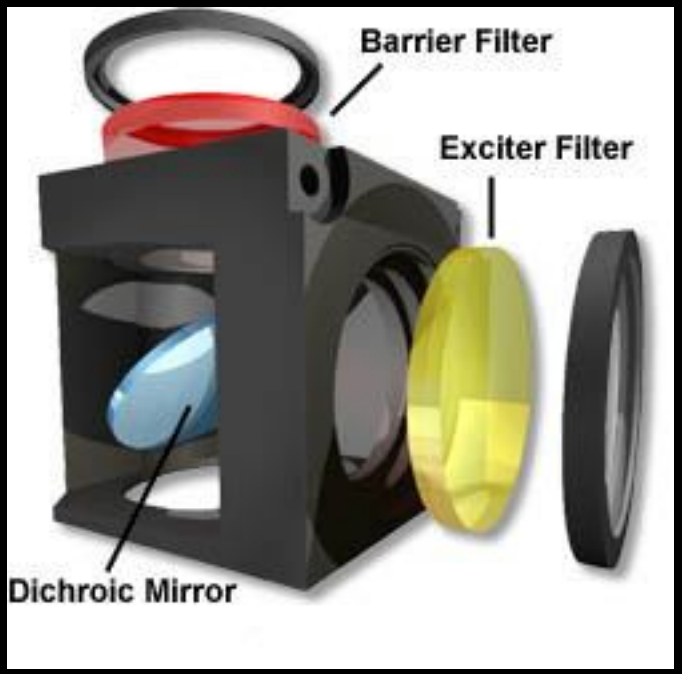
inverted

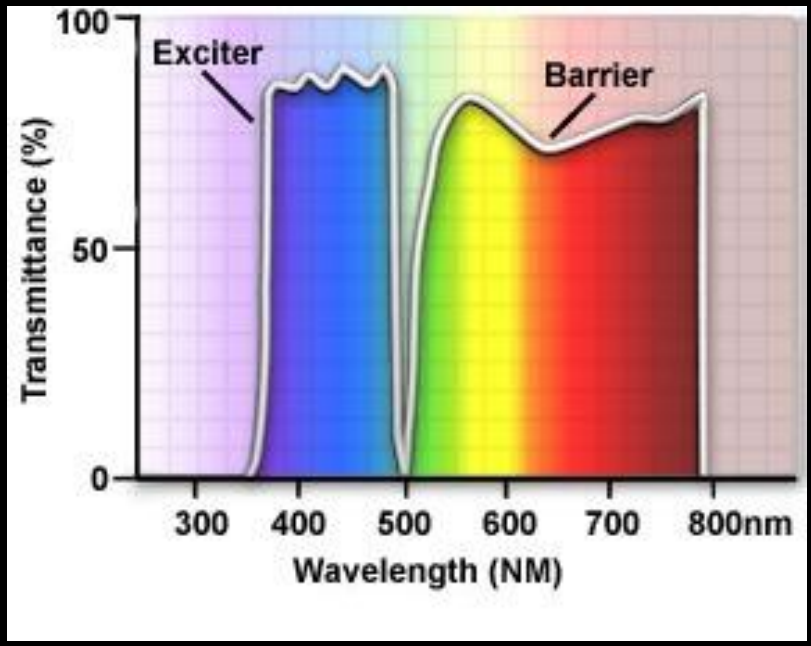
Fluorescence Microscope



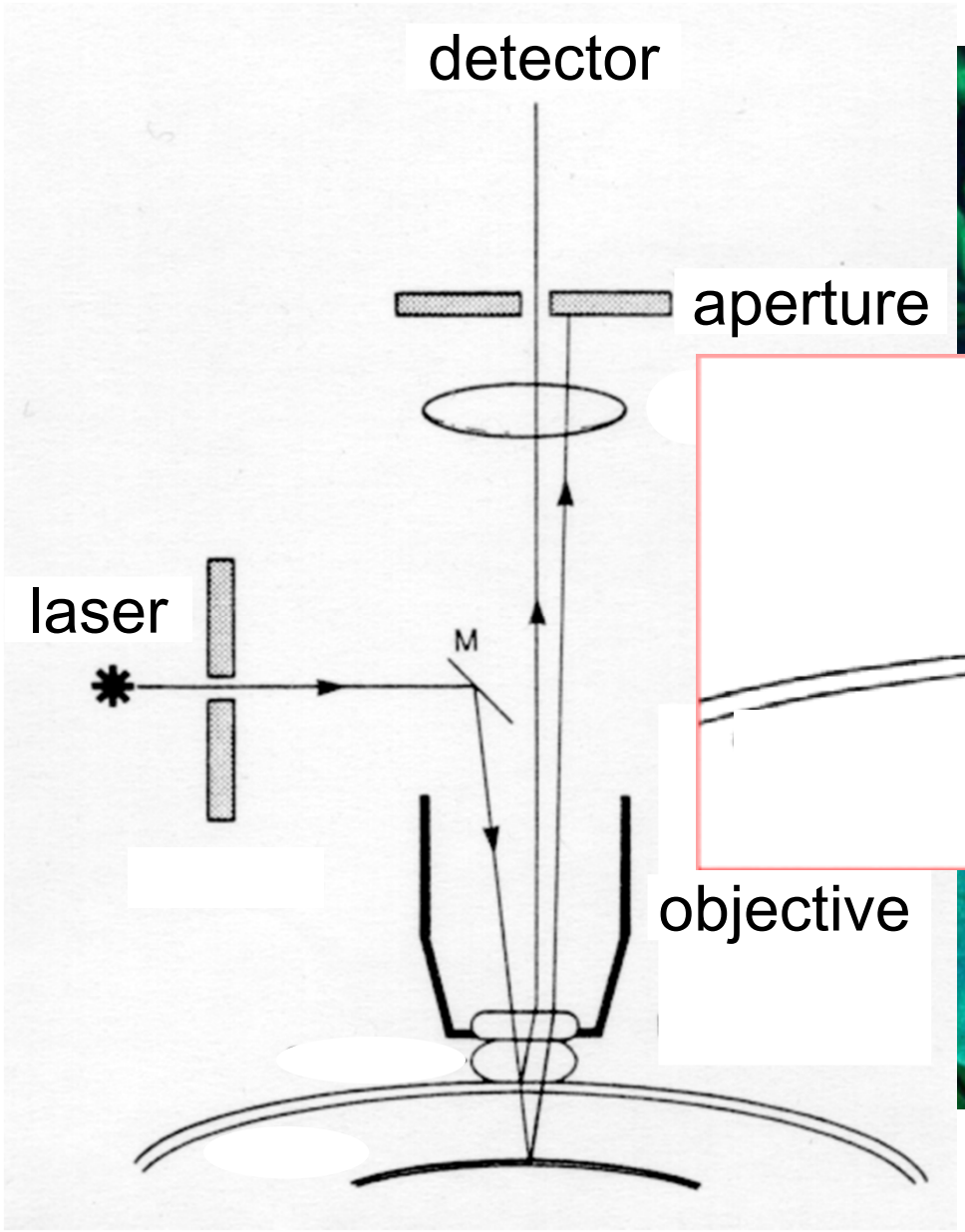

Mercury Arc Lamp
UV
IR
DETECTOR
filter block

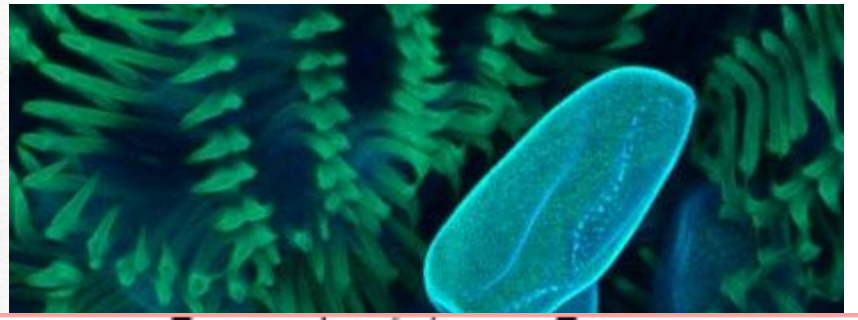
Filter Sets of Fluorescence Microscopy

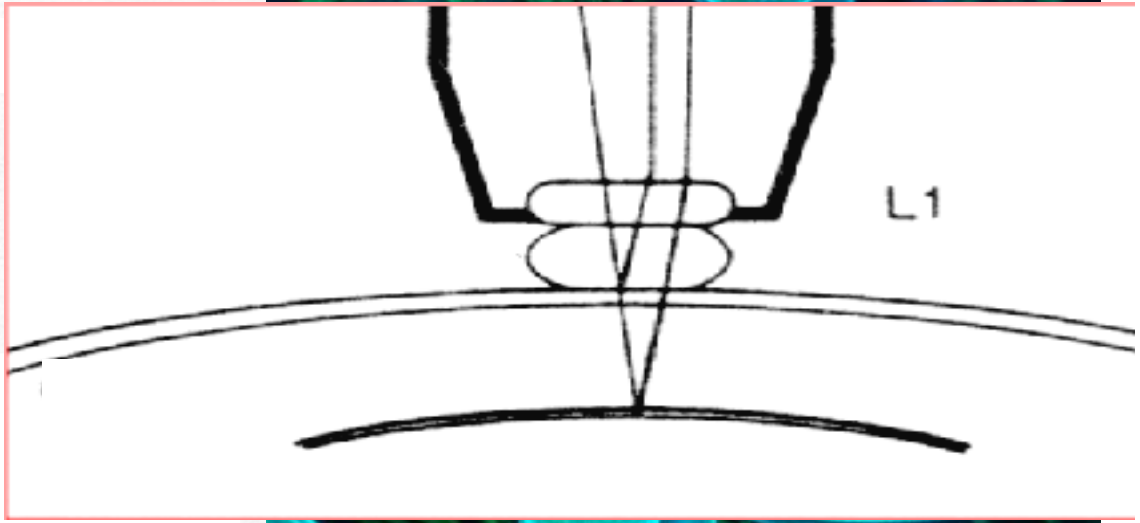


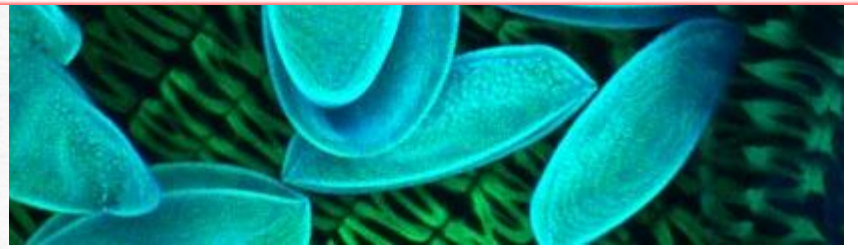


