

Object visualization in fluorescence microscopy

MMIB 2022

Jan Valečka, Light Microscopy Core Facility

Fluorescence microscopy

ground truth



bright field

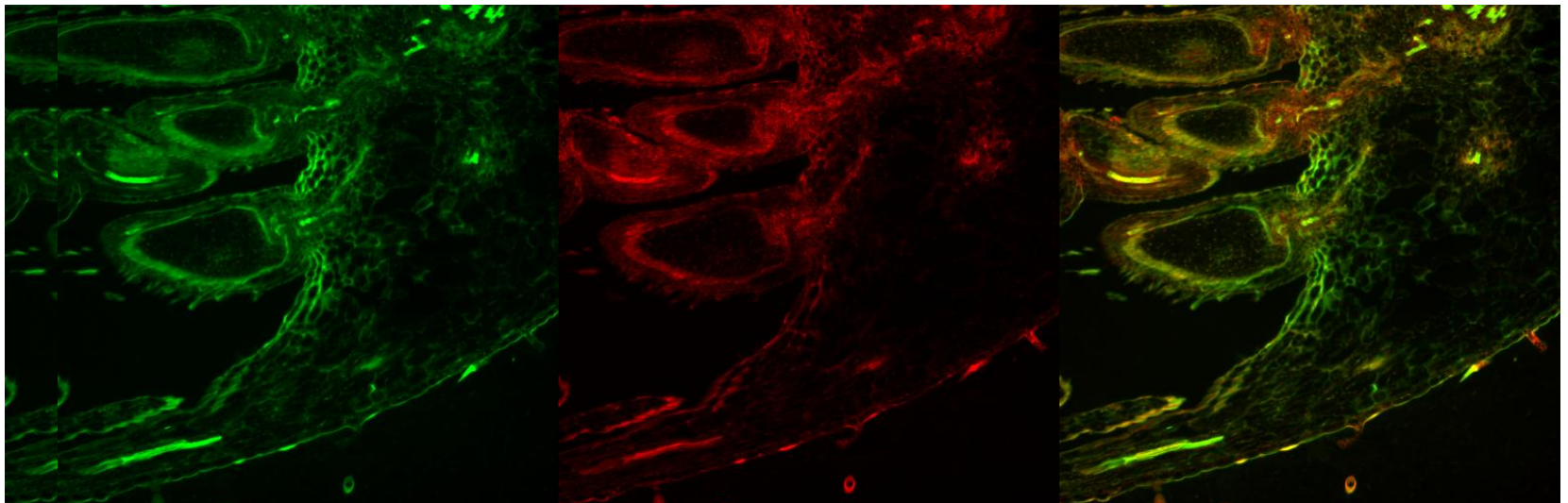
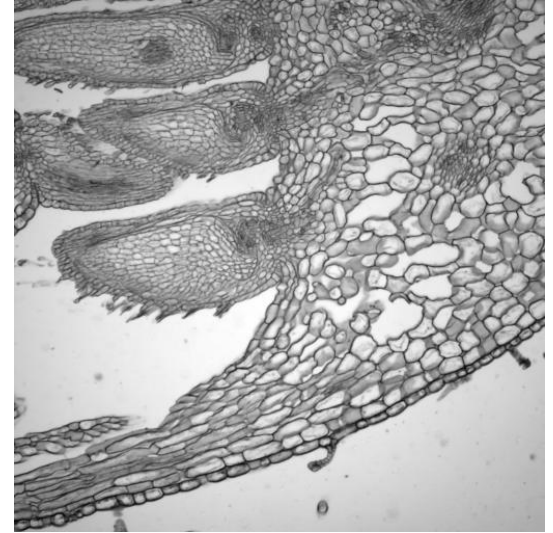


fluorescence

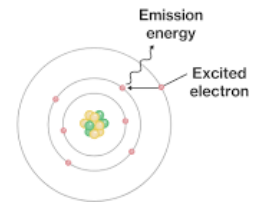
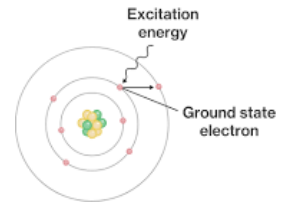
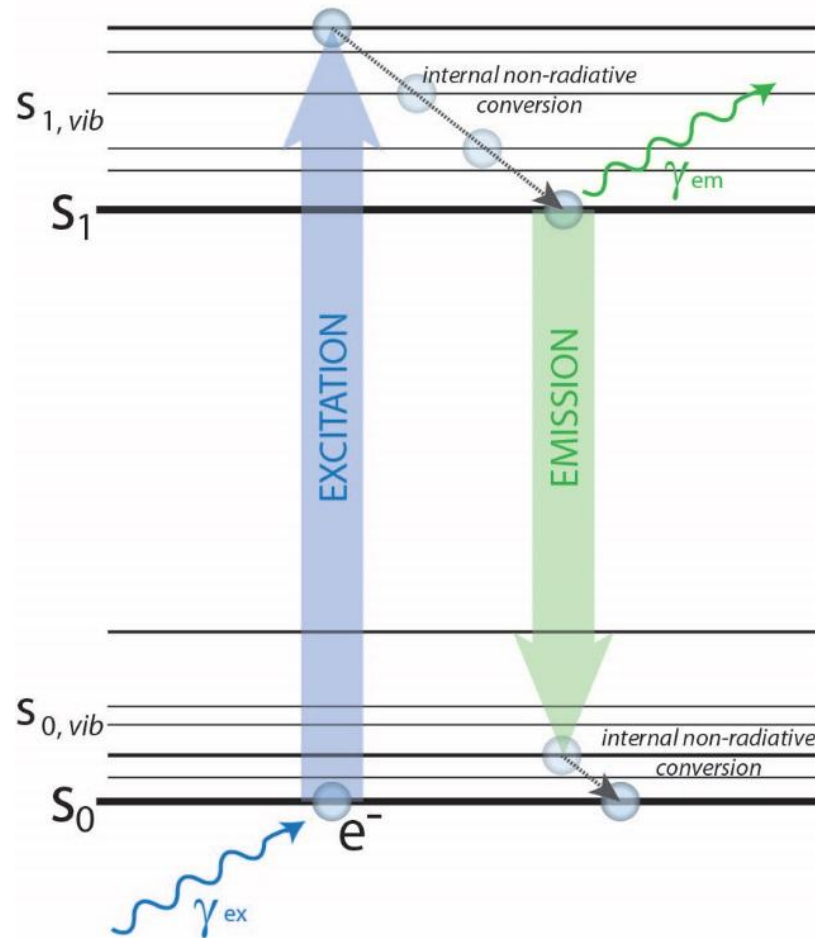


Fluorescence microscopy

- Advantages:
 - Contrast
 - Specificity
 - Multiple colours
- Disadvantage
 - Staining is necessary



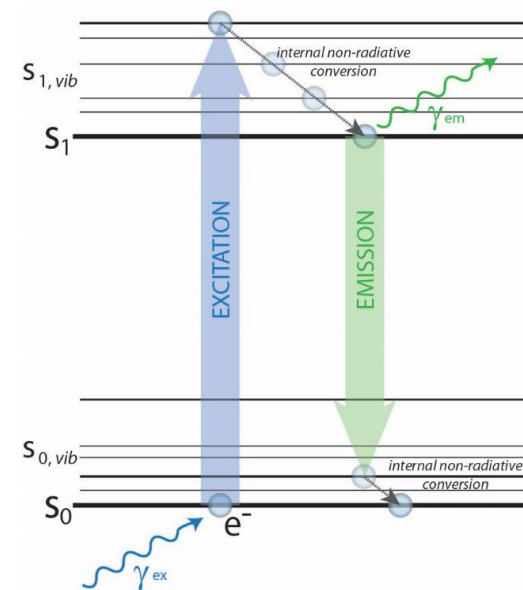
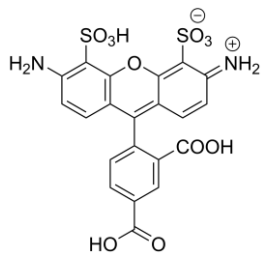
Fluorescence



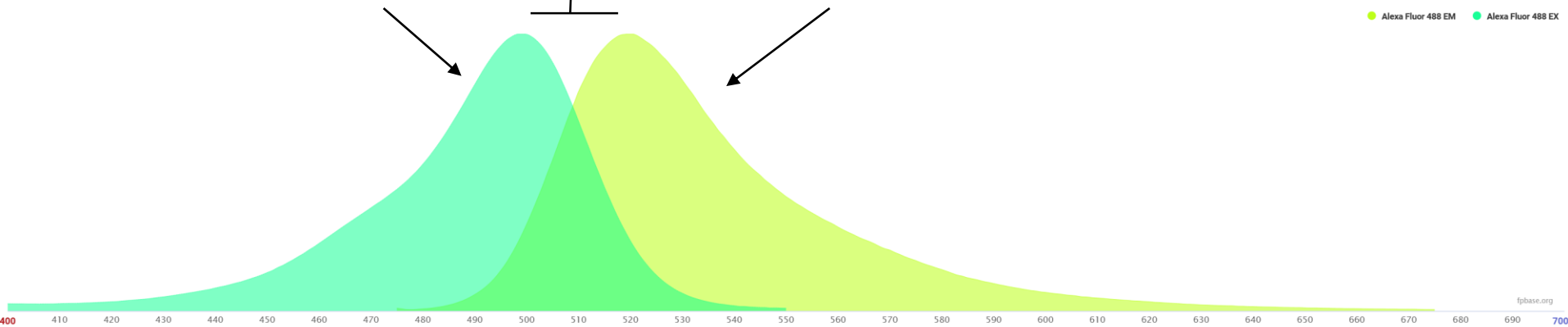
Wikipedia: „**Fluorescence** is the emission of light by a substance that has absorbed light or other electromagnetic radiation.“

Fluorescence spectrum

- Alexa fluor 488

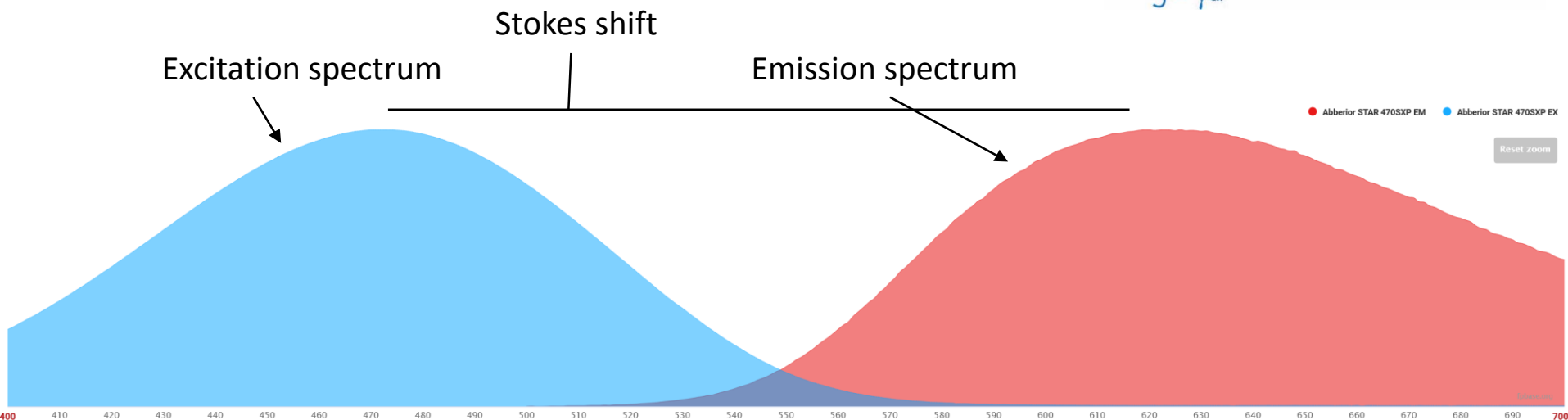
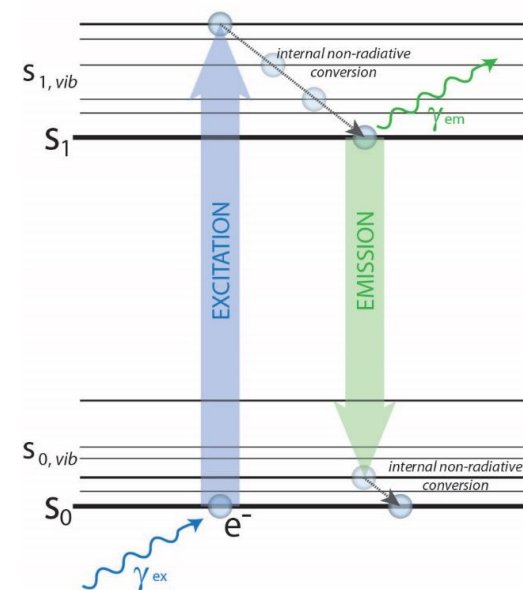
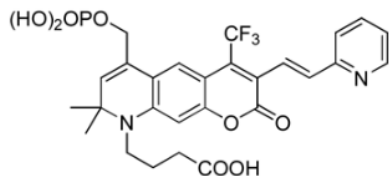


