607SM - Techniche avanzate di indagine microscopica

Advanced microscopy techniques – 6CFU, 2002/23, 1st semester

Part1:

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Tuesday 11-13, Aula 1C, Ed H3 + Thursday 14-16, Aula 5A EdH2-H2bis (colleg H3)

LECTURE 2-3

1. Optical microscopy

1.1. Basics

- Why optical microscopy?
- Image formation; magnification and resolutiont;
- diffracted limited resolution;
- Optical aberrations and image quality;
- Digital camera image acquisition (formats, properties, SNR)

1.2. Phase imaging techniques – 1h

- Phase contrast and differential interference contrast (DIC);
- Quantitative phase imaging: digital holographic microscopy.

1.3 Other techniques to image non-stained samples - 1h

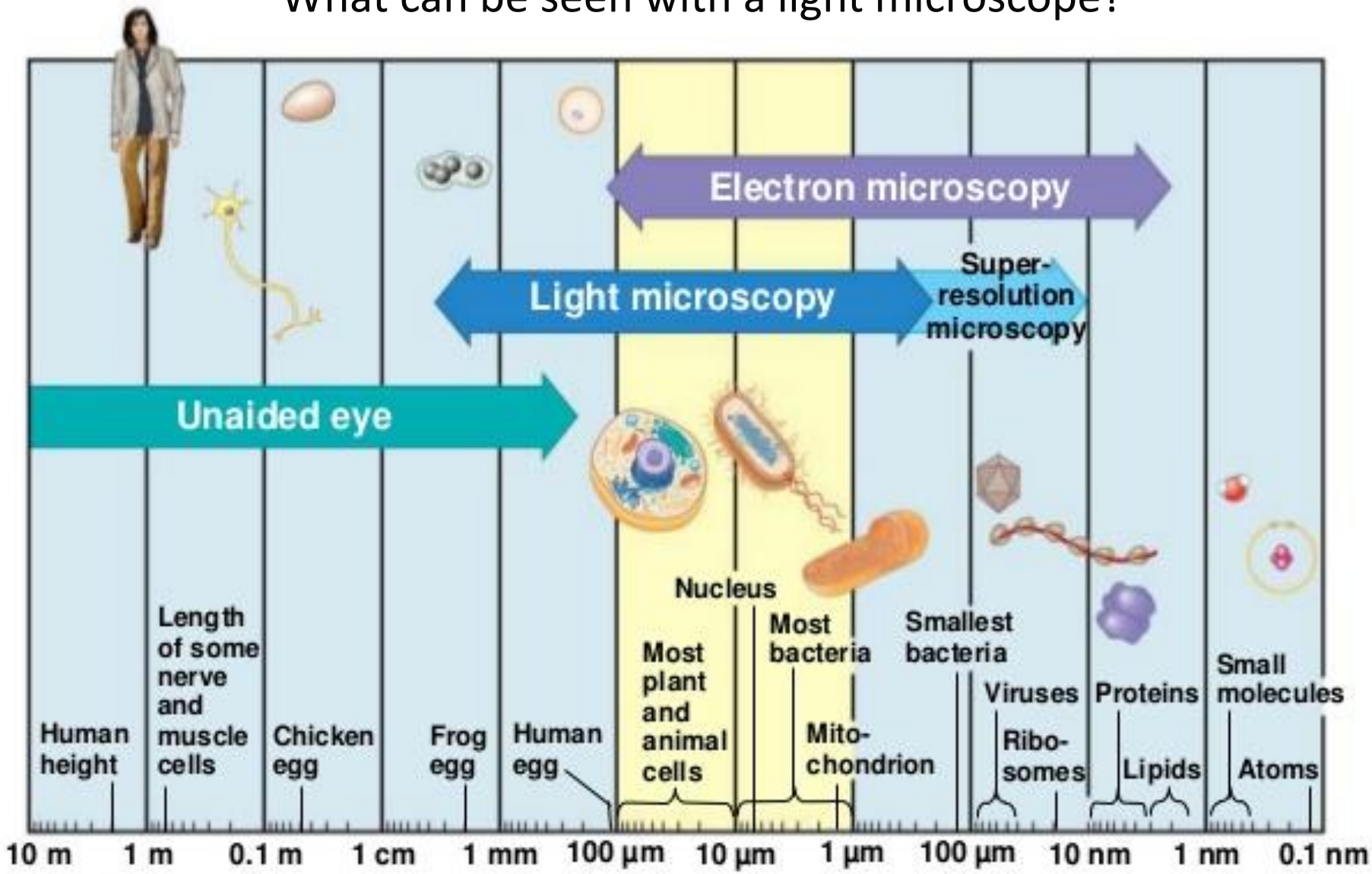
- Dark field microscopy
- Polarization microscopy
- Non linear microscopy

Optical microscopy:

- uses light in visible range (wavelength 400 - 700 nm) to image samples with details as small as 200 nm (~ half wavelength).
- recently, new techniques have been developed to improve the resolution and resolve details as small as 20 nm in living cells
- moreover, light has been employed to manipulate cells and their components (optical tweezers and scalpels) and measure piconewton forces (force spectroscopy)

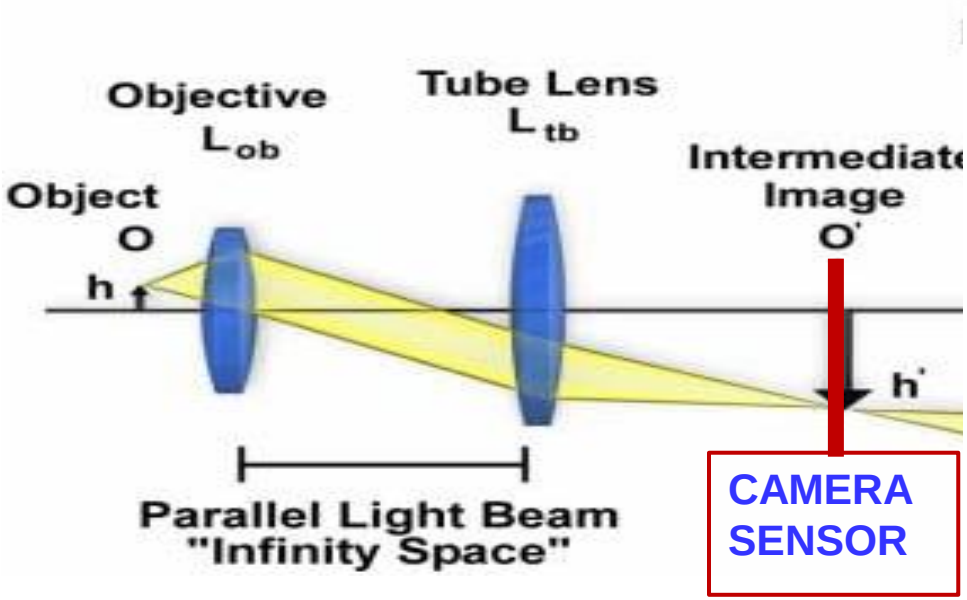
Figure 6.2c

What can be seen with a light microscope?



Magnification vs resolution in brightfield microscopy

Image formation in the optical microscope



- The object is positioned in the focal plane of the **OBJECTIVE**, hence its image (through the objective) is projected to infinity.
- The **TUBE LENS** 'brings' this image from infinity to its focal plane, forming a magnified image, called intermediate image. in its focal plane.
- The intermediate image can be observed through the EYEPIECE or it is directly captured by a CAMERA SENSOR and displayed on a monitor.

For a more detail discussion about image formation through a lens, a mirror, or a combination of lenses see Blackboard examples and:

Geometric / Ray OPTICS

https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwitlPvS2cT6AhWFsKQKHROGBkQQFnoECBQQAQ&url=https%3A%2F%2Fweb.njit.edu%2F~gary%2F234h%2Fassets%2FPhys234h_Lecture04.ppt&usg=AOvVaw0XB_XY0i5ICOG1c622lXn4

Uploaded ppt !!!

Geometric Optics by Anderson

Magnification

Objective magnification	$M_{OB} = \frac{f_{TL}}{f_{OB}}$	4X – 120 X
Microscope magnification	$M_M = M_{OB} M_{EY}$	50 X – 2000 X
EY – Eyepiece; DC – Digital Camera	$M_M = M_{OB} M_{DC}$	

Note:

Magnification is different from Resolution !!!

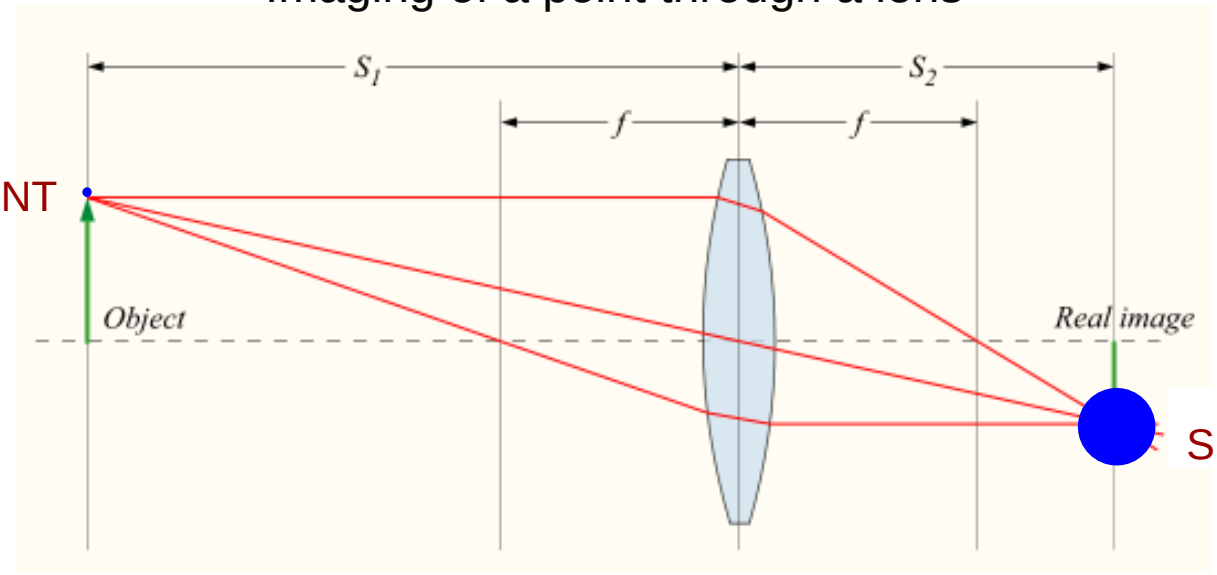
i.e. High magnification does not always mean a better resolved image

Resolution

Resolution describes the ability of an optical system to **resolve details** of the object that is being imaged.

Due to the diffraction of light through an optical system with finite size, a **POINT** object is imaged into a **SPOT** rather than a point.

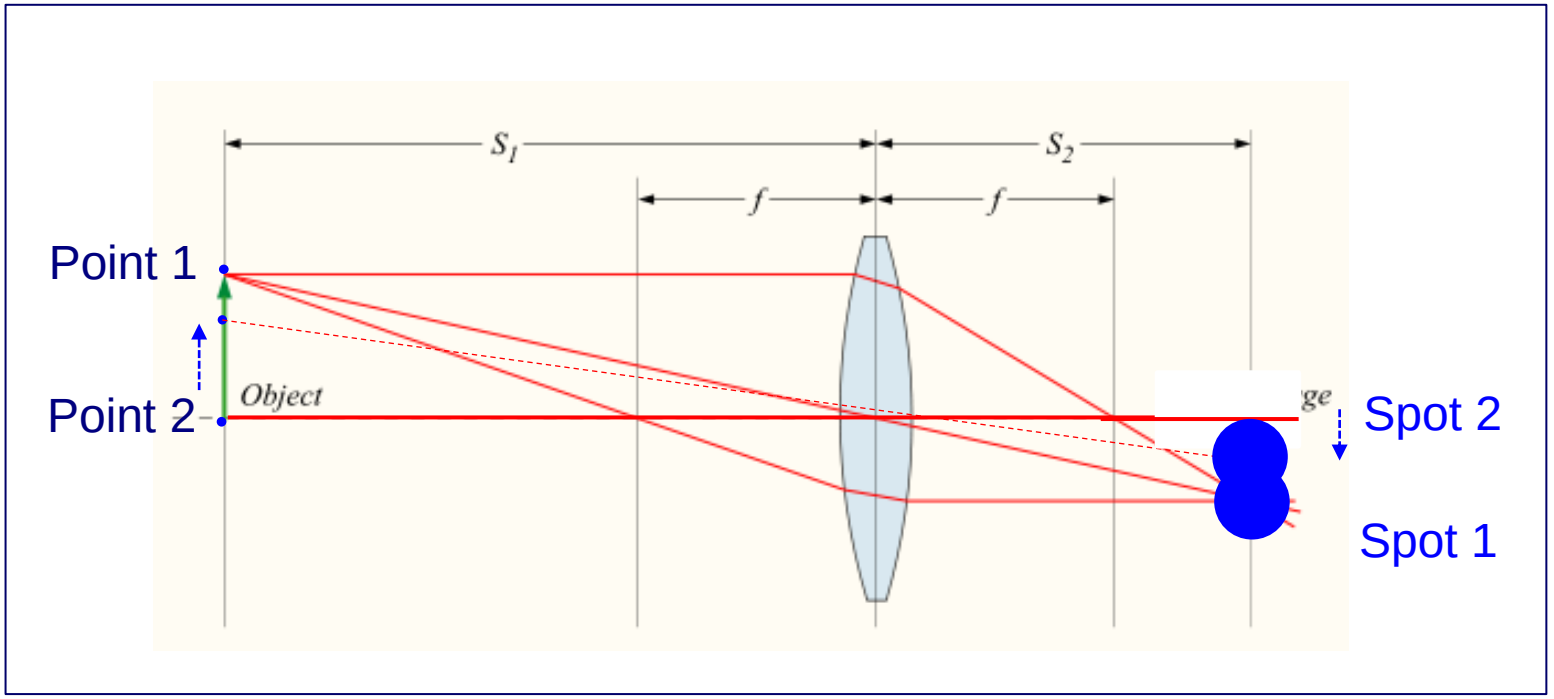
Imaging of a point through a lens



LARGER the LENS, SMALLER the SPOT !

SMALLER the SPOT, BETTER the RESOLUTION !

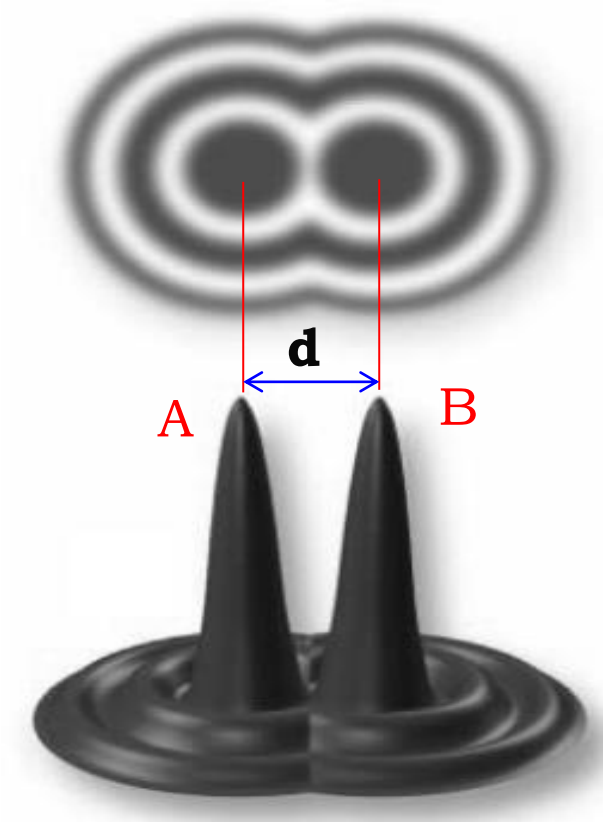
Imaging TWO points through a lens



When the two points are close each other, their images (two spots) overlap and hence they can not be separated (resolved)!

Resolution criterion

The resolution, r, is defined as the shortest distance between two points on a specimen that can still be distinguished by the observer or camera sensor as separate entities.



A and B are separated if: **d** > **r**

Rayleigh criterion

$$r = 0.61\frac{\lambda}{NA}$$

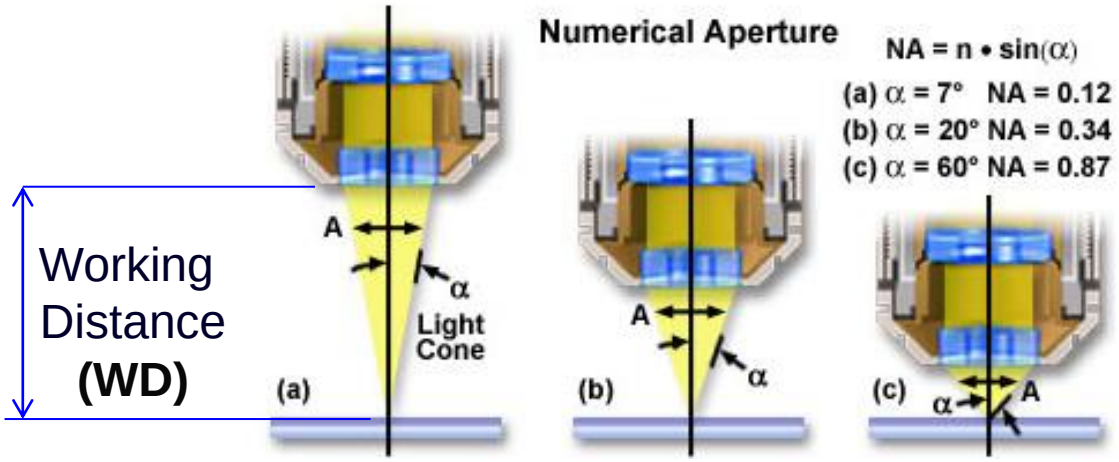
NA- Numerical Aperture

Estimating the lateral resolution of a microscope objective (lens):

NA = 1.5, λ = 400 nm

→ **r ~ 200 nm**

Resolution – Numerical Aperture – Working Distance



$$NA = n \cdot \sin \alpha$$

Immersed objectives NA > 1

Oil (n=1.515), Glycerin (n=1.47) or Water (n=1.33)

Higher NA → better lateral Resolution

Note: WD decreases when NA increases !!!

