Light microscopy in life sciences

BIOENG-518
2025

École
polytechnique
fédérale
de Lausanne

Image Acquisition

- What are images?
- How are images generated?
- What are digital images?
- Why must we know and care about it?

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Image Acquisition

- Which types of image generation do you know?
- Which types of imaging devices do you know?
- Which technological developments have been transformative for imaging?
- What is the difference between an analogue and a digital image?

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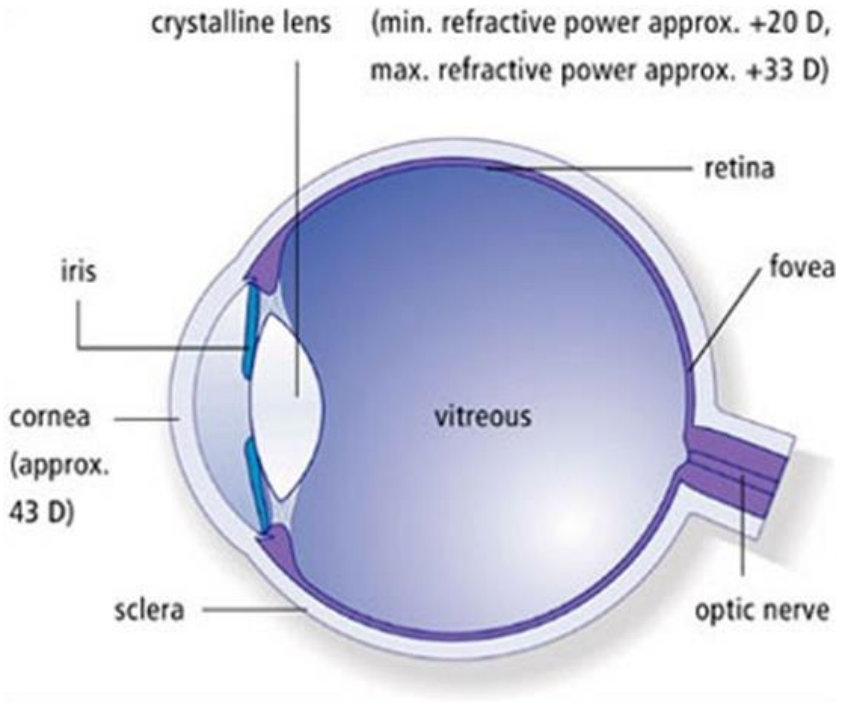
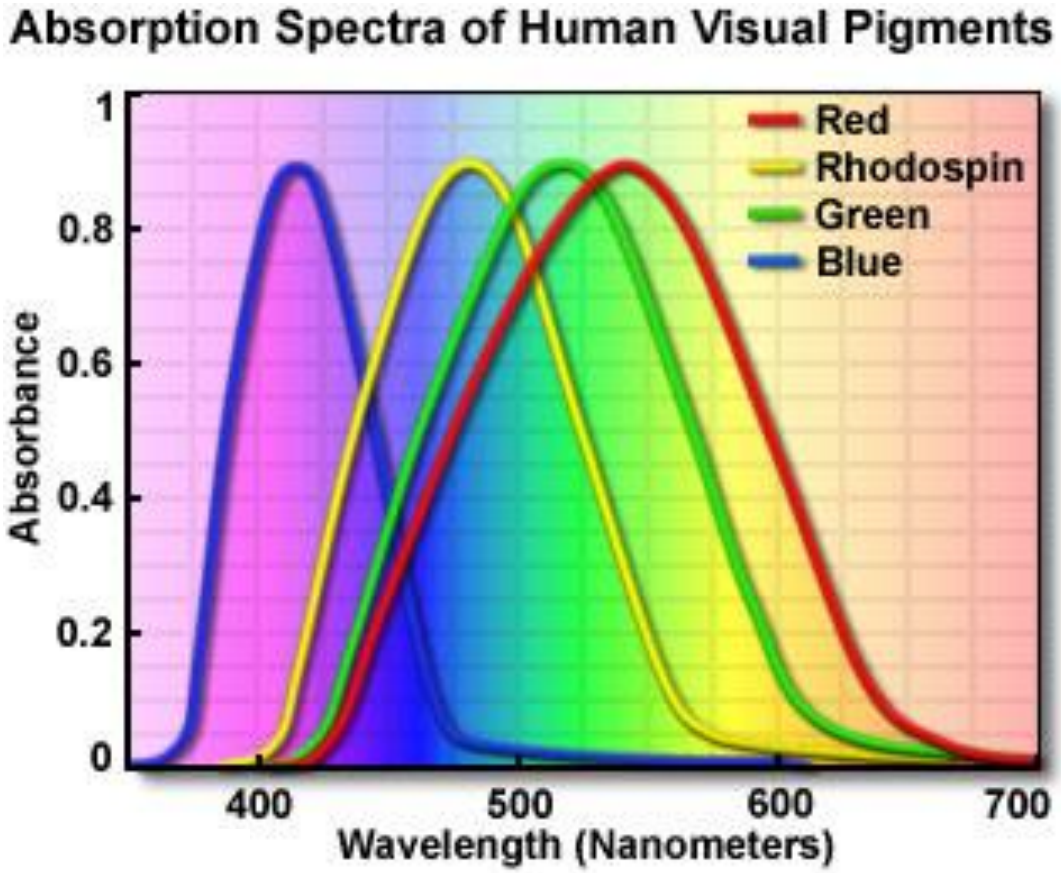
Human eye





Most perfect sensor for light detection up to now

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Microscopy meets Biology

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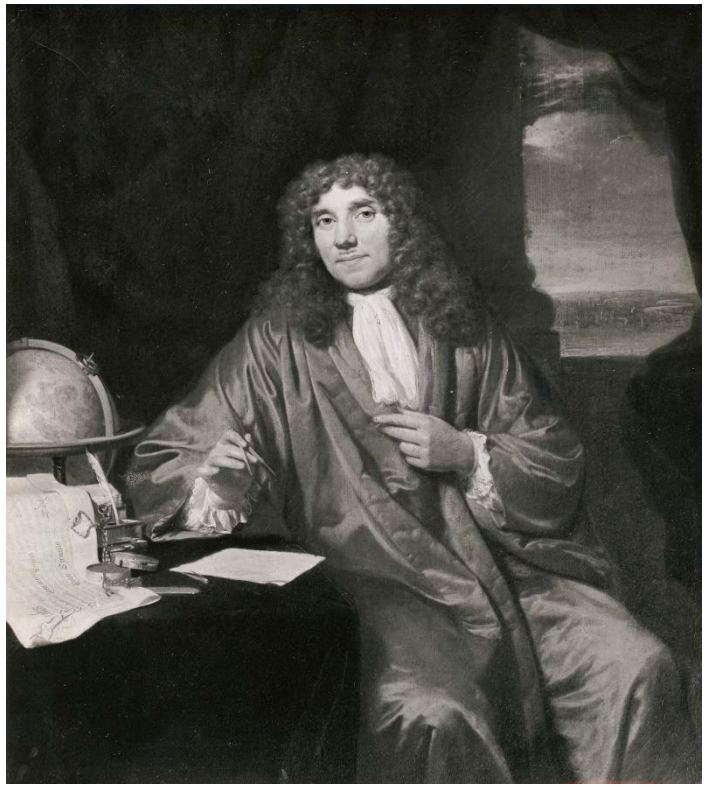
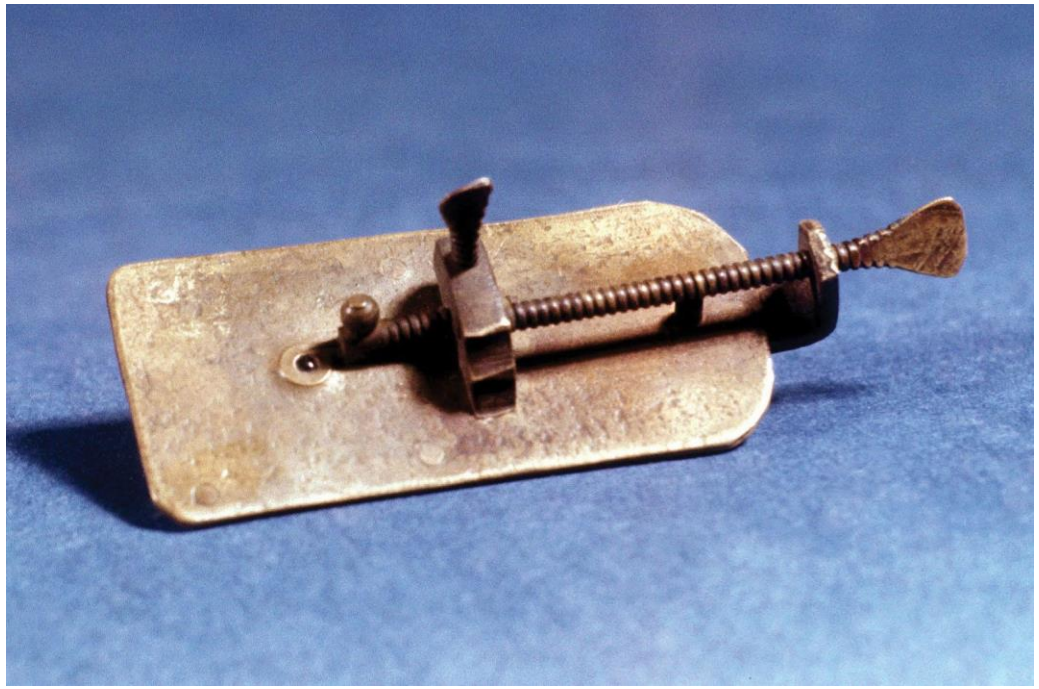
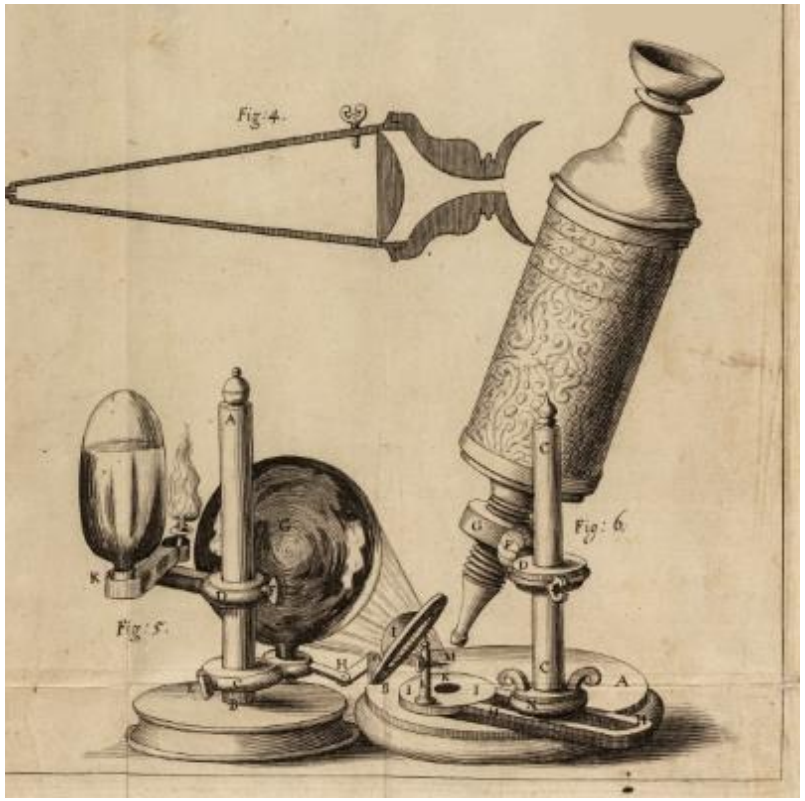


Antonie van Leeuwenhoek
October 24th, 1632 - August 26th, 1723

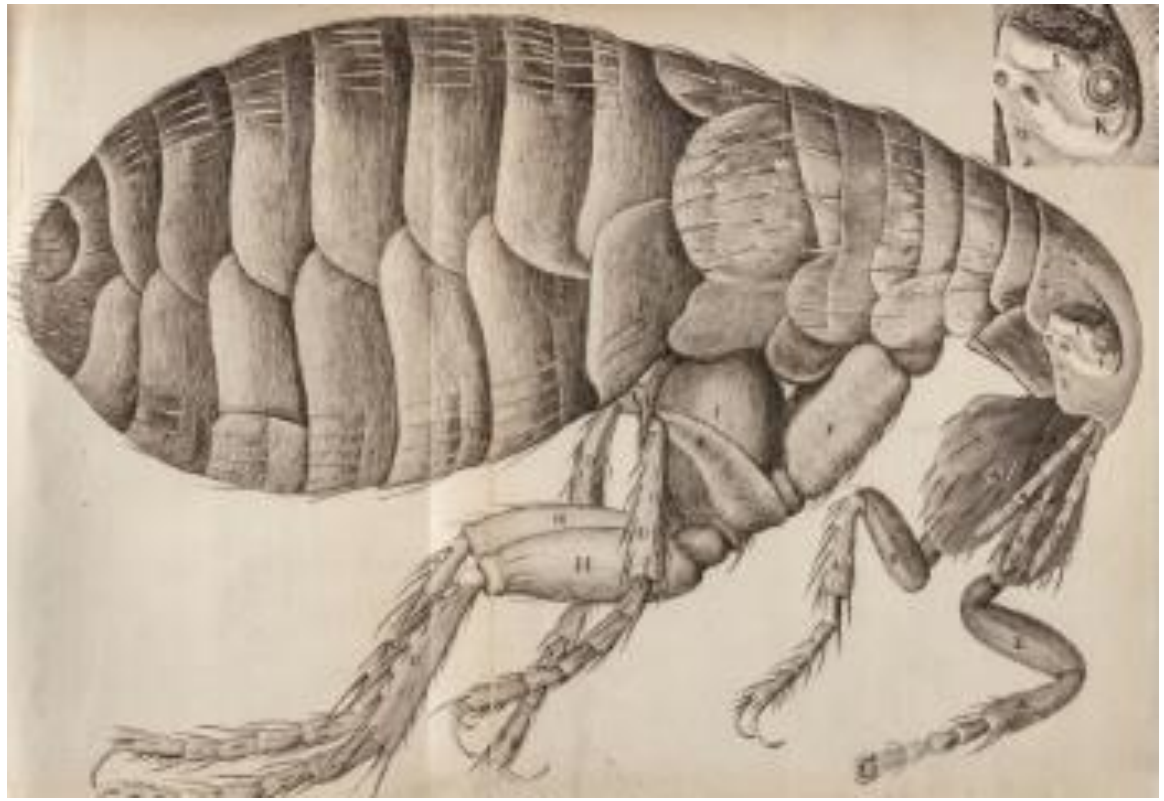


Philosophical Transactions 1683.
The first representation of bacteria is to be found in a drawing by Leeuwenhoek.

Microscopy meets Biology

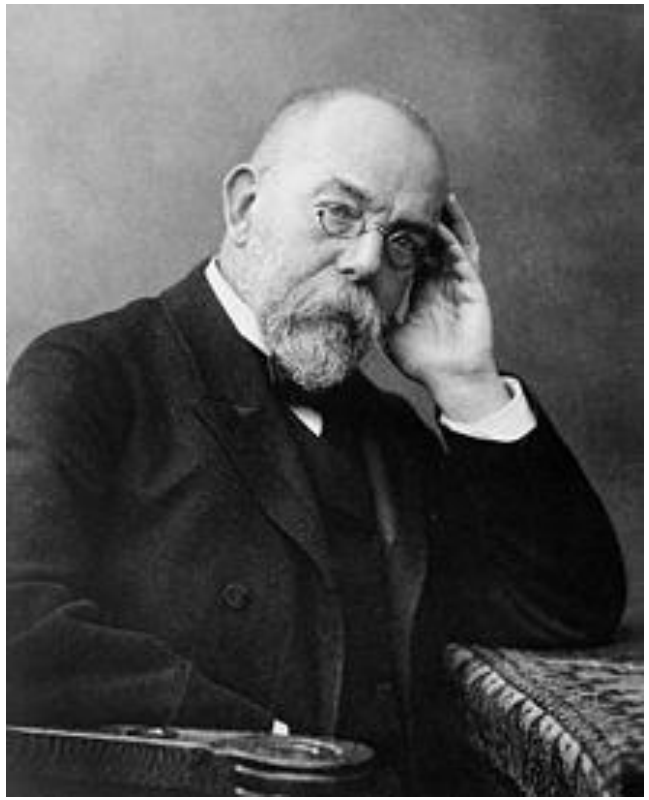


Robert. Hooke
July 18th, 1635 - March 3rd, 1703



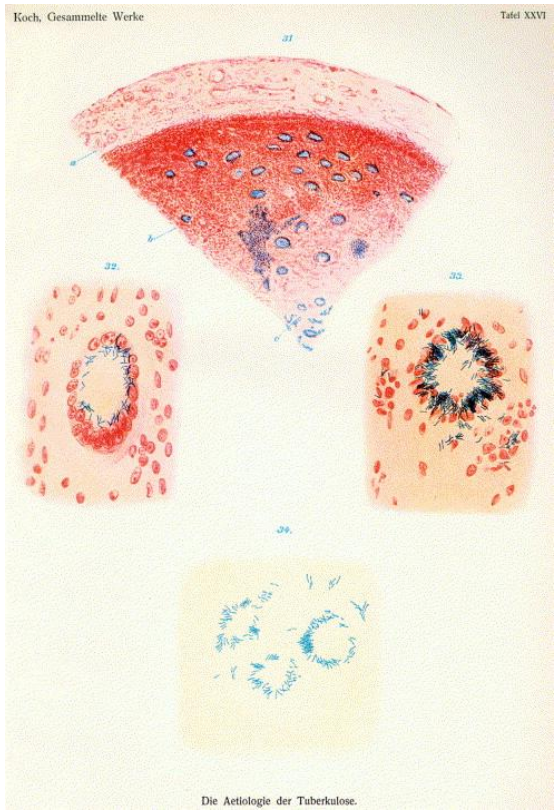
1665 Micrographia
https://royalsociety.org/blog/2020/07/micrographia-online/

Microscopy meets Biology



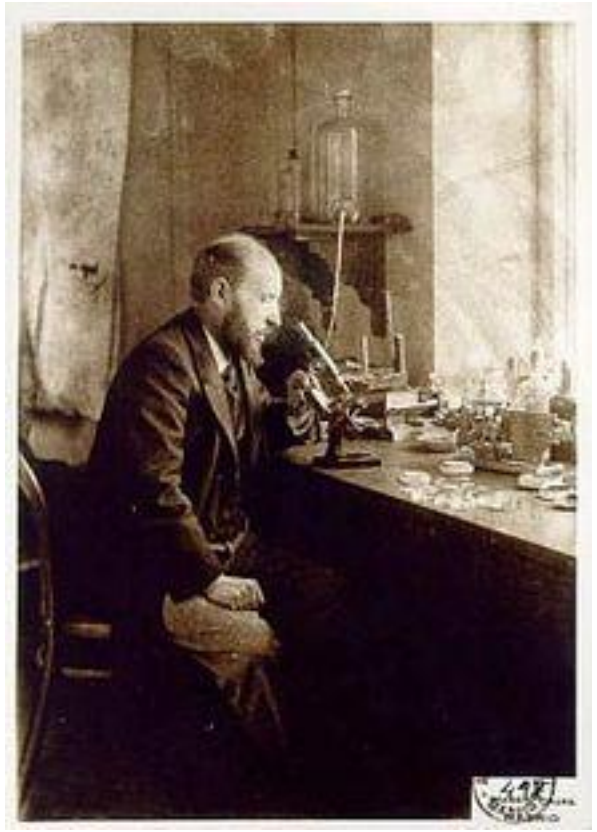
Heinrich Hermann Robert Koch
Dezember 11th 1843 - Mai 27th 1910
Nobel Prize in Physiology and Medicine 1905

Mikroskop
Carl Zeiss 1879

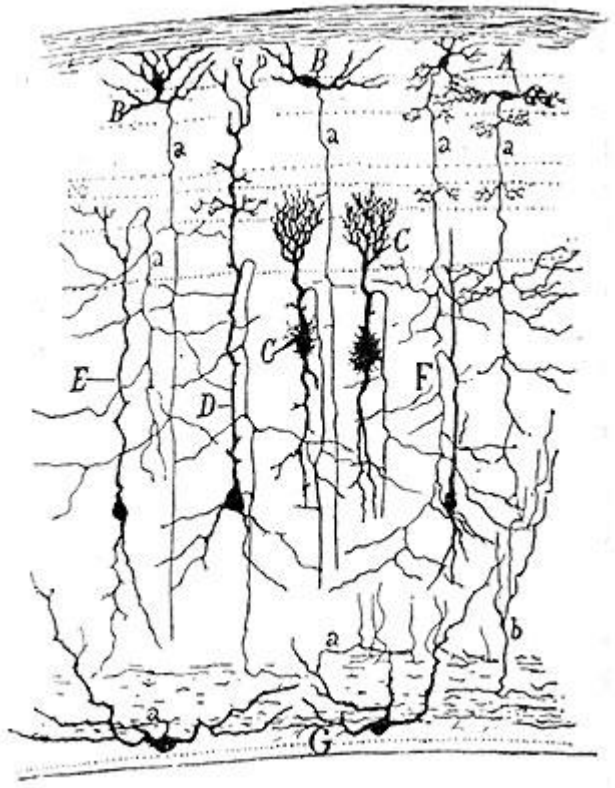


Koch's drawing of tuberculosis bacilli in 1882
(from Die Ätiologie der Tuberkulose)

Microscopy meets Biology



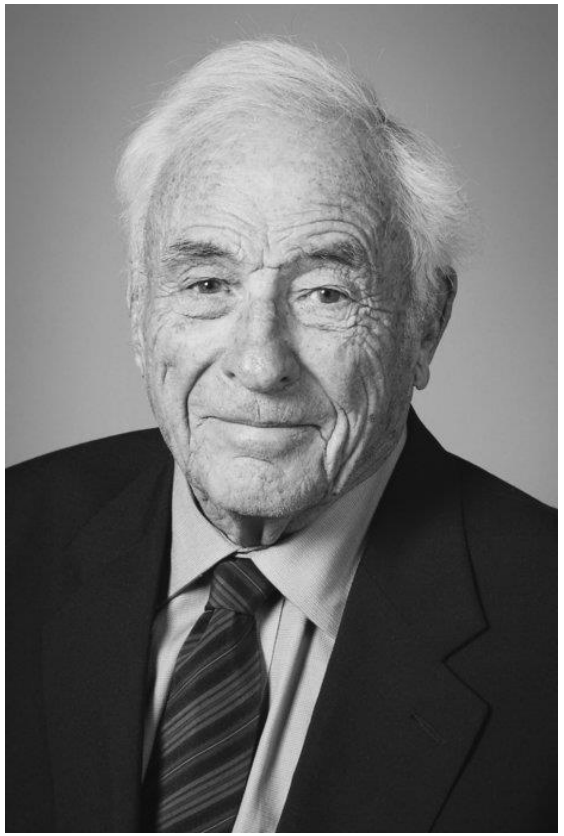
Santiago Ramón y Cajal
May, 1st 1852 –October 17th 1934
Nobel Prize in Physiology and Medicine 1906



Drawing of a section through the optic tectum of a sparrow, from "Structure of the nervous centres of the birds", by Santiago Ramon y Cajal, 1905.

Microscopy meets Electrical Engineering

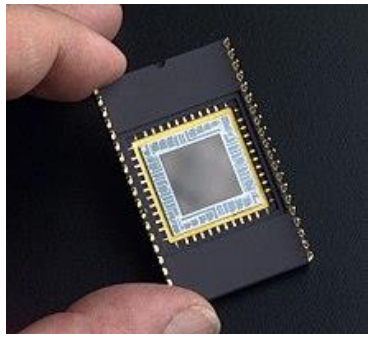
Nobel Price in Physics 2009



Willard S. Boyle
August 19th 1924 –May 7th 2011

George E. Smith
May 10th 1930 -

Charge-coupled device (CCD)



The basis for the CCD is the metal–oxide–semiconductor (MOS) structure, with MOS capacitors being the basic building blocks of a CCD, and a depleted MOS structure used as the photodetector in early CCD devices.

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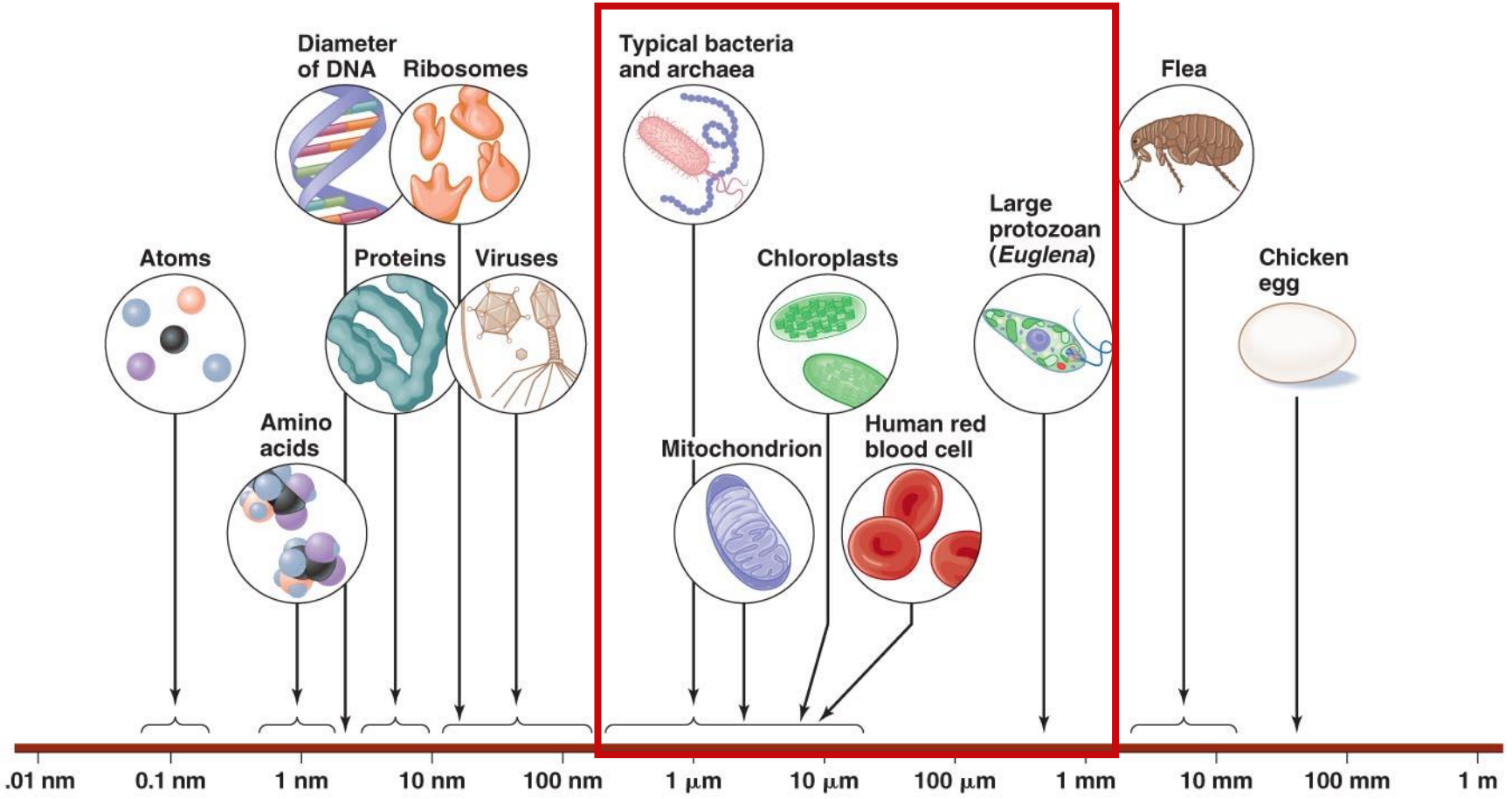
Light microscpy

- Advantages, Disadvantages

Light microscopy in Life Science



At a standard viewing distance (~ 250 mm), the smallest object we can see with the naked eye is ~80 μm.

