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Optic and Microscopy elements

Microscopy for dummies (or not)
Part 1

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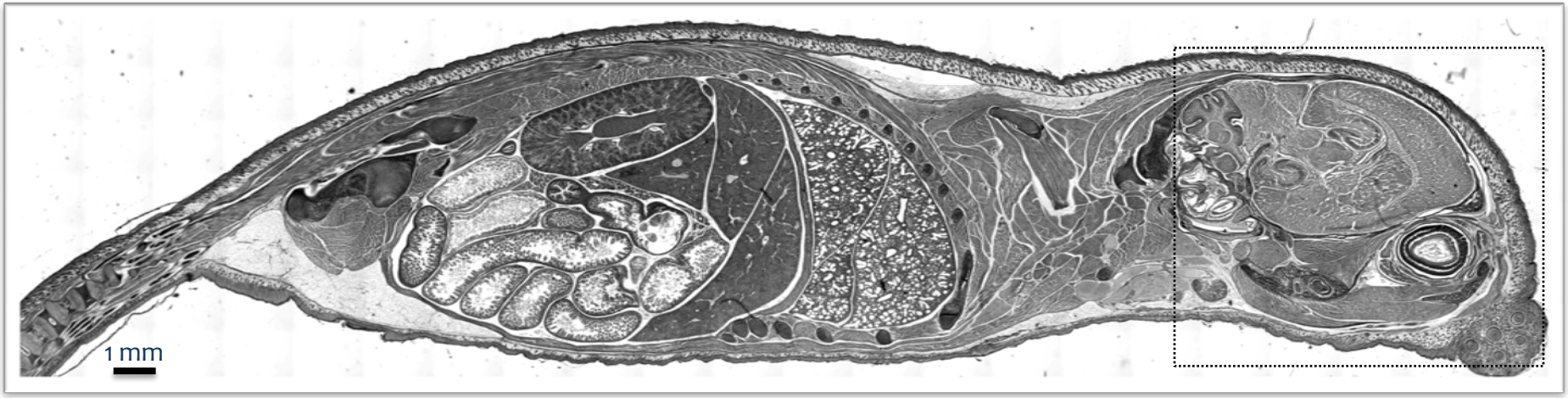
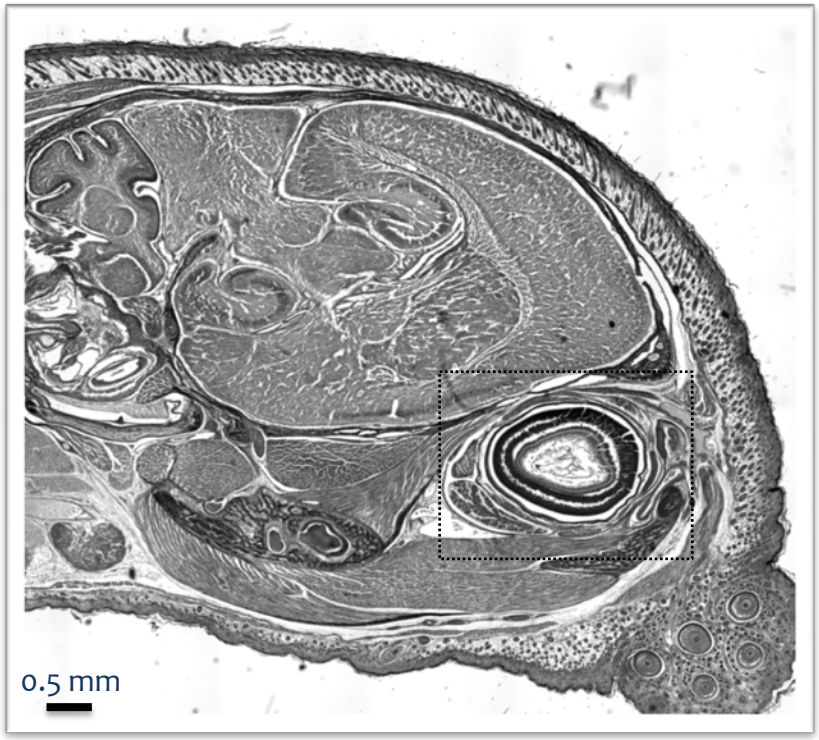
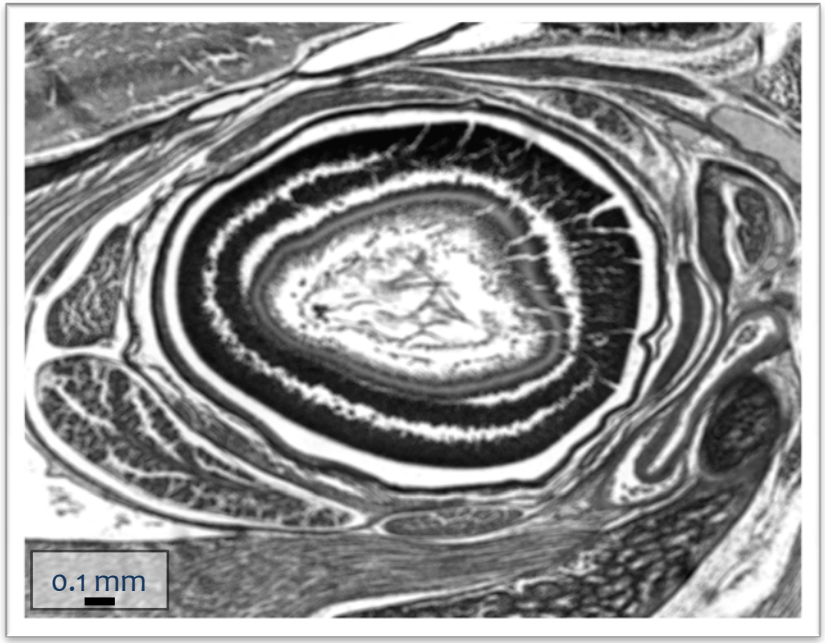
Some microscopic images

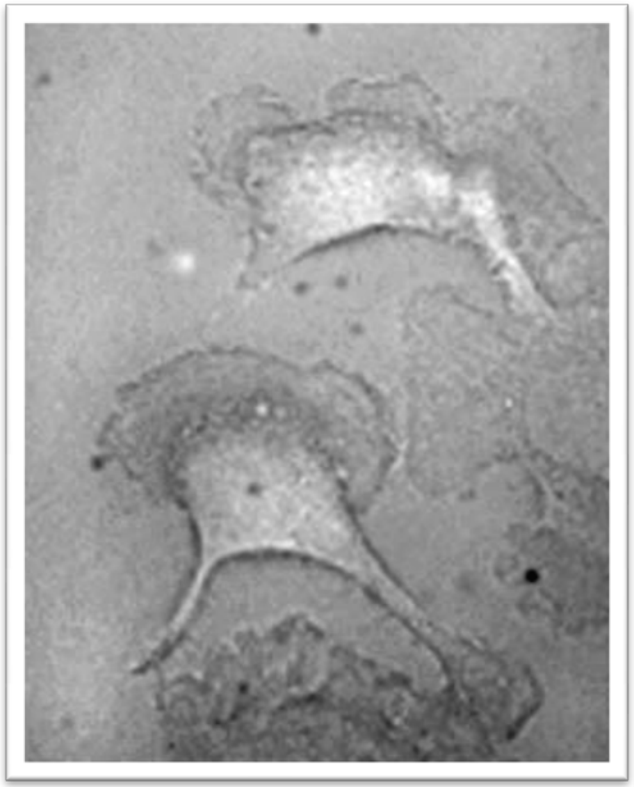




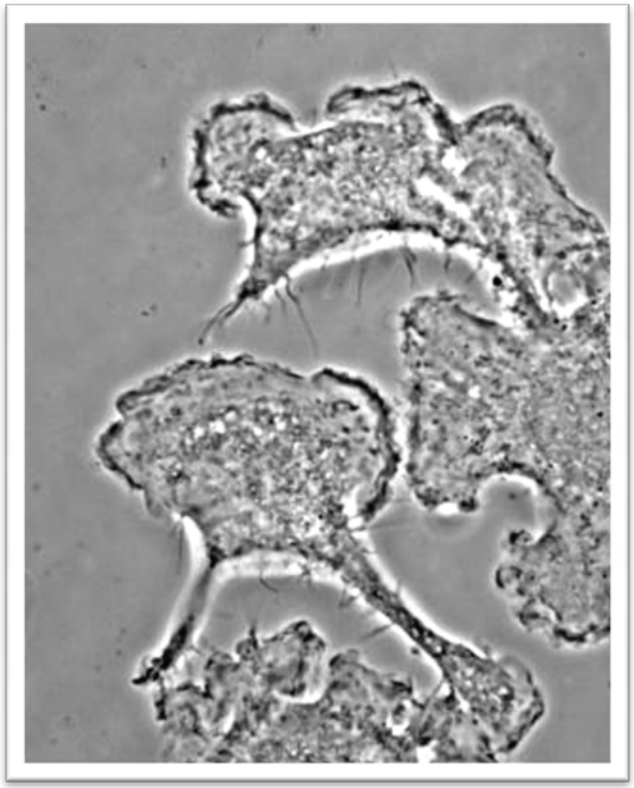


Some microscopic images

Bright field

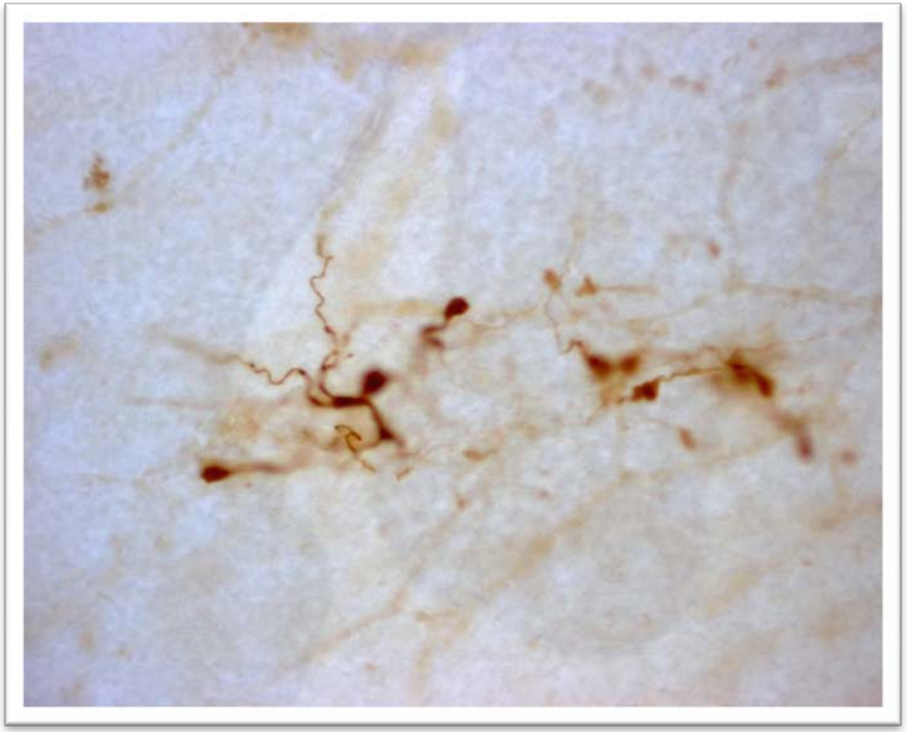


Phase contrast



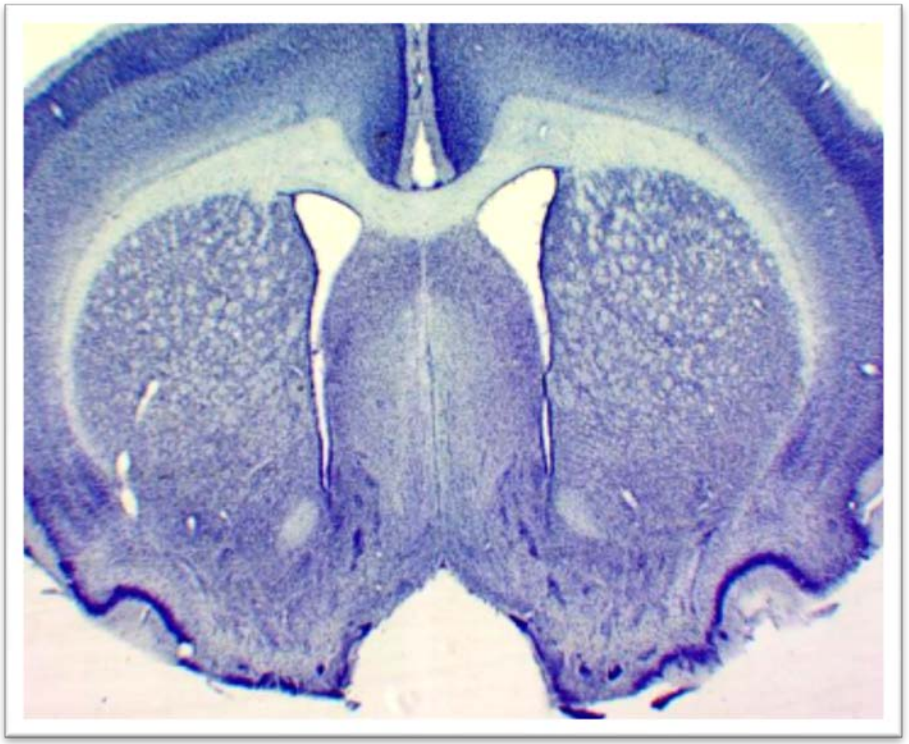
Differential interferential Contrast (DIC) Nomarski contrast

Some microscopic images



D.A.B

Cresyl



Program

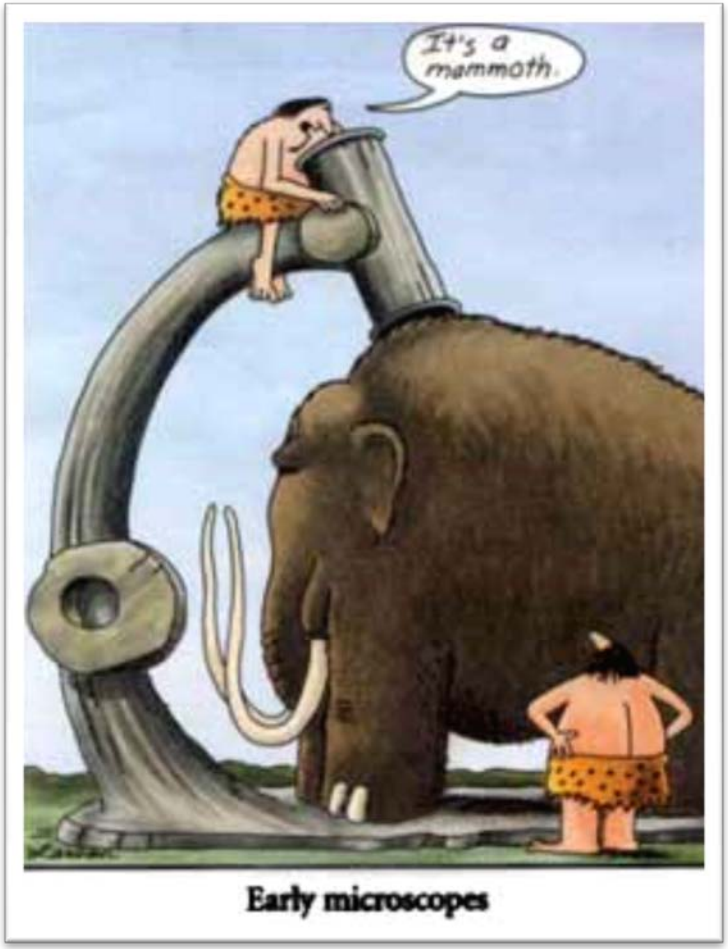
- General points
- Illumination
- Detection
- Aberrations and image deterioration
- Resolution and acquisition
- Contrast improvements

General points

Microscopy have multiple goals:

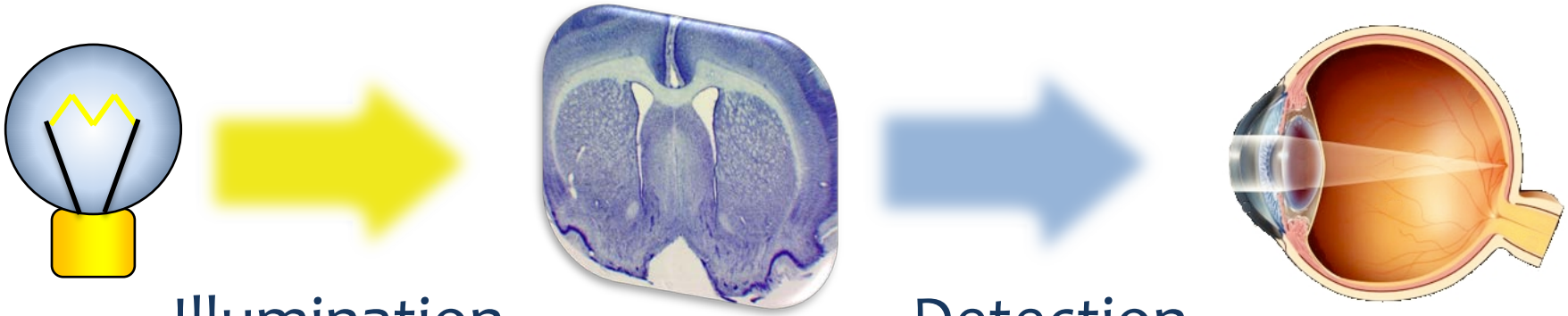
- **Make details visible** with human eyes or numeric camera
- **Resolve details** in images,
- **Measure and detect** light signals,
- Give **enlarge** image of small objects.

Observations often occur at the end of the protocol. We have to take into account, since the beginning of the experiment, of microscope limitations and constraints.



General points

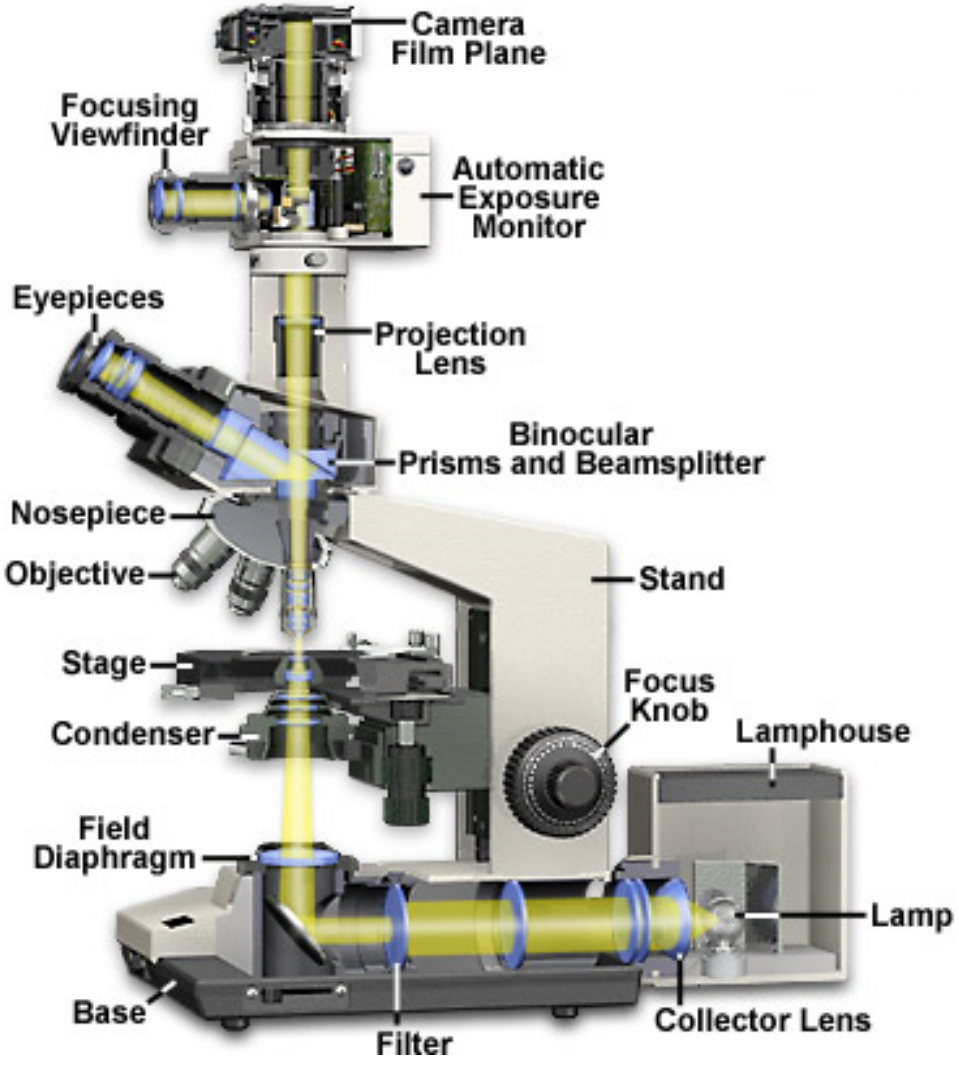
- We are able to see any object uniquely through the light that originate from it.
- This light is generate:
  - From transmission, through the sample
  - From emission (secondary emitter: fluorescence) after excitation (from a primary light source or any other energy source).
- A microscope is thus compose of two parts:
  - Illumination
  - Detection