Microscope objective specs

http://www.microscopyu.com/

- **The magnification of the optical microscope is max 2500X**
- **The lateral resolution of the optical microscope is diffraction limited to 200 nm (i.e. half of the wavelength: $\lambda / 2$)**

Magnification is different from Resolution

i.e. a higher magnification does not guarantee a better resolution

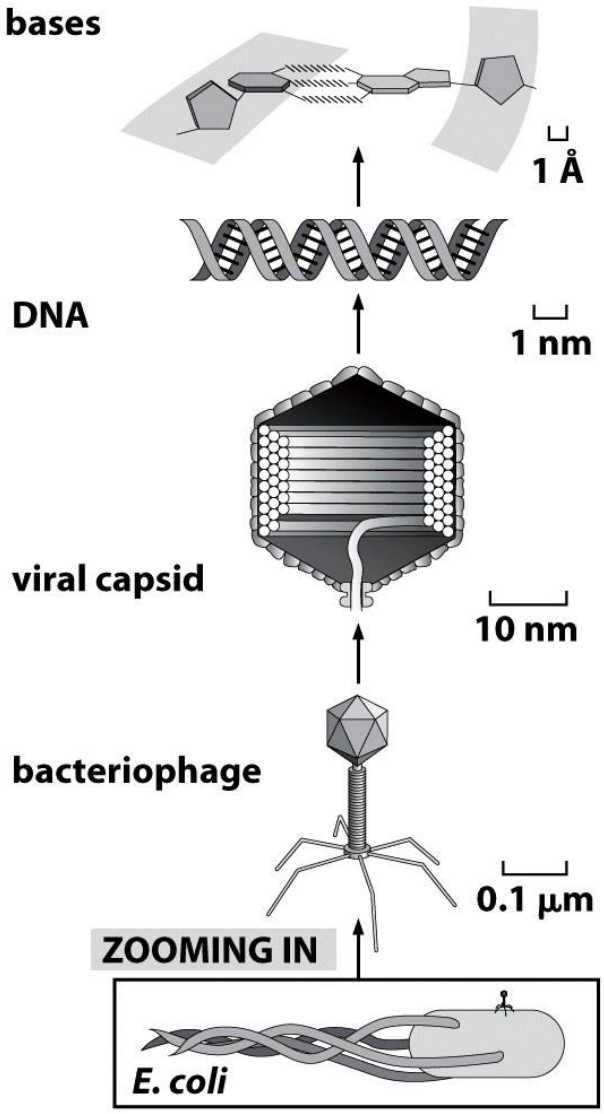
Lateral resolution $\Delta x \cong 0.5\frac{\lambda}{NA}$ ~ 200 nm

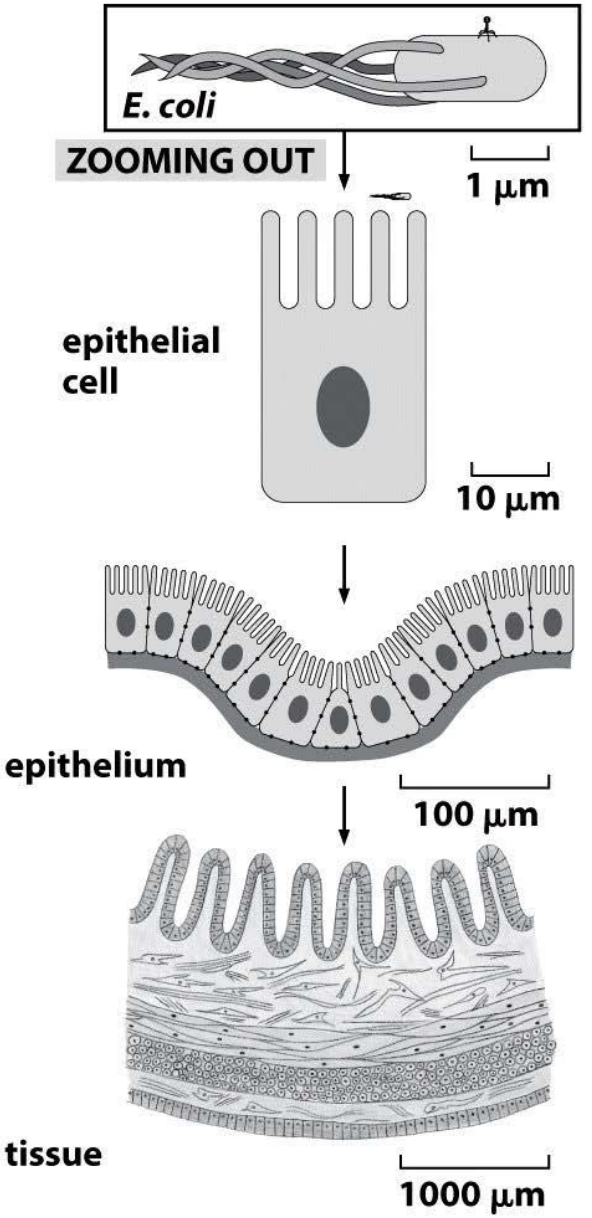
Axial resolution $\Delta z \cong 0.5\frac{\lambda n}{NA^2}$ ~ 300 nm

$\Delta z \approx \frac{n}{NA}\Delta x \longrightarrow \Delta z > \Delta x$

Lateral resolution is better than the axial resolution

Biological Scale and Size





Optical Microscopy

http://learn.genetics.utah.edu/content/begin/cells/scale/

Optical aberrations and image quaòity

Geometric and chromatic abberations

Optical aberrations:

deviation of the image from the ideal image given by paraxial approximation

Paraxial optics:

propagation of the rays of light close to the optical axis.

Example:

a point object is not transformed in a point image but in a line or a spot.

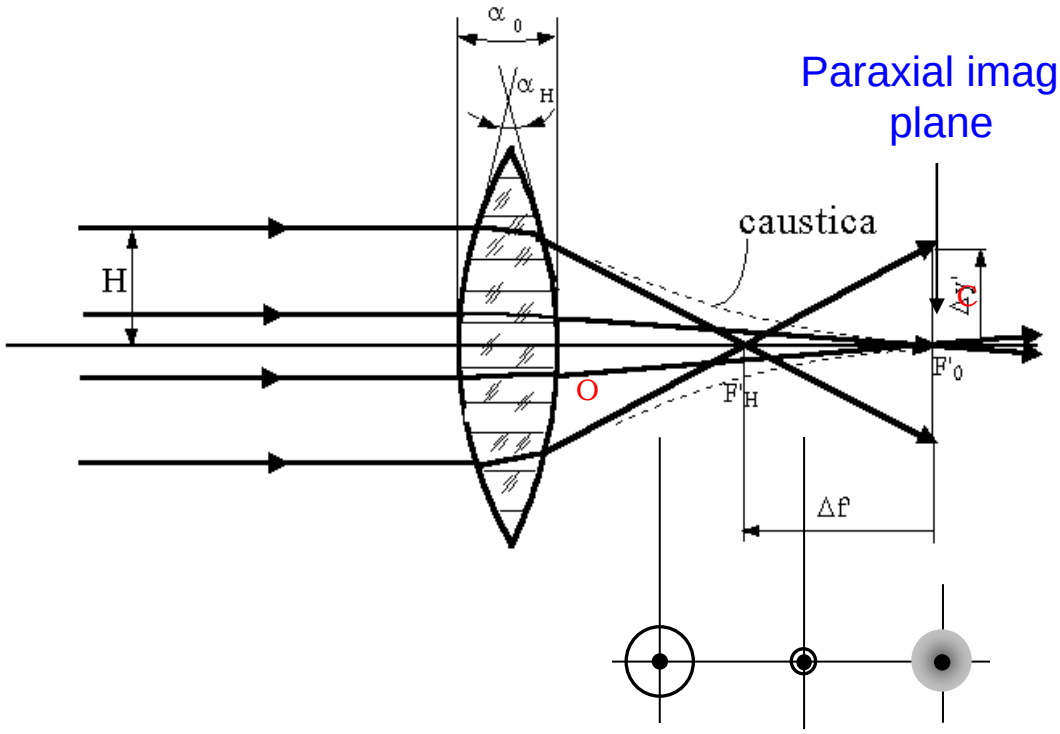
Geometric aberrations: due to the shape of the optical elements

Chromatic aberrations: due to the wavelength of the rays of light forming the image

Nice applets:

http://www.microscopyu.com/tutorials/java/aberrations/astigmatism/index.html

Geometric abberations: spherical aberration



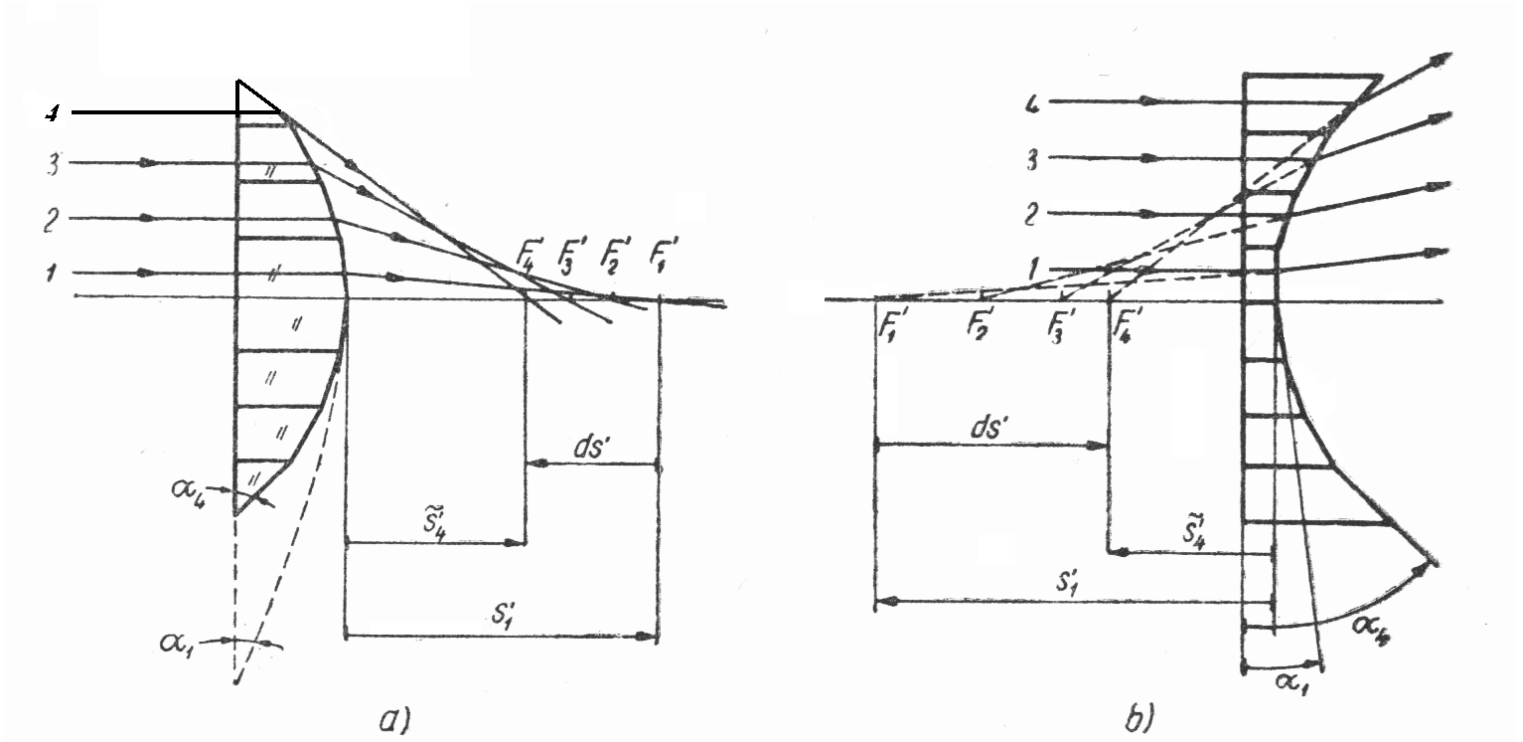
Rays passing through the outer part of the lens are focused closer than the paraxial rays.

Axial spherical aberration is defined by the difference between the extreme postions of the focal points F'_O and F'_H

This difference can be explained considering the 'prism' effect:

the emergent ray is deviated by an angle $\delta_i = (n - 1)\alpha_i$ with respect to the incident ray

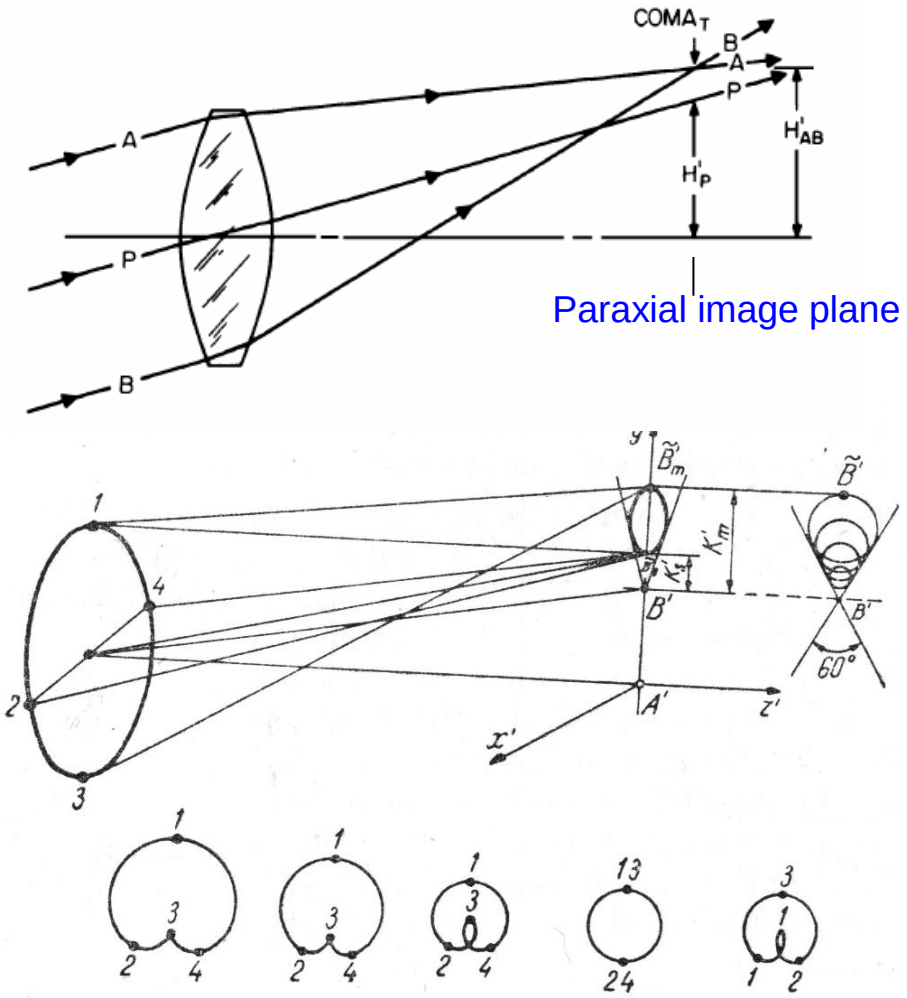
Spherical aberration



The sign of the spherical aberration for a positive lens is opposite to that of a negative lens → combining positive and negative lenses represents a solution to reduce spherical aberration by compensation.

Another solution is to keep the rays propagating as close as possible to the optical axis.

