Fluorescence and confocal microscopy

Basic principles and applications

Mgr. Ivana Andrejčinová

437403@mail.muni.cz

Conventional Light vs. Fluorescence Microscopy

Light source: halogen lamps

- low-contrast specimens

(cell almost transparent)

- additional optical mechanisms

(Phase contrast, DIC..)

- use of dyes

(crystal violet, eosin..)

- **High-intensity light source:** metal halide lamps, light-emitting diodes (LEDs), lasers (confocal microscopy)
- Use of fluorophores
- Better resolution

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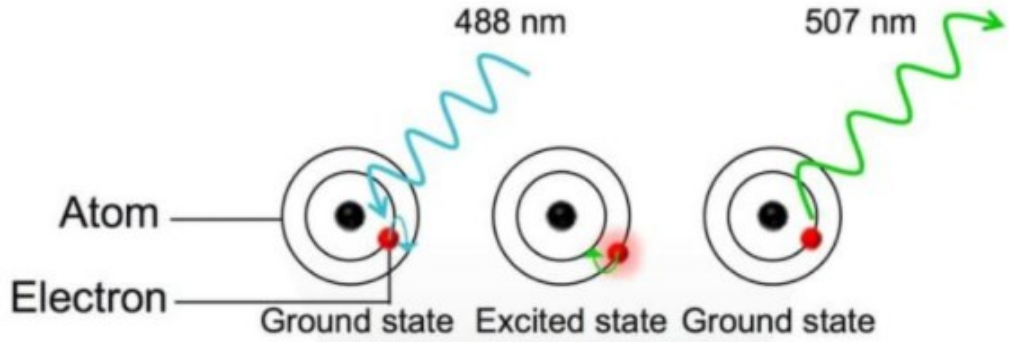
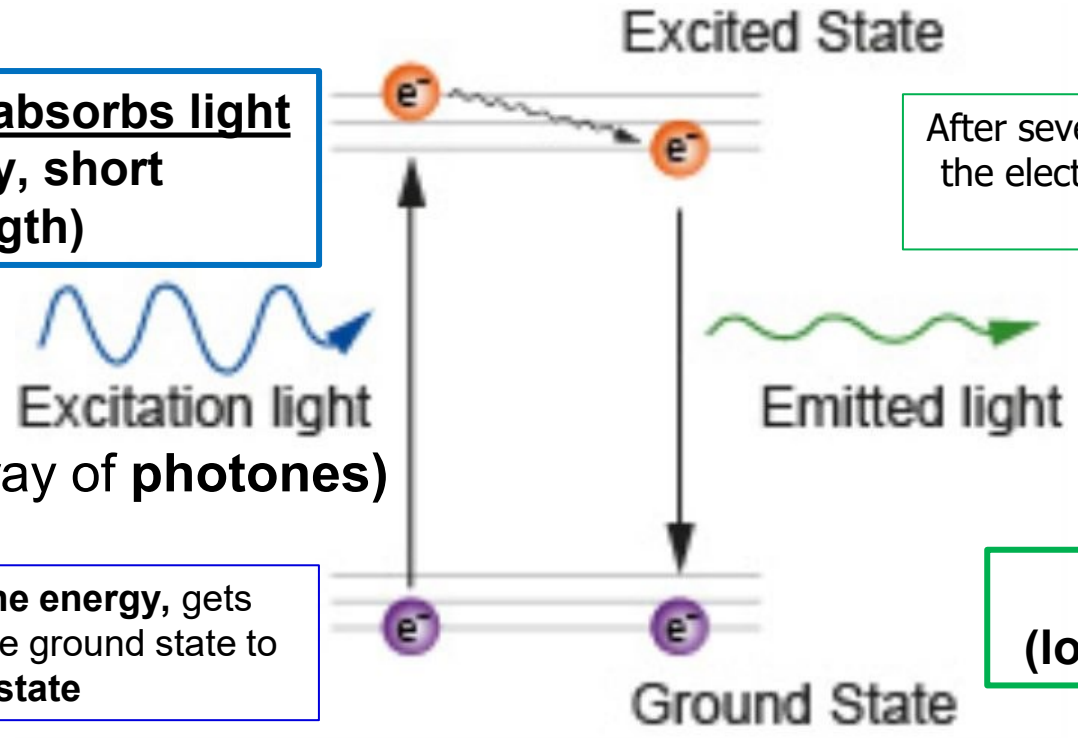
Principle of fluorescence

Fluorophore - chemical compound absorbing light at one wavelength and re-emitting it at another wavelength



Molecular system **absorbs light (high energy, short wavelength)**



After several nano-seconds (the fluorescence lifetime) the electrone will relax back to the ground state and **release the energy**

Electrone is hit by **photone energy,** gets **excited** and transits from the ground state to **higher energy state**

Molecular system **emits light (lower energy, longer wavelenght)**

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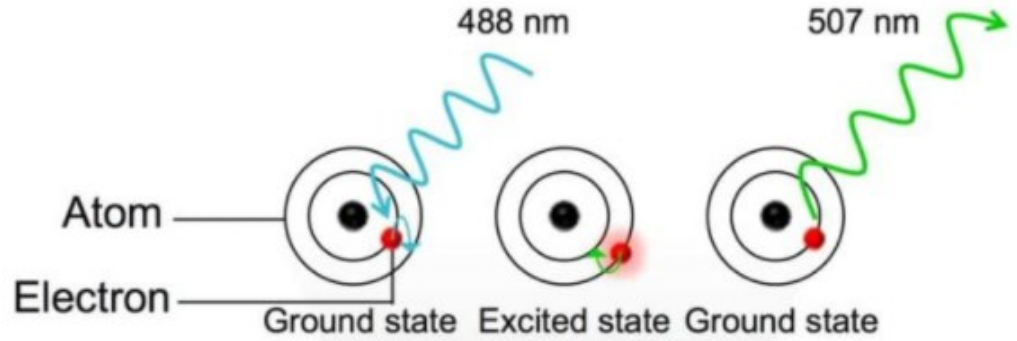
Principle of fluorescence

Fluorophore - chemical compound absorbing light at one wavelength and re-emitting it at another wavelength



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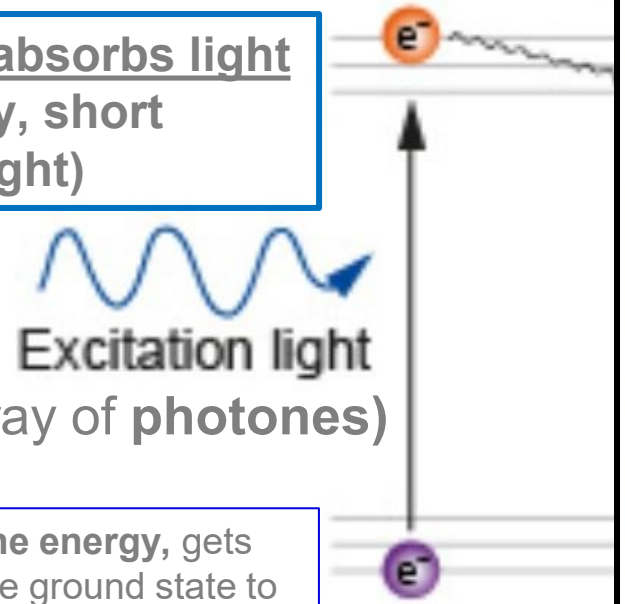
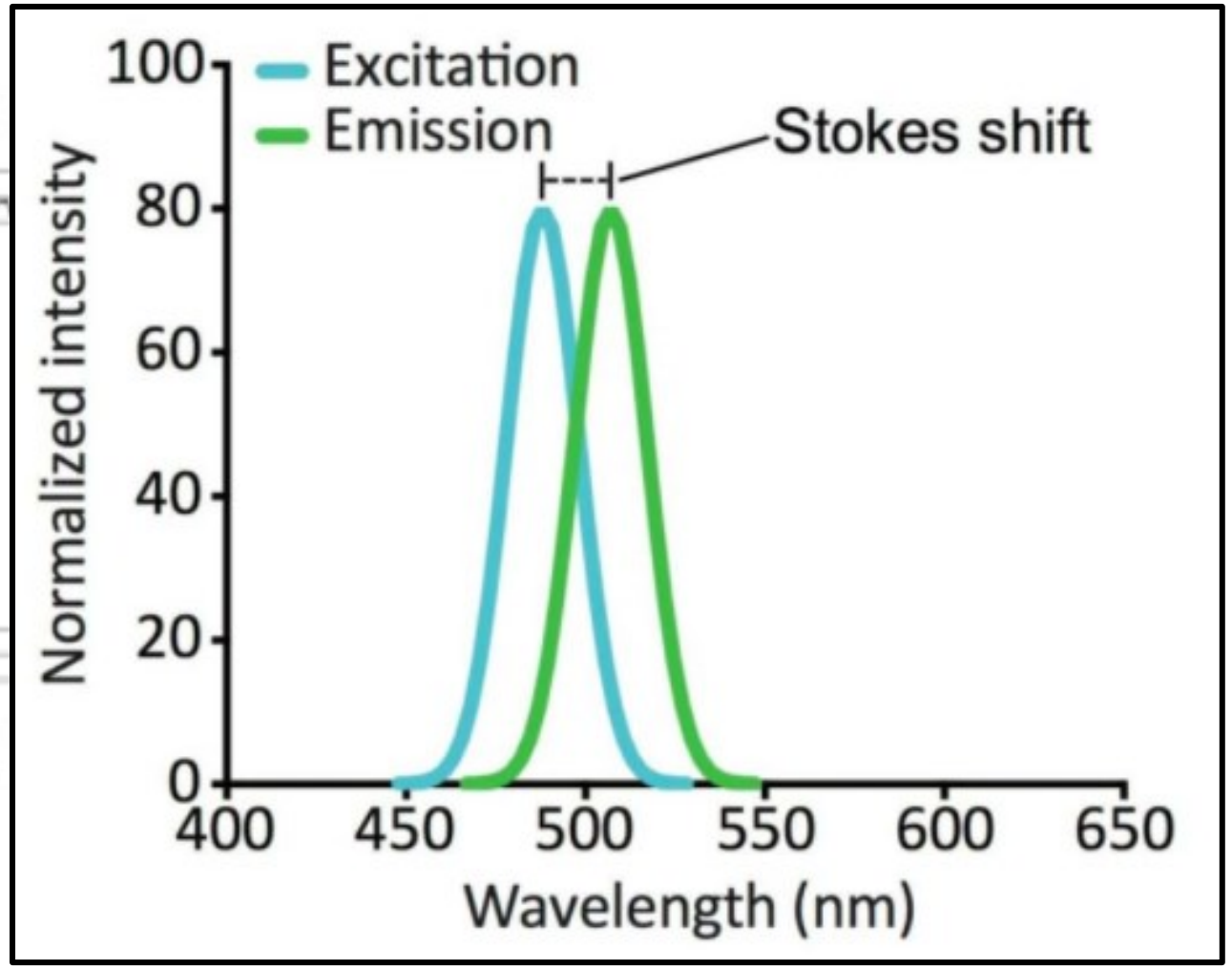


Excitation light
(ray of **photones**)

Electrone is hit by **photone energy,** gets **excited** and transits from the ground state to **higher energy state**