Excitation spectrum Stokes shift Emission spectrum



Fluorescence spectrum

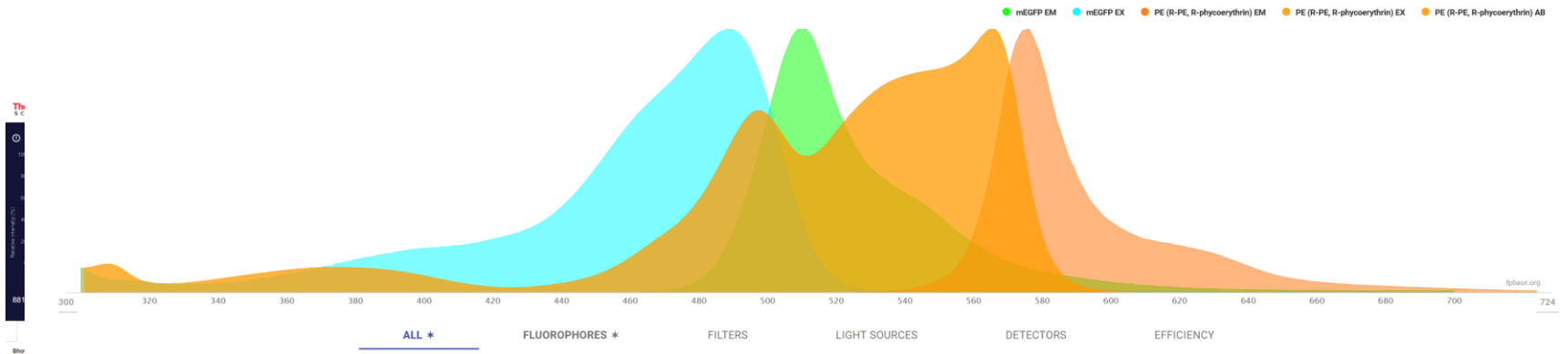
- Abberior STAR 470SXP



Spectra viewers



info tools explore sign in Search



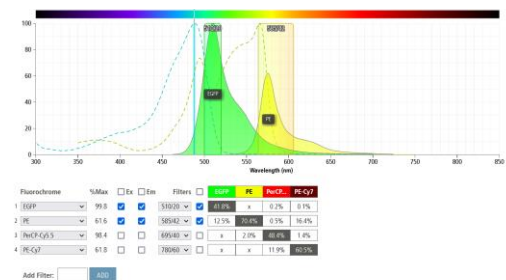
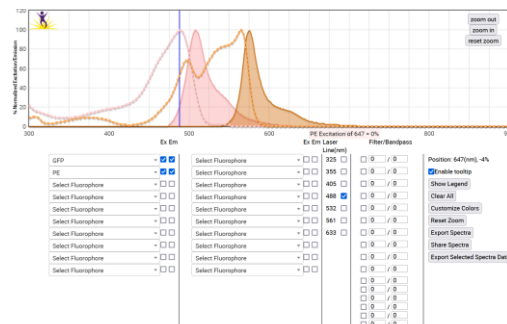
FLUORESCENT PROTEINS

mEGFP (QY: 0.6 / EC: 56,000)

DYES

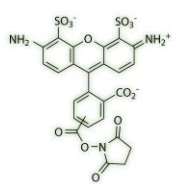
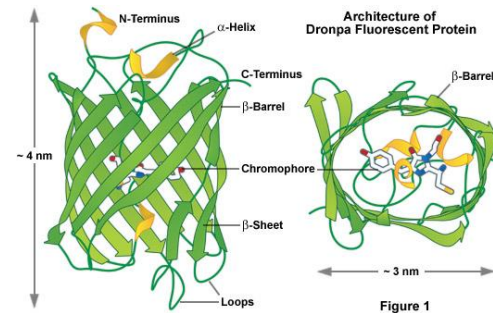
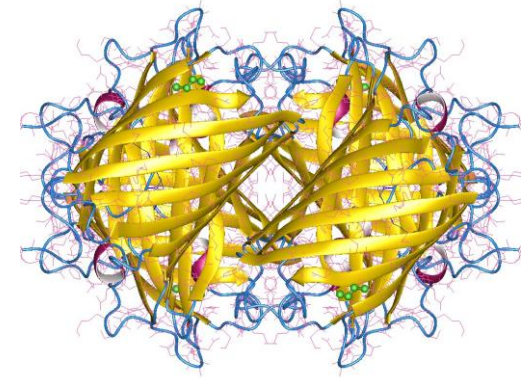
PE (R-PE, R-phycoerythrin)

Type to search...

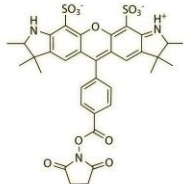


General groups of labels

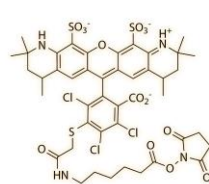
- Fluorescent proteins
- Small chemicals
 - Very diverse group
- Labelled antibodies



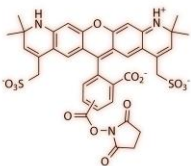
Alexa Fluor® 488



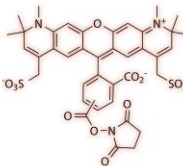
Alexa Fluor® 532



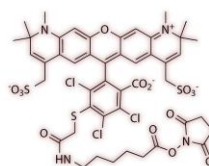
Alexa Fluor® 546



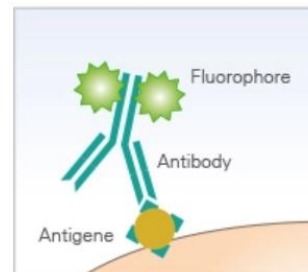
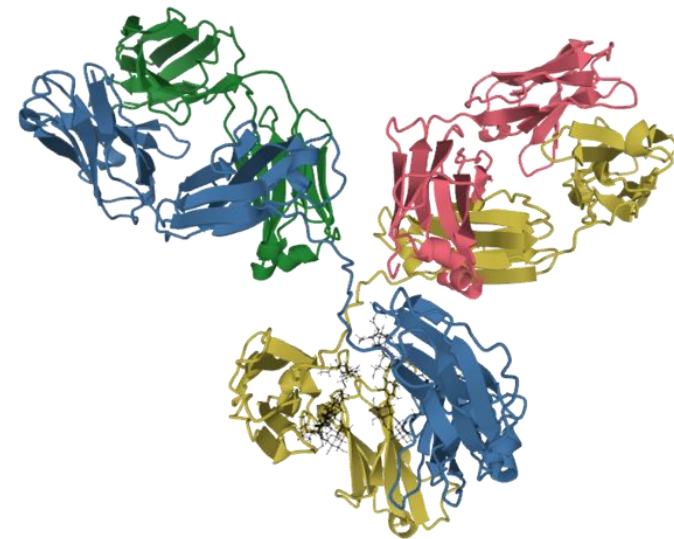
Alexa Fluor® 568



Alexa Fluor® 594



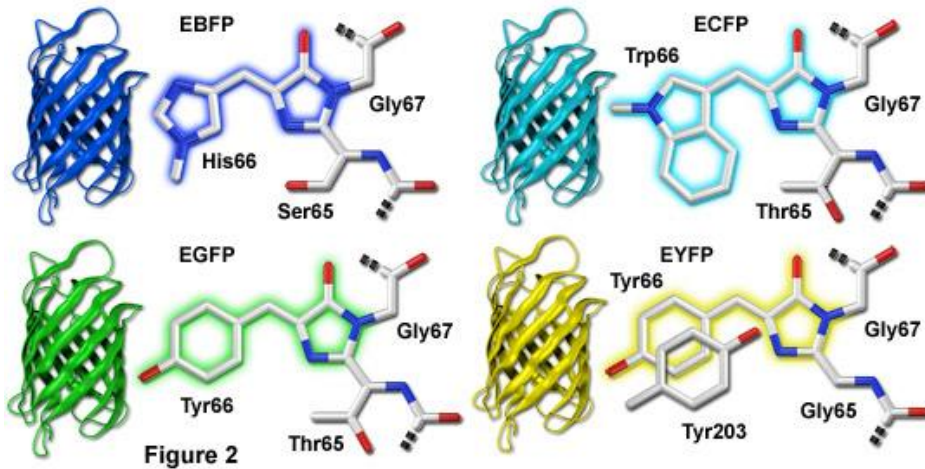
Alexa Fluor® 610



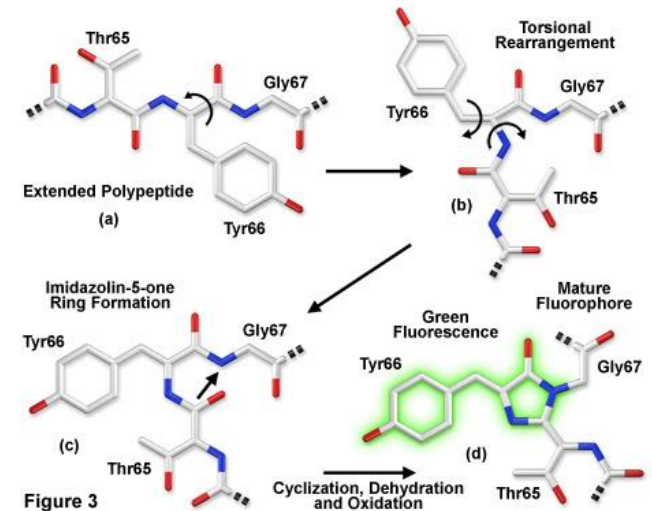
Live cells

- Genetically encoded markers
 - Fluorescent proteins
 - Brightness, maturation, monomer/oligomer, photostability (quantum yield, extinction coefficient, fluorescence lifetime)

Chromophore Structural Motifs of Green Fluorescent Protein Variants

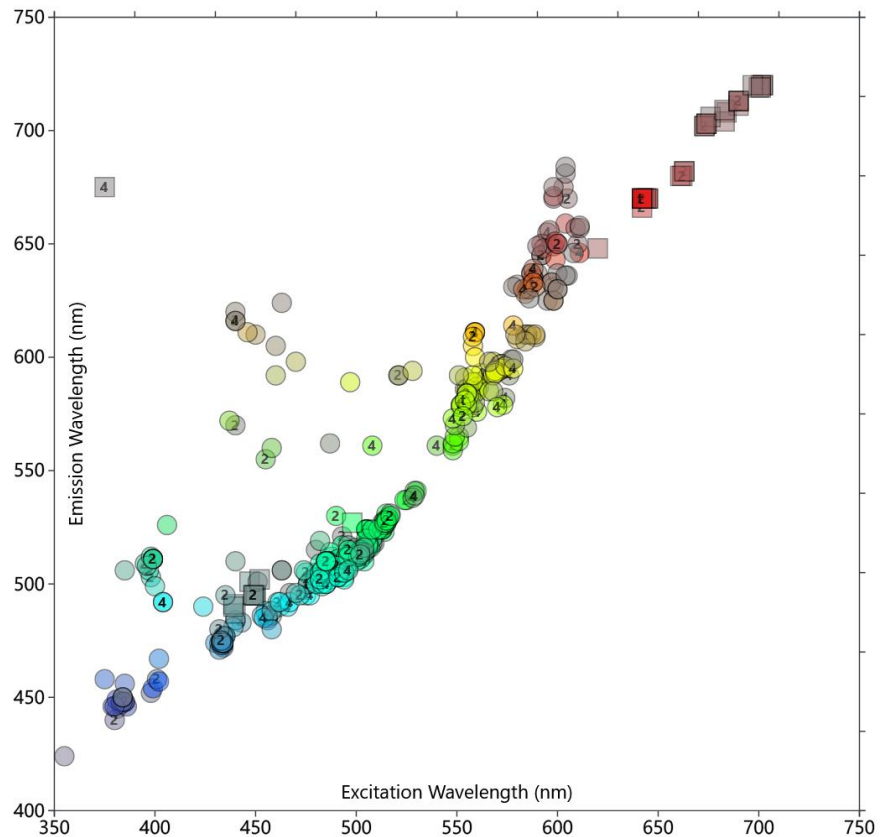


Maturation of the Enhanced Green Fluorescent Protein Chromophore



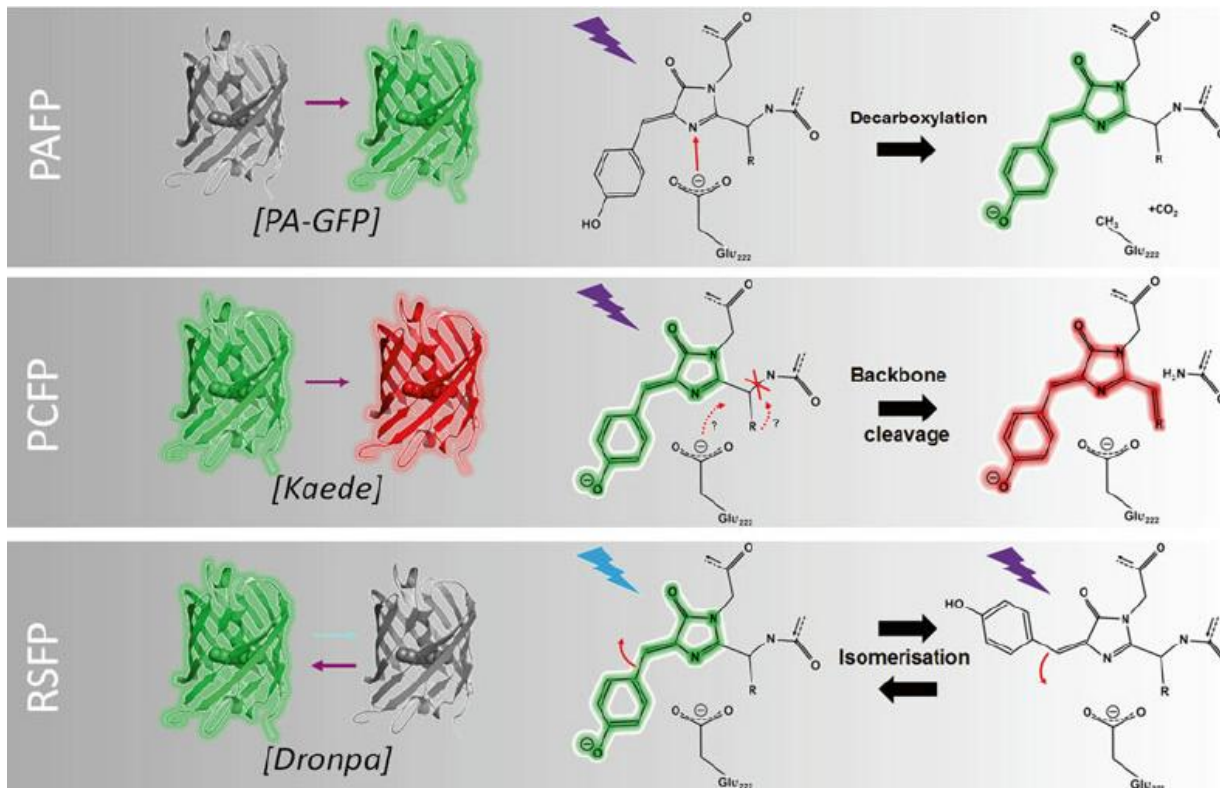
Live cells

- Genetically encoded markers
 - Fluorescent proteins
 - Various colours



Live cells

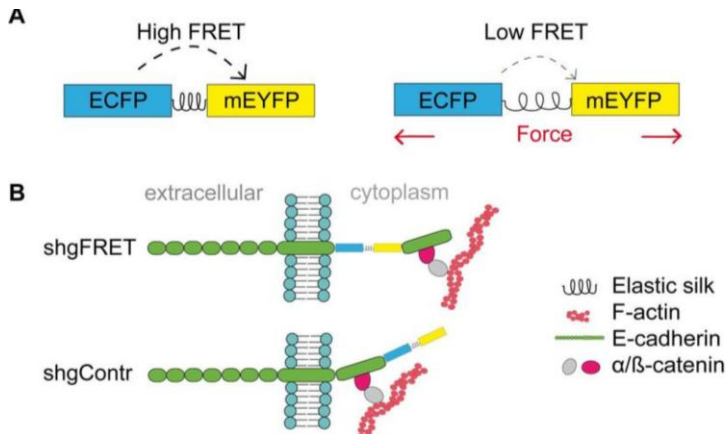
- Genetically encoded markers
 - Fluorescent proteins
 - Photoactivatable, photoconvertible, and photoswitchable



