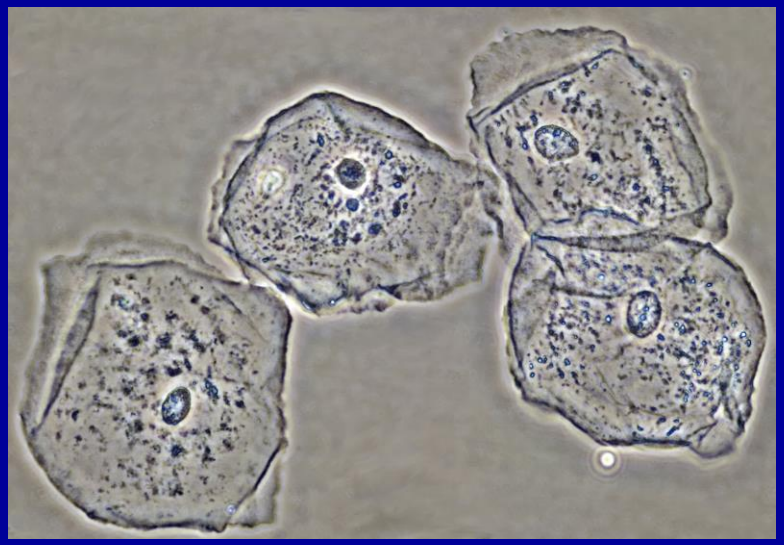
Manchester Microscopical & Natural History Society