Principle of fluorescence

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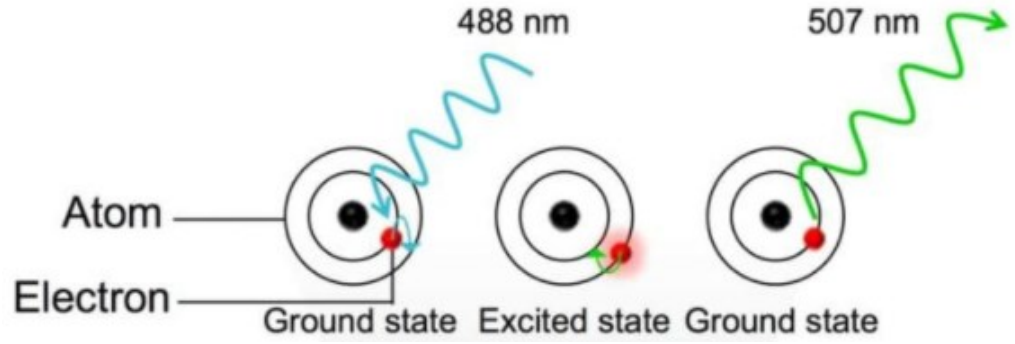
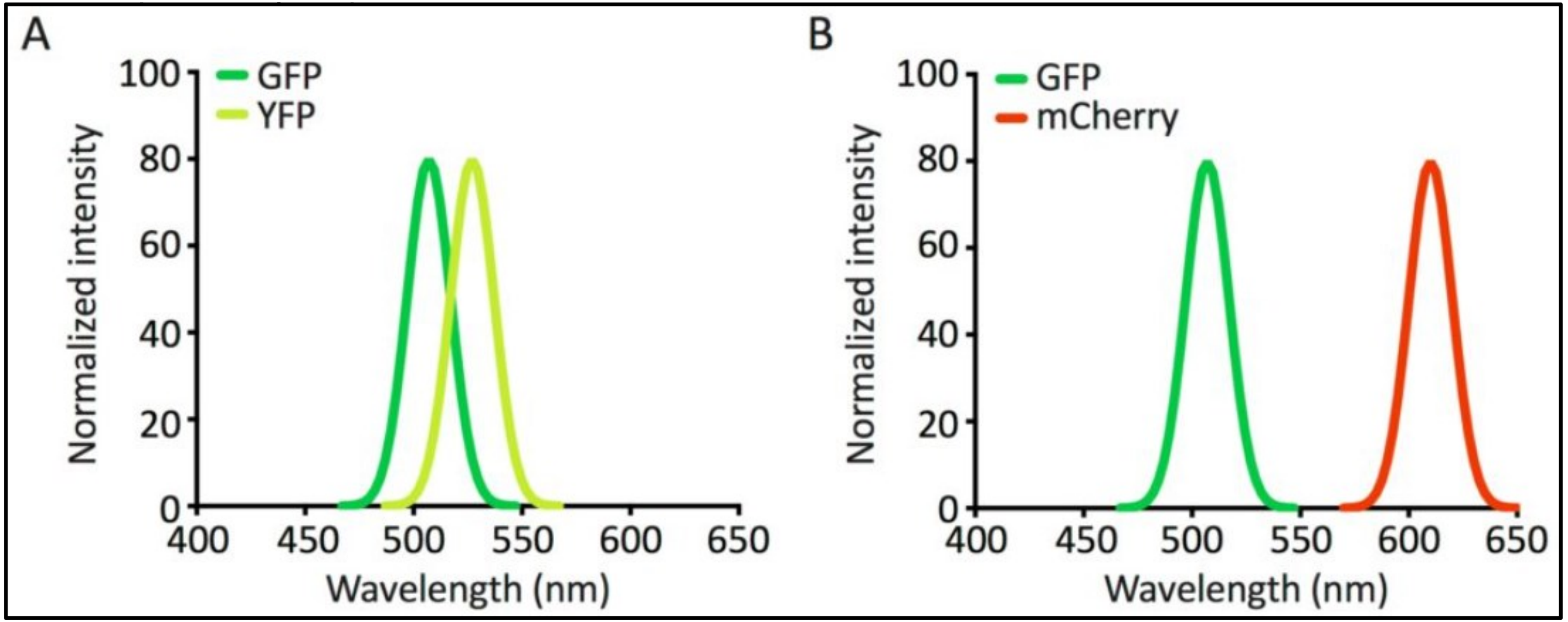
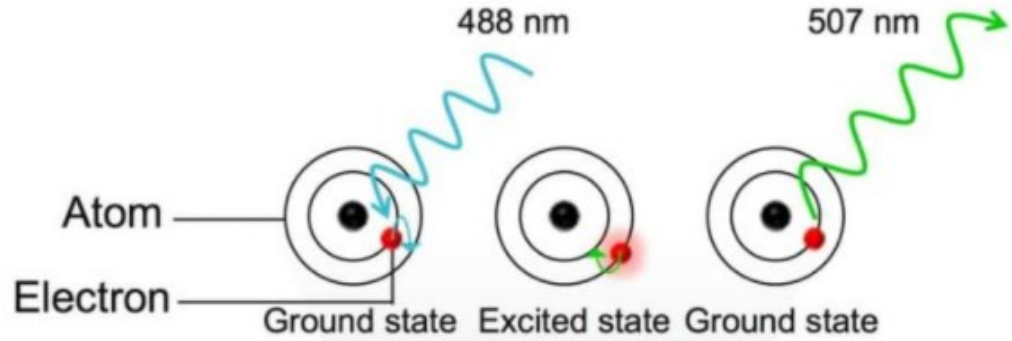
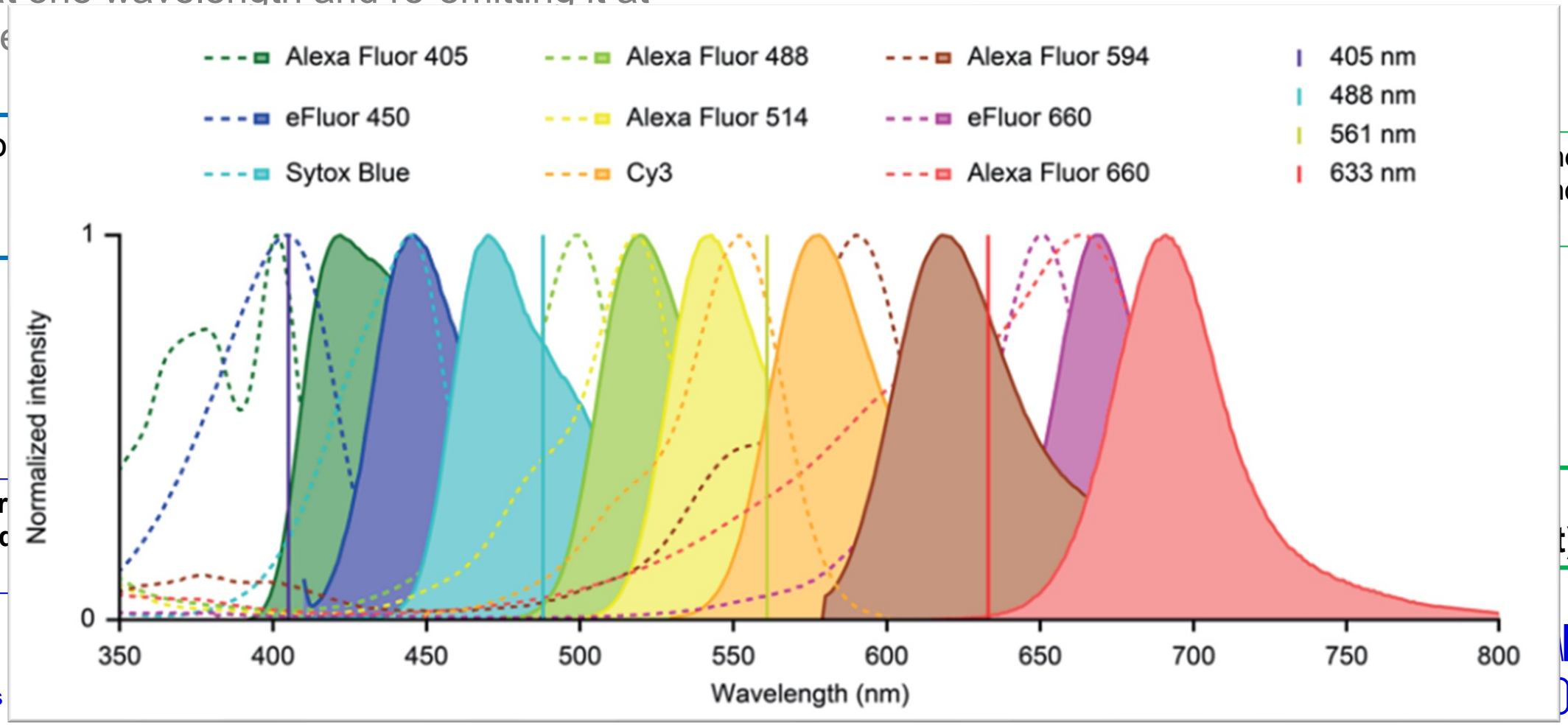




Principle of fluorescence

Fluorophore - chemical compound absorbing light at one wavelength and re-emitting it at anothe





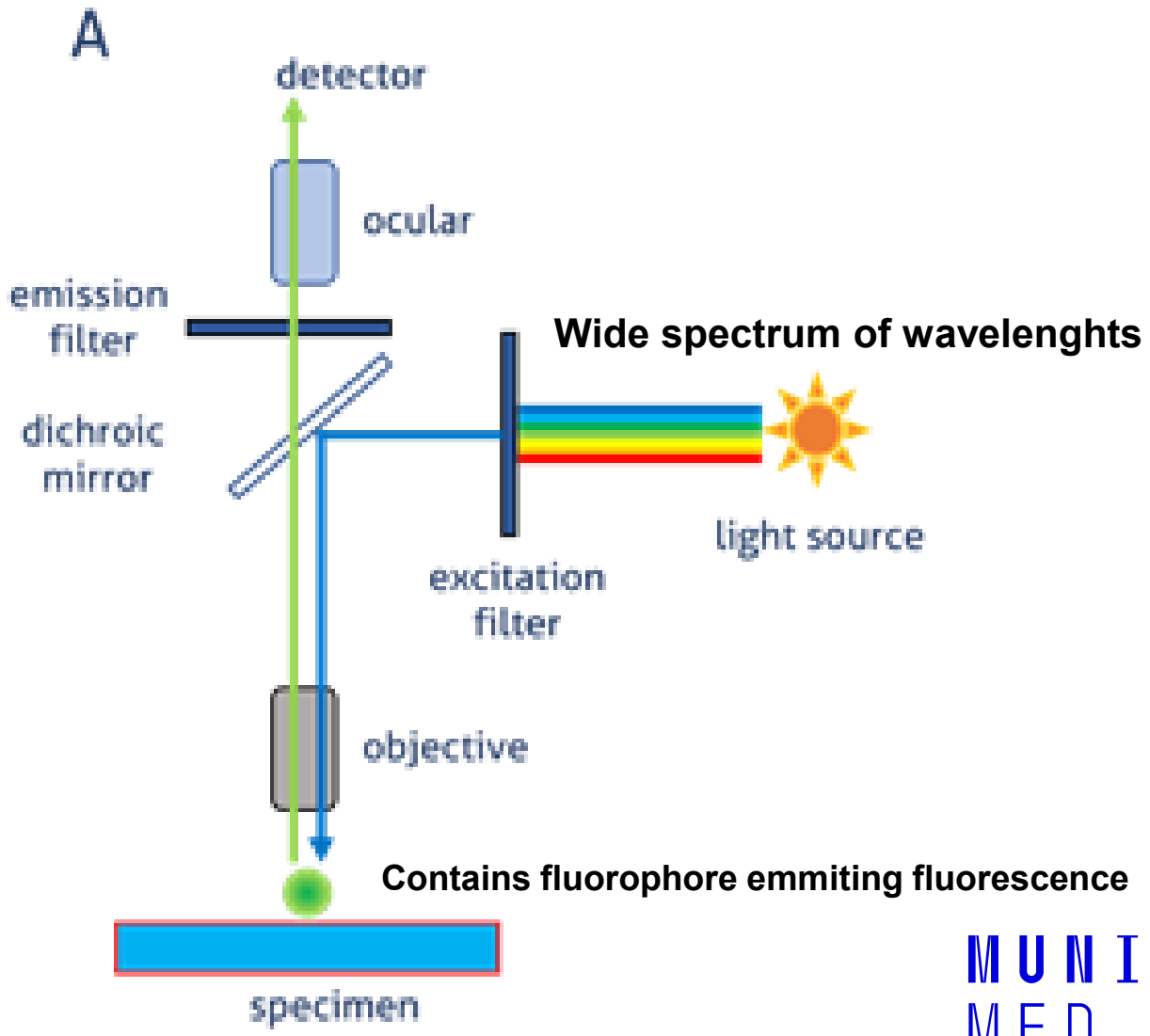
Widefield fluorescence microscopy

Essential components:

- **Excitation filter** – allows only light of specific wavelengths that excites the fluorophore to pass through
- **Dichroic mirror** – reflects one type of light and allows other type of light to pass through
- **Emission filter** – blocks excitation light and transmits emission light to the eyepiece/detector