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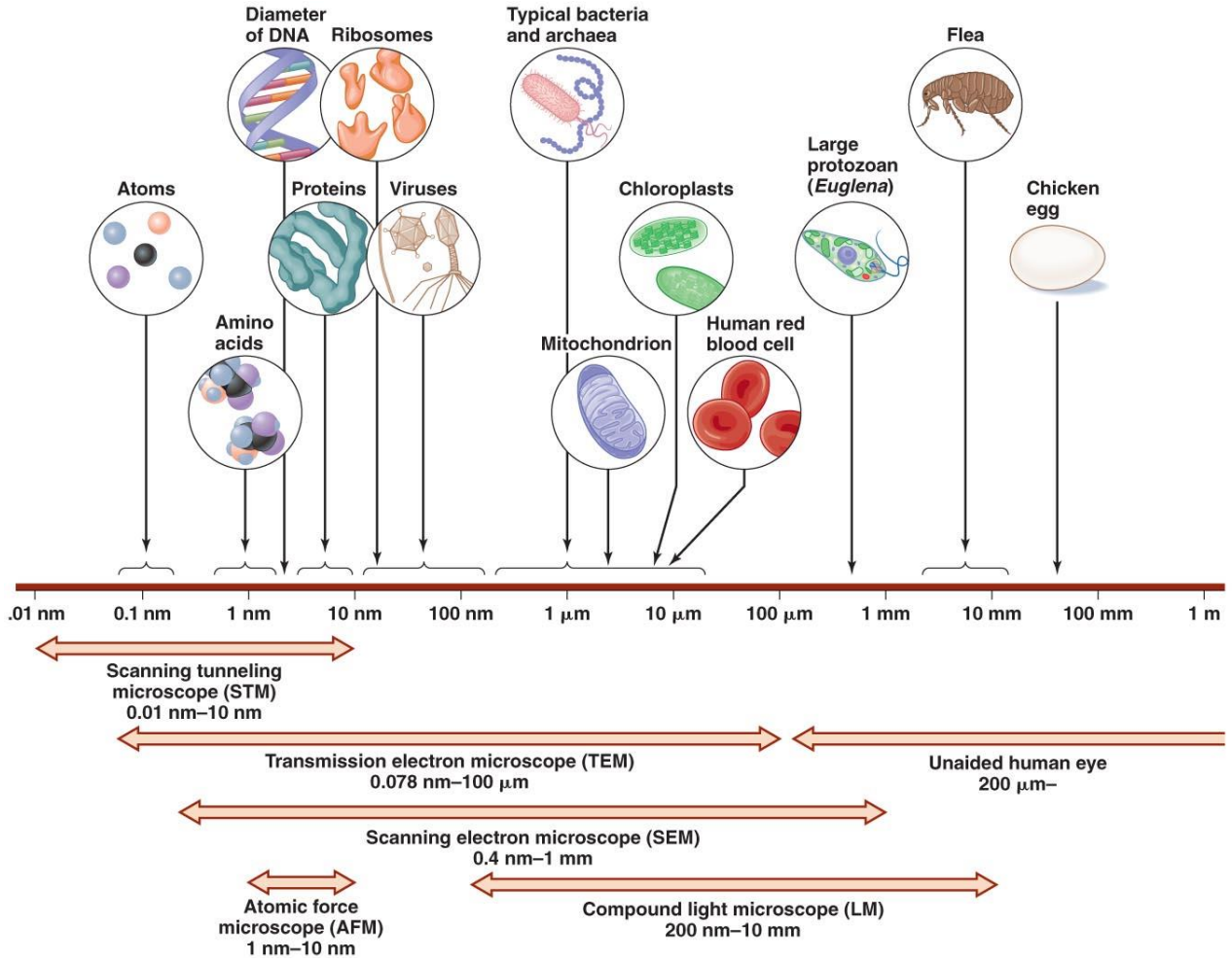
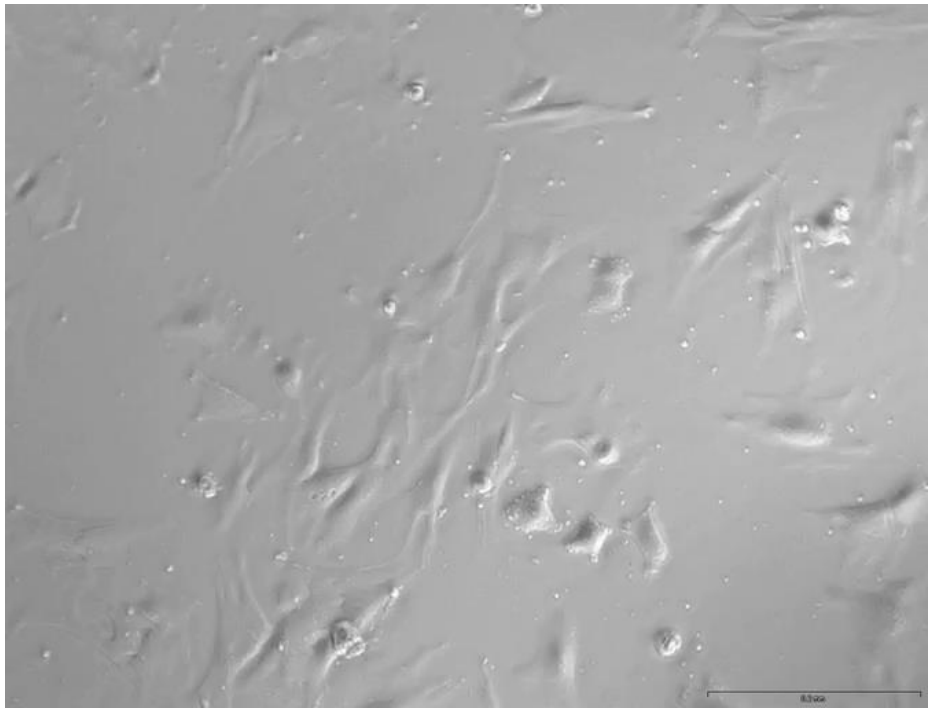
Resolution vs. dynamics





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Imaging

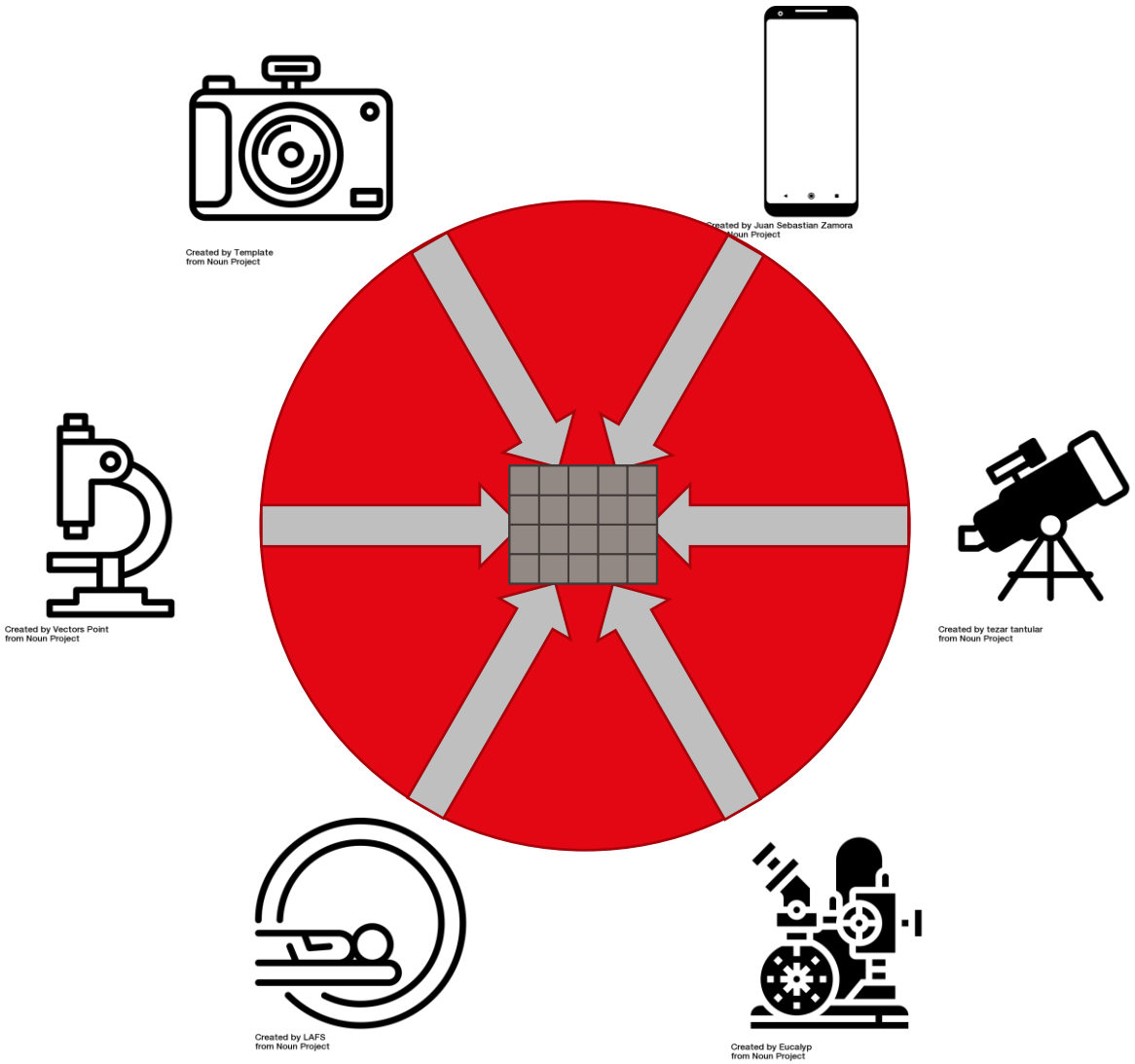
3D
continious space

Object

capture
device

2D
discrete space

Image



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Basics of lens-based imaging

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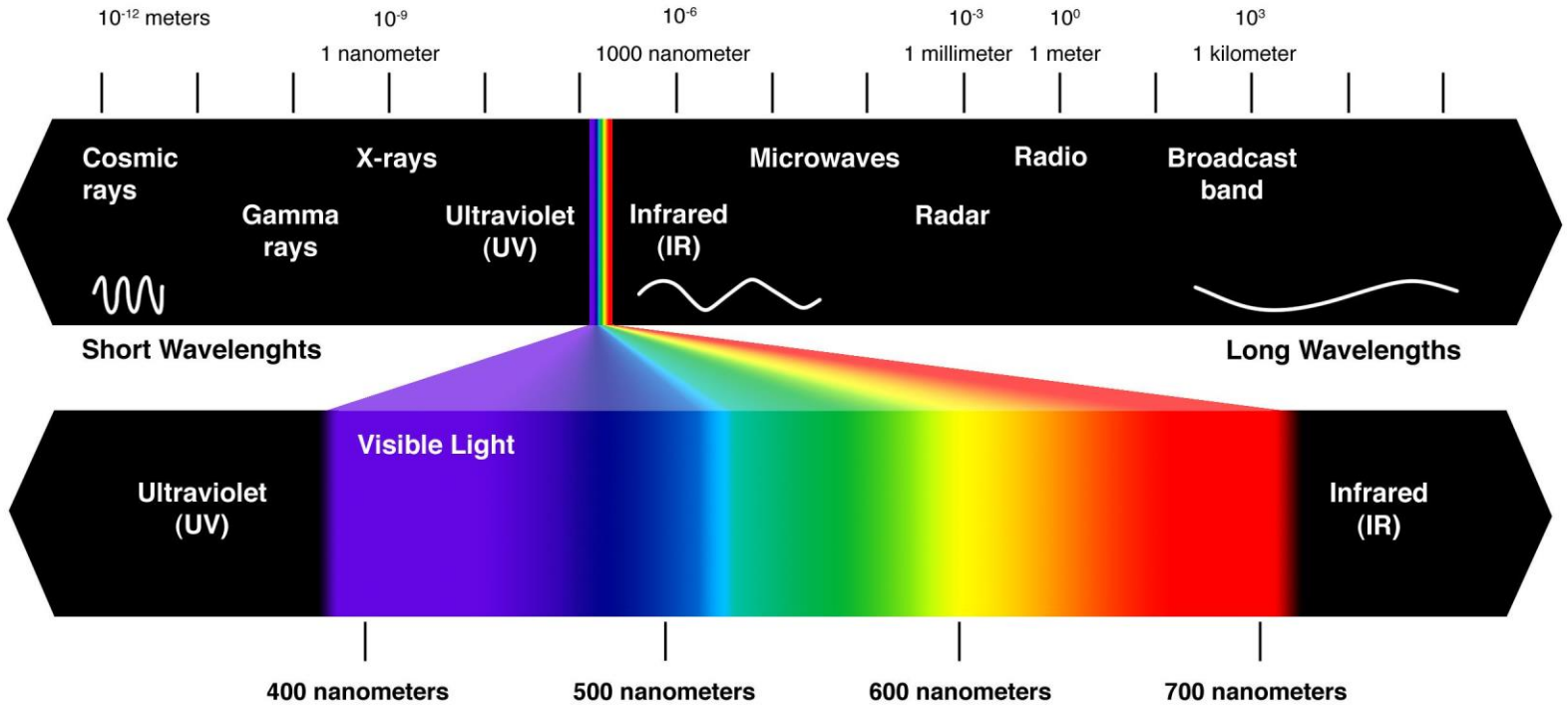
Electromagnetic spectrum



- λ for visible spectrum is 400 – 700 nm
- In order for ray optics to hold object size (e.g. lenses) should be >> λ

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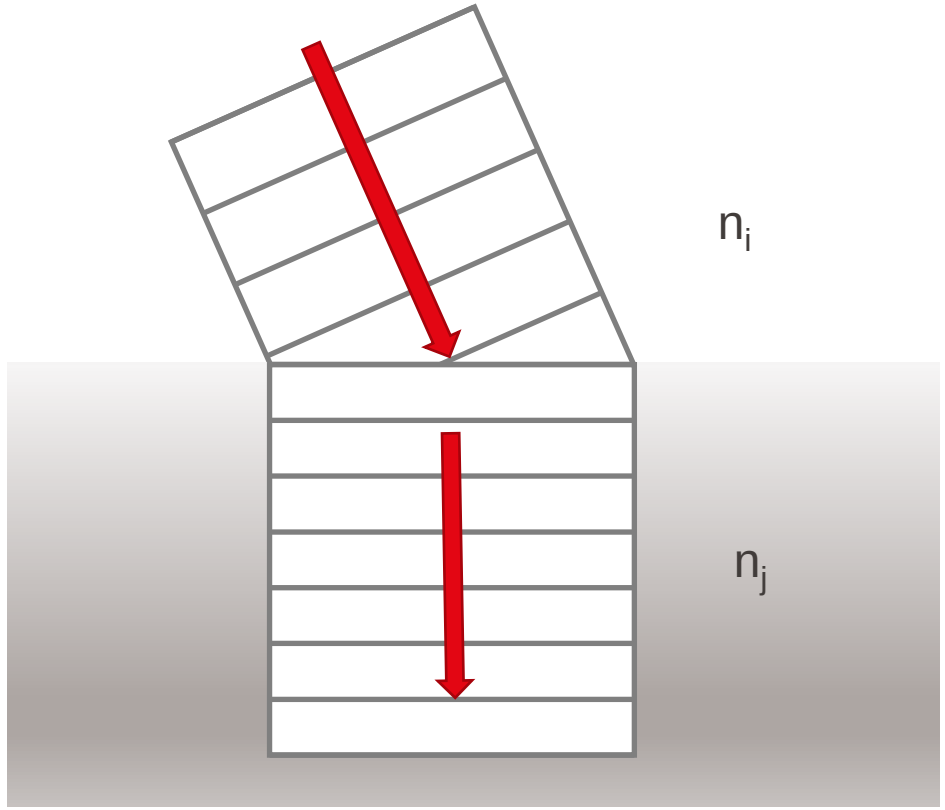
Refractive index *n*

Medium	Refractive index n	Velocity in medium / km*s^{-1}
Air	1.0003	299203
Water	1.33	225032
Glycerol	1.47	203600
Immersion Oil	1.518	197162
Glass	1.56-1.46	191854-204995

$$n_{medium} = \frac{velocity_{vacuum}}{velocity_{medium}} = \frac{2.992926 \cdot 10^8 \ \frac{m}{s}}{velocity_{medium}}$$

Speed of light in a material is less than its speed in vacuum



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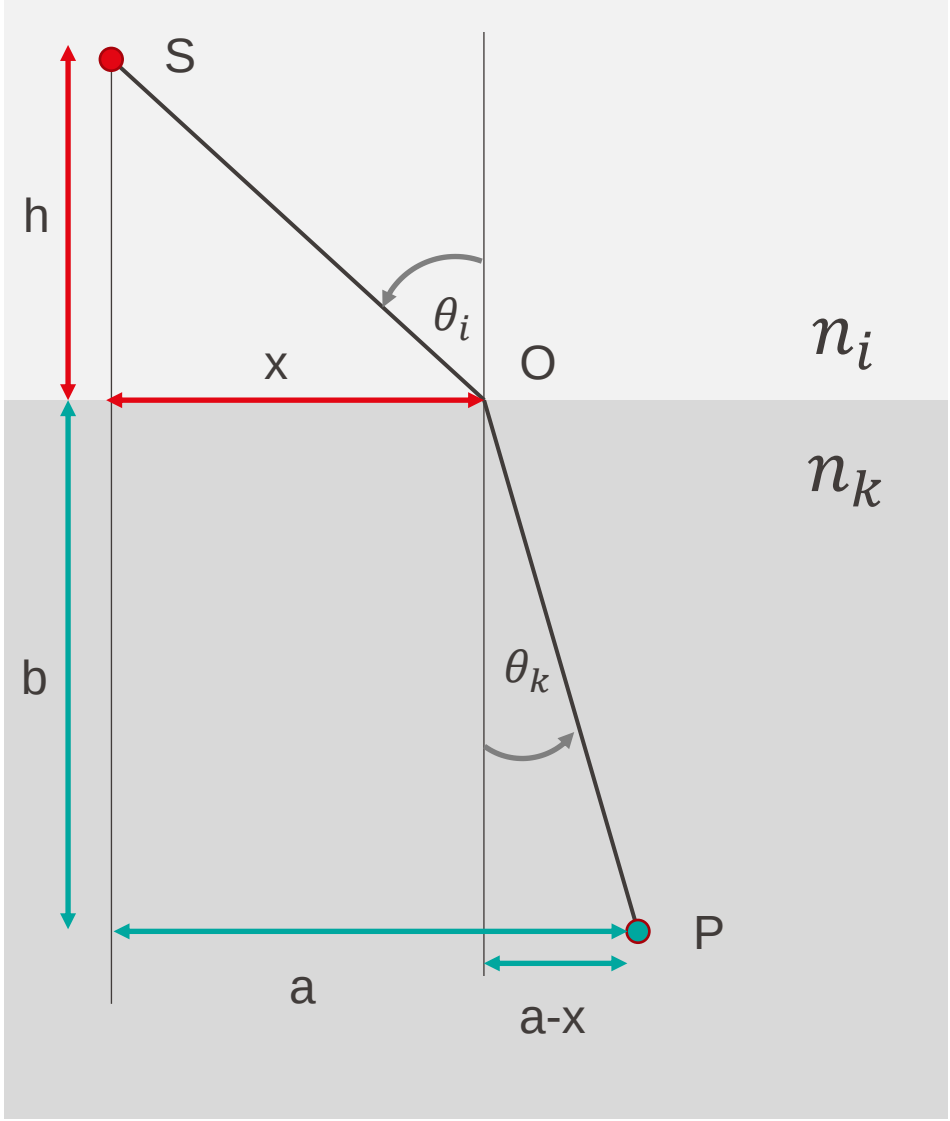
The Law of Refraction



modified from: E. Hecht, 7th edition, p. 225

$$t = \frac{\overline{SO}}{v_i} + \frac{\overline{OP}}{v_k}$$

$$t = \frac{\sqrt{h^2 + x^2}}{v_i} + \frac{\sqrt{b^2 + (a-x)^2}}{v_k}$$

$$\frac{dt}{dx} = \frac{x}{v_i\sqrt{h^2 + x^2}} + \frac{-(a-x)}{v_k\sqrt{b^2 + (a-x)^2}} = 0$$

$$\frac{\sin\theta_i}{v_i} = \frac{\sin\theta_k}{v_k}$$

Snell's Law

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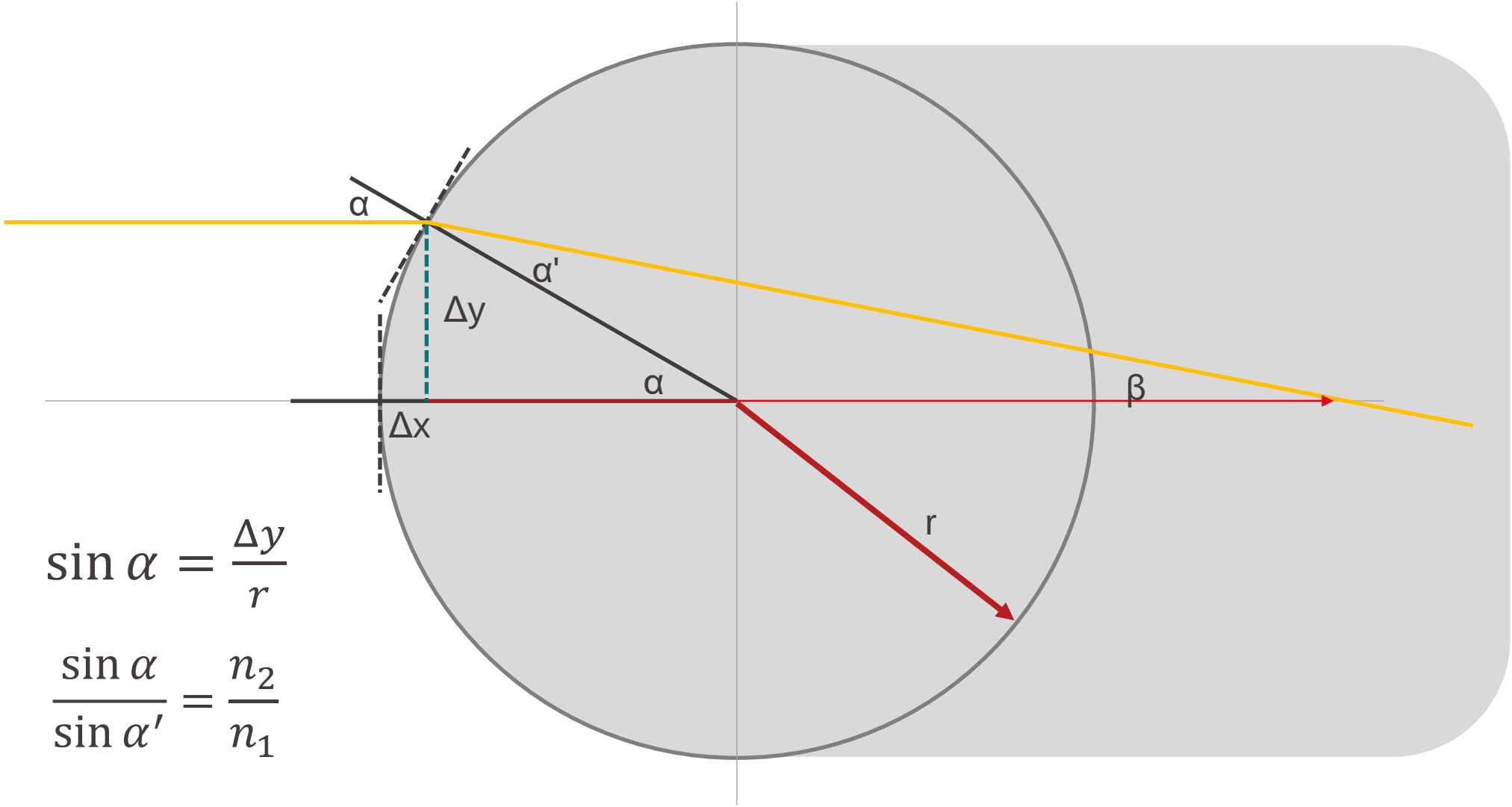
Refraction on curved surfaces



$$\sin\alpha = \frac{\Delta y}{r}$$

$$\frac{\sin\alpha}{\sin\alpha'} = \frac{n_2}{n_1}$$

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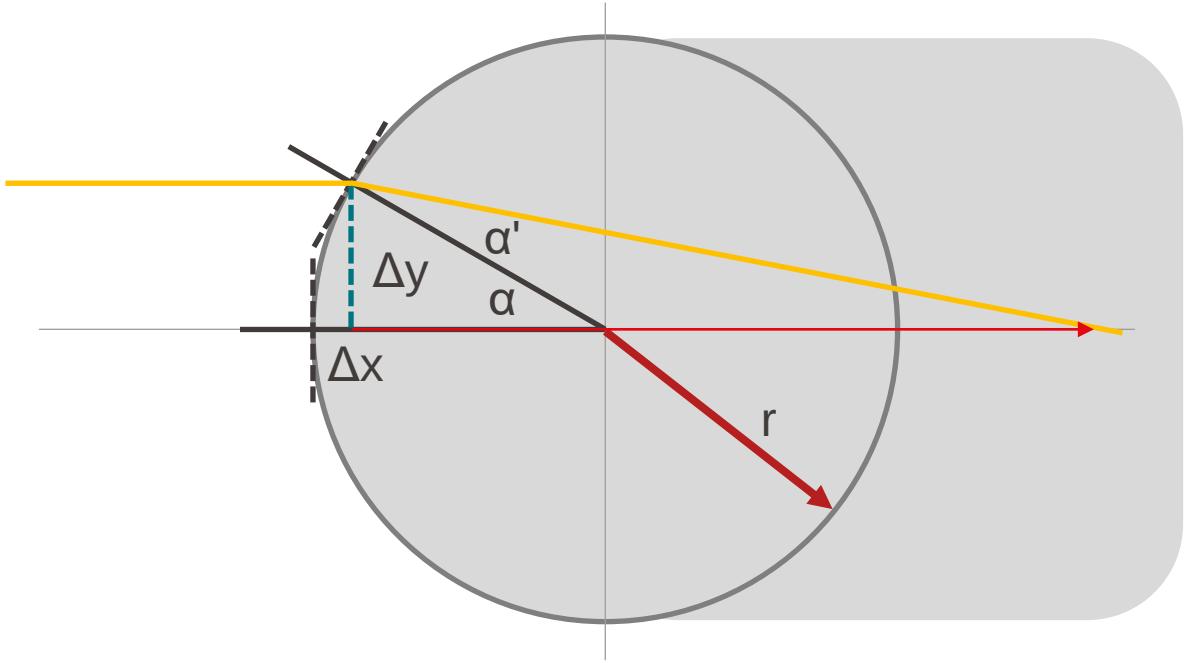
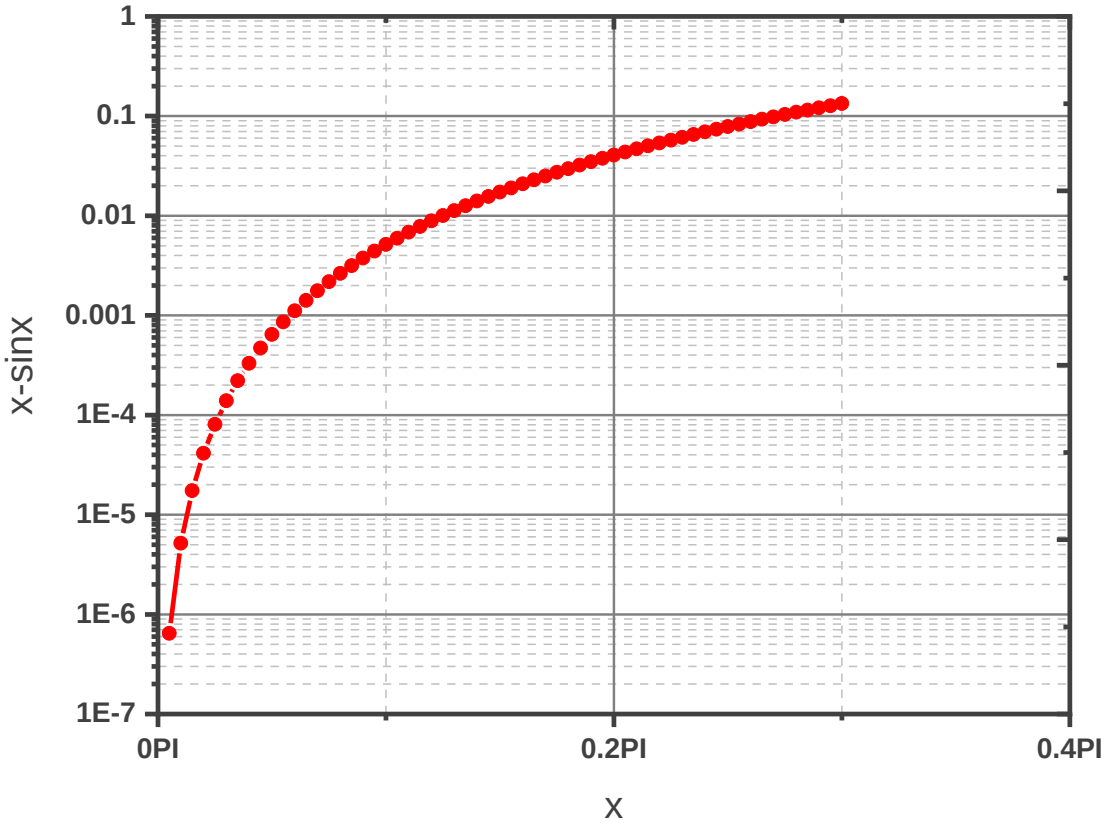
Refraction on curved surfaces





$$\sin\alpha \approx \alpha$$

$$\sin\alpha = \frac{\Delta y}{r} \qquad \frac{\sin\alpha}{\sin\alpha'} = \frac{n_2}{n_1} \qquad \alpha' = \frac{n_1}{n_2} \cdot \frac{\Delta y}{r}$$