Established 1880

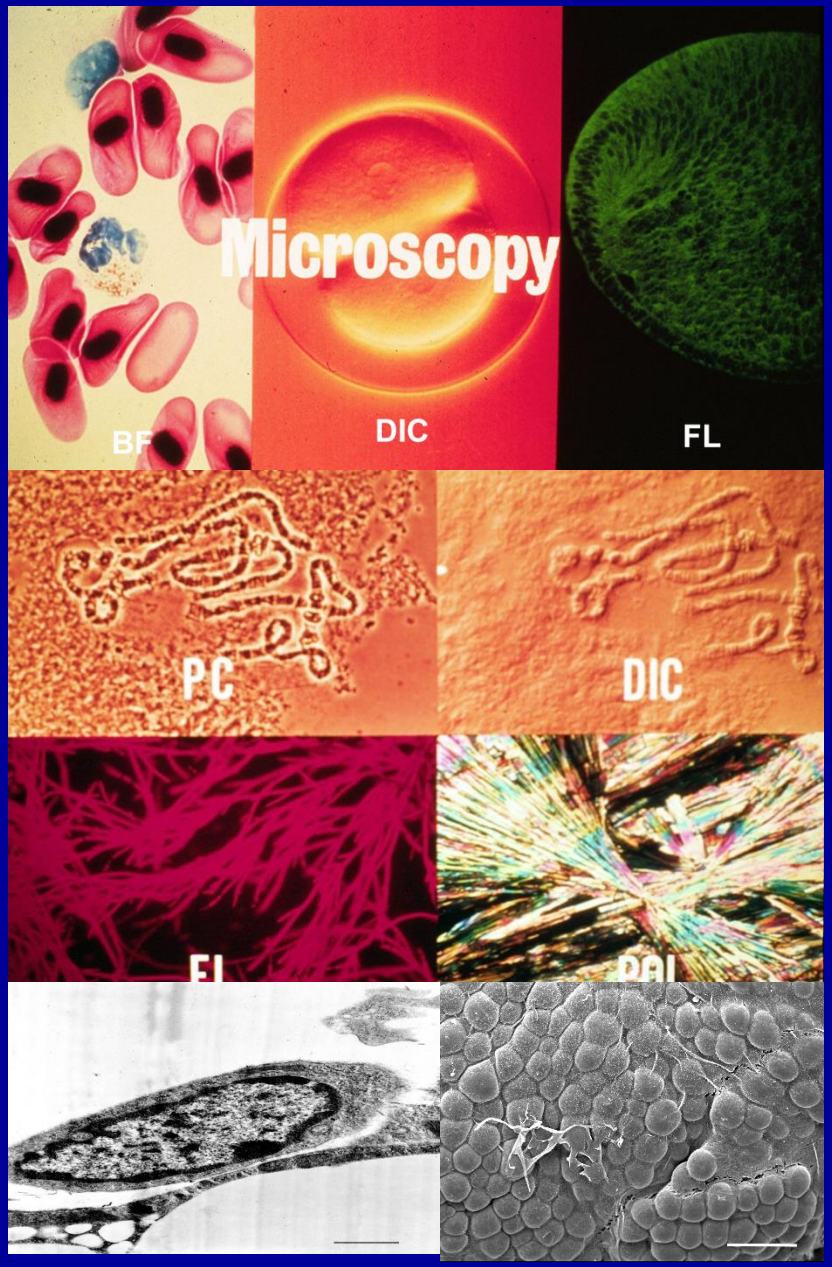
www.manchestermicroscopical.org.uk

Phase-Contrast Microscopy



Talk & Practical

***Mike Mahon**, November 23rd, 2024*

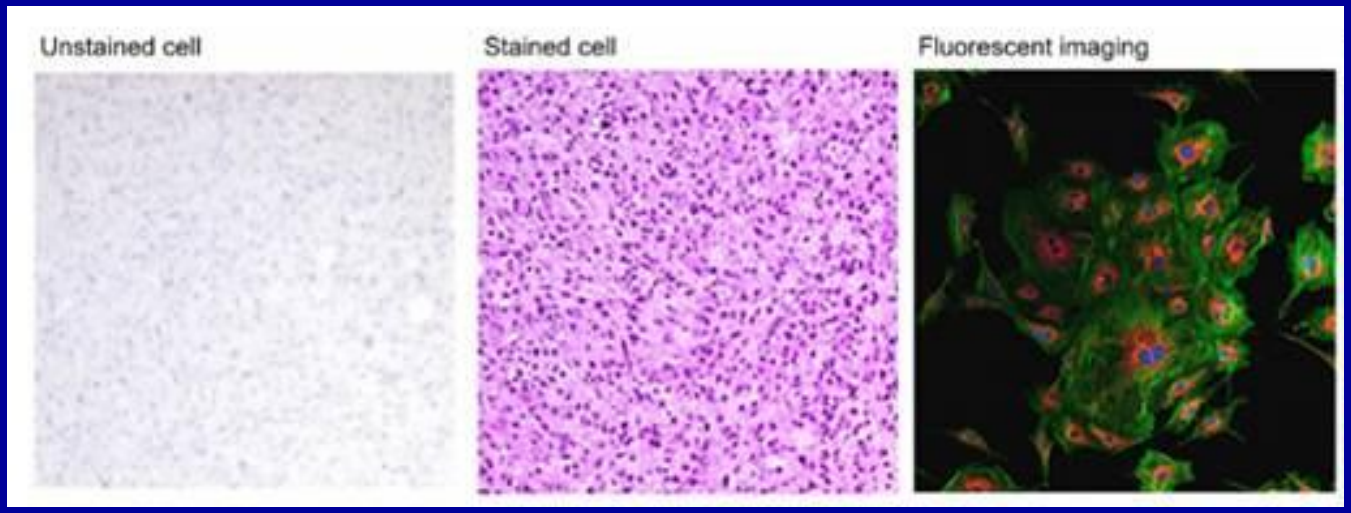


Which technique to use - & how ?