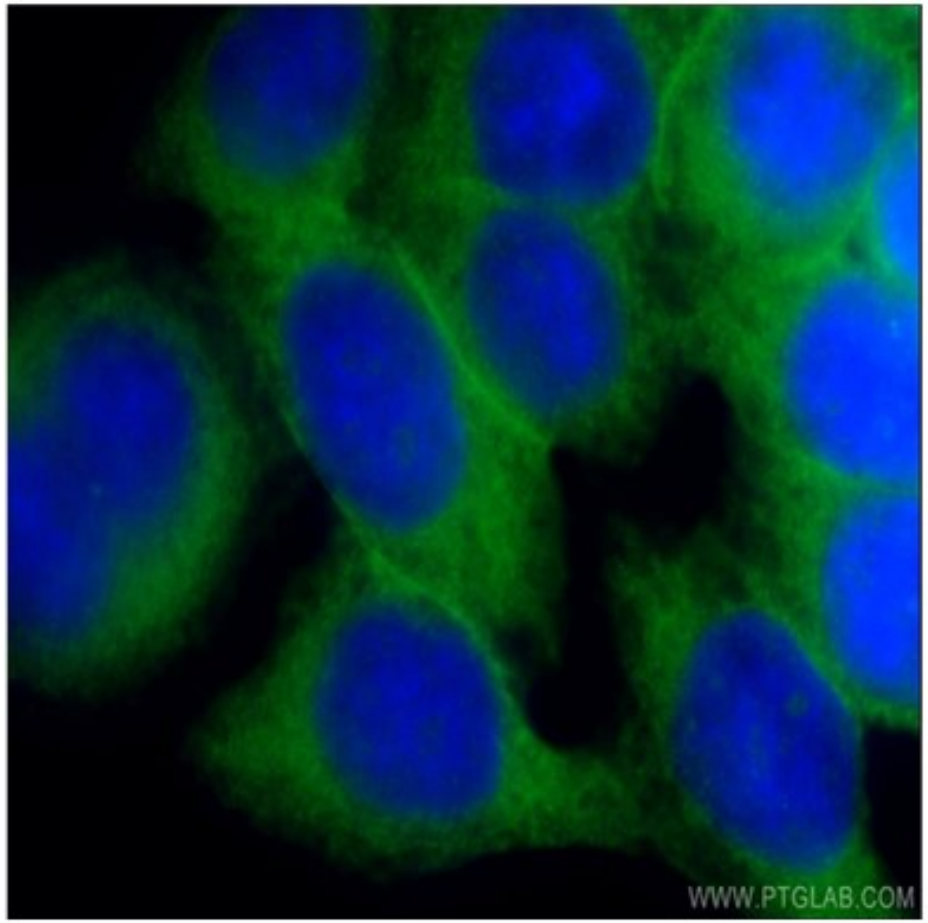
Reflected light has shorter wavelength

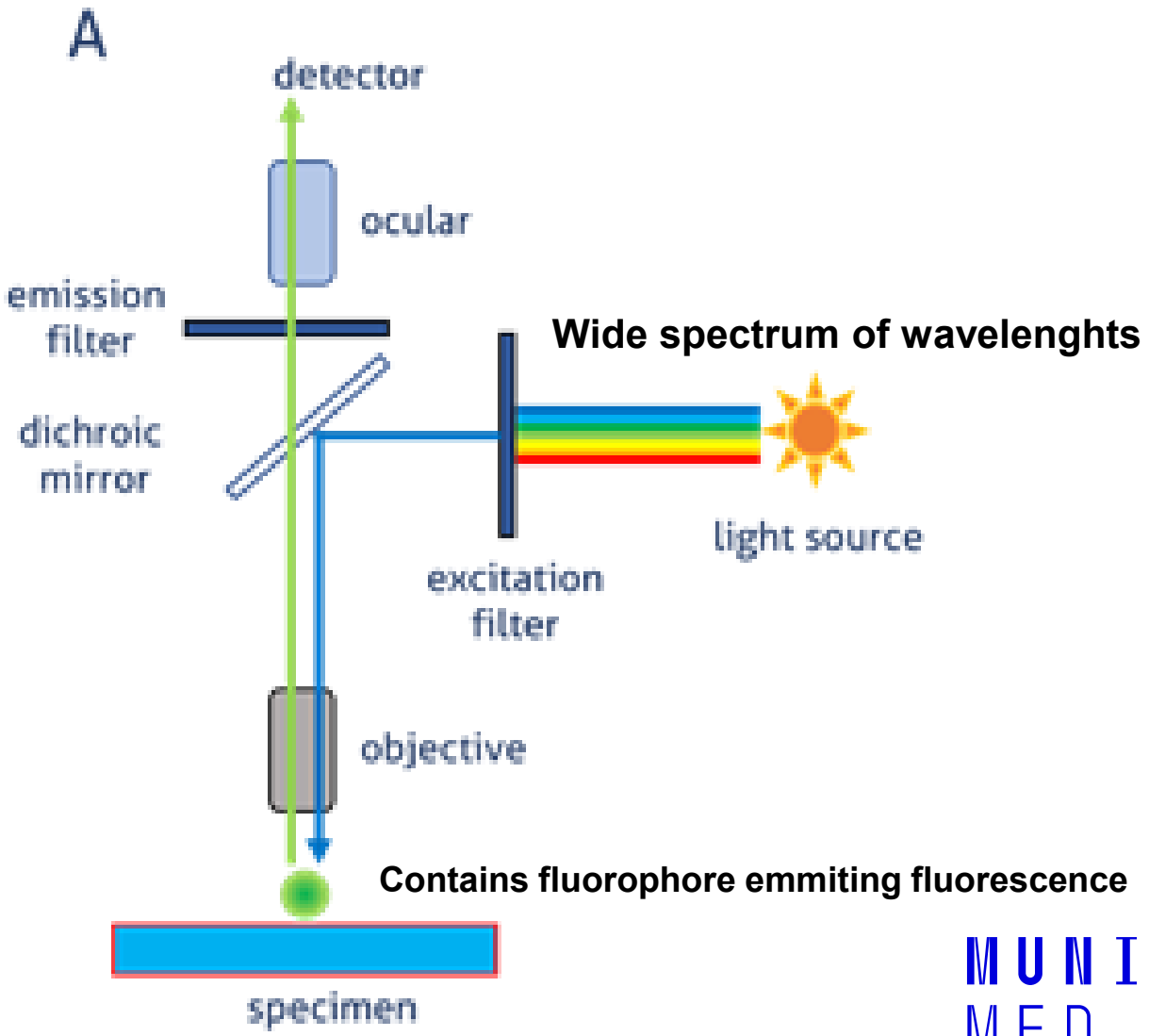
Passing light has longer wavelength (carries less energy than excitation light)



Widefield fluorescence microscopy

HeLa cells DAPI GLUT1





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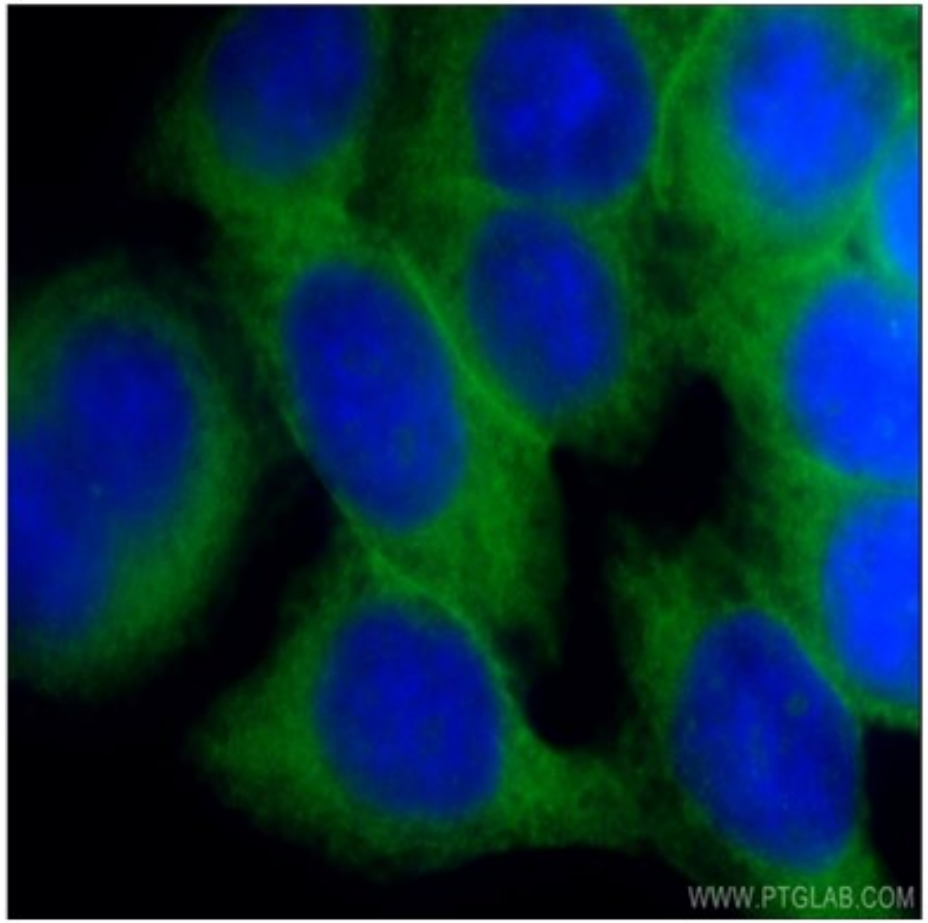

Limitations:

- the whole sample is illuminated
- Fluorescence detected not only from one specific focal plane, but also above and below it (out of focus signal)
- Light can be scattered (liquid-filled compartments)

↓

Background noise

- Problems when identifying specific subcellular localization of the target
- Not suitable for 3D structure modelling

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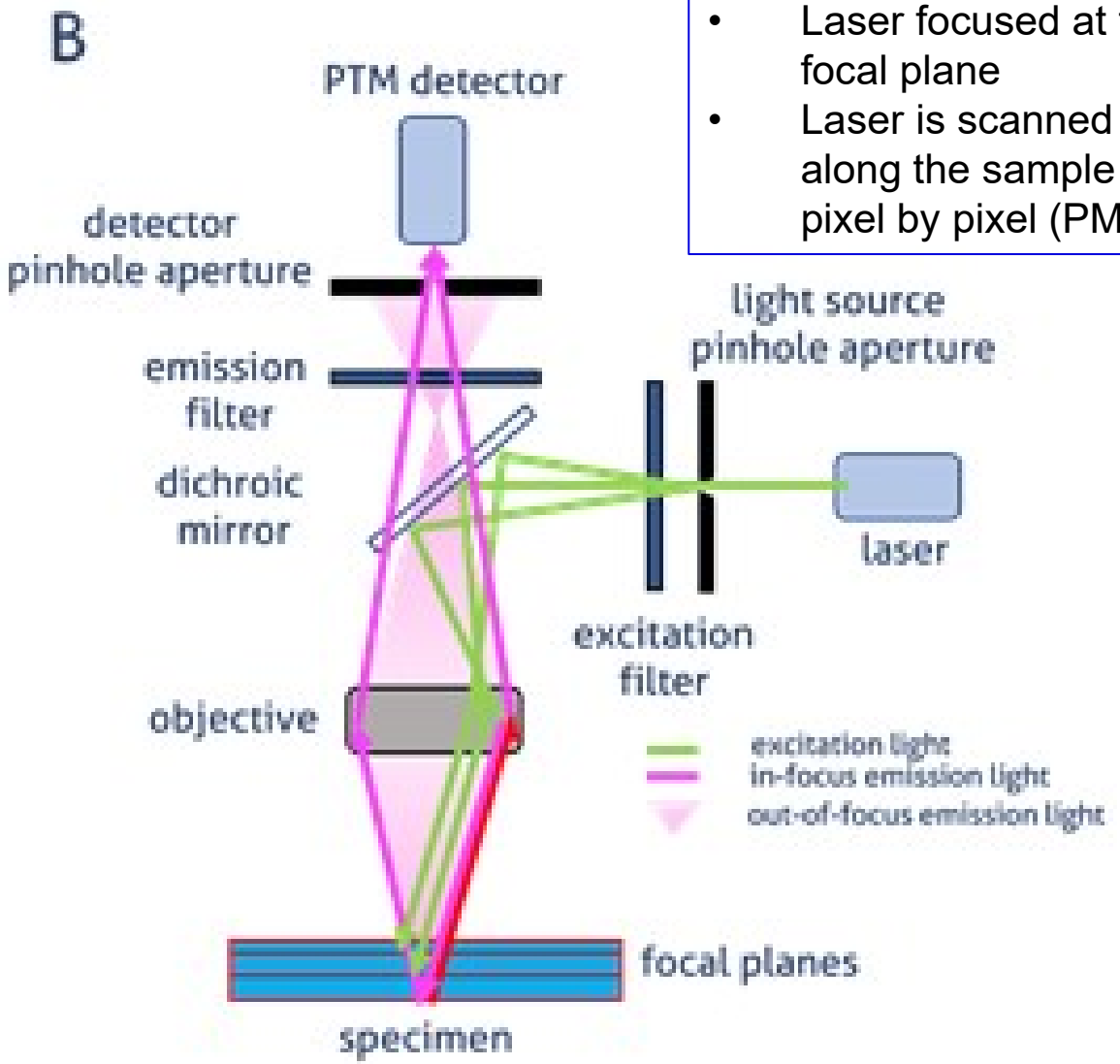
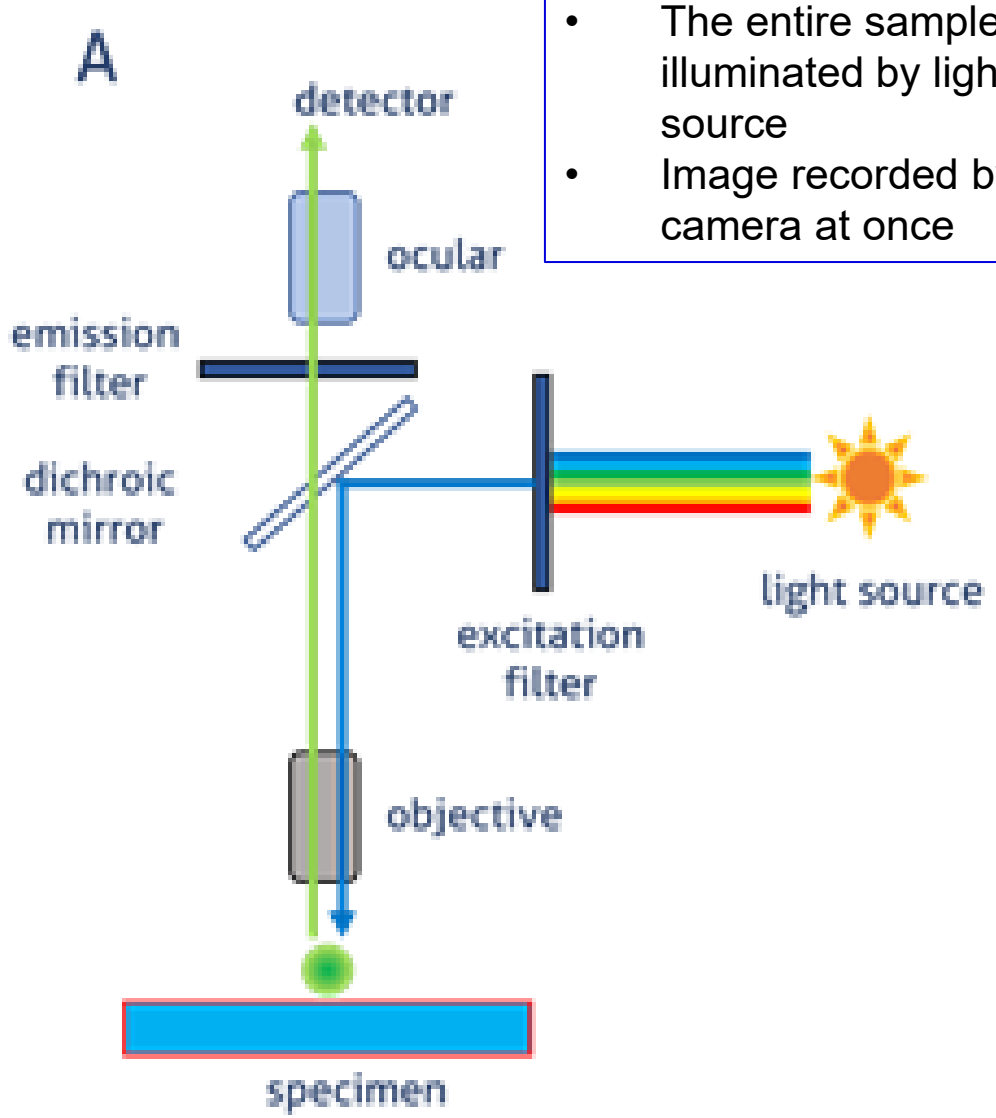
Confocal Laser Scanning vs. Widefield fluorescence microscopy