Geometric abberations: coma



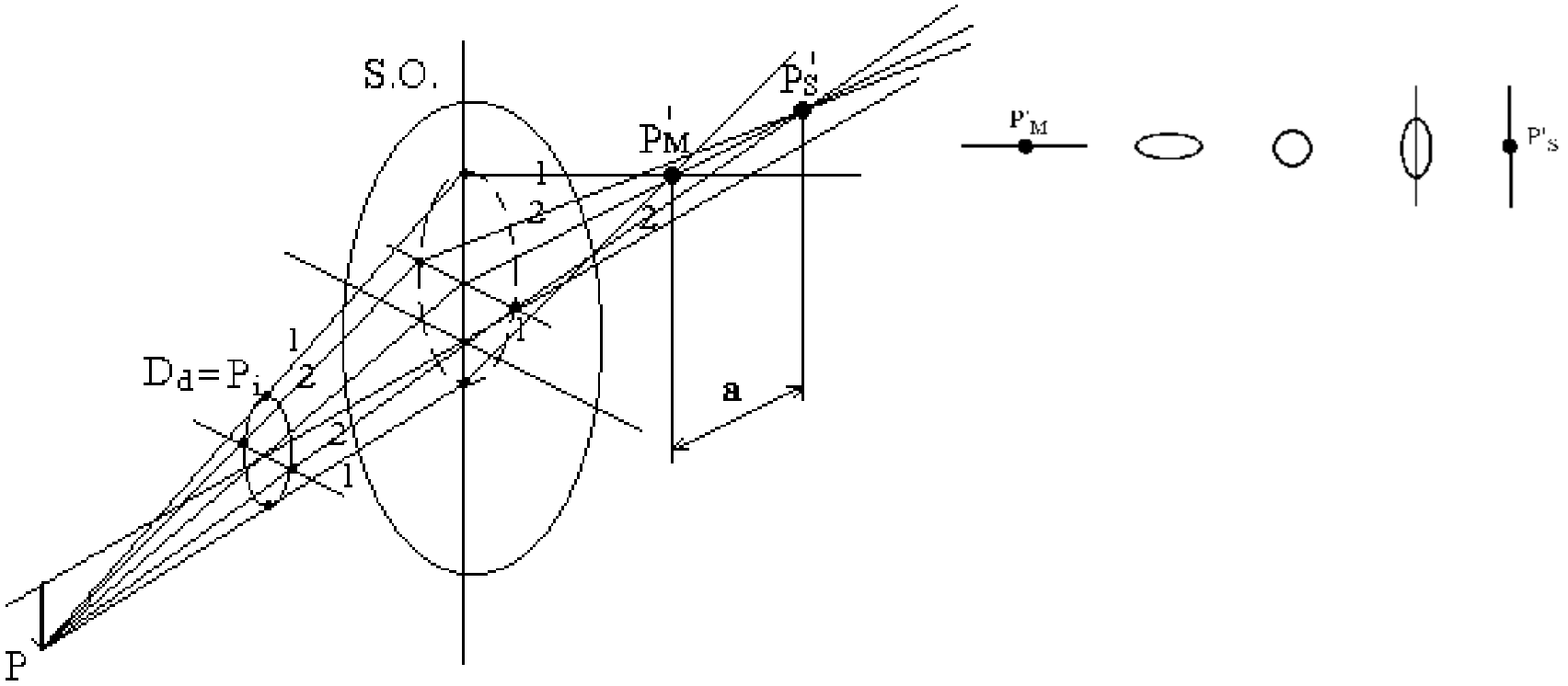
Coma: the modification of the magnification with the aperture of the optical system.

It occurs when extra axial obiect points are imaged with tilted parallel rays.

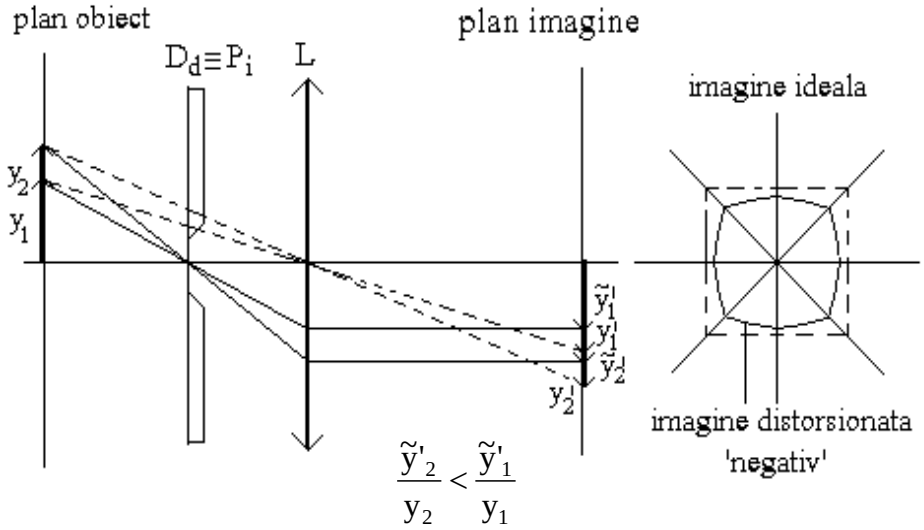
Geometric abberations: astigmatism

Astigmatism: the image of an extra axial point object is different for different planes (meridian, sagital) → the image is no more a point.

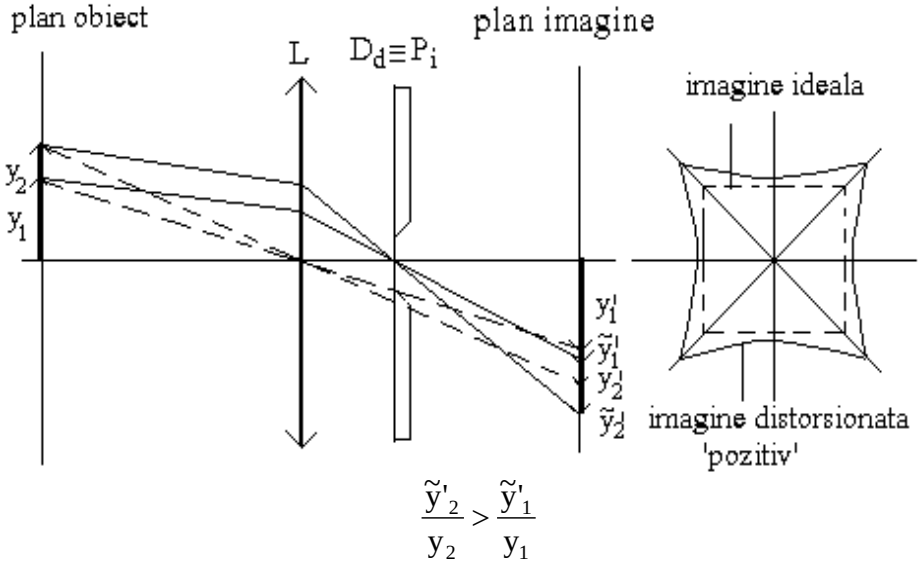
It occurs when extr axial points are imaged with meridian and sagital rays.



Geometric abberations: distortion



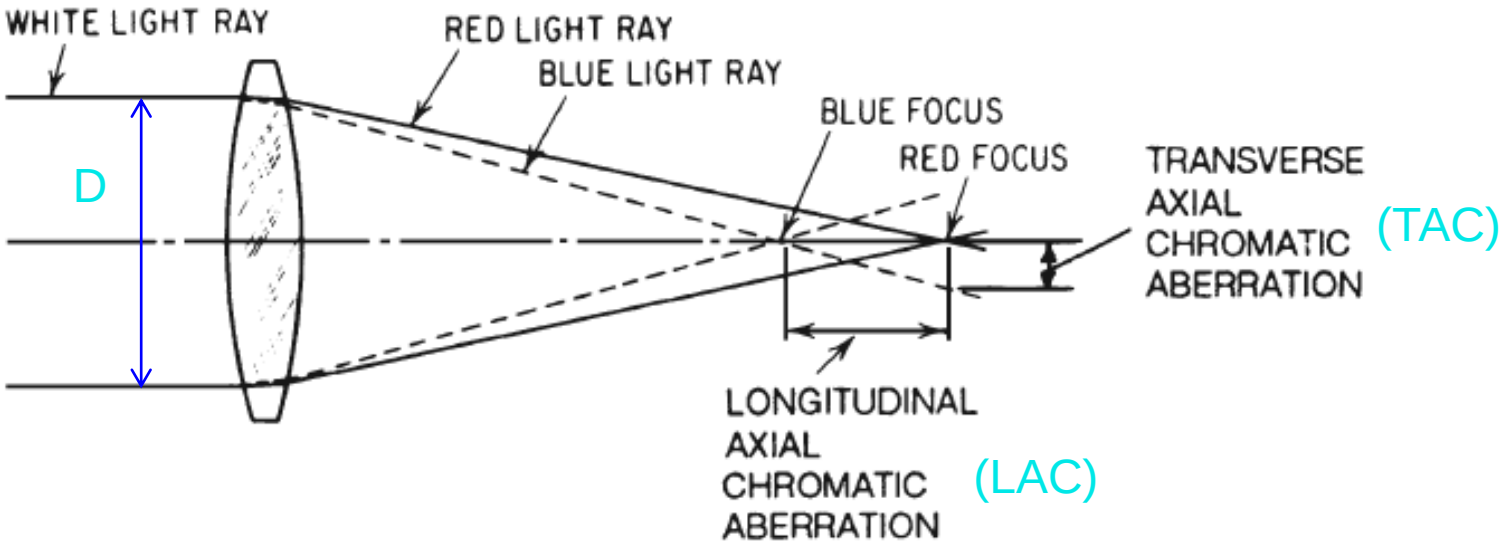
Distorsion is a function of the position of the aperture diaphragm with respect to the lens.



Its value is proportional with the cubic distance from the object point to the optical axis →

e.g. for a square the corners are distorted $2\sqrt{2}$ stronger than the middle of the line.

Chromatic aberrations



(8) $$\frac{\Phi_F - \Phi_C}{\Phi_d} = \frac{n_F - n_C}{n_d - 1} = \frac{1}{\nu} = V$$

d (λ= 0.5876 μm), C (λ= 0.6563 μm) F (λ=0.4861 μm)

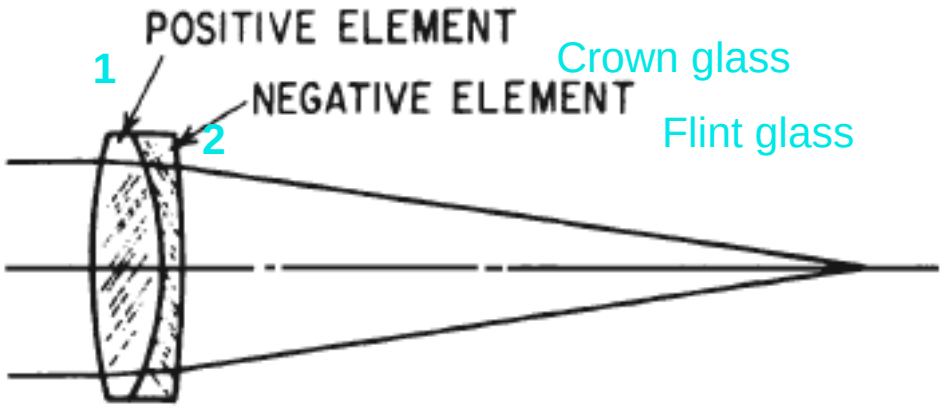
ν – relative dispersion, V – Abbé's number

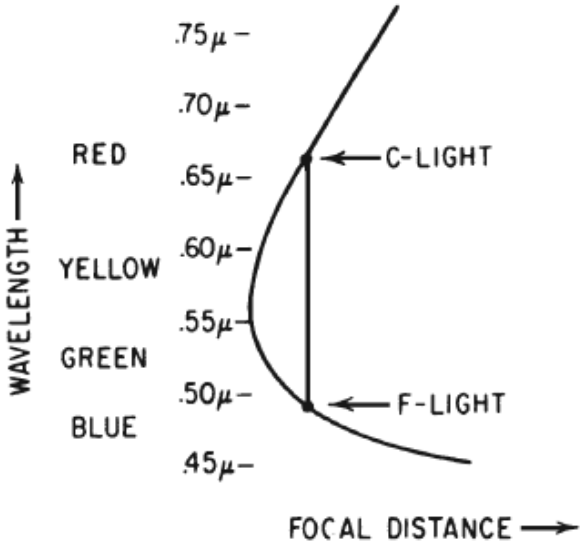
(9) $$f_d \frac{f_C - f_F}{f_C f_F} \cong \frac{f_C - f_F}{f_d} = \frac{1}{\nu} = V \longrightarrow LAC = f_C - f_F = \delta f = \frac{f_d}{\nu} = f_d V$$

$$TAC = \frac{D}{4\nu} \qquad (10)$$

Achromatic doublet

By combining two lenses: one positive in Crown glass and the other negative in Flint glass, the focal length will be the same for two wavelengths





Lens combination $\frac{1}{f} = \frac{1}{f_1} + \frac{1}{f_2}$

Achromatic equation $\frac{\delta f}{f^2} = \frac{\delta f_1}{f_1^2} + \frac{\delta f_2}{f_2^2} = 0$

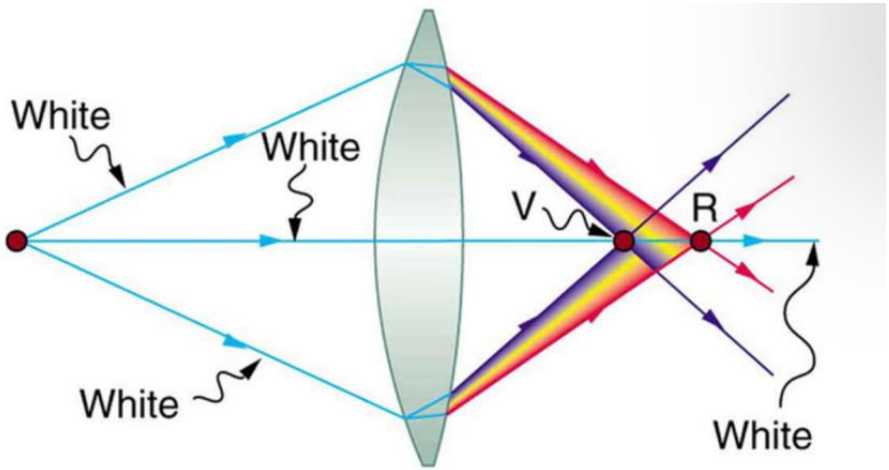
Chromatic aberration $\delta f = fV$

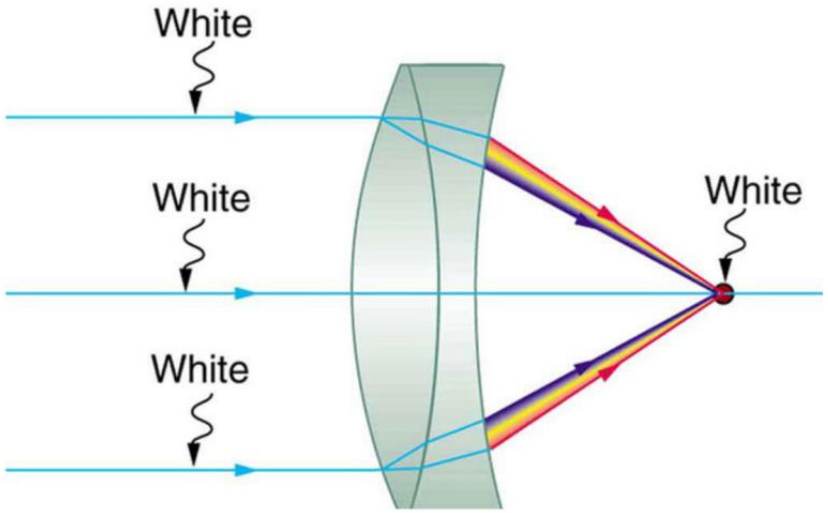
$$\frac{1}{f_1} = \frac{1}{f}\frac{V_2}{V_2 - V_1}$$

$$\frac{1}{f_2} = -\frac{1}{f}\frac{V_1}{V_2 - V_1}$$

Numerical example: f= 150 mm, V_2= 60, V_1= 40 → f_1=50 mm, f_2= -75 mm

Chromatic abberation and achromatic doublet





Digital camera image acquisition (formats, properties, SNR)

Common requirements for digital cameras in microscopy:

- accurate morphological representation of the specimens
- appropriate spatial resolution
- appropriate signal to noise levels
- accurate capture of dynamic events
- high sensitivity for minimum perturbation of the pecimens
- appropriate dynamic range

These requirements depend upon the Sample, Optics, **camera Sensor**

Digital cameras - sensors:

CCD - Charge **C**oupled **D**evice

EM-CCD – Electronic **M**ultiplying **CCD**

CMOS - Complementary **M**etal **O**xide **S**emiconductor

Camera Sensor

- Chip layout (pixel size, frame rate).
- Signal sampling
- Noise and Signal to Noise Ratio (SNR)

Chip Layout

Pixel size: a good sensitivity requires a large pixel size; on the other hand, a good spatial resolution requires a small pixel size ➔ a trade off must be found.

Usual ranges: CCD: 2 to 7.5 µm, EMCCD: 8 to 24 µm, CMOS: 2 to 7.5 µm

Chip size: determines the camera's field of view.

Usual ranges:

CCD: 4.74 x 3.55 mm to 15.16 x15.16 mm (small to medium)

EMCCD: 3.072 x 2.072 mm to 13.3 x13.3 mm (small to medium)

CMOS: 6.97 x 2.23 mm to 16.6 x14 mm (medium to large)

Frame rate: the maximum number of frames that can be captured in one second

Usual ranges:

CCD: 3-100 fps @ 1362 x1024 pixels

EMCCD: 8-500 fps @ 800x600 pixels

CMOS: 500 fps @ 1700x1600 pixels (up to 5 Mfps for ROI of 128 x 128 pixels)

Note: If a high frame rate is necessary, beside the sensor choice, the PC and data transfer are also very important (e.g. 2 sec recording of images 512x512 pixels with 8 bit depth / pixel at 1000 fps produces a file > 1 GB)

Signal Sampling

It is given by the pixel size and defines the sensor resolution. This should match the optical resolution: $Rr = 0.5\,\lambda / NA$, which at the sensor plane becomes:

$Rs = M \cdot Rr$ with M being the magnification.