- Light Microscopy
  - bright field / reflectance
  - dark field
  - polarising
  - **phase contrast**
  - interference
  - fluorescence
  - computerised / confocal
- Electron Microscopy
  - Transmission +
  - Scanning +
- Scanning Probe/Stylus

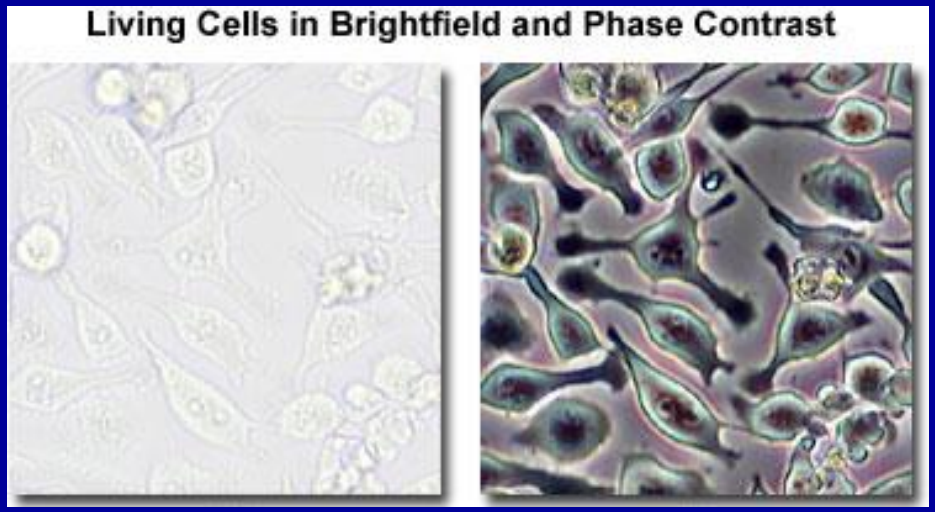
The Problem ?

Unstained biological cells and tissue slices are hardly visible under the microscope.



Add contrast by –
killing
fixing
embedding
staining
labelling

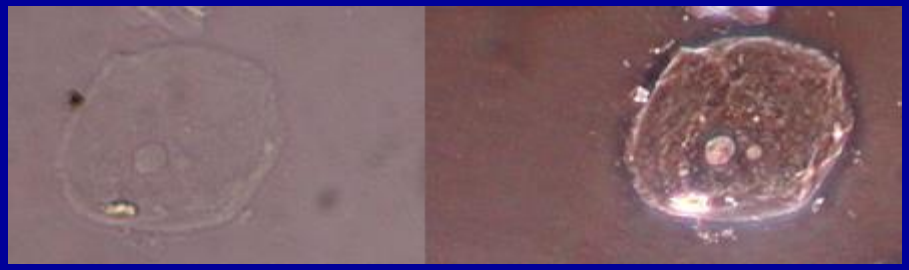
--ve Not natural !



Or view directly by **Phase Contrast**

++ Living, dividing, moving, …

Add Contrast Optically



Microscopy Landmarks

- <1600s magnifying lens
- 1600s simple microscopes
- 1650s compound microscopes
- 1830s expansion of light microscopy + pol, DF
- 1900-80 UV & fluorescence microscopy
- **1930-1950s phase contrast & interference**
- 1930-60 electron microscopes developed
- 1980s confocal microscopy
- 1990s-2010s scanning probe microscopies
- 2010s super-res, µCT, X-ray, 3D, AI microscopies,...

1892 Bratuscheck

History

Frits Zernike
(1888-1966)