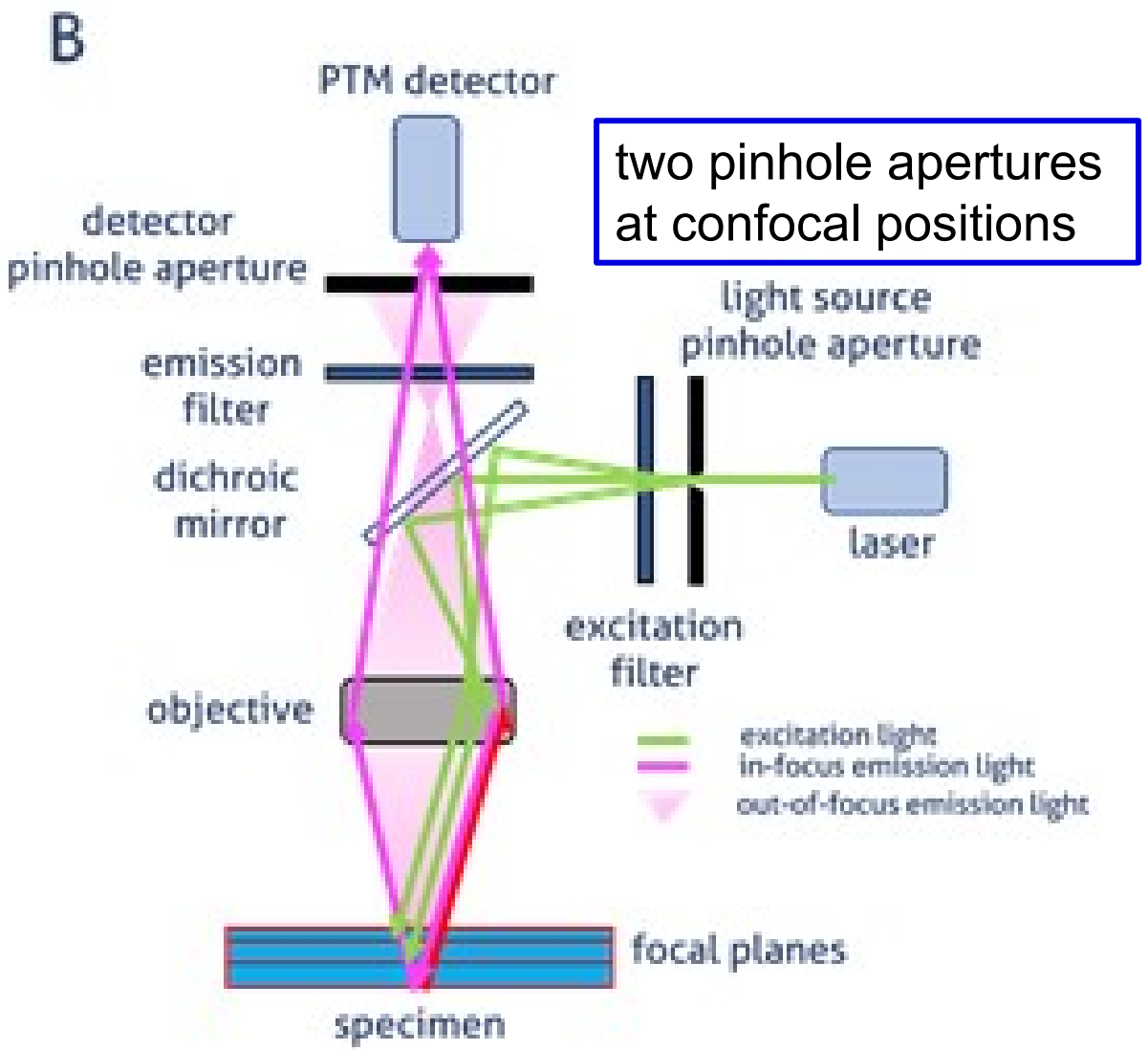
- Laser focused at the focal plane
- Laser is scanned along the sample pixel by pixel (PMTs)



- The entire sample illuminated by light source
- Image recorded by camera at once



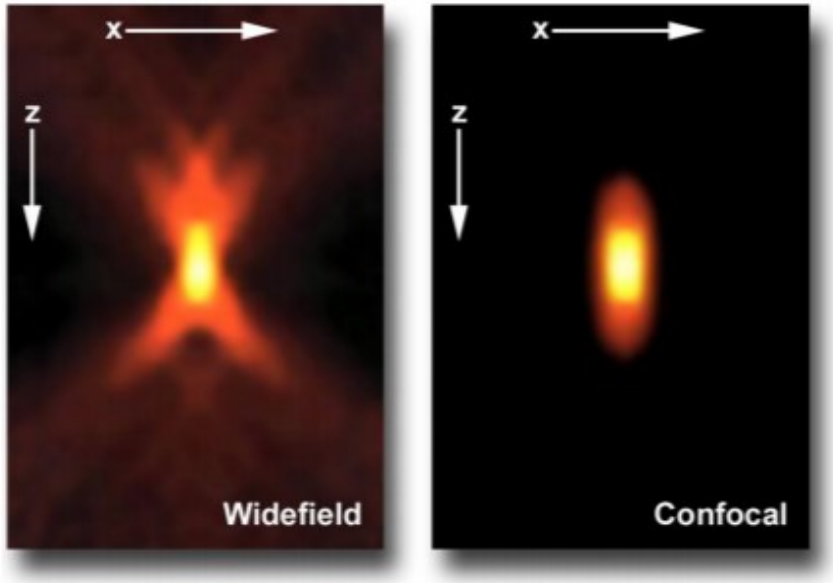
Confocal Laser Scanning microscopy (CLSM)



two pinhole apertures at confocal positions

Additional components:

- **Laser** – bright source of pinpoint illumination
- **1. pinhole** – focuses the beam of light on the **specific small part of the sample**
- **2. pinhole** – positionned in the focal plane, selects the light coming from the targetted point of the sample (reduces background illumination)



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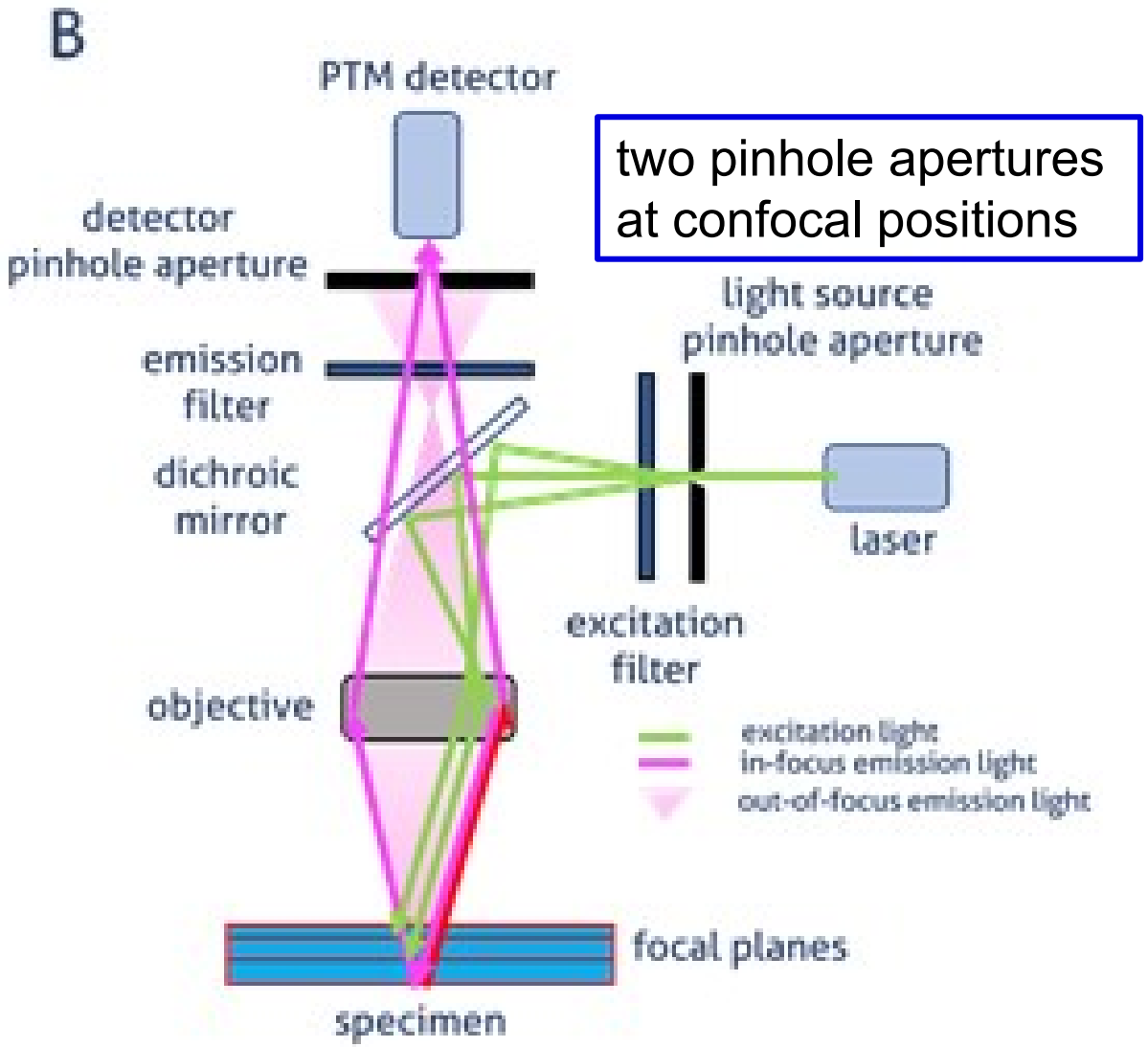

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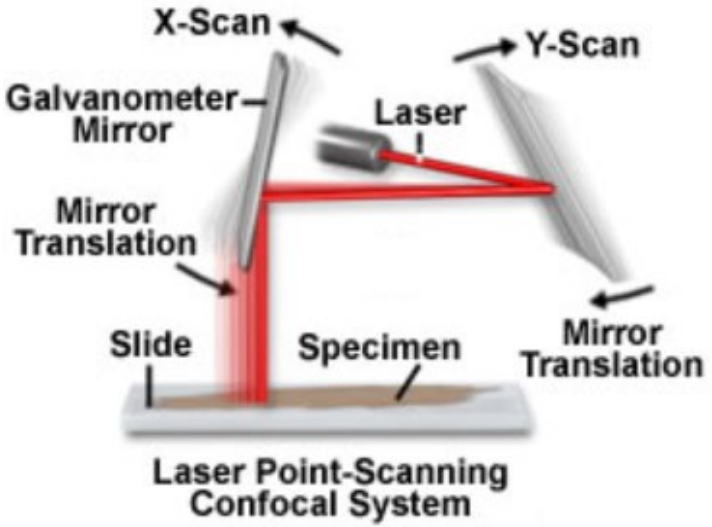

Laser scanning

- Motorized mirrors (moving laser across the sample)

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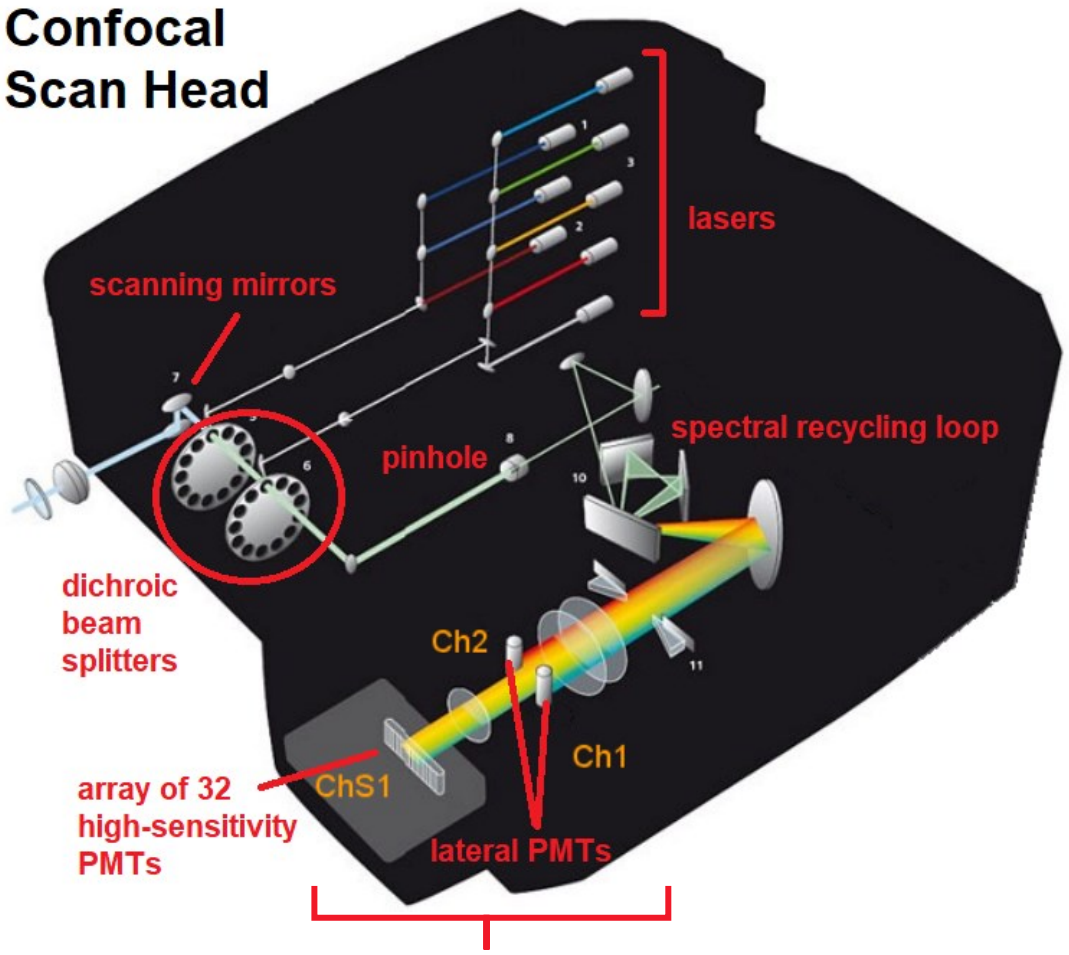
Confocal Laser Scanning microscopy (CLSM)