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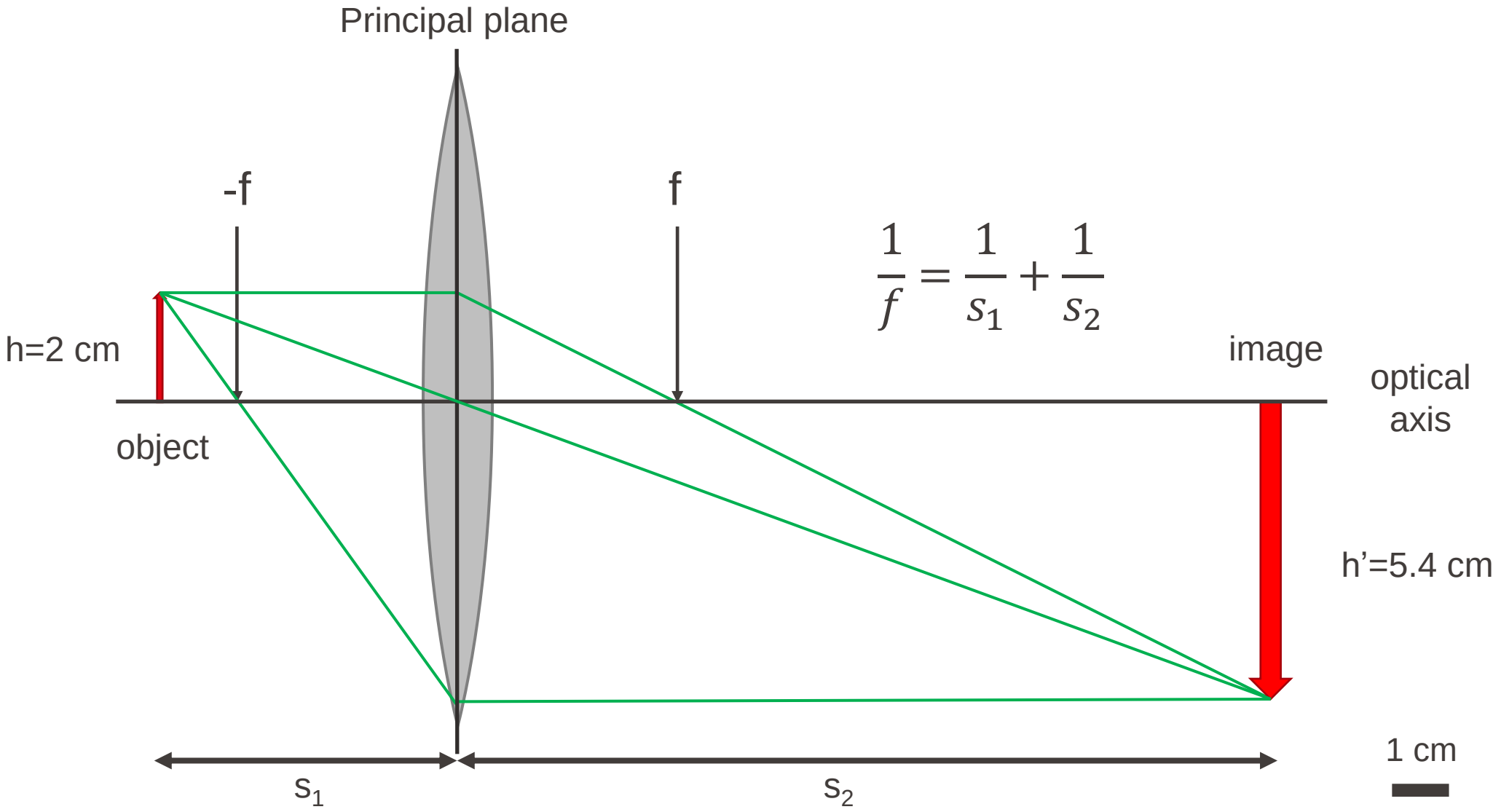
Applications of lenses

Image formation

Real Image Formation



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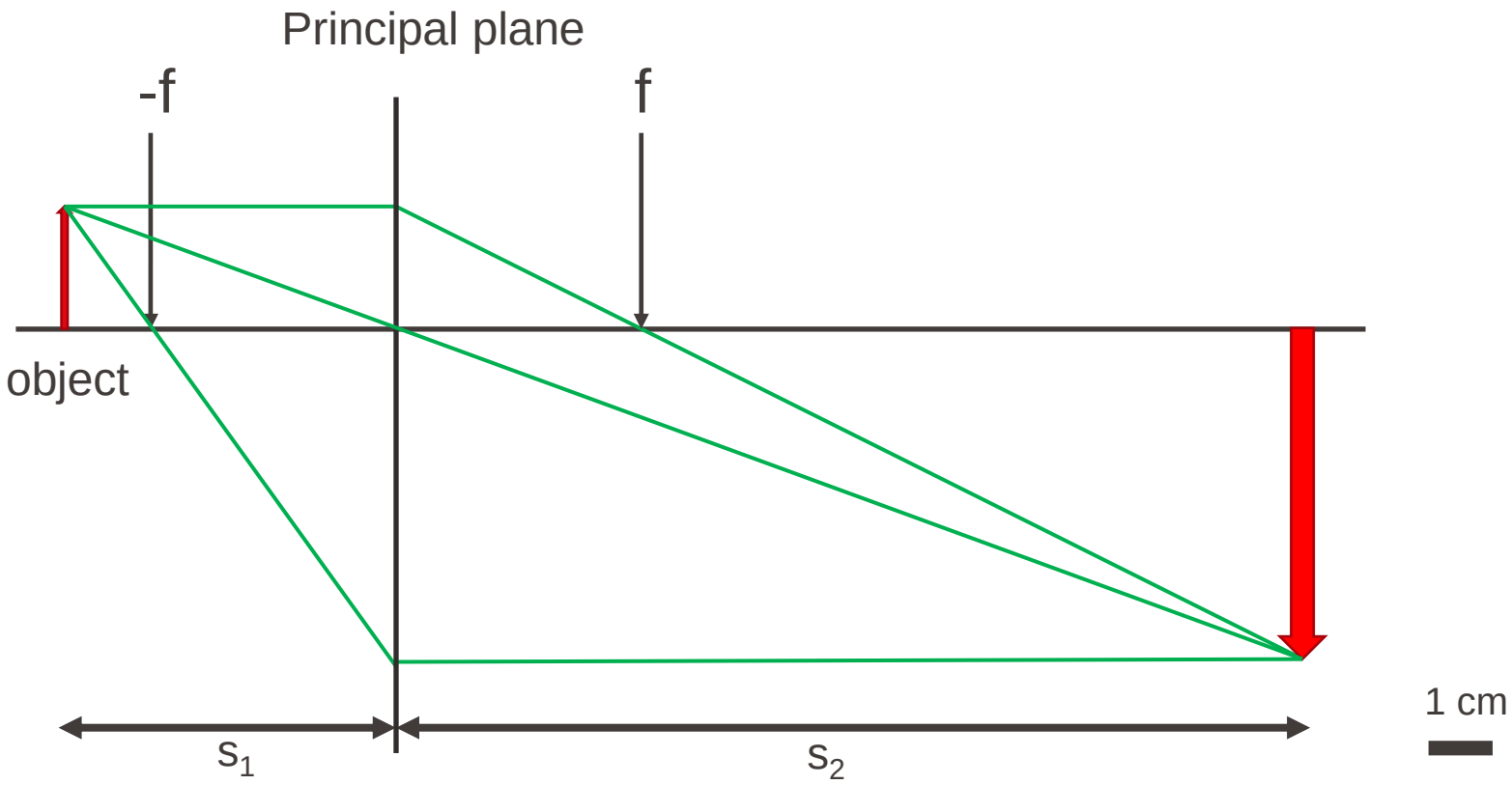
Real Image Formation



$$\frac{1}{f} = \frac{1}{s_1} + \frac{1}{s_2}$$

$$s_1 = 5.5\ cm$$
$$s_2 = 14.9\ cm$$
$$f = 4.0\ cm$$

$$1/s_1 = 0.182\ cm^{-1}$$
$$1/s_2 = 0.067\ cm^{-1}$$
$$1/f = 0.25\ cm^{-1}$$

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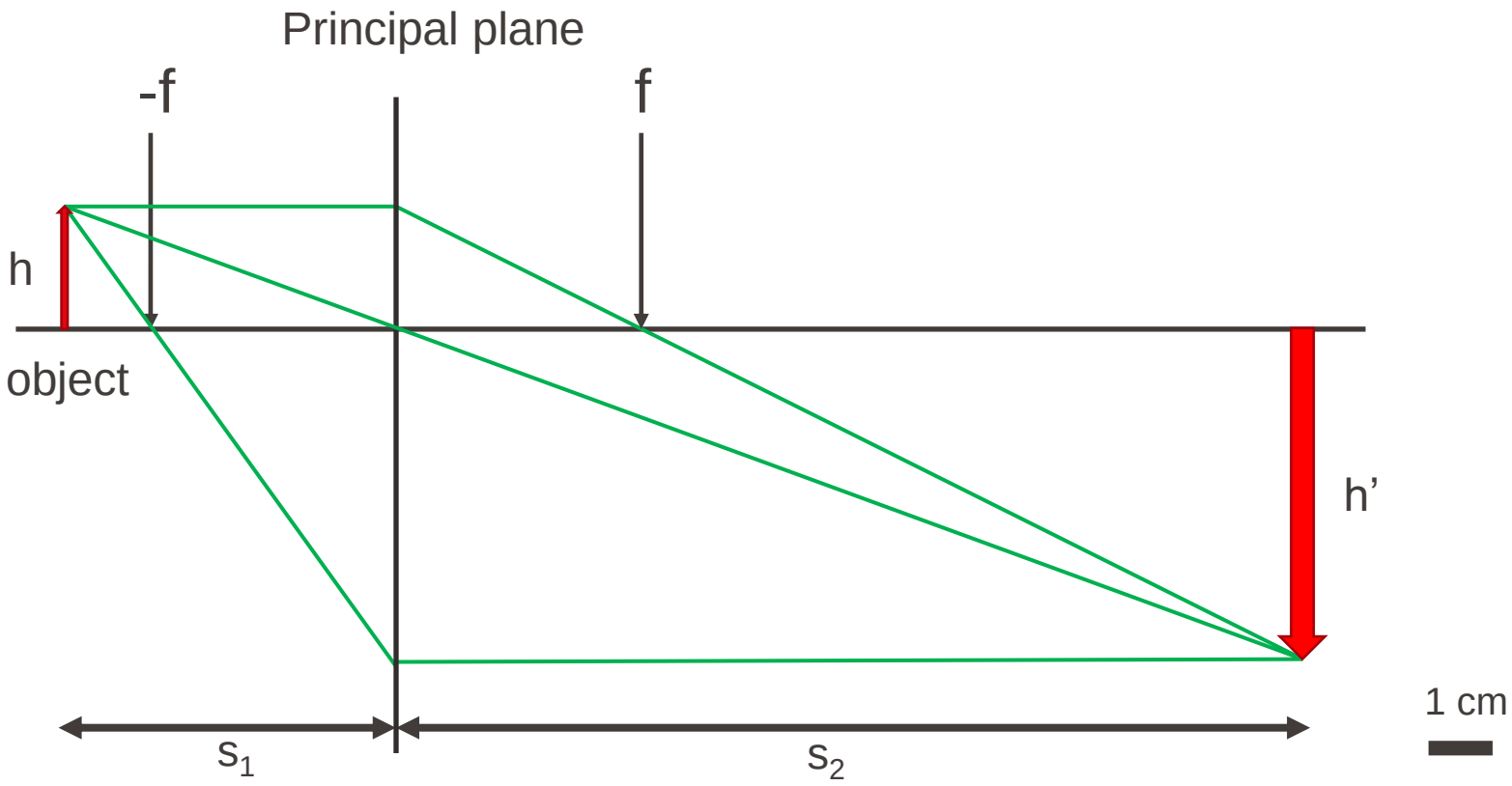
Real Image Formation



$s_1 = 5.5\ cm$
$s_2 = 14.9\ cm$
$f = \ \ 4.0\ cm$

$$M = \frac{s_2}{s_1} = 2.7$$

$$M = \frac{h'}{h} = 2.7$$

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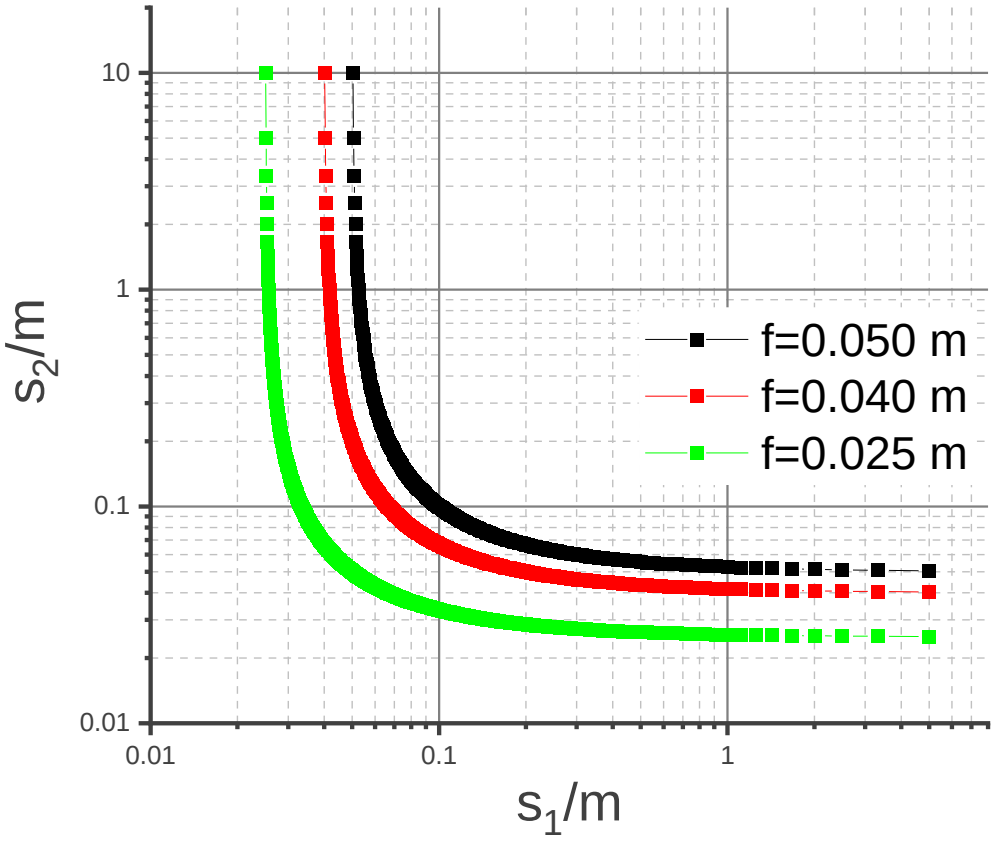
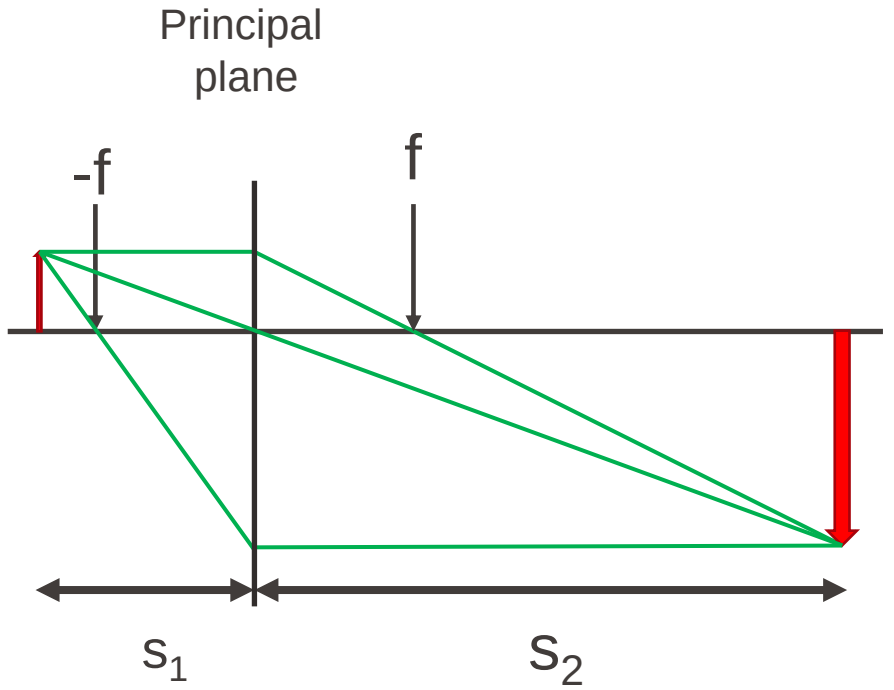
Image plane

$$\lim_{s_1 \to f} s_2 = \infty$$

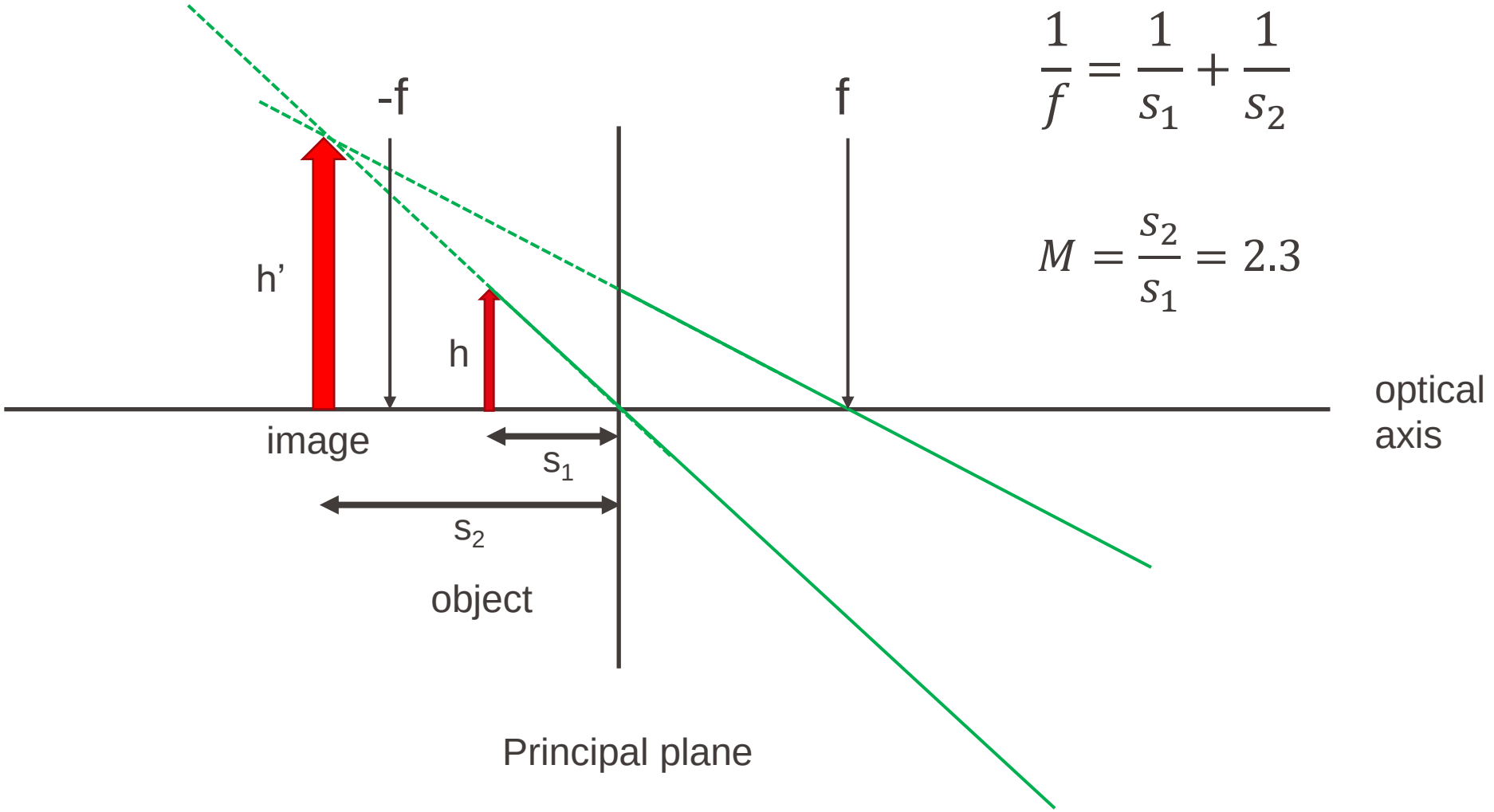




$$\lim_{s_1 \to \infty} s_2 = f$$

$$\frac{1}{f} = \frac{1}{s_1} + \frac{1}{s_2}$$

Virtual Image Formation



$$\frac{1}{f} = \frac{1}{s_1} + \frac{1}{s_2}$$

$$M = \frac{s_2}{s_1} = 2.3$$

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Limitations of light microcopy

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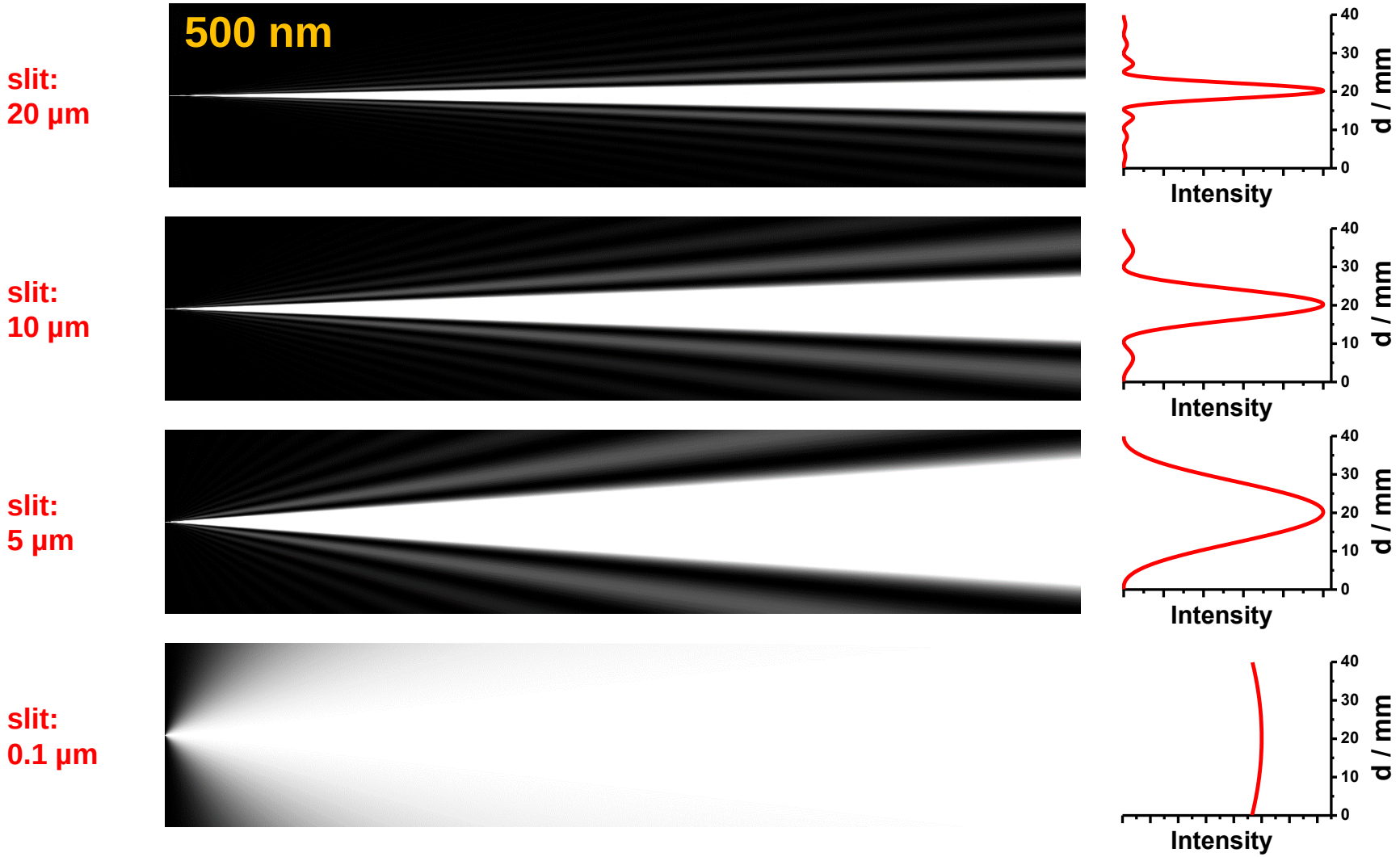
Diffraction Single Slit



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Diffraction Limited Resolution

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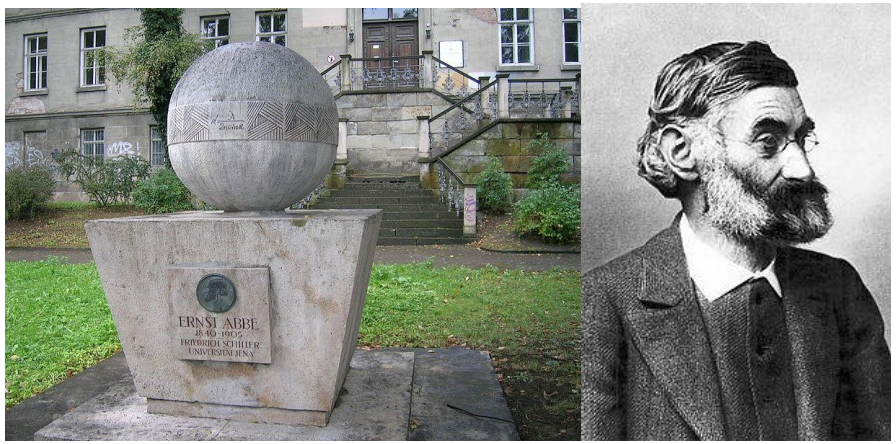

$$d = \frac{\lambda}{2 \cdot (n \cdot \sin\theta)}$$

$$NA = (n \cdot \sin\theta)$$

Ernst Abbe
1840-1905

"According to Abbe, a detail with a particular spacing in the specimen is resolved when the numerical aperture (**NA**) of the objective lens is large enough to capture the first-order diffraction pattern produced by the detail at the wavelength employed. In order to fulfill Abbe's requirements, the angular aperture of the objective must be large enough to admit both the zeroth and first order light waves."