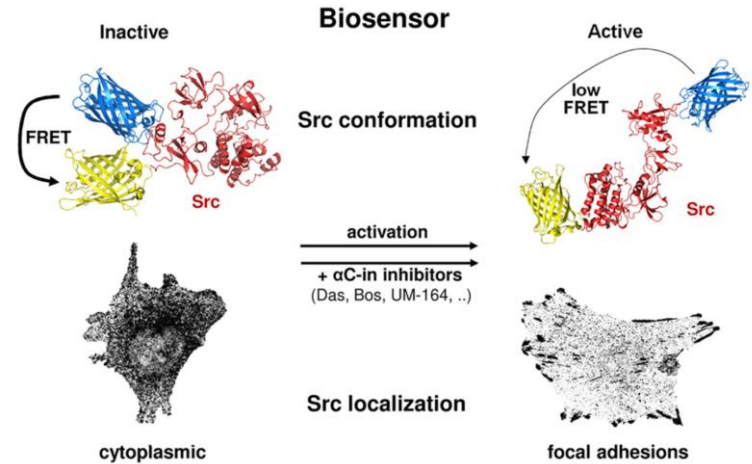
Live cells

- Genetically encoded markers
 - Fluorescent proteins
 - FRET detectors

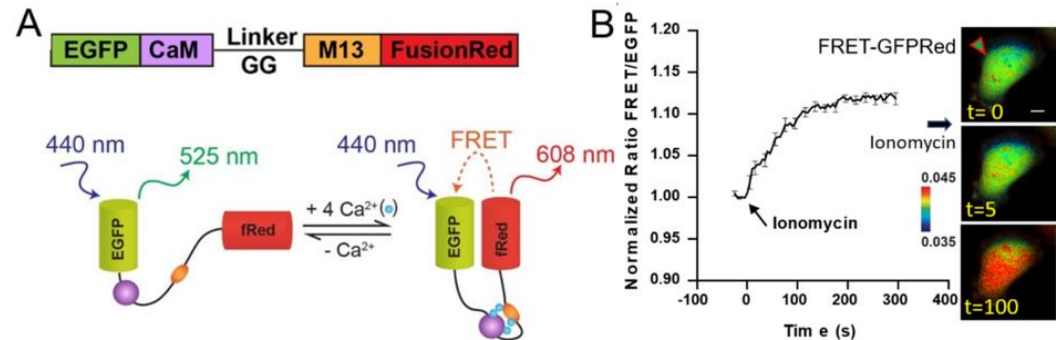
Tension



Active/inactive conformation

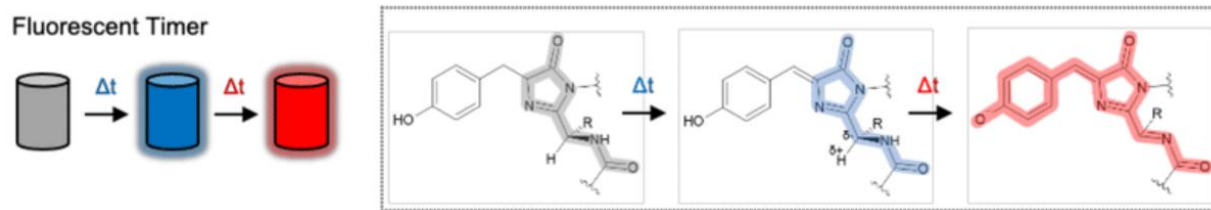


Calcium levels

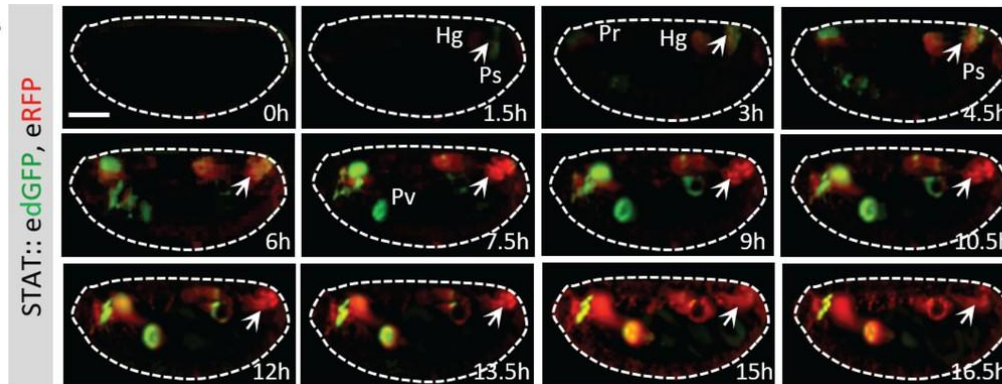


Live cells

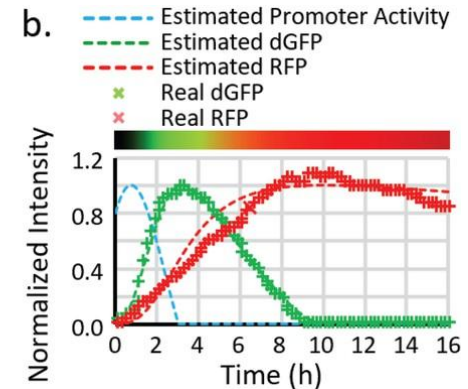
- Genetically encoded markers
 - Fluorescent proteins
 - Timer



a.

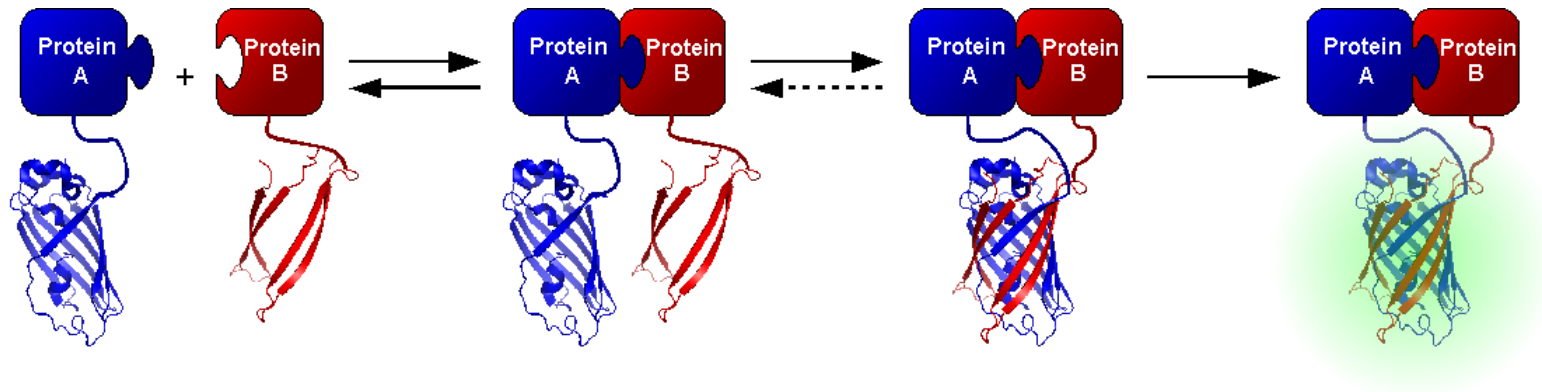


b.



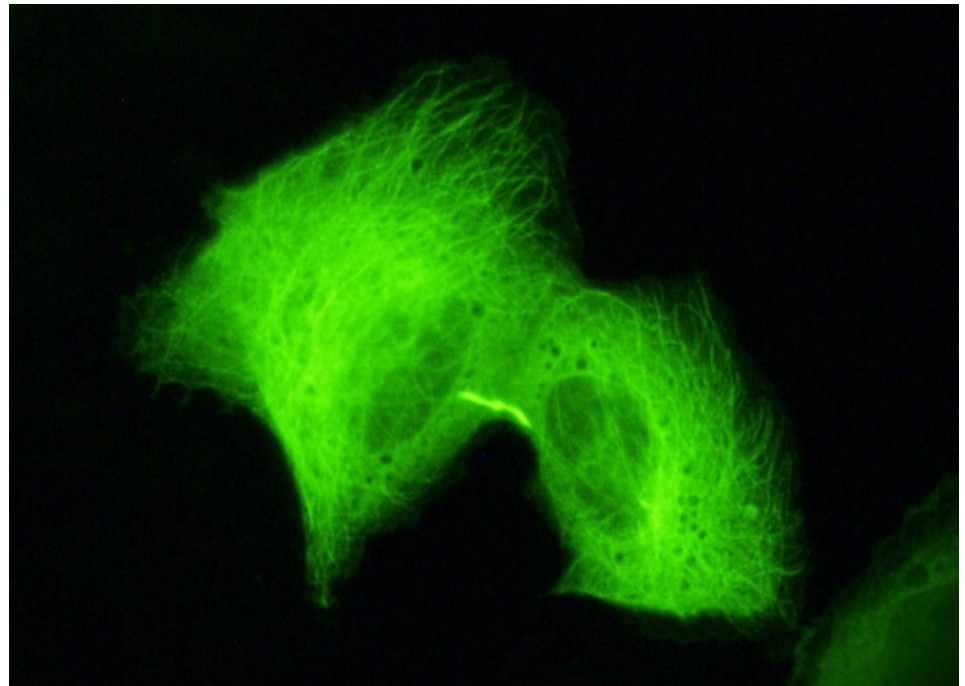
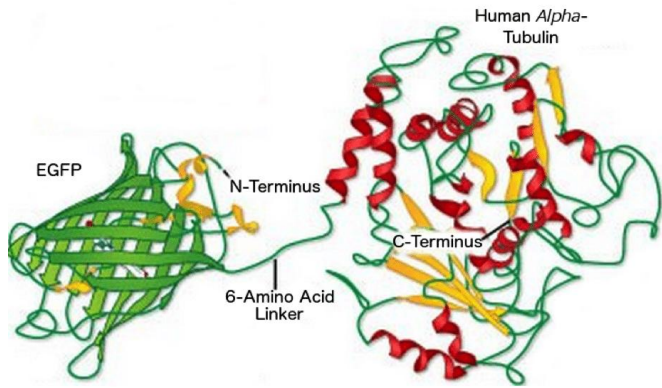
Live cells

- Genetically encoded markers
 - Fluorescent proteins
 - BiFC – bimolecular fluorescence complementation



Live cells

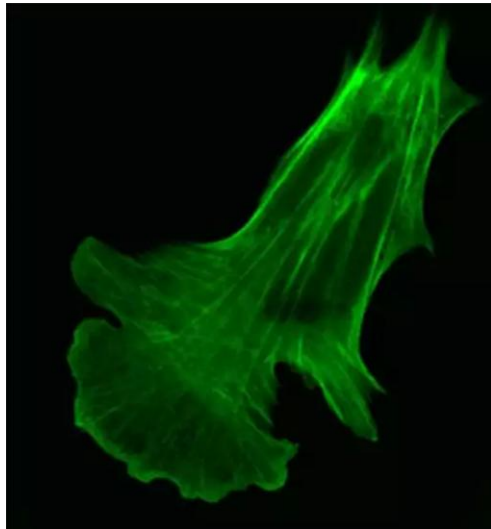
- Genetically encoded markers
 - Fusion fluorescent proteins
 - Protein-fluorescent protein



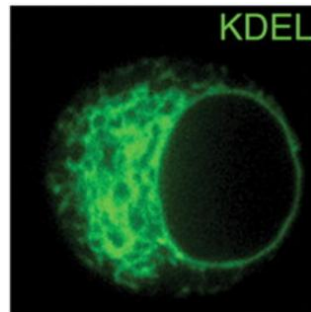
Live cells

- Genetically encoded markers
 - Fusion fluorescent proteins
 - Small targetting tags