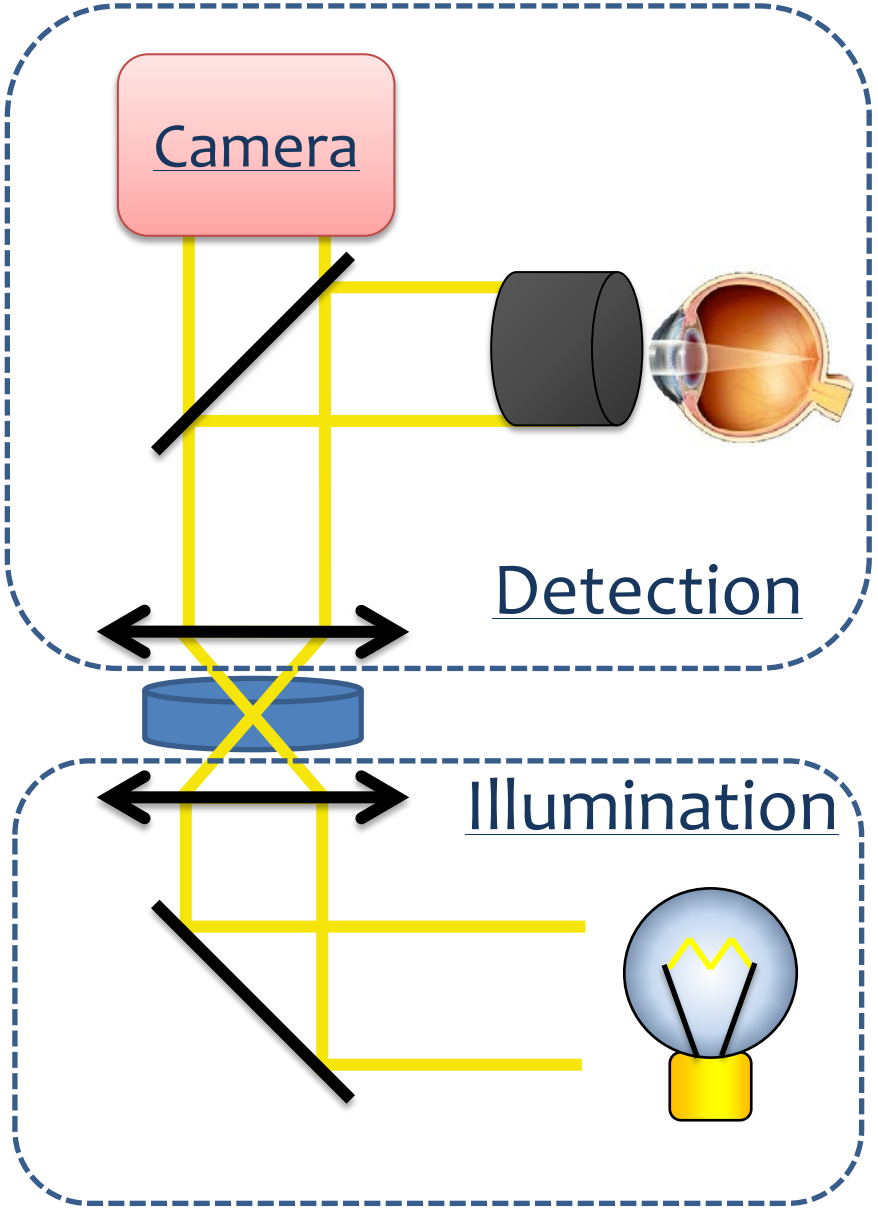


Illumination Detection

General points

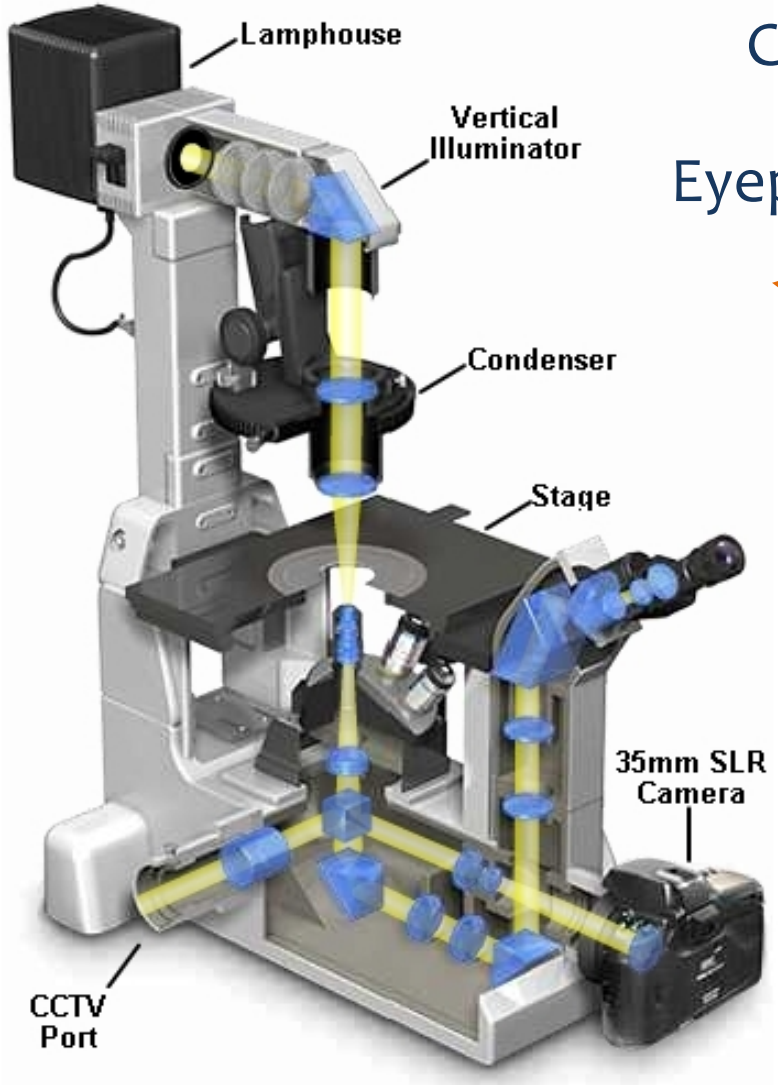
Upright microscopes

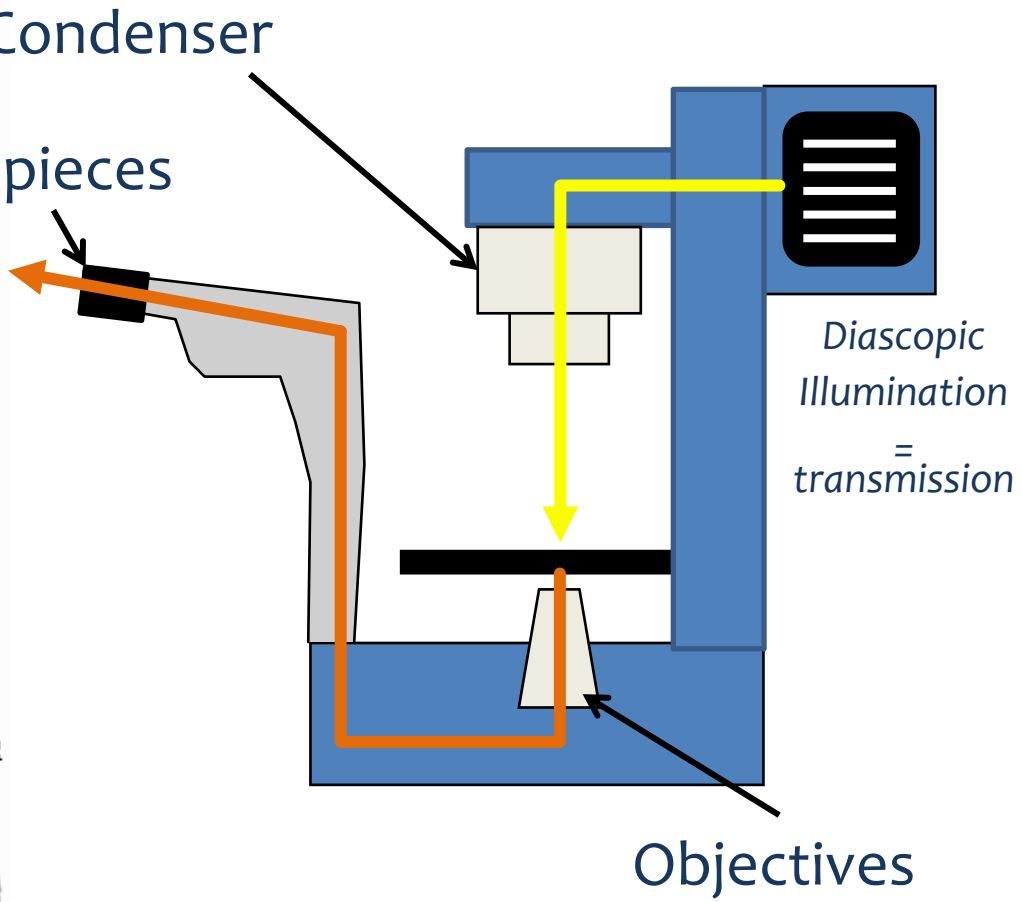




General points

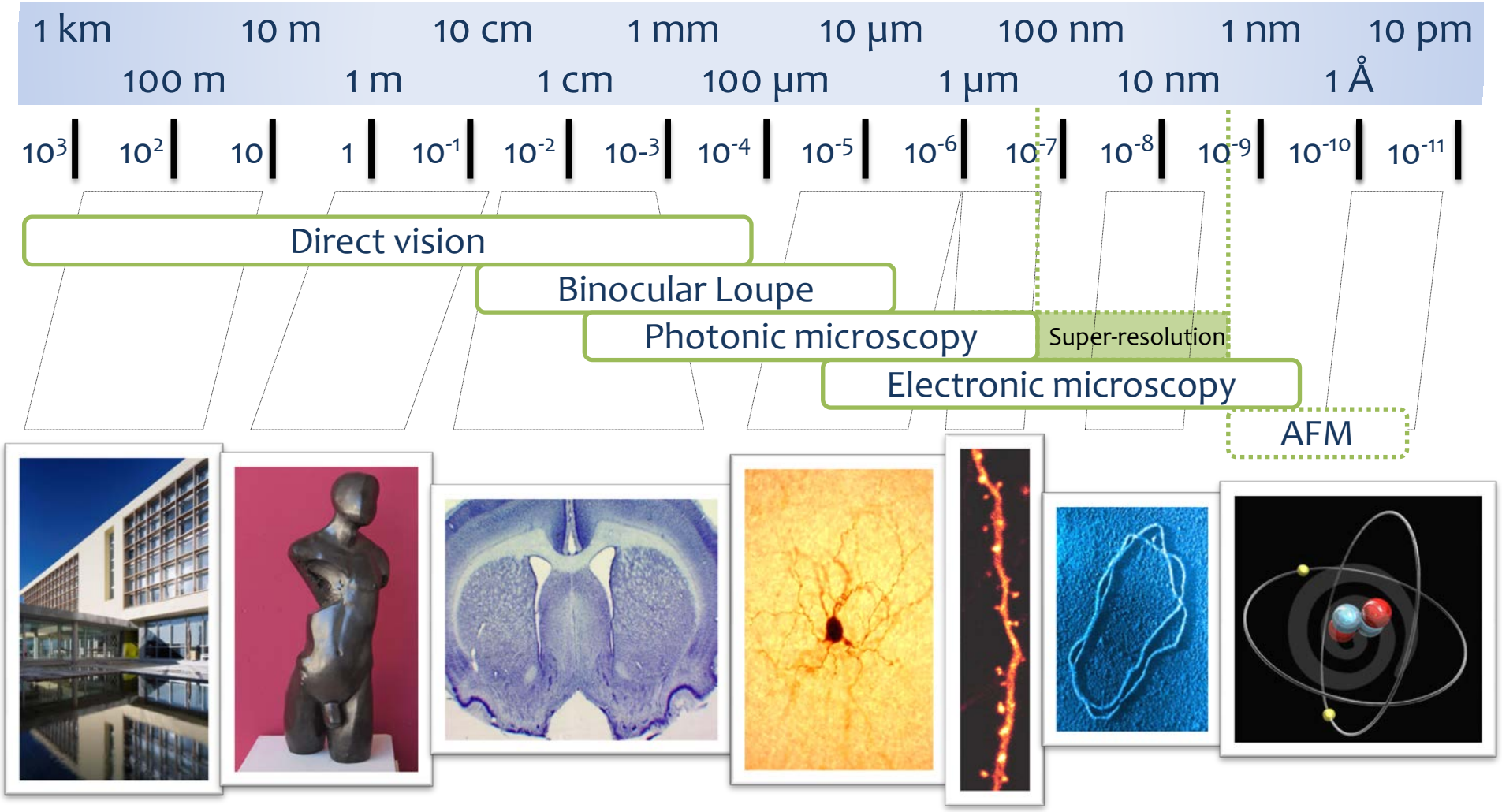
Inverted microscope





General points

Orders of magnitude



General points

Light nature and optics

Optics is the branch of physics which involves the behavior and properties of light, including its interactions with matter and the construction of instruments that use or detect it.

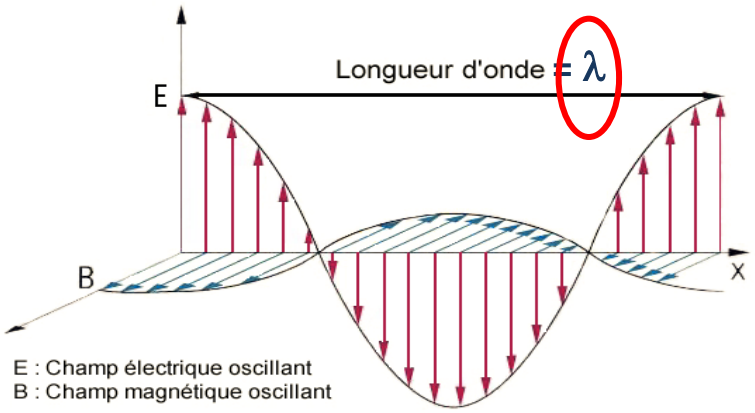
Old discipline, last more than 1000 year BC (Egyptian, Greeks...)

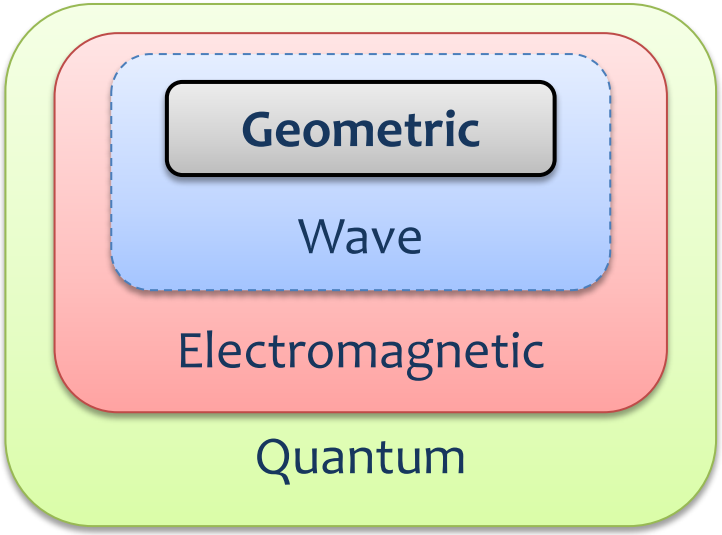
- In a first time, based on geometric optics: light rays travel in straight lines and bend when they pass through or reflect from surfaces: reflexion - refraction (Al-Hazem - Kepler - Snell - Descartes).
- End XVIIth, beginning of hostilities on light nature between:
  - Corpuscle theory that achieved to quantum optics in the XXth century: color, energy (Aristote - Euclide - Newton - Planck - Einstein)
  - Wave optics since XVIIIth that explain diffraction, interference, polarity (Huygens - Young - Fresnel - Maxwell) .
- Wave/corpuscle duality was theorized in 1927 by N. Bohr and closed the debate while giving birth to quantum electrodynamics.

General points

Light nature and optics

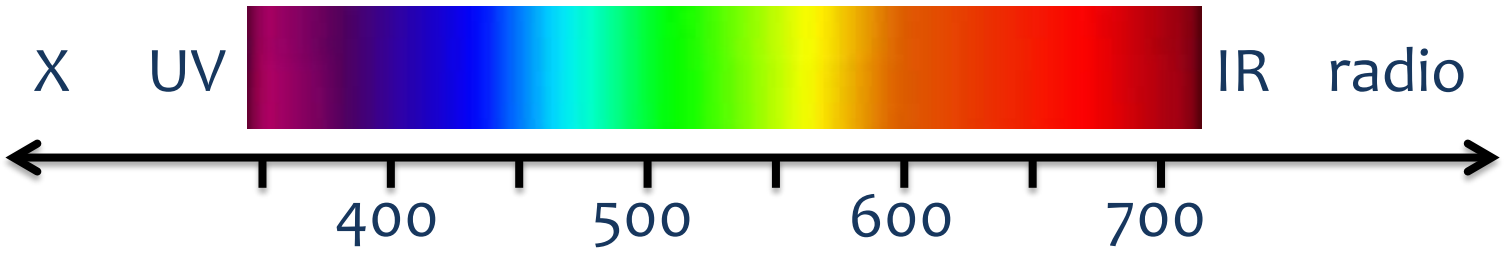
Light is composed of photons: γ
They propagate in straight lines as electromagnetic waves (Maxwell,1870).





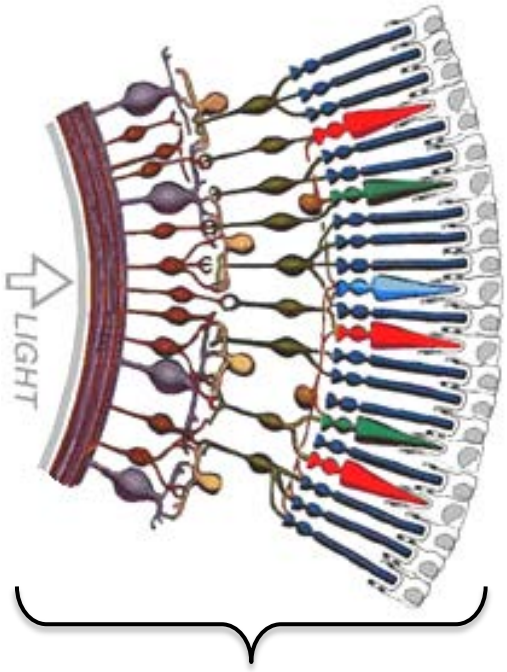
The wavelength give the color of emitted light.

Wavelength λ (in nm)



General points

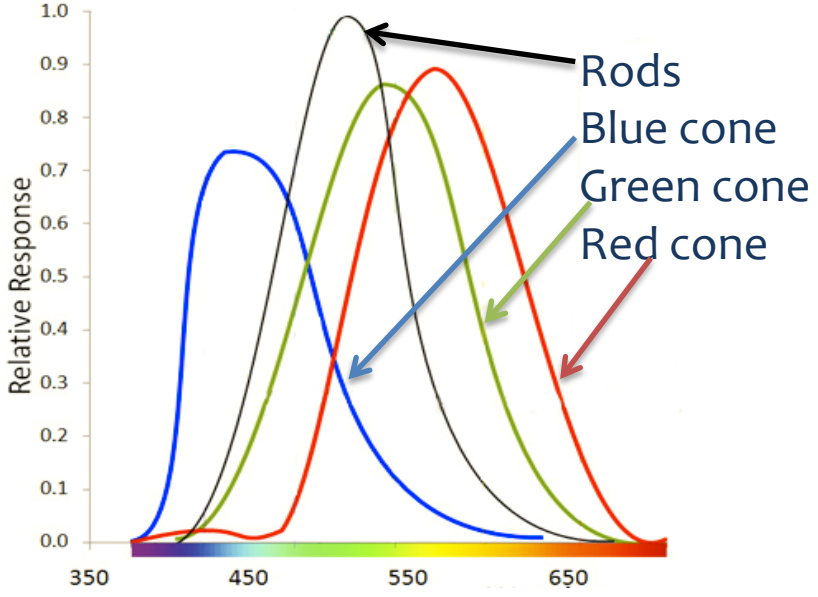
Physiology of vision (reminding ?!?)



Rétina +/- 300 µm

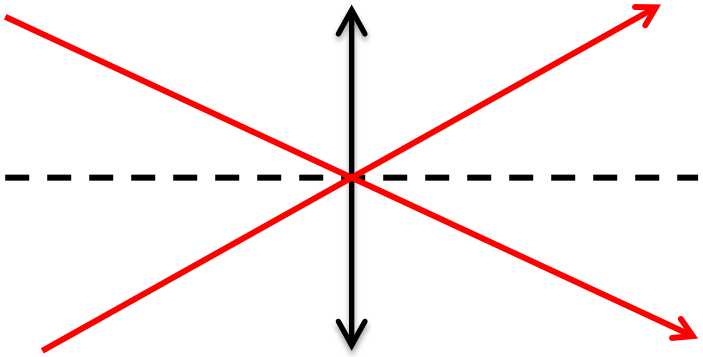
Eye resolution is link to the retina structure: Images of two points should impact two distinct photoreceptors in order to be distinguish. Minimal distance of two receptors is 2,2 µm. The resolution limit is affected by many parameters but in regular conditions it is around 3.10^{-4} radian = 1' arc that is about <150 µm from a distance of 25 cm.

The retina contains two major types of light-sensitive cells used for vision: the rods and the cones. Rods cannot distinguish colors, but are responsible for low-light monochrome vision. Cones are responsible for color vision.

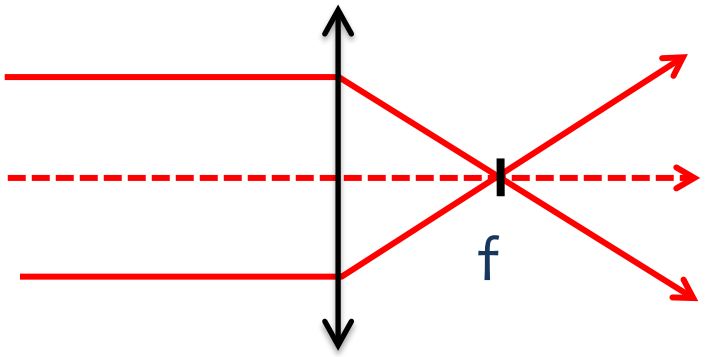


General points

Optic tracks: lens (convex)



Light rays that go through the center of the lens are not deviated.

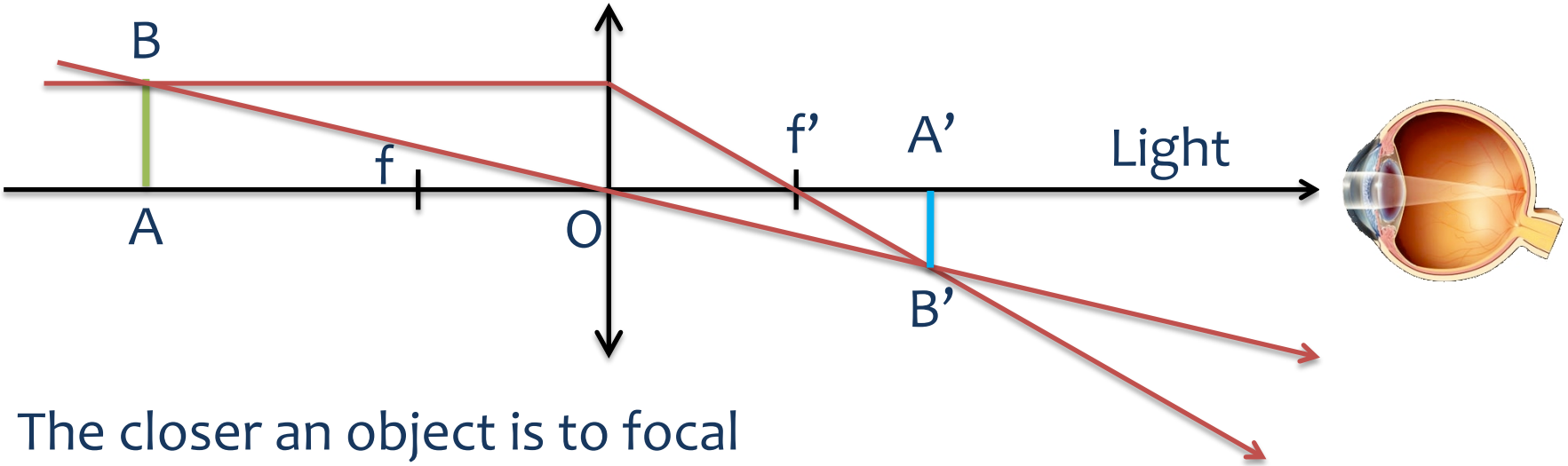


Light rays parallel to the central optic axe converge to the image focal points.

Gaussian conditions: Light rays might cross optics close to the center and as less as possible tilted comparing to the optical axis (paraxial rays).

General points

Geometrical optics (reminding ?!?)



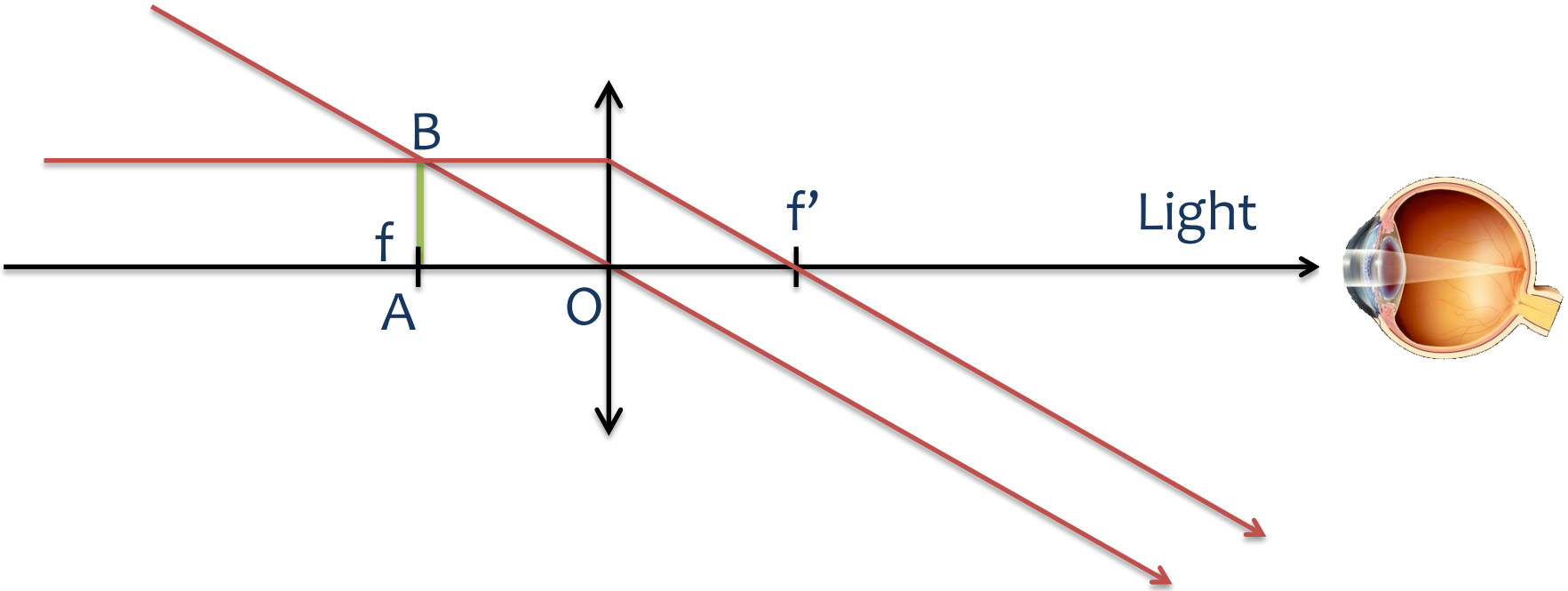
The closer an object is to focal point the bigger the image will be.
If the object is before the focal point, the image will be inverted.

Magnification of the lens: $\gamma = \frac{\overline{OA'}}{\overline{OA}} = \frac{\overline{A'B'}}{\overline{AB}}$

General points

Geometrical optics (reminding ?!?)