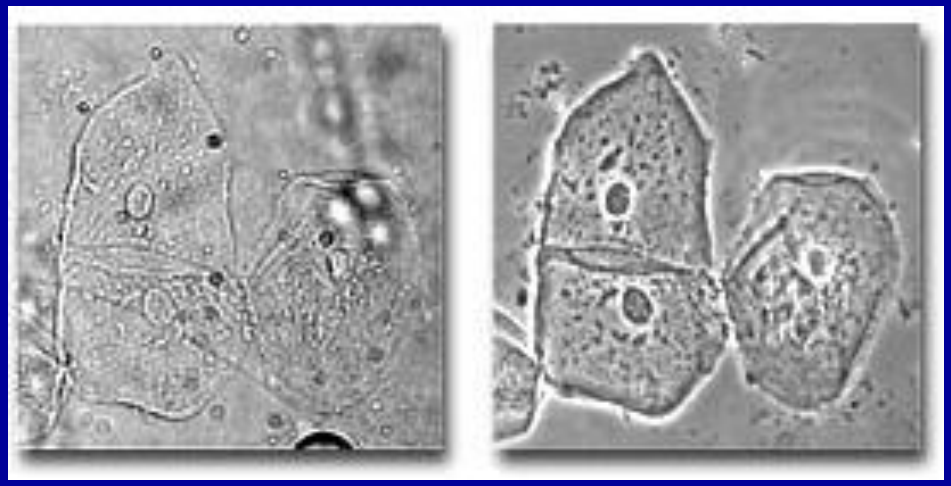
- 1930s Theory/Invention
  Diffraction gratings > Microscopes
- 1942 Zeiss
- 1953 Nobel Prize