http://micro.magnet.fsu.edu/optics/timeline/people/abbe.html

Resolution

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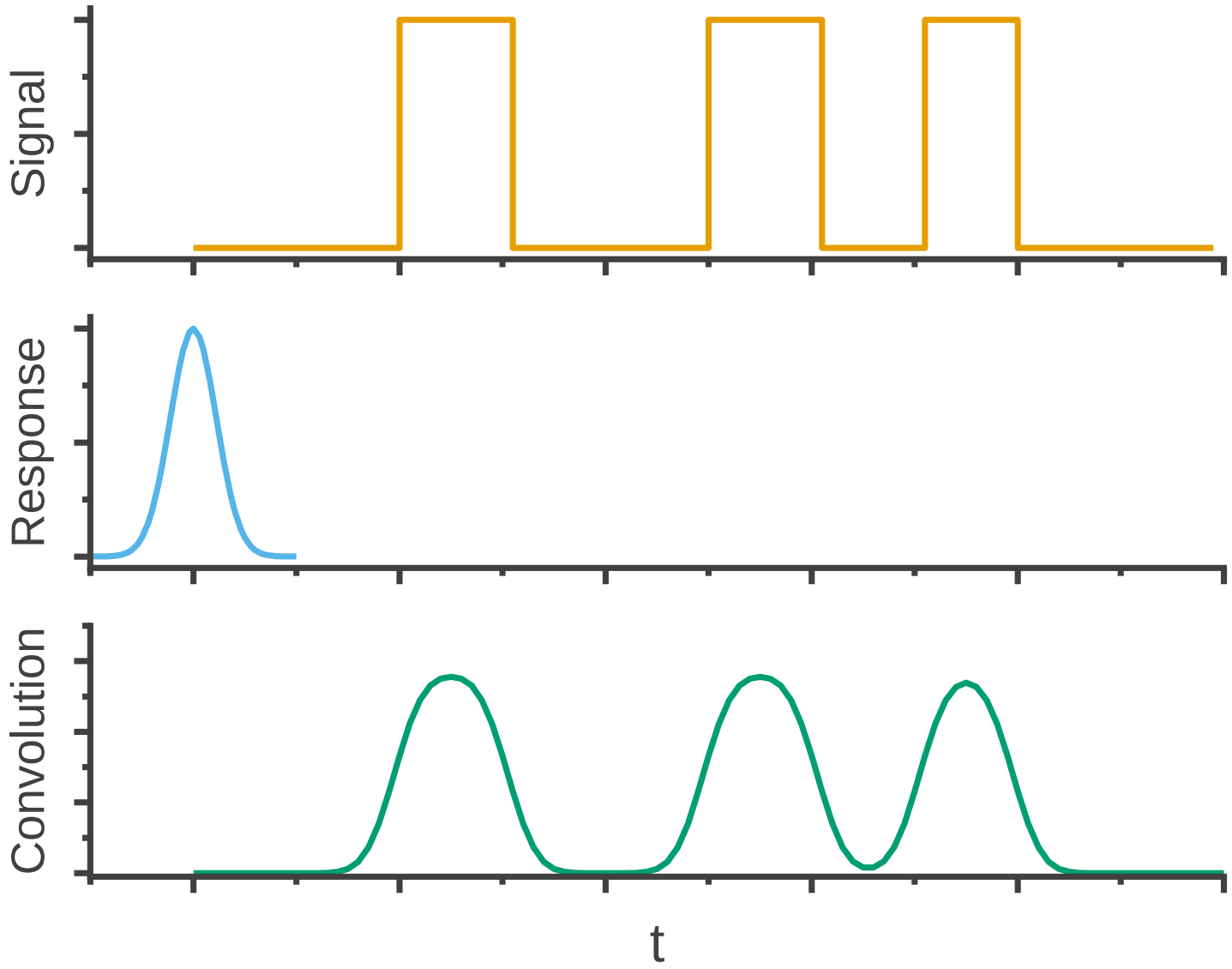
Convolution



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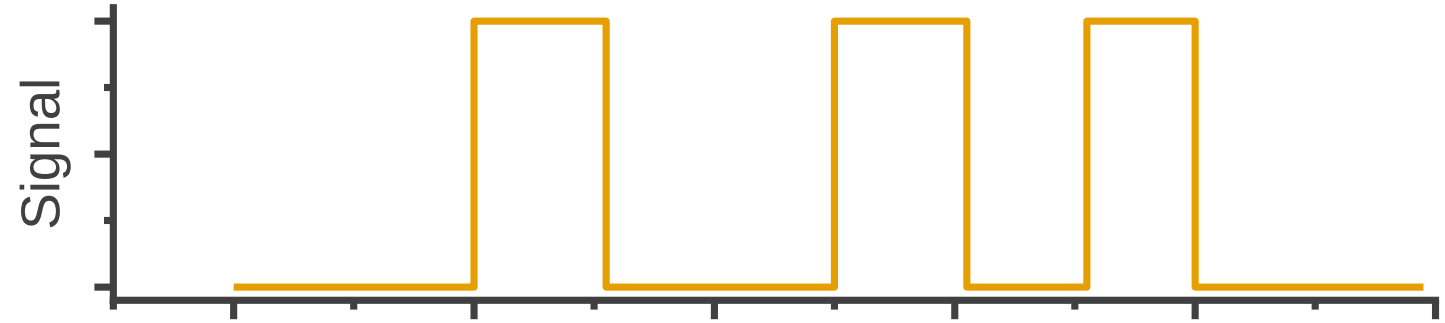
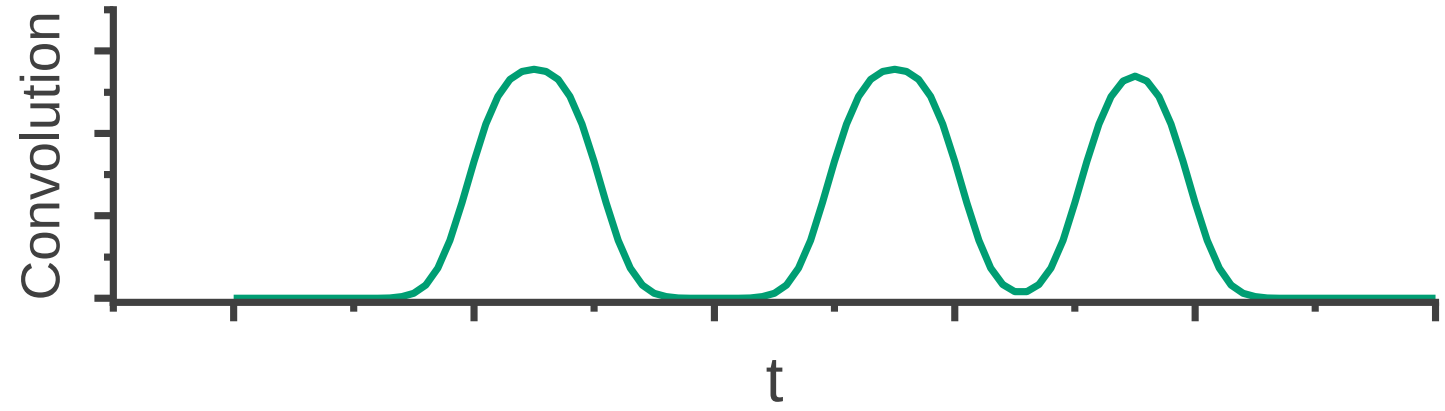
Convolution



$$f * g = \int_{-\infty}^{\infty} f(\tau)g(t-\tau)d\tau$$



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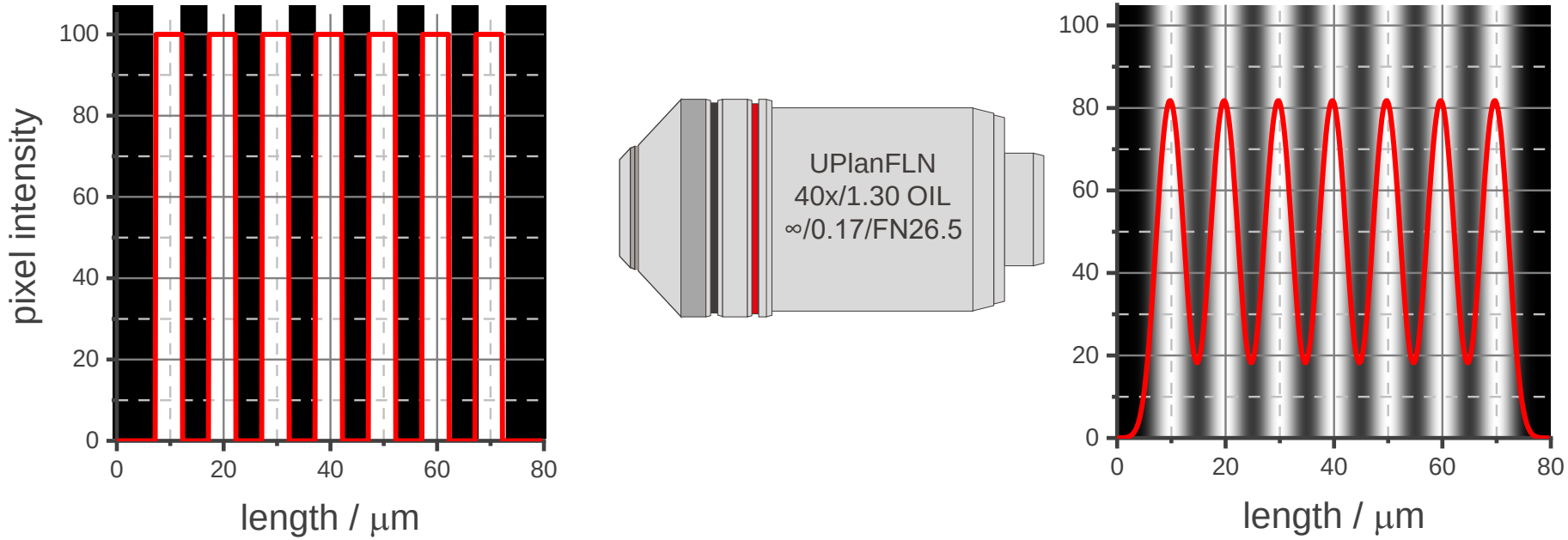
Low pass filtering



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Contrast

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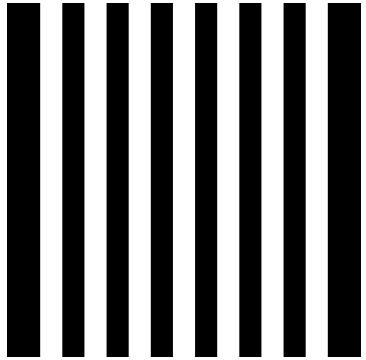
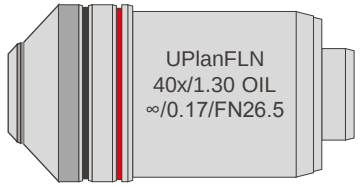
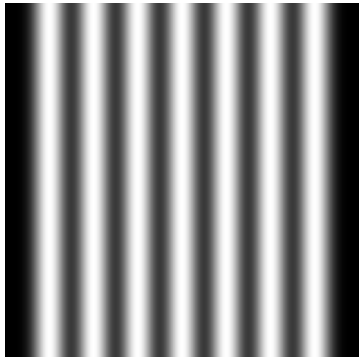
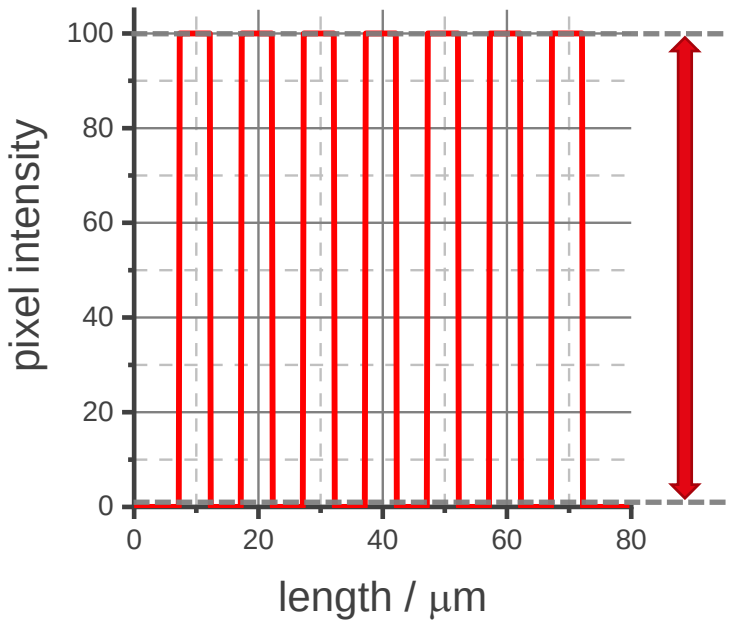






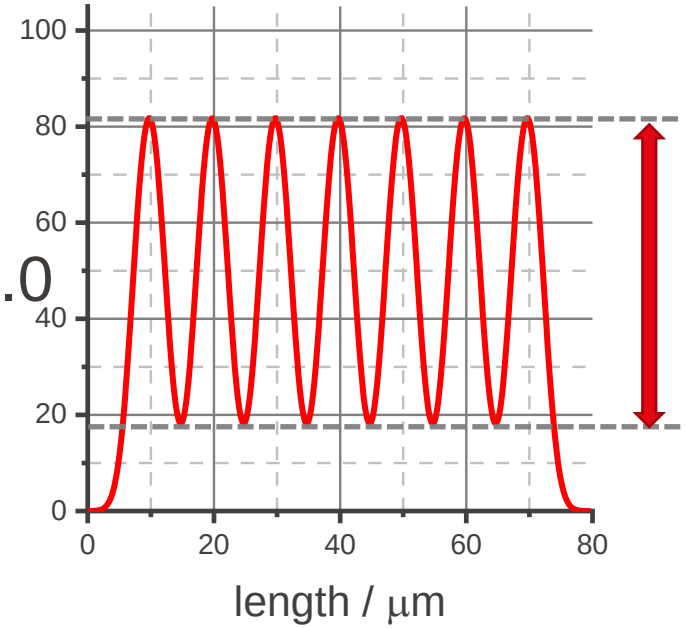
$$modulation = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$



I_{max}

m = 1.0

I_{min}



I_{max}

m = 0.62

I_{min}

Contrast

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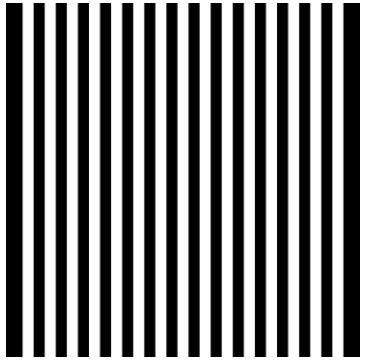
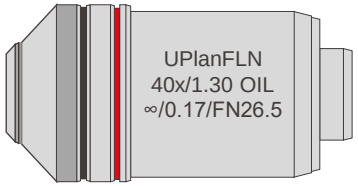
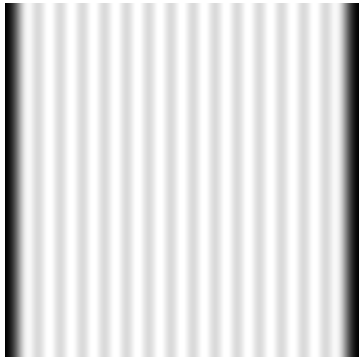
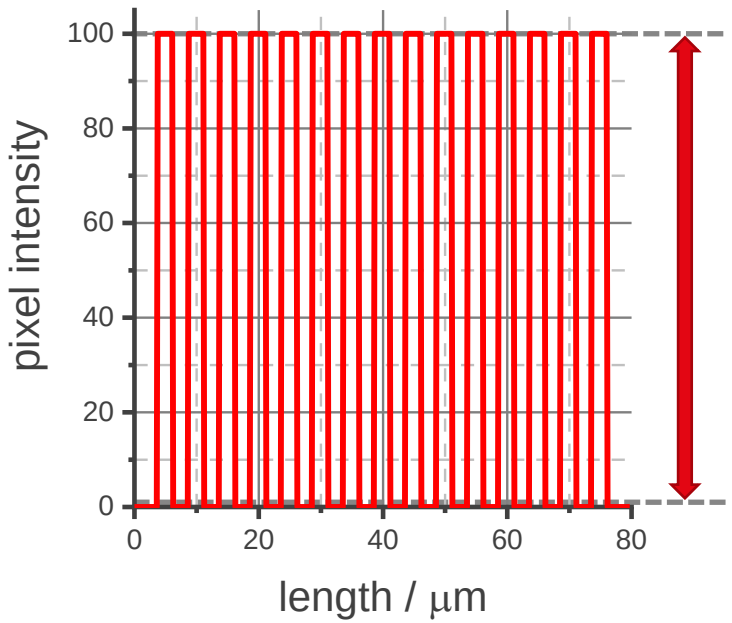






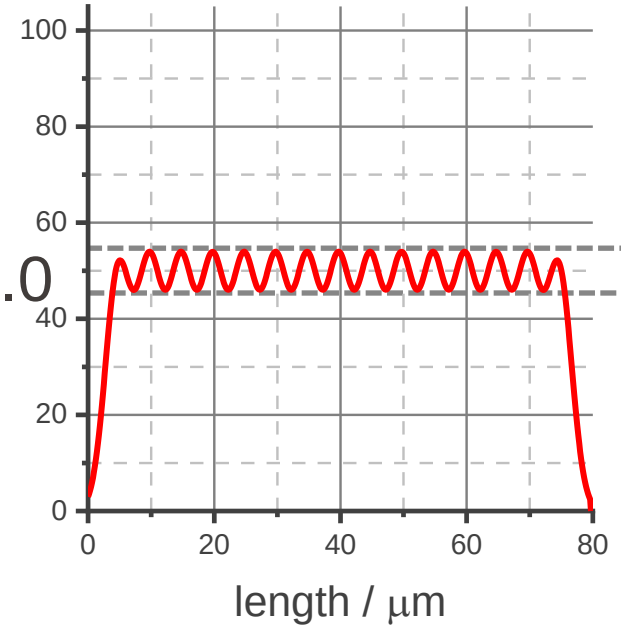
$$modulation = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$



I_{max}

m = 1.0

I_{min}



I_{max}

m = 0.08

I_{min}

Modulation Transfer Function

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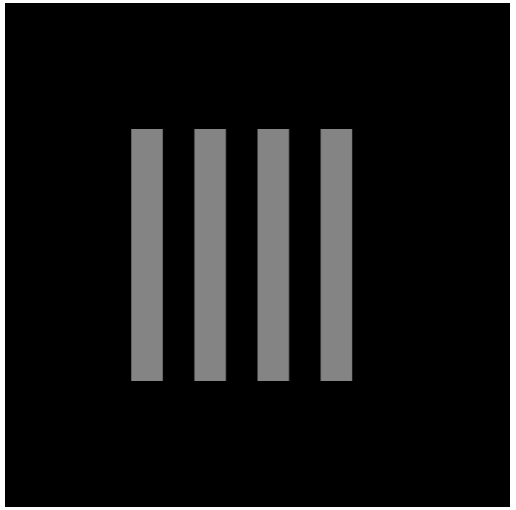
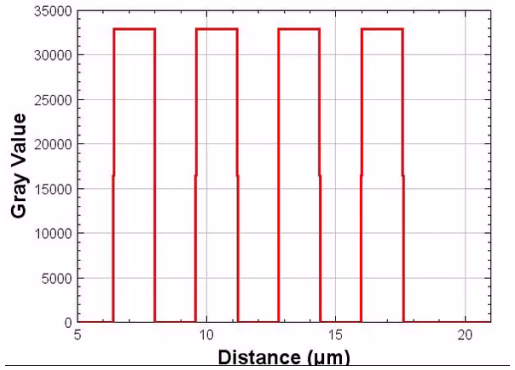
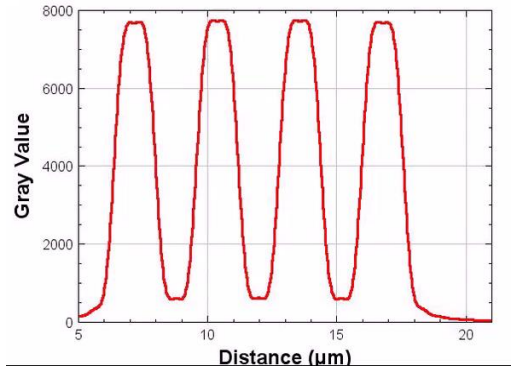
Object

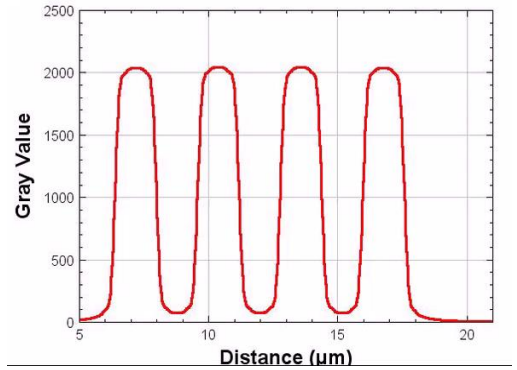




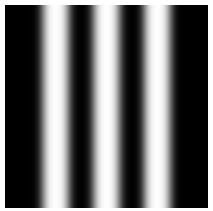
NA 0.4



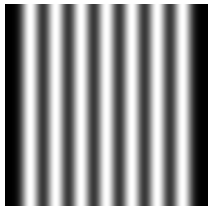
NA 0.8



Modulation Transfer Function

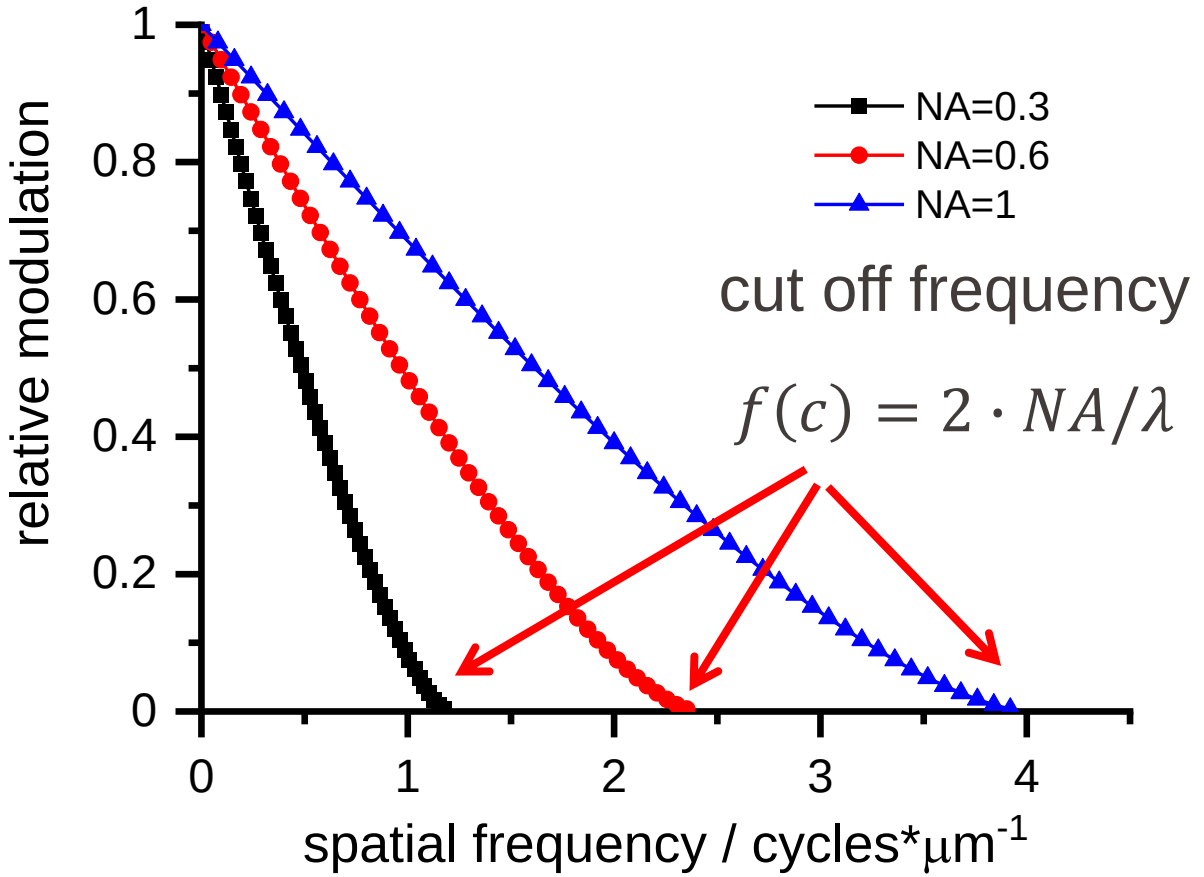


low spatial frequencies
high contrast



medium spatial frequencies
medium contrast

high spatial frequencies
low contrast



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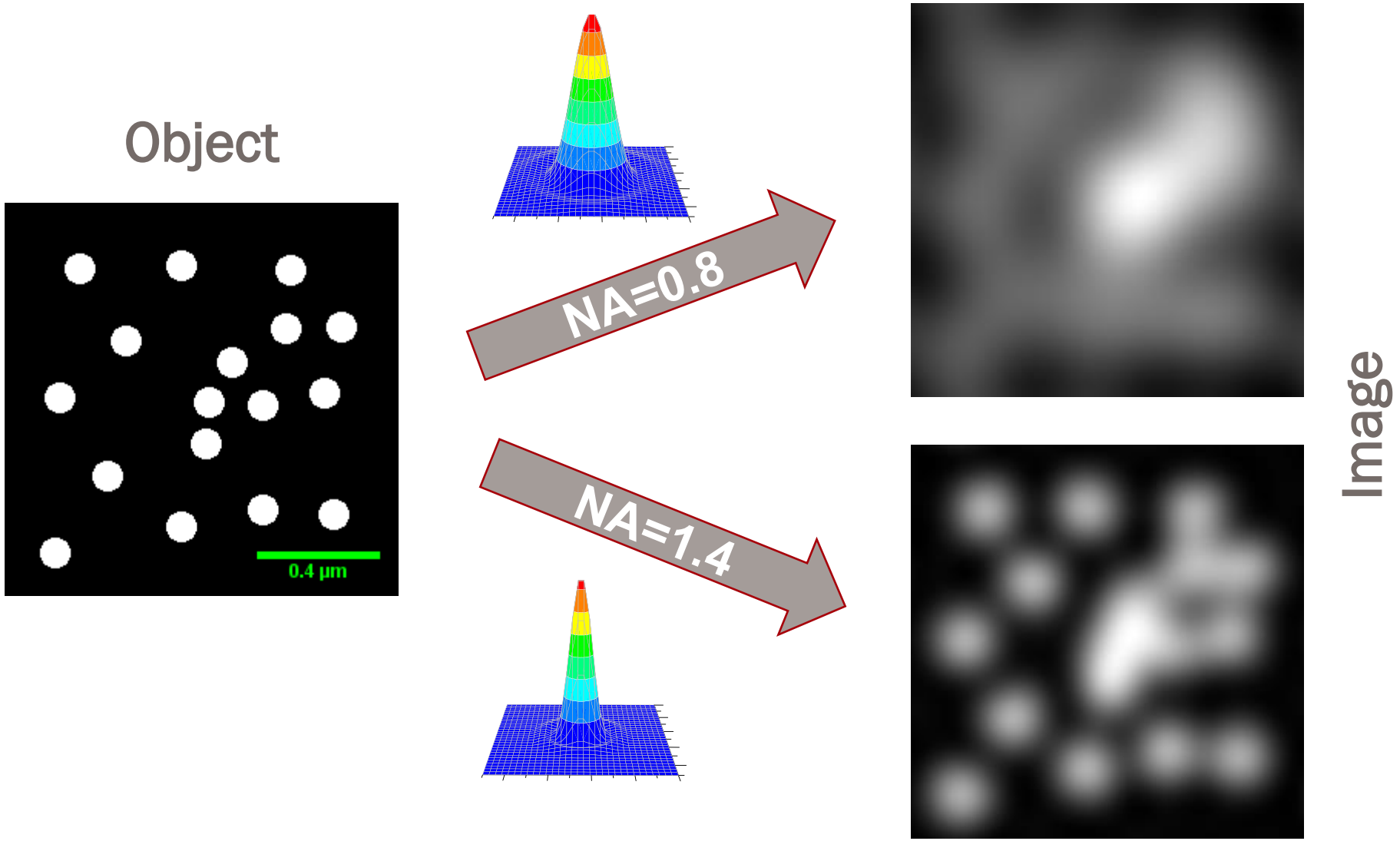
Low pass Filtering