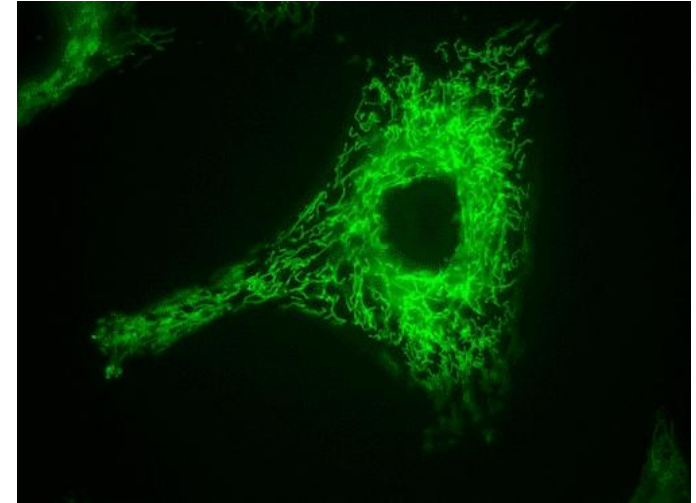
actin



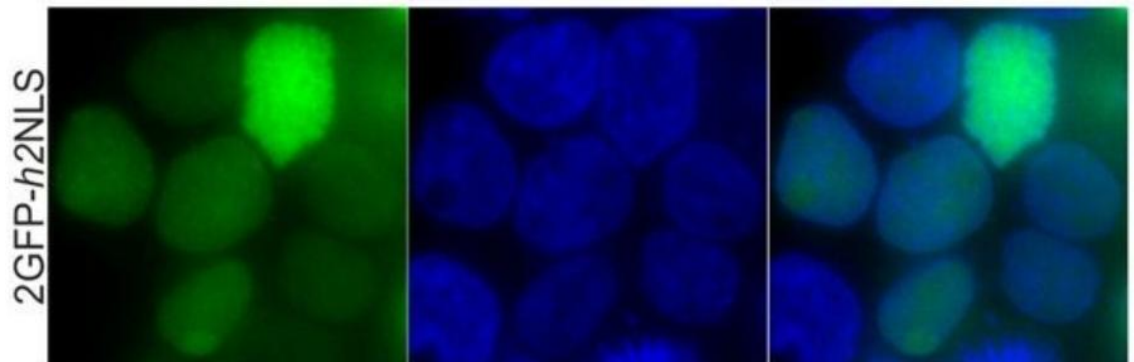
ER



mitochondria

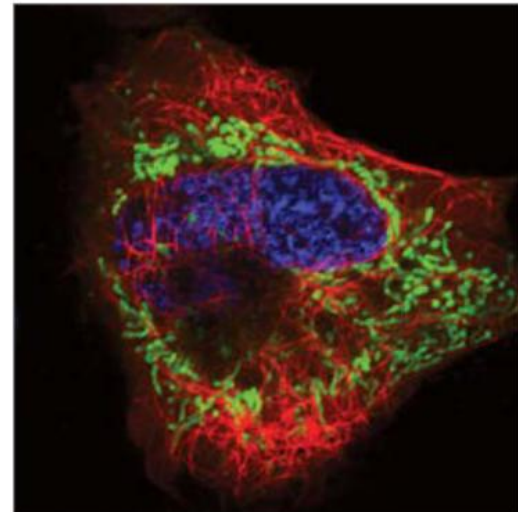
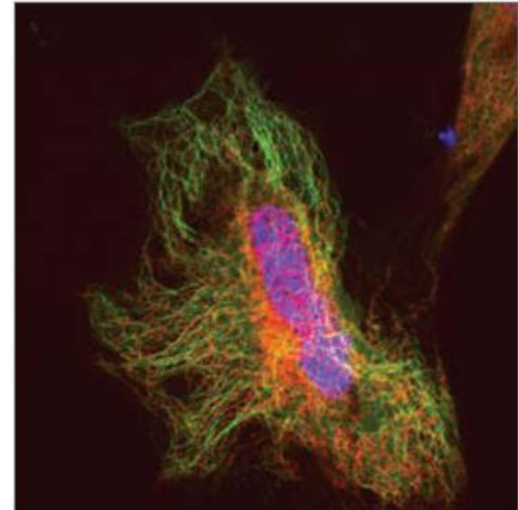
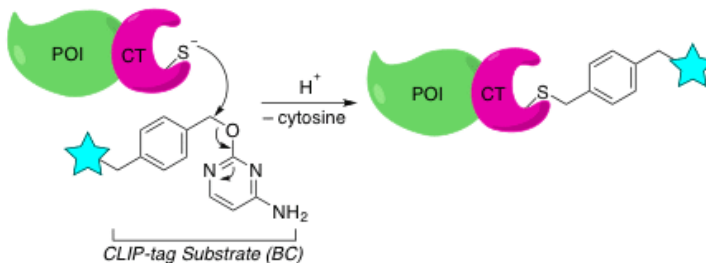
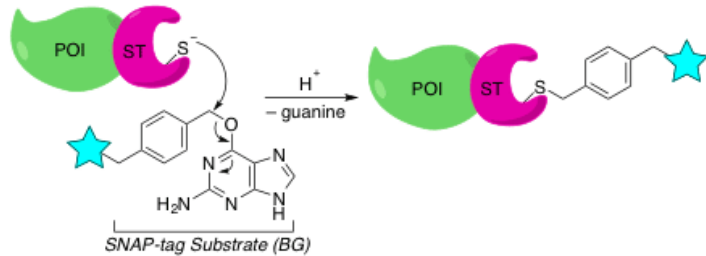


nucleus



Live cells

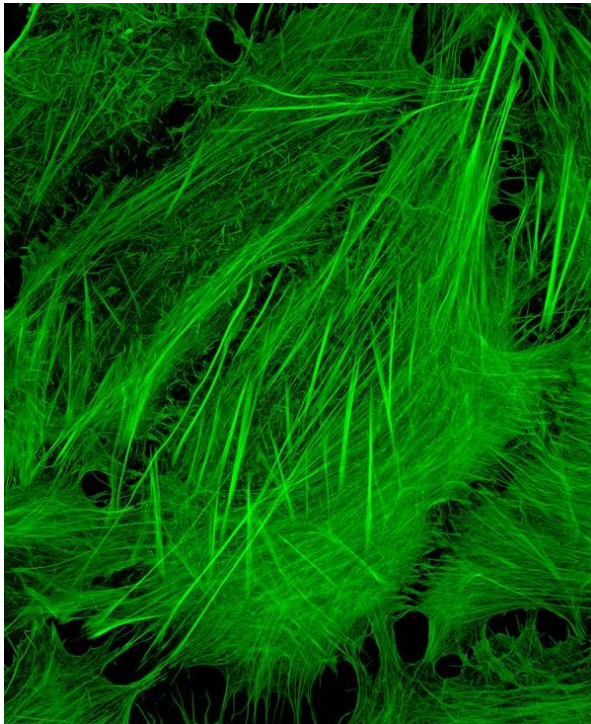
- Genetically encoded markers
 - Other tags (Halo, SNAP, CLIP)
 - Staining by external dyes



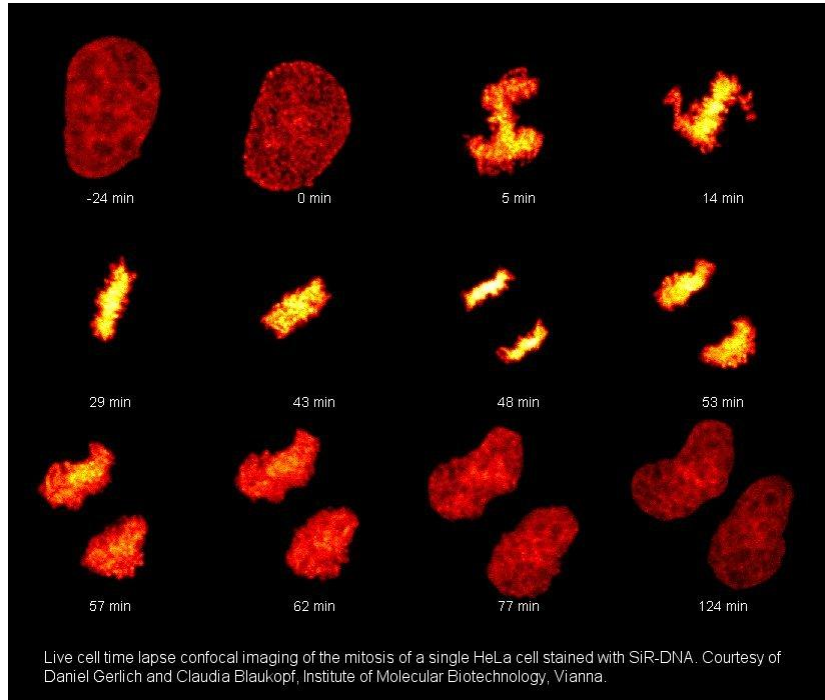
Live cells

- Small molecules
 - Organelle markers

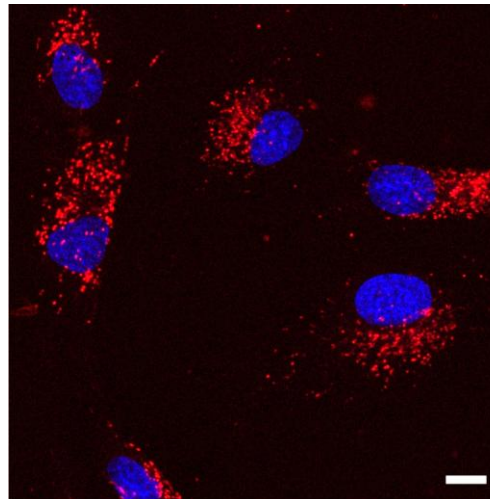
phalloidin



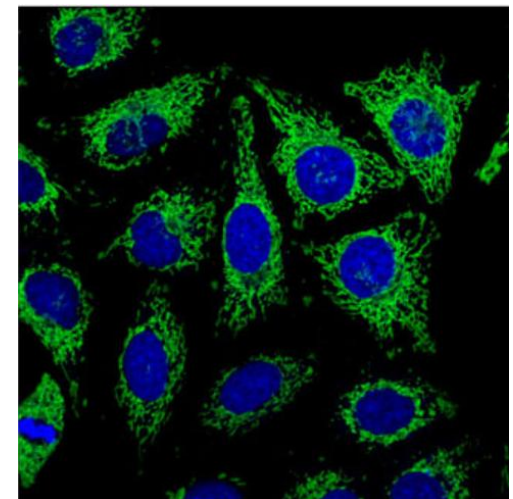
SiR-DNA



lysotracker

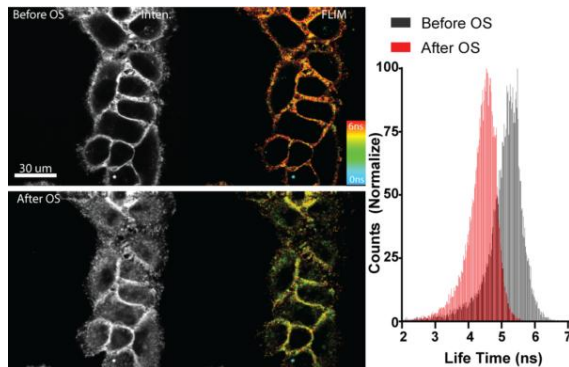
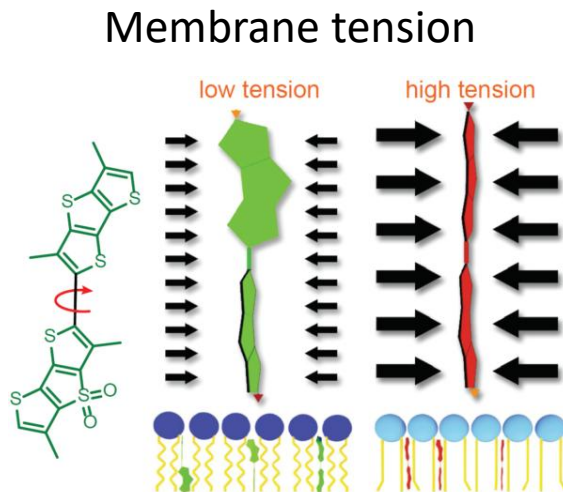


mitotracker

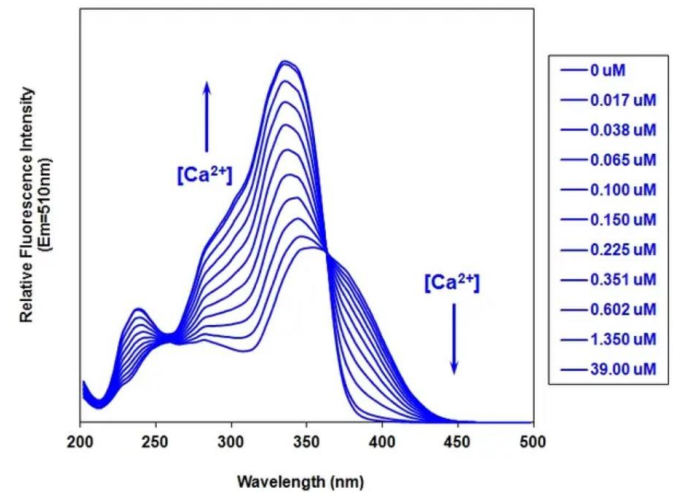
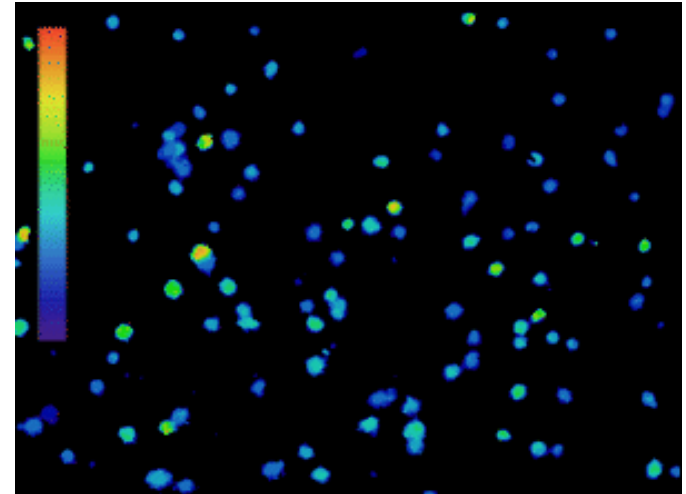


Live cells

- Small molecules
 - Condition detectors



Ca²⁺ concentration



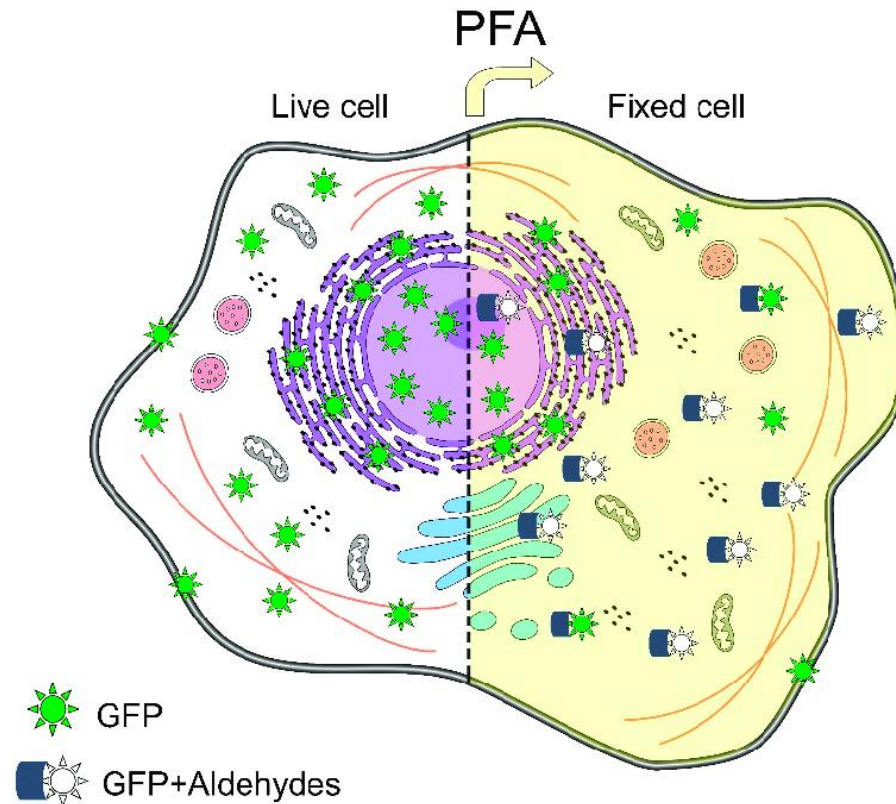
Sample preparation and acquisition

- Living cells in a dish
 - Phototoxicity
 - Speed – live cells move



Fixed samples

- Fluorescent proteins etc.