Confocal Laser Scanning Microscopy



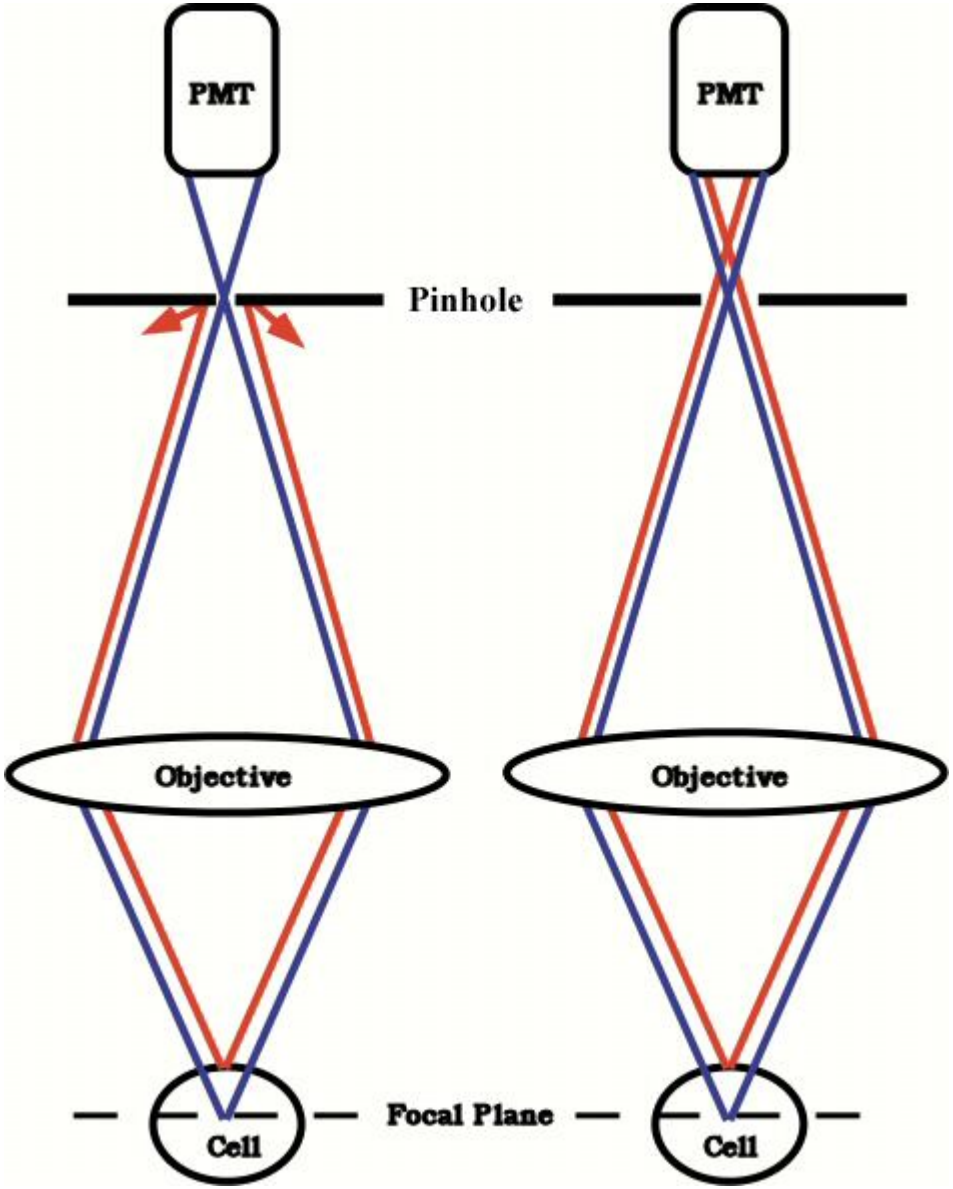




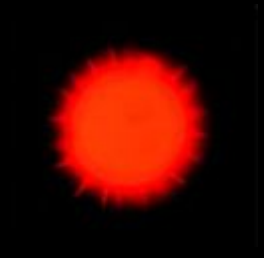


Immature pollen and endothecium cells of *Tradiscantai virginiana*

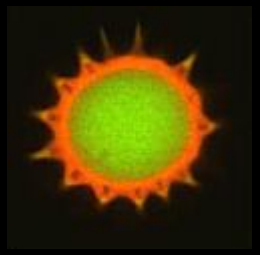
Confocal Laser Scanning Microscopy



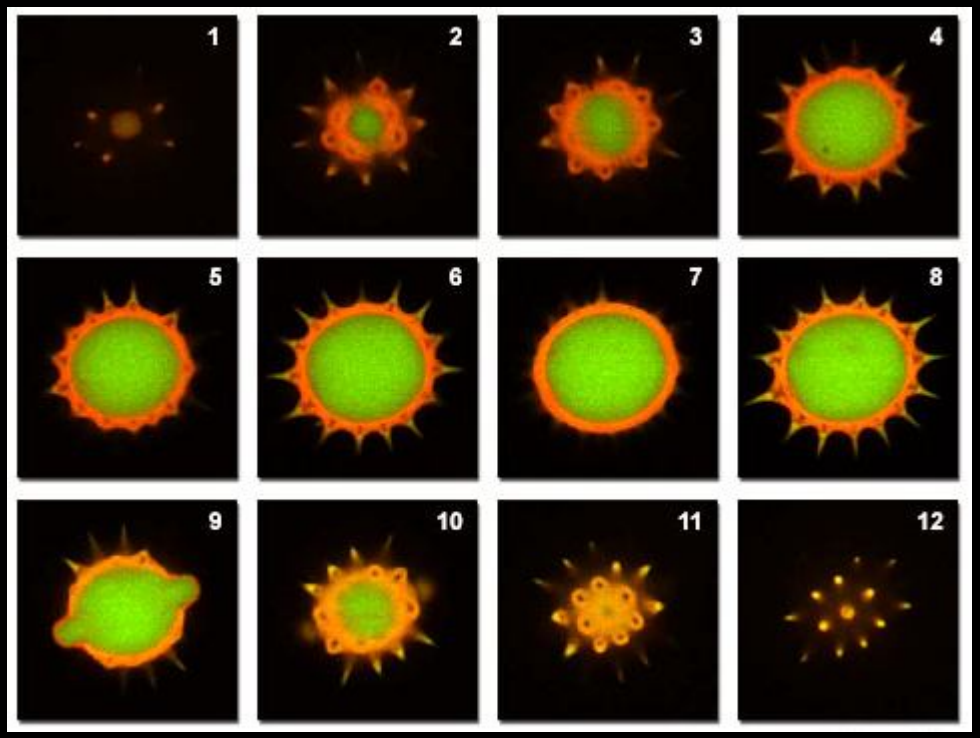
Optical Sectioning with confocal microscopy