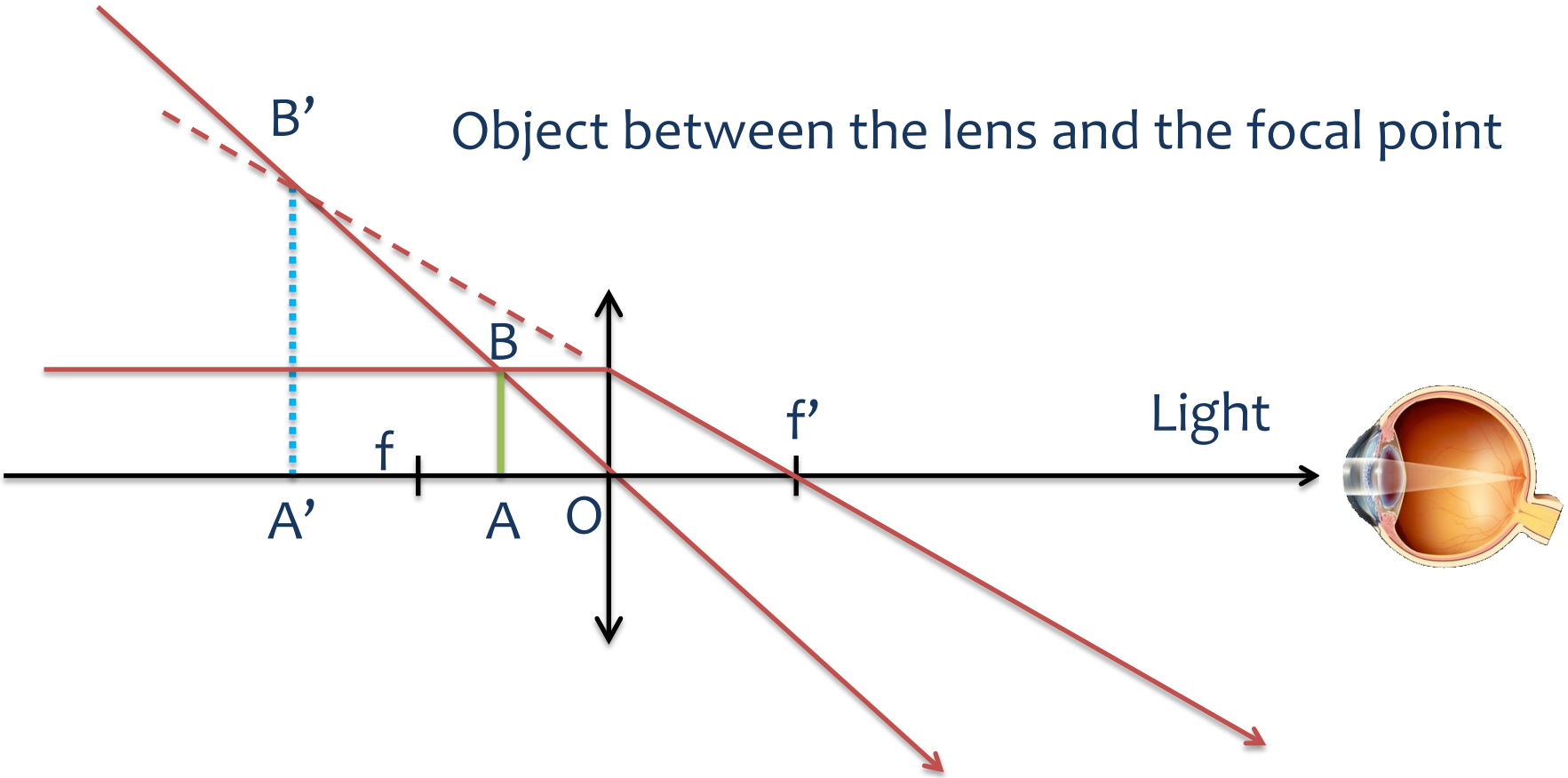
Object at the level of focal point



Inversed image at infinity

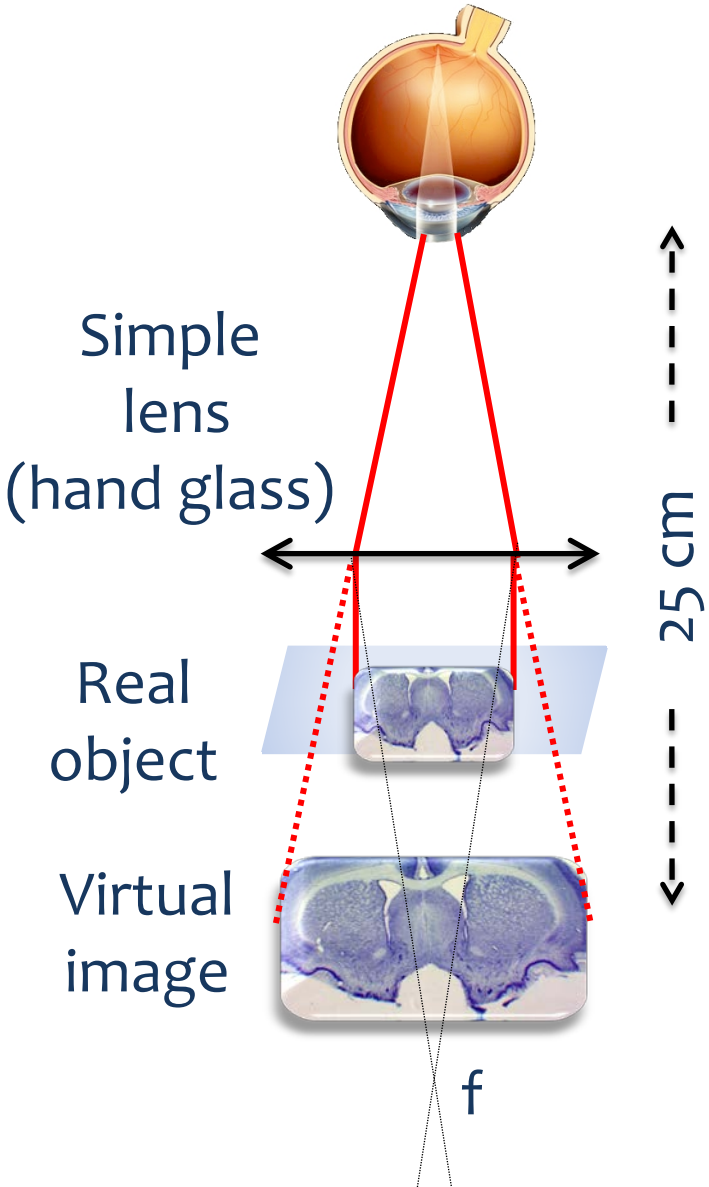
General points

Geometrical optics (reminding ?!?)



Virtual image in the same orientation as AB

General points



Practically

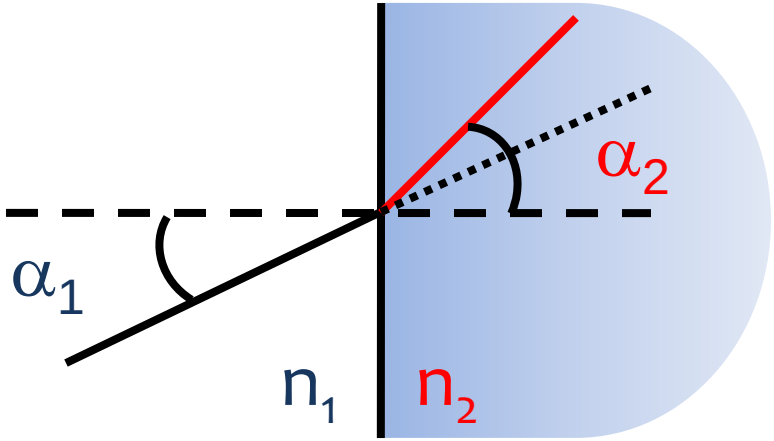
Simple lens (hand glass): eyes perception of the image as it is far as 25 centimeters.

Virtual image (non-inverted): the image appear in the same side of the objective as the object, it can't be seen in a screen.

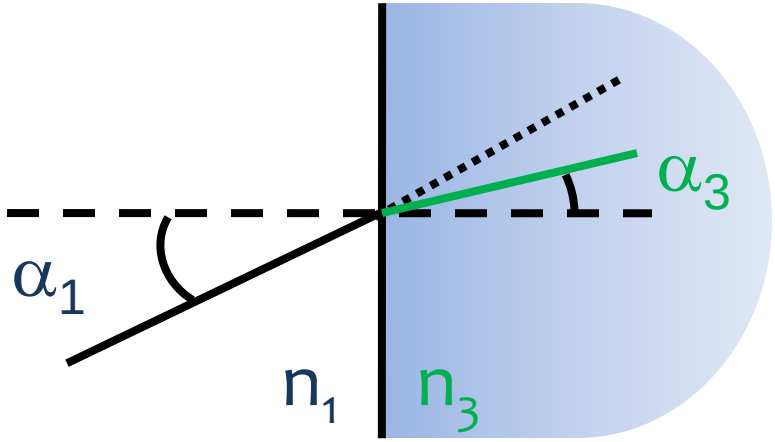
Transmitted light pass through the slice and enter in the lens to be refracted and focalized to produce the image detected by the retina.

General points

Interfaces and refraction



$n_1 > n_2$



$n_1 < n_3$

Nature 455 p 299 : Farewell to Flatland by Ortwin Hess

$$n_1 \sin\alpha_1 = n_2 \sin\alpha_2$$

Snell-Descartes' law

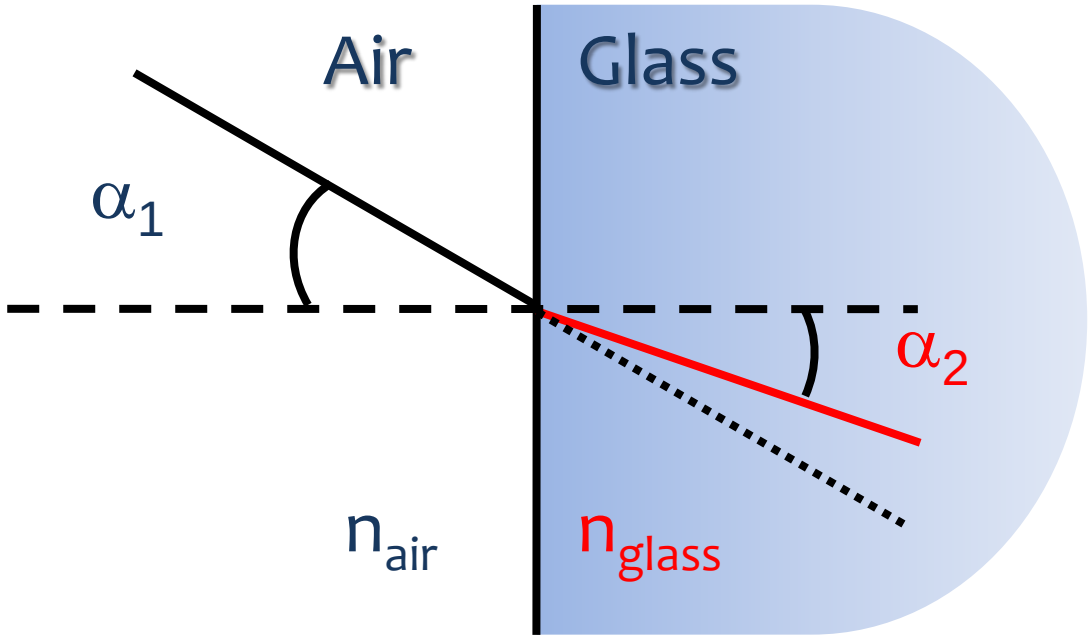
General points

Interfaces and refraction

$$\sin\alpha_1 = n_2/n_1\ (\sin\alpha_2)$$

Example : $\alpha_1 = 30°$ $n_{air}/n_{glass} = 1/1{,}52$

$\alpha_2 = 19{,}2°$



Optical index (n) relative values for some materials

Air	1,00029
Glace	1,31
Eau	1,333
Alcool éthylique (C_2H_5OH)	1,36
Quartz fondu (SiO_2)	1,4584
Tétrachlorure de carbone (CCl_4)	1,46
Térébenthine	1,472
Benzène (C_6H_6)	1,501
Plexiglas	1,51
Verre crown	1,52
Chlorure de sodium (NaCl)	1,544
Verre flint léger	1,58
Polystyrène	1,59
Disulfure de carbone (CS_2)	1,628
Verre flint dense	1,66
Verre flint au lanthane	1,80
Zircon ($ZrO_2.SiO_2$)	1,923
Fabulite ($SrTiO_3$)	2,409
Diamant (C)	2,417
Rutile (TiO_2)	2,907
Phosphure de gallium	3,50

General points

Interfaces and refraction n : optical index

$$n = c/\nu \text{ so } n \geq 1$$

c : light speed in vacuum

ν : wave speed in the conducting material

$\nu_{Vacuum} = c = 299\,792\,458\ m \cdot s^{-1} \approx \nu_{air} = 299\,704\,764\ m \cdot s^{-1}$

so $n_{Vacuum} = 1$ and $n_{air} = 1.0002926$

$\nu_{water} = 224841533\ m \cdot s^{-1}$ so $n_{water} = 1.33335$

$\nu_{glass} = 197603687\ m \cdot s^{-1}$ so $n_{glass} = 1.51714$

$$\nu = f(\varepsilon, \mu) = f(\lambda, P, T\ldots)$$

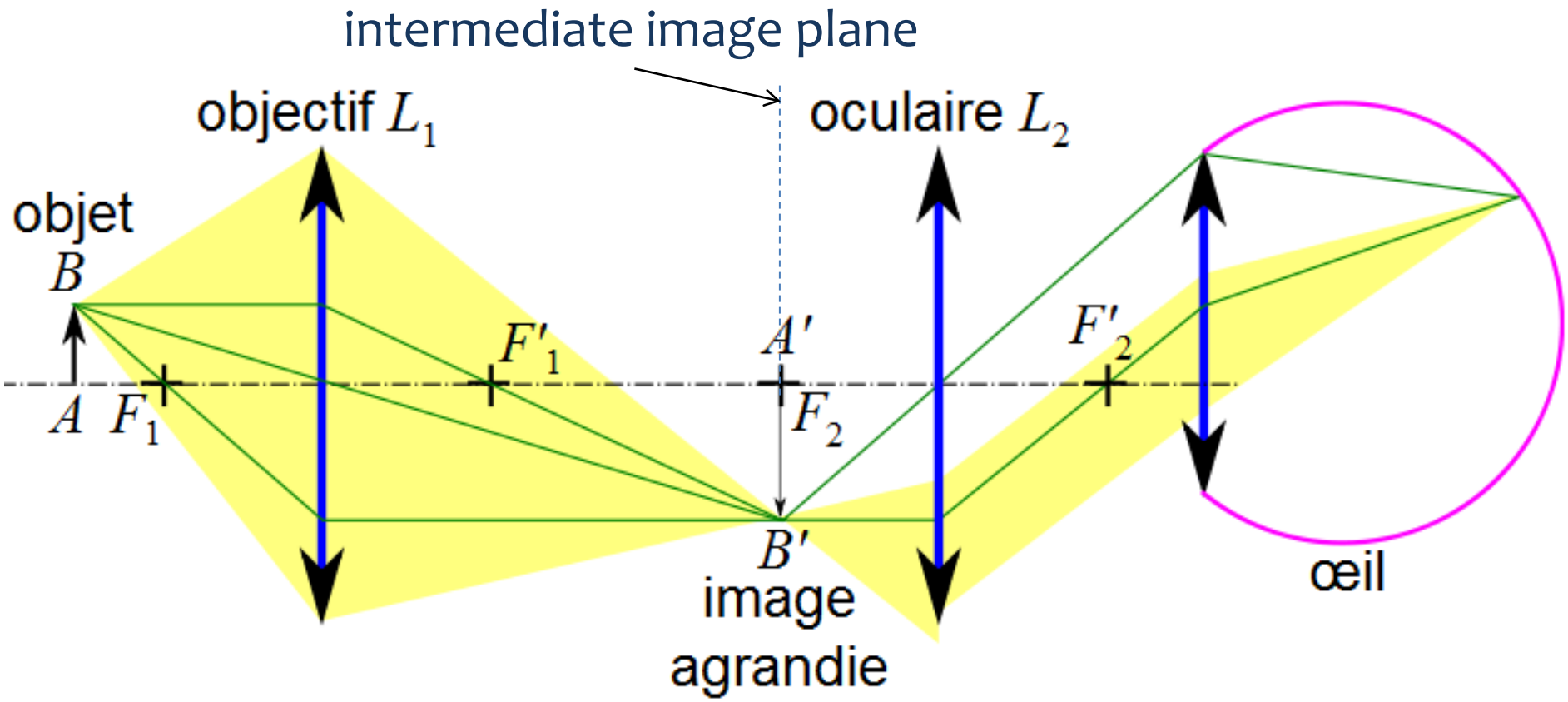
$\varepsilon\mu\nu^2 = 1$

ε : dielectric permittivity

μ : magnetic permeability

General points

Microscope principle



The objective (L1) give a magnified inverted real image A'B' of the object AB. This image play the role of a real object for eyepiece (L2) and produce a virtual enlarged image that is seen at infinity while A'B' is place in the F2 lens focal plan of eyepiece.

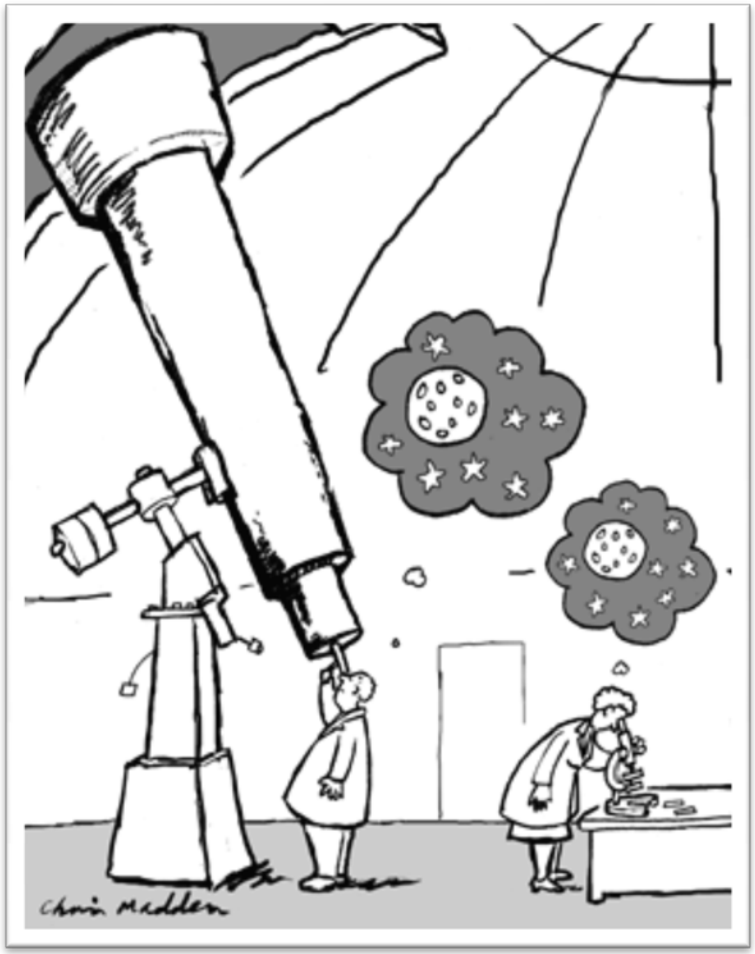
General points

Microscope parts

- Body
- Condenser
- Objectives
- Eyepiece /camera

Important concepts

- Numerical aperture
- Resolution
- Aberrations



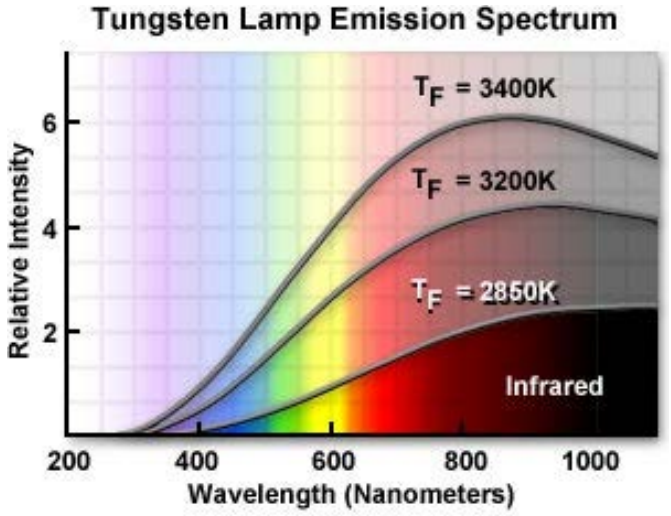
Program

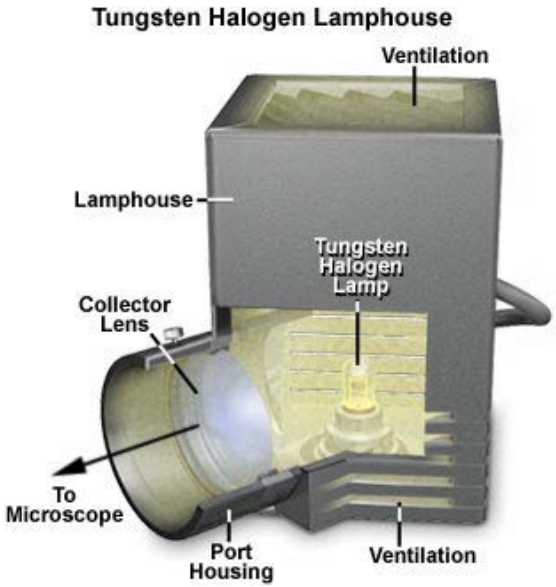
- ✓ General points
- ➢ Illumination
- ➢ Detection
- ➢ Aberrations and image deterioration
- ➢ Resolution and acquisition
- ➢ Contrast improvements

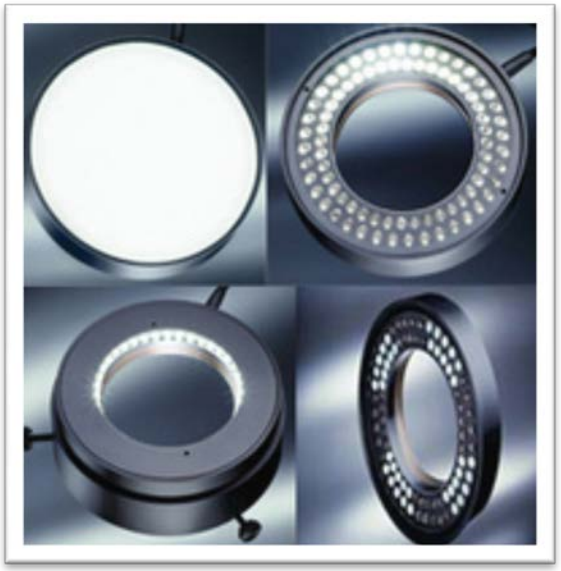
Illumination

Light sources

During a long time, light sources have been the sun or candles. It consist now of halogen lamp with 12 V Tungsten filament bulb and began to be replace with white LED.







Illumination

Condenser

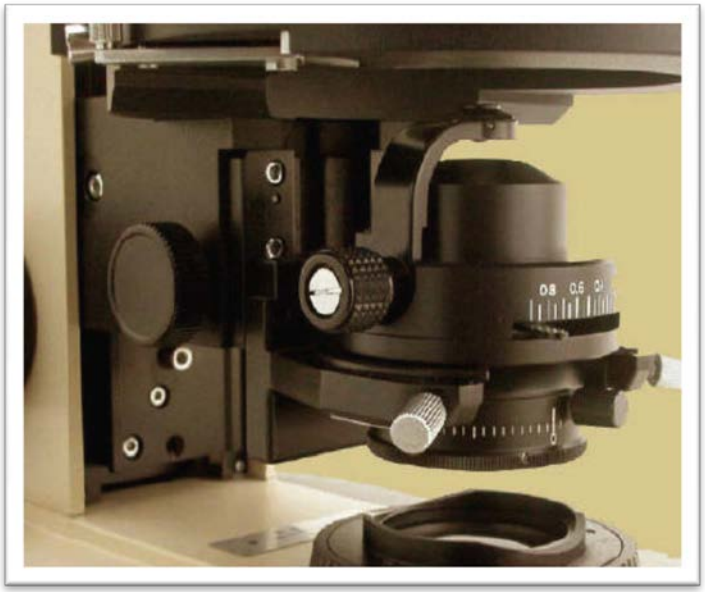
Condenser function is to concentrate the light on the sample.

Microscopes for beginner (or young students) are generally condenser less, where a concave mirror or white LEDS substitute it.

The field lighten by the condenser should be as homogenous as possible.