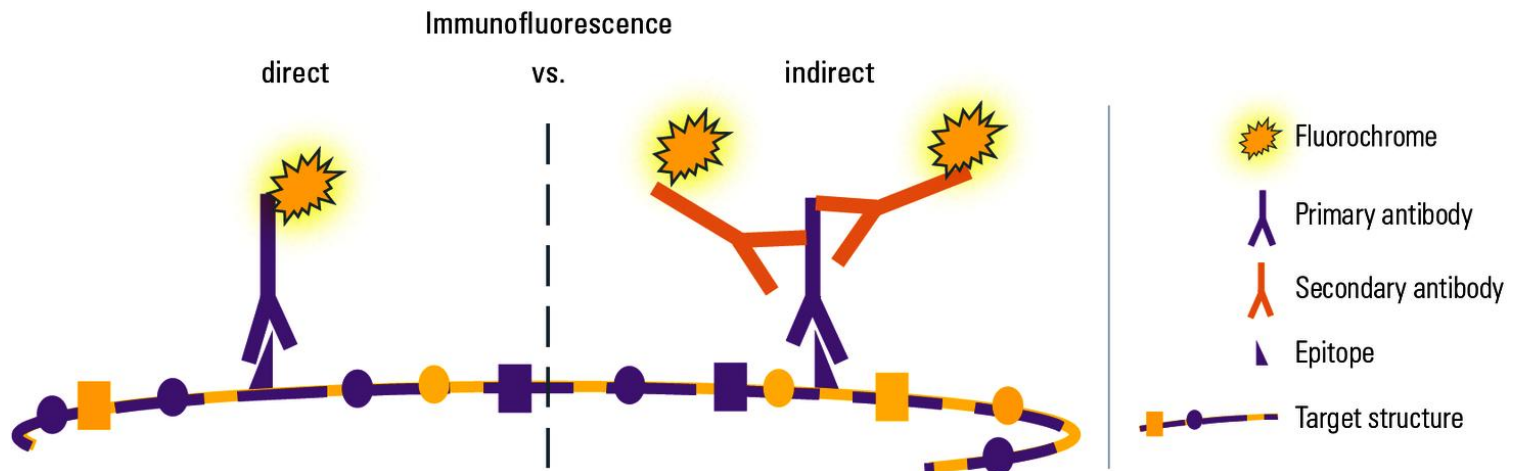


Fixed samples

- Antibodies

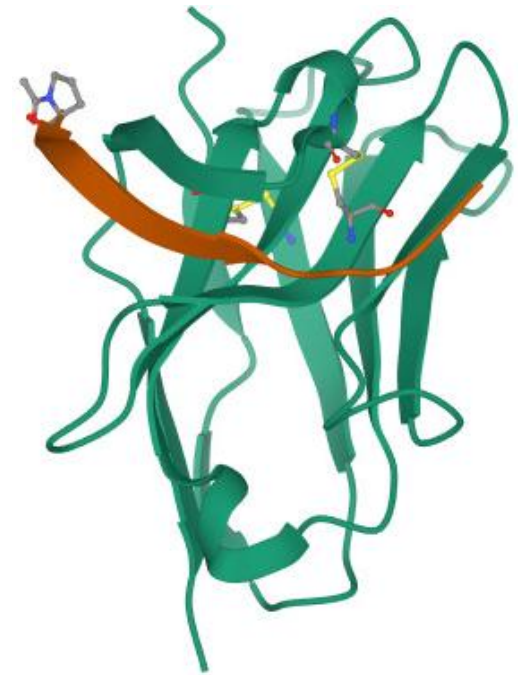
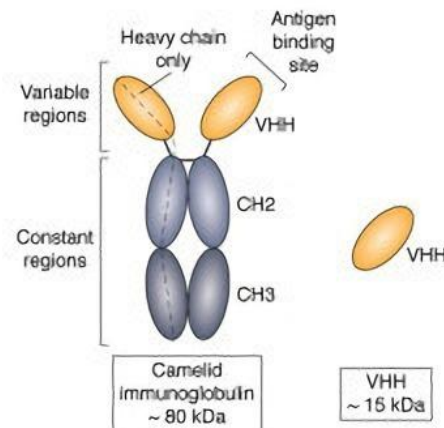
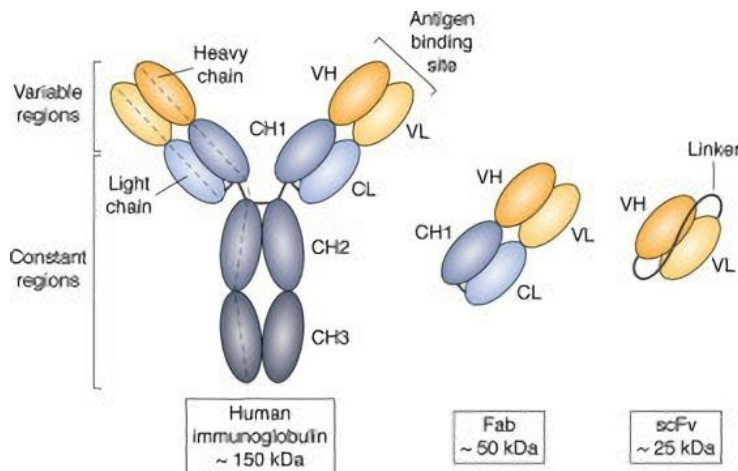
- Conjugated primary
- Secondary
 - Beware cross-reactivity

- Anti-protein
- Anti-tag (His, FLAG,...)



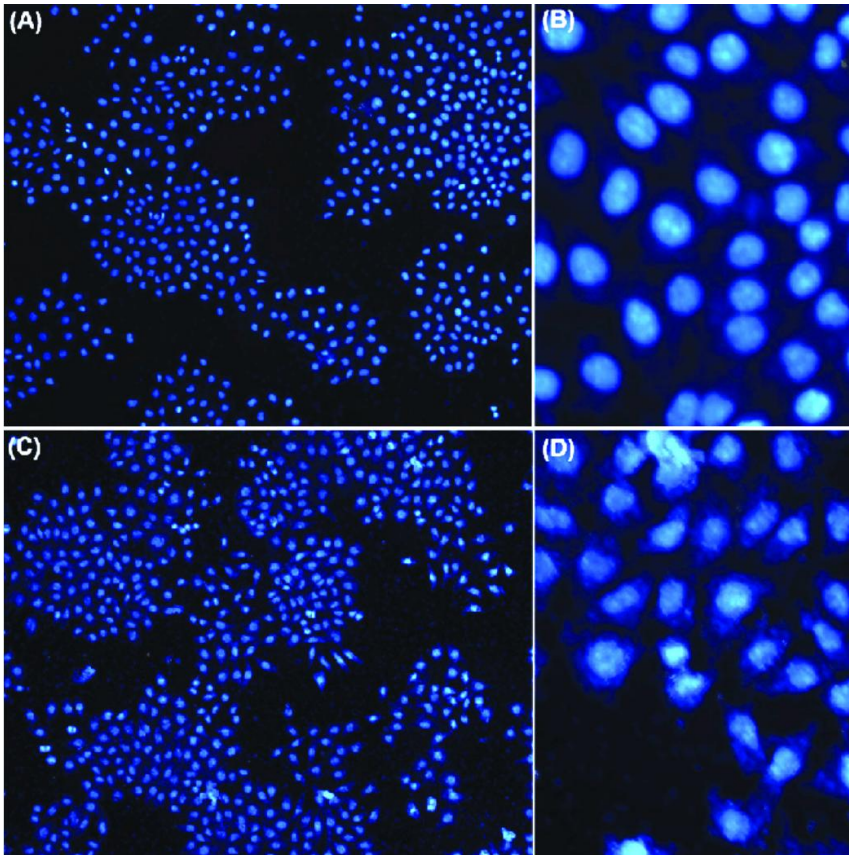
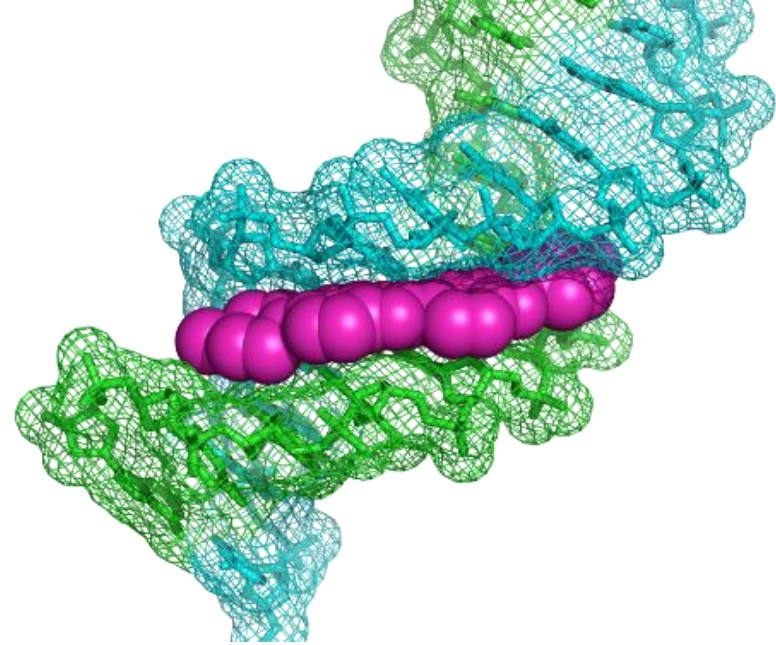
Fixed samples

- Nanobodies
 - Super-resolution
 - Tissues



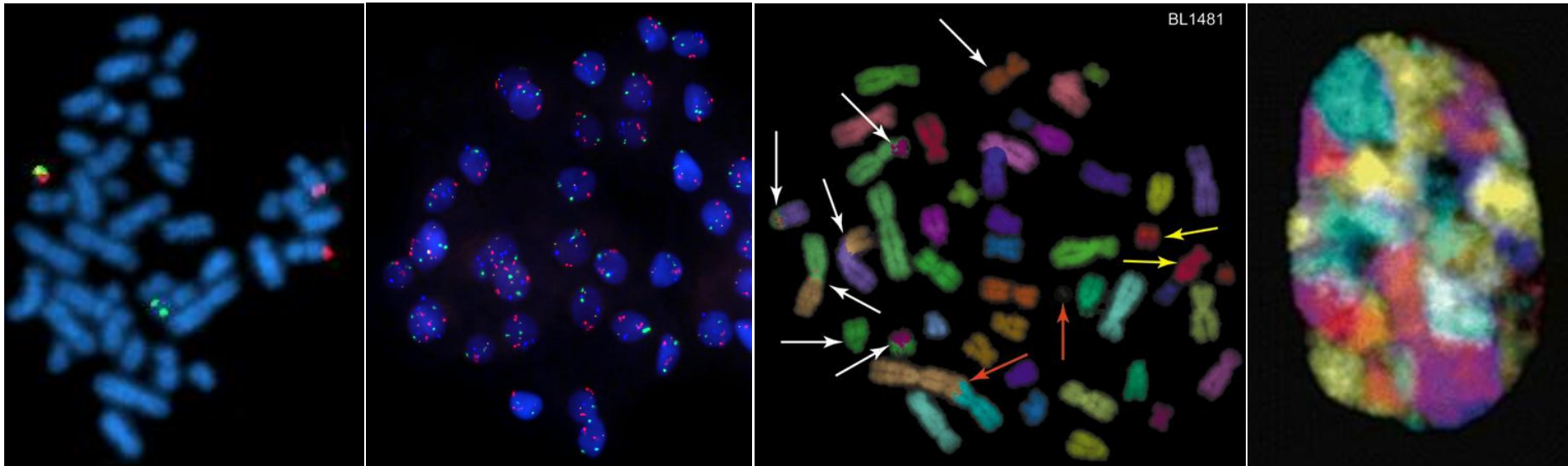
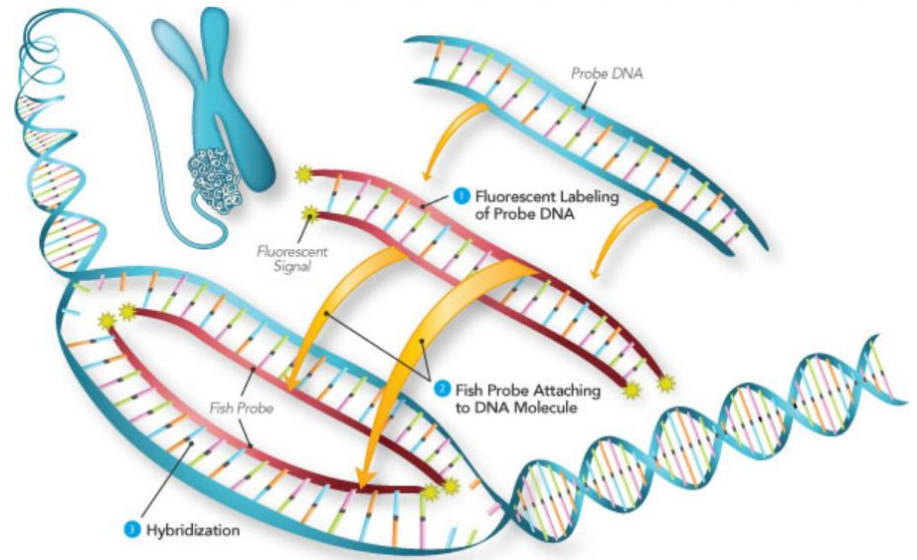
Fixed samples