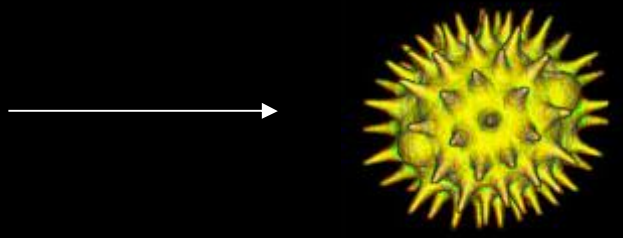


conventional



confocal

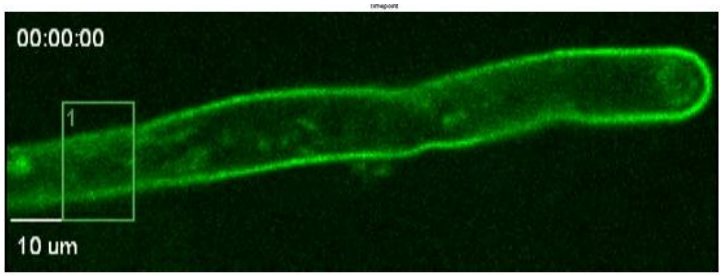




Advanced Applications of Confocal Microscopy
Protein Dynamics and Interaction

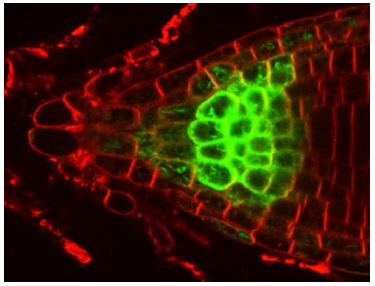
A) Bleaching techniques

FRAP, iFRAP, FLIP



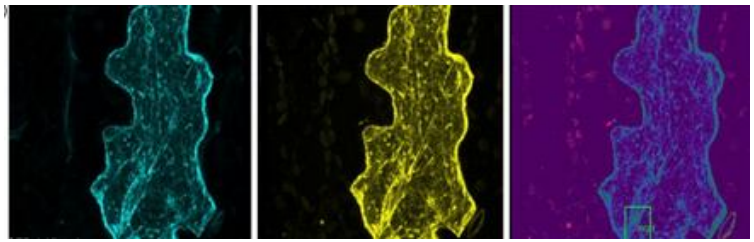
B) Photoconversion techniques

PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

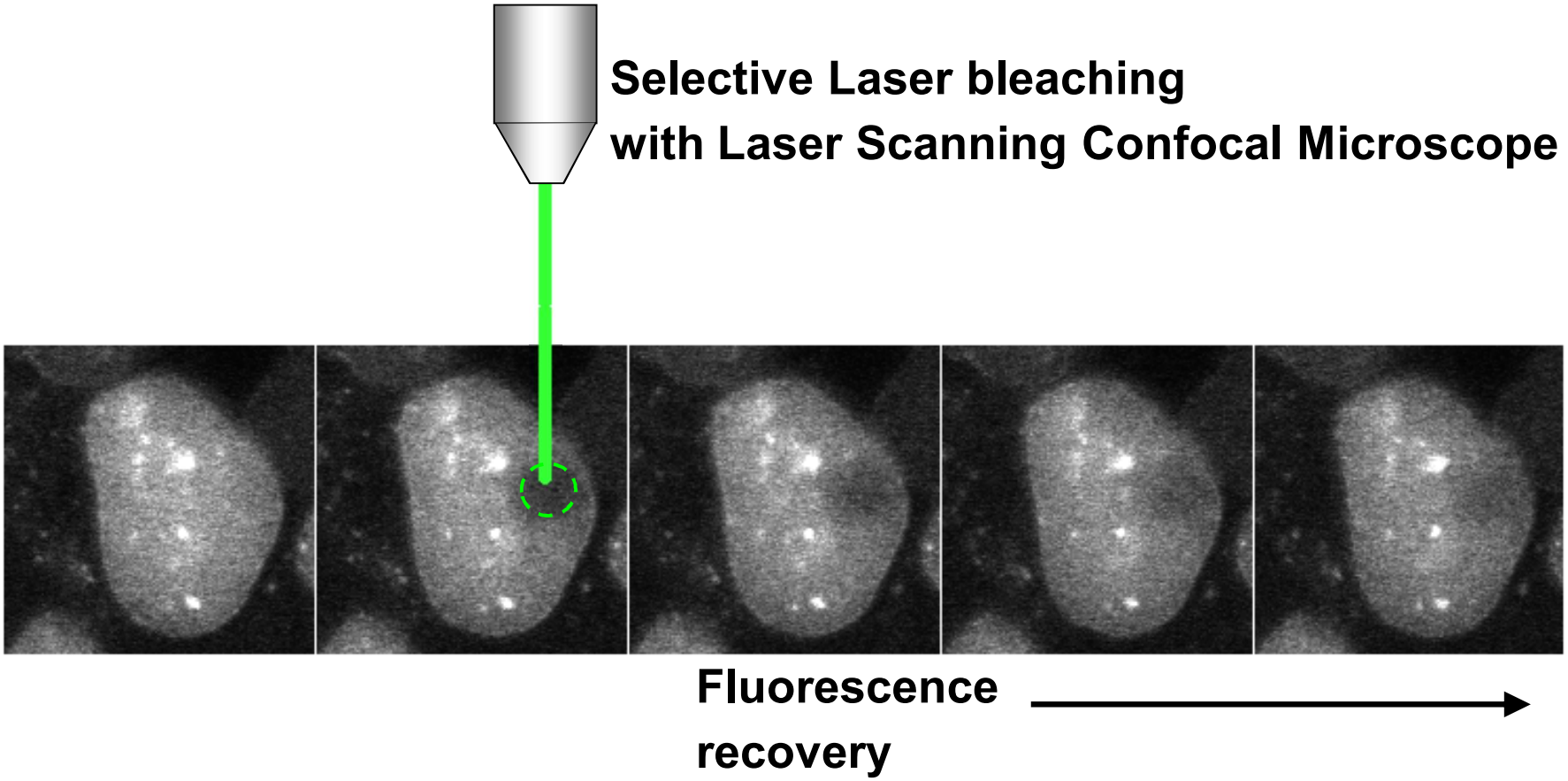


C) Protein-protein Interactions

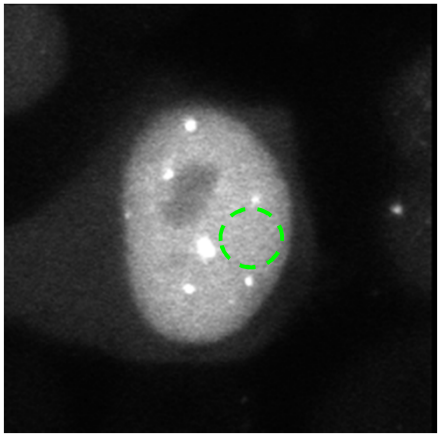
FRET, BiFC



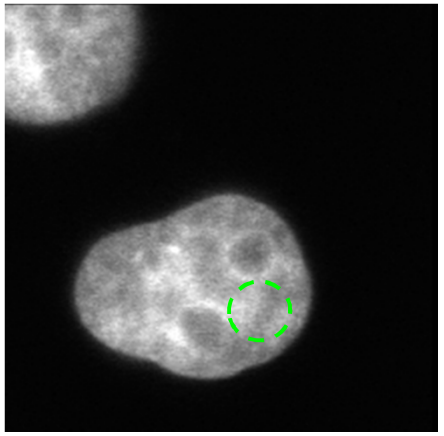
FRAP: Fluorescence Recovery After Photobleaching