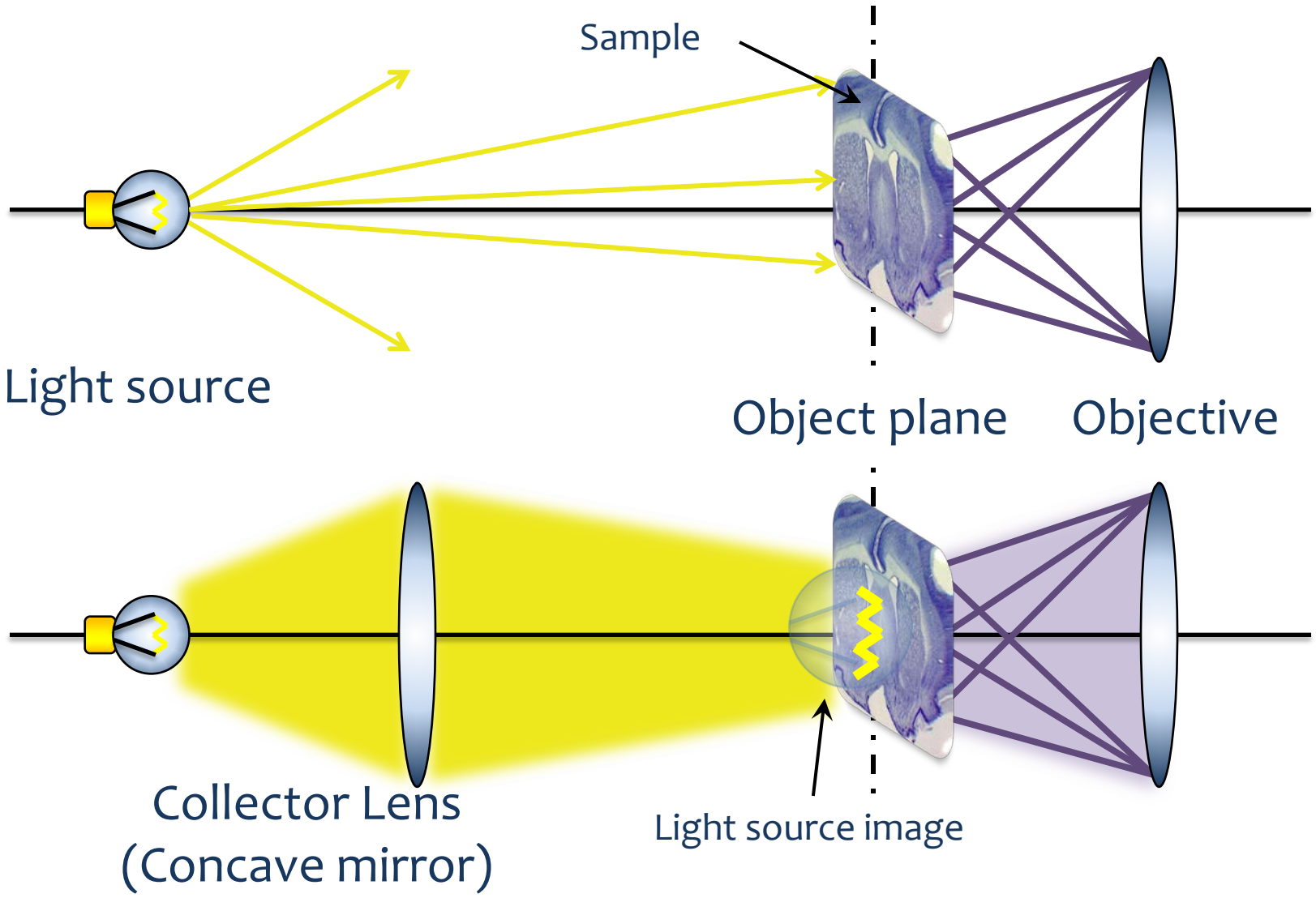
Condenser numerical aperture play a role on object illumination quality.

It should match with the objective one. There is some oil immersive condenser for NA>1 objectives.



Illumination

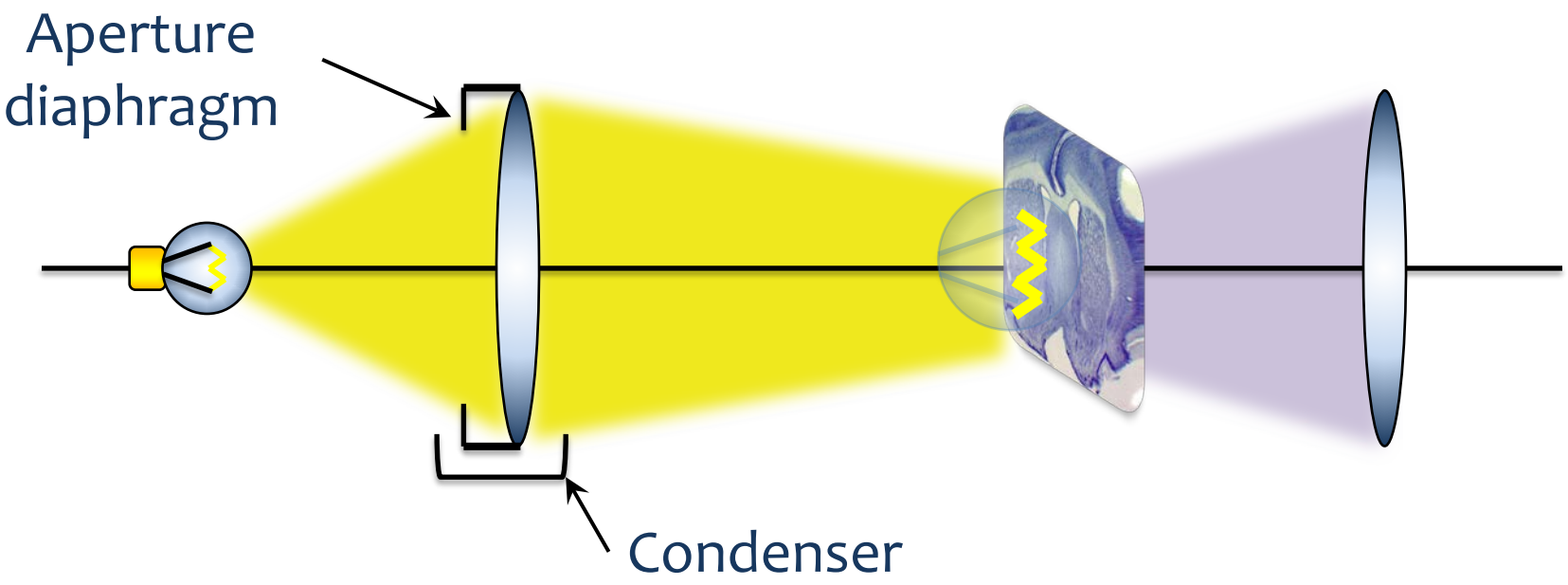
Critical illumination



Illumination

Critical illumination

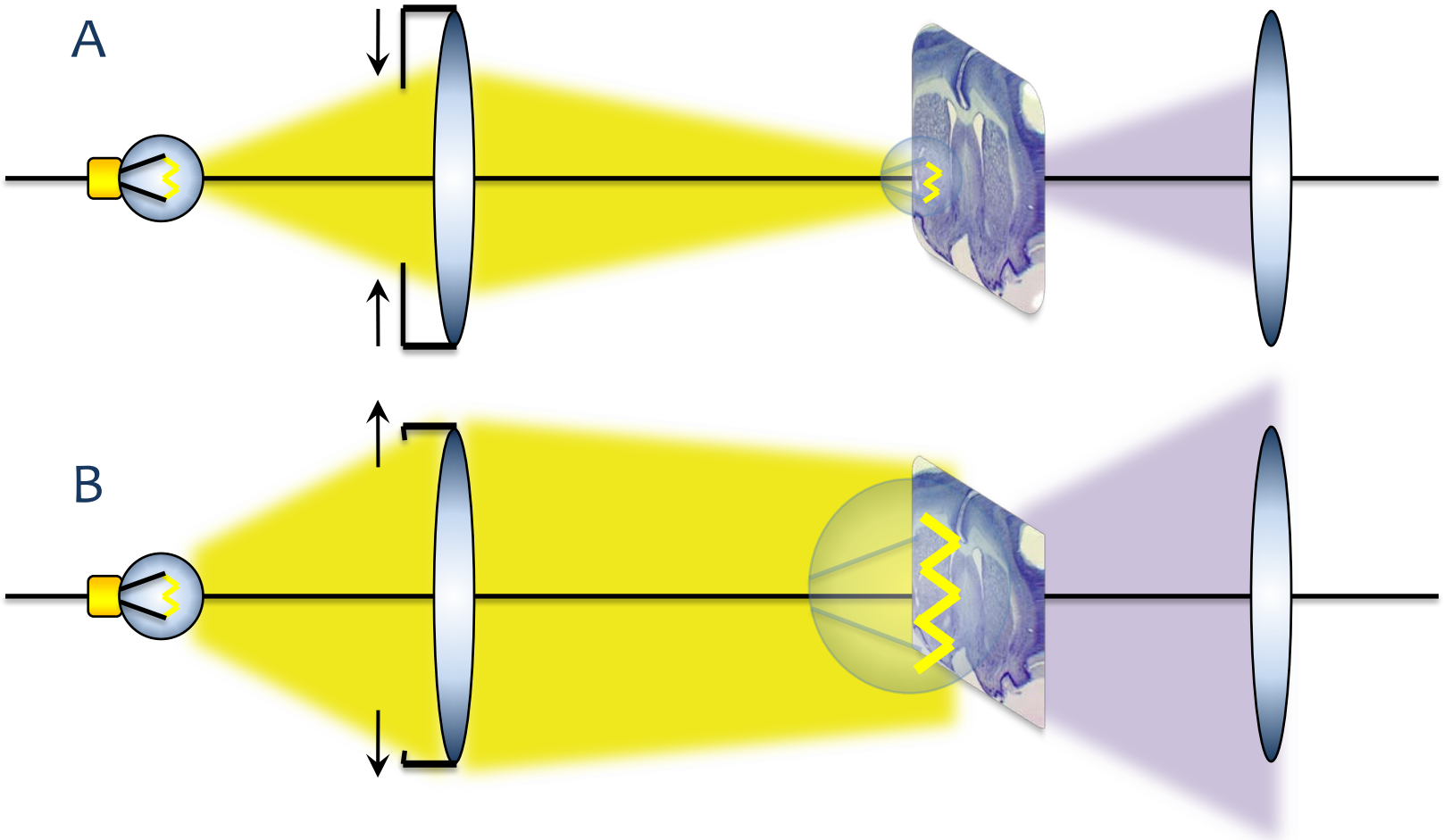
Simpler and less expensive, critical illumination focuses an image of a light source onto the specimen for bright illumination. Critical illumination generally has problems with evenness of illumination as an image of the illumination source (for example a halogen lamp filament) is visible in the resulting image. Homogenous light sources such as a flame or the sun give more even illumination.



Illumination

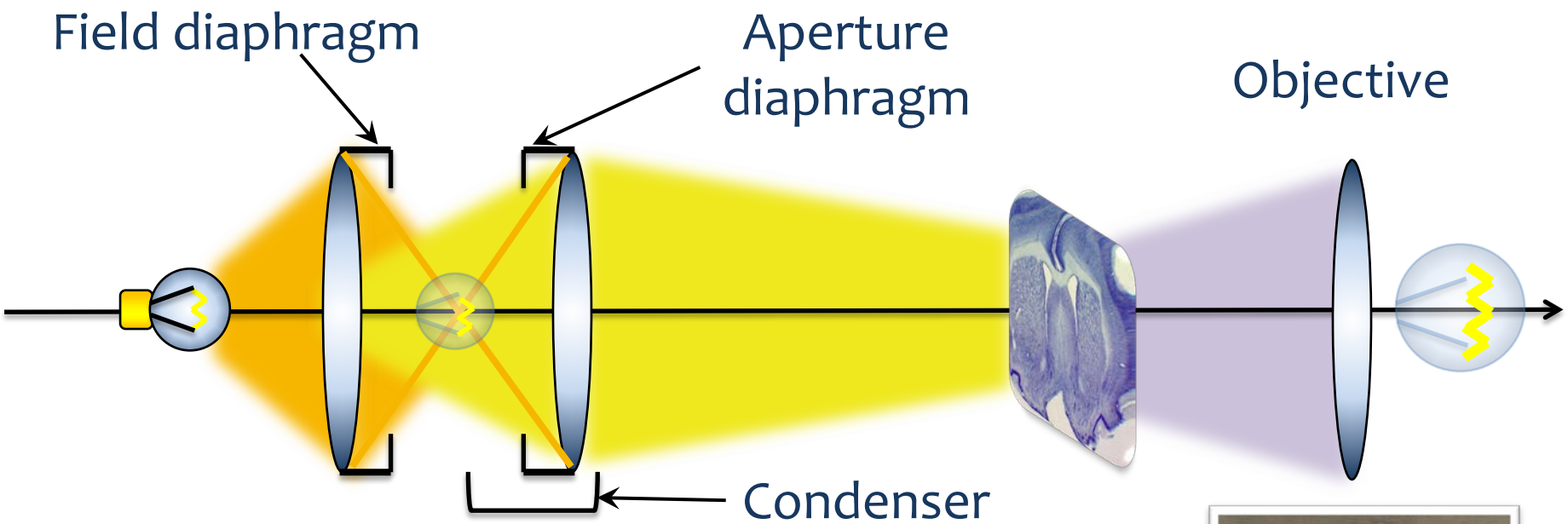
Critical illumination

Wrong tuning of the aperture diaphragm:
A) The objective lens entrance is not filled (resolution loss) or B) all the light do not go in the objective (luminosity and/or contrast loss).



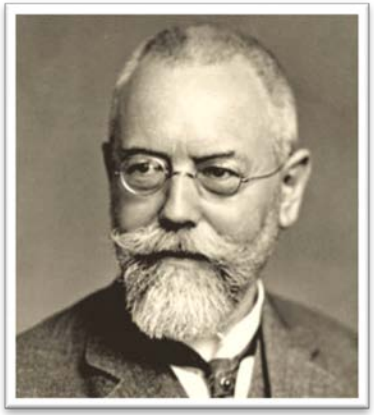
Illumination

Köhler illumination



Correct tuning obligatory:

- It give homogenous lightning of the sample,
- contribute to reach the best lateral resolution
- involve in depth of field and in image contrast.



August Köhler
(1866-1948)

Illumination

Diaphragm places

Halogen transmitted light lamphouse

Field diaphragm

Condenser positioning screws

Aperture diaphragm

Condenser

Grey (neutral densities) and/or colored filters

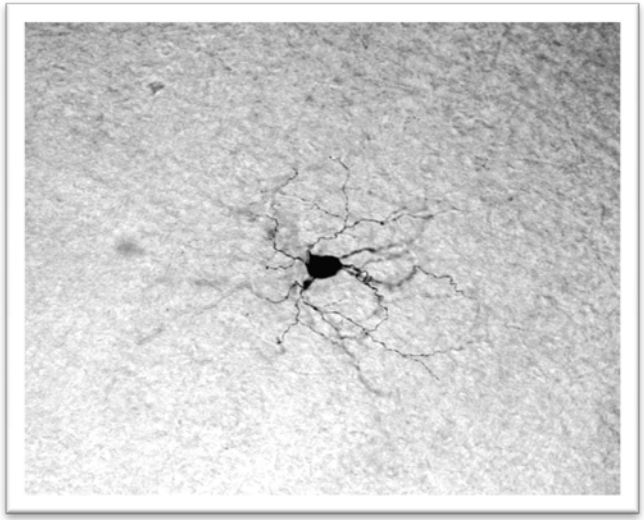
Condenser axial setting screw

Inverted microscope

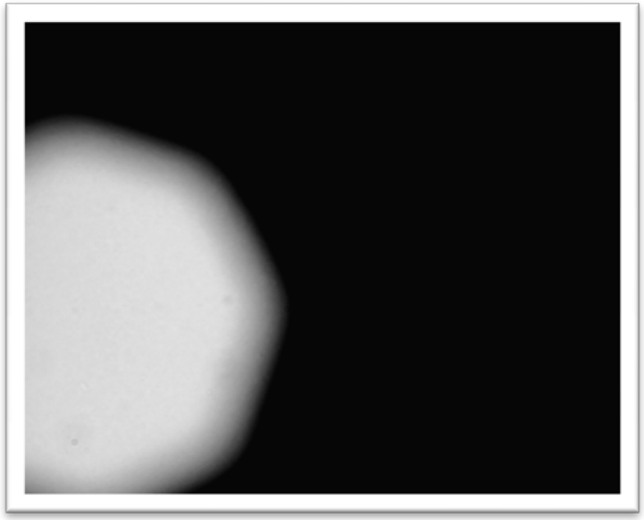
Illumination

Köhler setting (migth be done for each objective)

1) Open both diaphragms (field and aperture) and focus on a sample with low magnification objective.



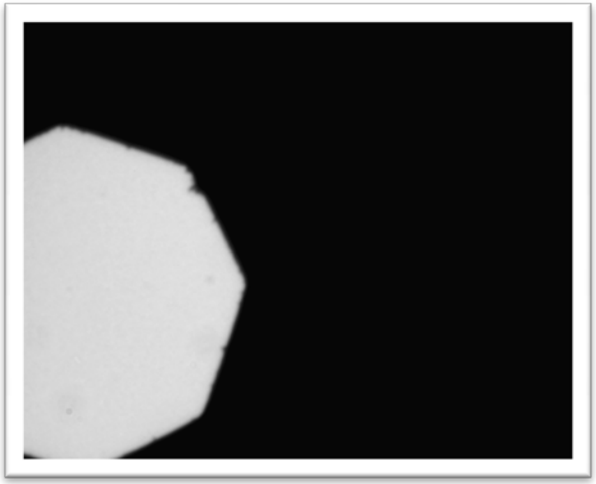
2) Move out the sample and close the field diaphragm in order to see the polygonal light area, blurry and not centered.



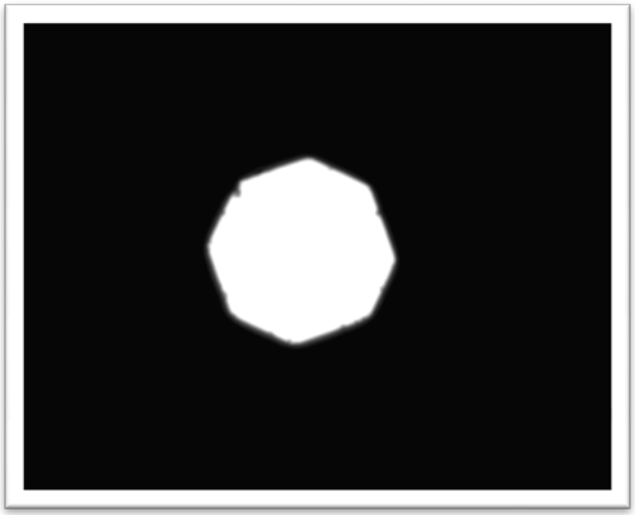
Illumination

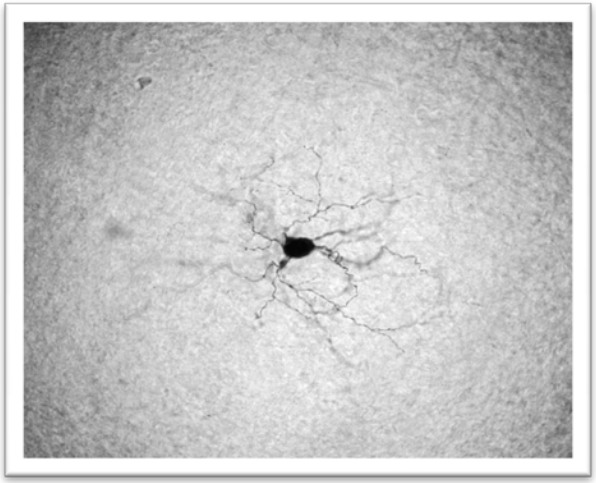
Köhler setting

3) Get the borders of the polygonal shape sharp playing with condenser focus screw.



4) Centered the polygon in the visible field with condenser positioning screws.





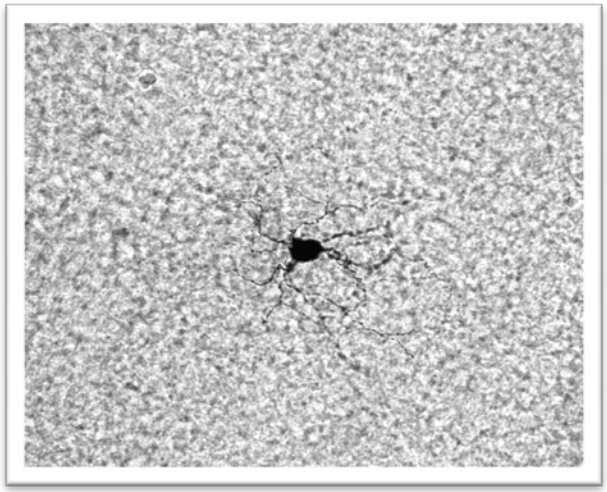
Illumination

Köhler settings

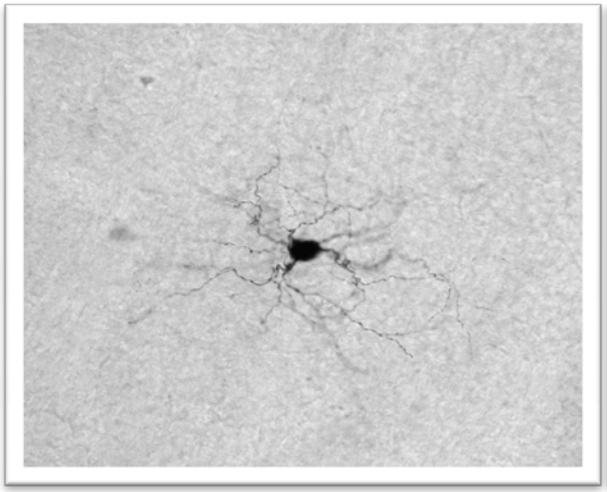
5) Open **field diaphragm** until perception limit of the eyepiece (tangential) in order to check alignment, open it at the end.

6) Set the **aperture diaphragm** if necessary to contrast or to play with depth of field.