From the sampling (Nyquist) theorem we get :

$$1/p = fs \geq 2 \cdot fmax = 1/Rs \quad \rightarrow \quad p \leq 0.5 \cdot Rs \quad \rightarrow \quad SF = Rs/p \geq 2$$

where: p is the pixel size, fs the sampling frequency, fmax the max frenquency in the image and SF the sampling factor

Example

Table 1: Sampling Factors For Different Cameras (< 2.0 is undersampled)							
MAG	Coupler	NA	Resolution Limit (@ sample in microns)	Resolution Limit (@ detector in microns)	3.5 micron pixel	6.5 micron pixel	14 micron pixel
10	1	0.45	0.75	7.46	2.13	1.15	0.53
20	1	0.75	0.45	8.95	2.56	1.38	0.64
40	1	0.95	0.35	14.13	4.04	2.17	1.01
40	1	1	0.34	13.42	3.83	2.06	0.96
40	1	1.3	0.26	10.32	2.95	1.59	0.74
60	1	1.2	0.28	16.78	4.79	2.58	1.20
60	1	1.3	0.26	15.48	4.42	2.38	1.11
100	1	1.4	0.24	23.96	6.85	3.69	1.71

Sufficient Sampling Insufficient Sampling

Sampling factor FS ≥ 2.0 → good sampling

Sampling factor FS < 2.0 → undersampled

Noise (σ) and Signal to Noise ratio (SNR)

Table 1: Types of Noise

	Type of Noise	Description
σ_d	Dark Noise	Dark current is a time-dependent signal generated on the sensor when no light is present due to heat which causes random generation of holes and electrons in the depletion region of the sensor. Dark noise is the fluctuation in this signal.
σ_r	Read Noise	Noise contributed by the amplifier during the conversion of the analog signal to digital signal.
σ_n	Shot Noise	Fluctuation in signal due to the quantum properties of photons. The number of photons measured at any given point in time can fluctuate by plus or minus the square-root of the measured signal.

They can be considered as independent,

so the total Noise is given by:

$$\sigma = \sqrt{\sigma_d^{\,2} + \sigma_r^{\,2} + \sigma_n^{\,2}}$$

Signal to Noise Ratio

$$SNR = \frac{\Phi}{\sigma} = \frac{S_d}{N}$$

$$\frac{\text{Detected (signal) photons}}{\text{Noise}}$$

CCD, CMOS:

$$\sigma = \sqrt{\sigma_d{}^2 + \sigma_r{}^2 + \sigma_n{}^2}$$

EMCCD:

$$\sigma = \sqrt{\sigma_d{}^2 + \psi^2\sigma_r{}^2 + \psi^2\sigma_n{}^2}$$

Excess - noise factor ψ due to the impact ionization through which electronic amplification is achieved

Example: pixel performance of the perfect camera

$$SNR = \frac{QE * S}{\sqrt{F_n{}^2 * QE * (S + I_b) + (N_r/M)^2}}$$

Shot Readout

where:

QE: quantum efficiency

S: input signal (photon/pixel)

F_n: noise factor

N_r: readout noise

M: EM gain (=1 for CCD/CMOS)

I_b: background

N_d: dark noise (not included, assumed to be negligible)

For a perfect detector (QE=1, Nr=0; Fn=1):

$$SNR = \frac{1 * S}{\sqrt{1^2 * 1 \times S + (0/1)^2}} = \sqrt{S}$$

Even with a perfect detector we have noise !

This is the photon shot noise and is a function of photon statistics.

In other words, until we have light (photons) we have noise.

To improve SNR we have to collect as many photons as possible.

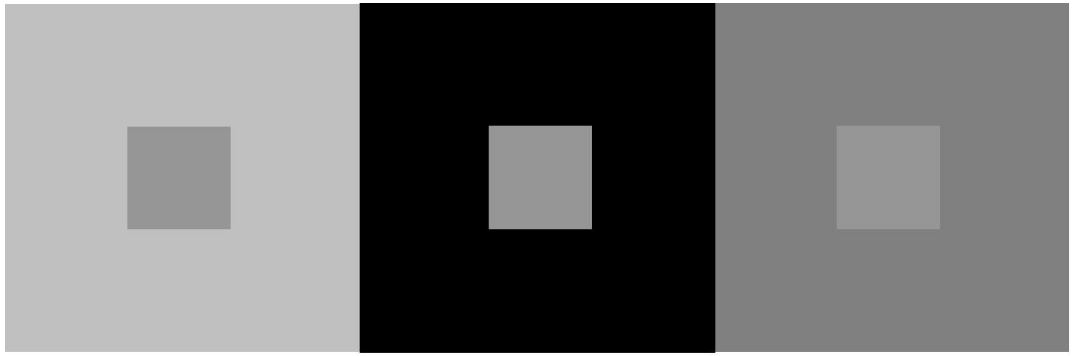
Phase contrast in light microscopy

Image contrast in brightfield microscopy

The contrast is formed by different absorption of light by medium and sample, creating an image with different intensity levels.

An useful image (with features that can be well distinguished) needs to have enough brightness and good contrast:

$$\textbf{Contrast} = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$



Which center grey box is lighter ?

Cells are almost transparent to visible light, hence it is difficult to obtain a good contrast in brightfield (based on absorption / transmission of light).

However, light is characterized also by PHASE and POLARIZATION which is influenced by the sampe, and hence carries information about it.

$$\boldsymbol{E} = \boldsymbol{A} \cdot e^{i\varphi}$$

E – intensity of the electrical (optical) field
Note that ***E*** is a vector; light polarization is related to the vector orientation
A – amplitude (vector) ; φ – phase

This information is usually lost because all existent sensors of light (e.g. retina photoreceptors, photodiodes) can detect only the intensity of light.

I – intensity of light $\quad I = |E|^2 = |A|^2 \quad$ because $|e^{i\varphi}|=1$

To exploit the phase or the polarization information

we need to convert it into intensity.

Exploiting the phase of light to enhance image contrast

The phase contrast method exploits **phase retardation of light** by different regions of the sample and the conversion of the phase difference into intensity.

$$\boldsymbol{E} = \boldsymbol{A} \cdot e^{i\varphi}$$

The phase of light: $\varphi = 2\pi \frac{OP}{\lambda}$ *λ – light wavelength*

OP – Optical Path $OP = t \cdot n$

n – refractive index of the material (sample or medium)

t – geometrical path (e.g. cell height)

Phase Contrast- principle

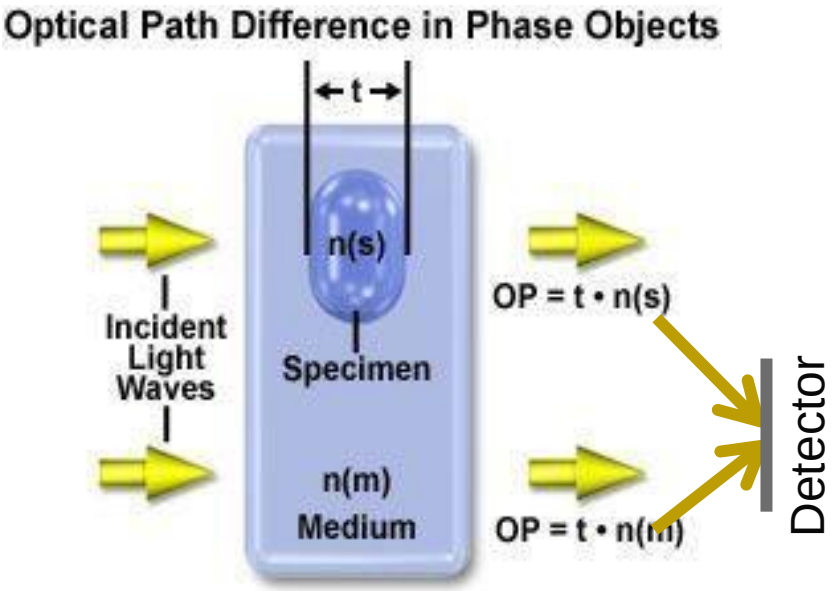
A pure phase sample, as a cell, does not absorb light but influence its phase.

The sample has a refractive index n(s) different from the refractive index n(m) of the medium in which the sampe is immersed.

Hence the optical paths (OP) of light through sample and medium are different:

$$OP(s) = t \cdot n(s) \quad ; \quad OP(m) = t \cdot n(m)$$

and so are the phase shifts / retardations: $\Delta\varphi(s) = 2\pi\, OP(s) / \lambda$; $\Delta\varphi(m) = 2\pi\, OP(m) / \lambda$



Unpolarized light and A(s)=A(m)= 1

$E(s) = e^{i\,\Delta\varphi(s)}$ $E(m) = e^{i\,\Delta\varphi(m)}$

The intensity on the detector:

$I = |E(s)+E(m)|^2 =$

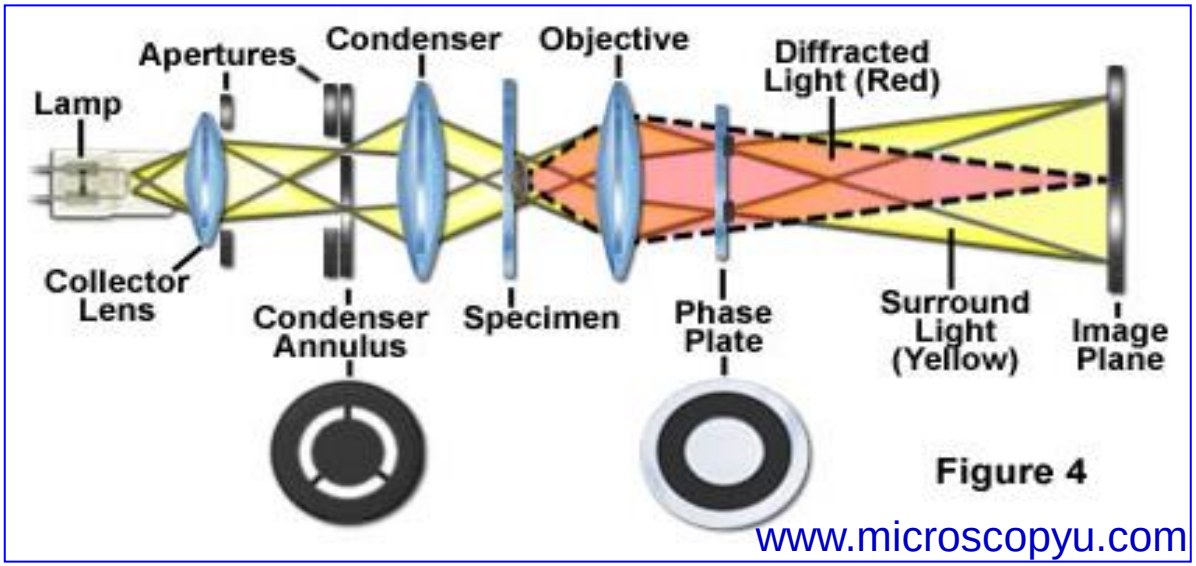
$=|E(s)|^2+|E(m)|^2+E(s)^*E(m)+E(s)E(m)^* =$

$= 2+2\cos(2\pi\, OPD / \lambda)$ NOT just 2

OPD - Optical Path Difference:

OPD= OP(s)- OP(m)= t(n(s))-n(m))

Phase contrast implementation



Using the condenser annulus, the specimen is illuminated obliquelly.

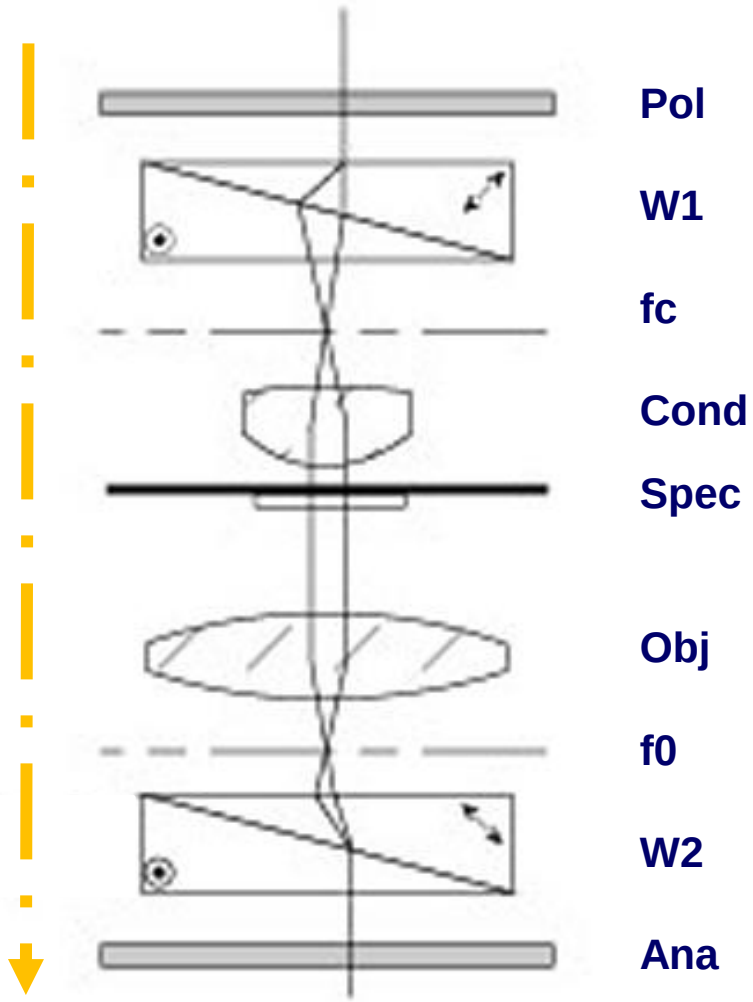
The light which is not intercepted by the specimen is focused onto the ring of the phase plate, which introduces a phase shift. The light reaching the specimen is focused on the image plane. Most of this light does not pass through the phase ring.

Phase contrast is obtained in the image plane from the interference between the light intercepted by the specimen and light not intercepted.

The Nobel Prize in Physics 1953 was awarded to Frits Zernike for “his demonstration of the phase contrast method, especially for his invention of the phase contrast microscope".

Another method: Differential Interference Contrast (DIC) method

DIC is an imaging method generating intensity contrast in the specimen's image by exploiting phase differences between the light passing through pairs of points of the specimen very near to each other.



Nomarsky DIC implementation:

Uses polarized light and anisotropic prisms

Pol: polarizer

W1, W2: Nomarski prisms

fc: condenser's focal plane

Cond: condenser lens

Spec: specimen

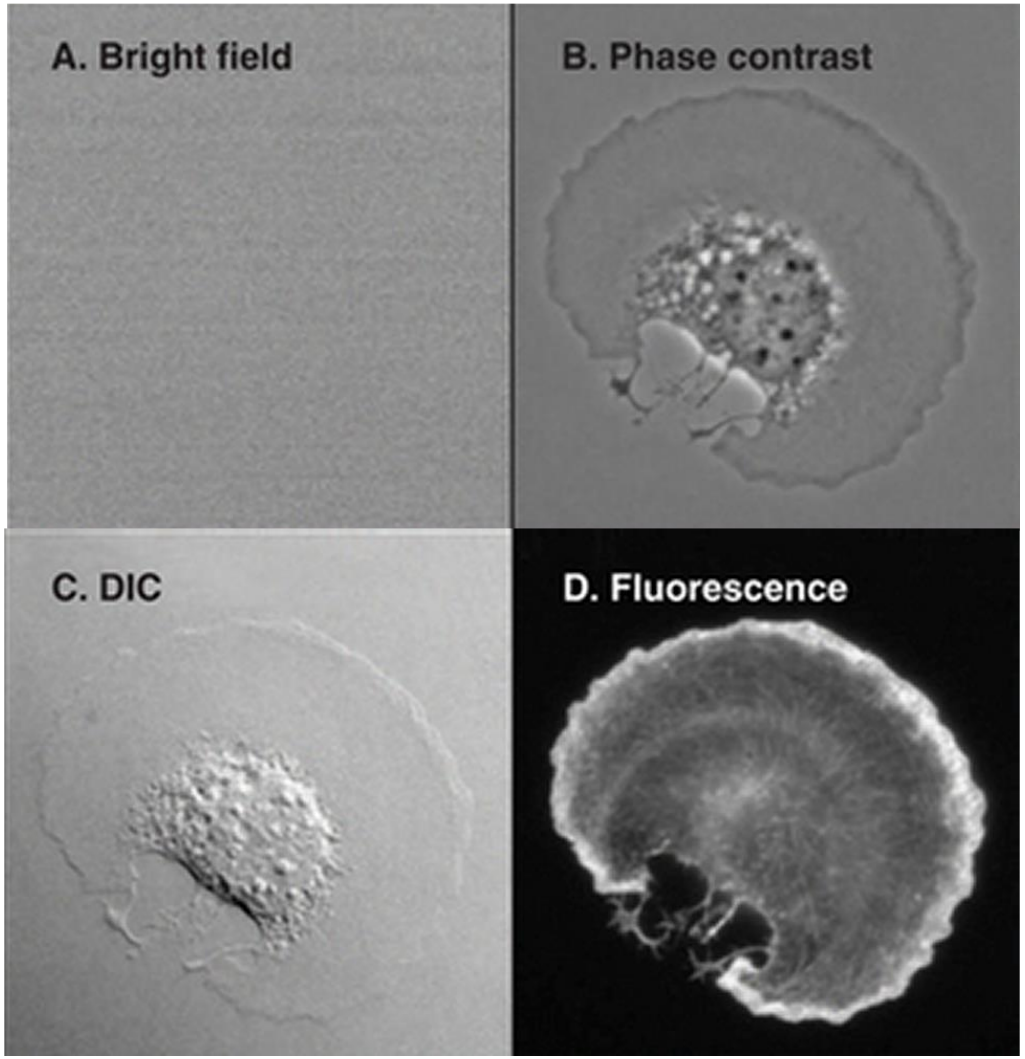
Obj: objective lens

f0: back focal plane of the objective lens

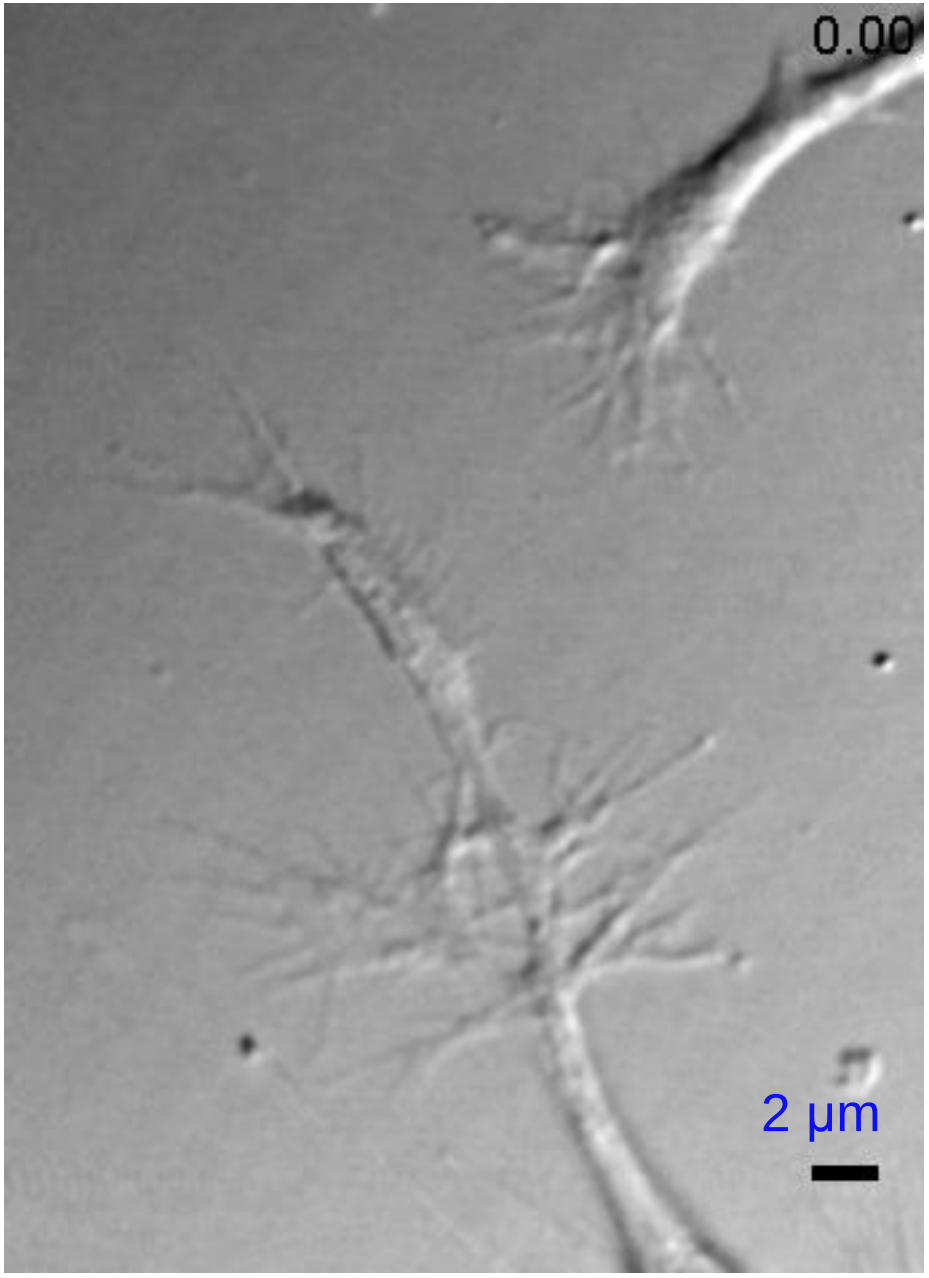
W2: Nomarski-modified Wollaston prism

Ana: polarizer (Analyzer)

Comparison - contrast enhancement by phase imaging



Images of a spread mouse 3T3 cell grown in tissue culture on a microscope slide, then fixed and stained with rhodamine-phalloidin, a fluorescent peptide that binds actin filaments.



Imaging neuronal growth cones with DIC

movie

F. Difato *et al* (2006) OM-Lab & SISSA

Quantitative phase imaging: digital holographic microscopy

Application Ex: measuring cell height and volume

Phase contrast microscopy is a qualitative technique, using the phase shifts to enhance the contrast and improve the image quality.

Since white light illumination is used (*λ : 400 – 800 nm*) phase contrast microscopy does not allow to measure the optical paths, which contain information about the sample height (t) and material (n(s)).

$$\Delta\varphi = 2\pi\, OPD / \lambda$$

OPD= t (n(s))-n(m))

Optical Path Difference

To get quantitative information on the phase, monochromatic light should be used → coherent sources – lasers

→ quantitative phase microscopy or digital holographic microscopy

Digital holographic microscopy (DHM)

DHM is a modern technology allowing quantitative-phase imaging of phase objects,

DHM is non-destructive, marker-free technique, derived from optical holography (OH).

DHM includes two steps: RECORDING and RECONSTUCTION .

RECORDING

An interference pattern is recorded on the digital camera. A laser is splited in two beams: one passes through the specimen and is called object beam and the other, called reference beam, is sent directly to the camera sensor. The interference between the object beam and reference beam forms an interference pattern called digital hologram. This contains both amplitude and phase information about the object.

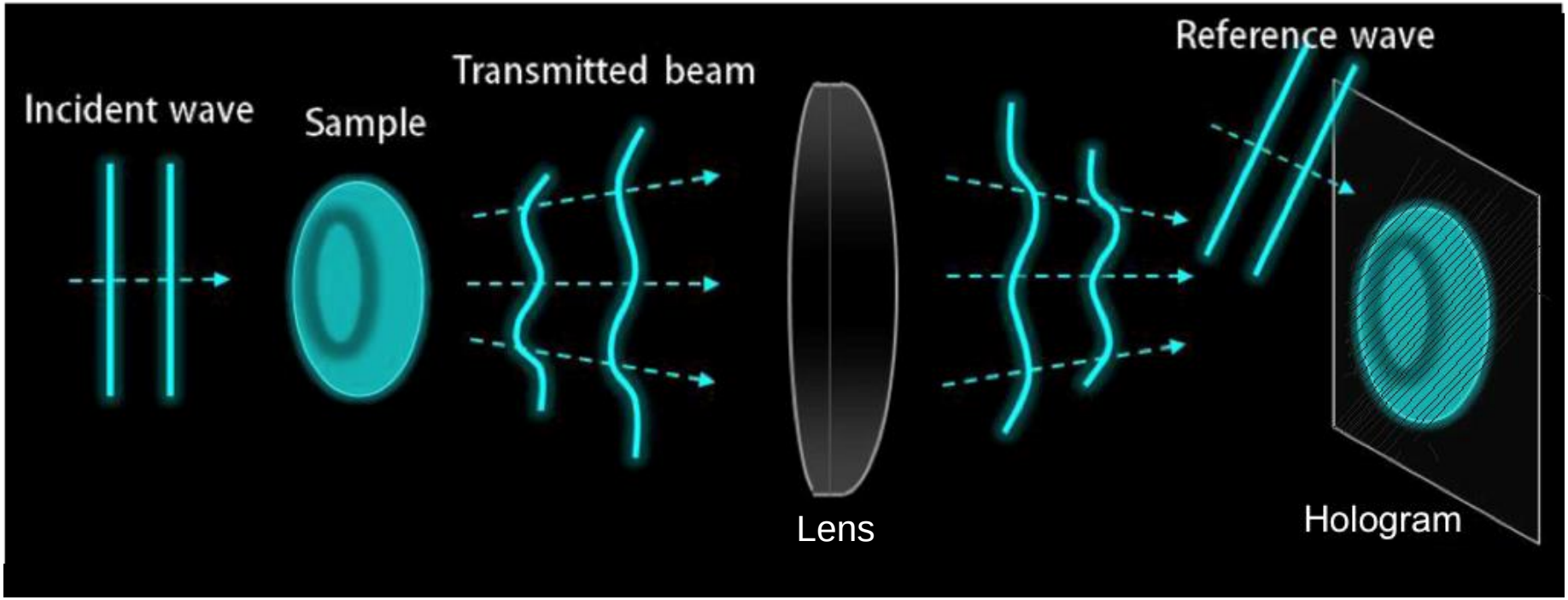
RECONSTRUCTION

The digital hologram is digitally processed to reconstruct the amplitude and phase information about the object.

Note: the difference between OH and DHM consists in the RECONSTRUCTION step which is DIGITAL in the case of DHM, while it is ANALOGIC (optical) in OH.

DHM – recording principle

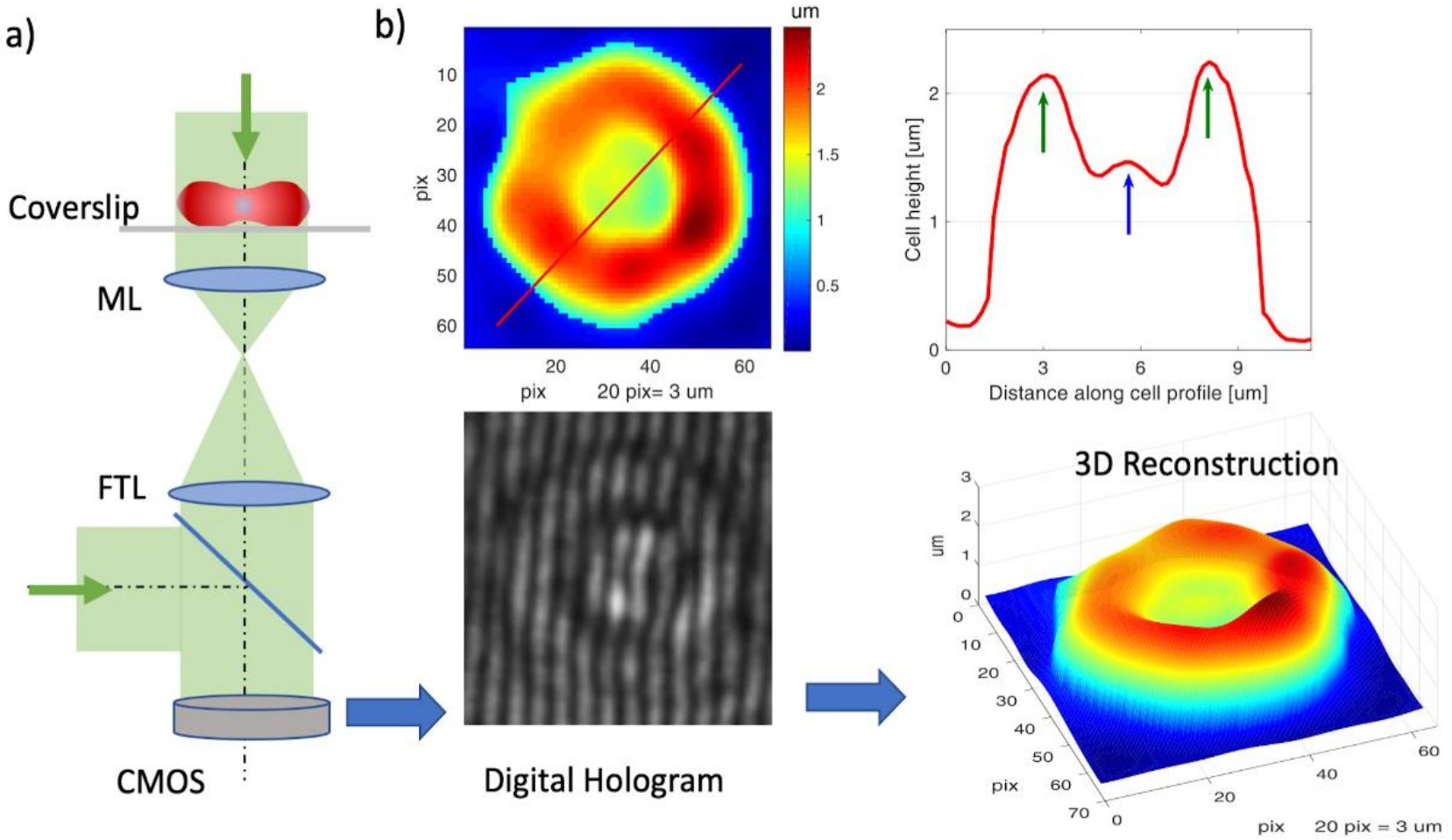
The digital hologram is obtained by the interference between the ‘transmitted beam’ and ‘reference’ waves



The incident and reference waves come from the same laser source

V. Mico et al, “Basic principles and applications of digital holographic microscopy”, Microscopy: Science, Technology, Applications and Education (2010).

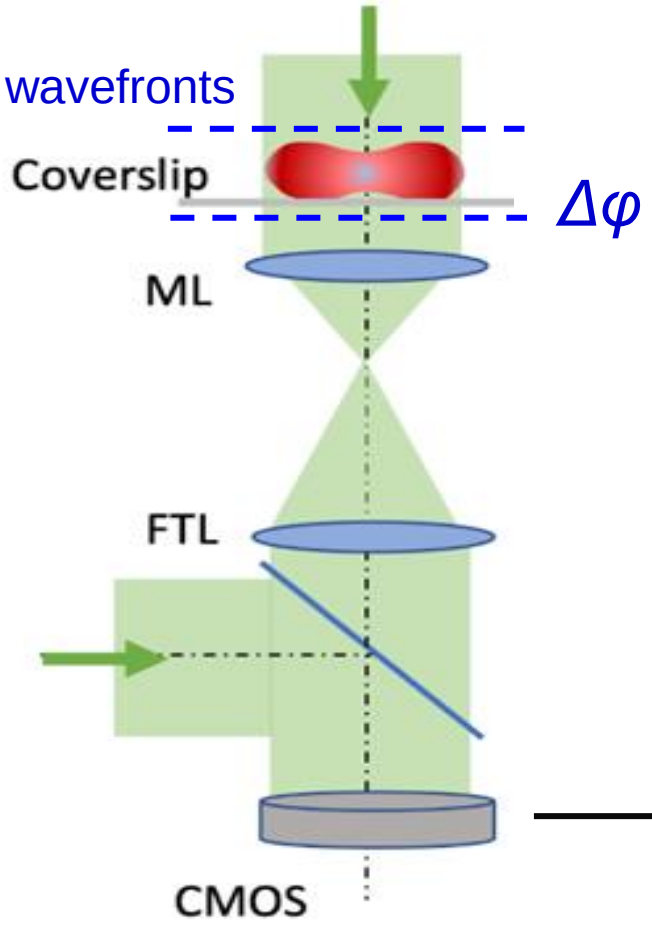
Example: Digital Holographic Microscopy (DHM) for 3D RBC imaging



a) Schematic of the DHM setup. Green arrows: laser beam; ML: Microscope Lens, TL: tube lens, CMOS: camera sensor;

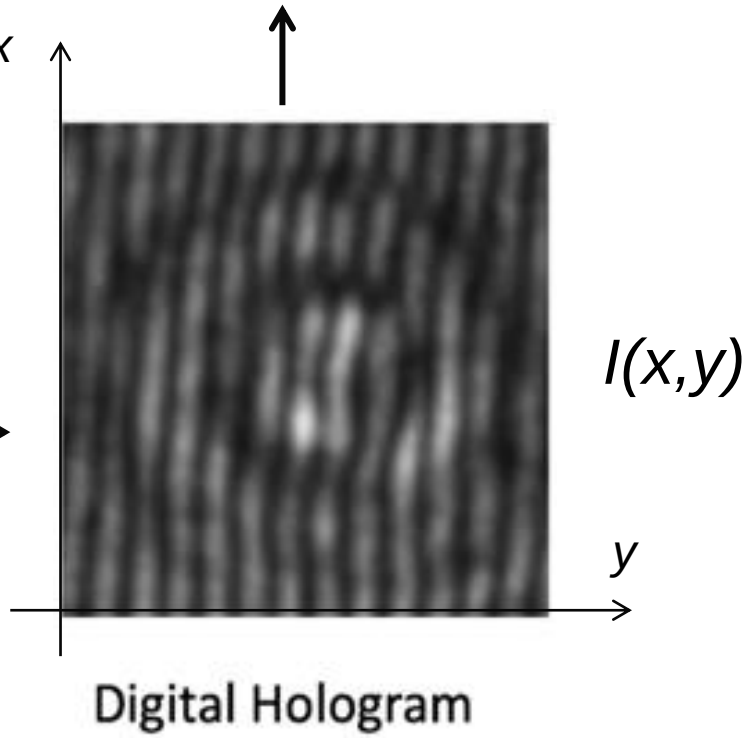
b) recorded Digital Hologram on CMOS and 3D Reconstruction (bottom); height profile of the cell (top right) along the red line shown in reconstruction (top left)

DHM – reconstruction principle



The intensity pattern *I(x,y)* is processed numerically to calculate the pase shift $\Delta\varphi(x,y)$ introduced by the specimen.