FRAP: Protein Mobility Comparison

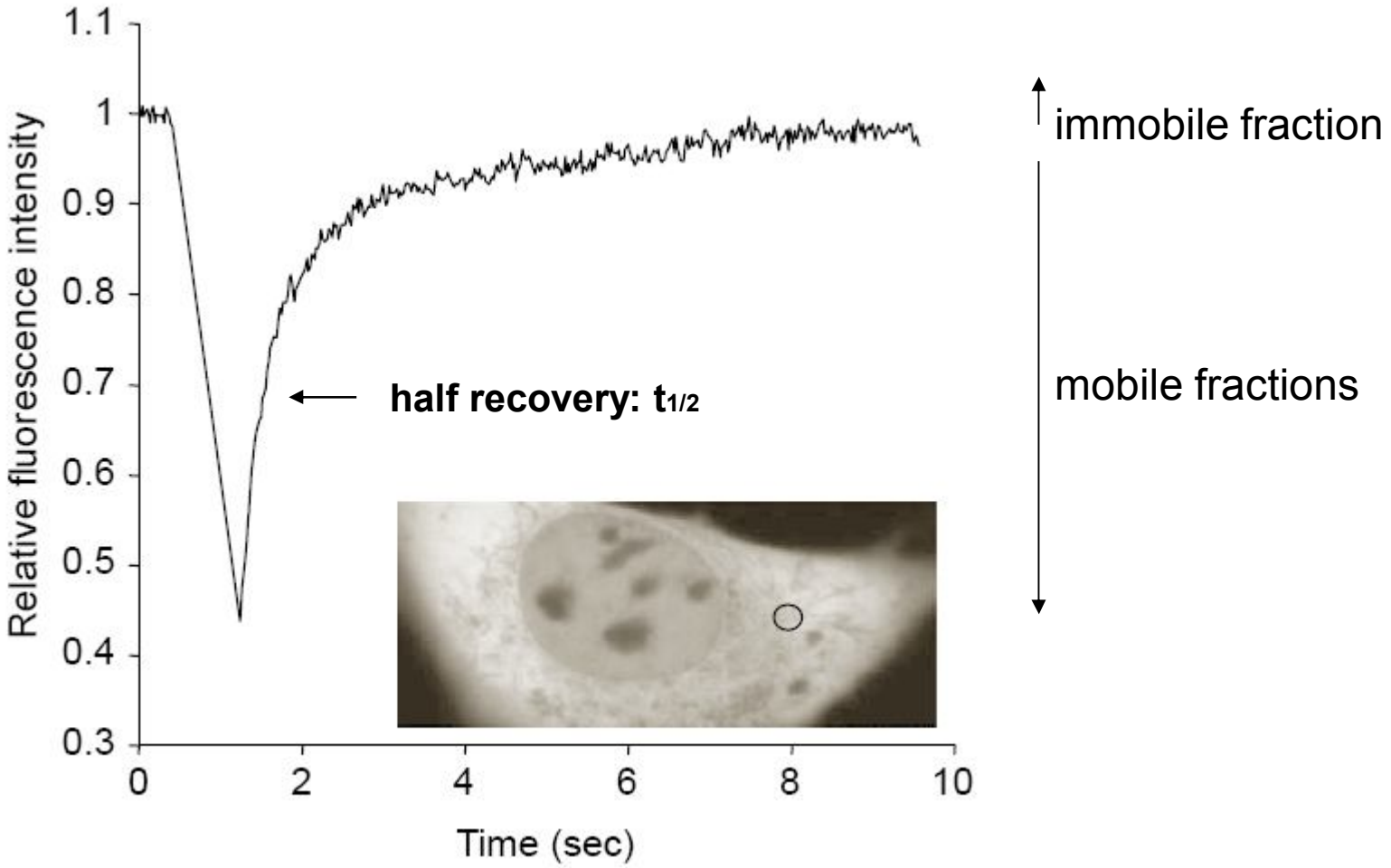


protein X

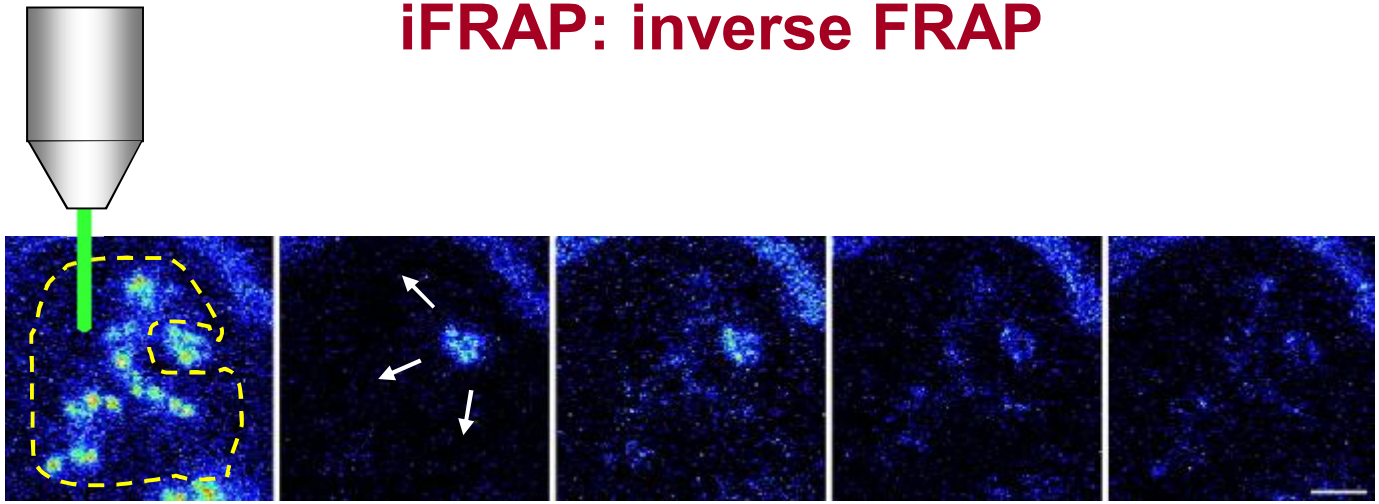


protein Y

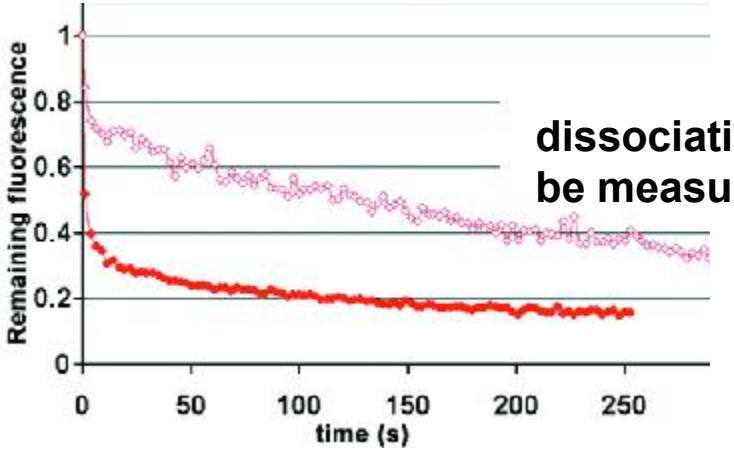
FRAP: Kinetics of Fluorescence Recovery



iFRAP: inverse FRAP

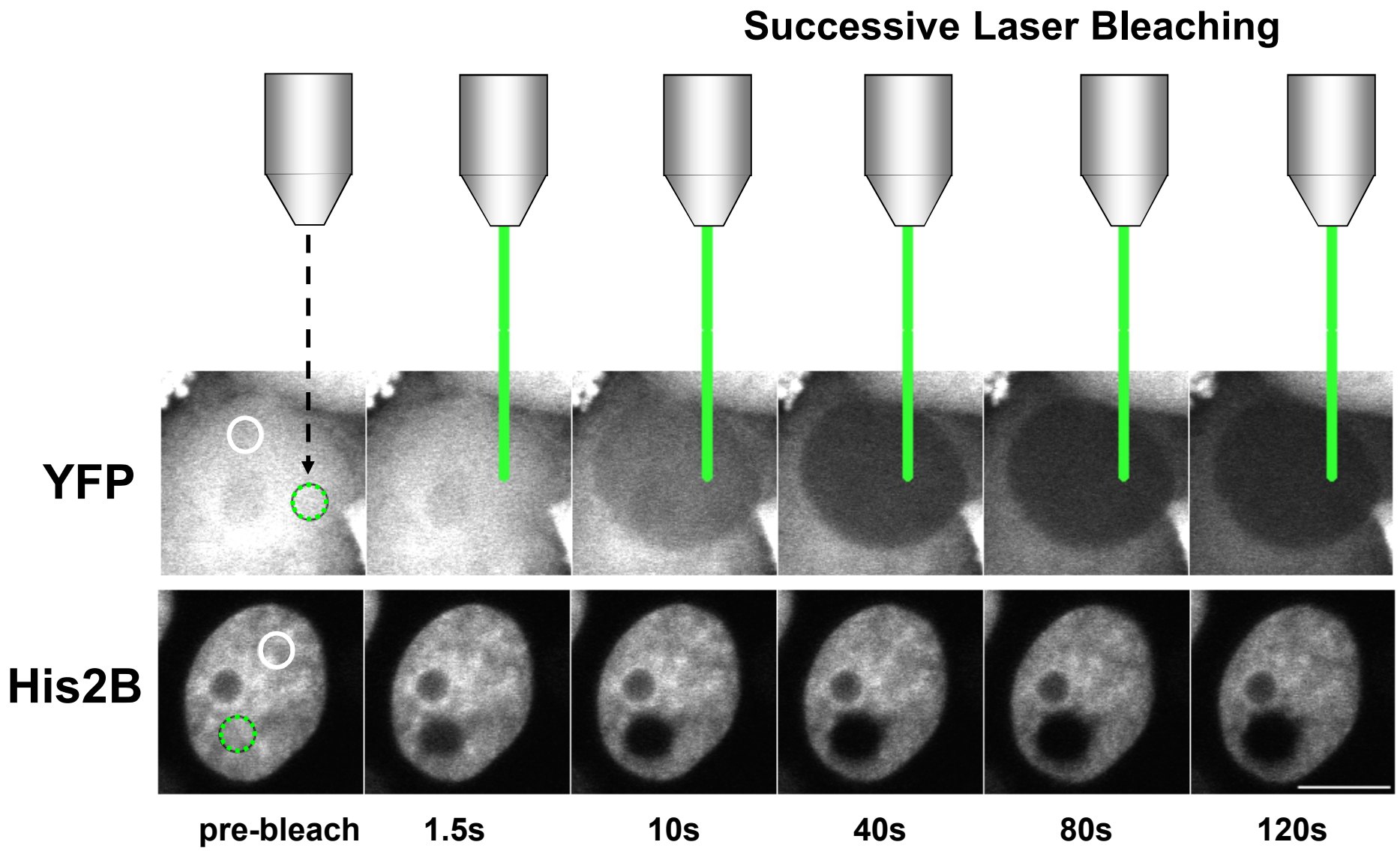


bleach everything else but the region of interest!

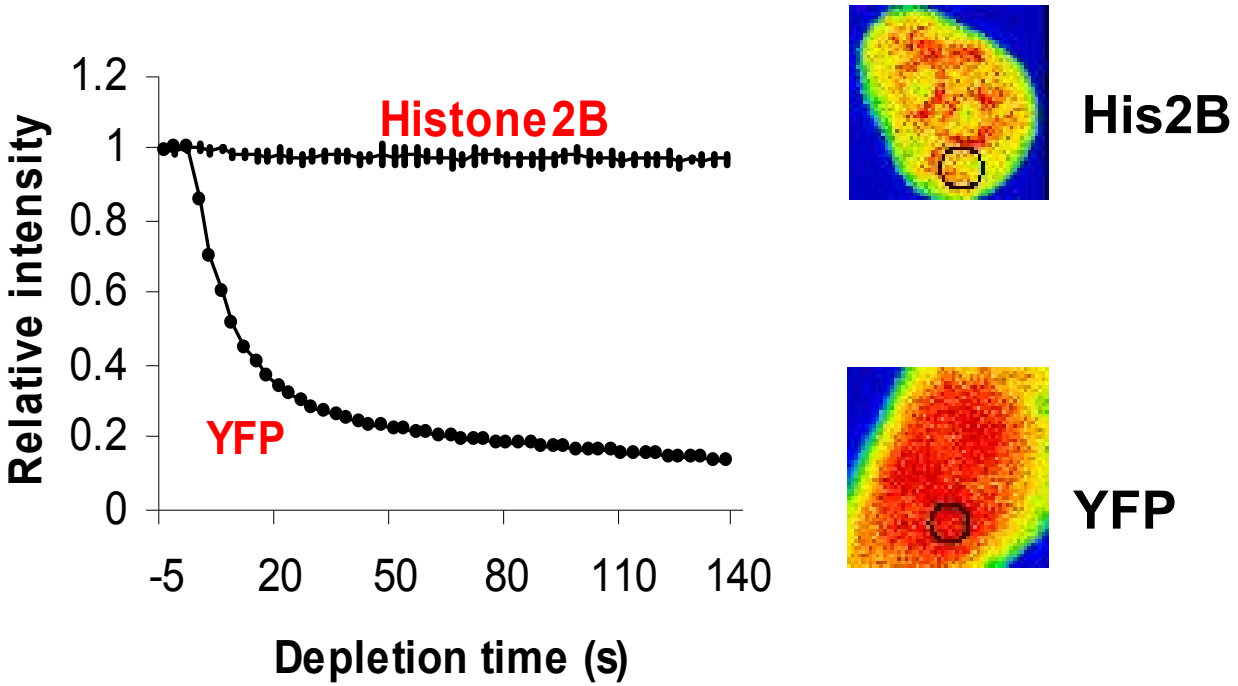


dissociation parameters of molecules can be measured

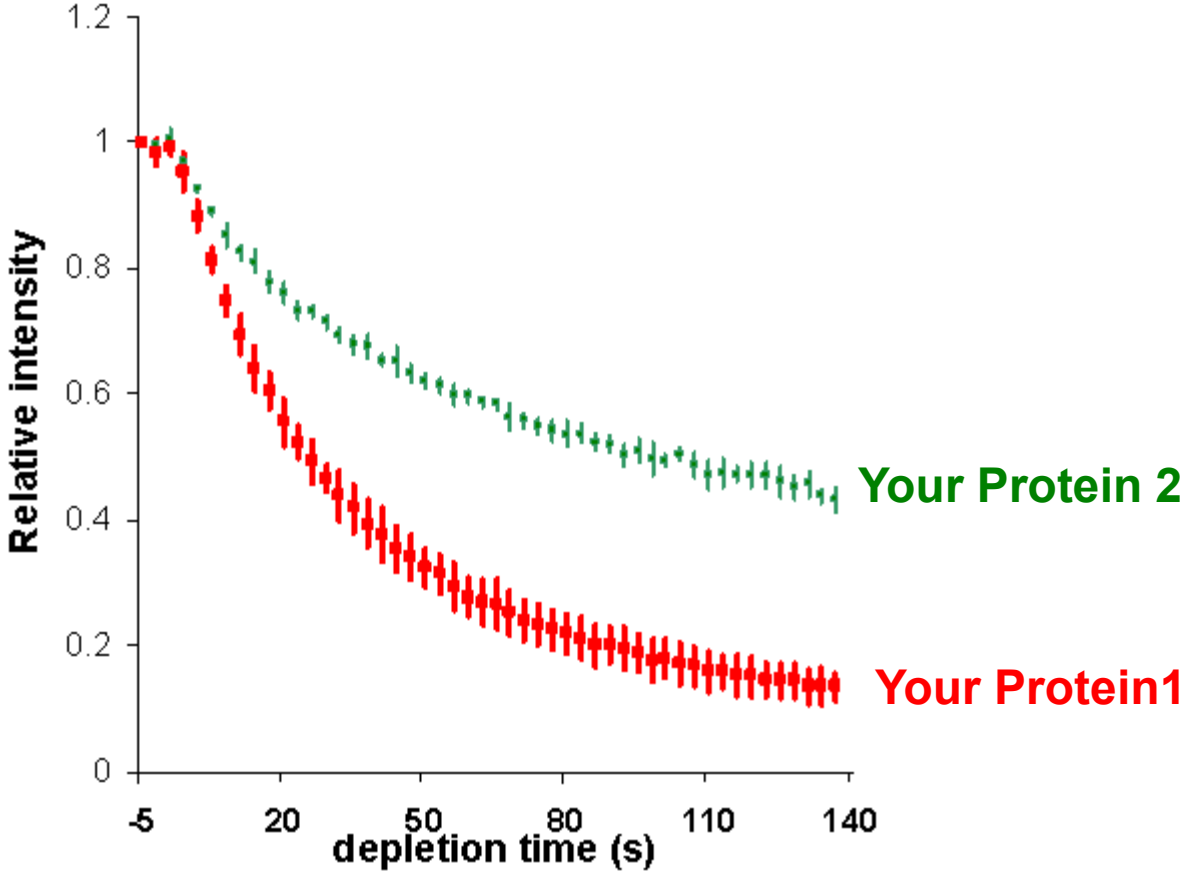
FLIP: Fluorescence Loss In Photobleaching