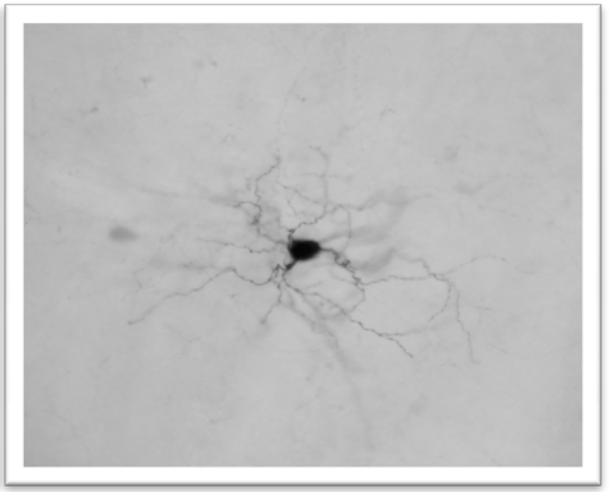
Closed



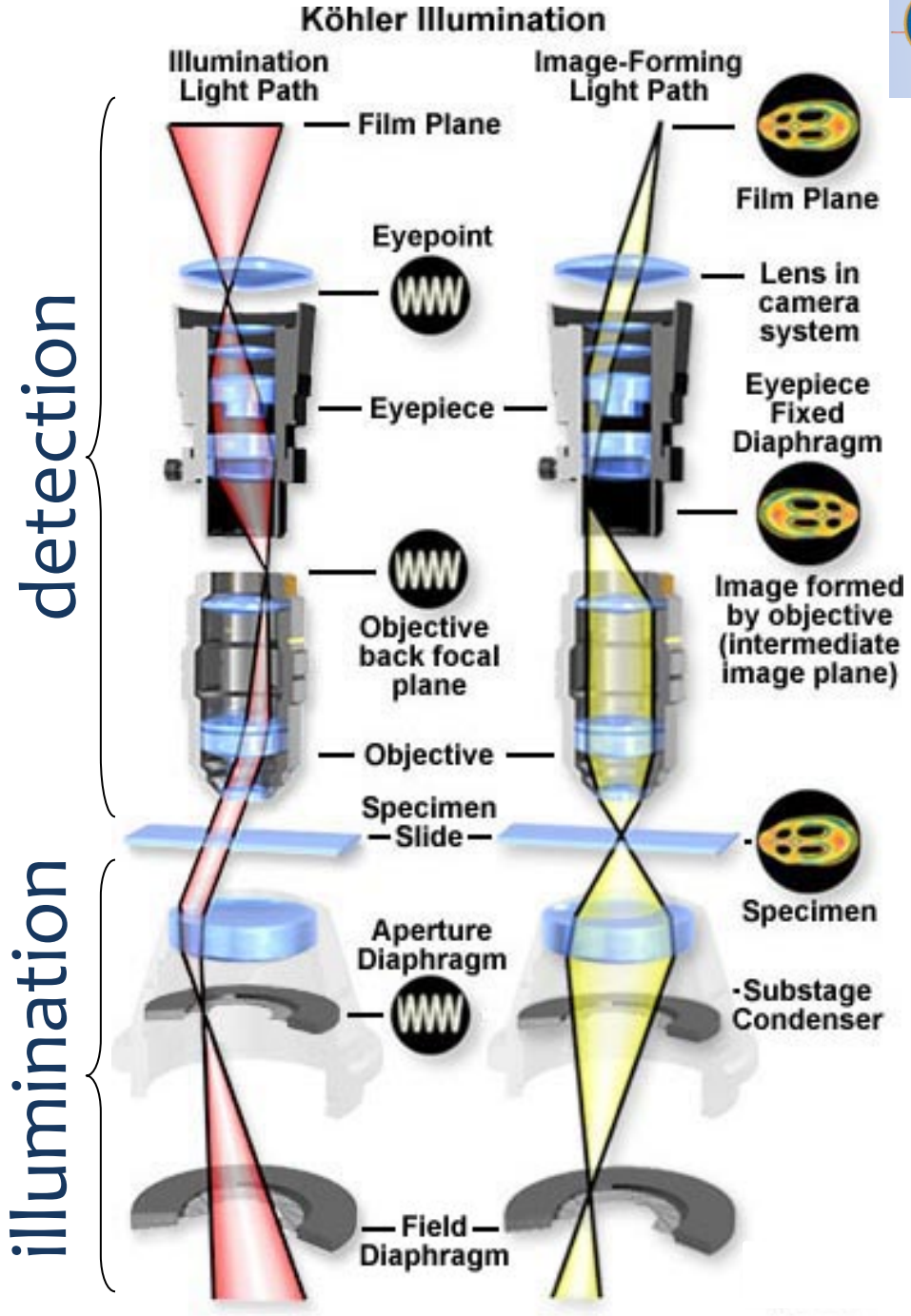
Median



Open



Illumination



Optical pathway

Optimal setting of

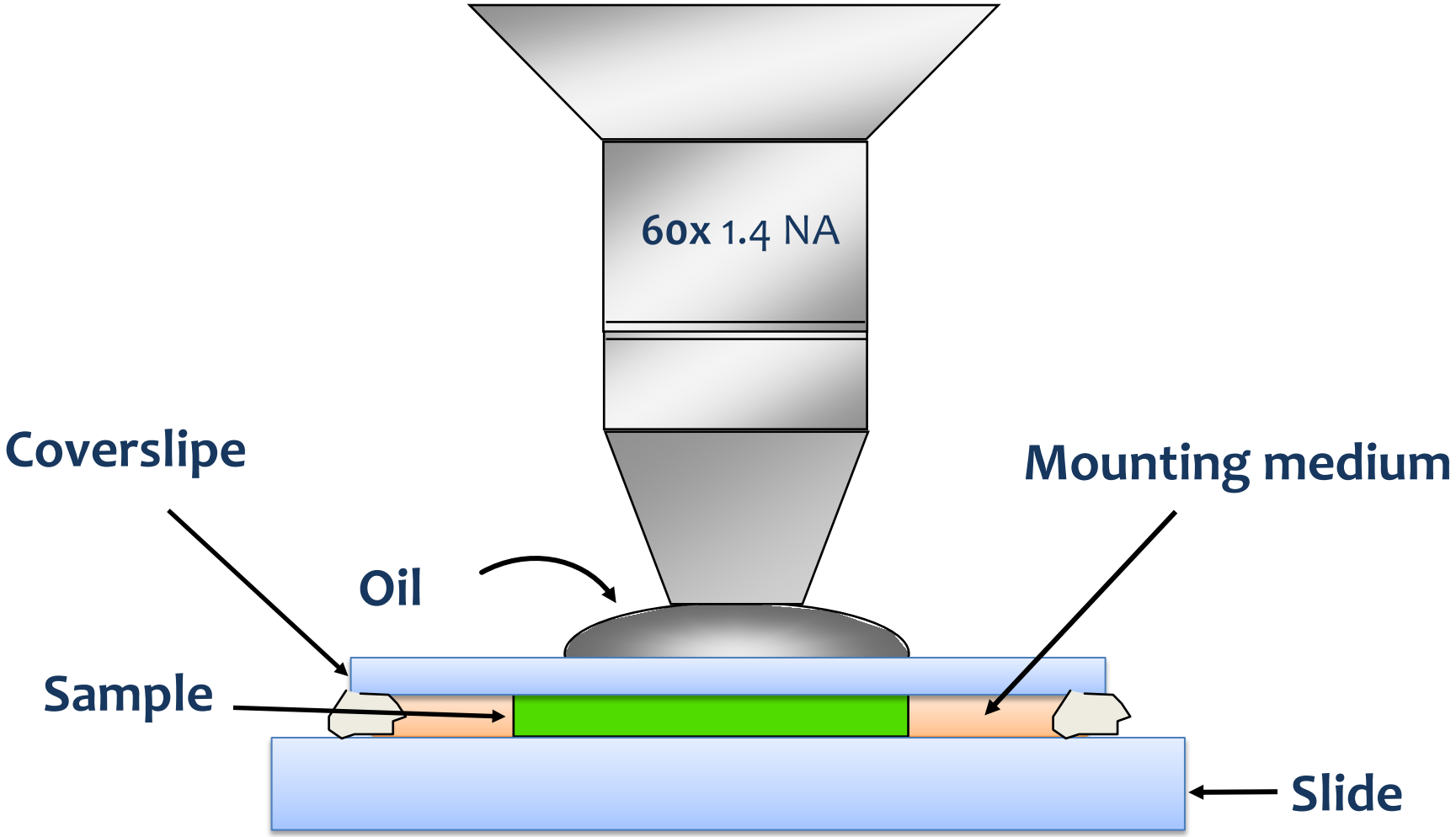
- lamphouse + collector
- diaphragms
- condenser
- objective
- eyepiece

Program

- ✓ General points
- ✓ Illumination
- ➢ Detection
- ➢ Aberrations and image deterioration
- ➢ Resolution and acquisition
- ➢ Contrast improvements

Detection

Objectives



Detection

Objectives

Fragile, complex and expensive (from 500 to more than 25,000 €), main point in the optical pathway, its quality (cleanliness) and specifications are directly involve on final image.

Detection

Technical specifications of objectives

- Brands: might not be mixed (screwing, optical corrections…)
- Magnification: 2.5 to 150X let's have the choice (color code, zoom…)
- Numerical aperture (NA): resolution, luminosity, DOF…
- Immersion medium: dry, water, oil (NA_{max}: 0.95 – 1.2– 1.47)
- Type (aberration corrections) : spherical, chromatic…
- Special optic properties: phase contrast, DIC…
- Coverslipe size correction: regular size 170 µm (could sometimes be adjustable, be careful of inverted microscope (petri dish = thick plastic))
- Working distance*: from last lens to focal point (from 150 µm to several millimeters)
- Depth of field (DOF)*: thickness of "optic slice", NA influenced

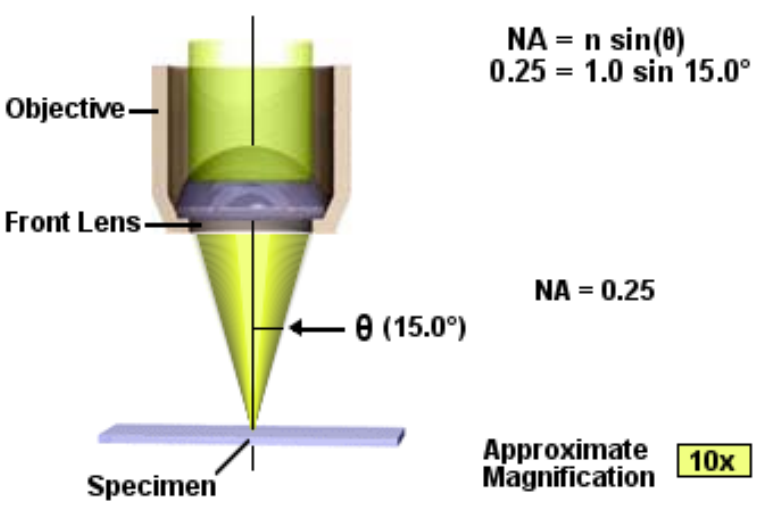
* Generally not written on objectives

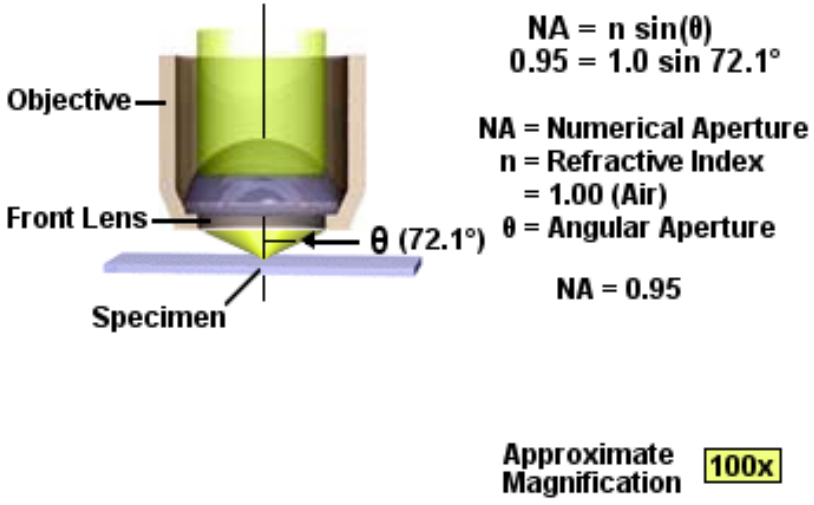
Detection

Numerical aperture

- Numerical aperture: NA=n.sin(θ)

  where n is optical index and θ the half-angle of cone detection.
- NA is not link to magnification, there are large NA with low magnification such as 20X water immersion objectives (Olympus NA=1 or more recently Zeiss and Leica with NA=1.05).
- The biggest is NA, the better is the resolution ($Res_{(nm)} = 0.61\ \lambda/NA$)
- The quality of illumination affect the NA.

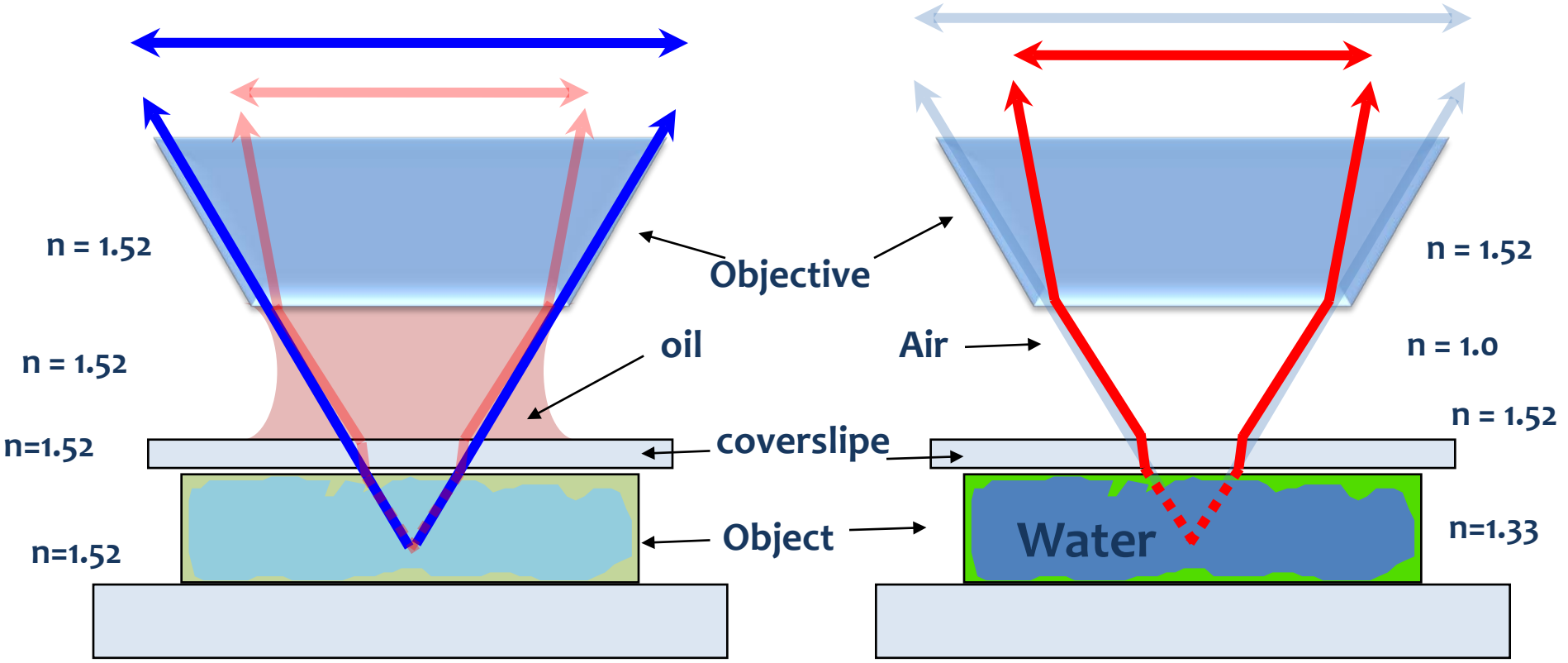




Detection

Immersion medium: n in real life

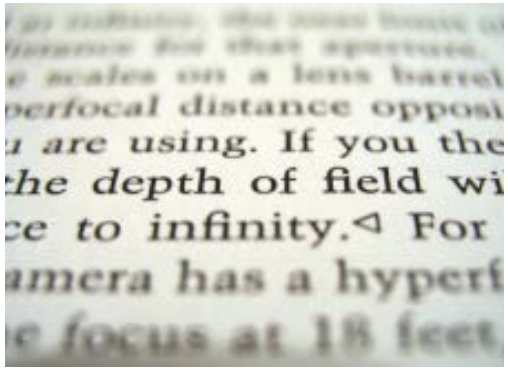
Index inhomogeneities degrade microscope resolving power and could induce chromatic aberrations for example (n=f(λ..))



Detection

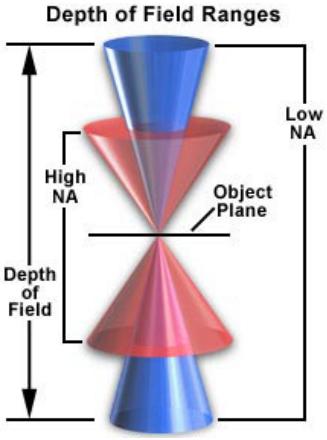
Depth of field - DOF

Distance between highest and lowest point in a sample that give on focus image (thickness of the optical slice).



Depending of the NA: decrease when NA increase.

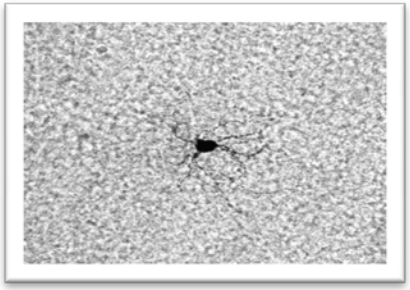
$$DOF = \frac{n\lambda}{NA^2}$$



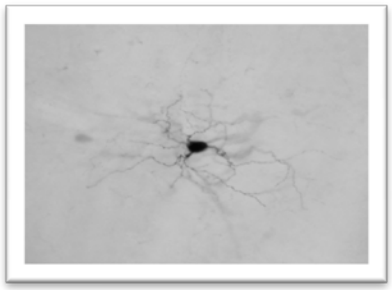
Example at 488 nm **: 5,4 µm** for a 10X **dry** objective (**NA 0,3**)
0,378 µm for a 63X **oil** immersion objective (**ON 1,4**)

Transmission light microscope NA is not only due to objective NA but the condenser is also involve: aperture diaphragm reduce illumination cone and by the way the NA.

Closed (large contrast)



Open (large resolution)



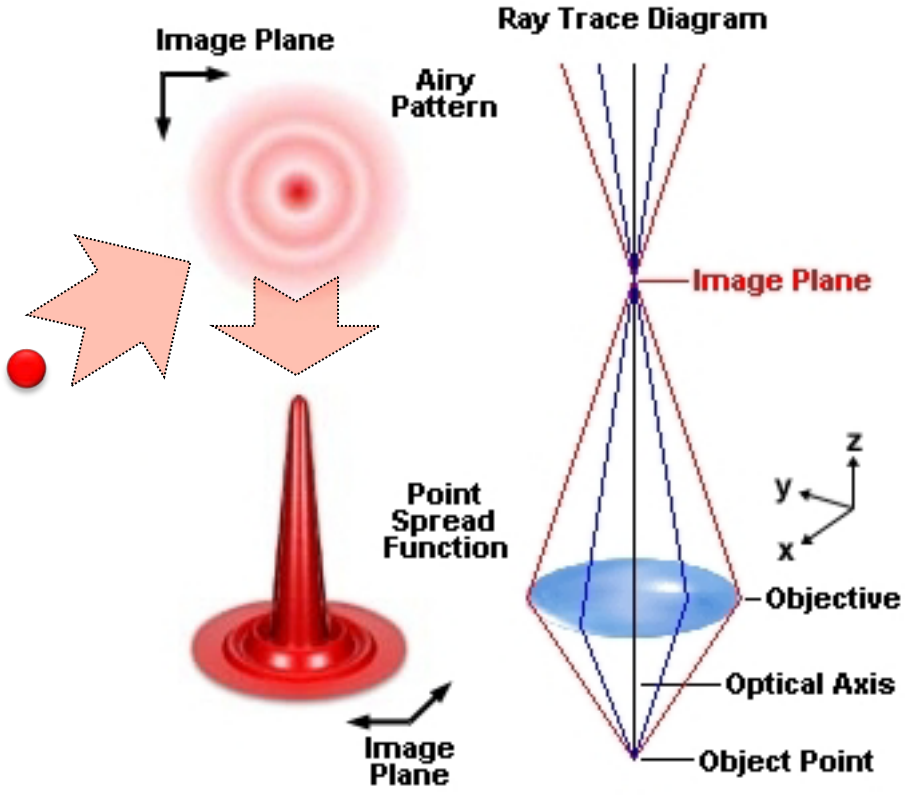
Program

- ✓ General points
- ✓ Illumination
- ✓ Detection
- ➢ Aberrations and image deterioration
- ➢ Resolution and acquisition
- ➢ Contrast improvements

Aberrations and image deterioration

Optical aberrations are deviation from Gauss conditions

- **Monochromatic aberrations**
  - Spherical
  - Coma
  - Astigmatism
  - Field curvature
  - Distortion
- **Chromatic aberrations**
  - Longitudinal
  - Lateral

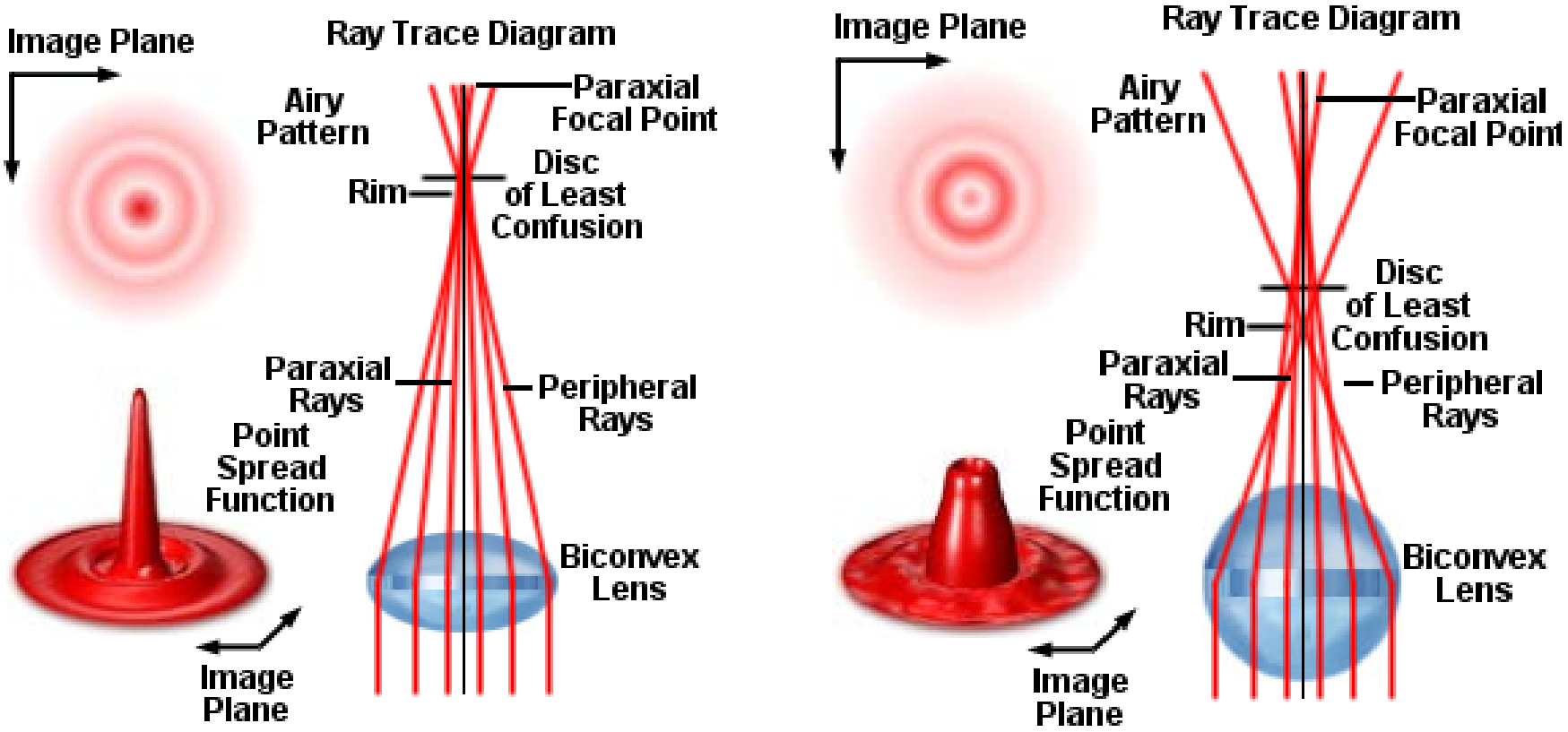


The image of a point is not a point !

We will come back on this later…

Aberrations and image deterioration

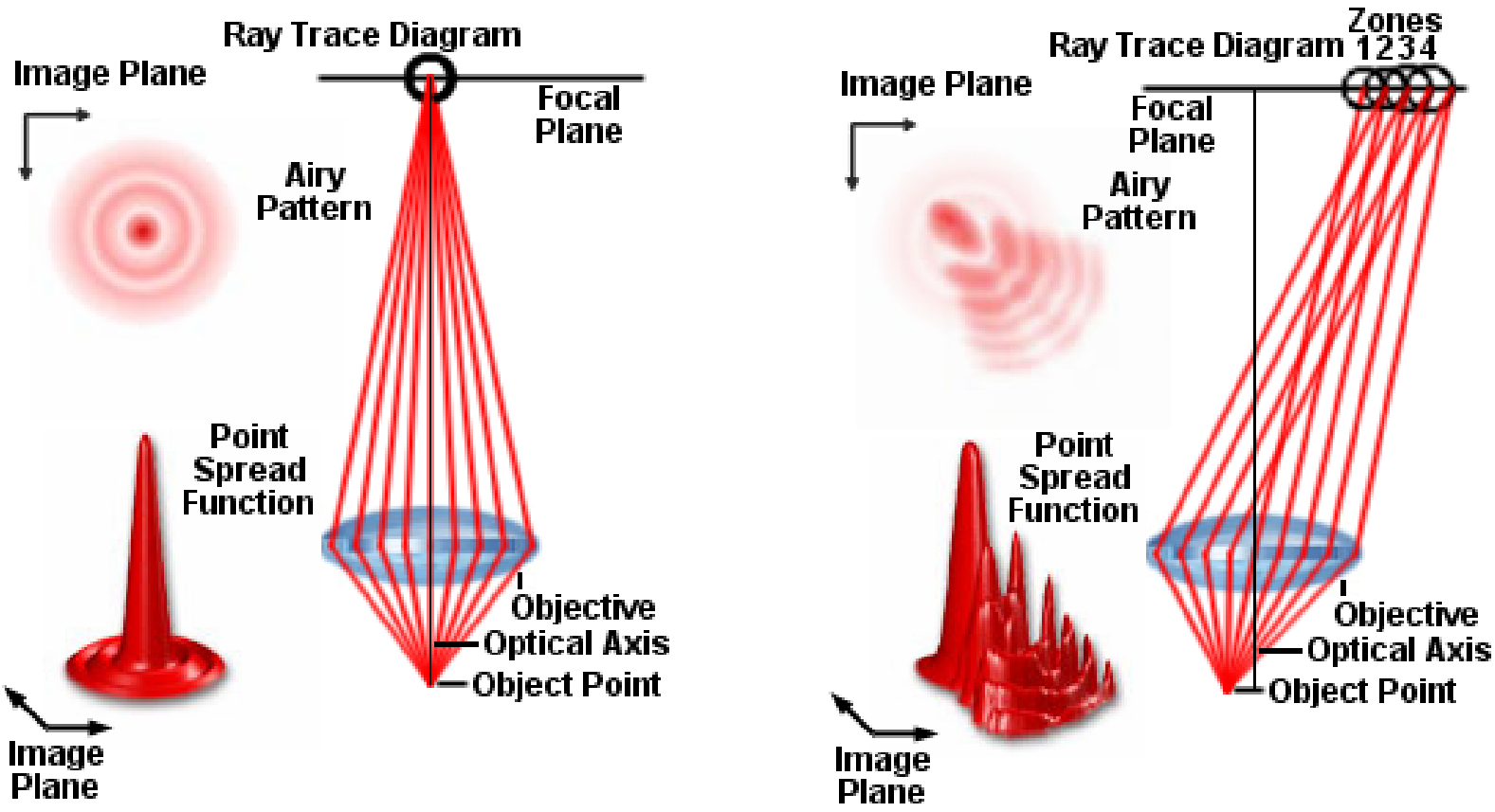
Spherical



Light rays that transmit through the periphery of a lens do not converge in the same place as those transmit close to the optic axial center.