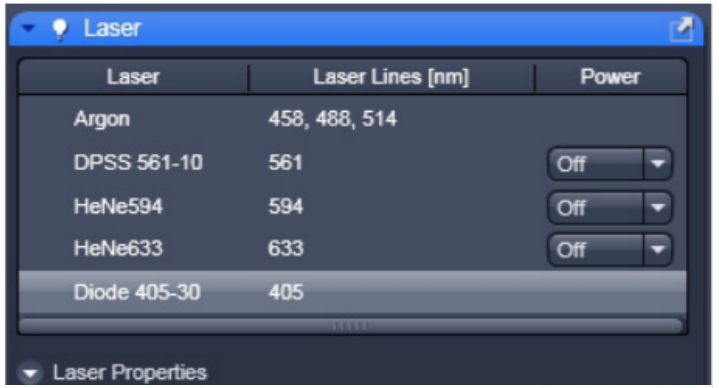
Scanning mirrors

- Motorized mirrors allow laser scanning of the sample along X- and Y-axis within the same focal plane

Photomultiplier tubes (PMTs)

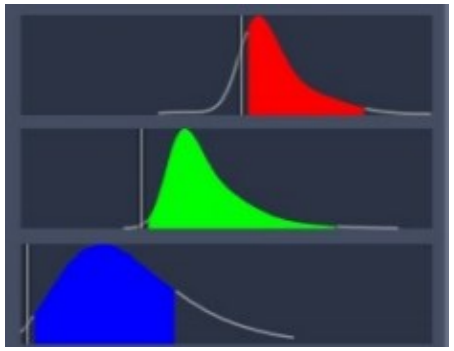
- collect photones and amplify signal (creating digital signal processed by computer)
  – generated image



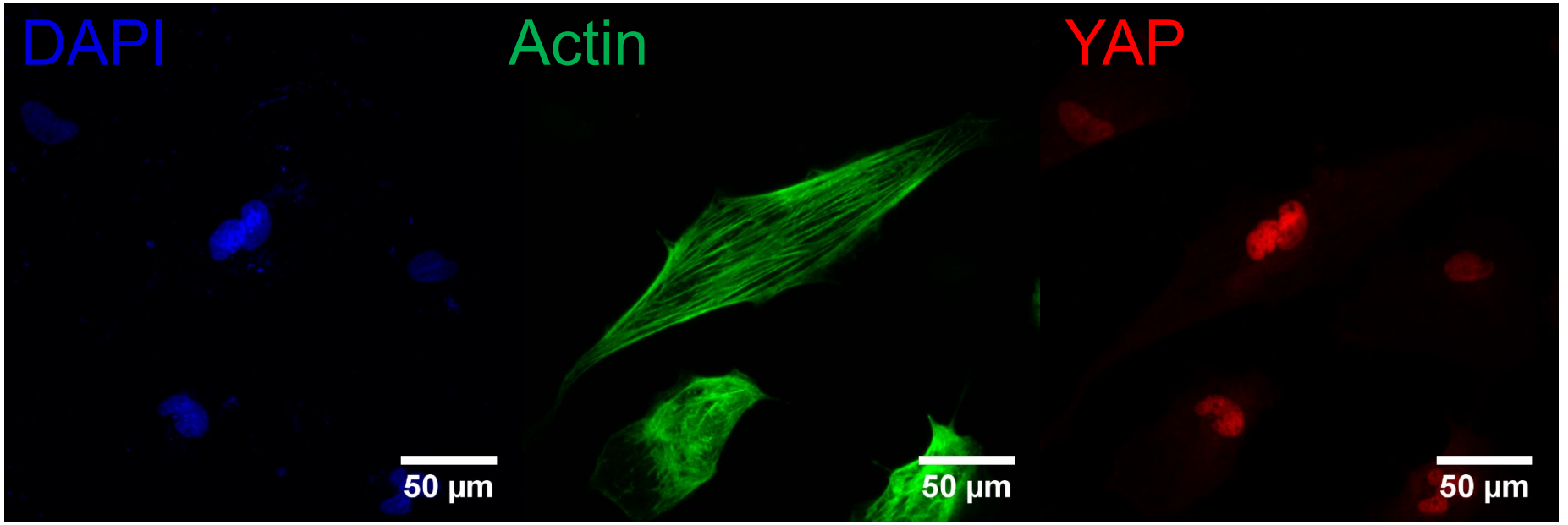


Confocal Laser Scanning microscopy (CLSM)

1. acquisition of signal from each channel separately - minimal bleed through (scanning along x and y)



cardiomyocyte



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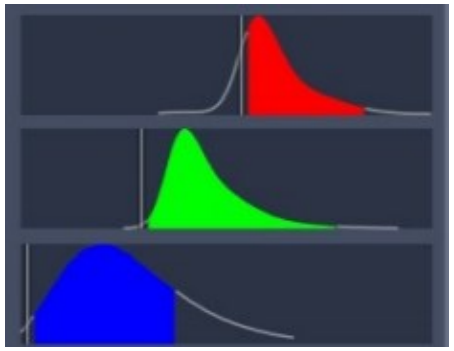
Confocal Laser Scanning microscopy (CLSM)

1. acquisition of signal from each channel separately - minimal bleed through (scanning along x and y)
2. software generates **merged image**



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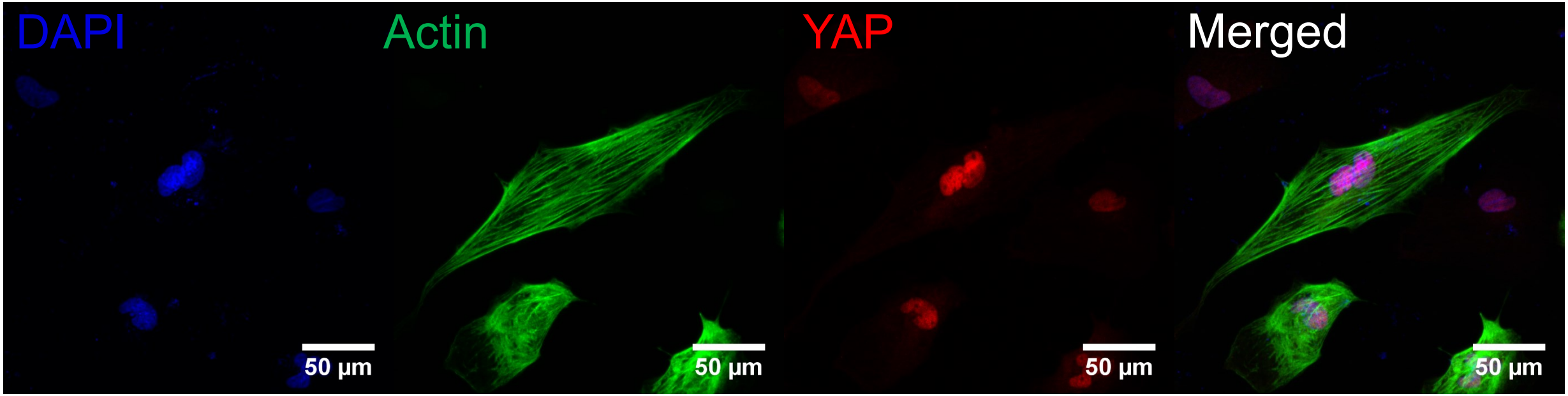

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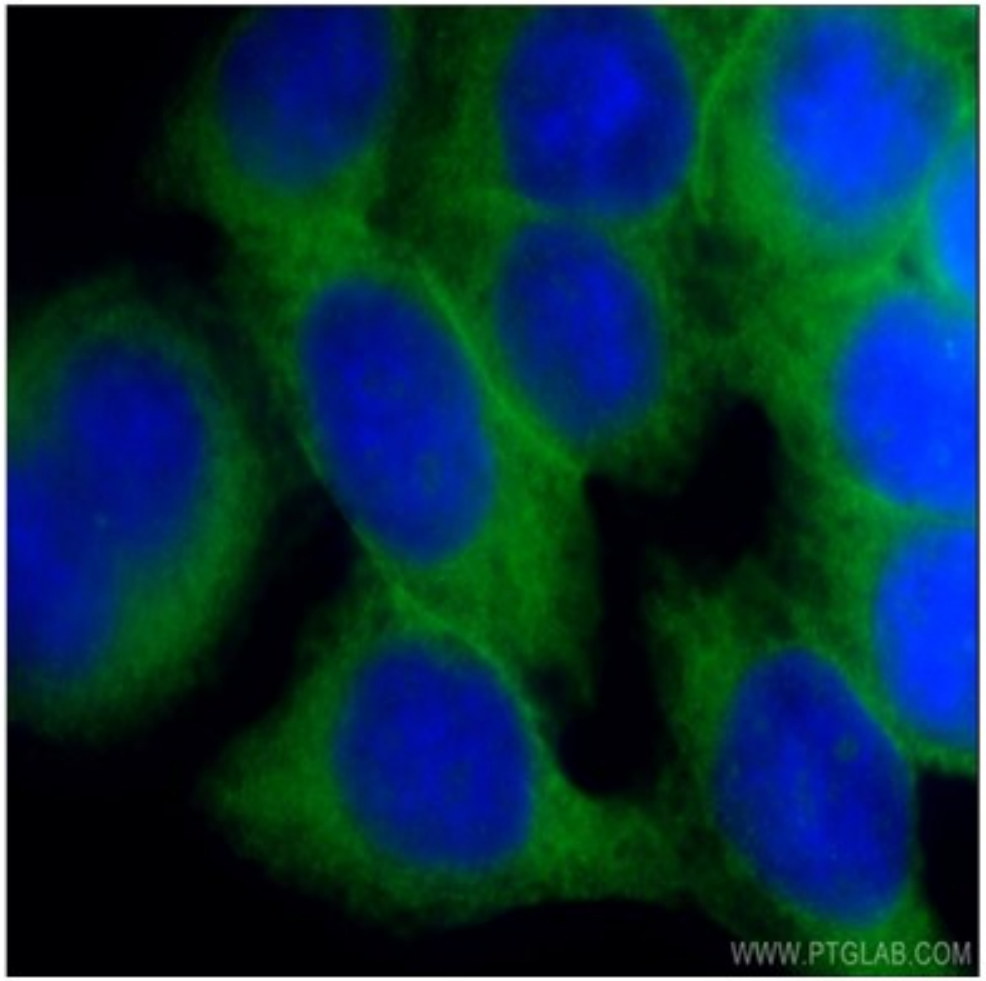
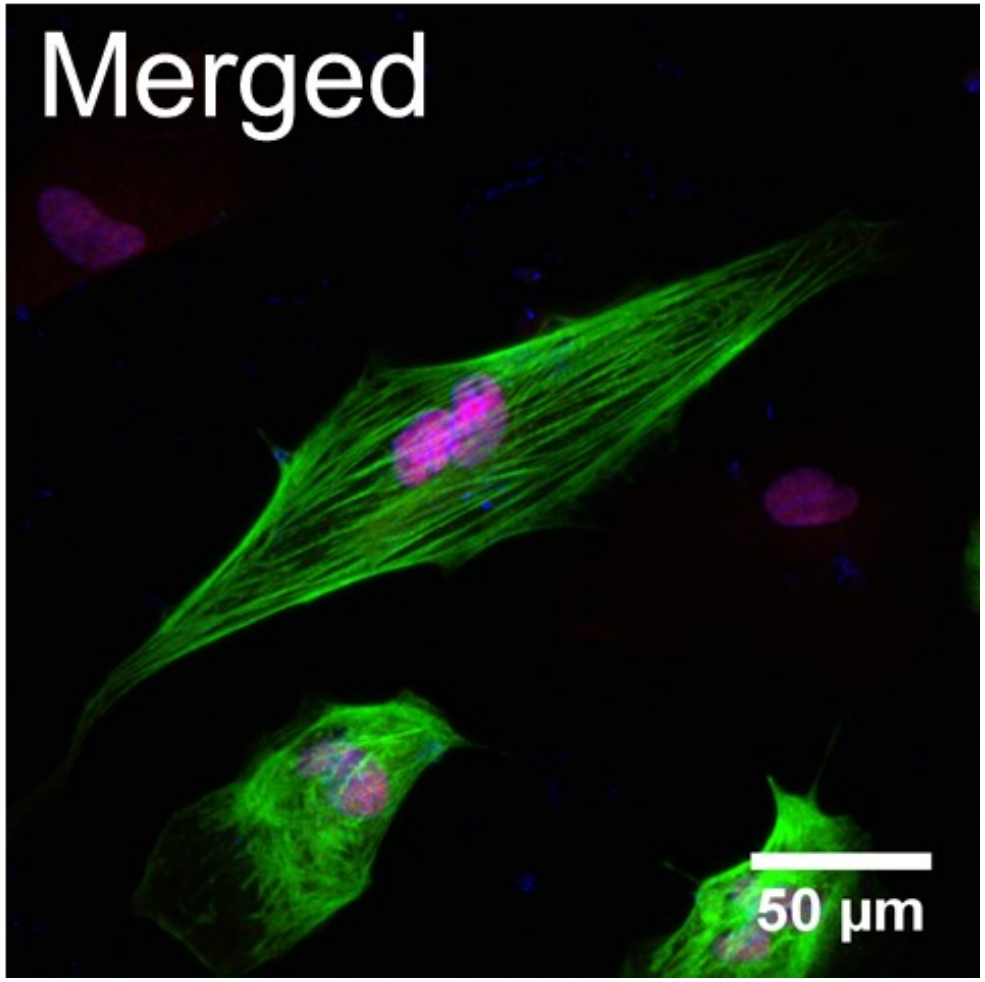
Resolution of images - widefield FM vs. CLSM