- Small molecules
 - DAPI etc. – nucleic acids



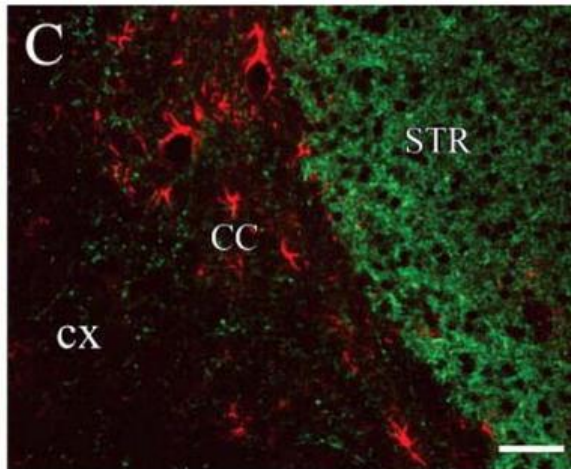
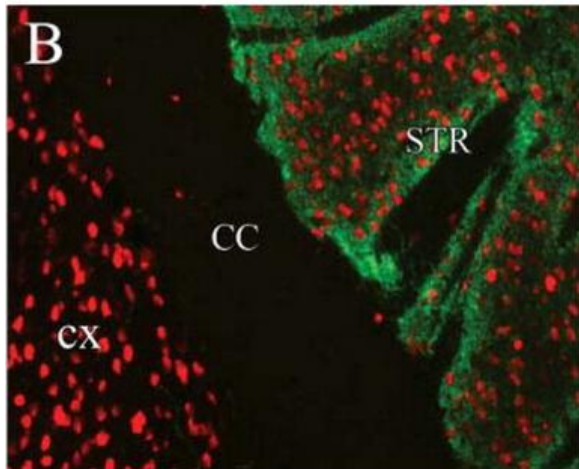
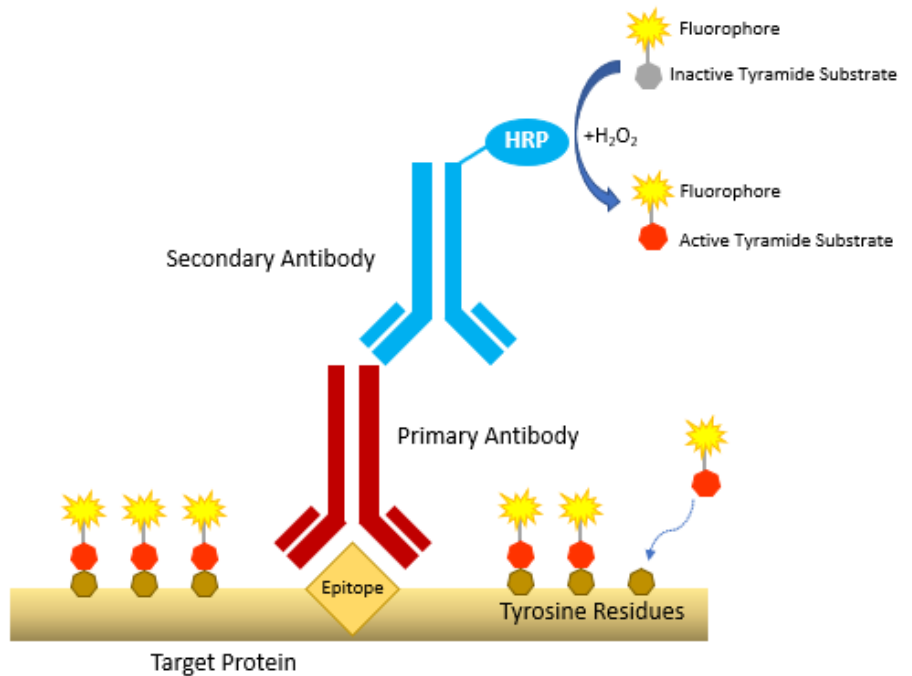
Fixed samples

- Small molecules
 - Oligos (FISH – fluorescent in-situ hybridisation)



Fixed samples

- Tyramide signal amplification
 - Amplification of rare targets

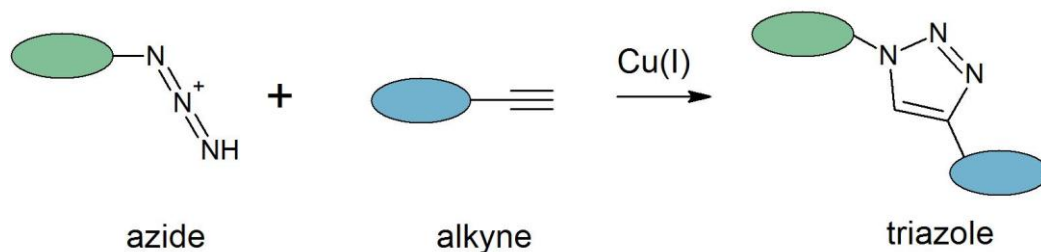


Fixed samples

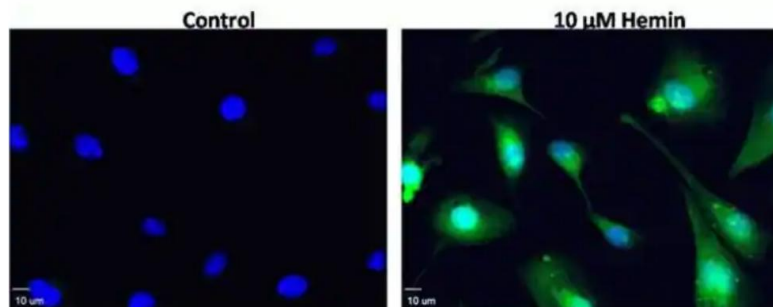
- Small molecules
 - Click chemistry



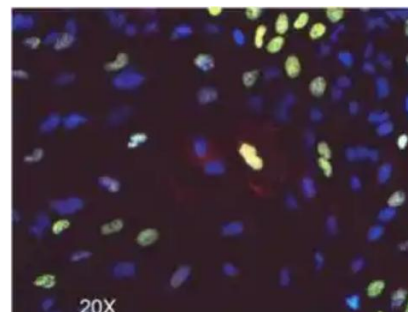
The Nobel Prize in Chemistry 2022 - Carolyn R. Bertozzi, Morten Meldal and K. Barry Sharpless “for the development of click chemistry and bioorthogonal chemistry”.



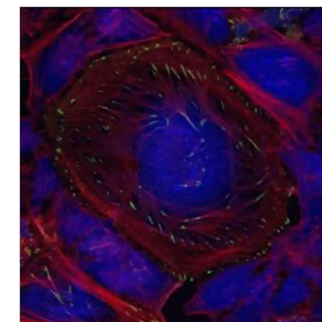
Lipid peroxidation (modified lipids)



Cell proliferation
(nucleotide analogue)

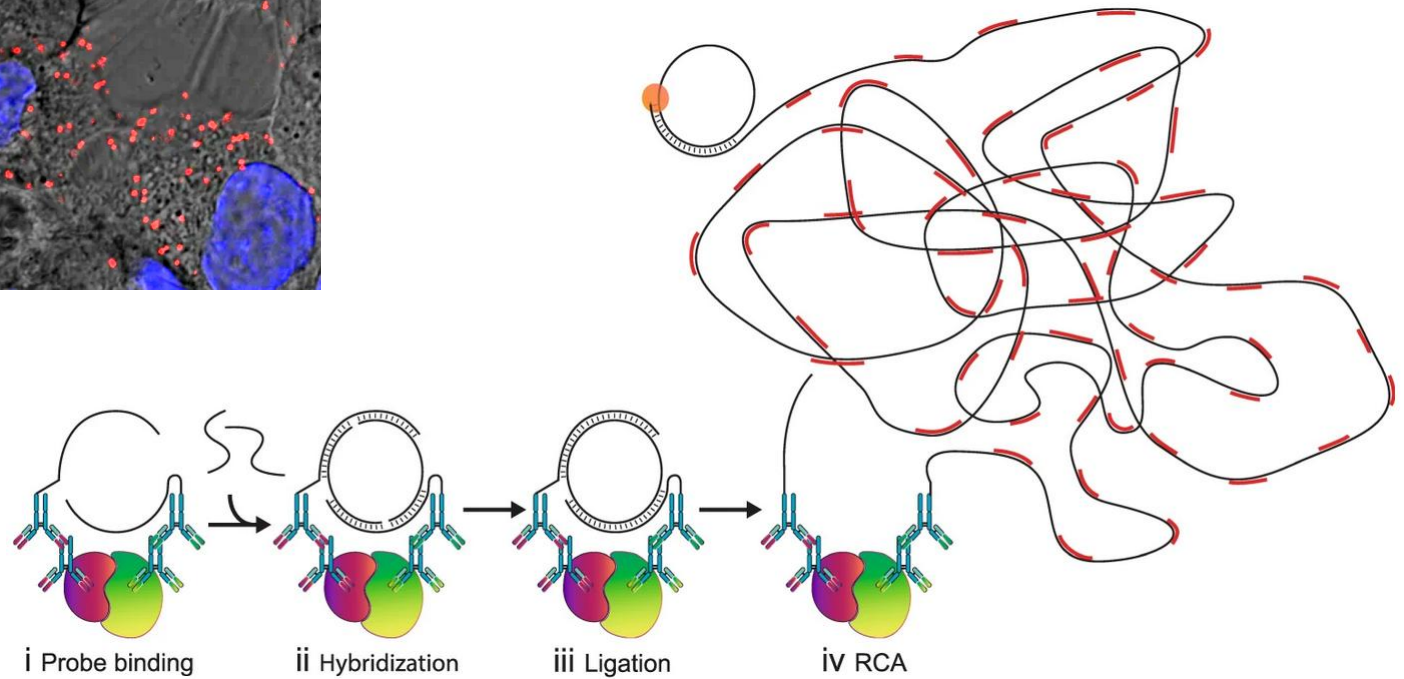
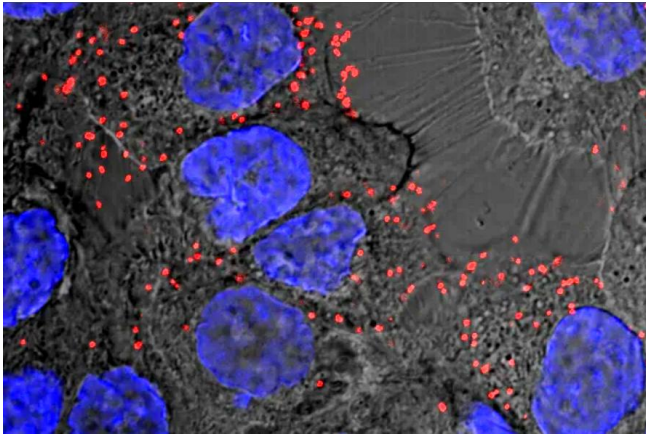


Protein synthesis
(methionine analogue or puromycin derivative)