Aberrations and image deterioration

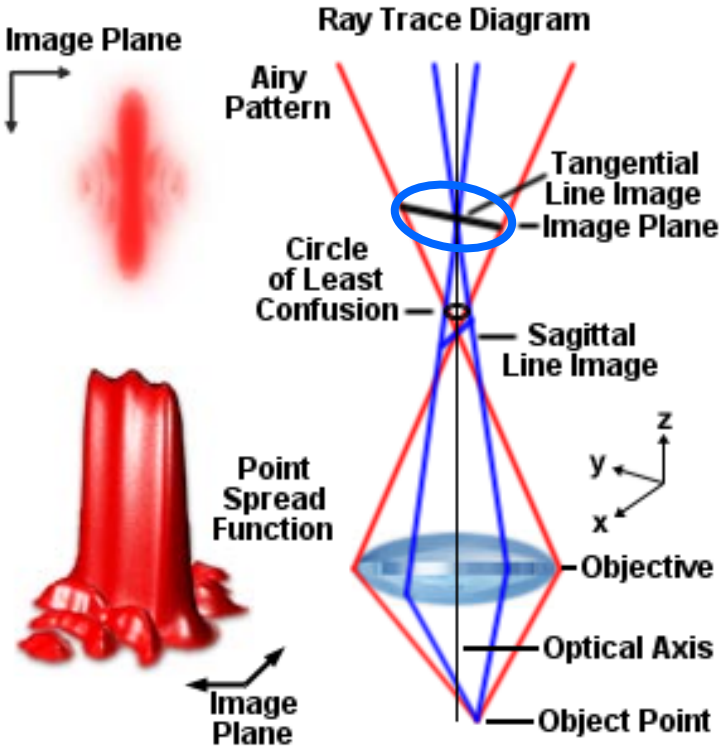
Coma

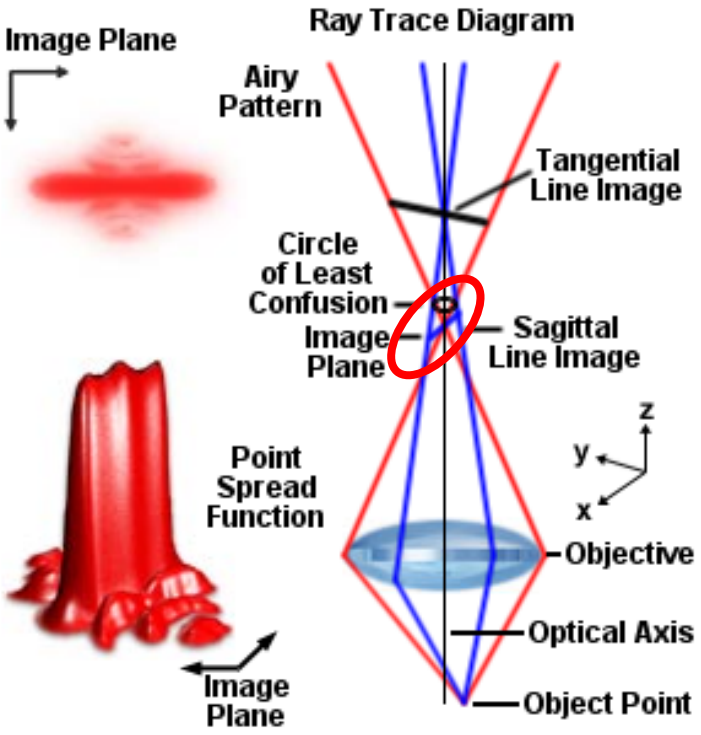


The image of a point through a tilted lens Vs. optical axis appear as a comet.

Aberrations and image deterioration

Astigmatism

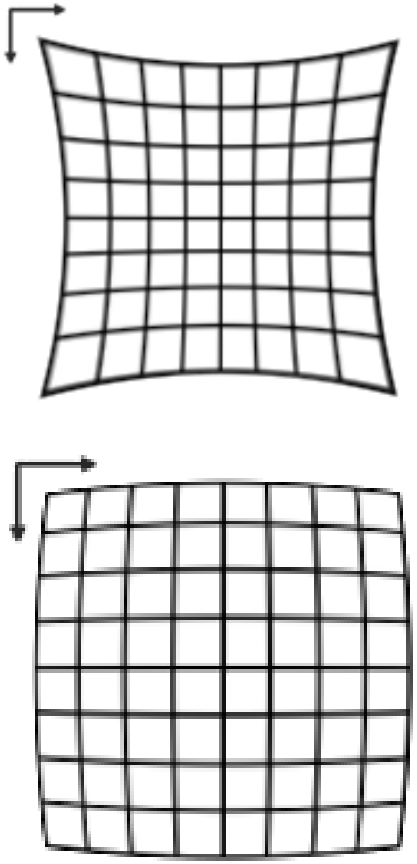


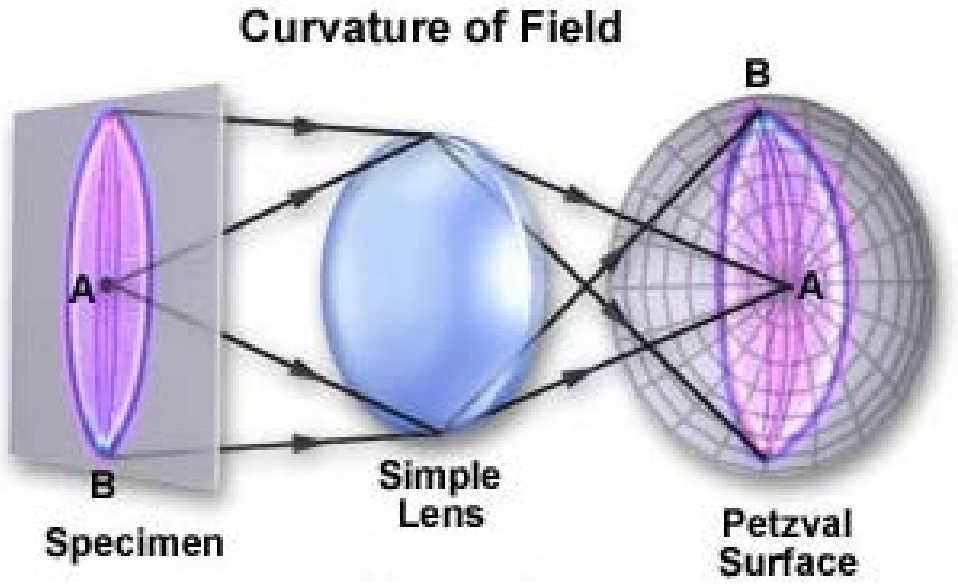


The image of a point out of optical axis appear as cross ; there are two focal points due to system asymmetry.

Aberrations and image deterioration

Geometrical / field curvature

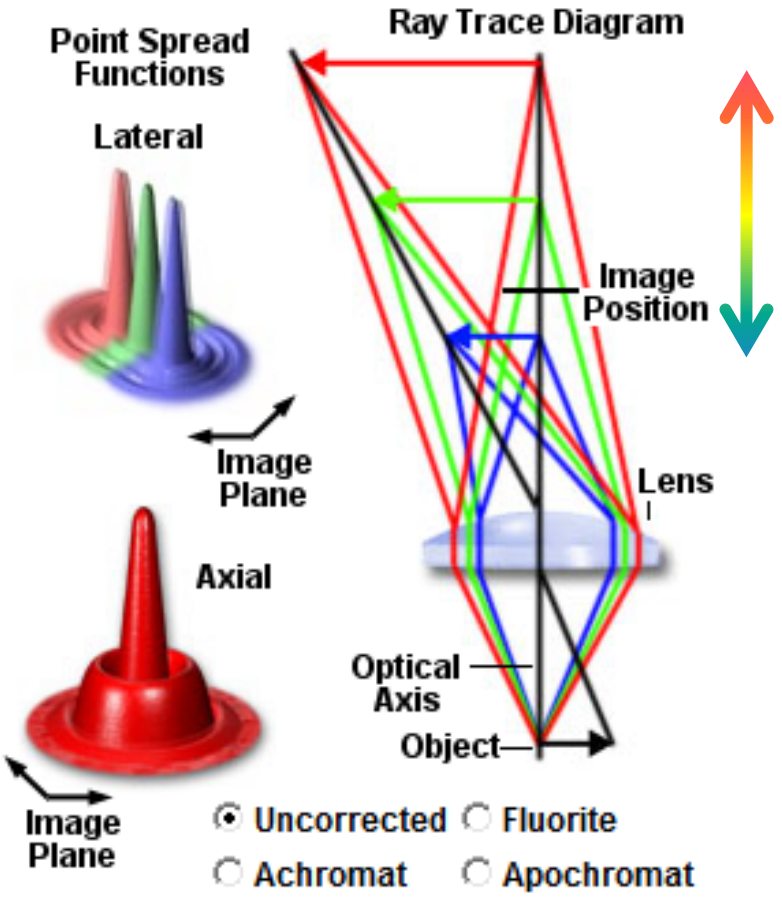


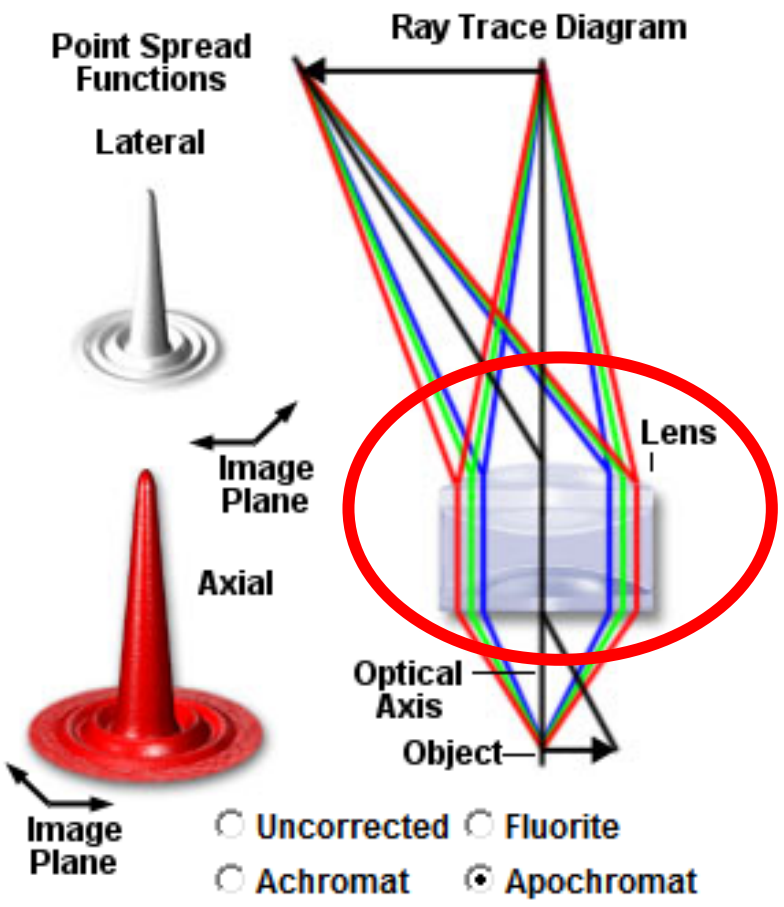


A flat image is not flat but a concave spherical surface (convergent lens) or convex (divergent lens).

Aberrations and image deterioration

Chromatic (axial et laterals)



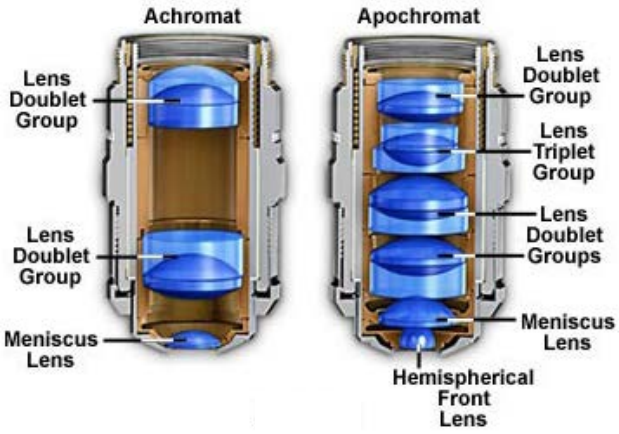


Due to refraction laws, the focal distance of a lens is link to n (optical index) and by the way to λ.

Aberrations and image deterioration

Aberrations correction:

- Use the center of the lens (field diaphragm, Gauss conditions)
- Aspherical lens (difficult to produce, adaptative optic)
- Association of multiple lens (convex and concave) in doublet or triplet…).



Objective types	Spherical aberrations	Chromatic aberrations	Field curvature
Achromat	1 color	2 colors	No
Plan Achromat	1 color	2 colors	Yes
Fluorite	2-3 colors	2-3 colors	No
Plan Fluorite	3-4 colors	2-4 colors	Yes
Plan Apochromat	3-4 colors	4-5 colors	Yes

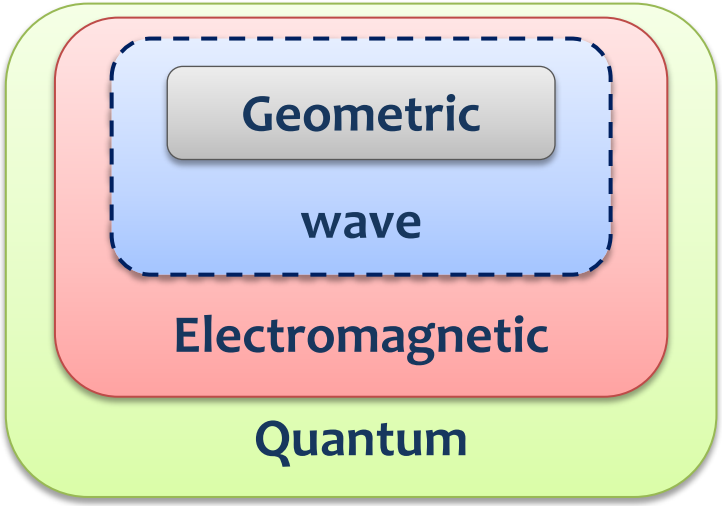
All these aberrations can be corrected with lens association and/or surface treatment:

Microscope objectives are complex, fragile and expensive.

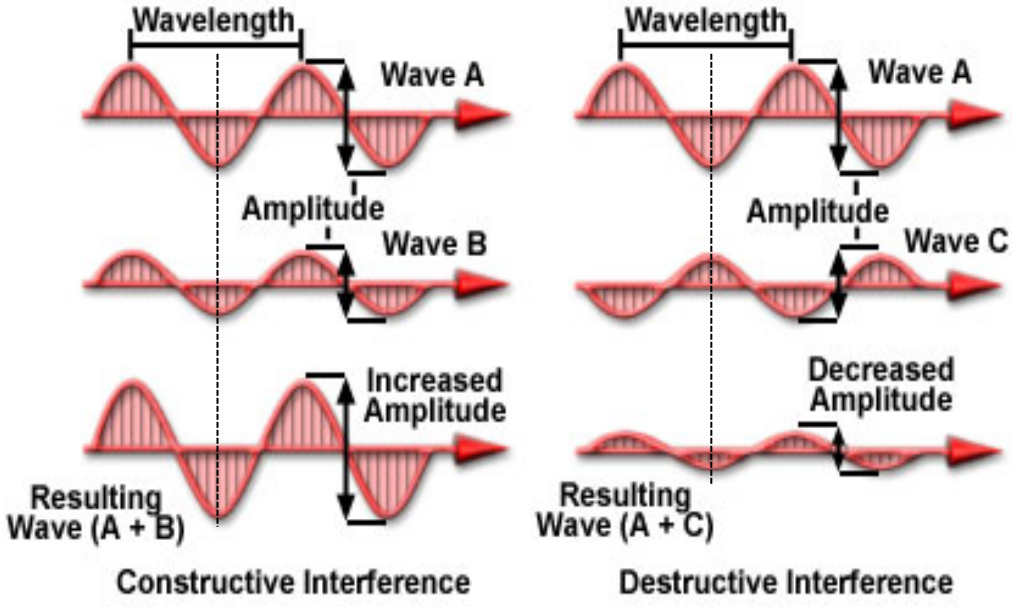
Program

- ✓ General points
- ✓ Illumination
- ✓ Detection
- ✓ Aberrations and image deterioration
- ➢ Resolution and acquisition
- ➢ Contrast improvements

Resolution and image acquisition



Interferences

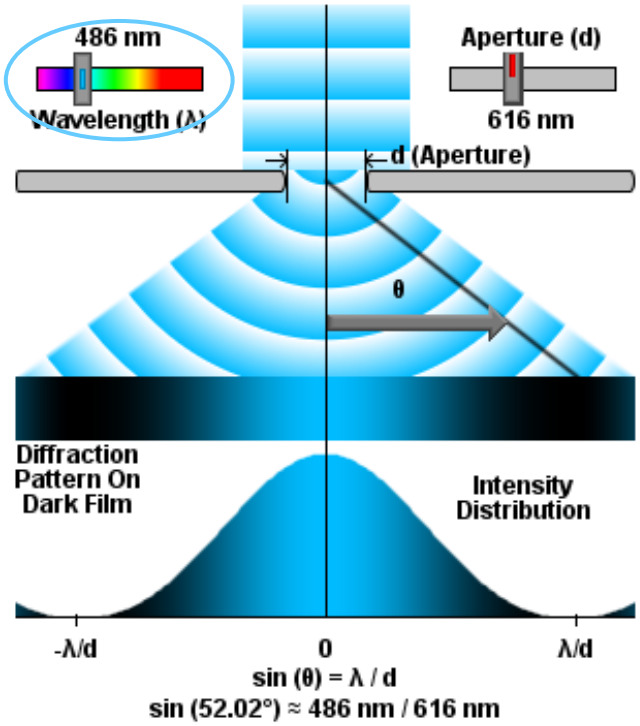


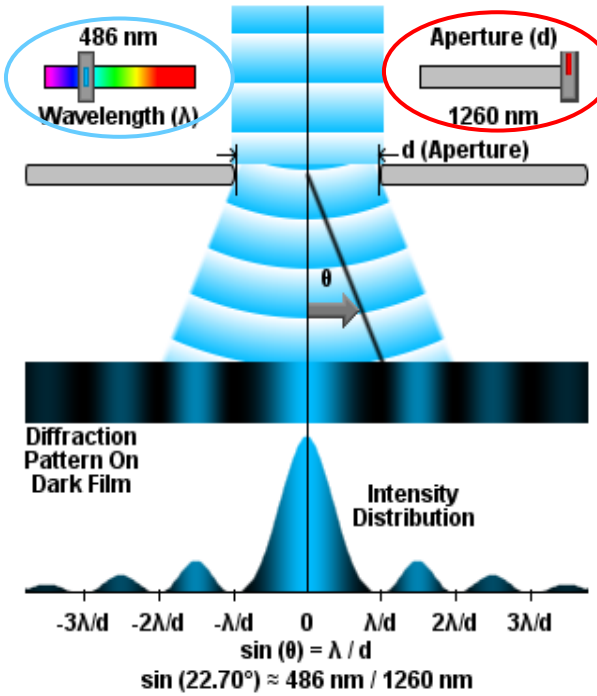
Each wave is characterize with a phase and an amplitude.
Two waves could create interferences if they propagate in the same direction and in the same vibration plan.

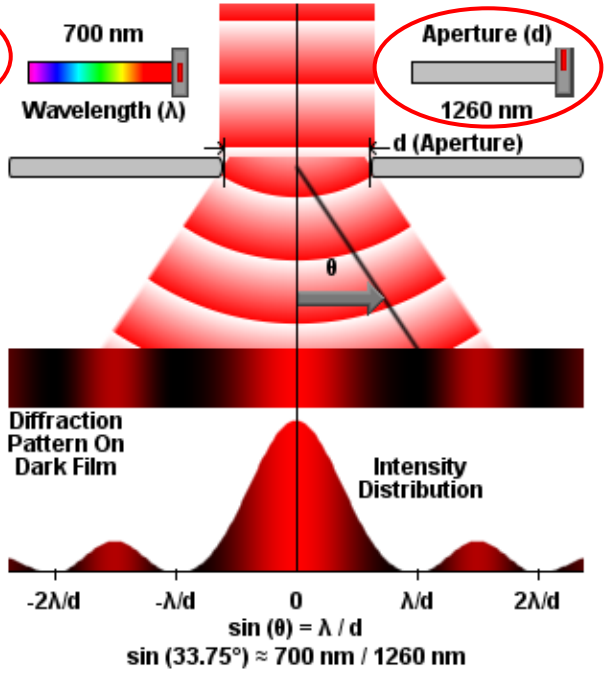
Resolution and image acquisition

Diffraction

Diffraction refers to various phenomena which occur when a wave encounters an obstacle (for example a slit or small hole), It will influence the phase and/or the amplitude of light.



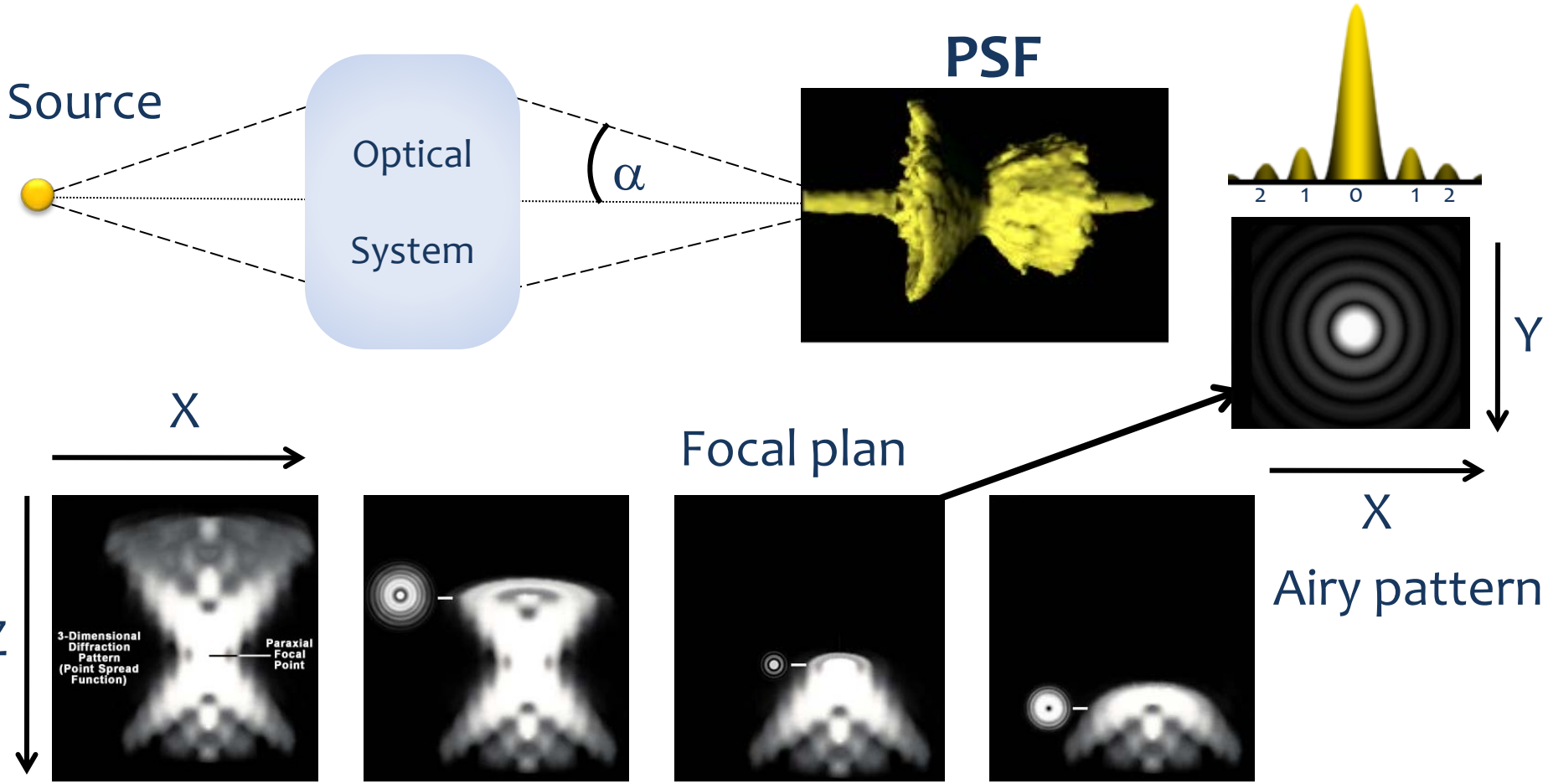




Light diffraction depend on the size of the hole and the wavelength.

Resolution and image acquisition

All optical system affect the source image through a Point Spread Function (**PSF**).



The image of a point is not a point !