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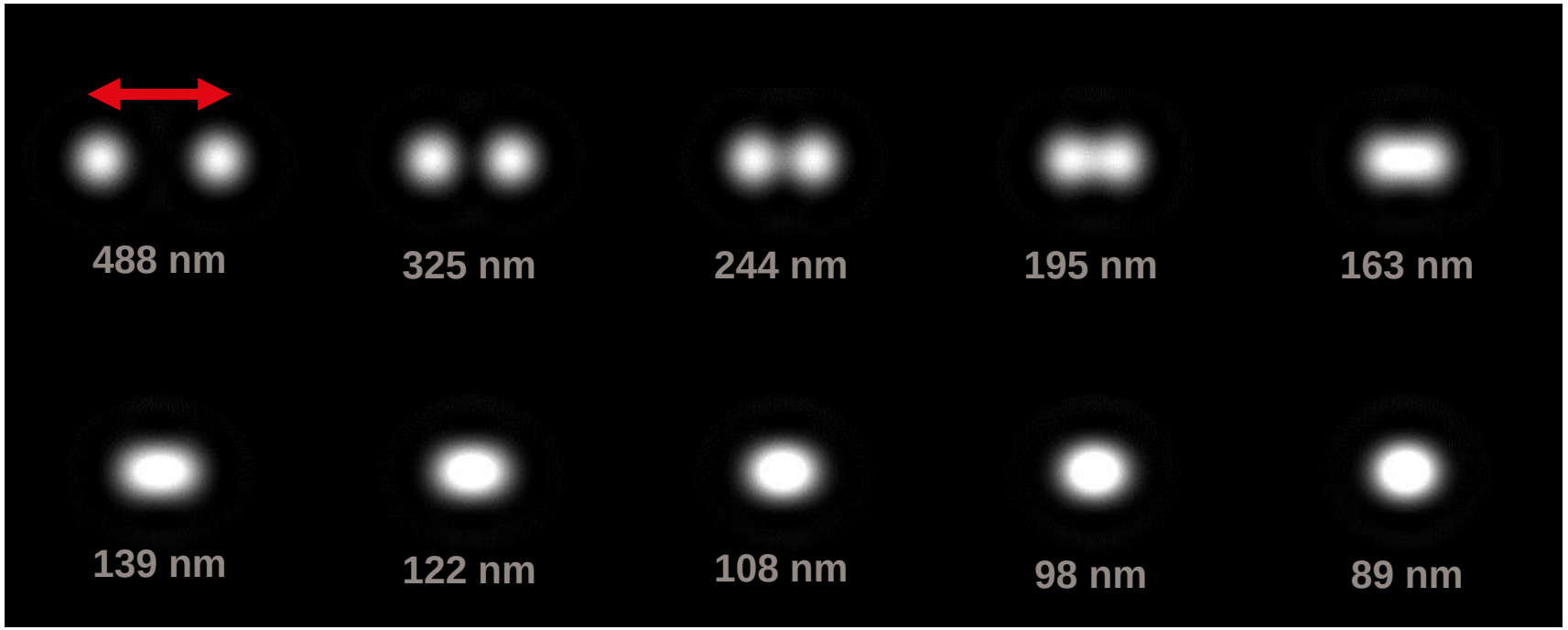
Resolution



wavelength: 488 nm NA=1.4

Rayleigh: 212 nm

Abbé: 174 nm

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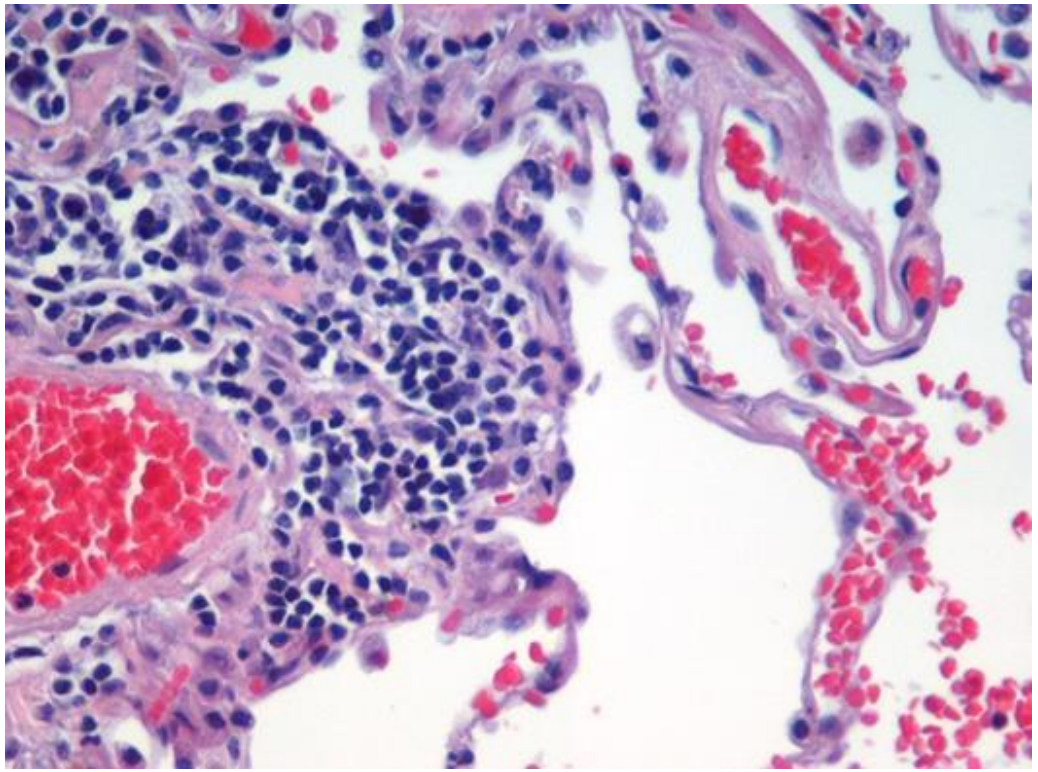
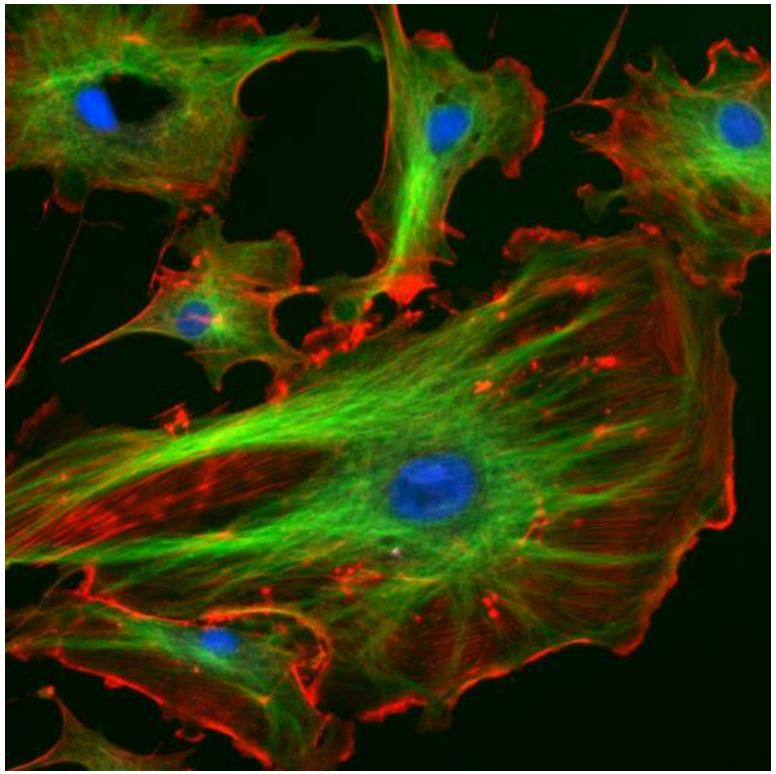
Describe the differences between the two images.

Absorption
Hematoxilin and Eosin staining (H&E)



Fluorescent dyes
Subcellular organelles



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Fluorescence microscopy

Basic principles

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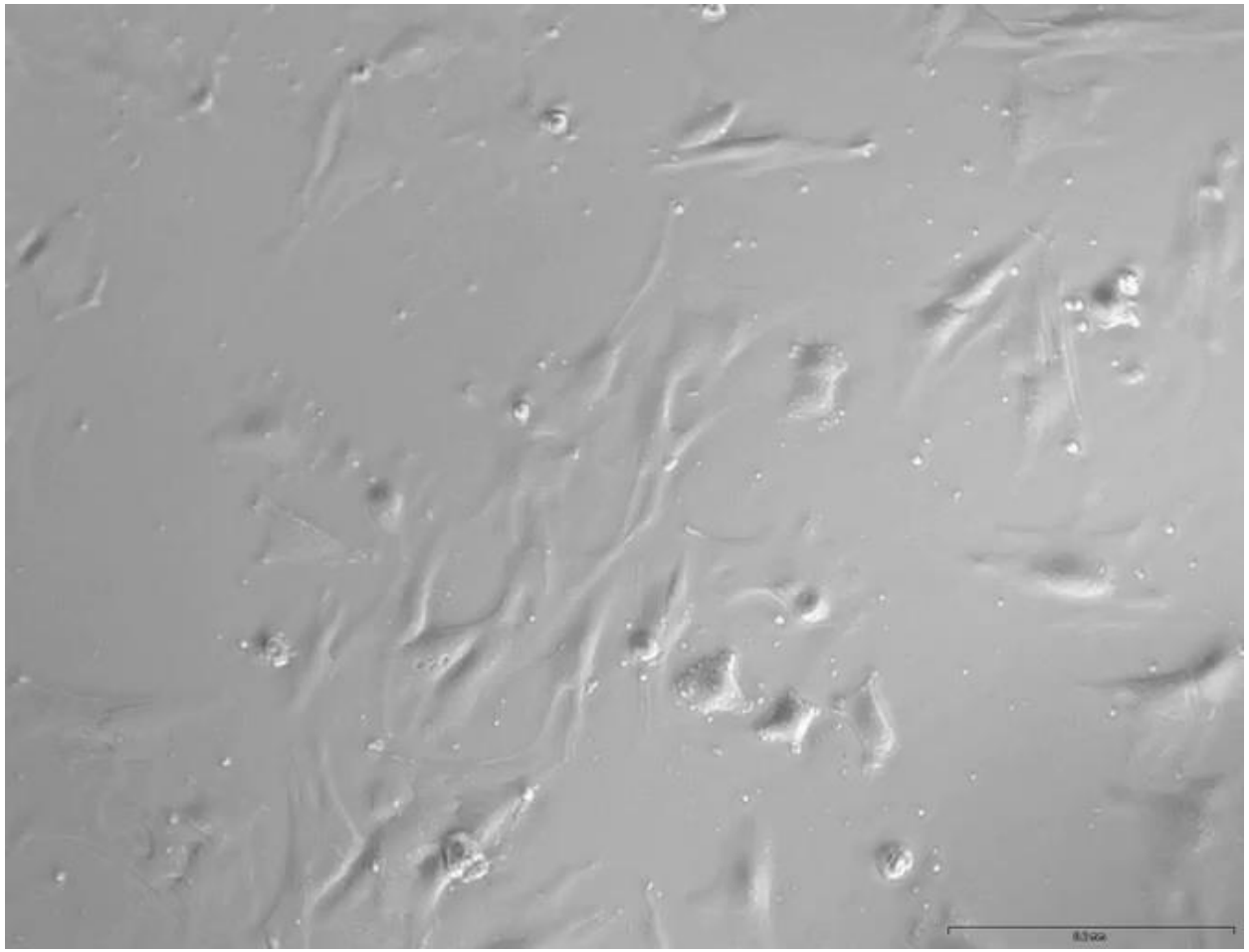
Unstained Specimen



Low contrast

Missing specificity

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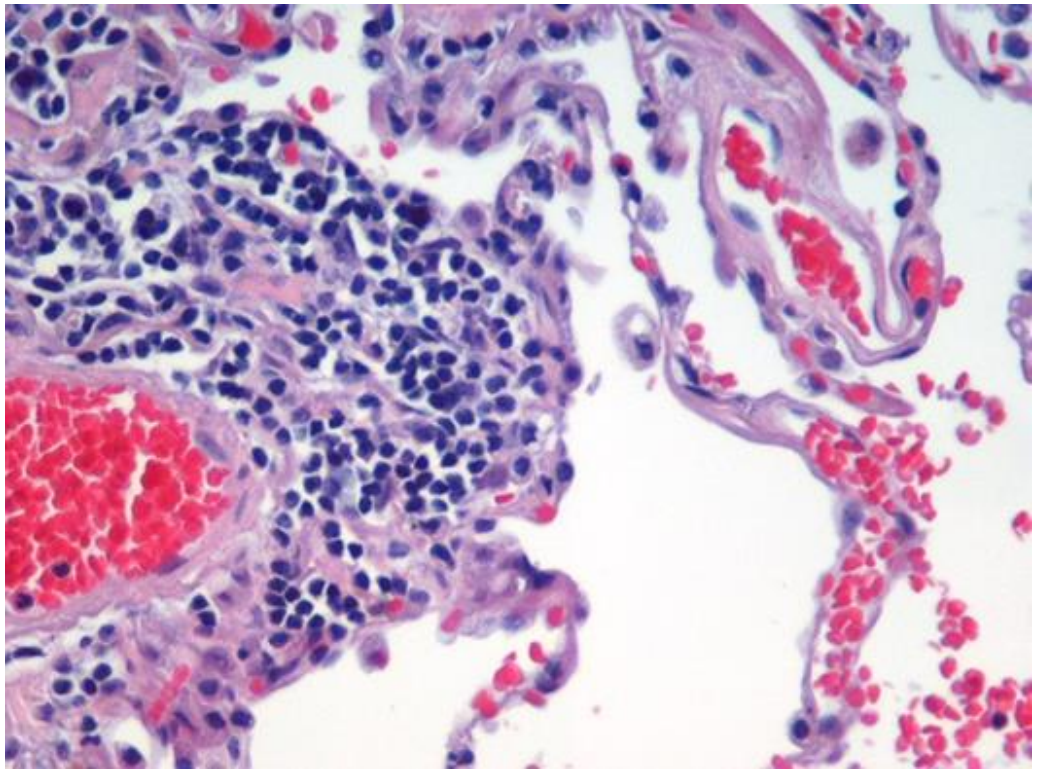
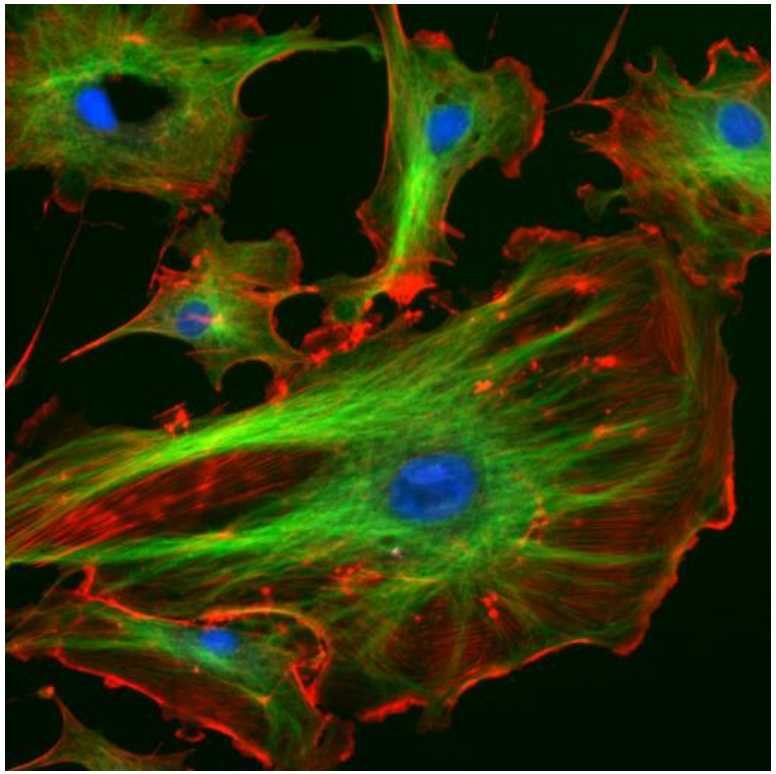
Different staining strategies

Absorption
Hematoxilin and Eosin staining (H&E)



Fluorescent dyes
Subcellular organelles



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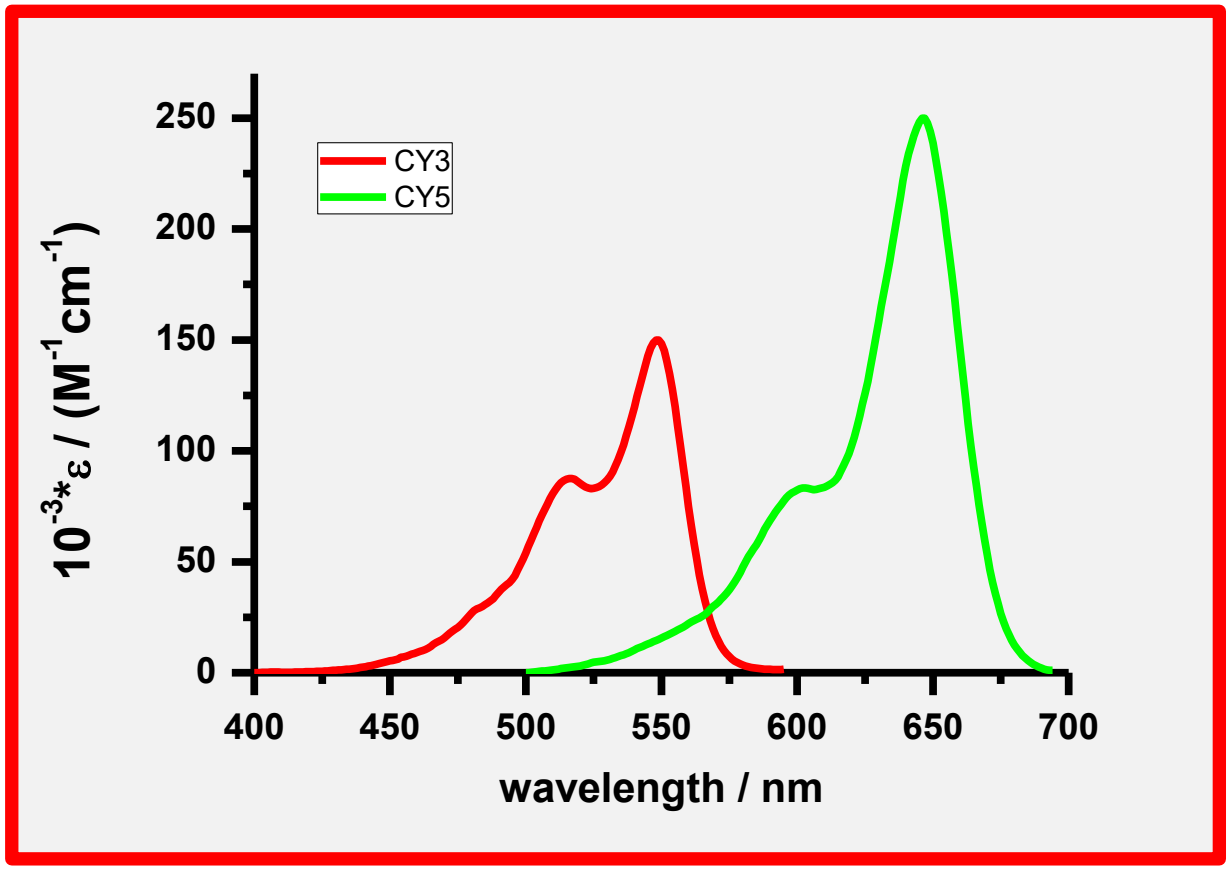
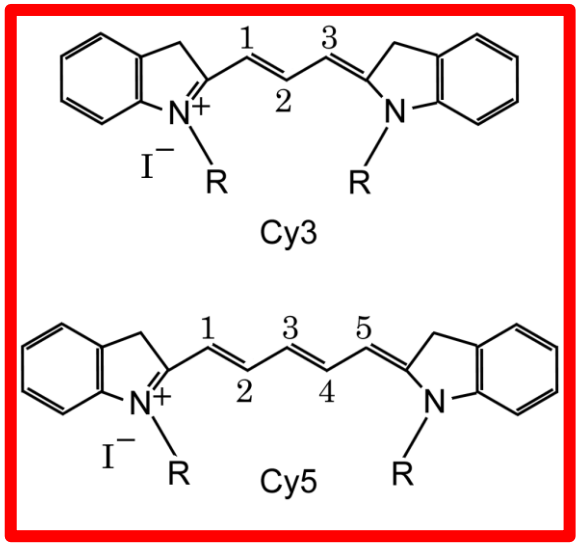
Absorption of molecules



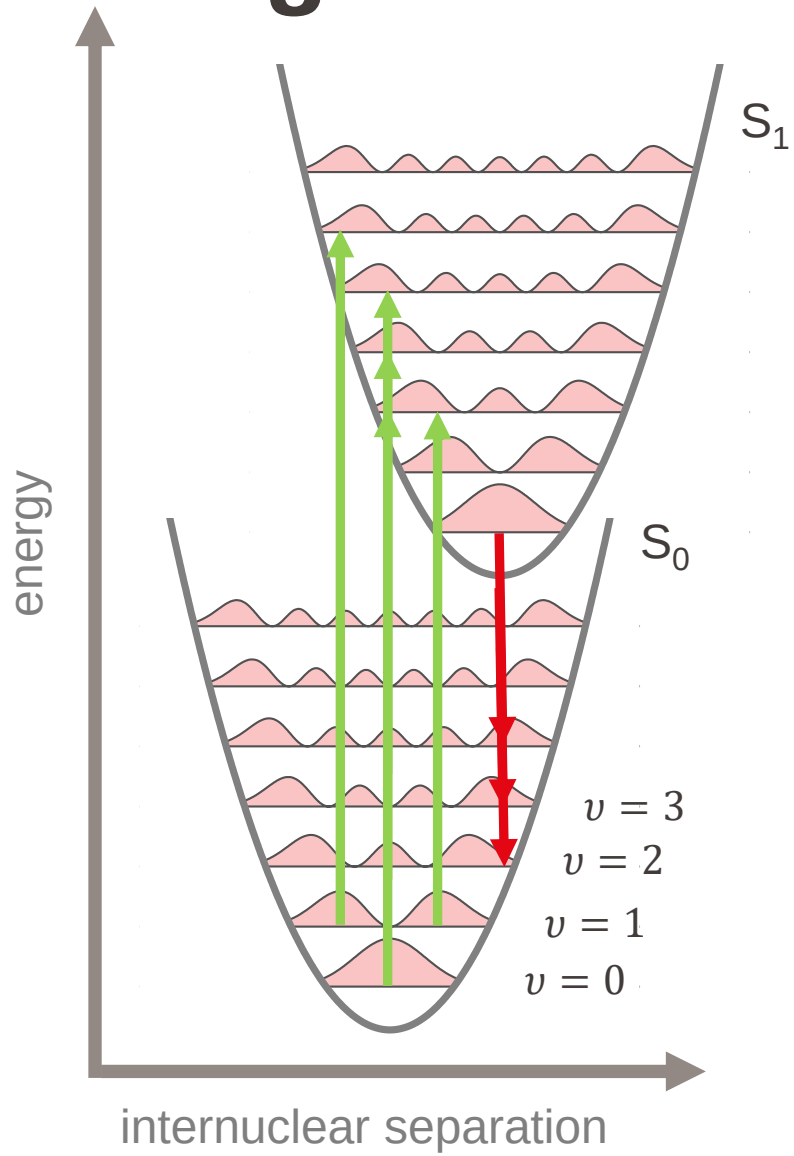


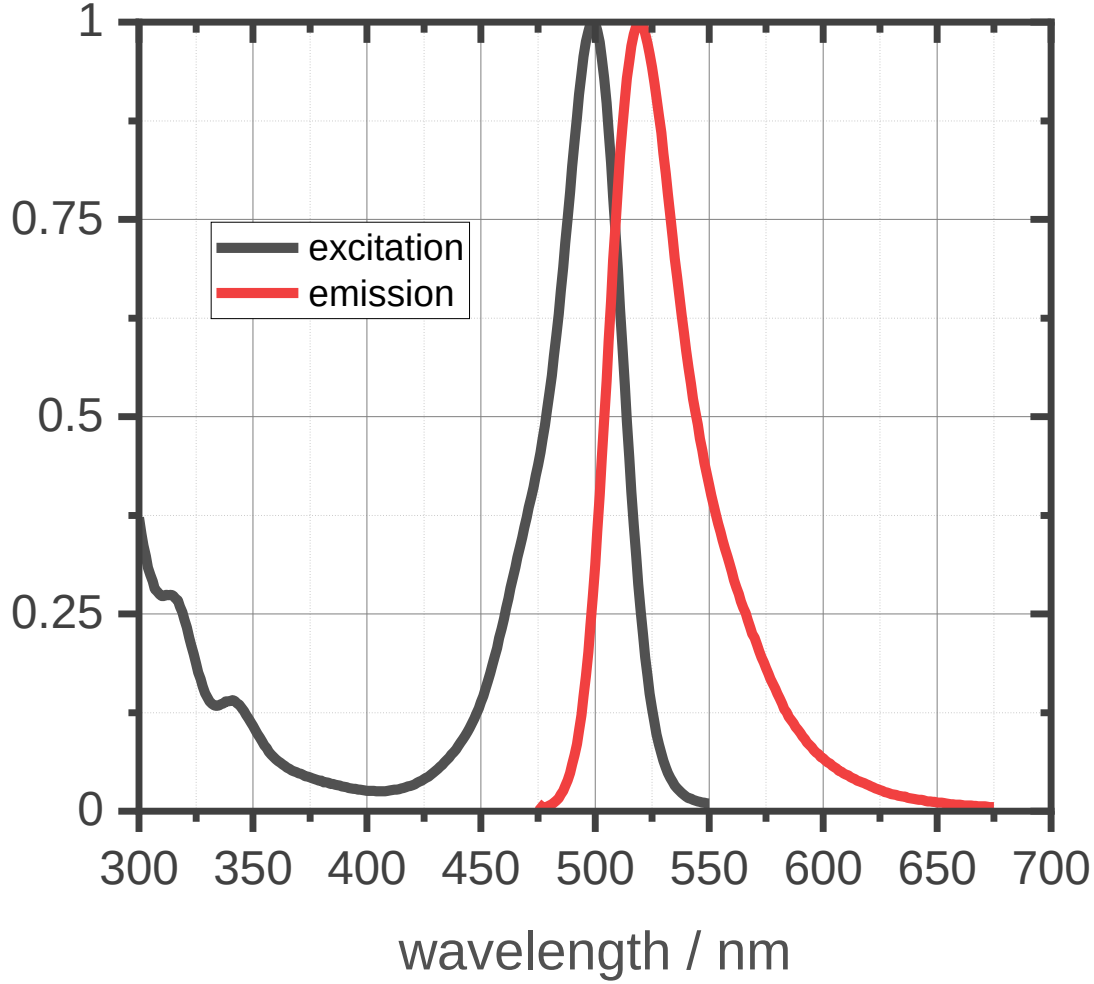
$$E_n = \frac{n^2 h^2}{8mL^2}$$

$$\Delta E = E_{n+1} - E_n = (2n+1)\frac{h^2}{8mL^2}$$

The absorption spectra of molecules (in solution) are not discrete.