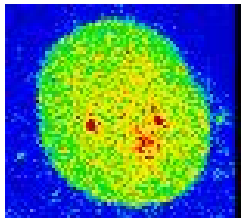


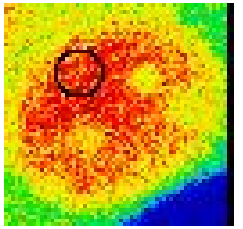
FLIP: Fluorescence Loss in Photobleaching



FLIP: Depletion Comparison

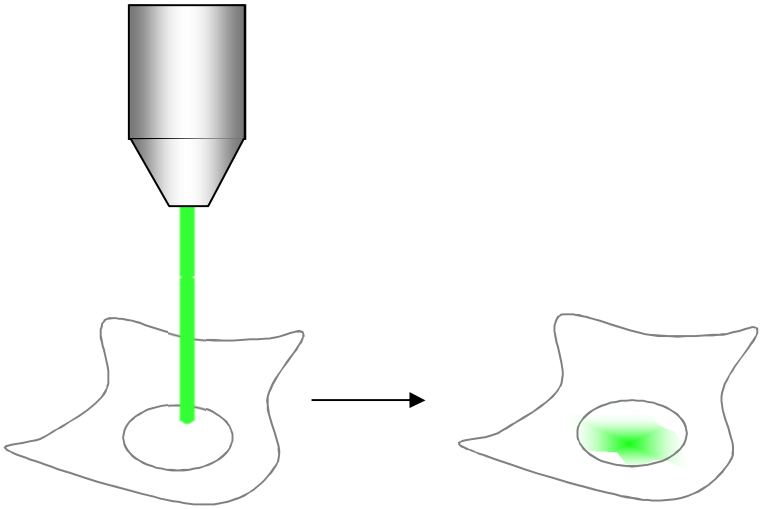




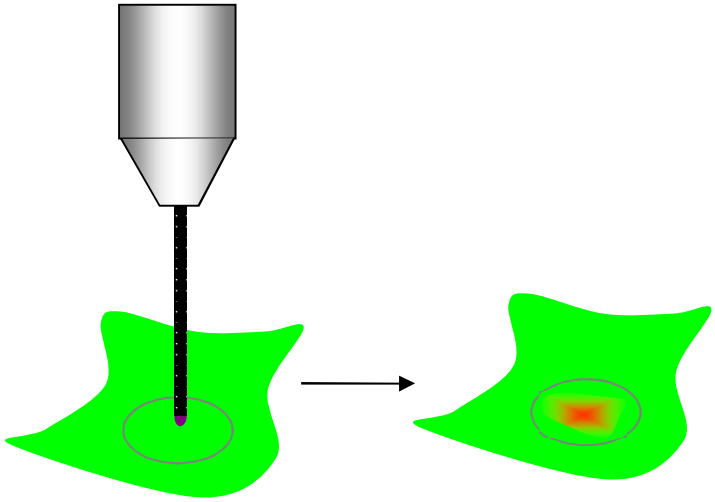


You can bleach with laser but,
lasers can also be used to “activate” or “photoconvert” a fluorescent protein...

Activatable and Photoconvertable fluorescent proteins: Highlighters

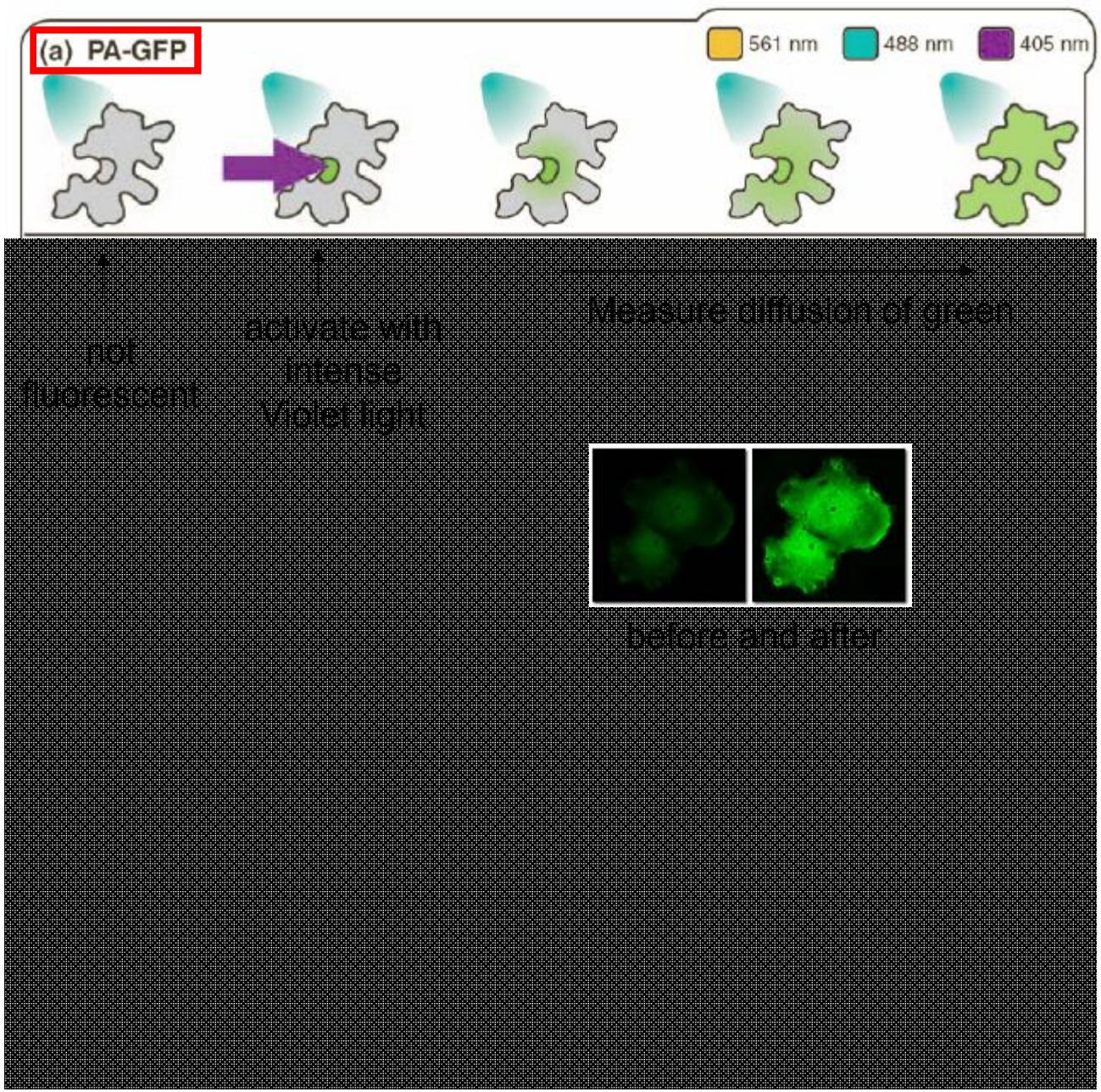


activation

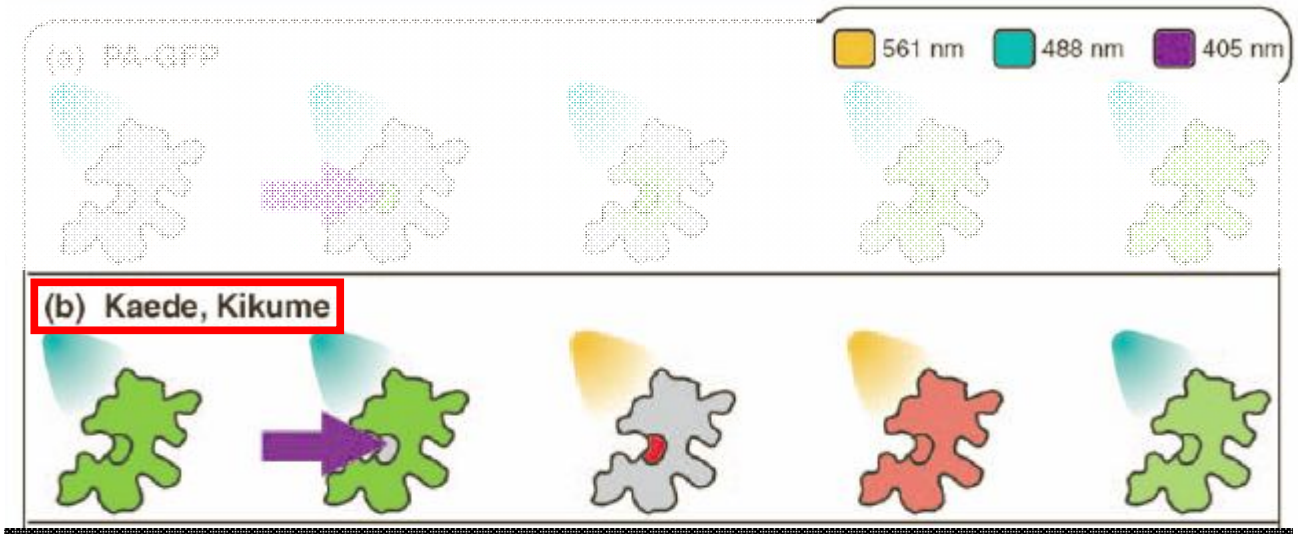


color conversion

B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

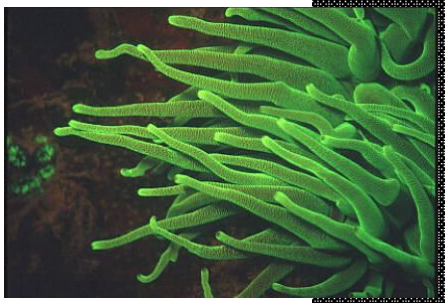


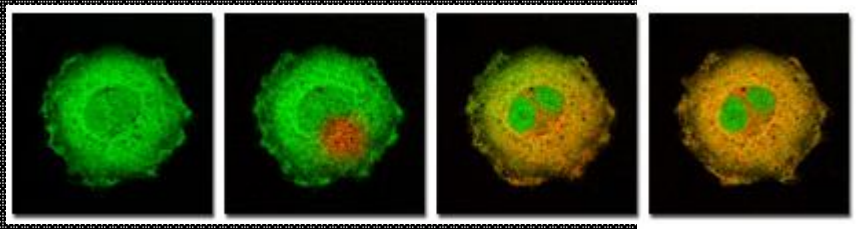
B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA