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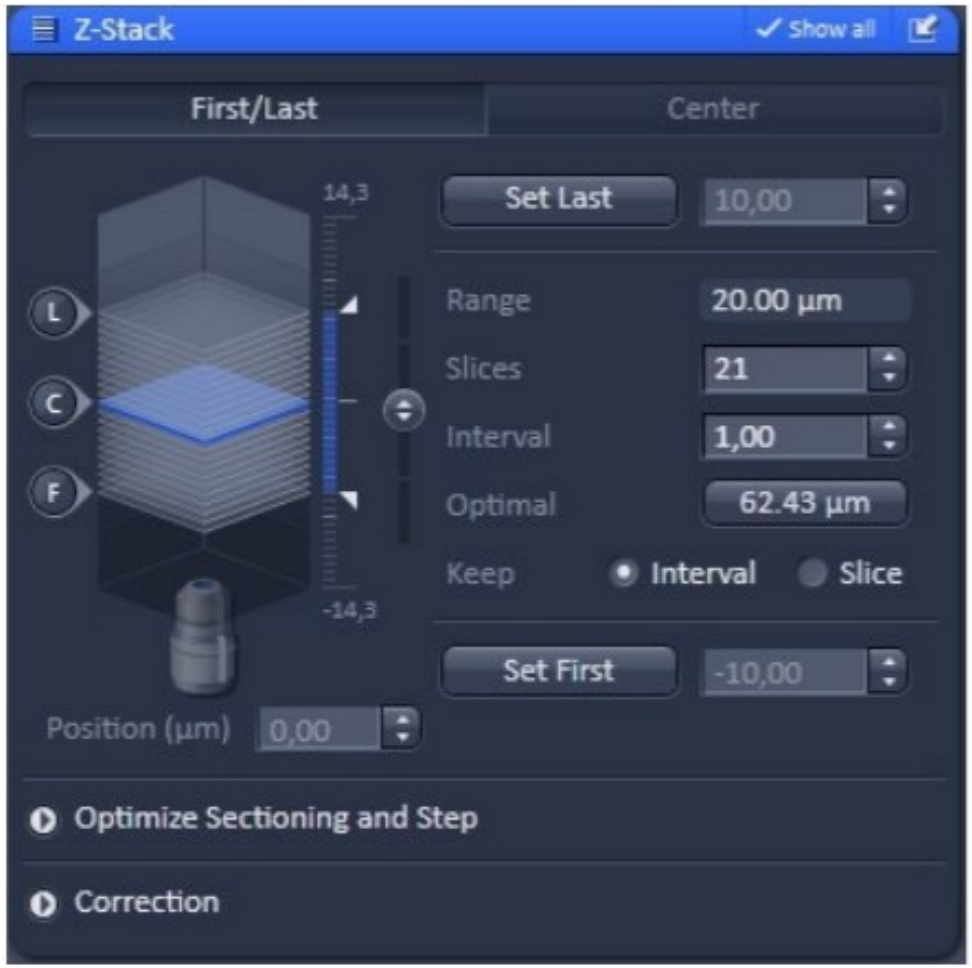
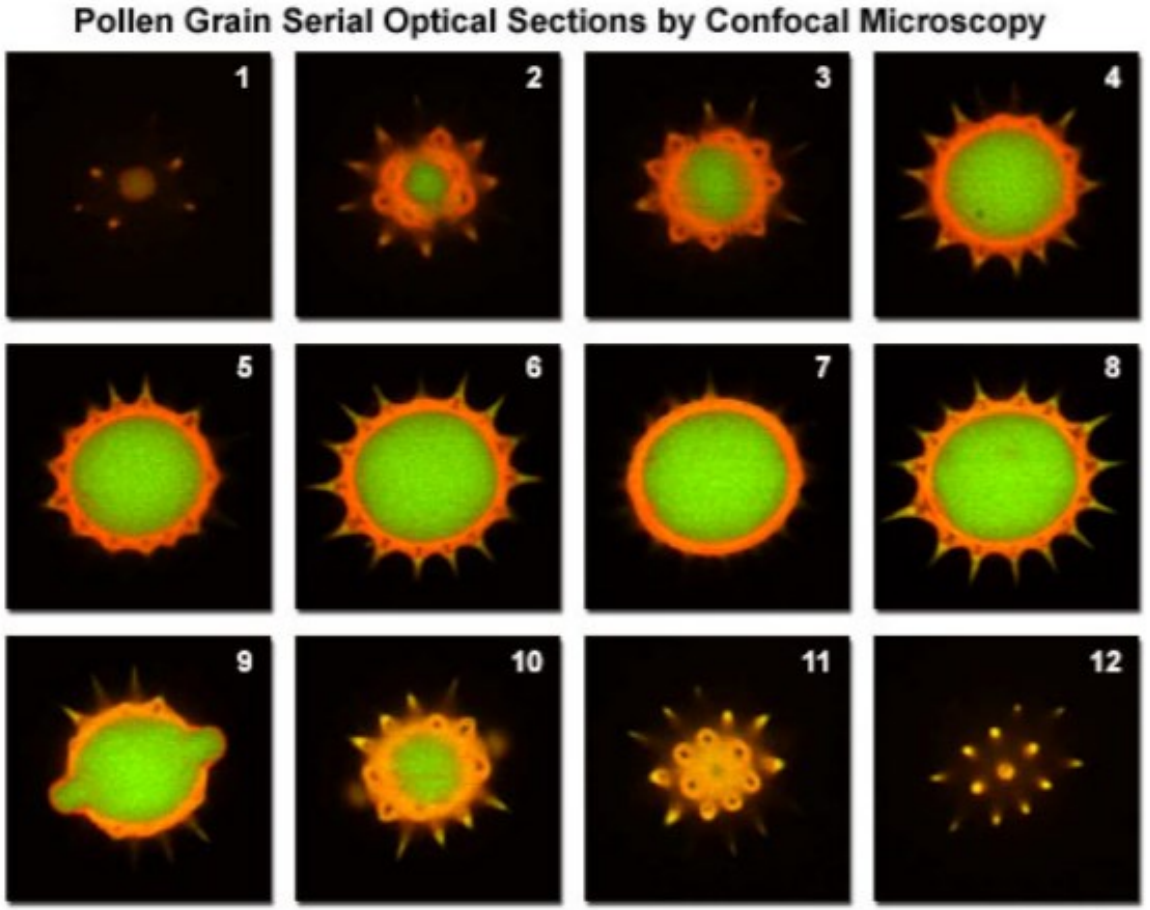
Key Advantage - Optical sectioning (CLSM)

- Clear images of **thin sections** within a **thick sample**





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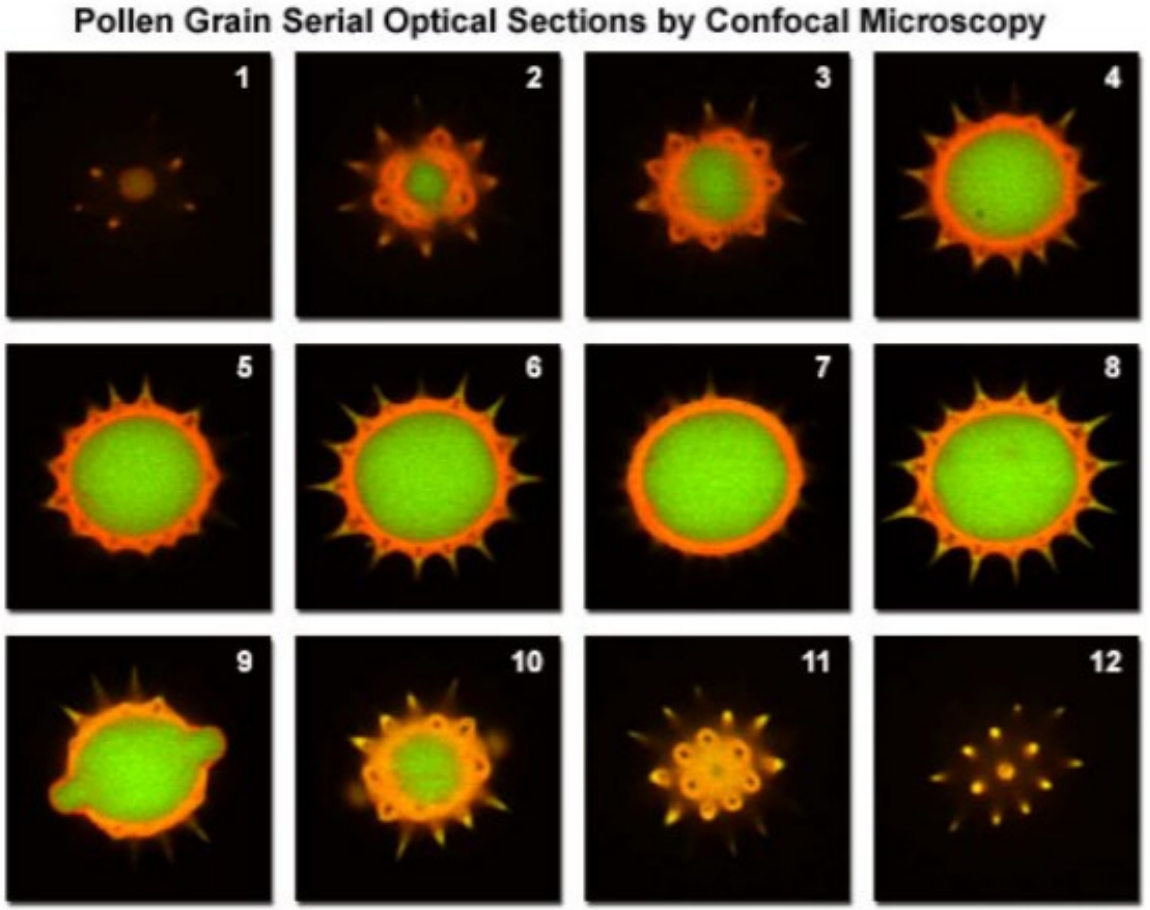

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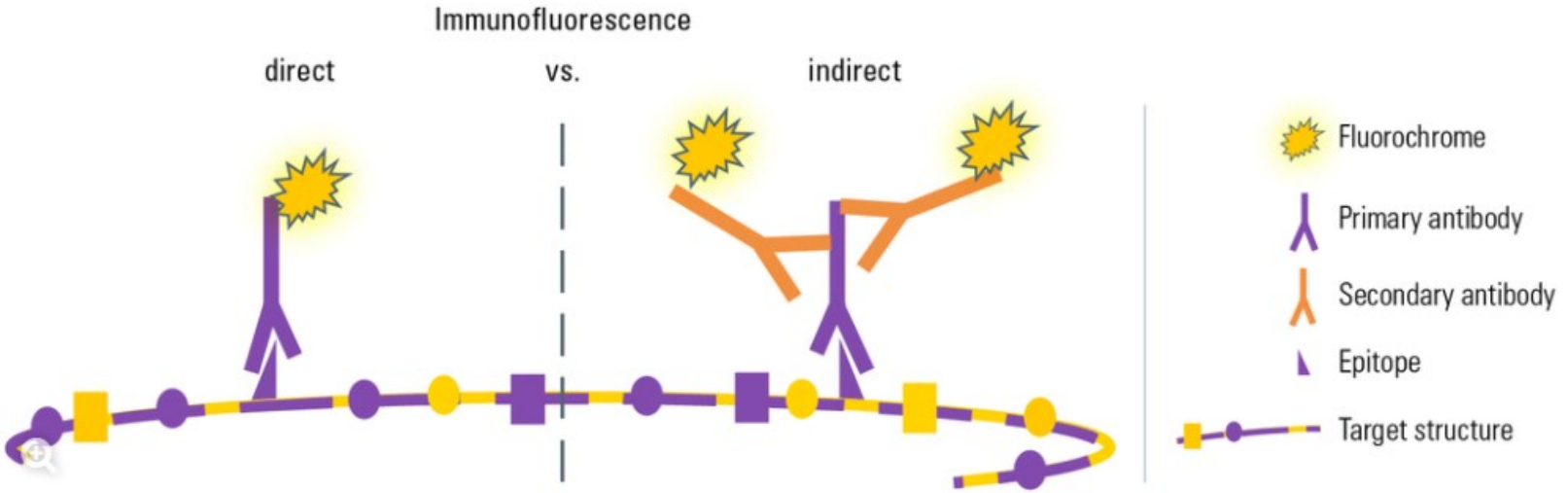
Advantages and Disadvantages of CLSM

- ✓ reduced background compared to widefield FM
- ✓ optical sectioning (0,5-1,5 um) of up to 100 um thick sample
- ✓ generation 3D images
- ✓ examination of living and fixed specimens (better resolution)

- – **Speed** !
- – Photobleaching of the sample (long-lasting acquisition)
- – Limitated number of excitation wavelengths (availability of lasers)
- – high cost of purchasing