Resolution and image acquisition

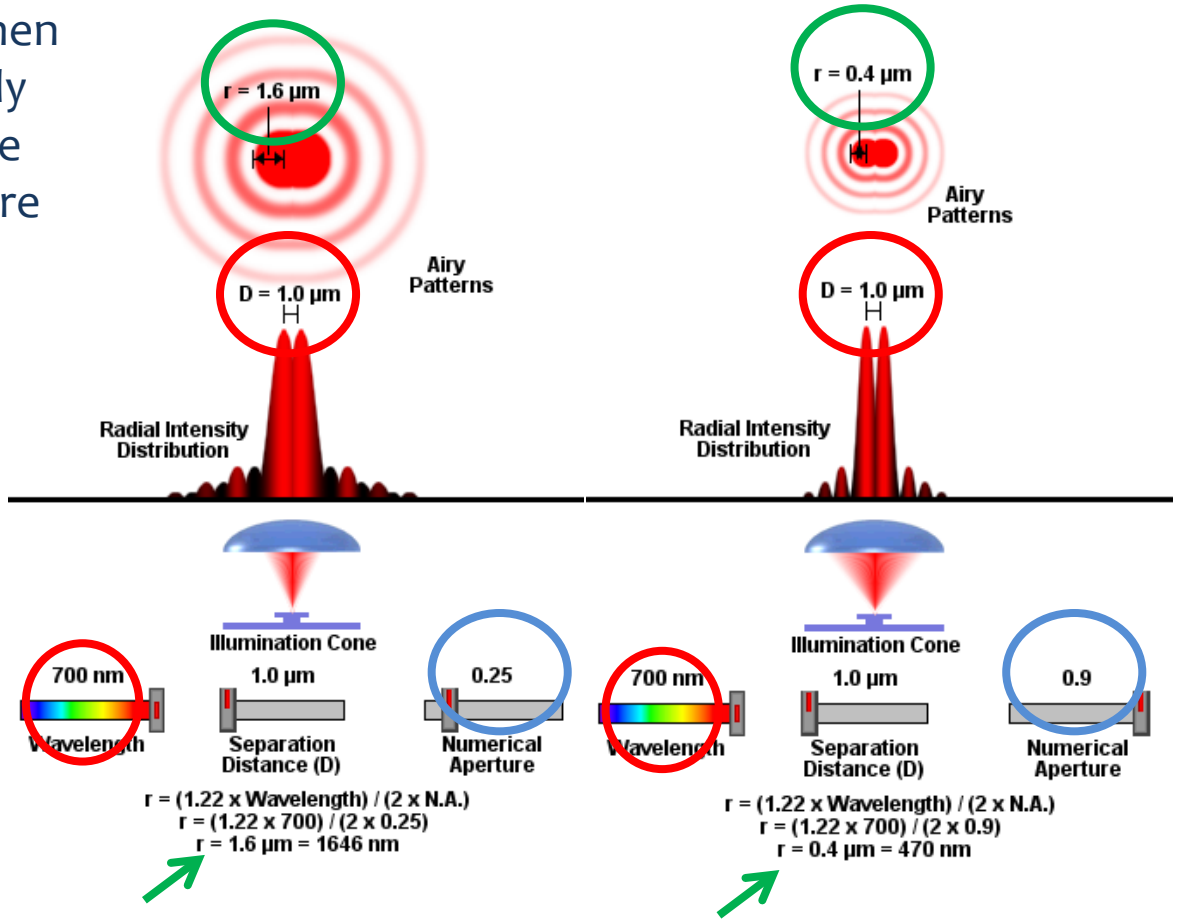
Optical resolution describes the ability of an imaging system to resolve details in the object that is being imaged. It is the distance between two distinct points seen as two individual PSF.

Two Airy patterns will appear as separated in the only condition when their energy curves are sufficiently separated to be distinguish by the detector (eye, camera…) i.e.: More than the radius of the Airy disc (harmonic o).

Rayleigh criteria

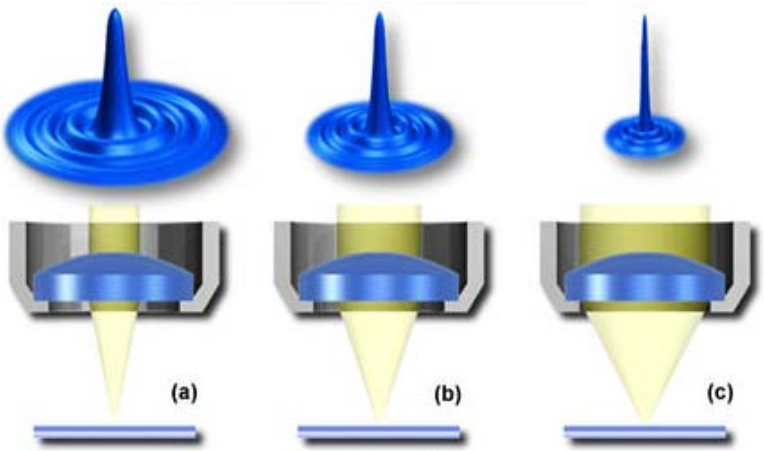
$$d \geq \frac{1{,}22\lambda}{NA(obj) + NA(cond)}$$

$$R_{fluo} = \frac{1{,}22\lambda}{2NA} = \frac{0{,}61\lambda}{NA}$$

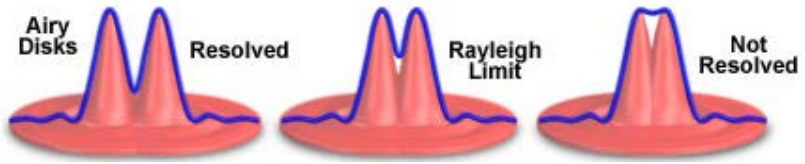


Resolution and image acquisition

PSF shape and resolution are function of NA



NA =0,12 NA =0,34 NA =0,87



The resolution is function of NA but not of the magnification.

For 488nm	5X dry NA= 0.15	10X dry NA= 0.3	20X dry NA=0.8	40X oil NA=1.3	60X water NA=1.2	60X oil NA=1.4
Resolution XY in µm	1.984	0.992	0.372	0.229	0.248	0.212

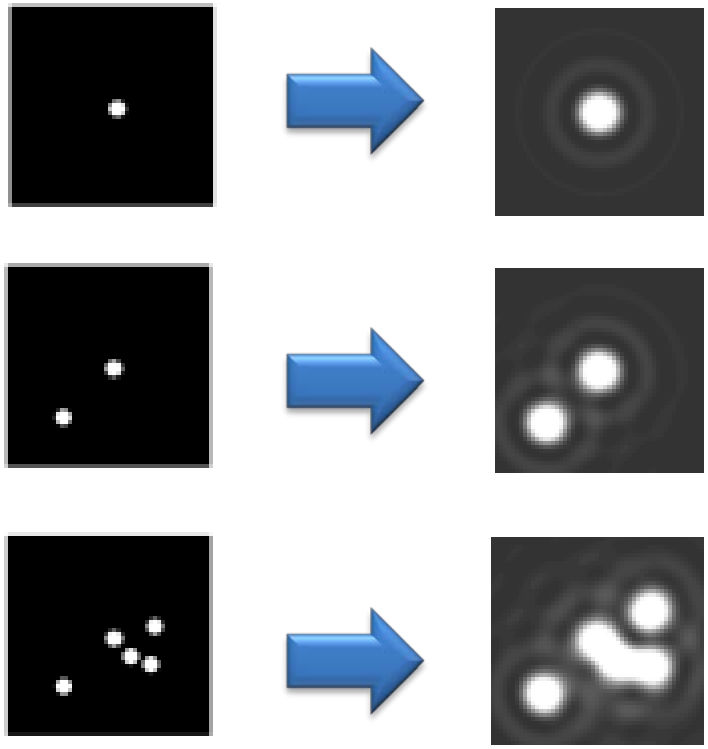
Resolution and image acquisition

Image construction

1 point

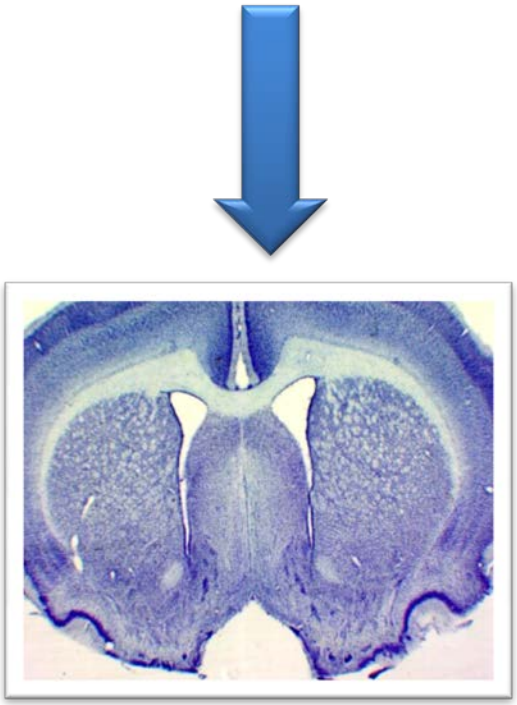
2 points

5 points



Source PSF

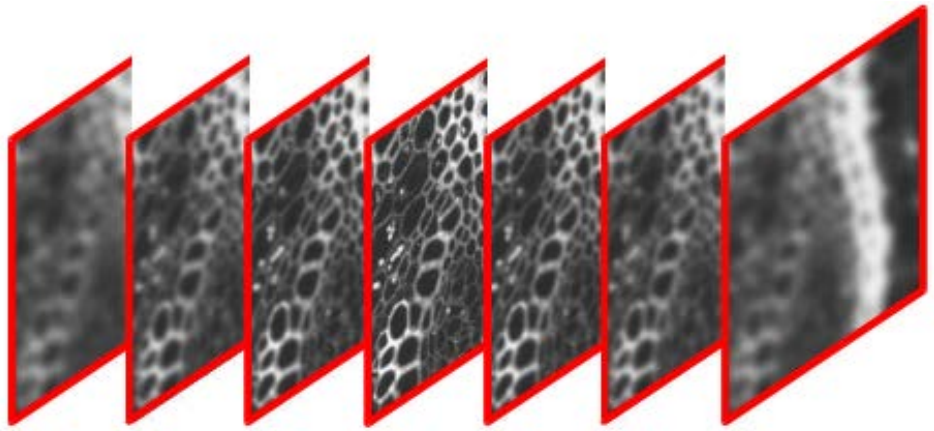
N points



Resolution and image acquisition

Axial resolution

$$R_{xz} = \frac{2\lambda}{NA\sin\alpha} = \frac{n2\lambda}{NA^2}$$

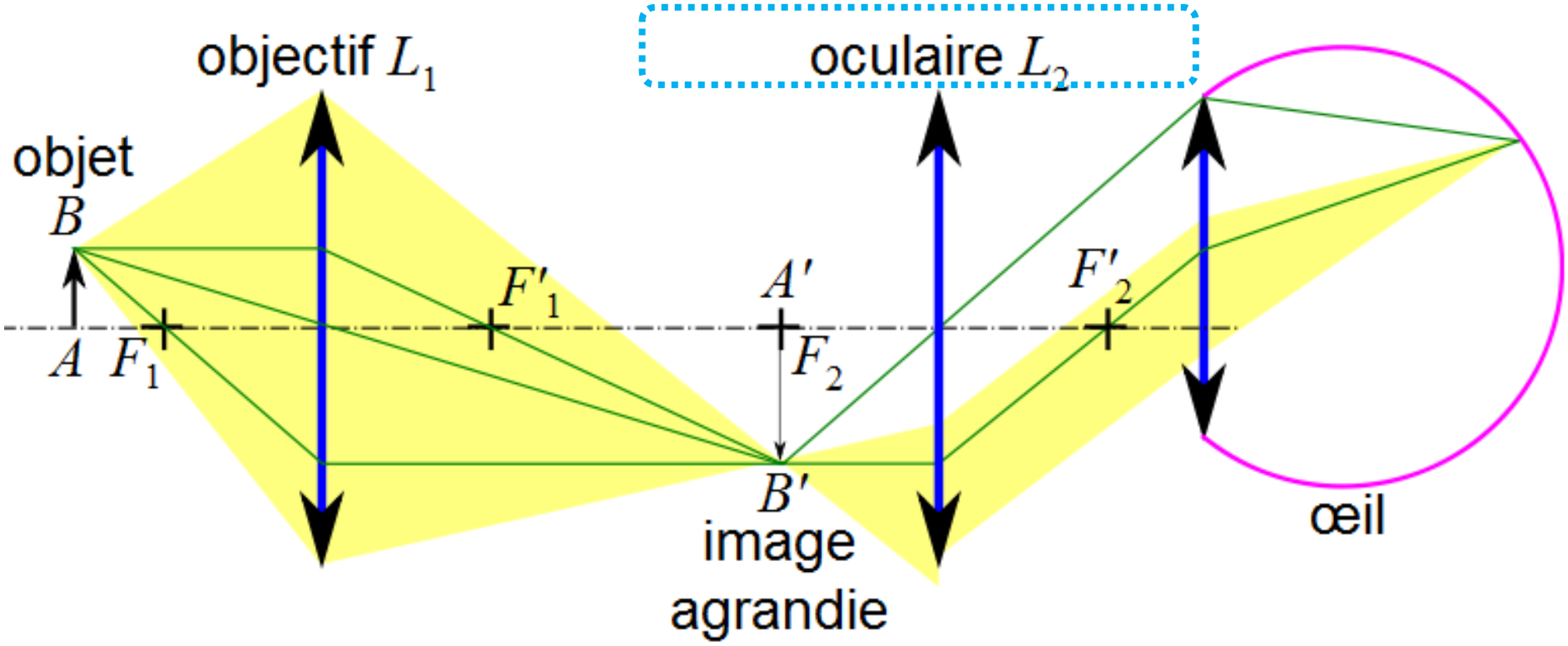


for 488 nm	5X dry NA= 0.15	10X dry NA= 0.3	20X dry NA=0.8	40X oil NA=1.3	60X water NA=1.2	60X oil NA=1.4
Resolution XZ in µm	43.378	10.844	1.525	0.878	0.901	0.757
α	8.6°	17.5°	53.1°	58.8°	64.5°	67.1°

Remind that $\alpha = \sin^{-1}\left(\frac{NA}{n}\right)$

Resolution and image acquisition

Detection

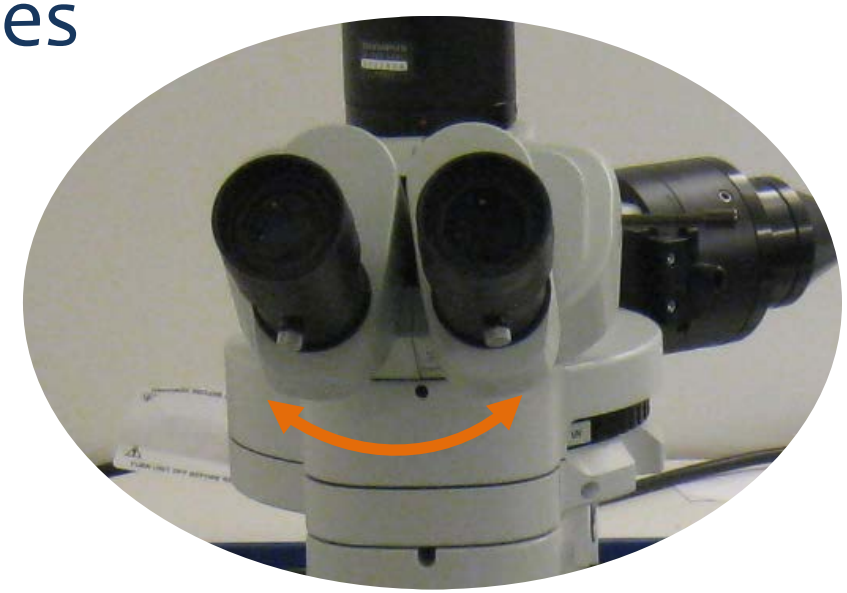


In a microscope, we observe the enlargement of the virtual image generated by the objective (composed microscope).

Resolution and image acquisition

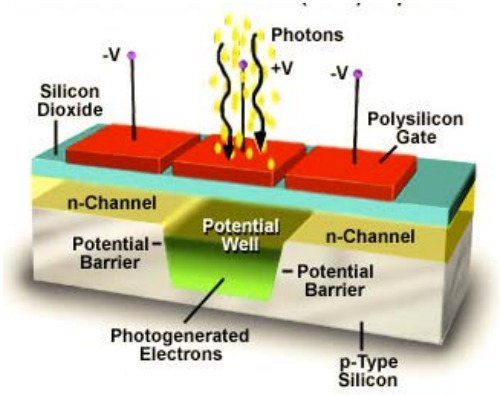
Eyepieces

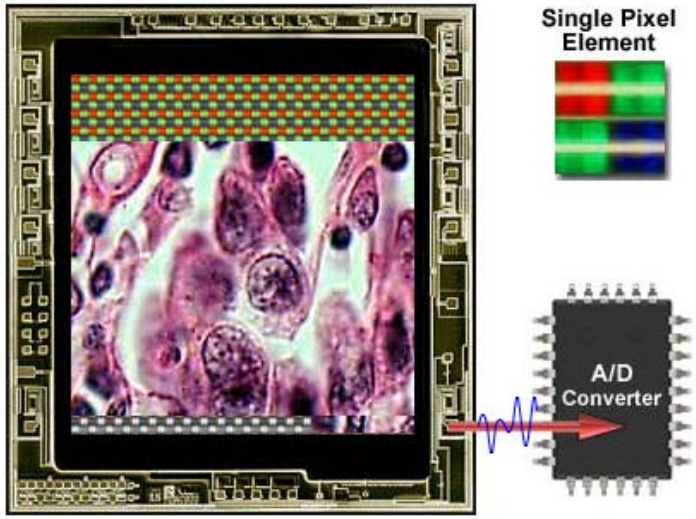
- It mainly has lens projecting function with a magnification from 5x to 20x.
- We have to set the distanced between each eyepiece to our own morphology
- Each one should also be focused individually to our own vision.
- The image is focus at about 1 cm from the last lens (the rubber pieces are dedicated for this space and to prevent dust).
- We can add filters and/or grids in the optical track.

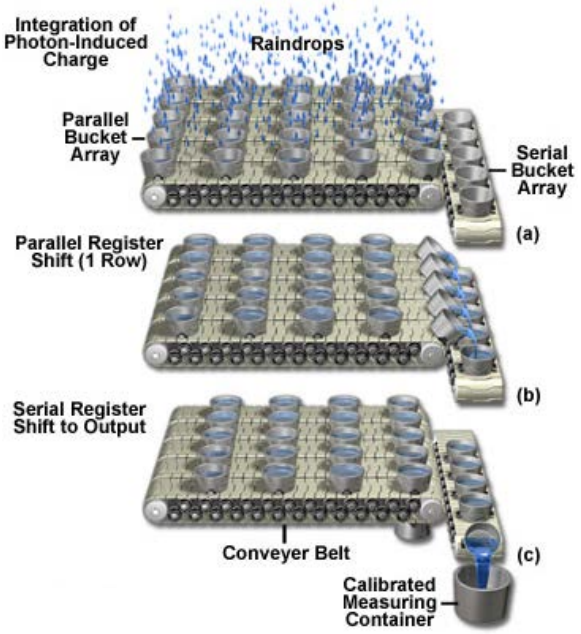


Resolution and image acquisition

CCD Cameras (Charged Coupled Device)





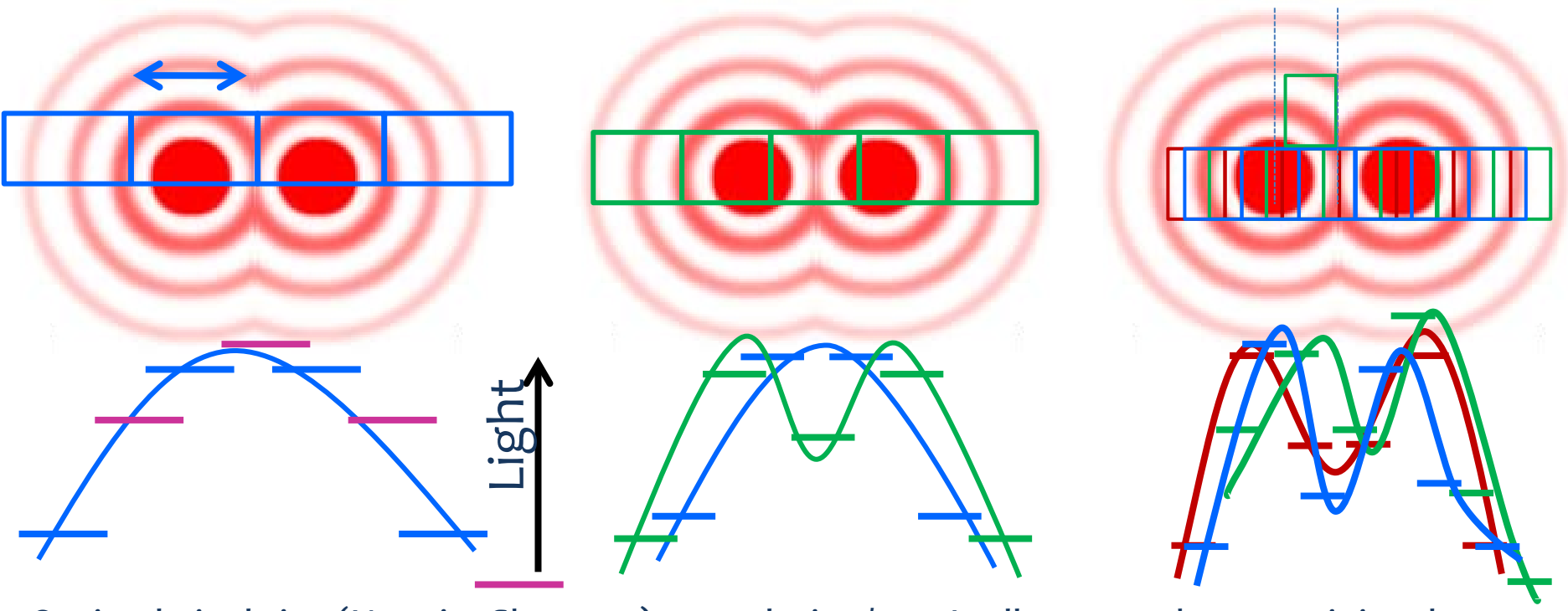


Camera specifications

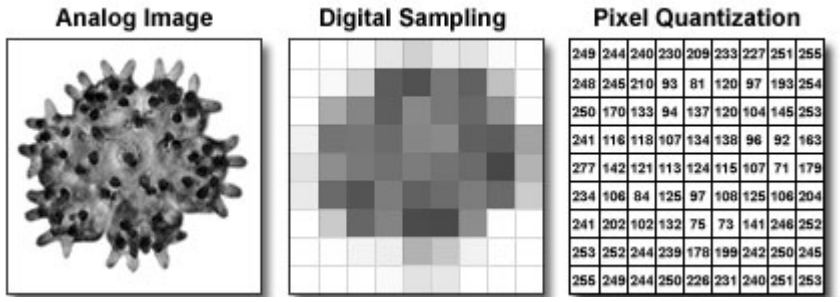
- Spatial resolution
- Time resolution: Sensitivity, transfer architecture, binning
- Quantum efficiency (q<1) : Material, technical and wavelength dependent
- Background noise
- Dynamic and bit depth
- Linearity: important for quantitative studies

Resolution and image acquisition

Spatial sampling



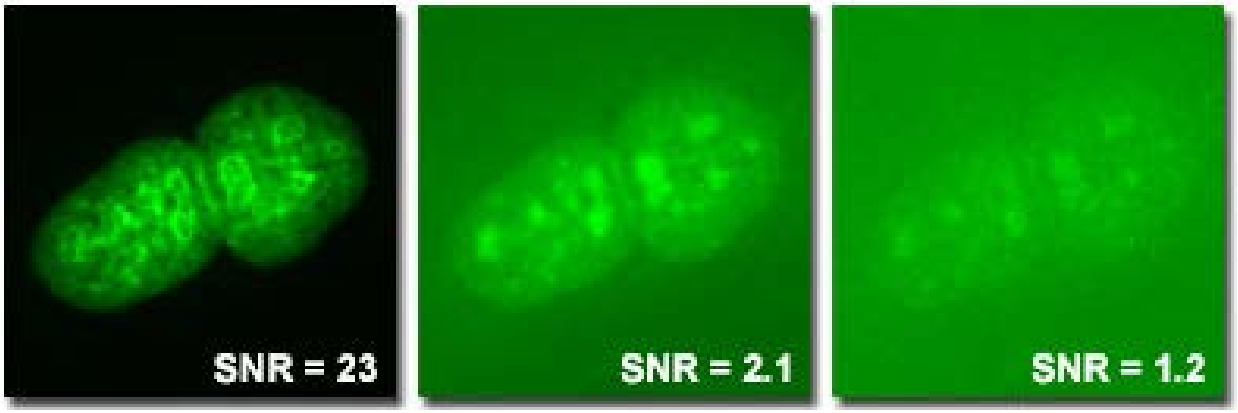
Optimal pixel size (Nyquist-Shannon) = resolution/2,3 : It allow us to have a minima between two maximas whatever the pixel positions (reading frame).