fluorescent proteins from anemones and coral

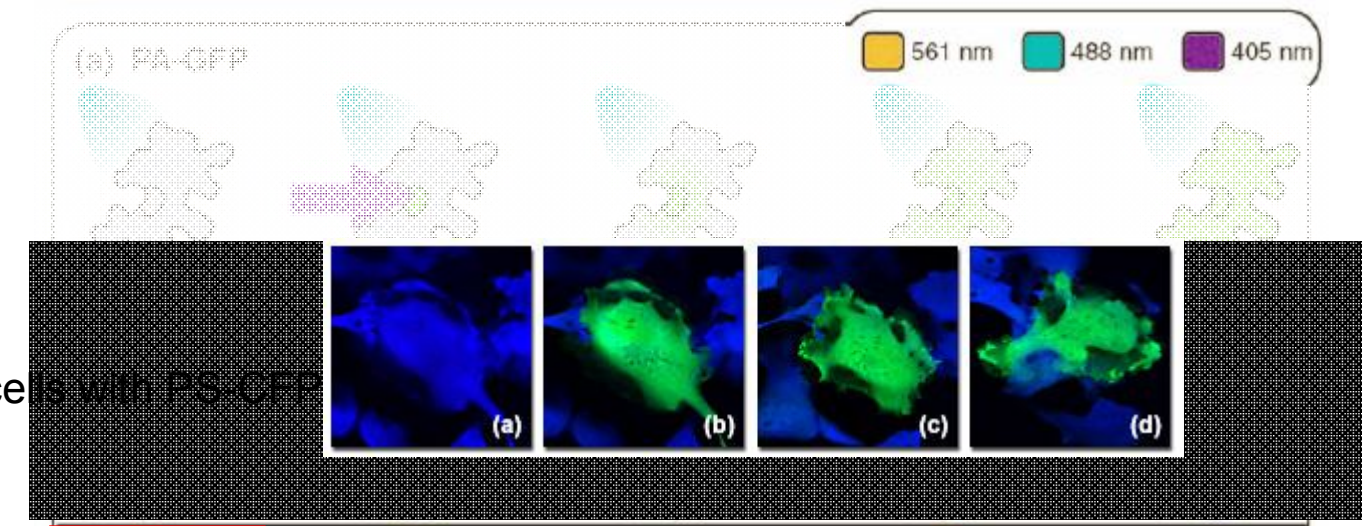
cleavage of peptide backbone changes the chromophore



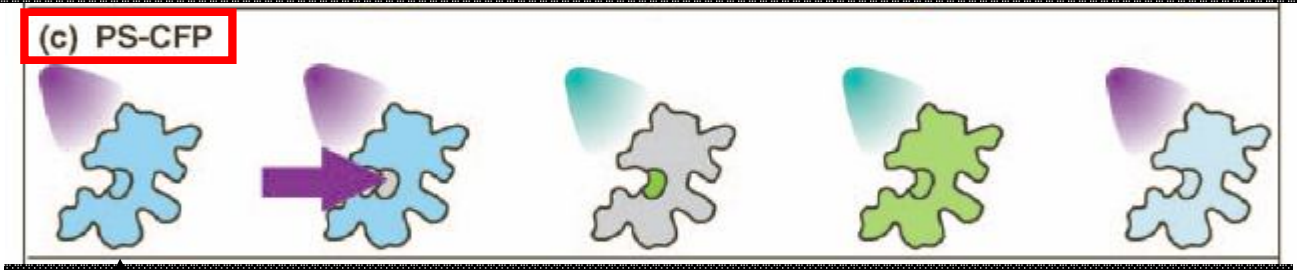


Cytoplasmic Kaede diffusion

B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA



tracking cells with PS-CFP



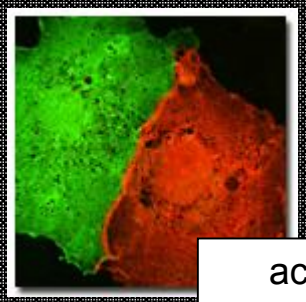
mutation of GFP

Cyan colored before conversion

turns green after intense violet illumination

B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

isolated from the coral Lobophyllia

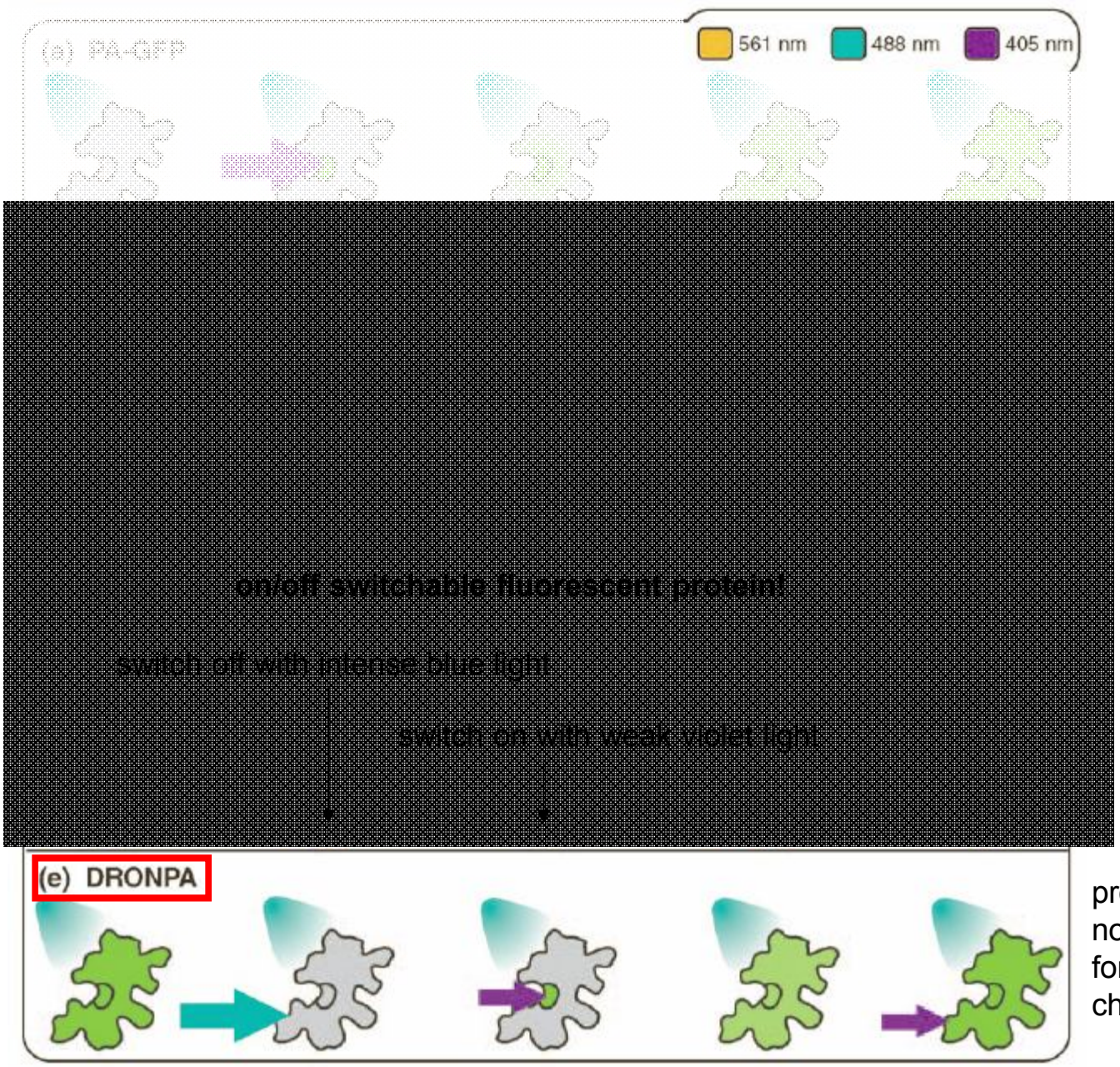


activated (red) Eos FP
not activated (green) Eos FP

Green colored before conversion

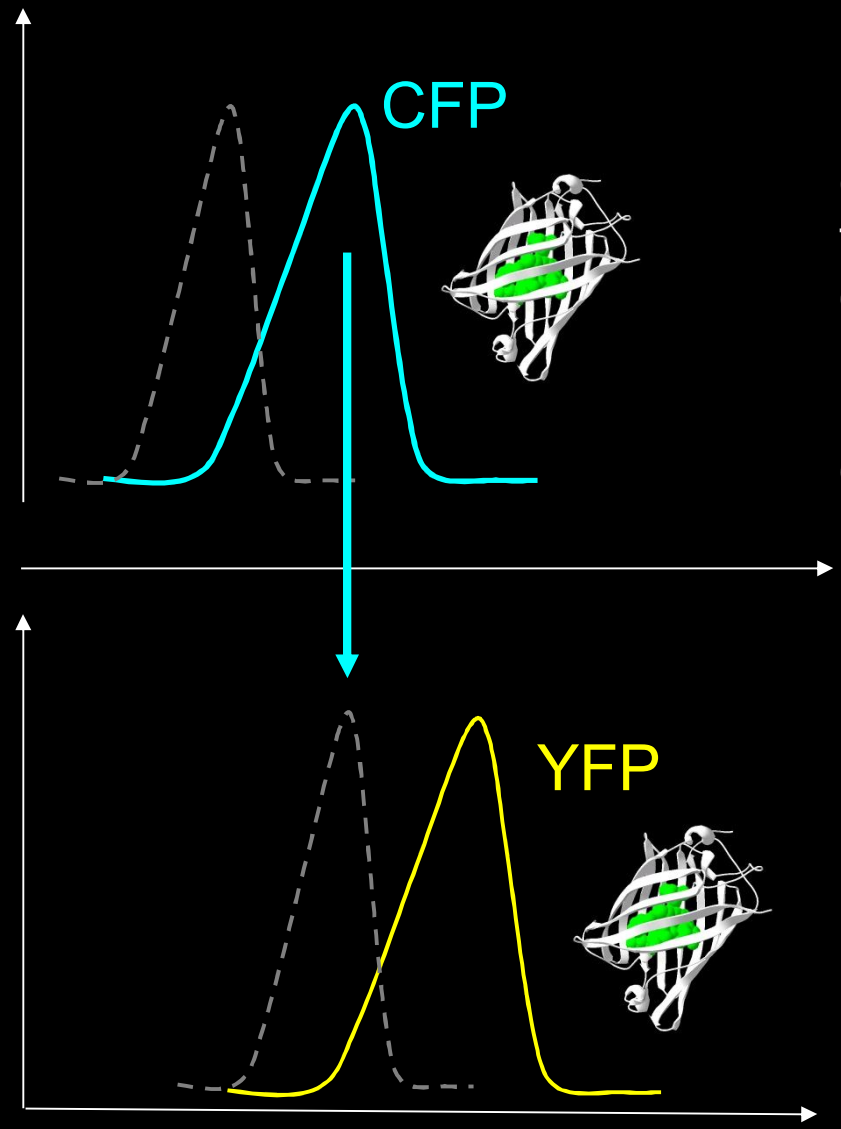
turns red after intense violet illumination