This processing includes Fourier transforms and image spatial filtering, to model light propagation.



The result of the numerical reconstruction is the pase shift (retardation) :

$\Delta\varphi(x,y) = 2\pi\, OPD(x,y) / \lambda$, with $OPD(x,y) = h(x,y)\,(n_c - n_m)$

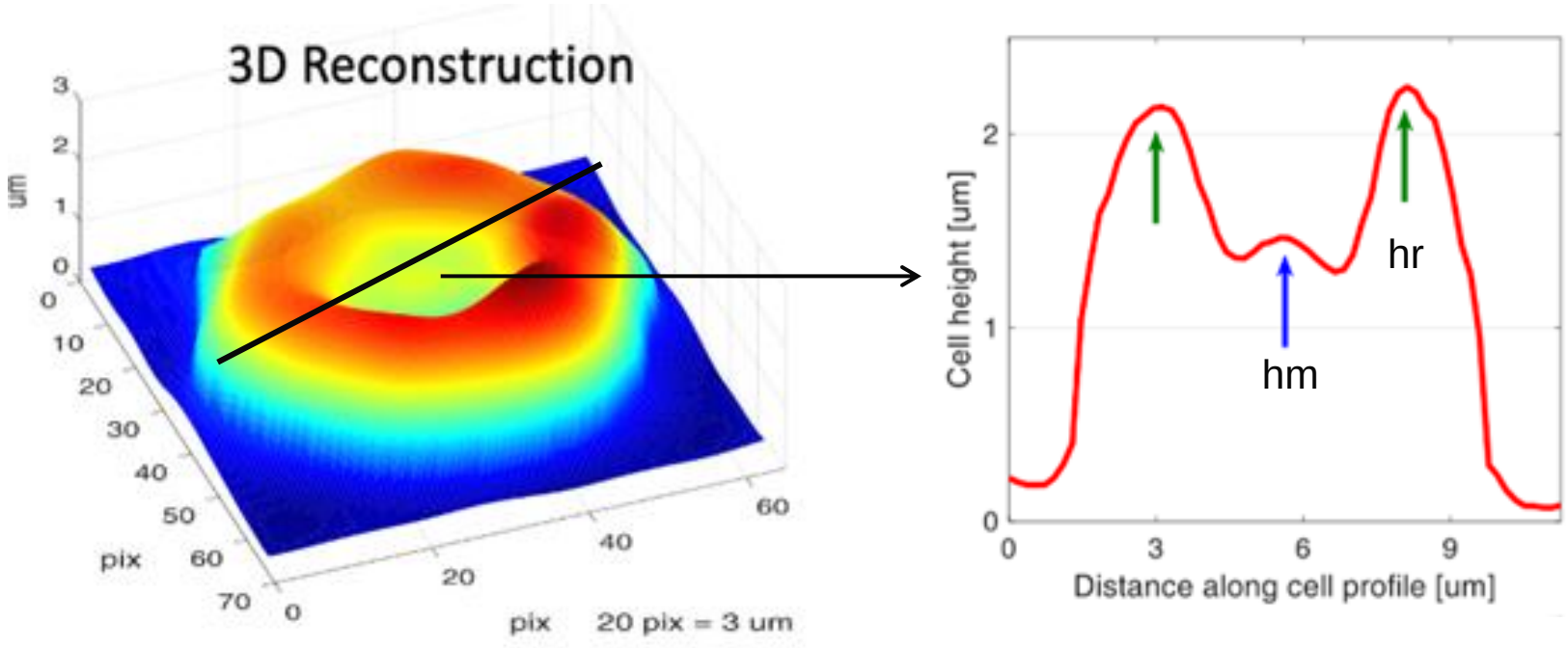
If we know the refractive index n_c of the cell and the refractive index n_m of the medium, we can calculate the optical **height of the cell**:

$h(x,y) = \lambda\, \Delta\varphi(x,y) / 2\pi\, (n_c - n_m)$

the optical **volume of the cell**:

$V = p \sum h(x,y)$, with p being the cell area corresponding to one pixel on the sensor

and other morphological parameters as **cell sphericity** CS= hm/ hr



DHM allows to measure cell height, volume and spheircity, parameters which are not possible to be determined by standard microscopy.

Example: characterization of *ex vivo* generated red blood cells (RBCs)

Cultured red blood cells (cRBCs) obtained under different conditions are compared with native RBC, from morphology point of view:

Morphology		**CA**	**CV**	**CS**	**MCH**	**hm**
		mean ± std	mean ± std	mean ± std	mean ± std	mean
Cells	n	um^2	um^3 (fL)	-	pg	um
nRBC	25	**55,42** ± 9,2	**95,2** ± 16,6	**0,57** ± 0,1	**25,24** ± 5	**1,72** ± 0.4
cRBC^{Plasma}	24	**41,05** ± 14,4	**125,5** ± 43,3	**1,04** ± 0,1	**31,17** ± 11,7	**3,06**± 0.6
cRBC^{HPL}	29	**70** ± 21,7	**107,1** ± 37,8	**0,671** ± 0,4	**28,1** ± 10,9	**1,53**± 0.3

Cell Area

Cell Volume

Cell Sphericity

Mean Corpuscular Hemoglobin

Cell mean height

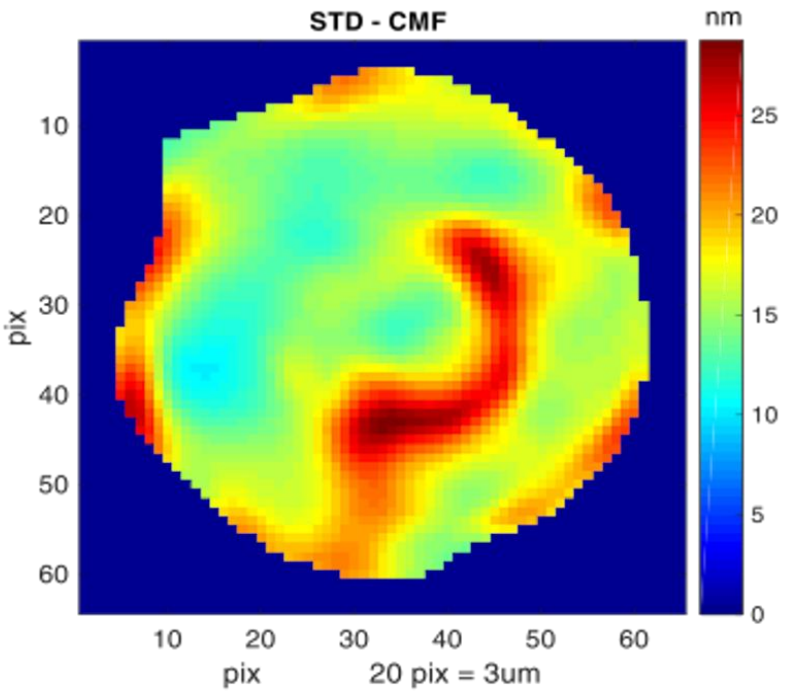
Bernecker et al, Cells 2021 in press

Morover, DHM makes it possible to measure cell membrane fluctuation (CMF) which is related to the viscoelastic properties of the membrane.

To determine CMF one calculates, for each pixel within the cell, the fluctuation of the cell height in time at high acquisition rate, then the corresponding standard deviation for each pixel of the cell, STD_pix_i .

The CMF value is calculated as the mean of STD_pix:

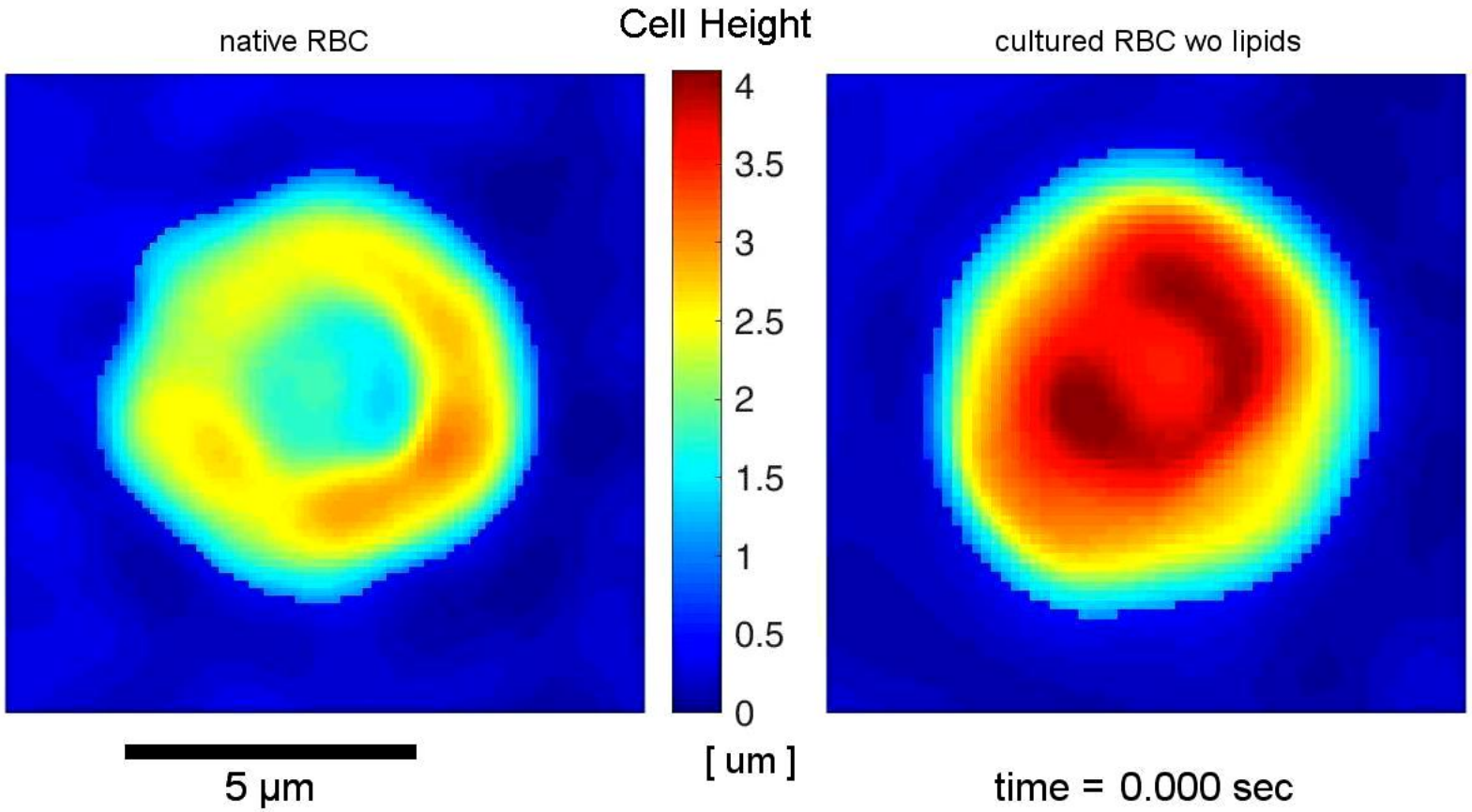
$$CMF = 1/N_p \sum_i STD\_pix_i$$



STD_pix - the standard deviation distribution over the cell area.

Note the nanometer sensitivity!

Cell Membrane Fluctuations

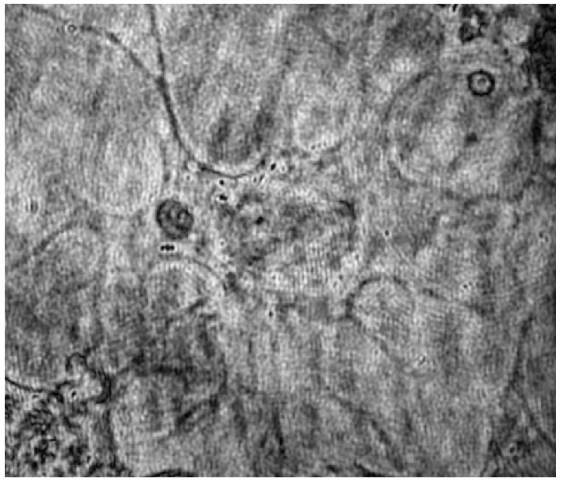


Video shows about 1 second of cell membrane fluctuations (height fluctuations) as measured with DHM

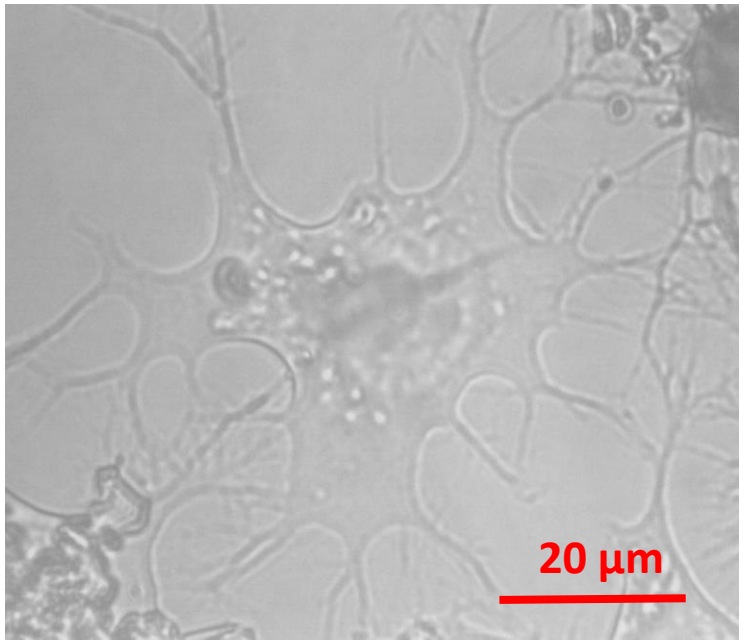
DHM example for differentiated type of neuroblastomaxglioma hybrid cell line, NG108-15, which has widely been used in in vitro studies instead of primary-cultured neurons.