Fluorescence Microscopy Applications

1. Immunostaining of fixed samples
   - **Direct**
   - **Indirect**



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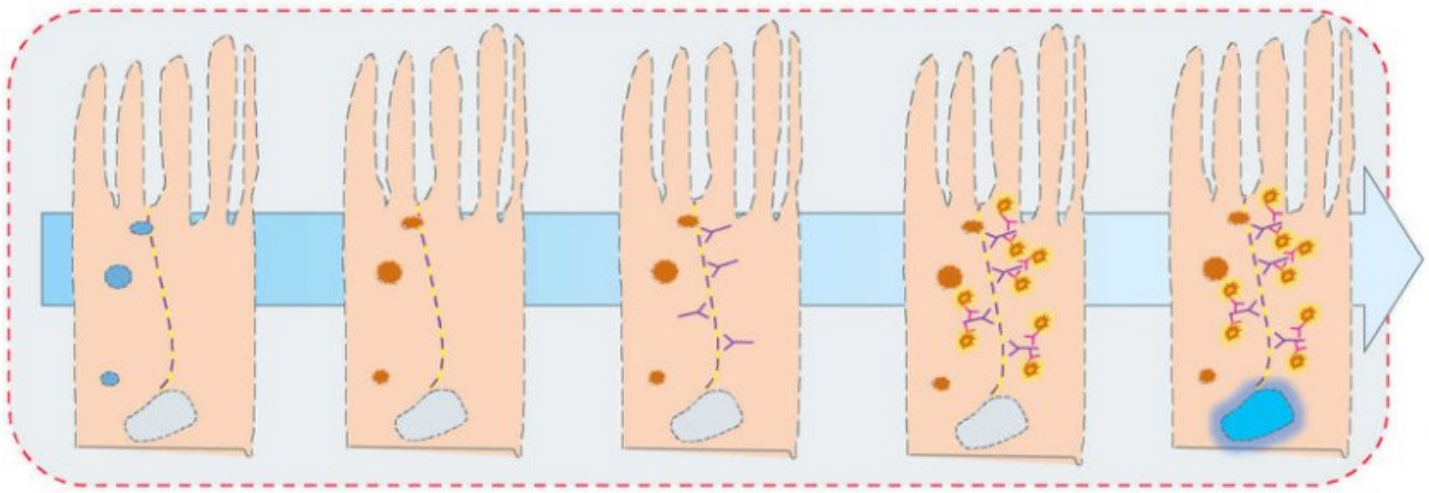
Fluorescence Microscopy Applications

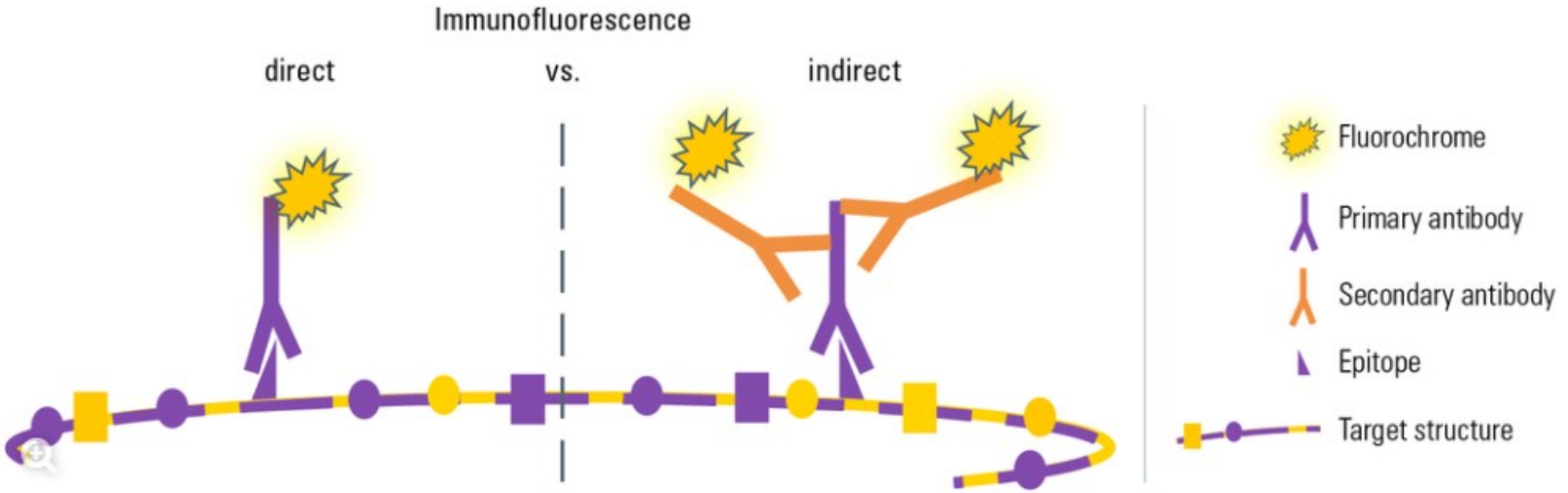
1. Immunostaining of fixed samples
   - **Indirect**
     1. Fixation and Permeabilization
        Cross-linkers (e.g. PFA) – form covalent chemical bonds between the proteins
        Organic solvents (alcohol, acetone) – remove lipids, dehydration of the tissue, protein precipitation
        Permeabilization reagents (Triton X-100, NP-40)
     2. Blocking (e.g. BSA) – prevents **unspecific binding** of primary Ab
     3. First Immunostaining – **primary Ab** recognizes and binds to the epitope of target protein
     4. Second Immunostaining – **secondary Ab** conjugated with a fluorophore binds to the **primary Ab (species-specific)**
     5. Nuclear staining (e.g. DAPI)
     6. Mounting medium (PBS, Mowiol)

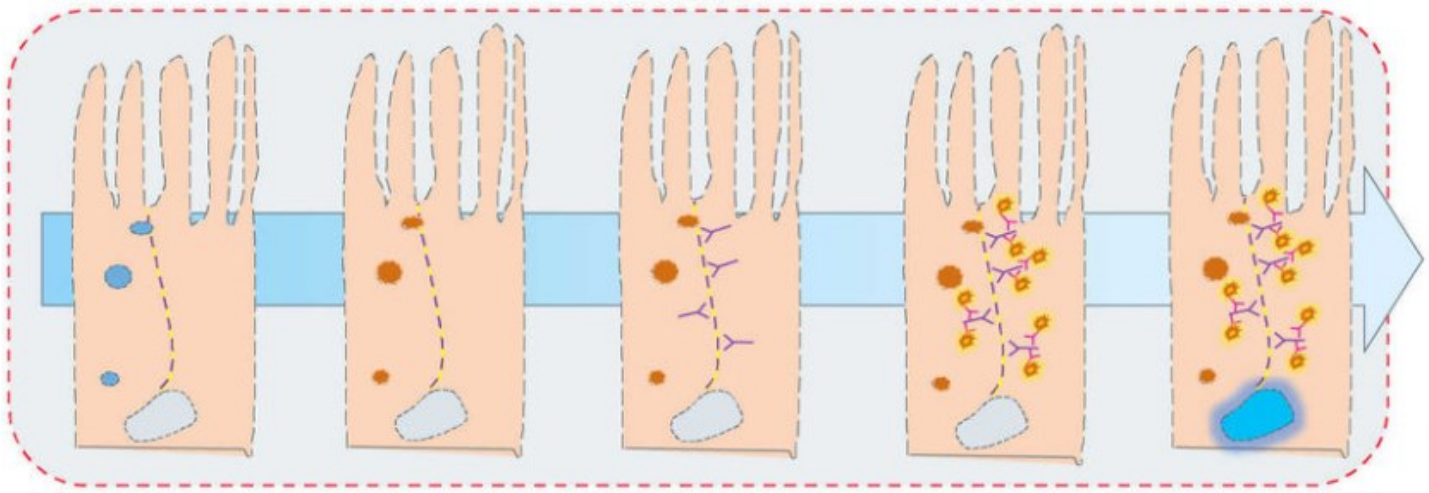


Indirect immunofluorescence staining

Fluorescence Microscopy Applications

1. Immunostaining of fixed samples
   - **Direct**
     - ✓ **Faster**
     - ✓ **Easier to handle** (rapid analysis, standardized experiments, useful for clinical practice)
     - - **Validated Ab with high-affinity (expensive)**
   - **Indirect**
     - ✓ **Flexibility in design** (combining different prim. Ab with different sec. Ab)
     - ✓ **Signal amplification**
     - ✓ **Economical procedure**
     - - **time-consuming workflow**





Indirect immunofluorescence staining

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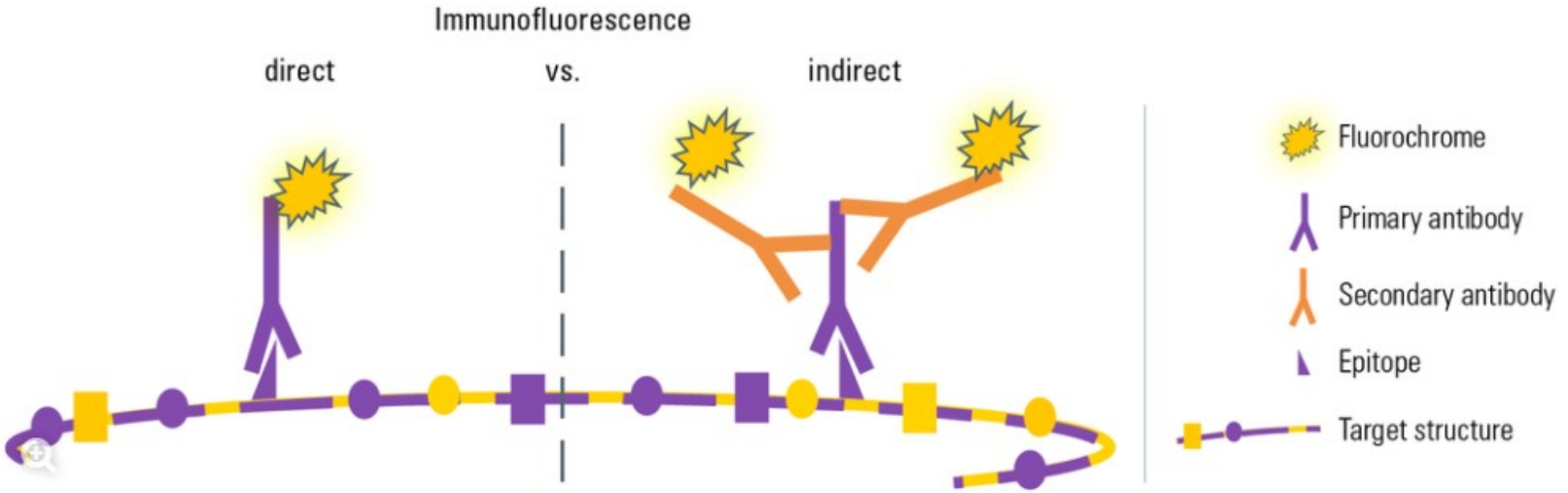
Fluorescence Microscopy Applications

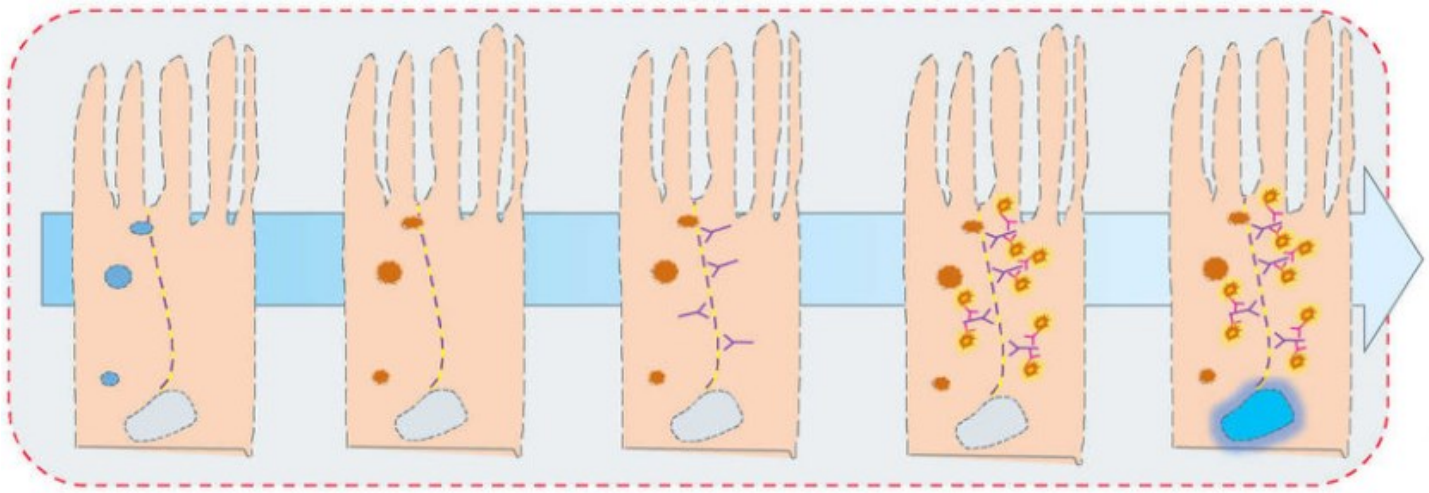
1. Immunostaining of fixed samples
   - **Direct**
   - **Indirect**

   ➢ Detection of several fluorophores
      ➢ organelle-specific localization
      ➢ co-localization