Example for a 100X 1.4 NA for 488 nm
Optical resolution 212 nm
Optimum pixel size≈92 nm
Camera size 6.45 µm /100 = 64.5 nm

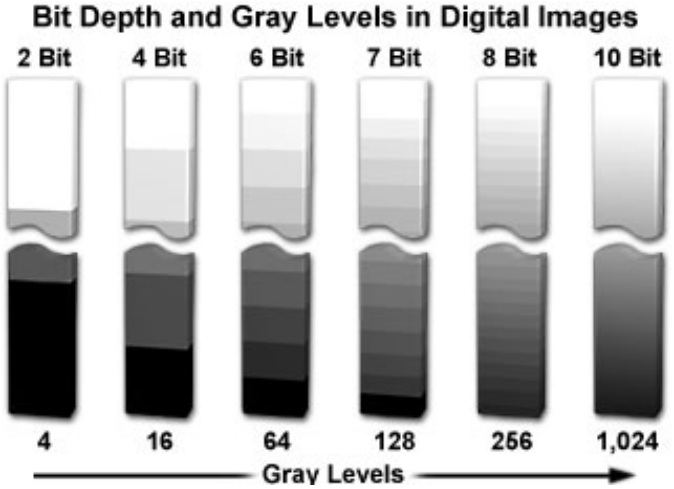
Resolution and image acquisition

Signal to Noise ratio and dynamic



Background noise:

- Photon noise: due to environment light.
- Dark noise: generation of e^- by thermal effect.
- reading noise: generate by the camera itself (electronic).



dynamic :

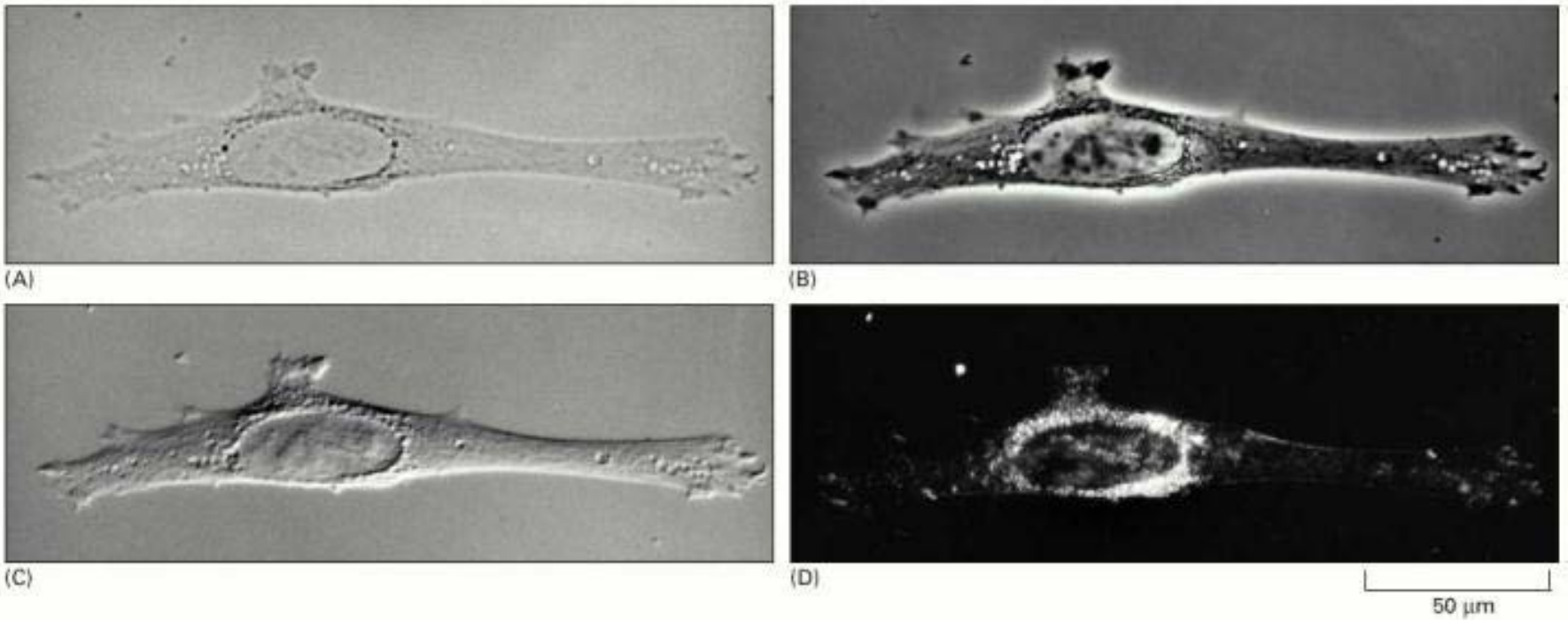
Proportional of the potential well capacity (binning) and the background. Example: 5 10^5 e^-/10 e^- give 5000:1 dynamic that is adapted for a 12 bits image characterized by 4096 level of grey.

Program

- ✓ General points
- ✓ Illumination
- ✓ Detection
- ✓ Aberrations and image deterioration
- ✓ Resolution and acquisition
- ➢ Contrast improvements

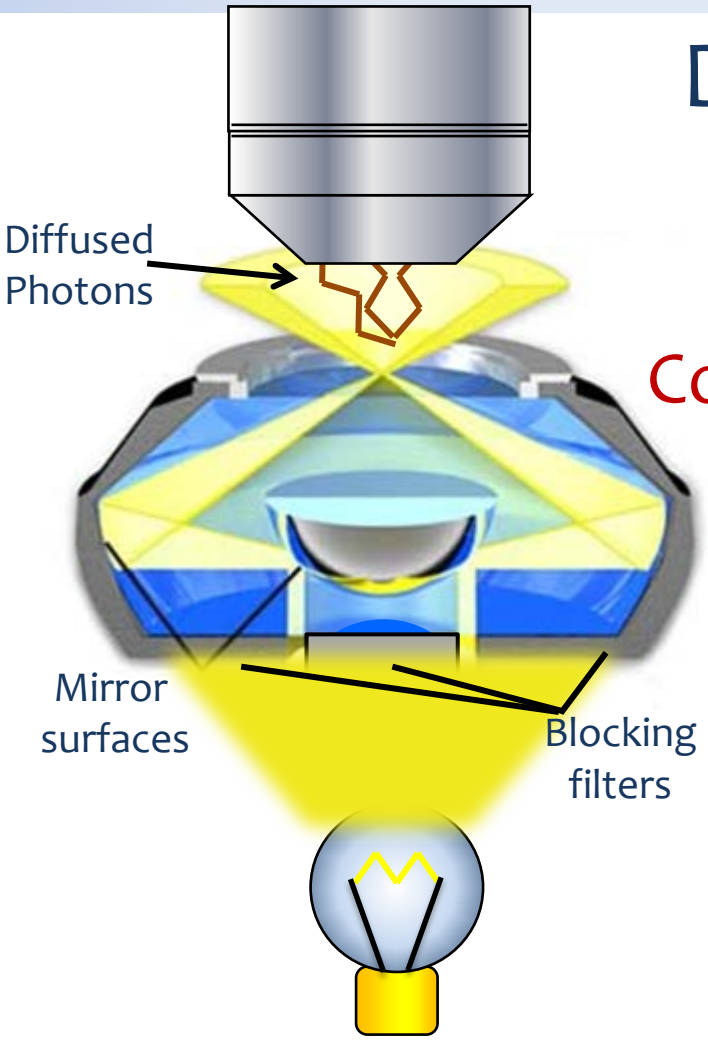
Contrast improvements

- Dark field
- Phase contrast
- Differential Interferential Contrast (DIC)

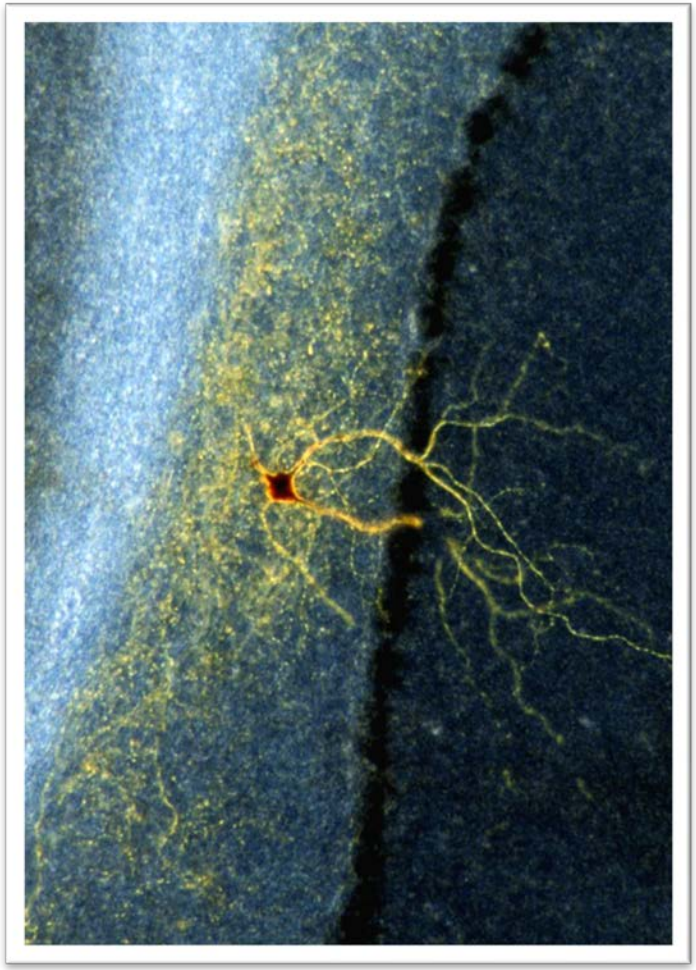


Contrast improvements

Dark field



Special Condenser



Mixed image with of a dark field and polarized illumination.
Authorized by Dr Steve Edgley
http://www.pdn.cam.ac.uk/groups/edgleylab/index.html

Only photons that were diffracted/diffused by the sample can be detected by the objective due to the structured light.

Contrast improvements

Phase contrast

Fritz Zernicke, Nobel Prize (1953). Phase contrast inventor

- Human eye is only able to see light intensity (amplitude) or color (wavelength) differences.

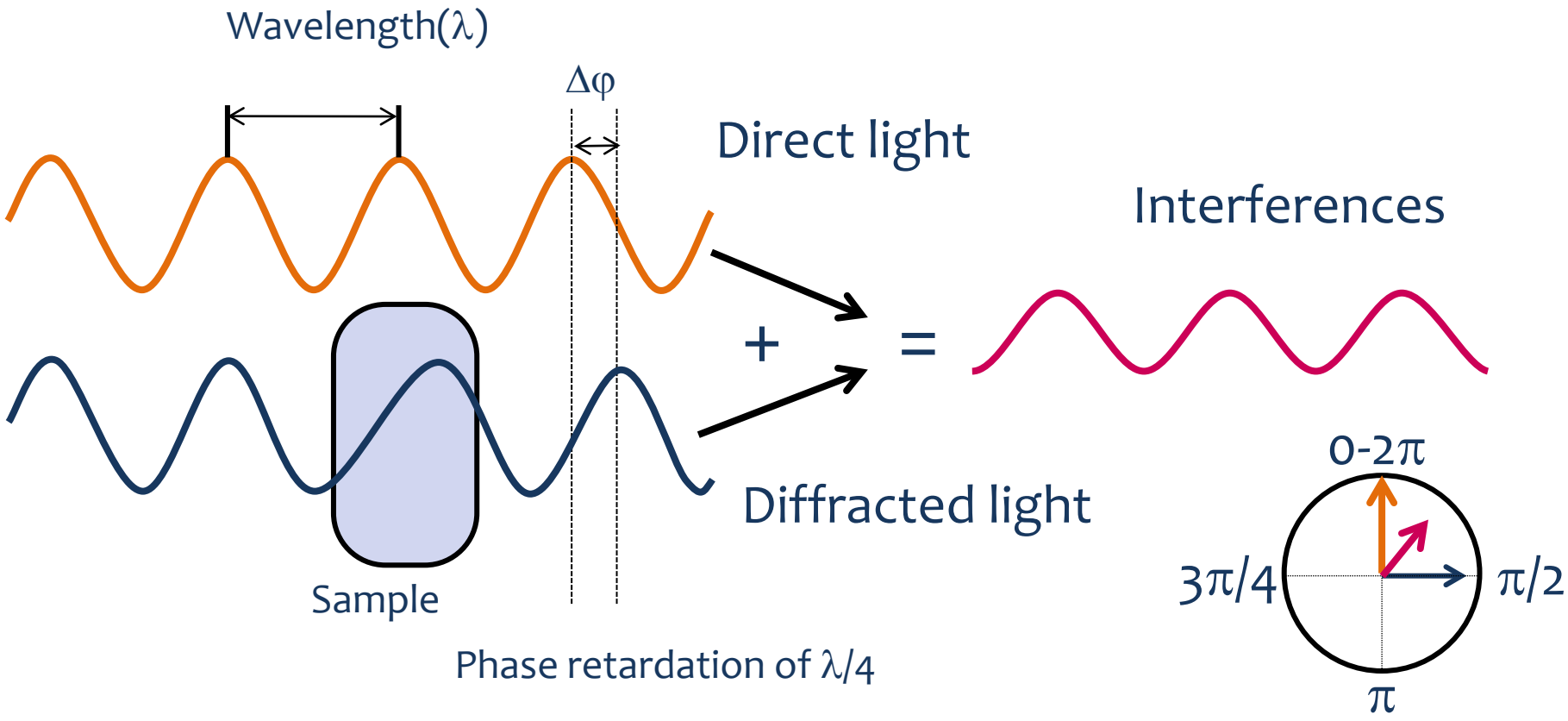
- Microscopic biological objects are generally transparent and offer low contrast because light flux transmitted by different parts of a cell are quite similar.

- In another way, optical index of these different cell parts are very variable that induce wave speed differences and by the way wave phase modulations.

- The goal of phase contrast microscopy is to transform wave phase modulations in intensity differences

Contrast improvements

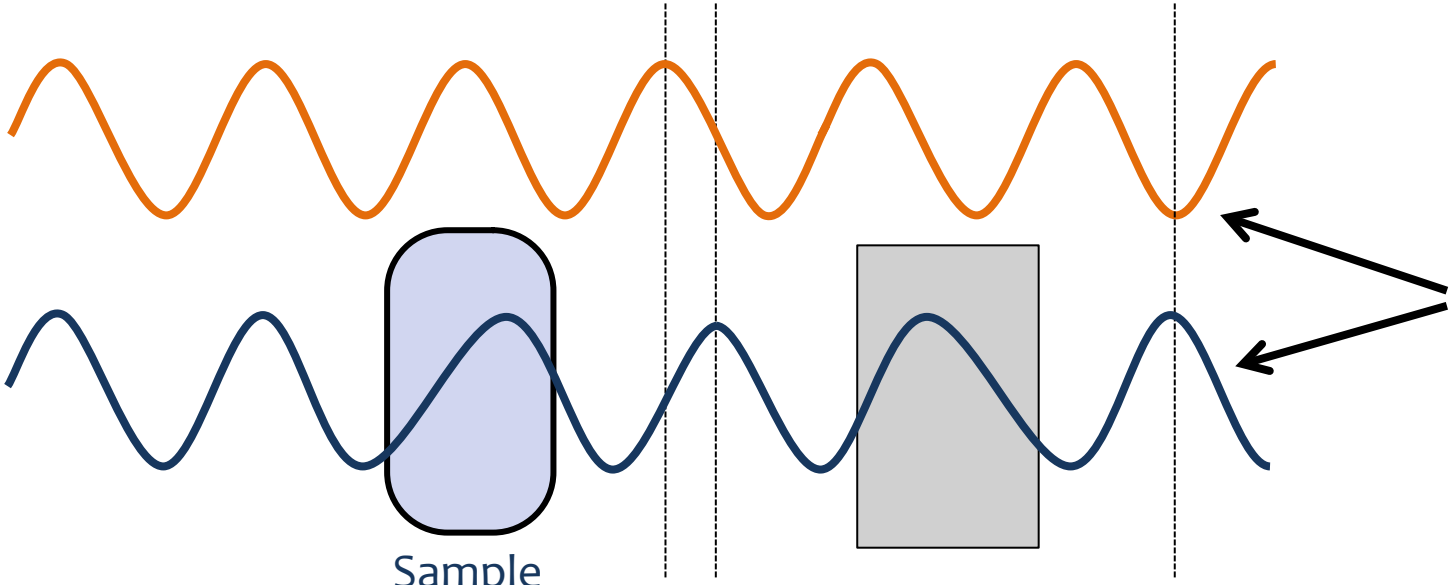
Phase contrast



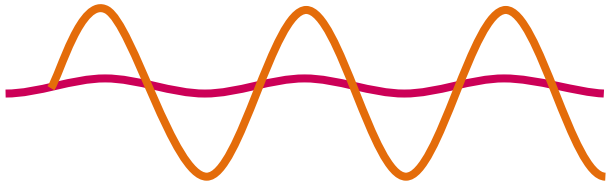
In average a biological sample induce a λ/4 phase retardation ($\pi/2$ in cosinus).
Interferences are to small to induce any visible contrast.

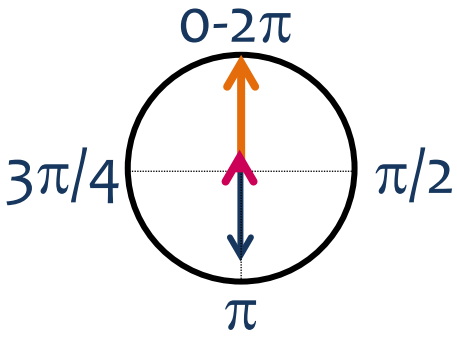
Contrast improvements

Phase contrast



A phase blade induces a supplementary
= λ/4 phase retardation

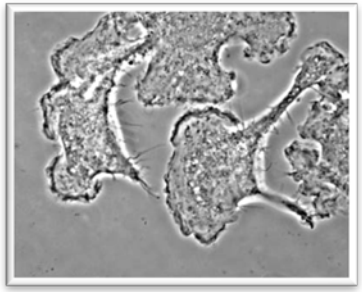




In order to obtain a maximal contrast, we might induce interferences with a λ/2 phase retardation (π in cosinus): In antiphase

Contrast improvements

Phase contrast

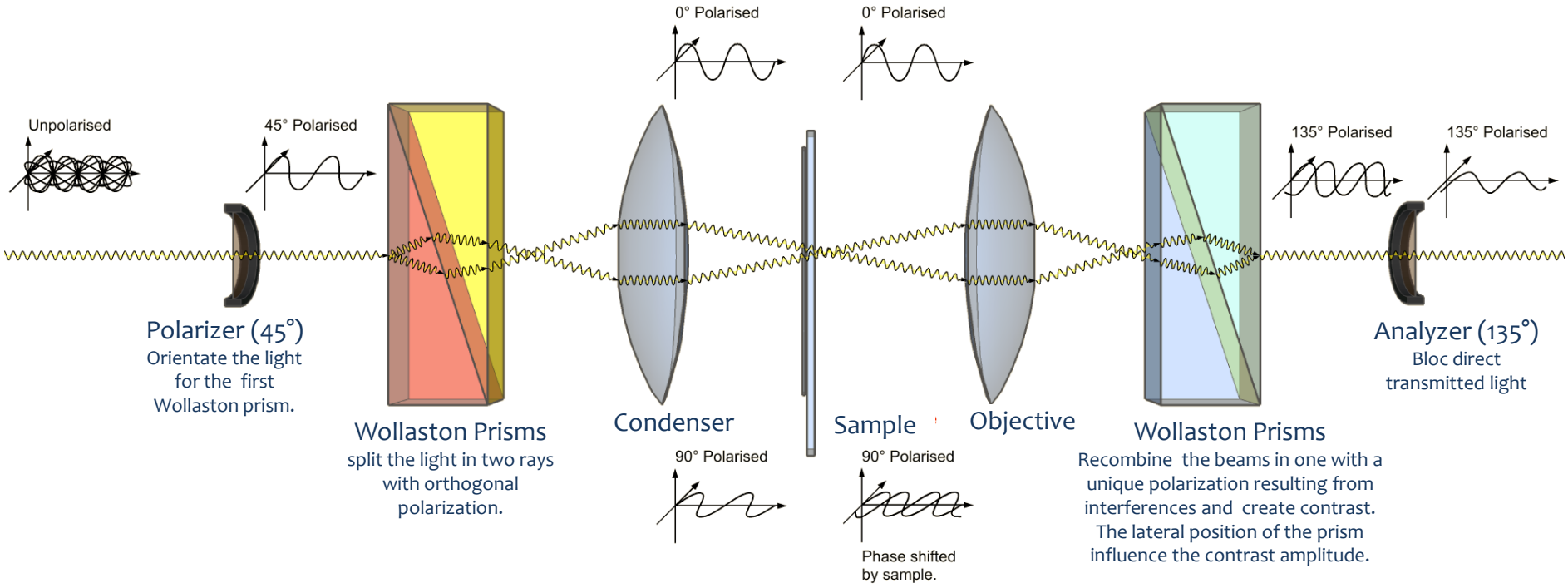


The directly transmitted light (annular - yellow) is largely diminished by the ring in the phase blade et interfere with diffracted light from the sample (grey in the scheme) to obtain large contrast in "black and white“ (destructive or constructive interferences : (+λ/4) or (-λ/4)).
We finally obtain an optimal differential phase of λ/2.

Contrast improvements

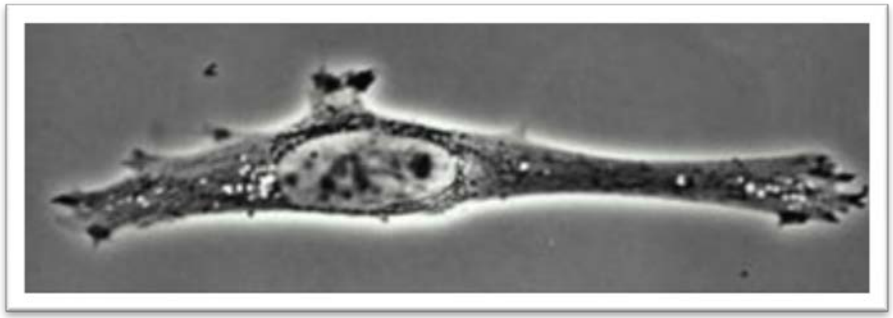
Differential interferential contrast: DIC (Nomarski)

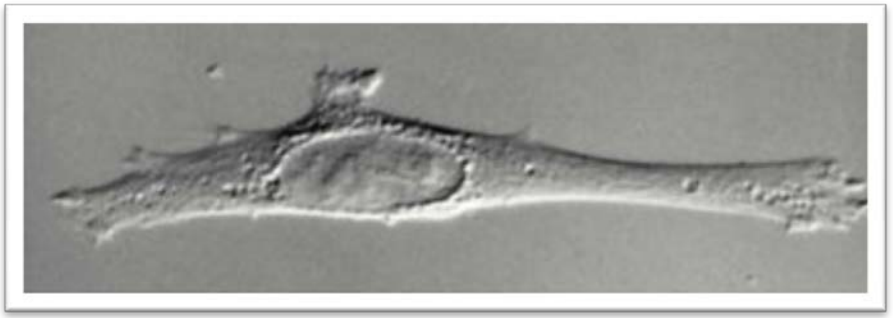
Wollaston prisms split the light in two rays with orthogonal polarization (birefringence). When they are recombine by the second Wollaston prism, there is some interferences with the two beams depending their optical pathways. It result a grey light if both beams get the same track and white or black if they have been deviated by the sample giving a razing illumination. This kind of contrast give in relief vision in sick brain slice (best for patch-clamp).



Contrast improvements

Phase contrast /DIC comparaison





Phase contrast

- Work with plastic Petri dish
- Sensitive of the optical pathway
- Halos (Black and white contrast) at the edge of visualized object
- Not sensitive to the orientation of the structures ("isotropic" contrast)
- Chipper

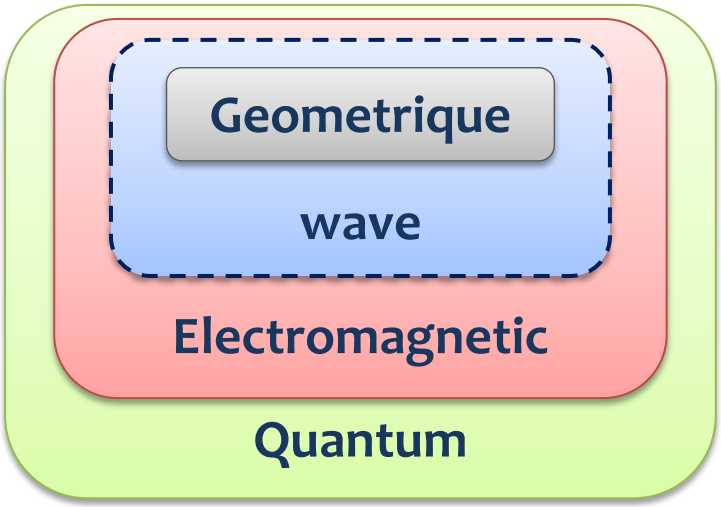
DIC

- work only with glass
- Sensitive to optical index variations
- No halos around the objects
- Use optimally the NA and give by the way the best resolution
- Sensitive to the orientation of the structures (pseudo-shadow effect)
- More expensive

End of the first round

Thanks to listen me till now !

Next step: fluorescence microscopy



Commentaries, Remarque, Improvements:
francois.michel@inserm.fr

Sources et Ressources

WEB

- Molecular expressions http://micro.magnet.fsu.edu/index.html
- Microscopy U (Nikon) : http://www.microscopyu.com
- Microscopy resource system (Olympus): http://www.olympusmicro.com/index.html
- A whole world of microscopy knowledge (Zeiss): http://zeiss-campus.magnet.fsu.edu/index.html
- Wikipédia: http://en.wikipedia.org/wiki/Main_Page

Imaging facilities

- PICSL http://www.picsl.univ-mrs.fr/ : MICROSCOPIE OPTIQUE (PDF indisponible)
- BIC http://www.bic.u-bordeaux2.fr/ : Microscopie à épi-fluorescence et microscopie confocale (PDF)
- Membres du GDR2588 CNRS (microscopie fonctionnelle des systèmes vivants) et du RTmfm (Réseau technique de microscopie de fluorescence multidimensionnelle).

Cours

- Arnaud Sergé (université de la Méditerranée – Marseille II)
- Yves Husson (Université Joseph Fourrier – Grenoble)
- Serge Monneret (Institut Fresnel, Marseille)
- Maxime Dahan (Laboratoire Kastler Brossel, ENS)
- François Waharte (institut Curie, UMR144)

Handbooks

- Fondamentaux d'optique et d'imagerie numérique à l'usage des microscopistes (C Cibert, ed. Cépaduès)
- Handbook of biological confocal microscopy, third edition (JB. Pawley ed. Springer)
- Fundamentals of light microscopy and electronic imaging (DB. Murphy, ed Wiley-liss)