DHM reconstruction

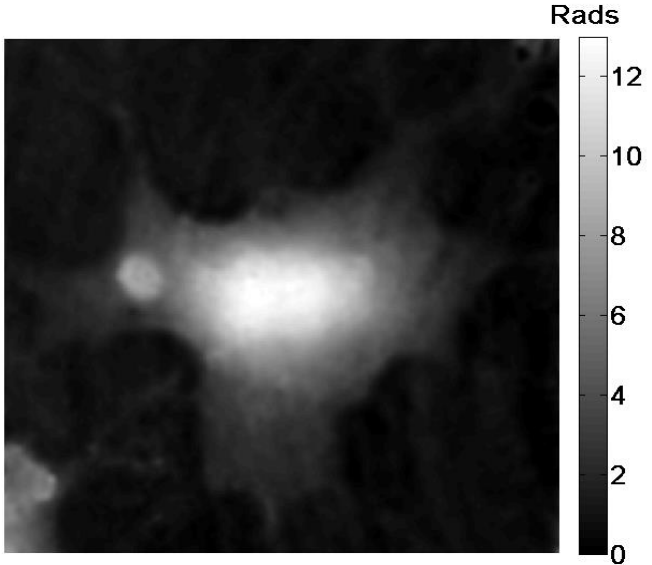
AMPLITUDE



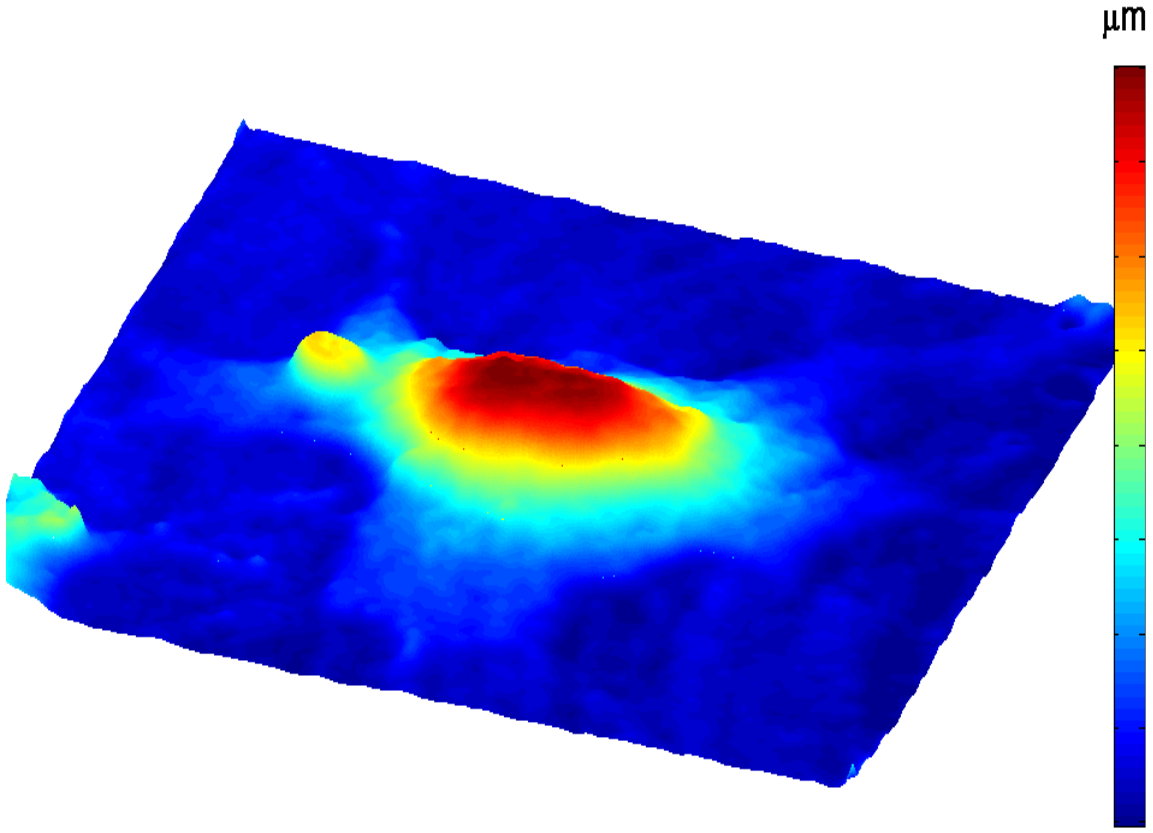
Brightfield image



PHASE



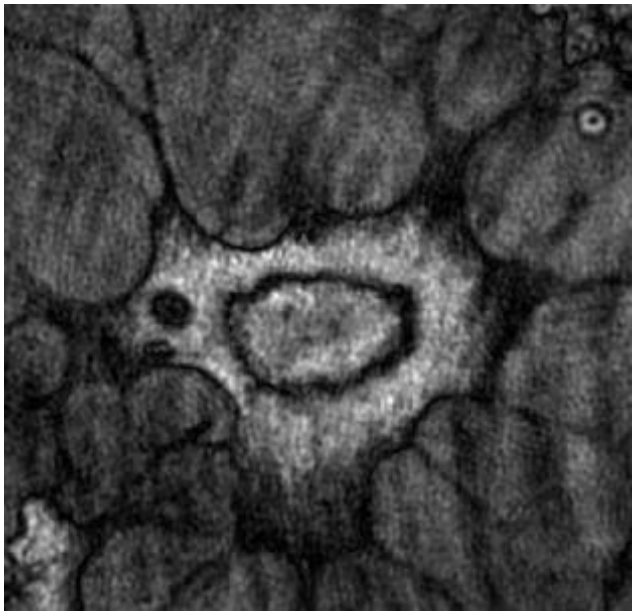
3D reconstruction



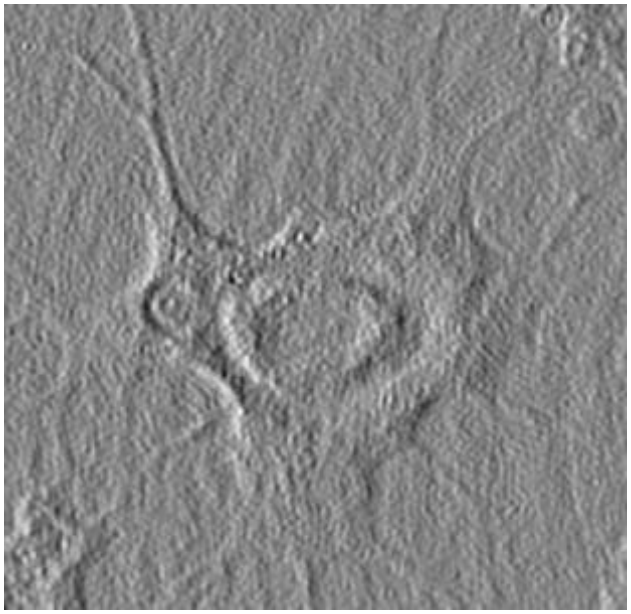
Hyphothesis:

$$\Delta n = n_{CELL} - n_{WATER} \approx 1.37 - 1.333$$

PHASE CONTRAST

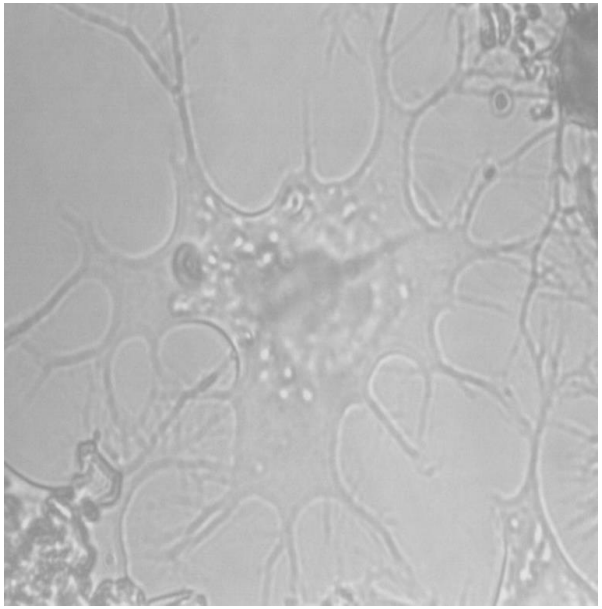


DIC



DHM can be used also for pase contrast imaging

BRIGHT FIELD



DHM advantages

- It is label free
- Allows measuring the height profile of the cell
- High axial sensitivity: 1-10 nm << 500 nm (the axial resolution in classic microscopy)

DHM drawbacks

- Low lateral resolution:~ λ / NA instead of 0.5 λ / NA
- Assumes the cell has an average refractive index.

A special technique has been introduce allowing DHM with lateral resoution < 100 nm

nature photonics

LETTERS

PUBLISHED ONLINE: 20 JANUARY 2013 | DOI: 10.1038/NPHOTON.2012.329

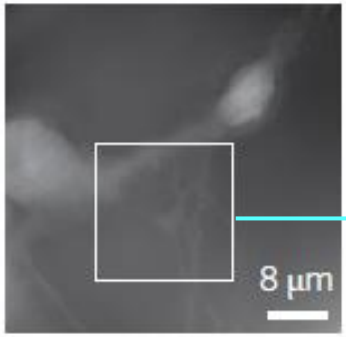
Marker-free phase nanoscopy

Yann Cotte[1]*, Fatih Toy[1], Pascal Jourdain[2], Nicolas Pavillon[1], Daniel Boss[1,2], Pierre Magistretti[2,3], Pierre Marquet[2,3] and Christian Depeursinge[1]

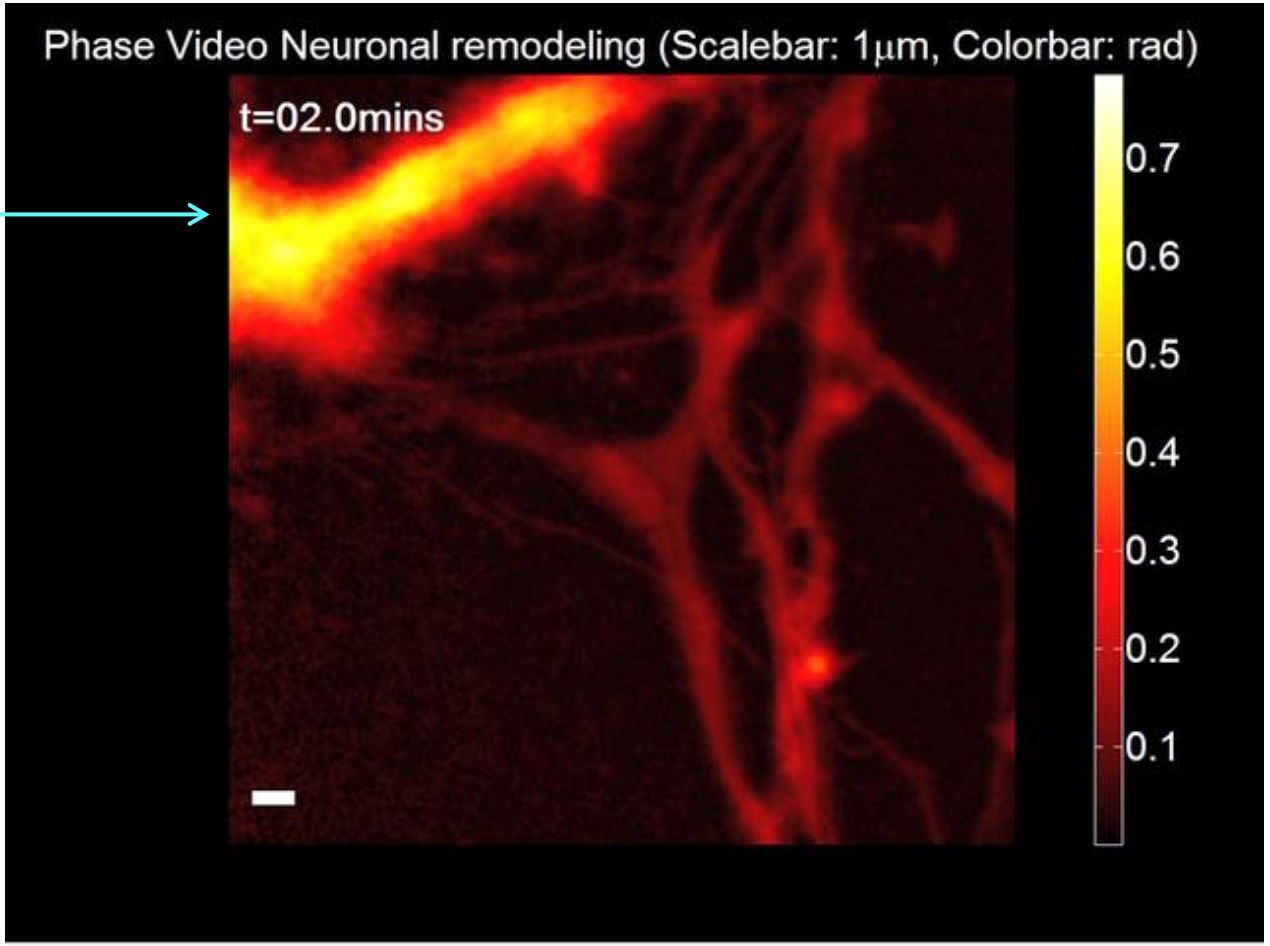
Thus, DHM allows direct imaging of unstained living biological specimens in non-invasive optical nanoscopy can achieve a lateral resolution of 90 nm by using a quasi-2π-holographic detection scheme and complex deconvolution.

We do not enter into technical details, just see an application for neuronal synapses.

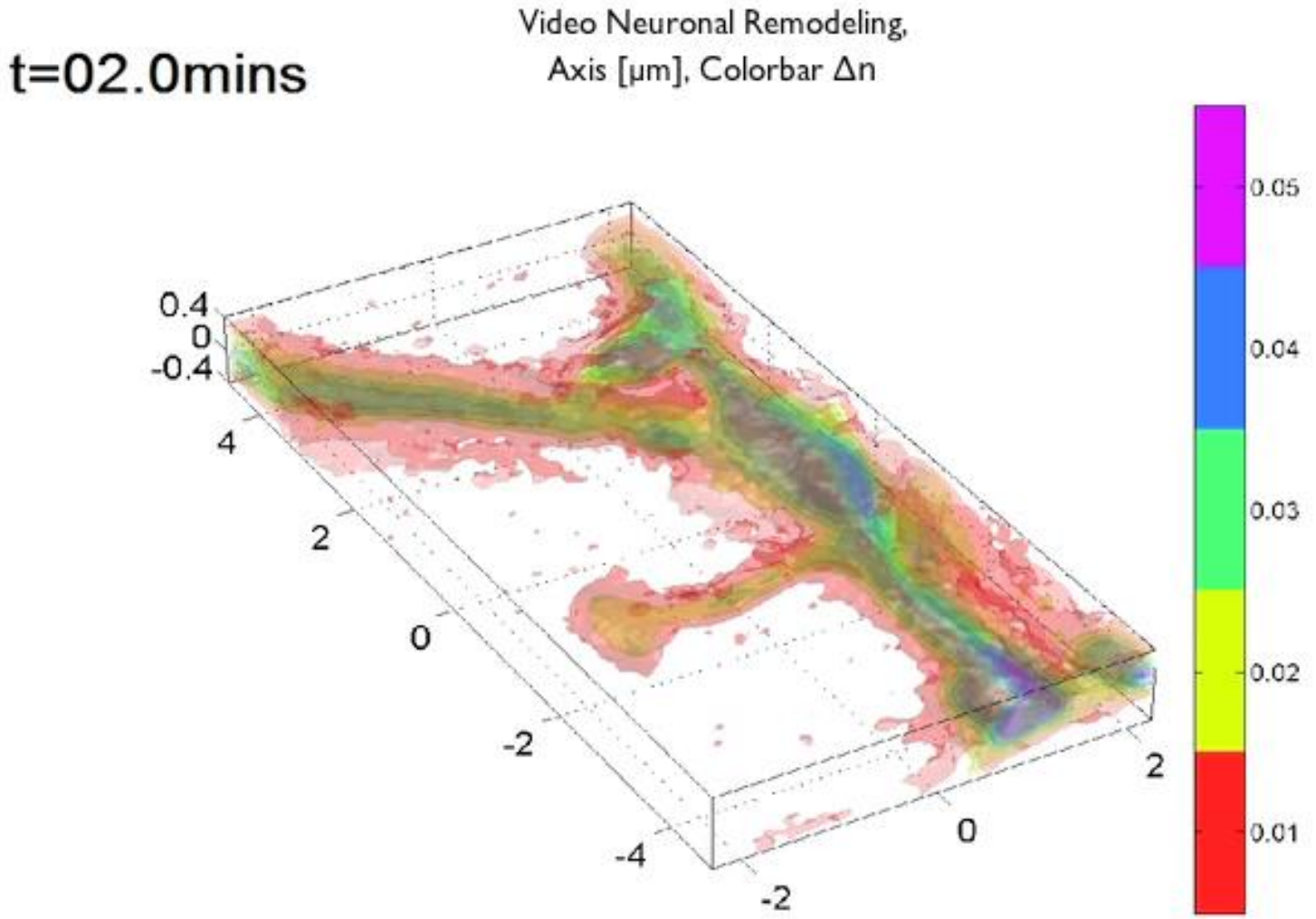
time-lapse imaging on five day-old live mouse cortical neurons



two neurons and their synaptic contact regions



Time-lapsed refractive index change Δn during filopodia formation of a neuronal spine (perspective view).

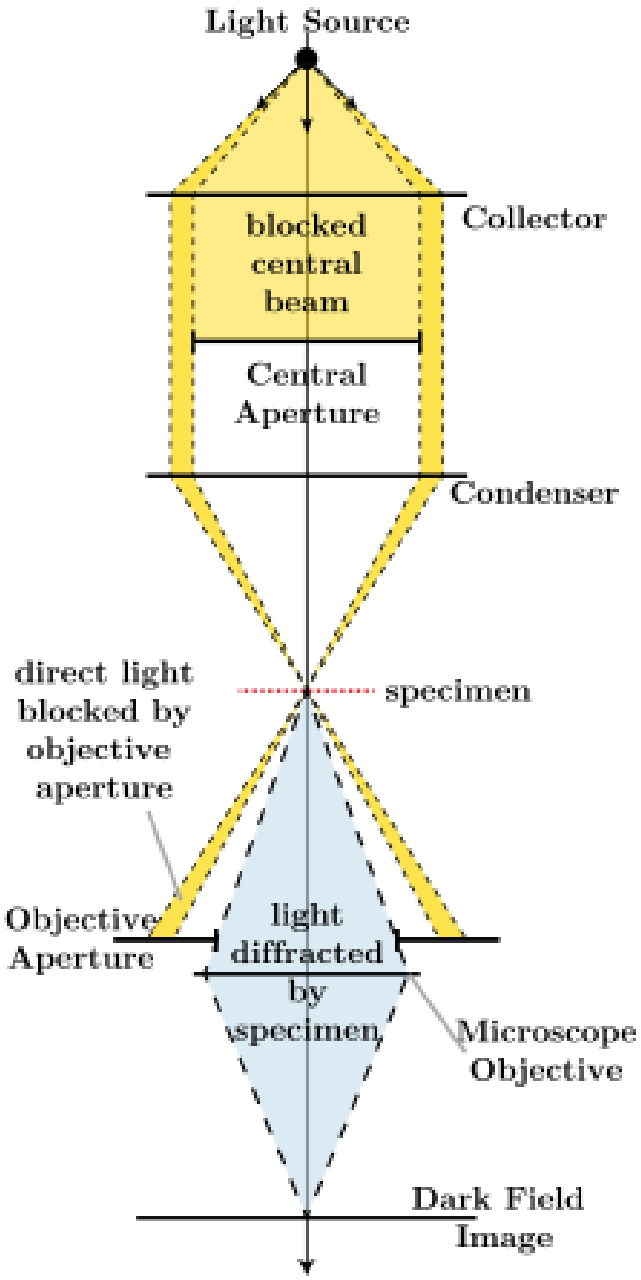


Samples were observed directly (see Methods) without the need for preliminary preparation steps such as fixation, membrane permeabilization or fluorophore incubation.

The absence of fluorophores removes any concerns regarding cytotoxicity, phototoxicity through bleaching and molecular oxidation, or phototoxicity from high-power laser intensities.