Franck Condon Energy diagram





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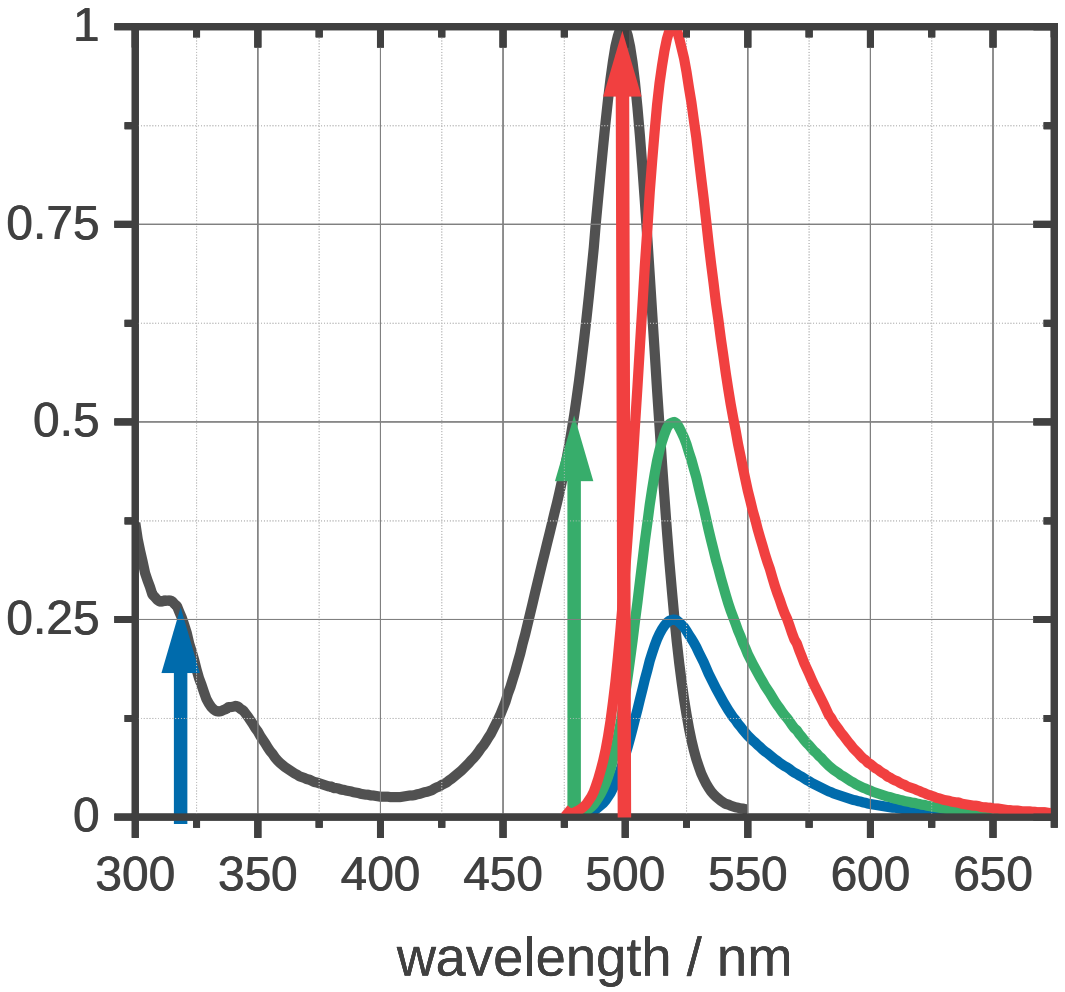
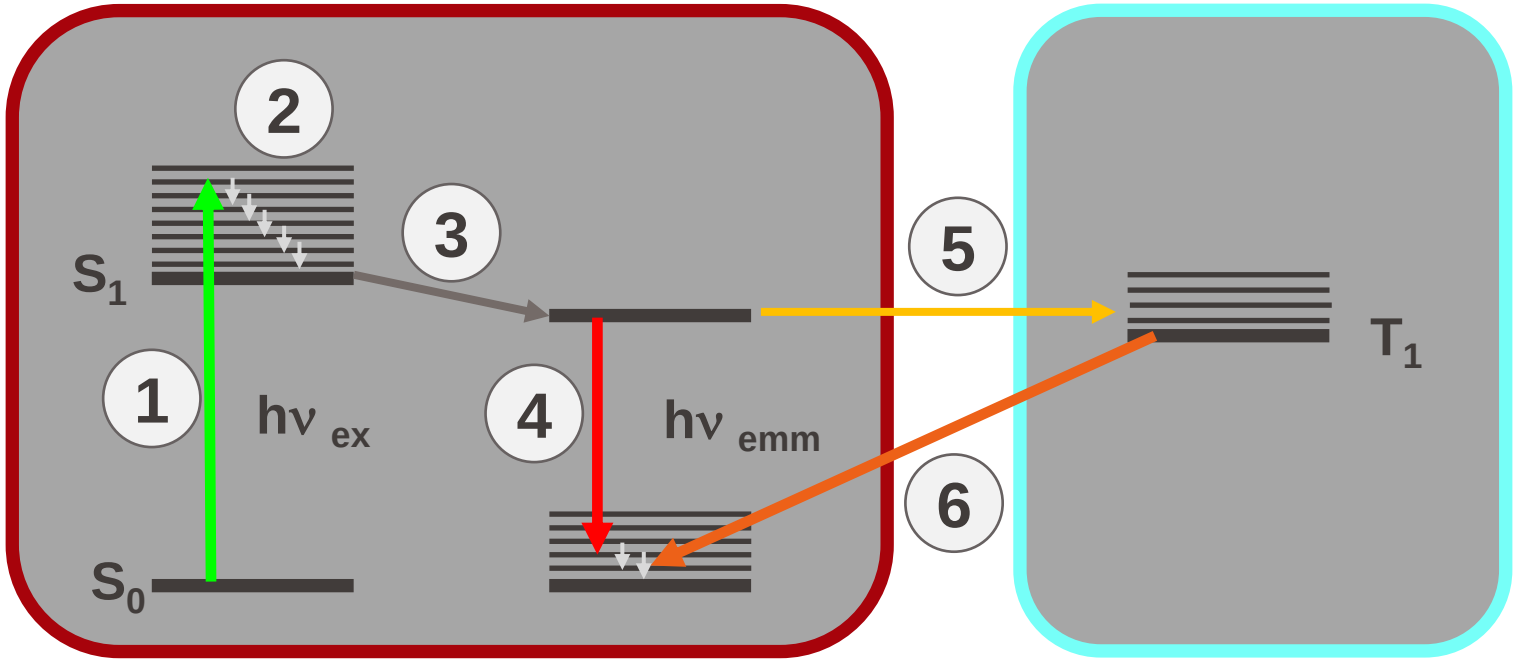


- **The profile of the emission spectra are independent of the excitation wavelength**

Simplified Jablonski Diagram



1. Excitation 10^{-15} s
2. Internal conversion 10^{-12} s
3. Solvent relaxation 10^{-11} s
4. Fluorescence 10^{-9} s
5. Intersystem crossing 10^{-9} s
6. Phosphorescence 10^{-3} s

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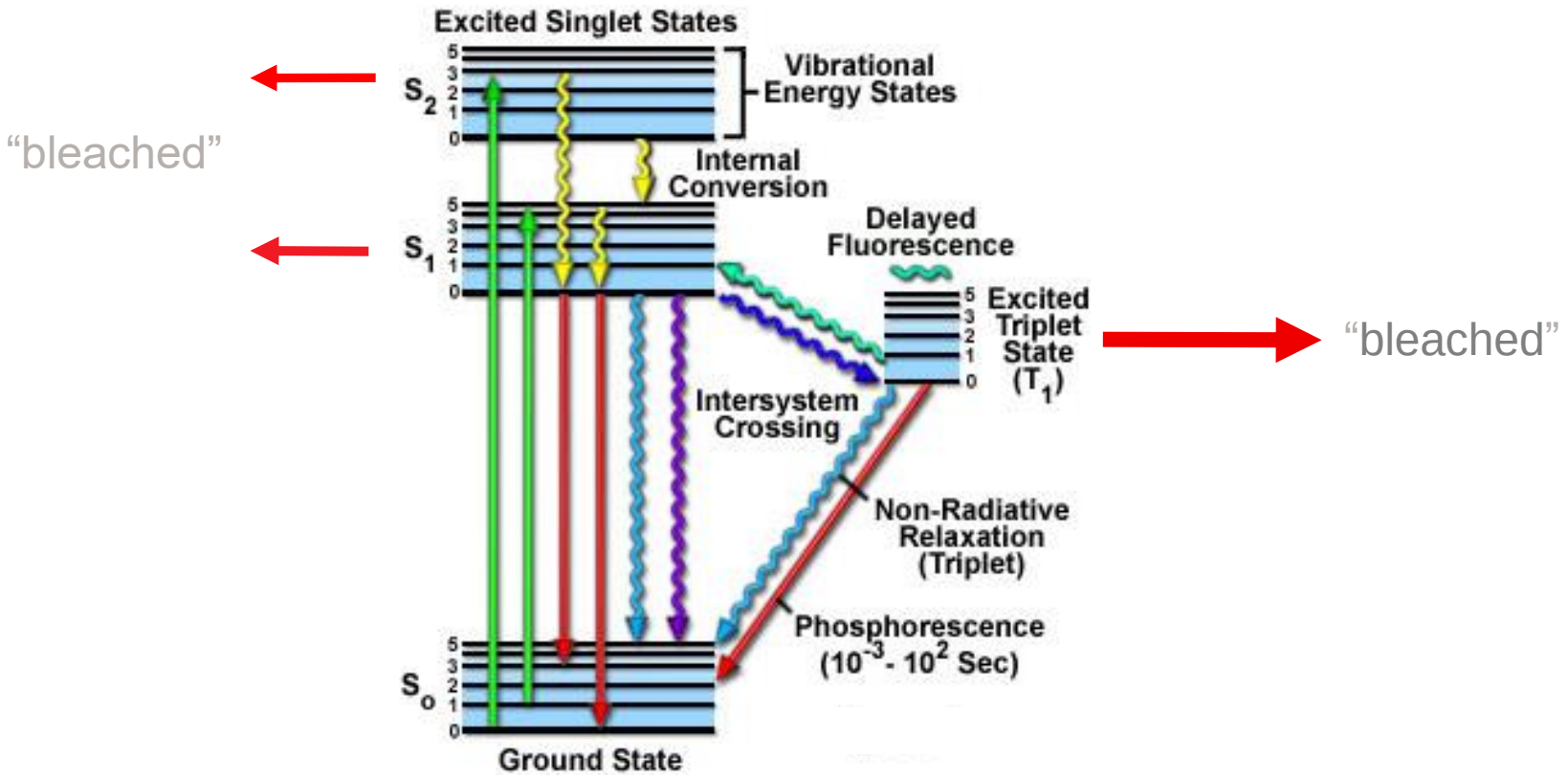
Bleaching

- Bleaching is irreversible (=fluorophore is destroyed)
- Bleaching is dependent on the excitation power
- Bleaching can also cause **photodamage**



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What is the intensity ratio between excitation light and emitted light in fluorescence microscopy?

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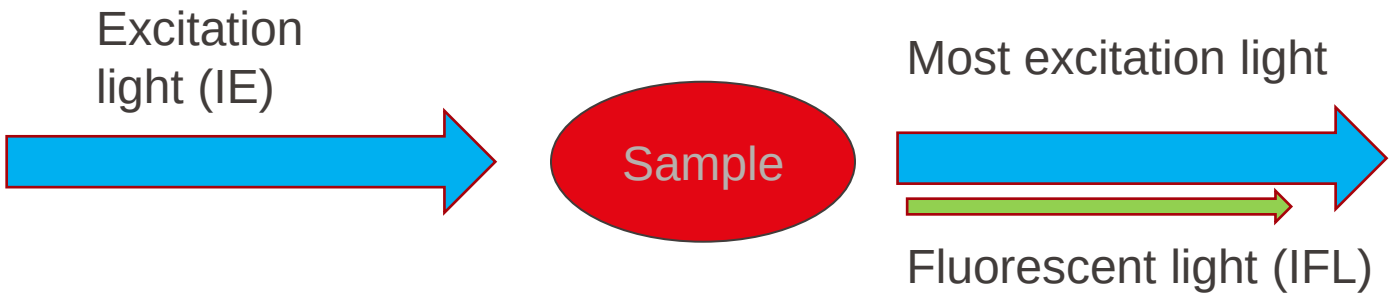
Fluorescence detection



IE/IFL = 10^4 for strong fluorescence

IE/IFL = 10^{10} for weak fluorescence (e.g. *in situ* hybrid.)

In order to detect the fluorescence at 10% background
the excitation light must be removed or attenuated by a factor up to ≈ 10^{11}

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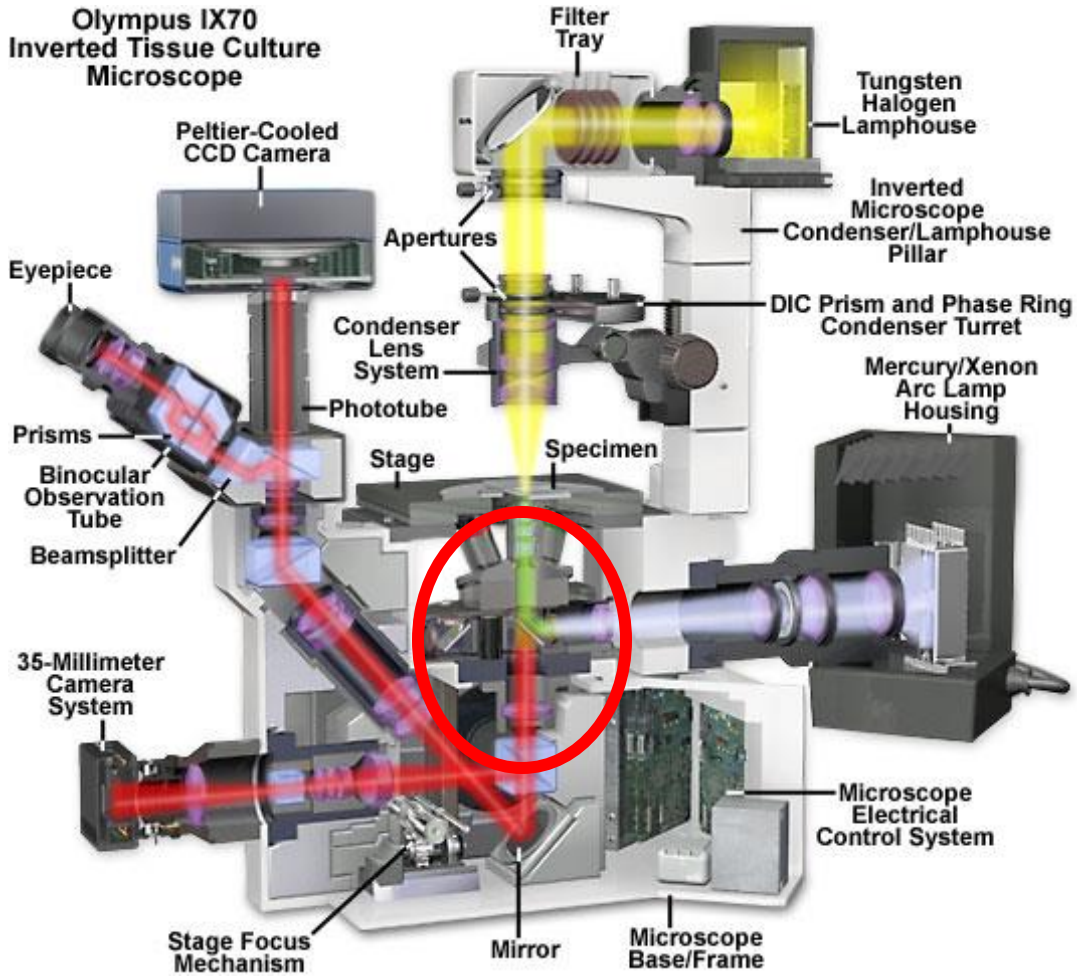
Implementation of Epifluorescence



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Axial resolution

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Depth of Field

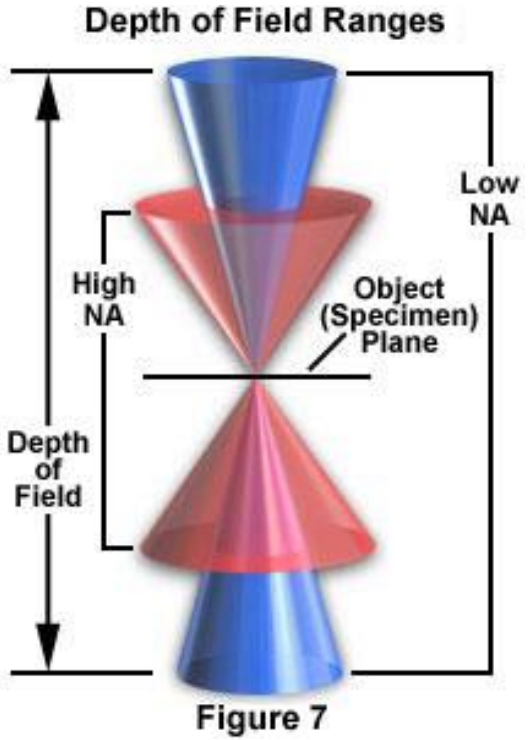

Figure 7

Magnification	Numerical Aperture	n	wavelength µm	depth of field µm
5	0.05	1	0.55	246.000
10	0.1	1	0.55	61.500
10	0.3	1	0.55	8.278
20	0.45	1	0.55	3.438
20	0.8	1	0.55	1.266
40	0.65	1	0.55	1.552
40	0.95	1	0.55	0.780
40	1.3	1.52	0.55	0.685
60	1.4	1.52	0.55	0.544
100	1.4	1.52	0.55	0.497

$$d_{tot} = \frac{\lambda \cdot n}{NA^2} + \frac{n}{M \cdot NA} \cdot e$$

Diffraction limited depth of field

Detection system (e=6.5 µm)

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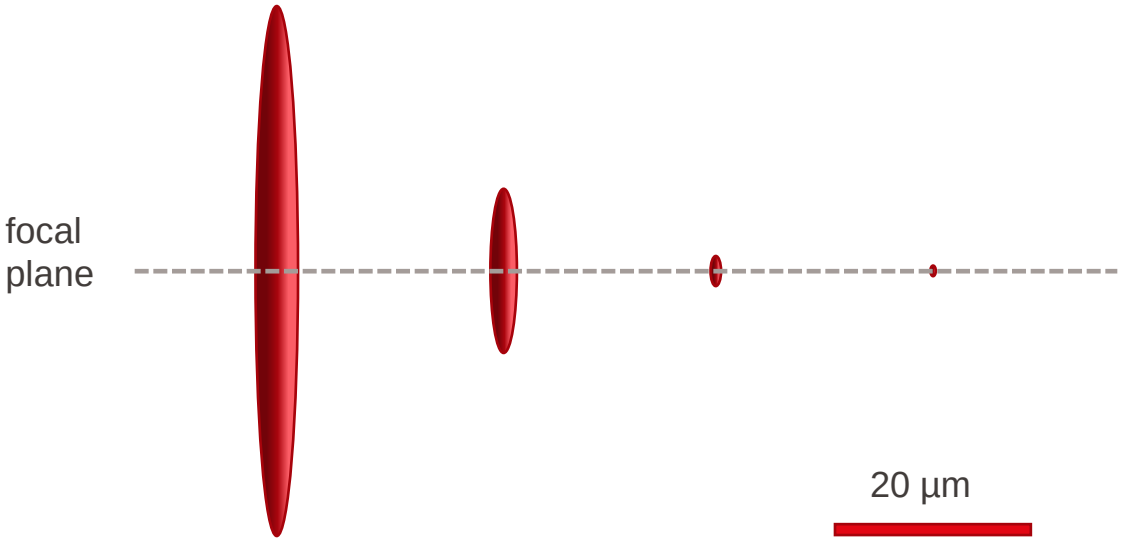
Optical Slicing



$$\mathrm{DoF} = \frac{\lambda \cdot n}{NA^2} + \frac{n}{M \cdot NA} \cdot e$$

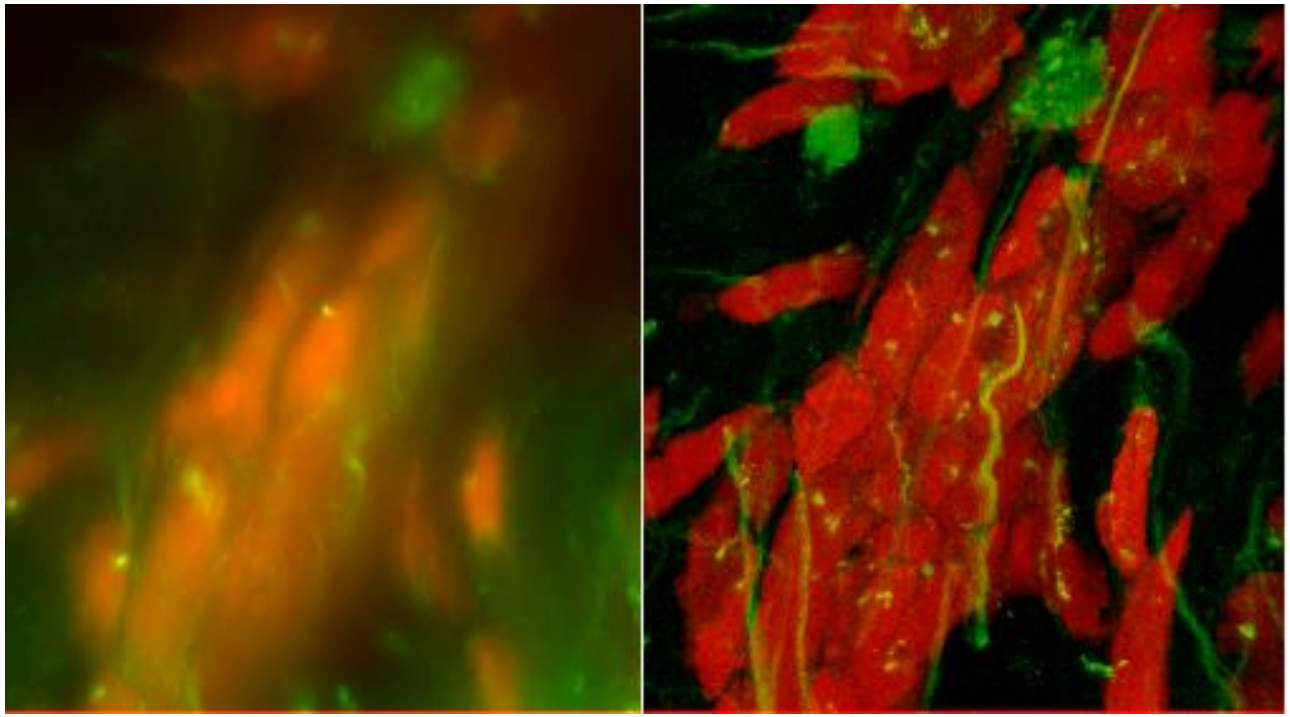
$$\text{Lateral resolution} = \frac{\lambda}{2 \cdot NA}$$

M	NA	n	DoF/µm	Lateral resolution/ µm
2	0.06	1	54.72	4.58
4	0.1	1	16.80	2.75
10	0.25	1	3.15	1.10
20	0.5	1	1.20	0.55
40	0.65	1	0.80	0.42
50	0.95	1.518	1.04	0.29
60	0.8	1	0.69	0.34
100	1.25	1.518	0.91	0.22
100	0.9	1	0.62	0.31

λ: wavelength 0.55 µm

e: pixel size camera 6.45 µm

Imaging of thick specimen



wide-field confocal

→ „out of focus“ light is bluring the image and thus reducing the contrast/resolution

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Imaging point by point

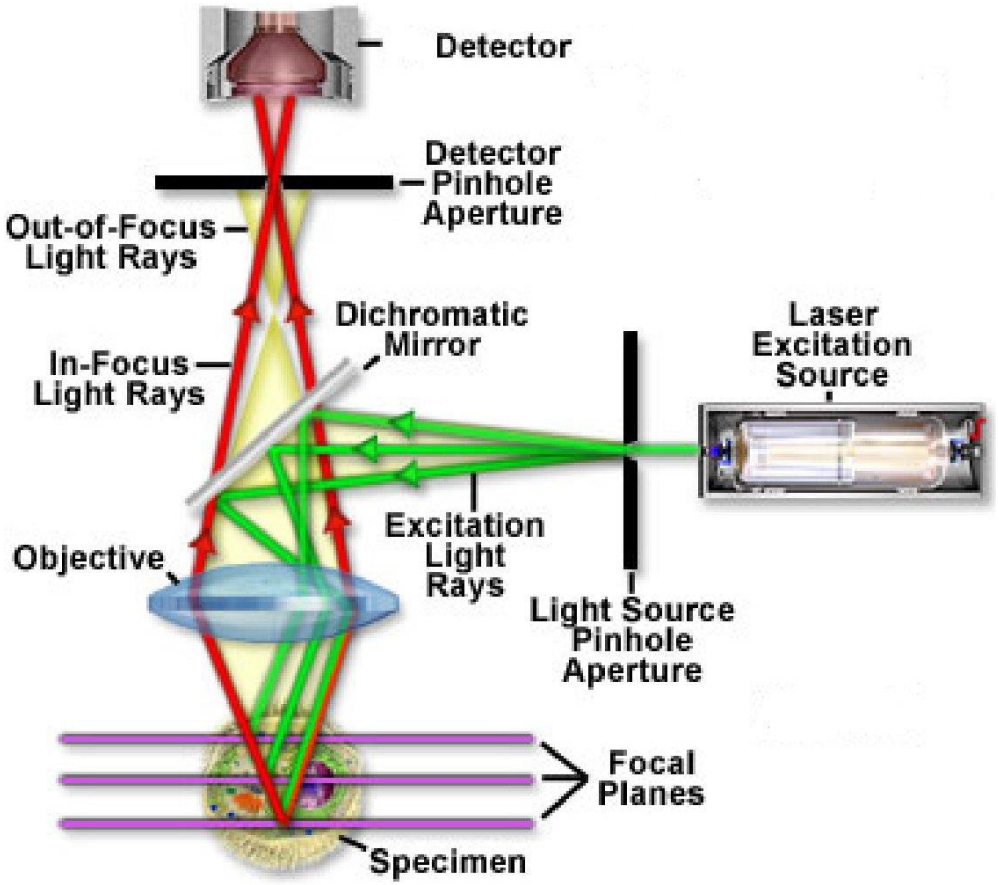
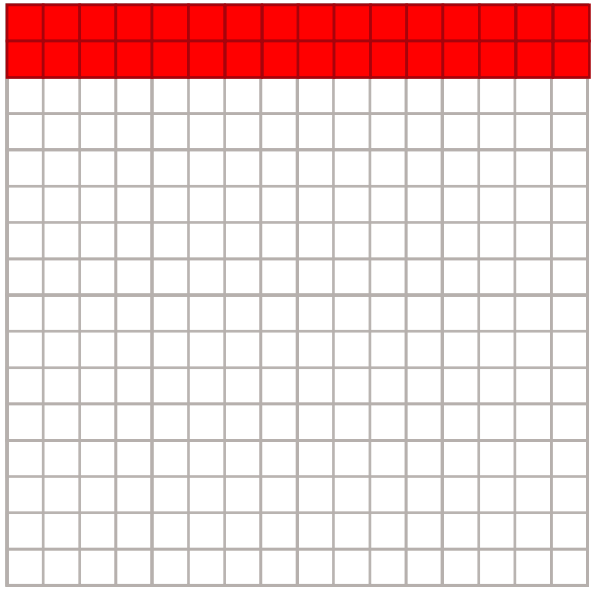


Image formation by reconstruction



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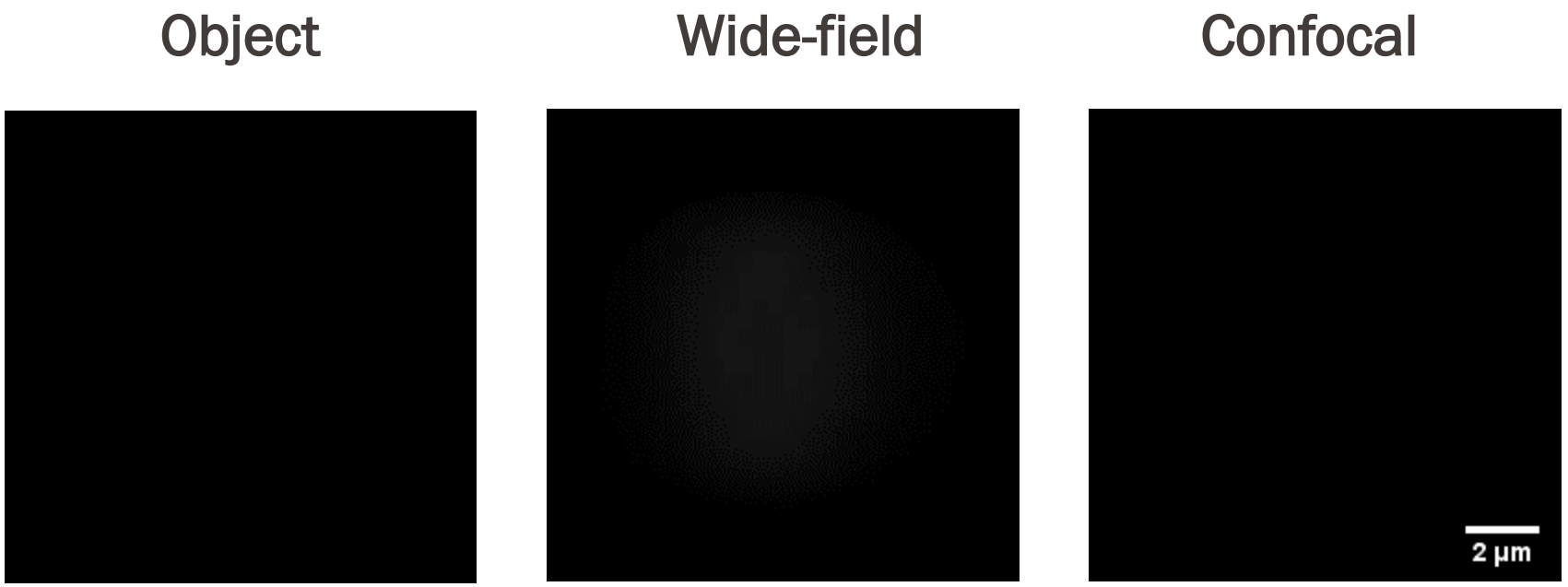
Optical slicing