Fixed samples

- Proximity ligation assay

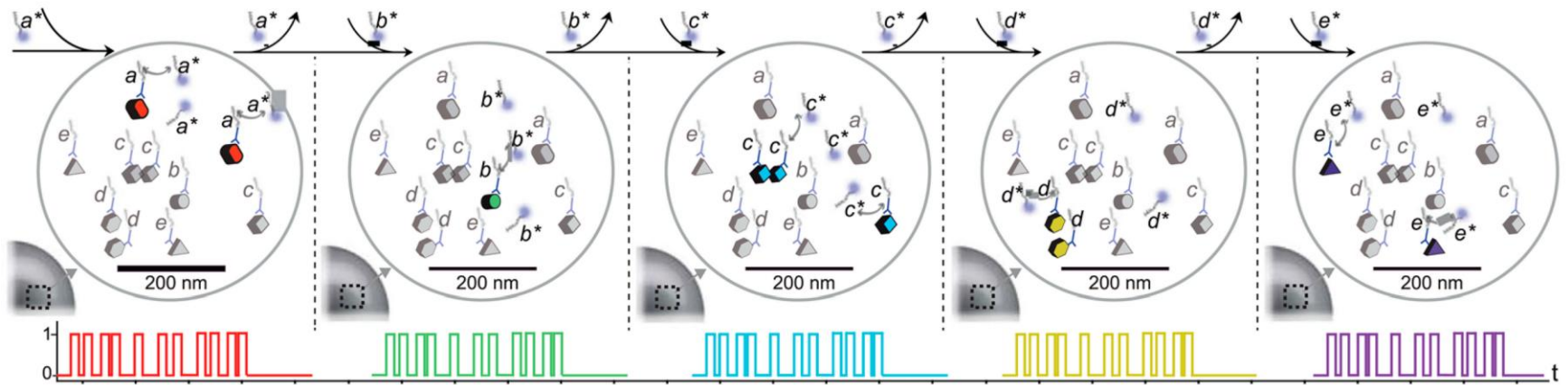


Fixed samples

- DNA-PAINT

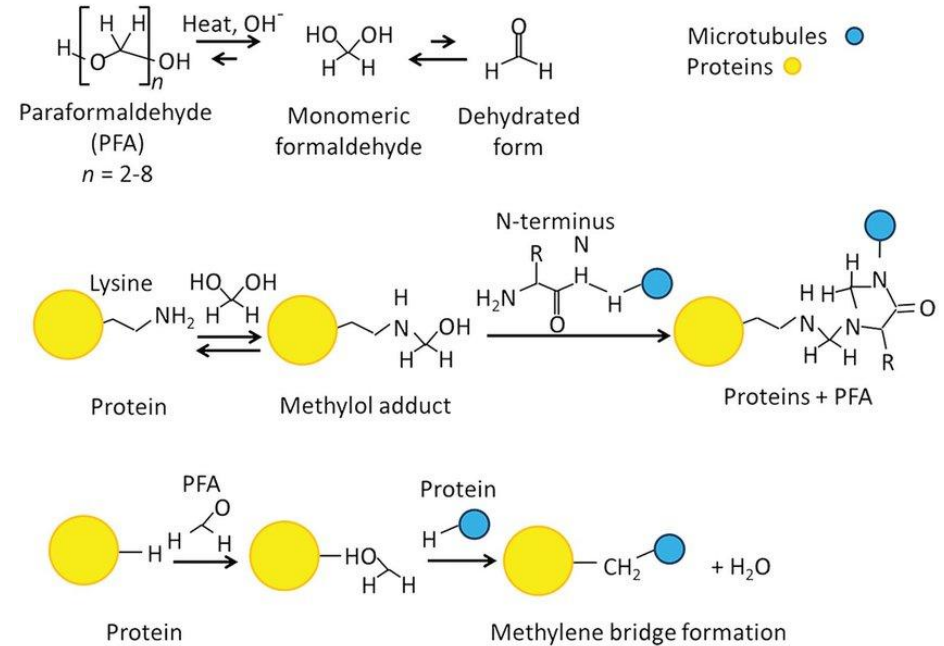
Exchange-PAINT

a b c d e



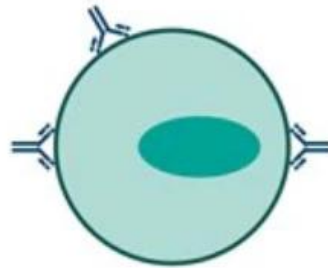
Sample preparation

- Fixation
- Permeabilisation
- Blocking
- Staining
- Washing
- Staining by secondary Ab
- Washing
- Mounting

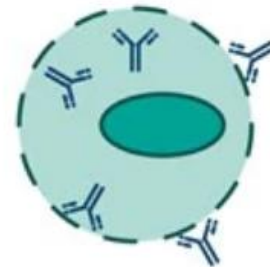


Sample preparation

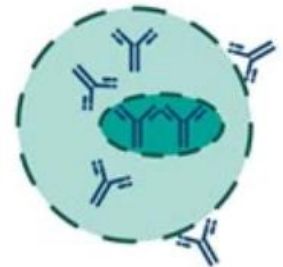
- Fixation
- **Permeabilisation**
- Blocking
- Staining
- Washing
- Staining by secondary Ab
- Washing
- Mounting



Unpermeabilized



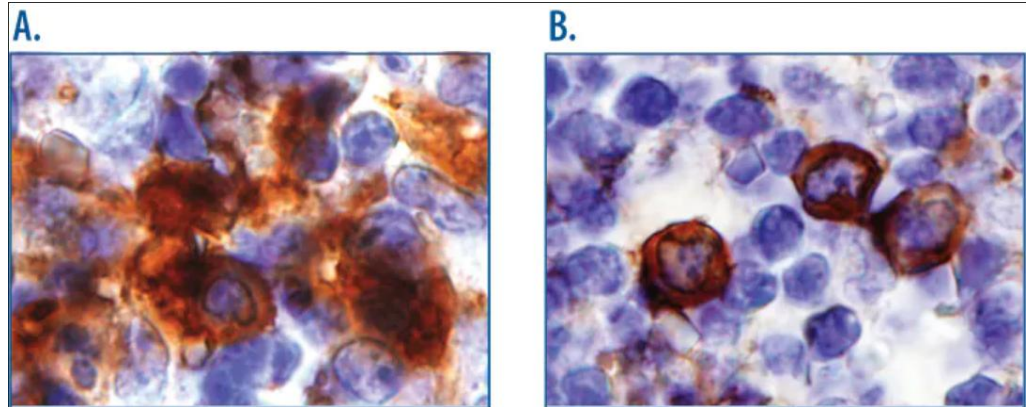
Digitonin



Triton X-100

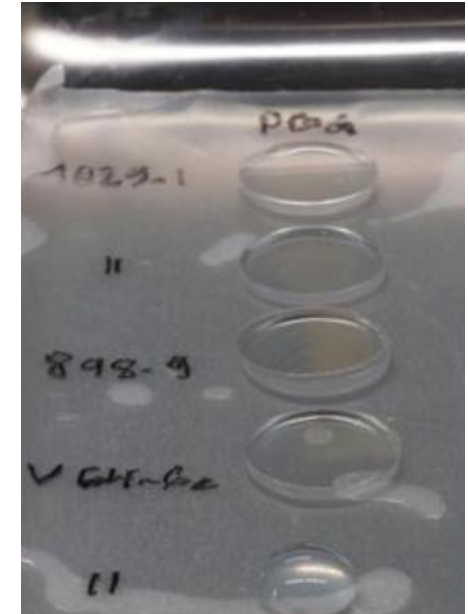
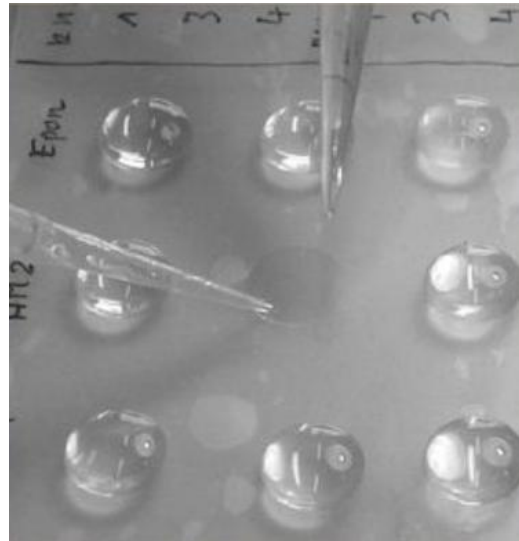
Sample preparation

- Fixation
- Permeabilisation
- Blocking
- Staining
- Washing
- Staining by secondary Ab
- Washing
- Mounting



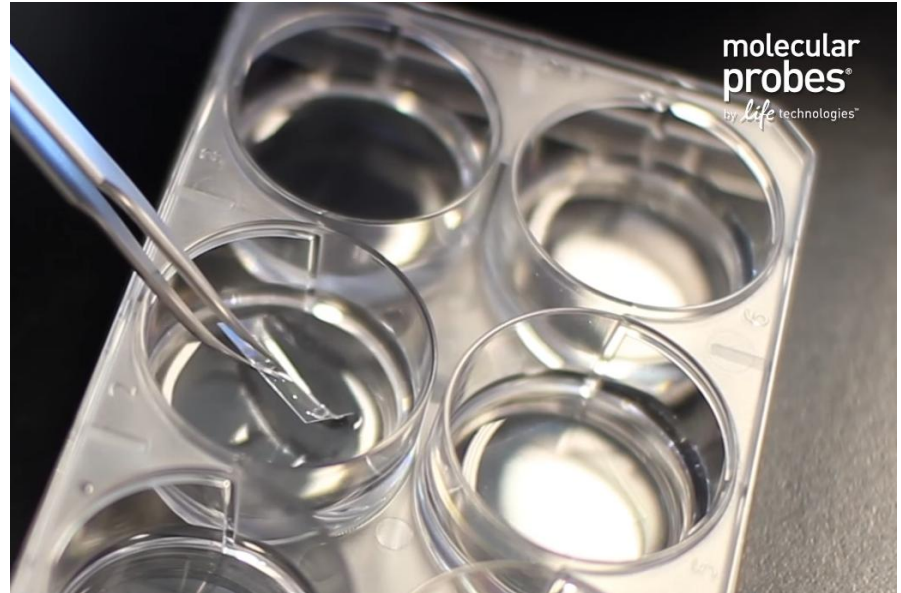
Sample preparation

- Fixation
- Permeabilisation
- Blocking
- **Staining**
- Washing
- Staining by secondary Ab
- Washing
- Mounting



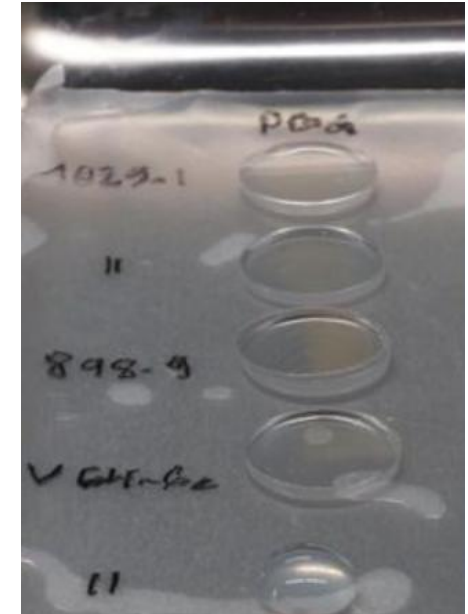
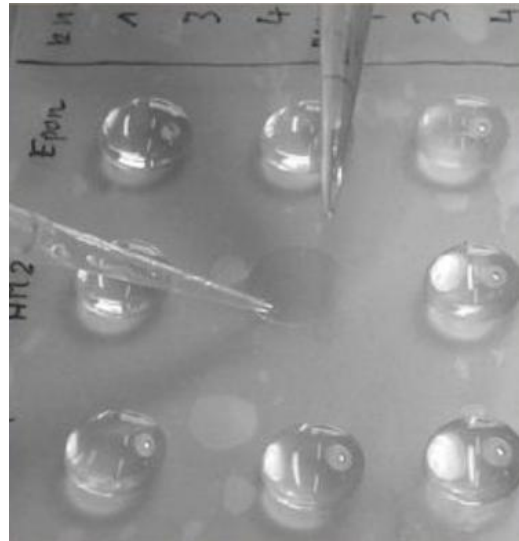
Sample preparation

- Fixation
- Permeabilisation
- Blocking
- Staining
- **Washing**
- Staining by secondary Ab
- Washing
- Mounting



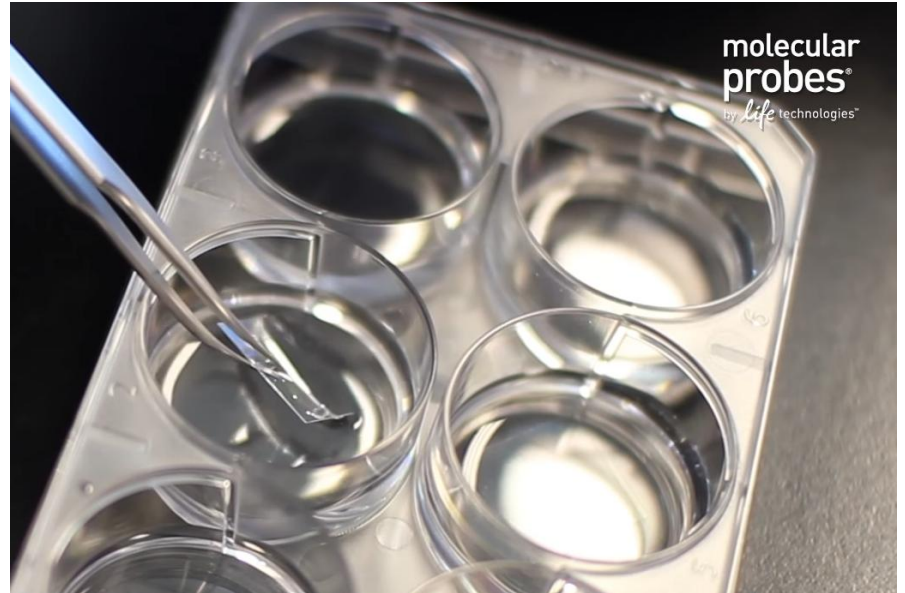
Sample preparation

- Fixation
- Permeabilisation
- Blocking
- Staining
- Washing
- Staining by secondary Ab
- Washing
- Mounting



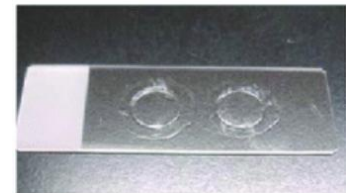
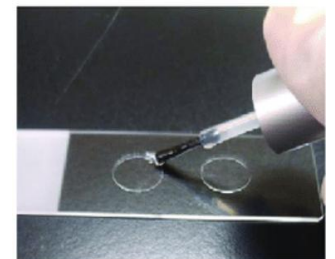
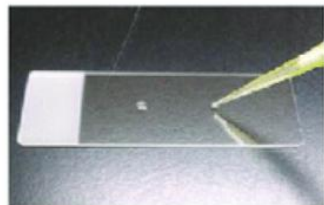
Sample preparation

- Fixation
- Permeabilisation
- Blocking
- Staining
- Washing
- Staining by secondary Ab
- **Washing**
- Mounting