Imaging nanoparticles by darkfield microscopy

Darkfield Microscopy

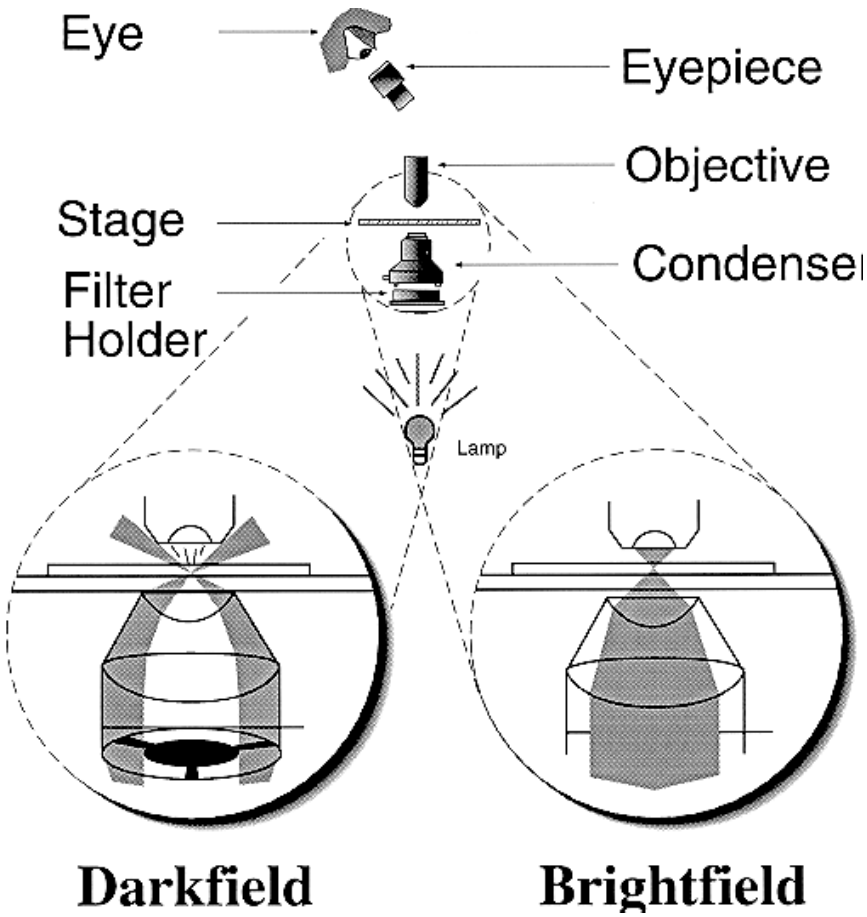


The light at the apex of the cone is focused at the plane of the specimen; as this light moves past the specimen plane it spreads again into a hollow cone. The objective lens sits in the dark hollow of this cone; although the light travels around and past the objective lens, no rays enter it.

The entire field appears dark when there is no sample on the microscope stage; when a sample is on the stage, the light at the apex of the cone strikes it; the image is made only by those rays scattered by the sample and captured in the objective an it appears bright against the dark background.

Single nanoparticles can be imaged !

Darkfield vs Brightfield Microscopy (DM vs BM)



DM and **BM** use

different illumination schemes:

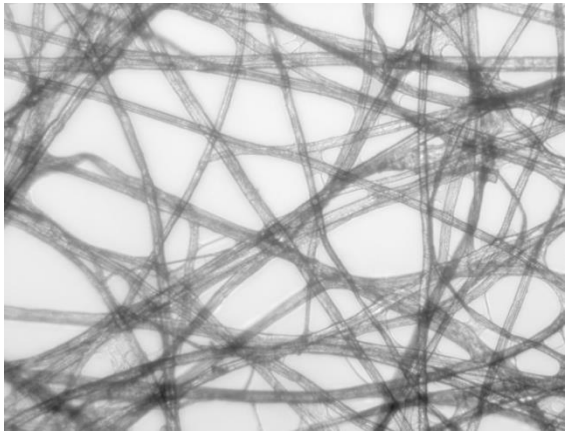
DM uses a darkfield stop to block the center of the beam of light, producing a **hollow cone of light** which does not directly enter the obj lens.

In contrast, a **solid cone of light** illuminates and enters the obj lens in **BM**.

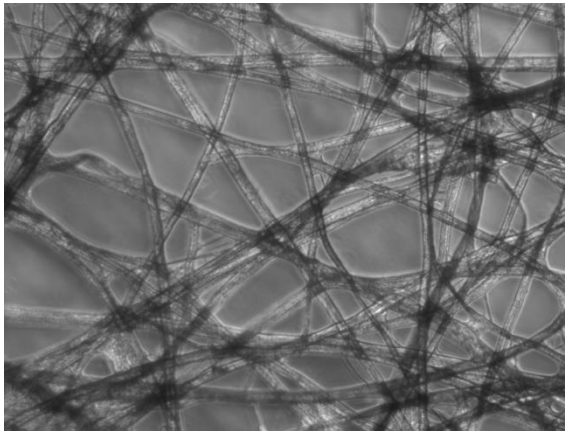
Comparison btw different microscopy techniques

Sample: tissue paper micrograph

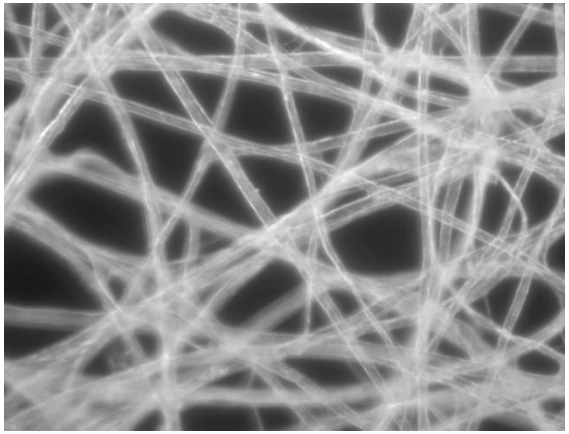
Brightfield



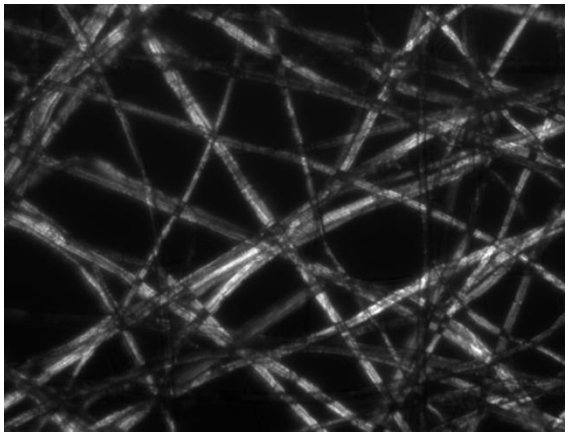
Phase Contrast



Darkfield



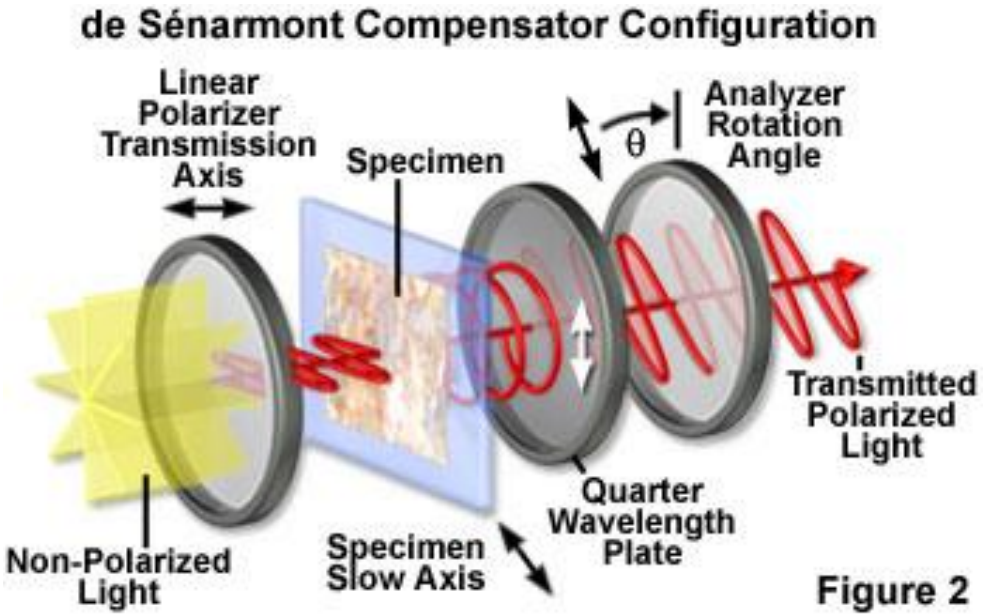
Polarization



?

Polarized light microscopy

Polarized light Microscopy (PM)

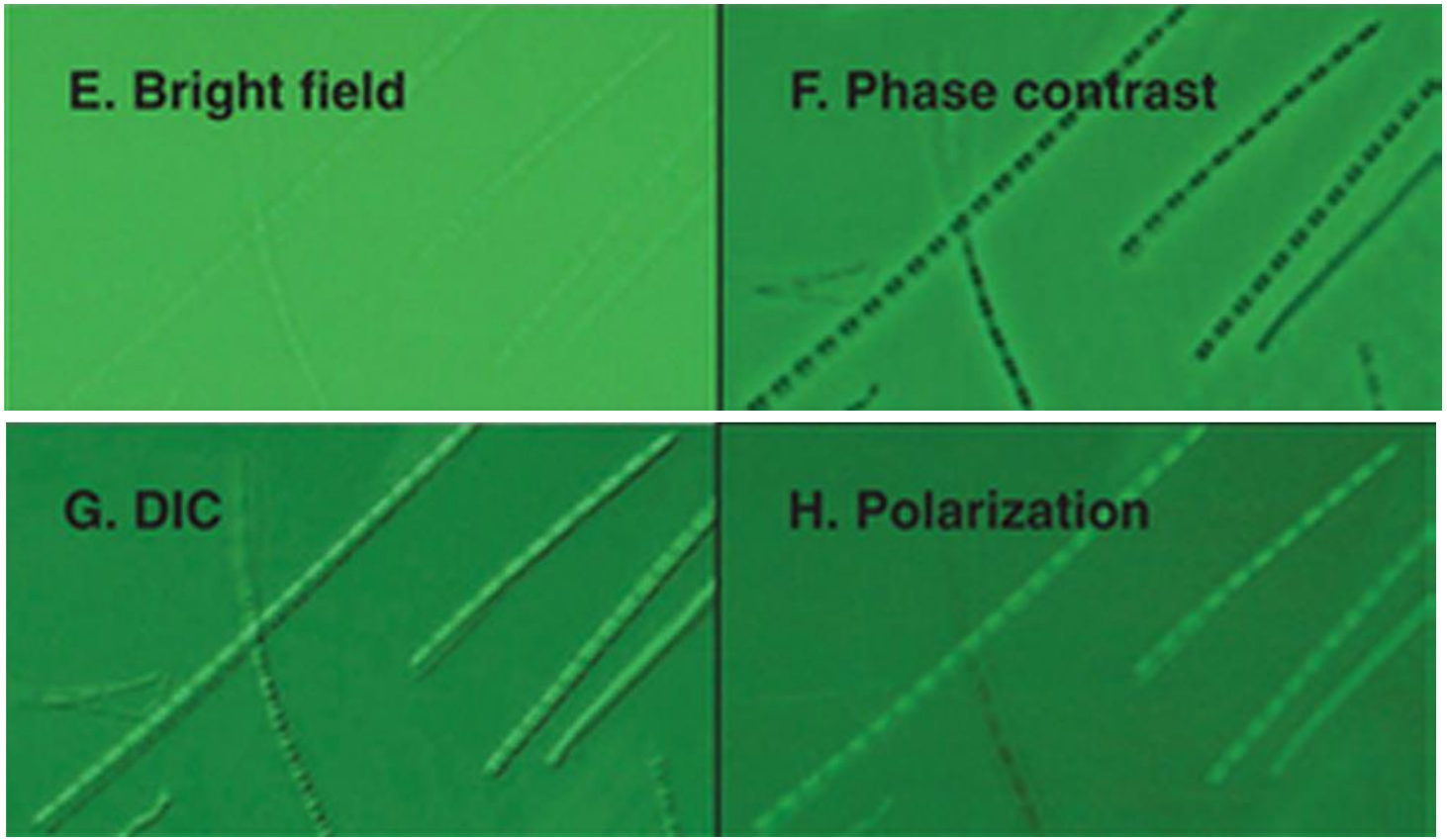


PM requires birefringent samples and polarized light.

A linear polarizer is used to illuminate the sample with linear polarized light.

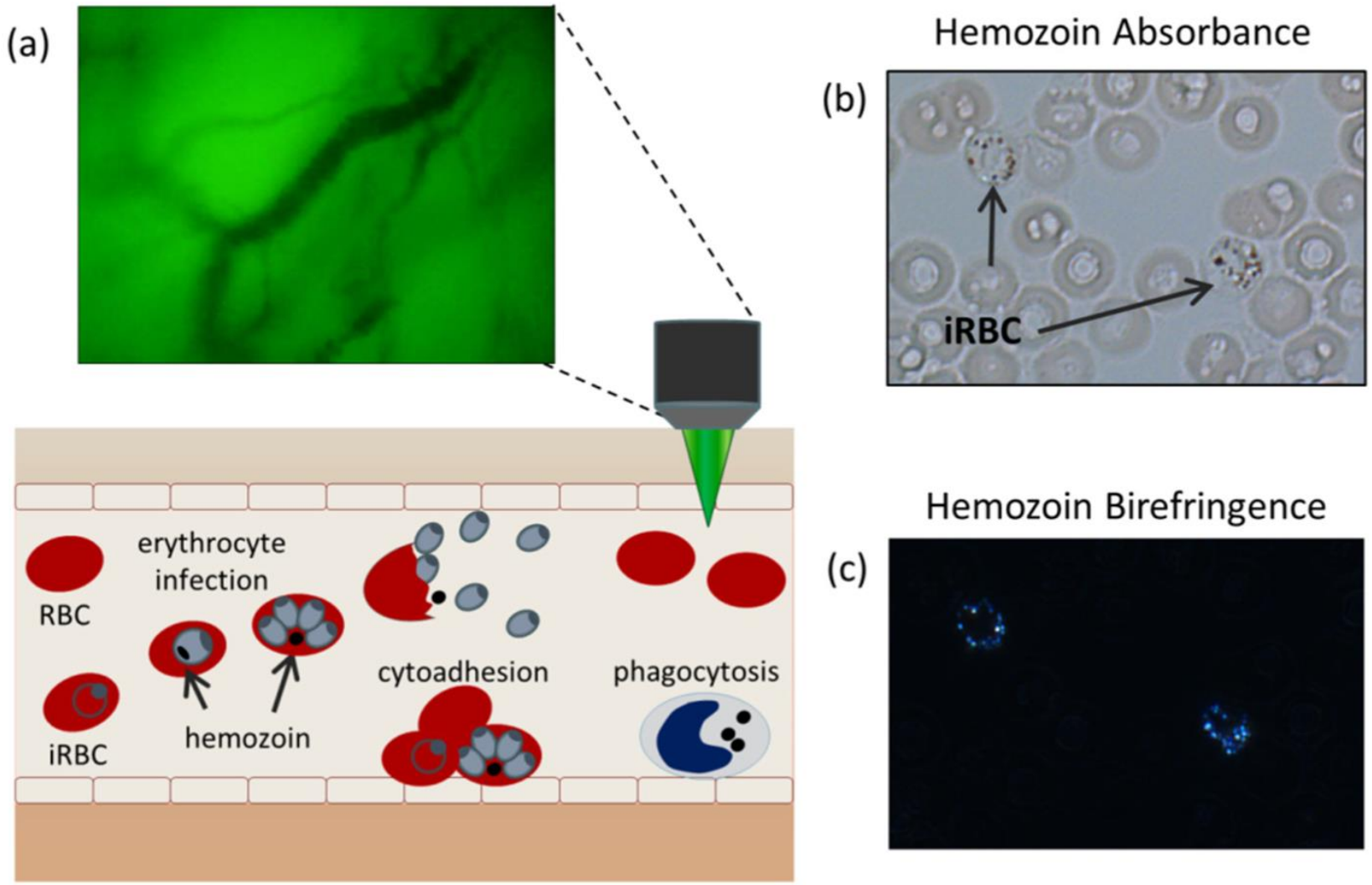
Due to its birefringence the sample changes the light polarization. This change is detected with an analyzer + compensator.

Applications: mineralogy, plant biology, blood cells



E–H, Micrographs of myofibrils isolated from skeletal muscle.
Contrast methods include bright field **(E),** phase contrast **(F),** differential interference contrast **(G),** and polarization (H).
The A-bands, consisting of parallel thick filaments of myosin (see Fig. 39-3), appear as dark bands with phase contrast and are birefringent (either bright or dark, depending on the orientation) with polarization.

Example: Hemozoin imaging for malaria detection



J. Burnett, J. Carns, and R. Richards-Kortum,
"In vivo microscopy of hemozoin: towards a needle free diagnostic for malaria,"
Biomed. Opt. Express **6**, 3462-3474 (2015).

LECTURE 2-3 SUMMARY

1. Optical microscopy

1.1. Basics

- Why optical microscopy?
- Image formation; magnification and resolutiont;
- diffracted limited resolution;
- Optical aberrations and image quality;
- Digital camera image acquisition (formats, properties, SNR)

1.2. Phase imaging techniques – 1h

- Phase contrast and differential interference contrast (DIC);
- Quantitative phase imaging: digital holographic microscopy.

1.3 Other techniques to image non-stained samples - 1h

- Dark field microscopy
- Polarization microscopy
- Non linear microscopy → we discuss with fluorescence micro