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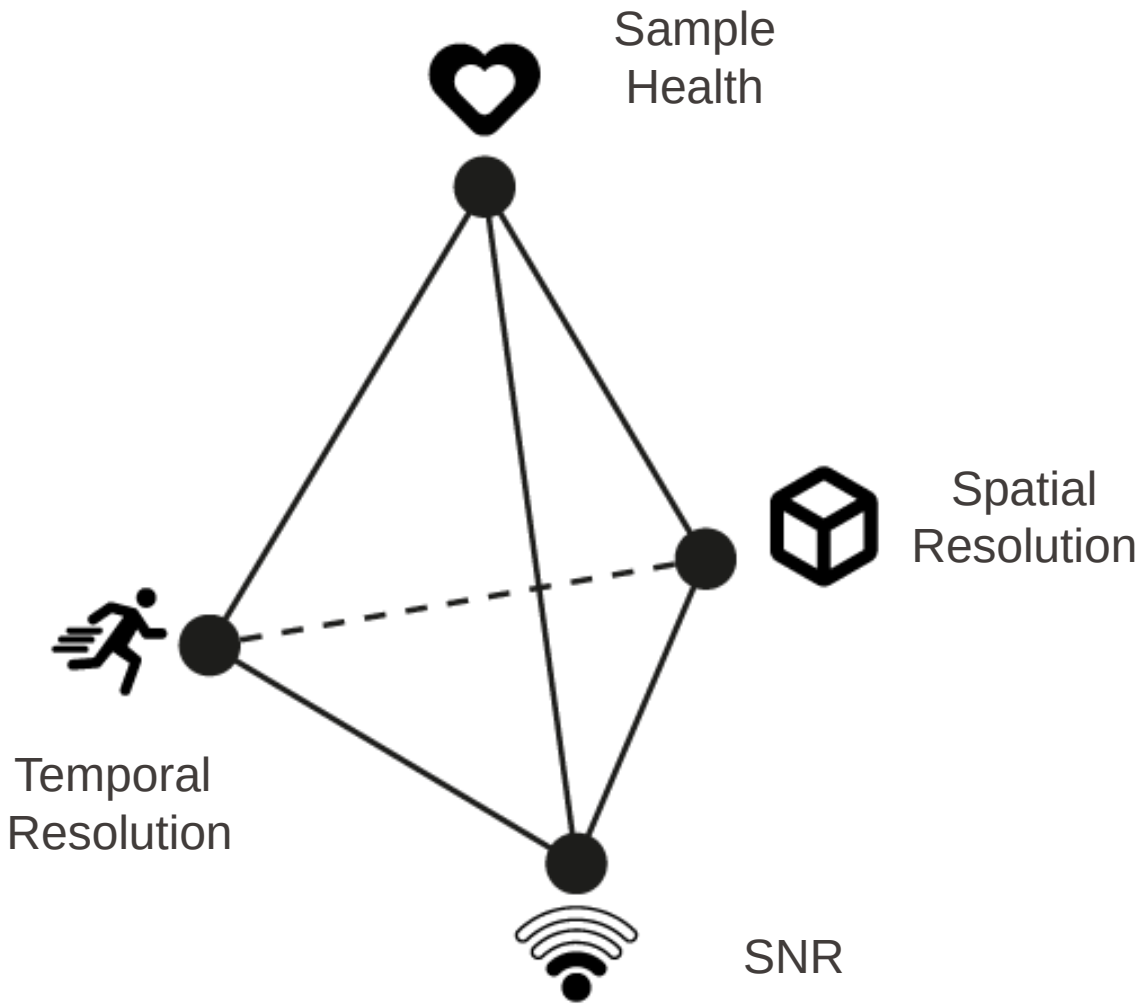
Imaging Frustration Pyramid



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Summary

- Light microscopy
  - Lens-based imaging
  - Diffraction limited
- Main modalities
  - Transmission
    contrast generation: absorption, scattering
  - Fluorescence
    contrast generation: Stokes shift
- Specificity
  - Dyes coupled to
    - antibodies
    - Target molecules
  - Fluorescent proteins

Imaging of tissue sections

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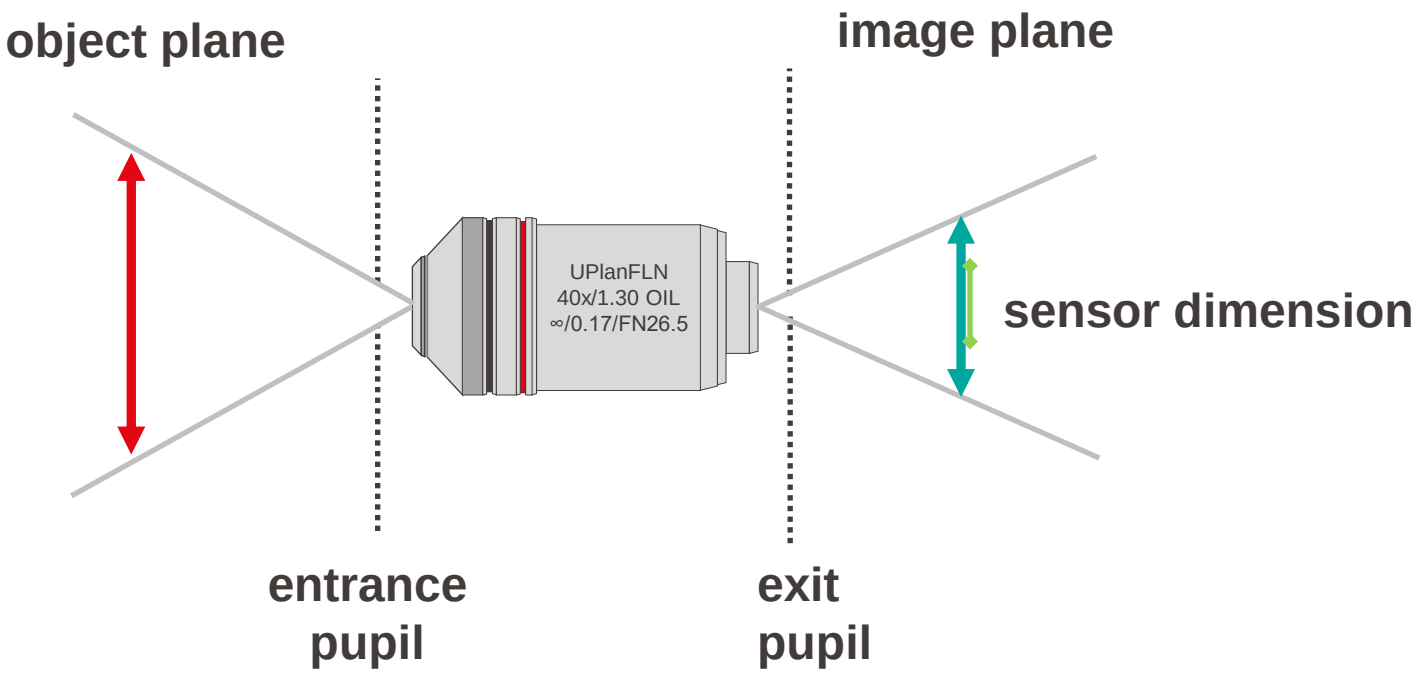
Field of view (FOV)



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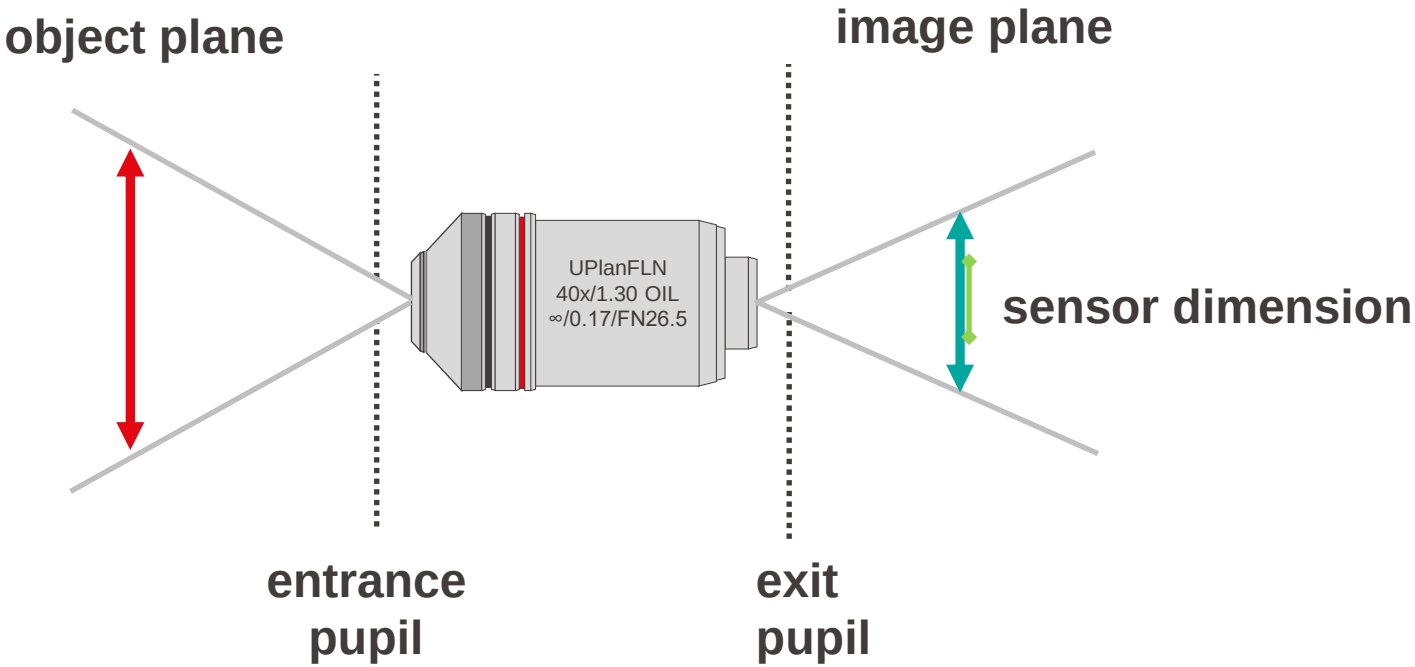
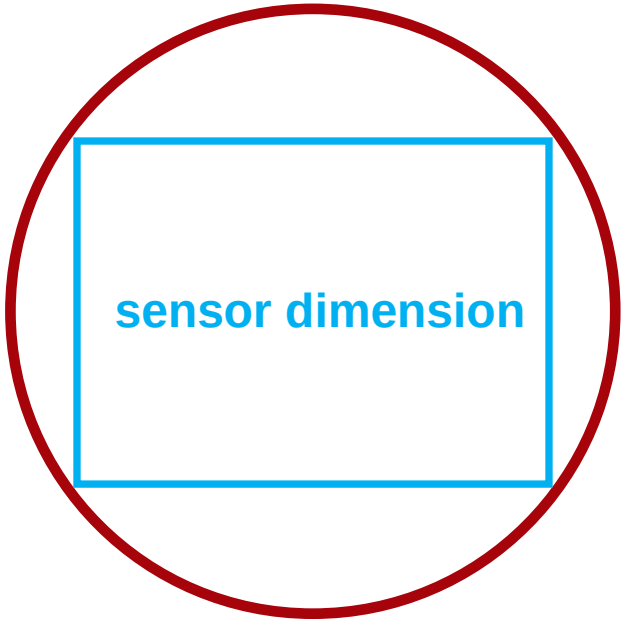
Field of view (FOV)





FOV lens

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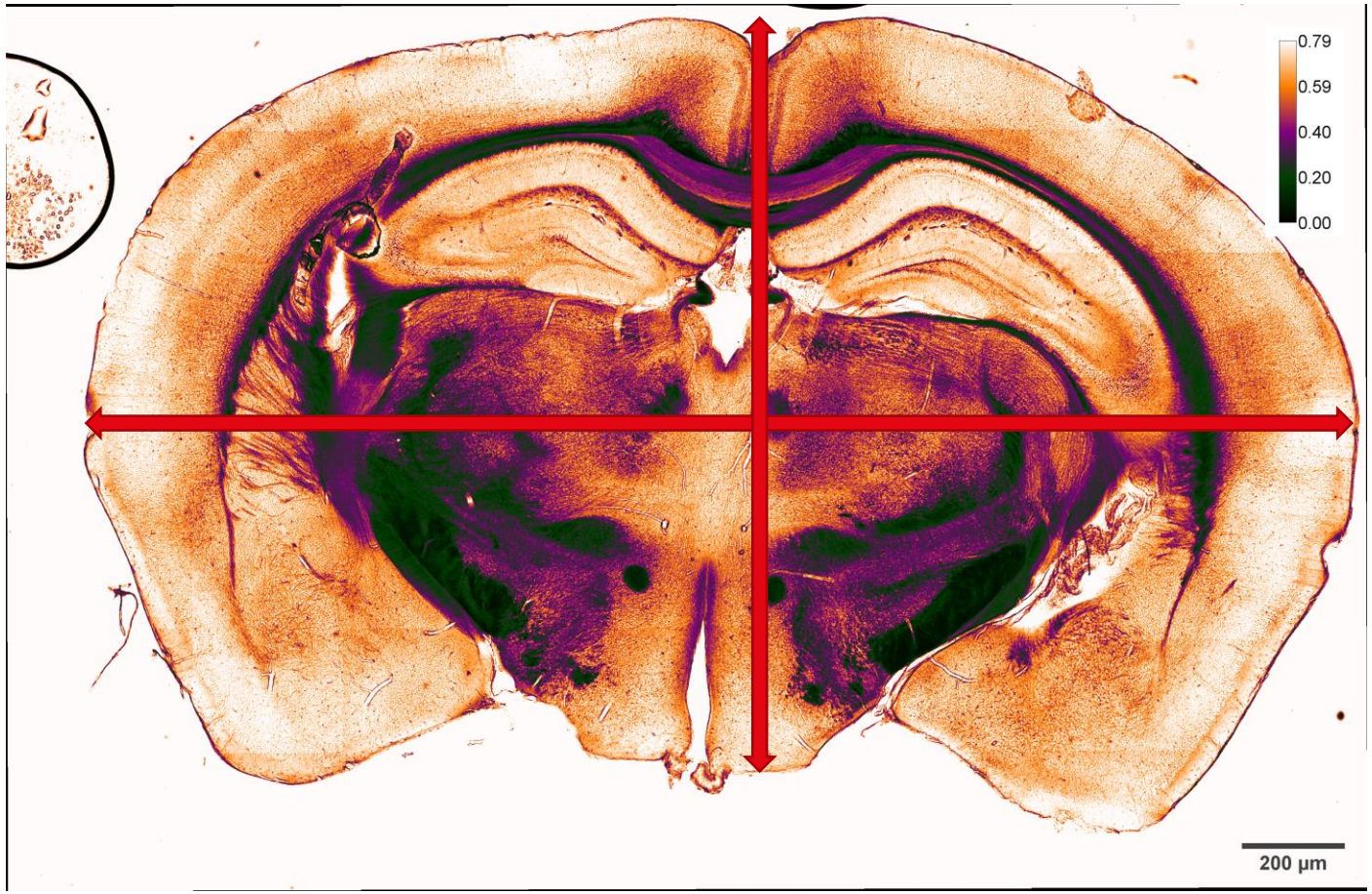
Motivation



FOV sCMOS camera (color)

20x

Magnification: 10x
Sampling: 0.32 µm/pixel

Magnification: 5x
Sampling: 0.64 µm/pixel

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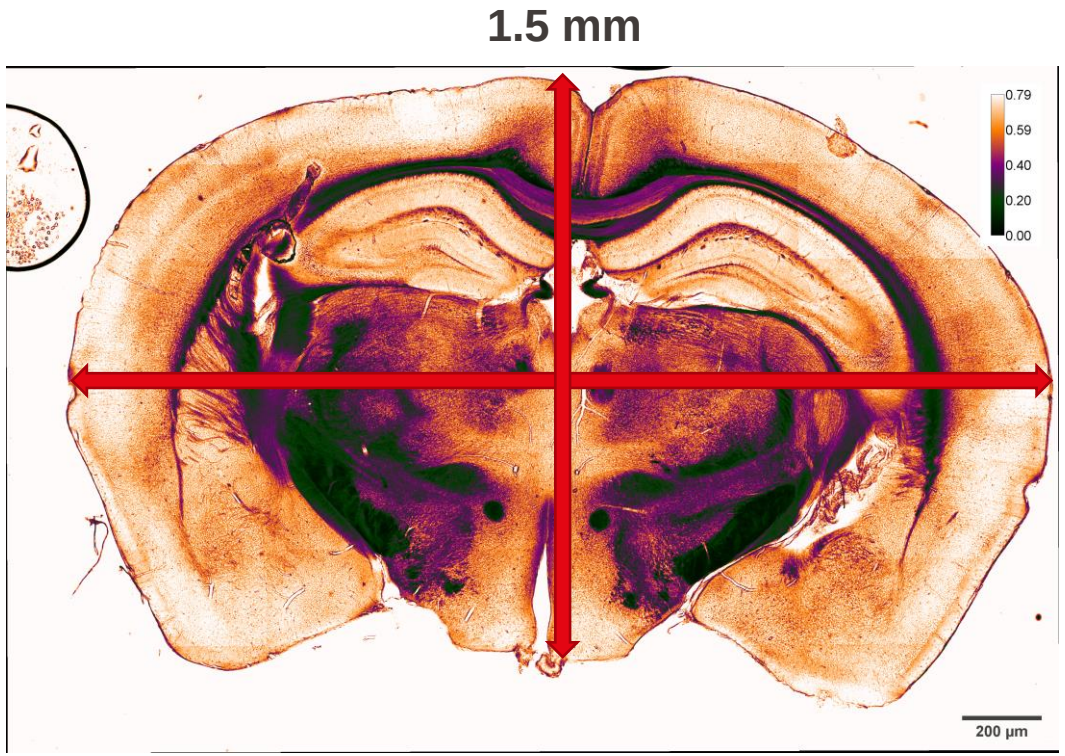
Motivation



FOV sCMOS color	Tiles	N.A. resolution	rel. bright-ness
40x	224	0.95 0.29 µm	0.71
20x	56	0.8 0.34 µm	1.0
10x	8	0.4 0.69 µm	0.5
5x	4	0.95 2.1 µm	0.11

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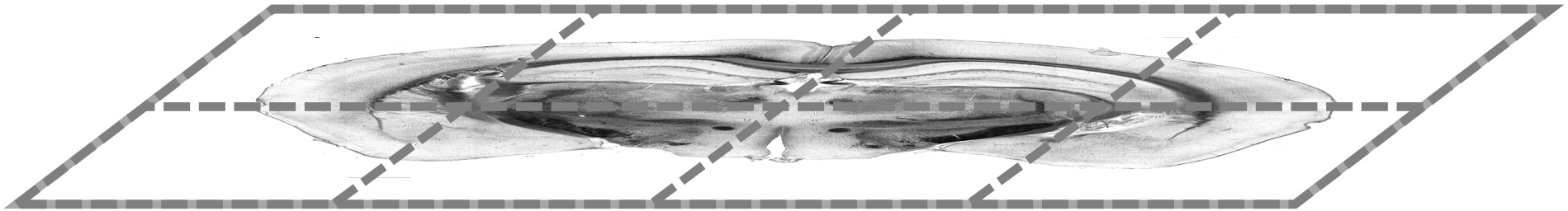
Tiled Imaging



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Tiled Imaging

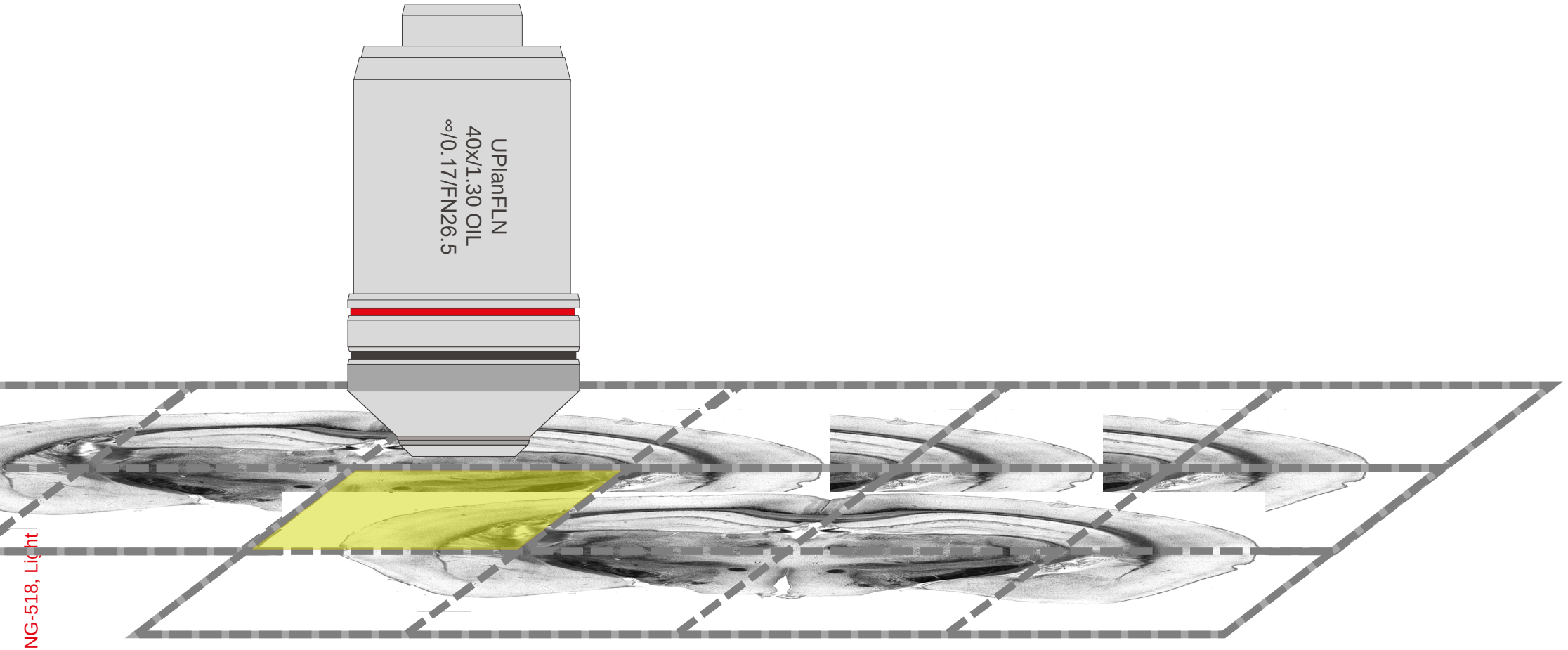

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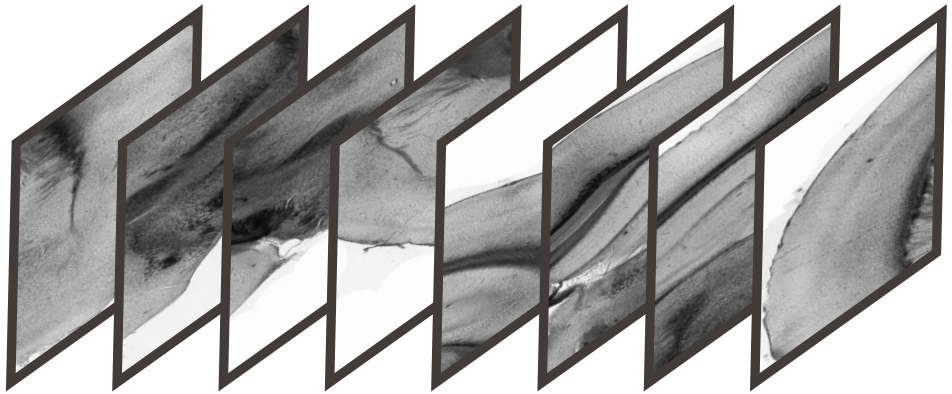
Image stack

Image Stack
2 x4



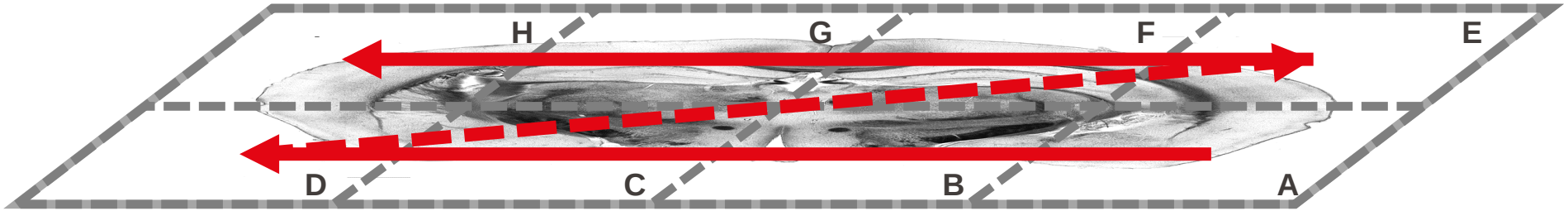


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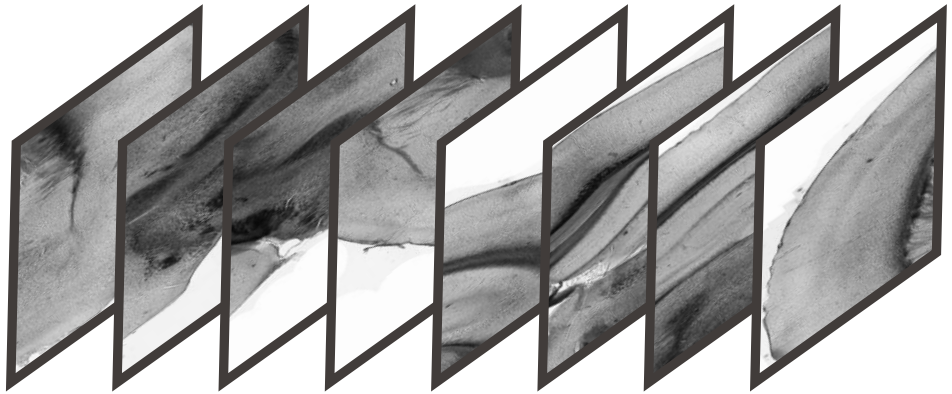
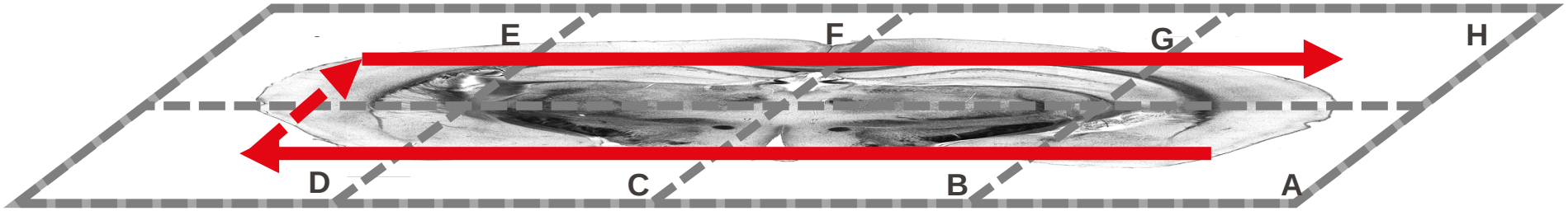
Image Stack