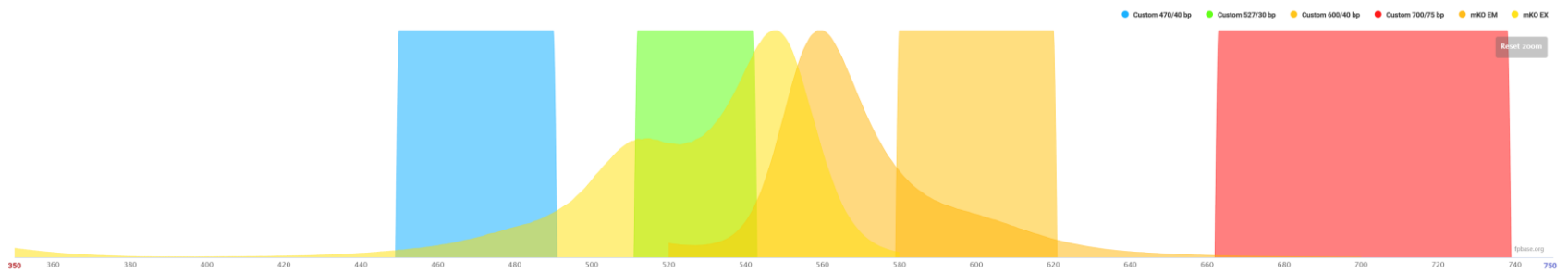
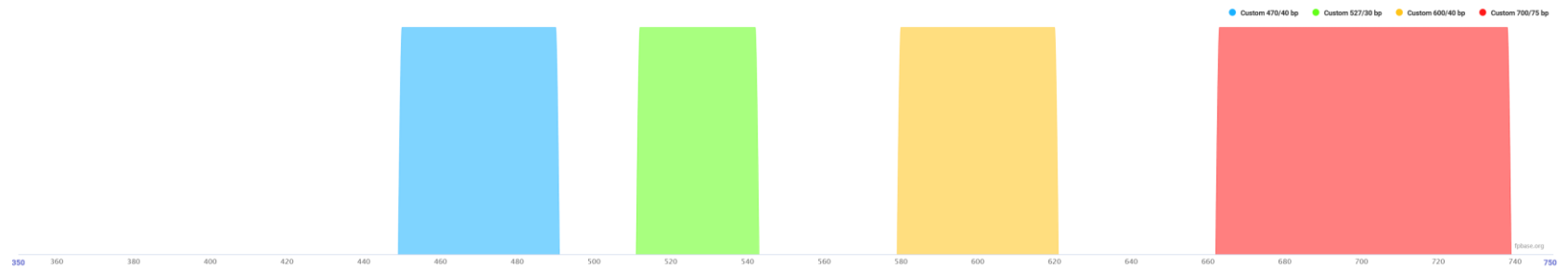
Sample preparation

- Fixation
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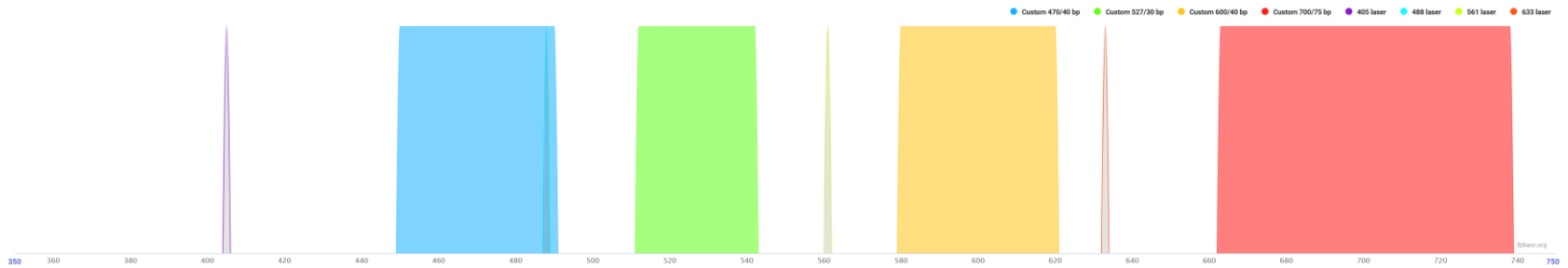
Fluorophore selection

- First – select a microscope
 - Filters

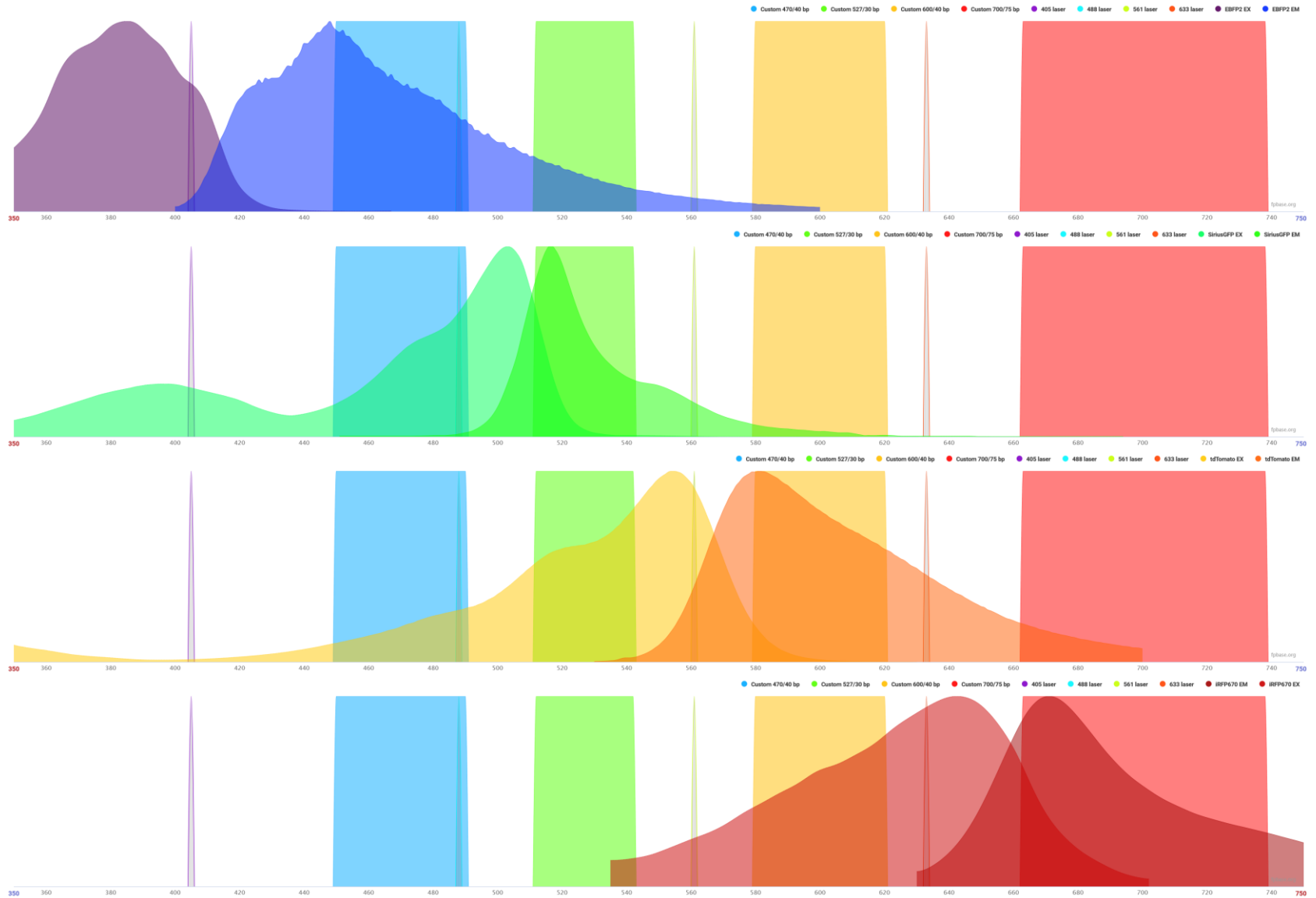


Fluorophore selection

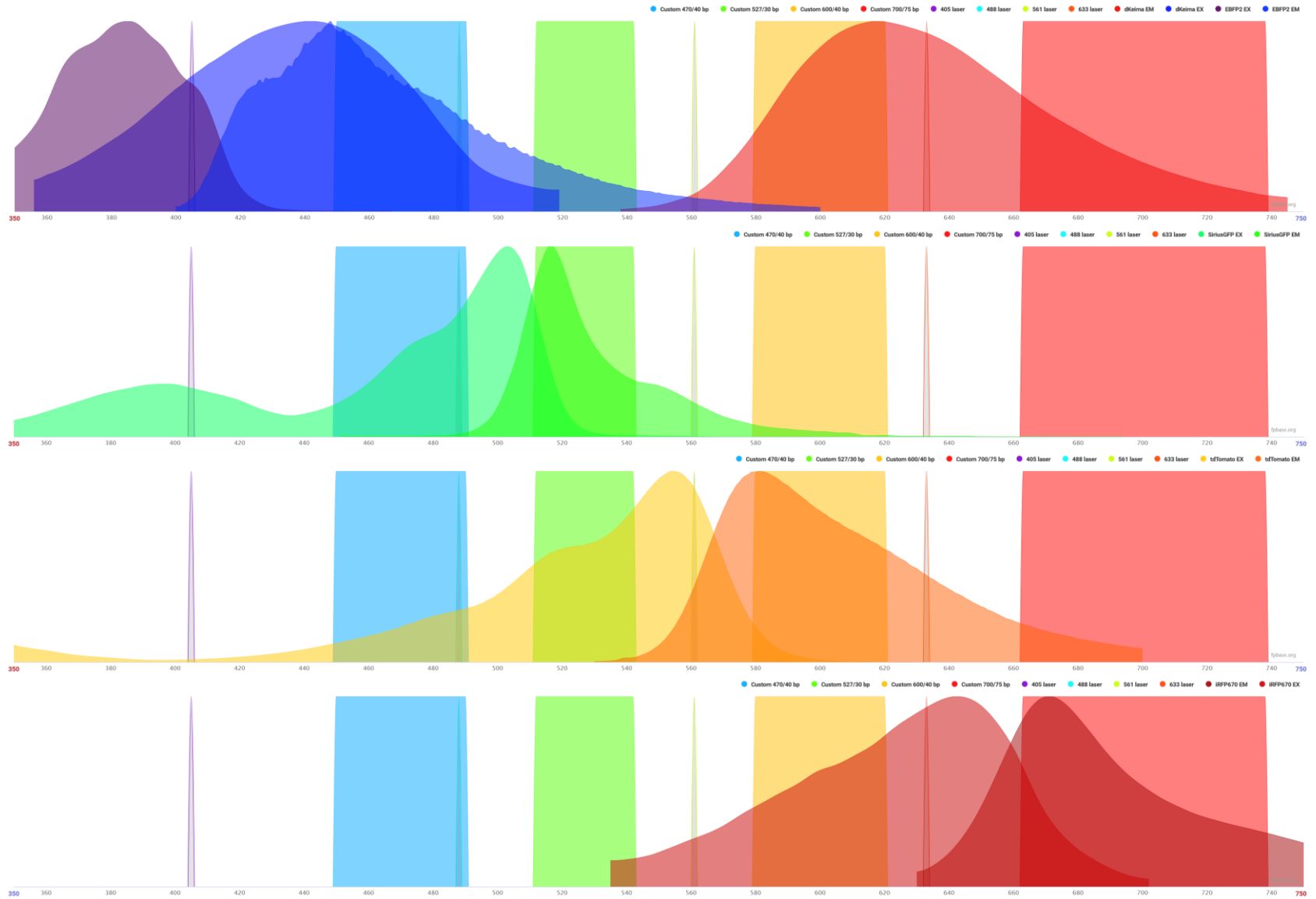
- Number of channels



Fluorophore selection



Fluorophore selection

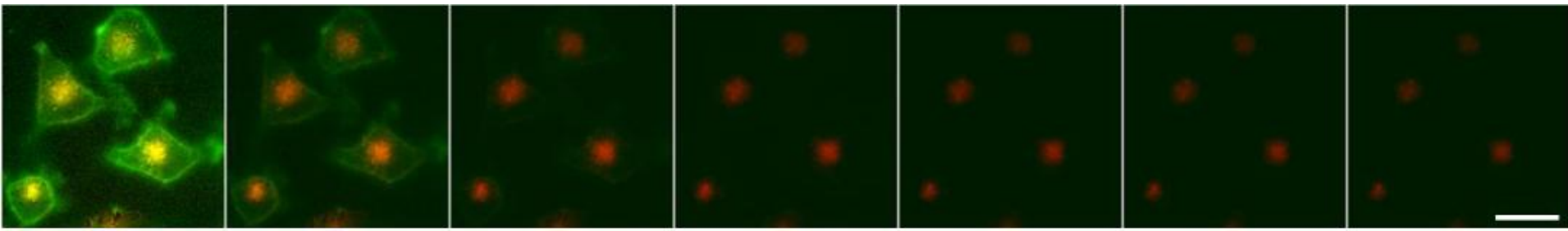
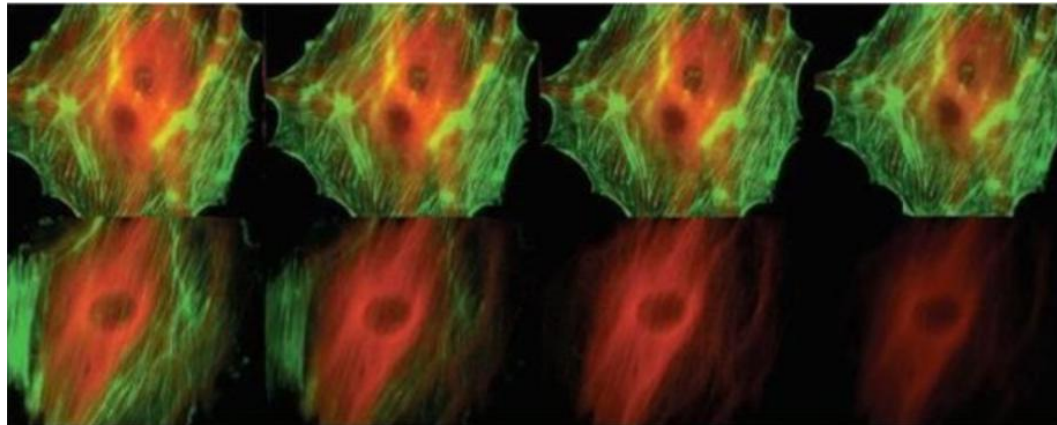


Fluorophore selection



Acquisition

- Time of acquisition
- Bleaching



Thank you for your attention!