B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA



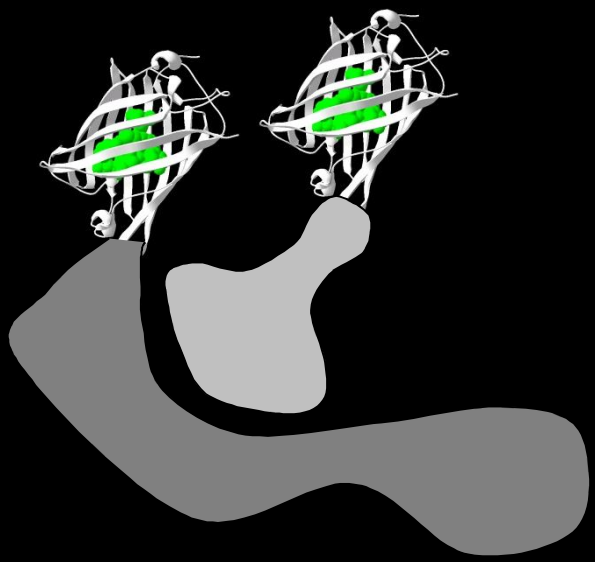
protonated and nonprotonated forms of the chromophore

FRET: Fluorescence Resonance Energy Transfer

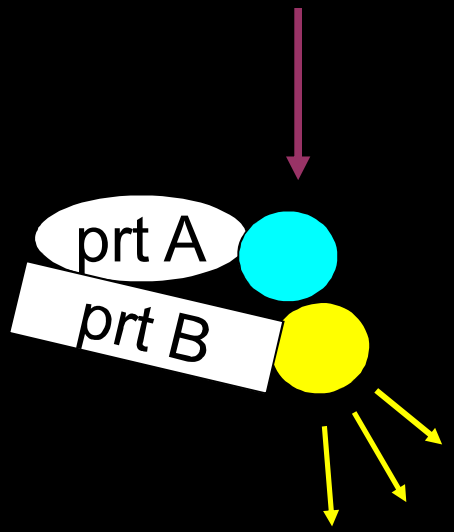


energy transfer in a non-radiative fashion, through long-range dipole-dipole interactions (e.g tuning forks)

distance should be 10nm or less

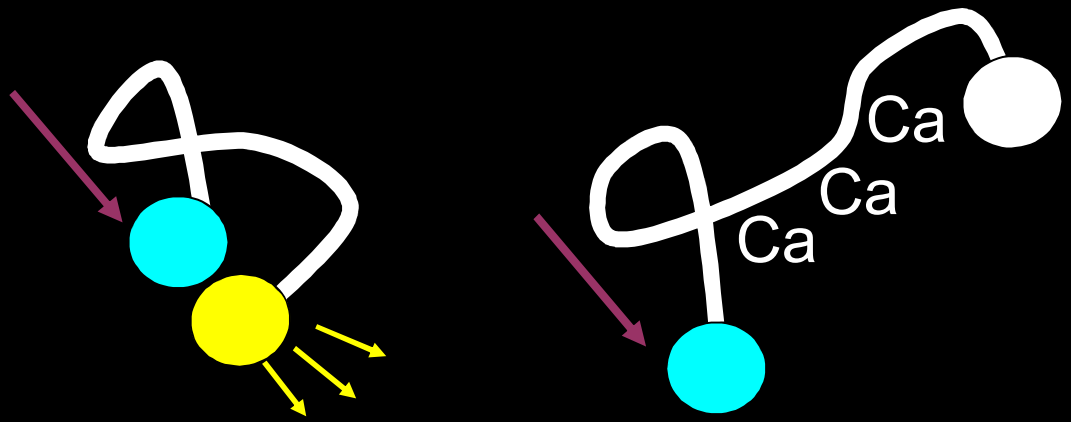


C) Protein-protein Interactions
FRET, BiFC



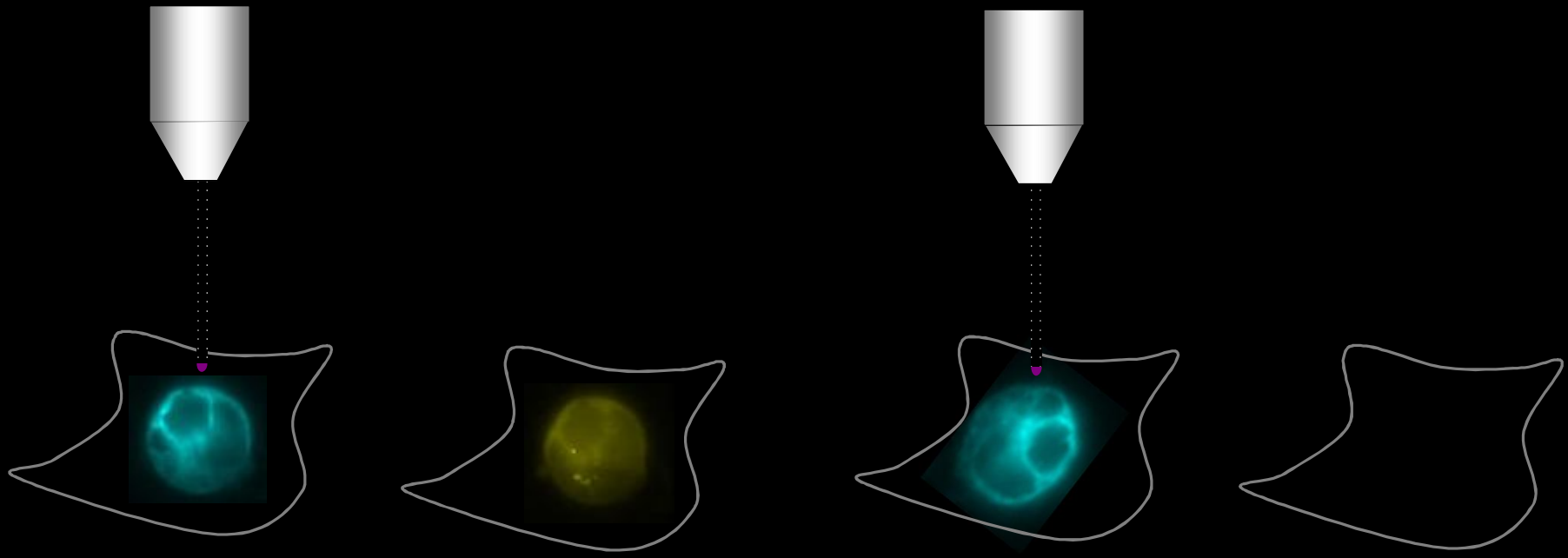
FRET pairs
CFP/YFP
GFP/ RFP
NewFP / NewestFP

protein-protein interactions



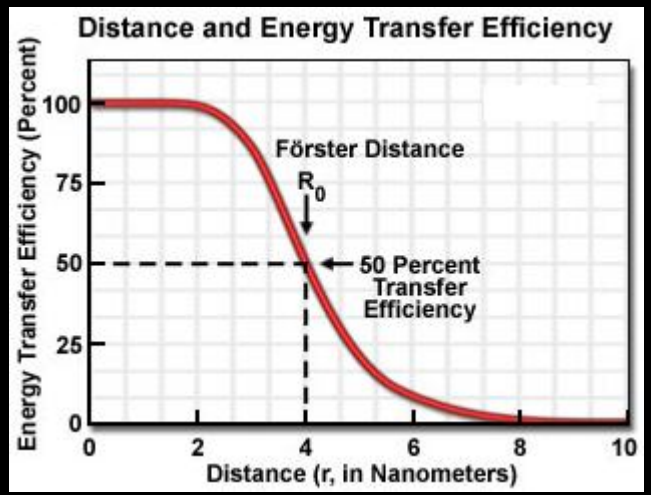
protein conformation changes

C) Protein-protein Interactions
FRET, BiFC



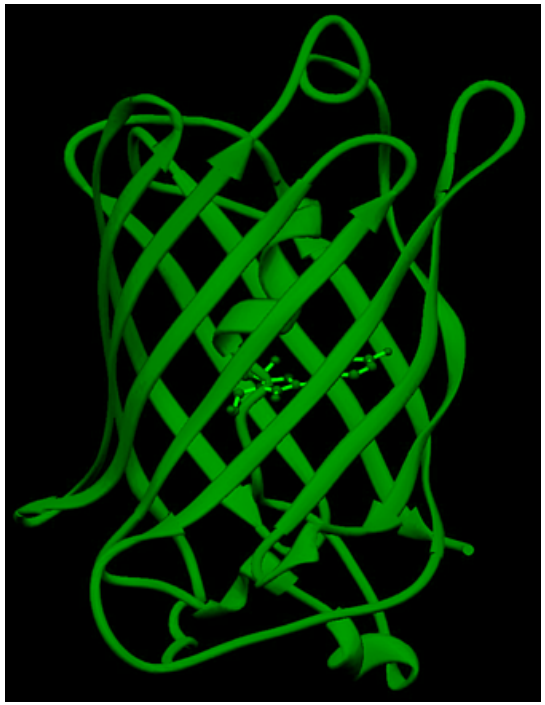
(YFP) conjugated protein is in close proximity