Image Stack
2 x4





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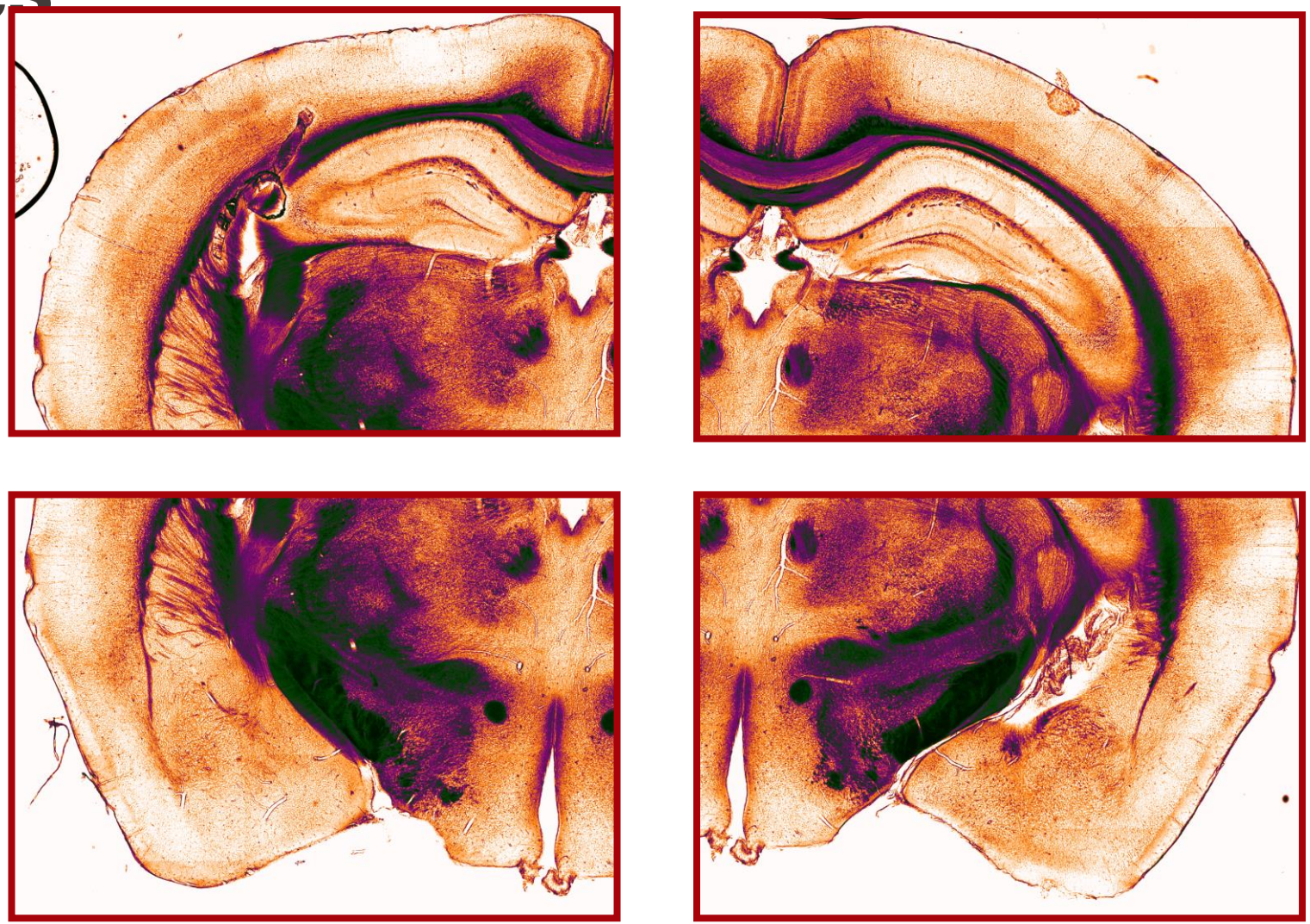
Alignment of individual tiles



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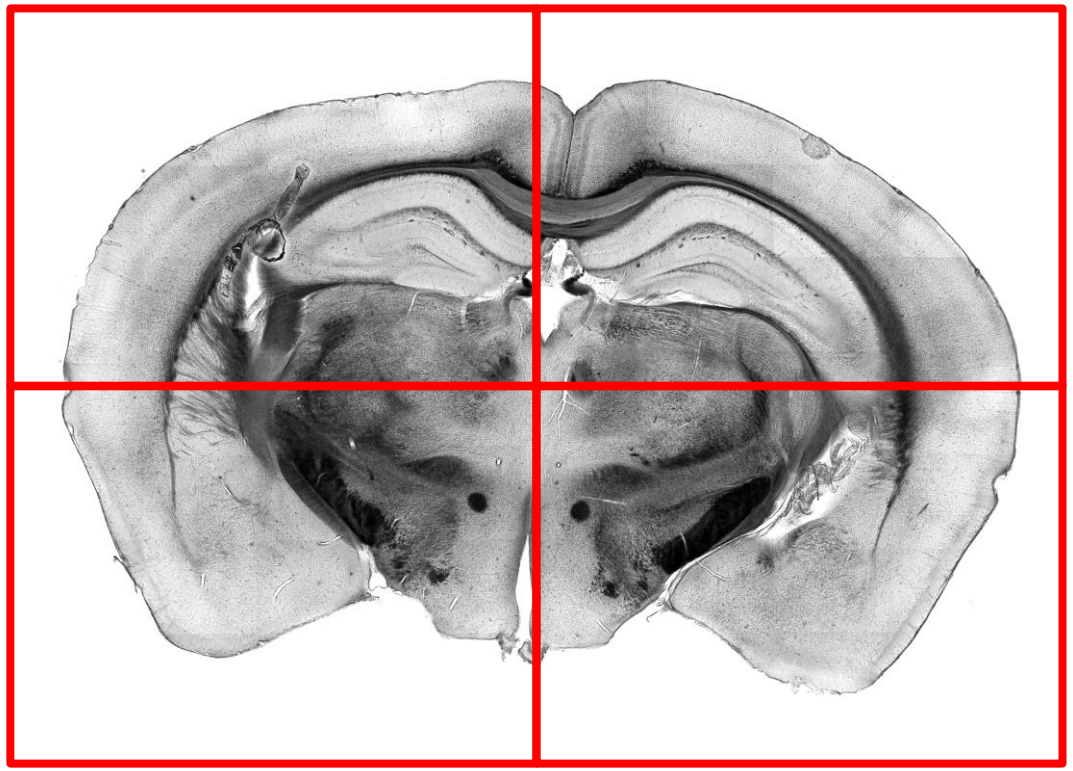
Alignment strategy



Alignment based on stage position (apriori knowledge)

- No post processing needed
- Limitation
  - stage precision
  - camera alignment
  - camera/sample orientation

In practice: used only for low resolution overview images.

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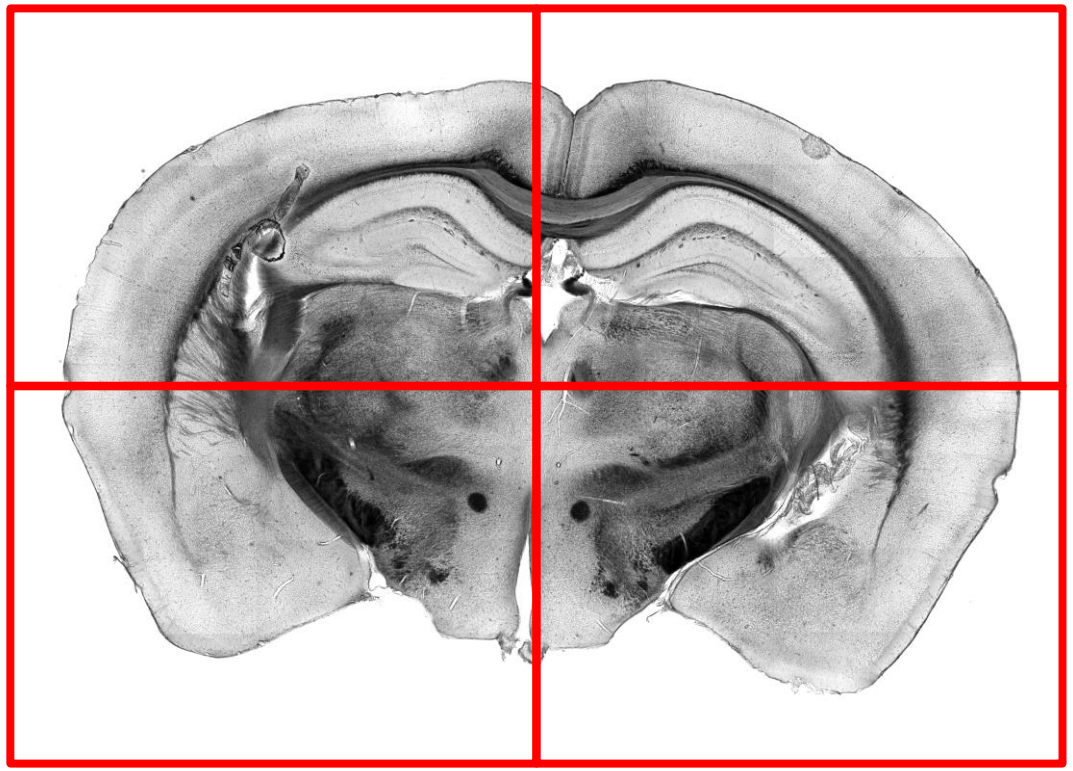
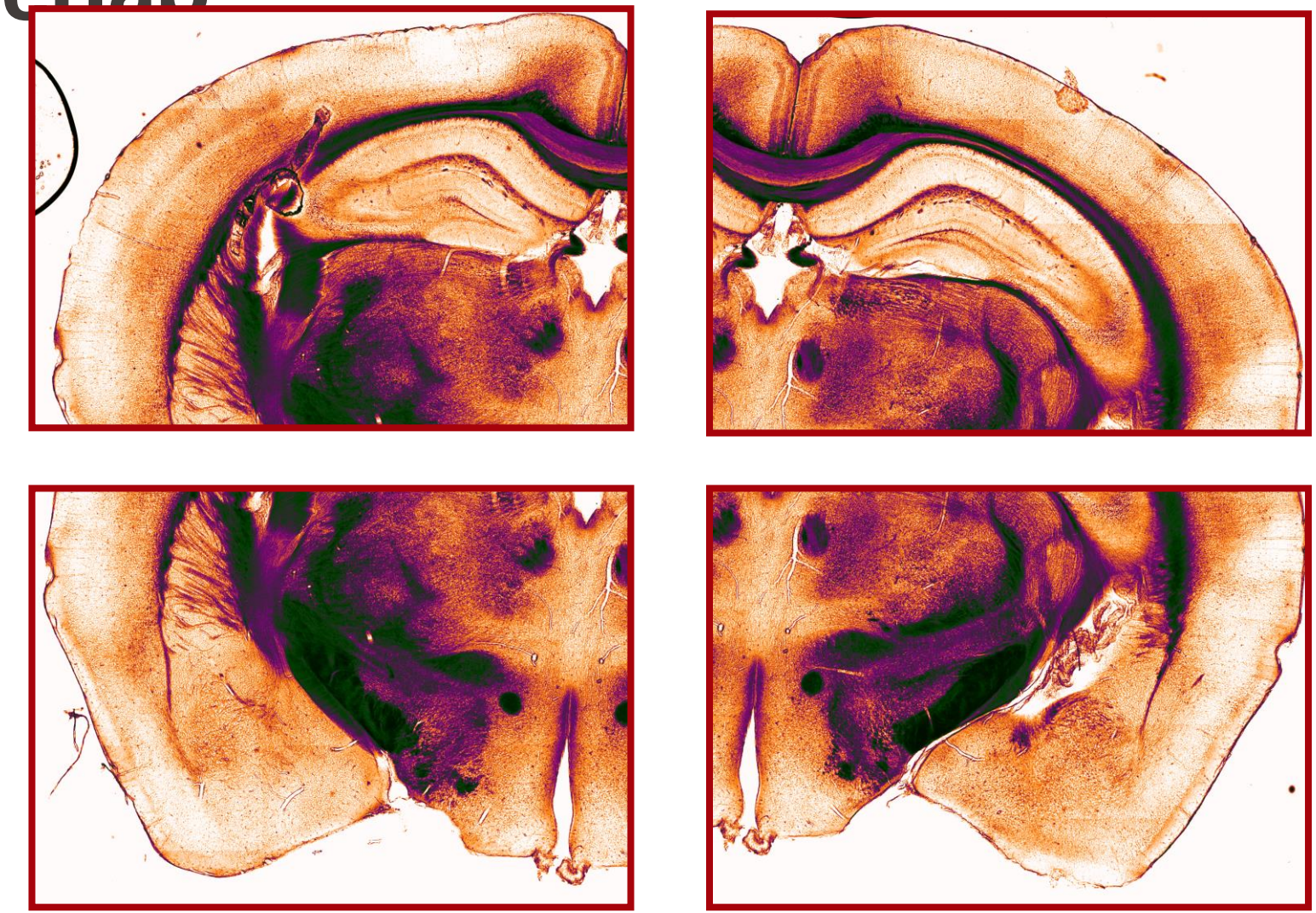
Alignment strategy



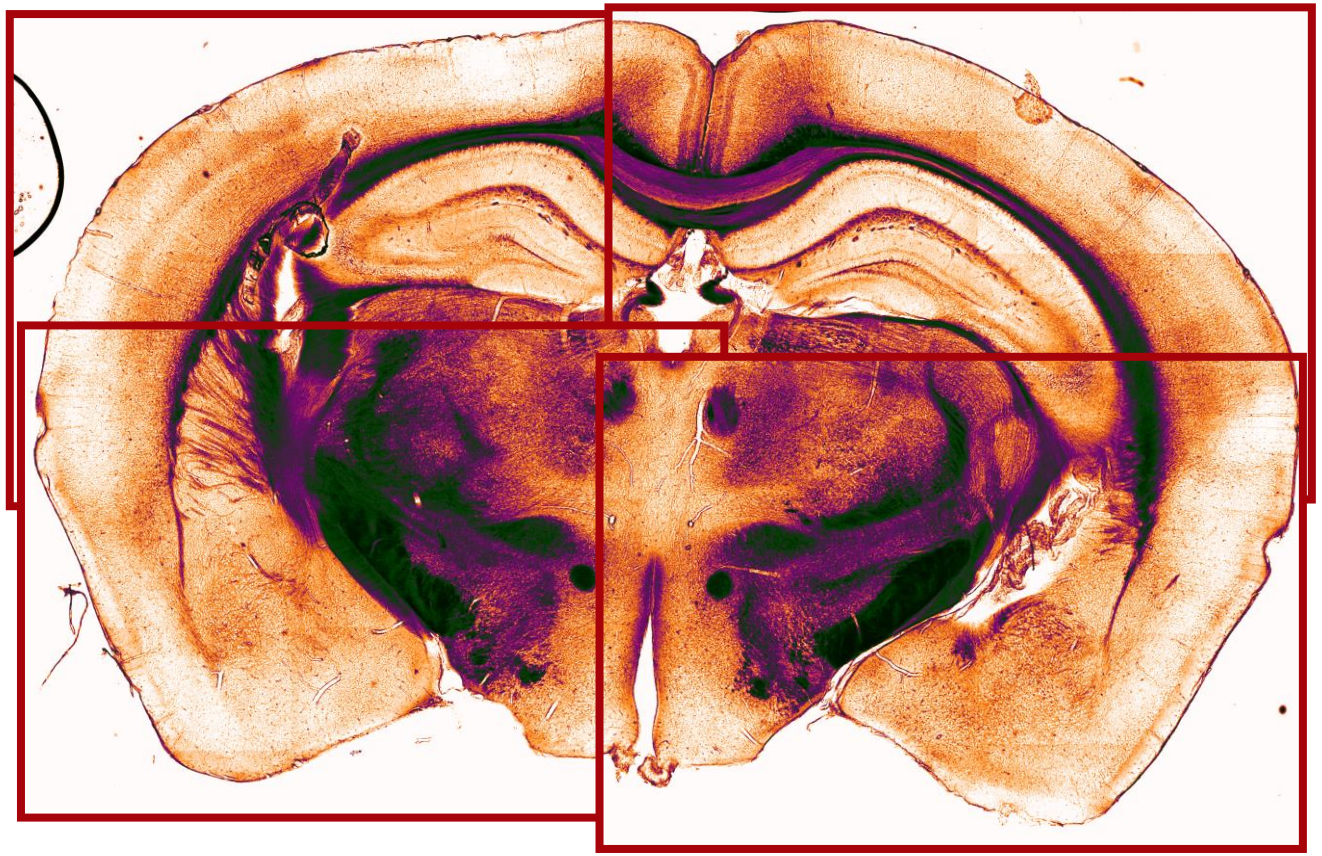
Alignment based on overlap

- Constant overlap/varying overlap
- Post processing needed
- High accuracy

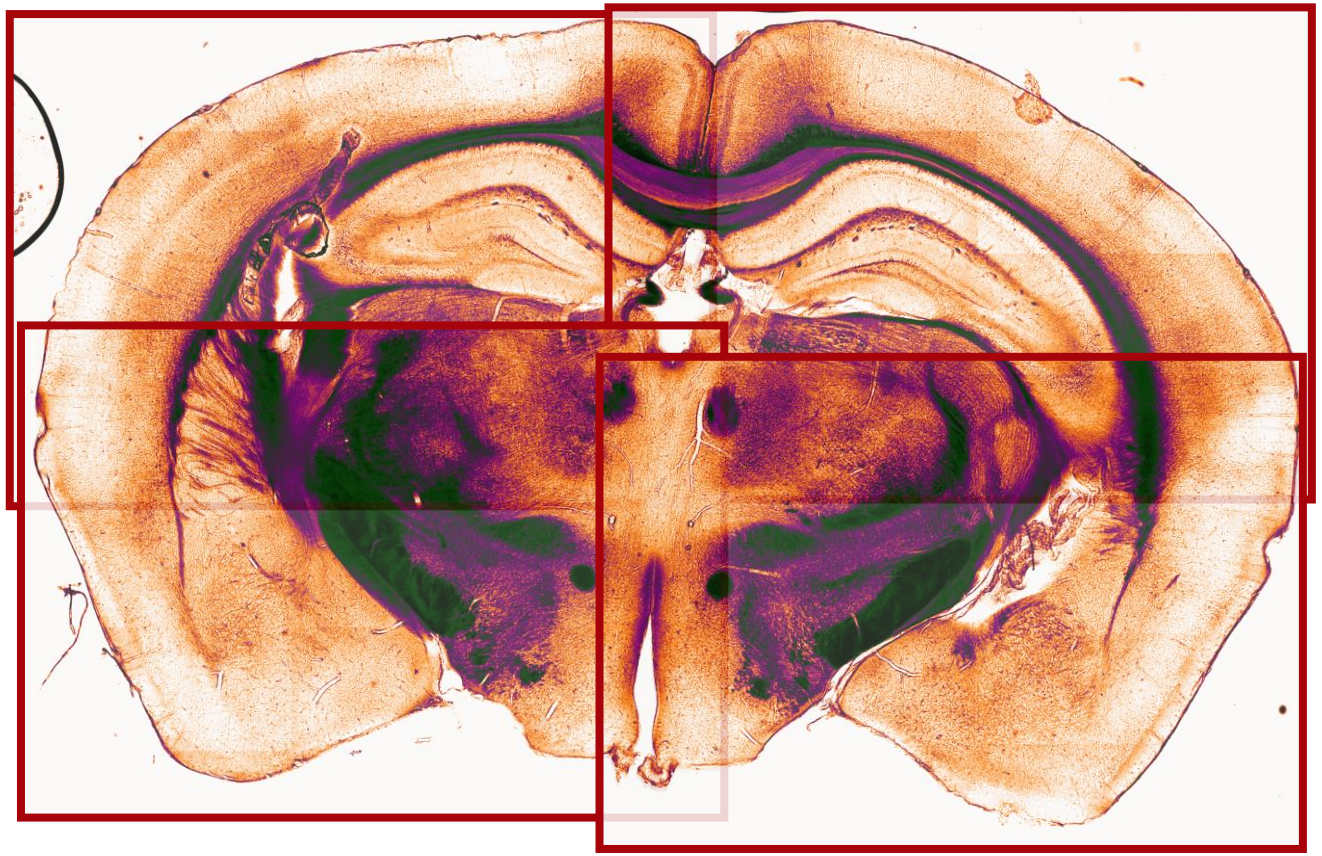
Alignment based on overlap



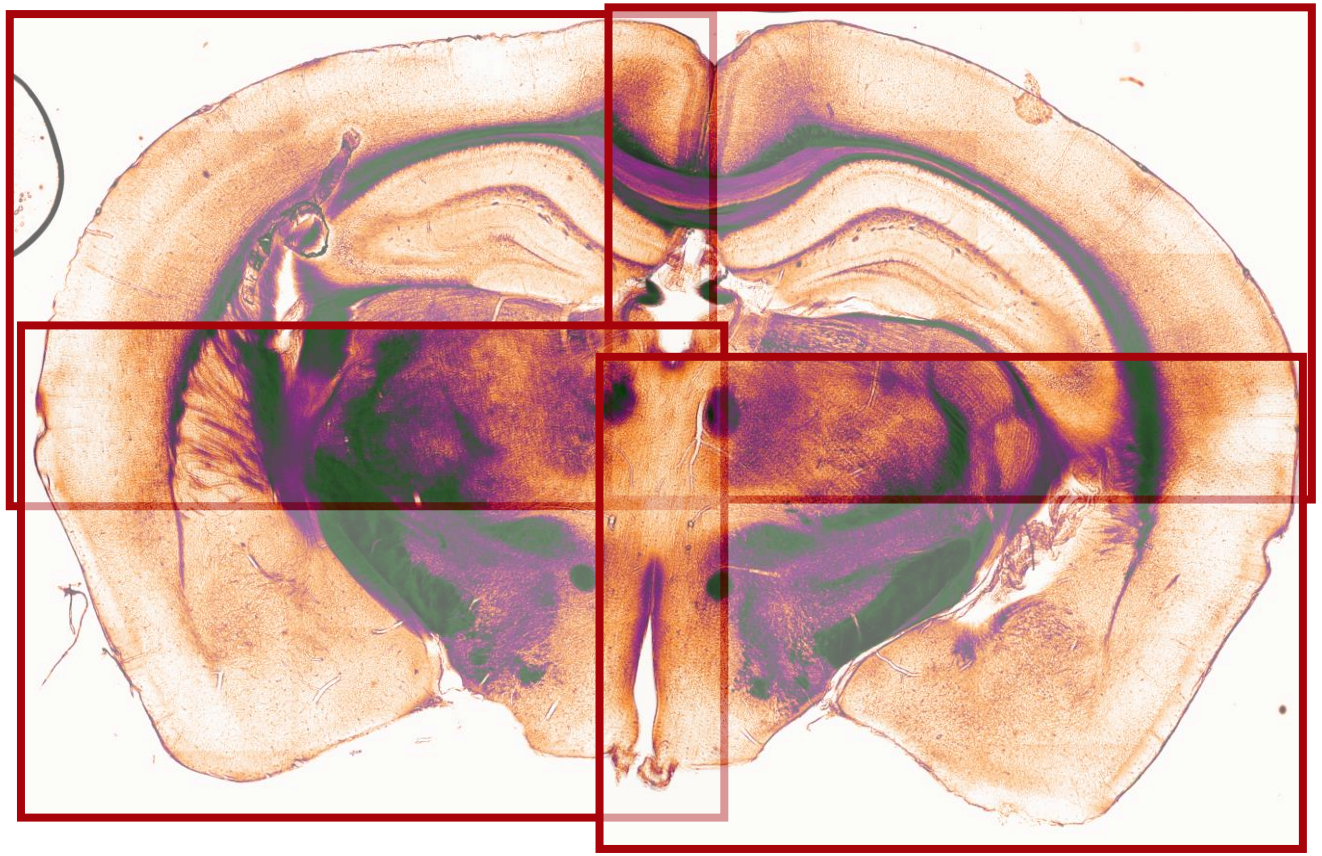
Alignment of individual tiles



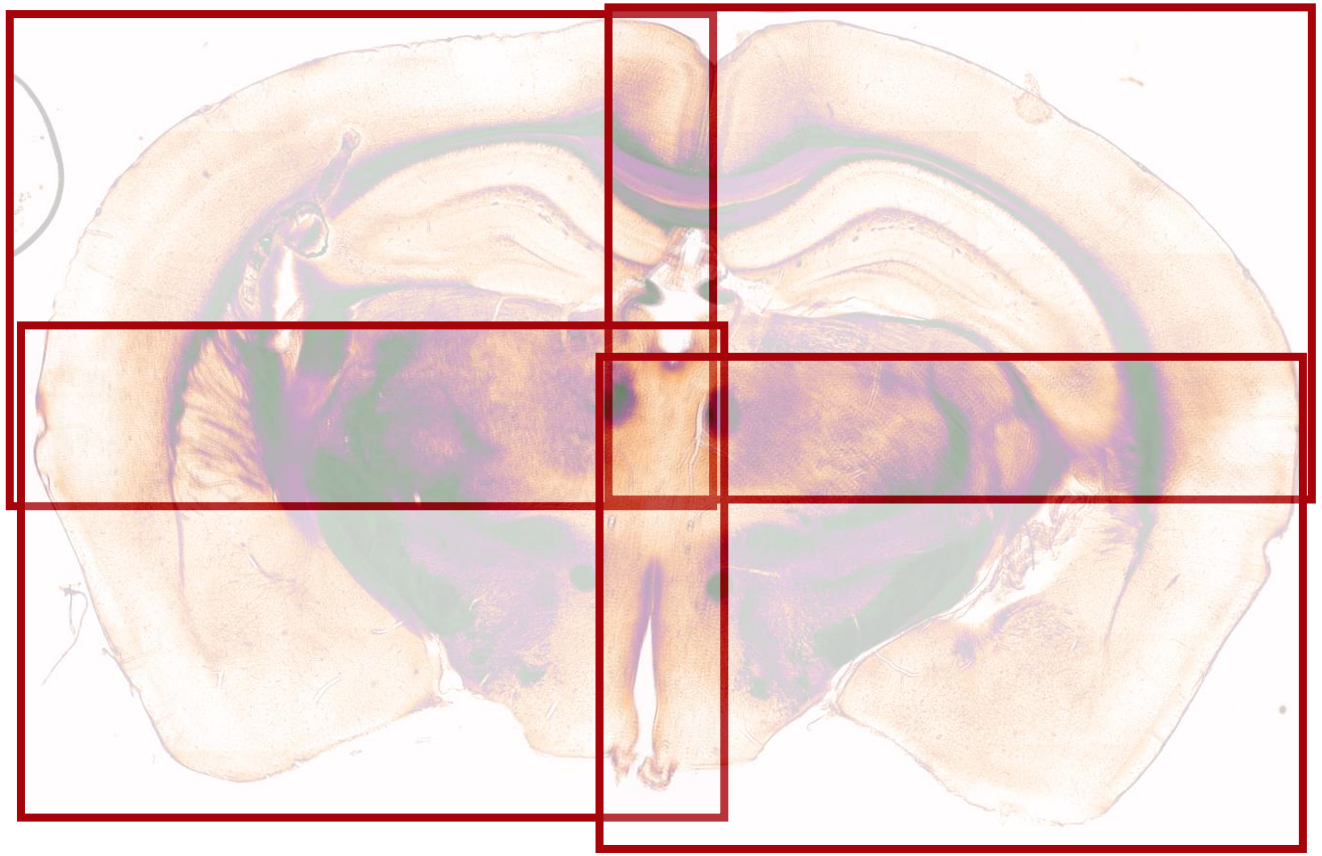
Alignment of individual tiles



Alignment of individual tiles



Alignment of individual tiles



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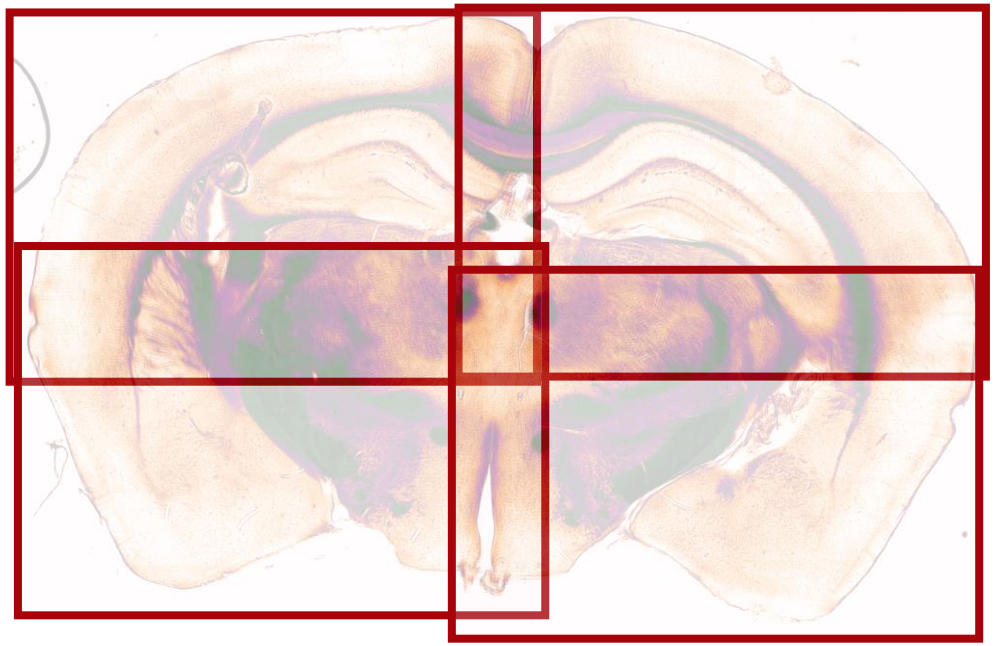
Stitching Tasks

- Define overlap region
- Align individual tiles
- Transformation
- Image fusion



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