         ➢ **FISH-ing for chromosomal abnormalities**

   ➢ 3D visualization of cells and tissue sections (100um) **(CLSM)**





Indirect immunofluorescence staining

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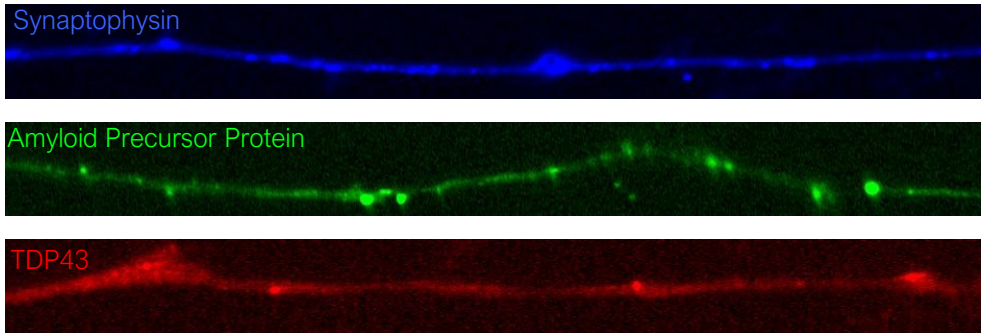
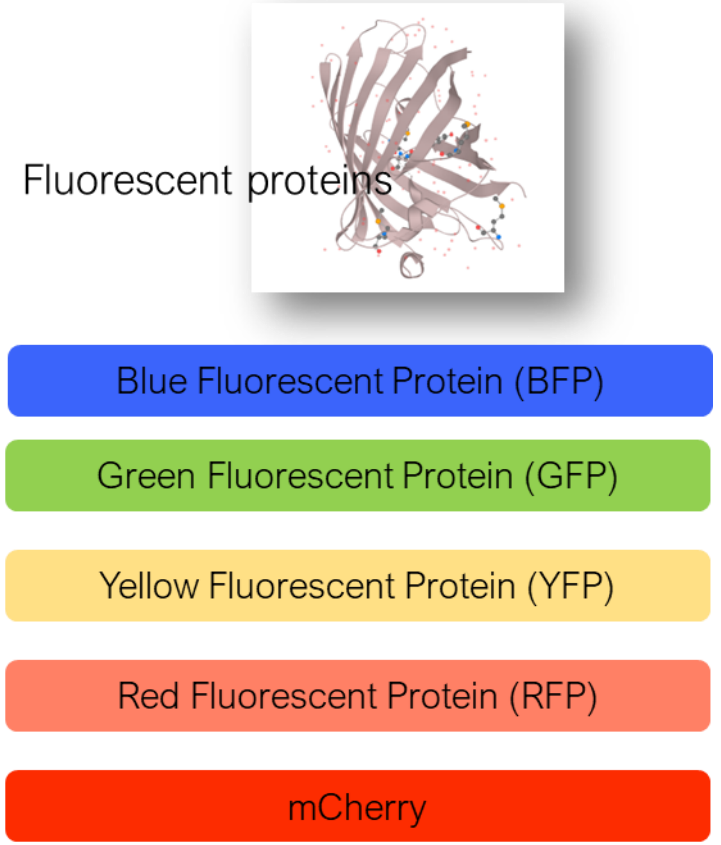
Fluorescence Microscopy Applications

2. **Live cell imaging**
   - Genetically encoded **GFP/FPs** (fluorescent proteins) fused with **protein of interest**
     - tracking localization (organelle-specific proteins), abundance or changes within the labelled protein over time or in response to treatments





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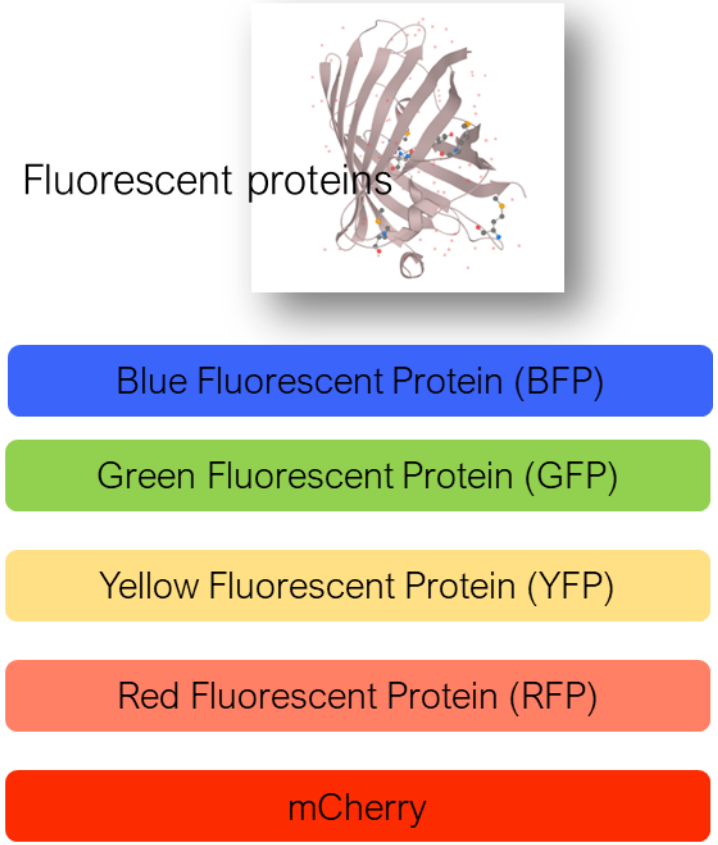
   - Organelle-specific dyes
     - **MitoTracker, LysoTracker, ER Tracker...**
   - Fluorescent DNA intercalating agents (fixed samples as well)
     - Hoechst (minor-groove binding dye, A/T-rich regions)



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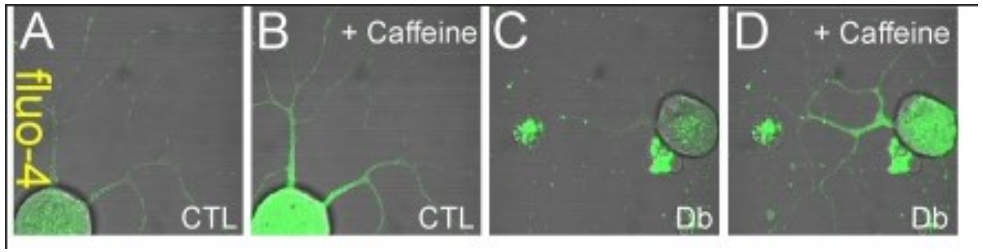
Fluorescence Microscopy Applications

2. **Live cell imaging**
   - Fluorescent dye detecting changes in
     - **ion** concentration (fluo-labelled chelators – concentration-dependent shift in spectral properties)
     - **voltage**
     - **pH** (phagocytosis assay)

Ca^{2+} levels changes



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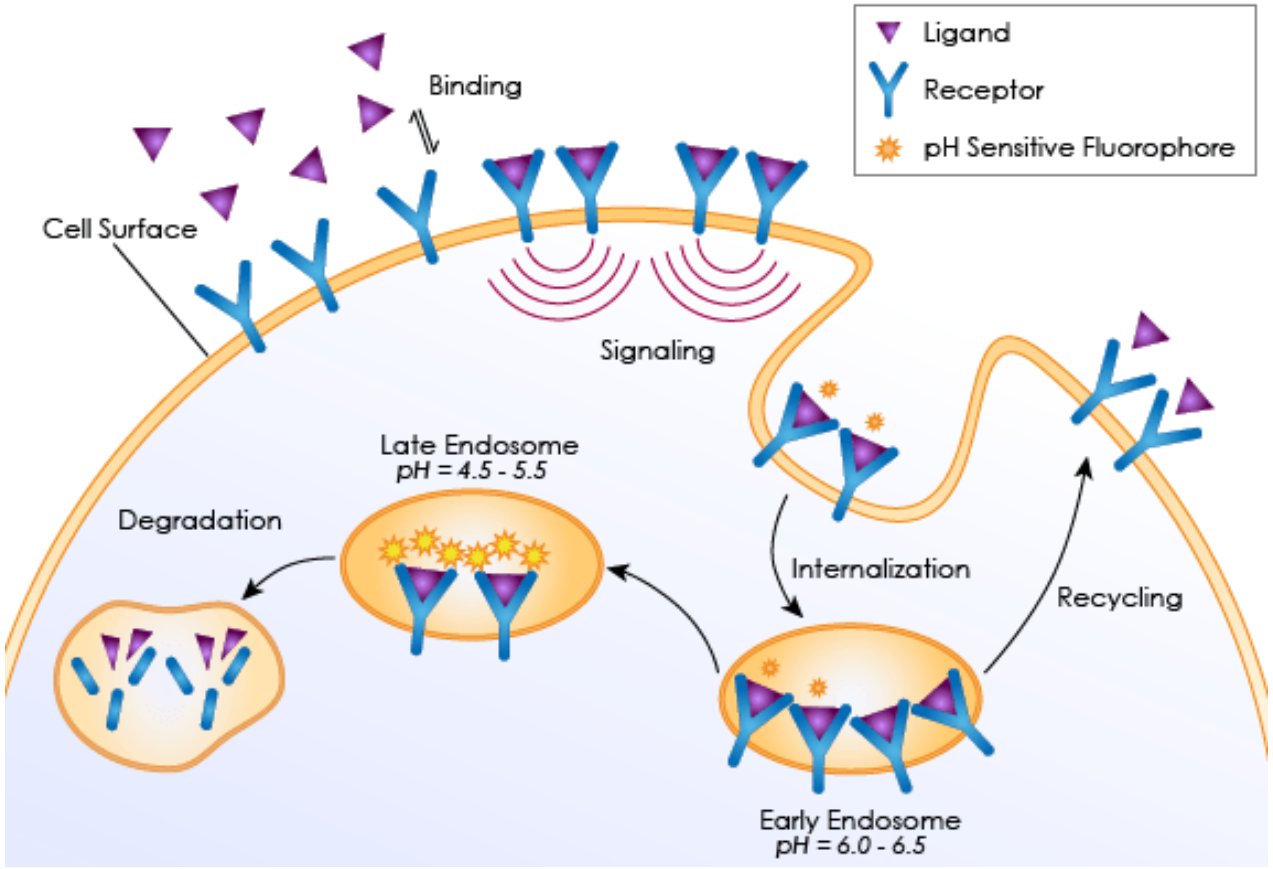

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- Fluorescent dye detecting changes in
  - **ion** concentration (fluo-labelled chelators – concentration-dependent shift in spectral properties)
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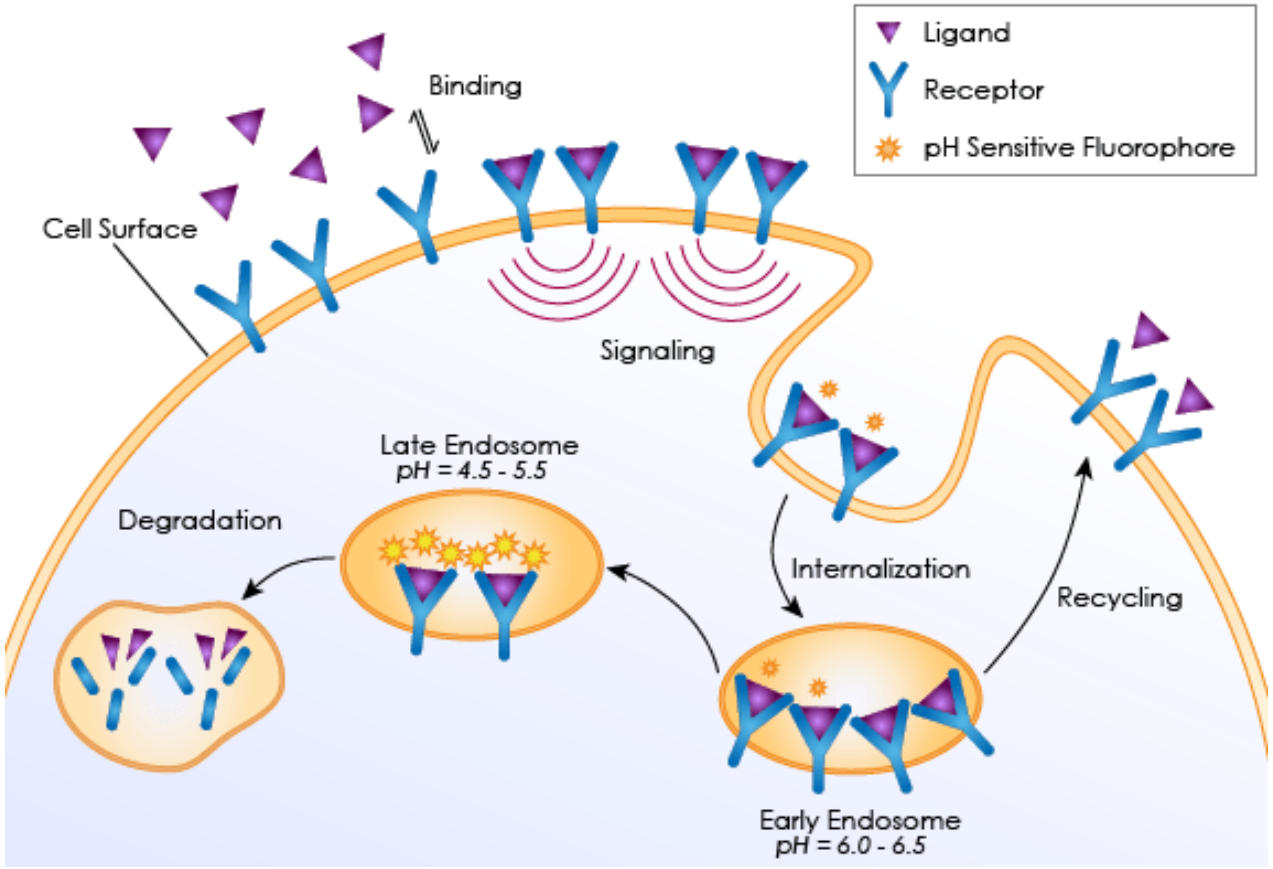
➢ 4D – time-lapse experiment

➢ in response to treatment

➢ cell cycle



Thank you for your attention

Mgr. Ivana Andrejčinová

437403@mail.muni.cz

Sources

https://www.esric.org/education/education-centre/principles-of-fluorescence
https://www.nexcelom.com/applications/celigo/fluorescent-assays/internalization-and-phagocytosis/
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https://www.leica-microsystems.com/science-lab/how-to-prepare-your-specimen-for-immunofluorescence-microscopy/
https://bitesizebio.com/33529/fluorescence-microscopy-the-magic-of-fluorophores-and-filters/
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