(YFP) conjugated protein is distant

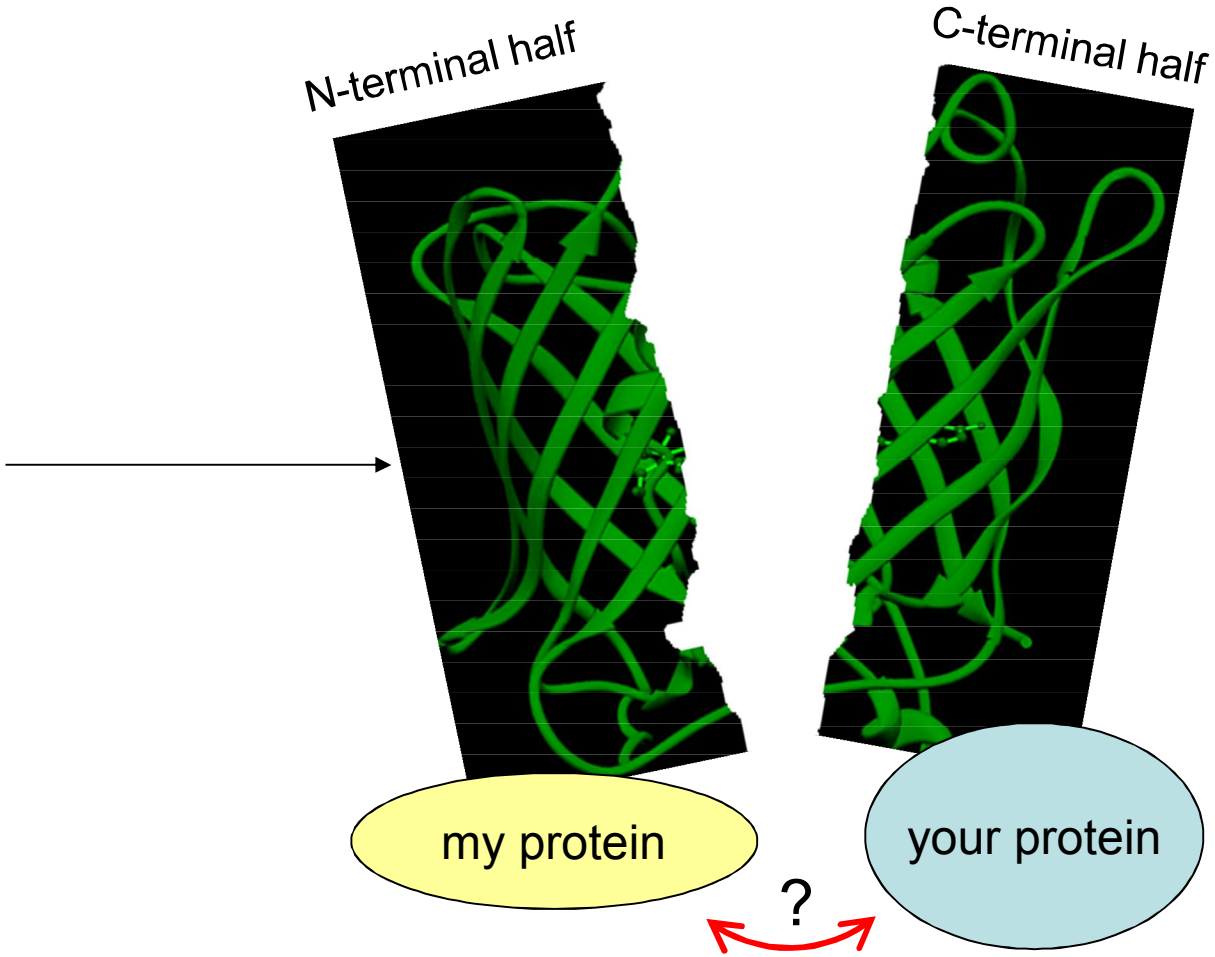


FRET efficiency

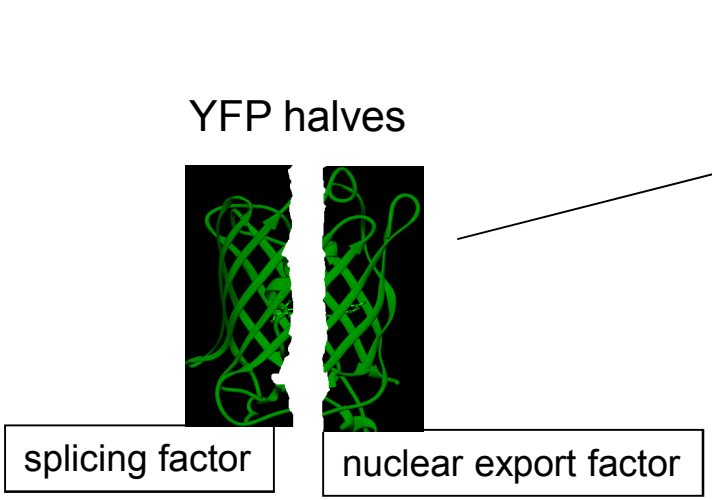
BiFC: Bimolecular Fluorescence Complementation

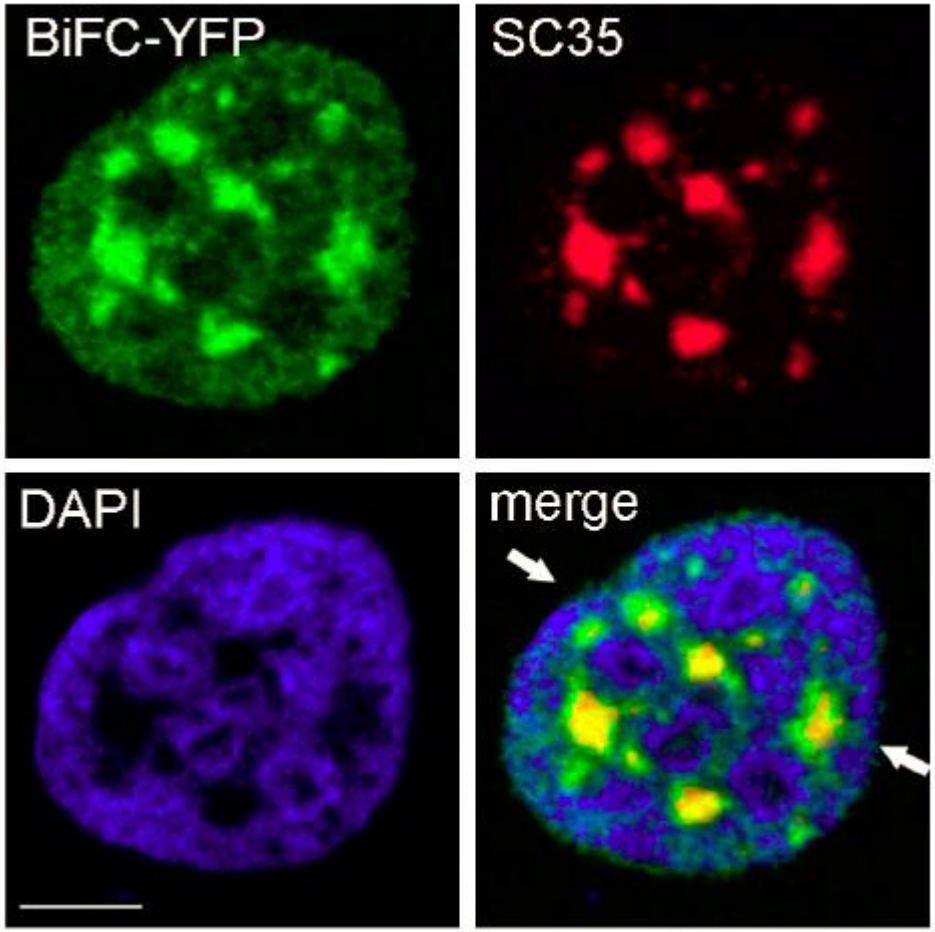


GFP

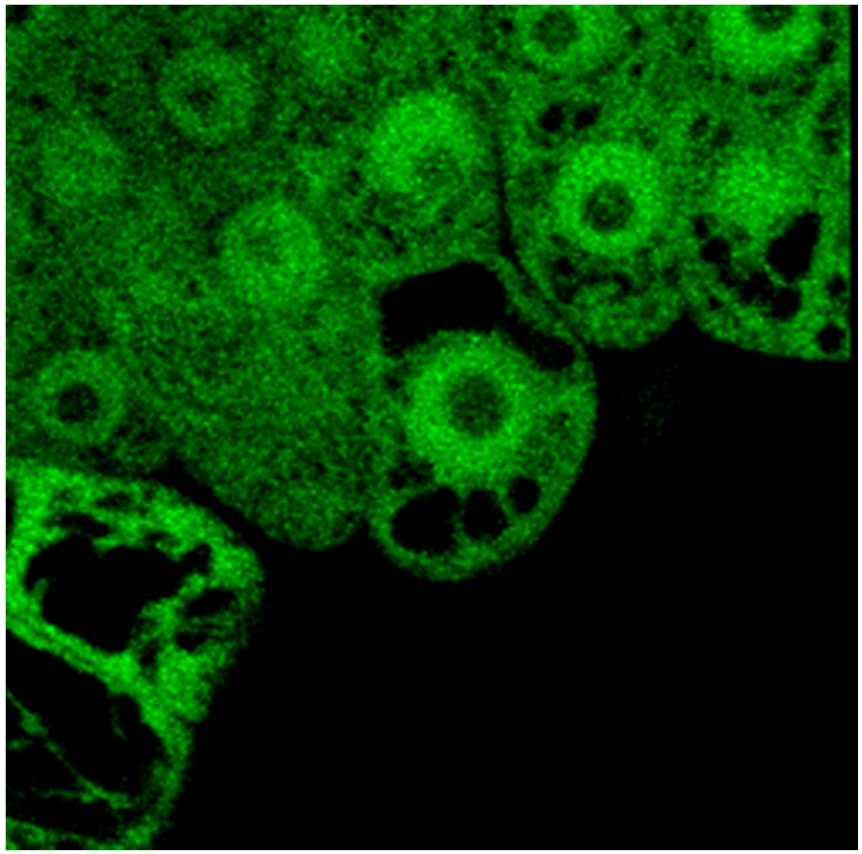


BiFC: Bimolecular Fluorescence Complementation





BiFC is easier than FRET as it requires less complicated setup and equipment.
However FRET is more suitable for reversible/dynamic interactions



Future is Fluorescent!