FRITS ZERNIKE

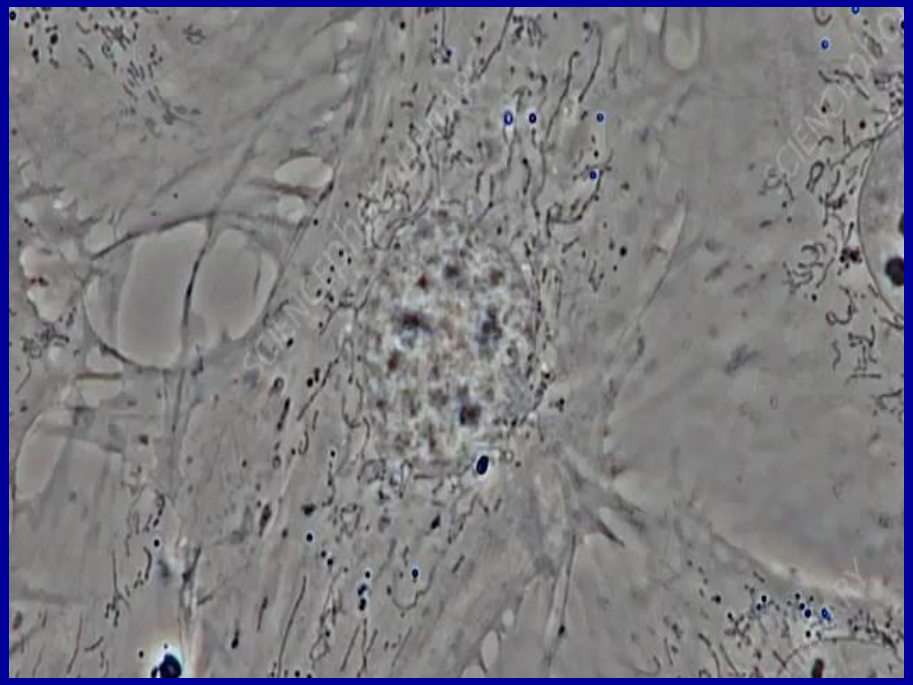
How I discovered phase contrast

Nobel Lecture, December 11, 1953

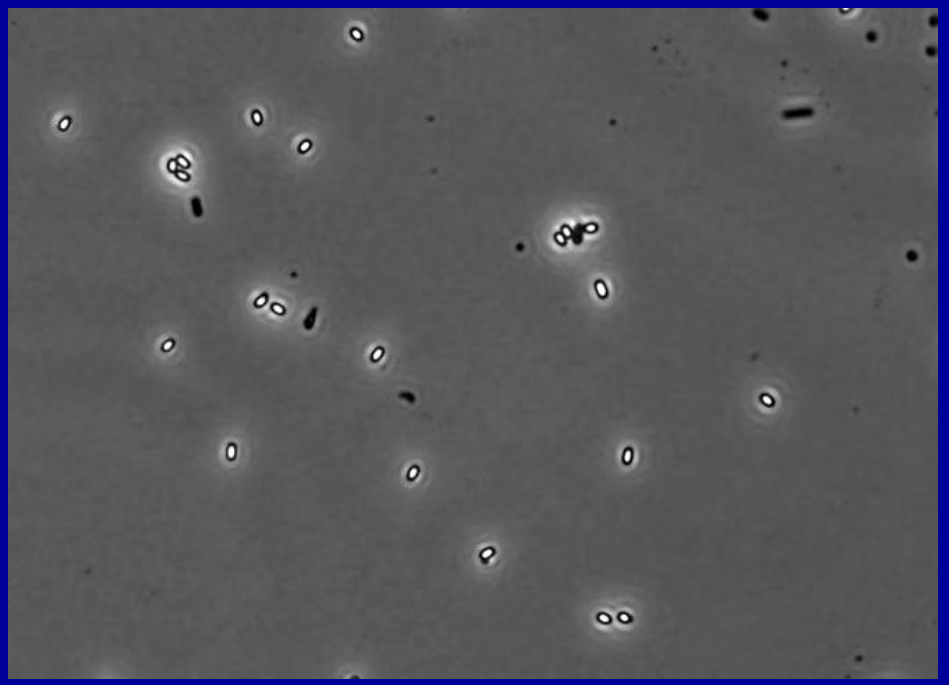
< Click image to view on web



Videos

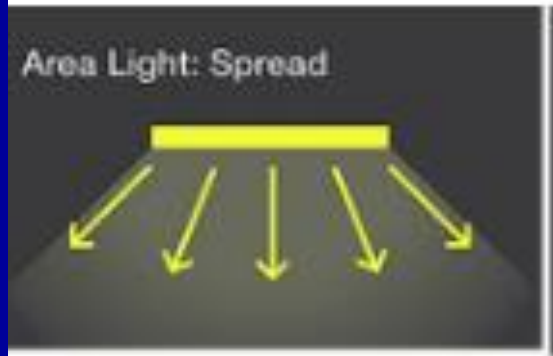


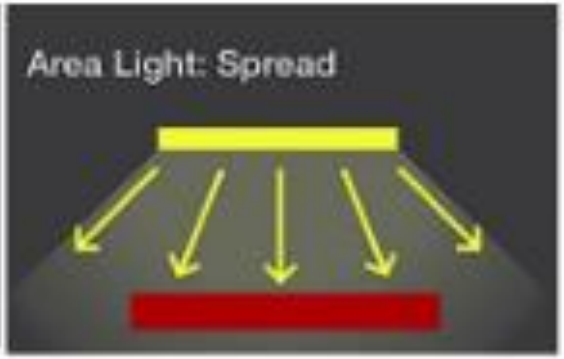
Cell division, timelapse light microscopy - Stock Video Clip - K005/7812 - Science Photo Library

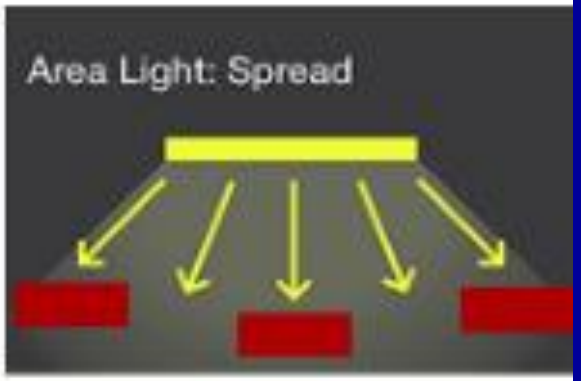


File:Live-Cell-Imaging-of-Germination-and-Outgrowth-of-Individual-Bacillus-subtilis-Spores-the-Effect-of-pone.0058972.s005.ogv - Wikimedia Commons

Historic time lapse movie by Dr. Kurt Michel, Carl Zeiss Jena (ca. 1943)





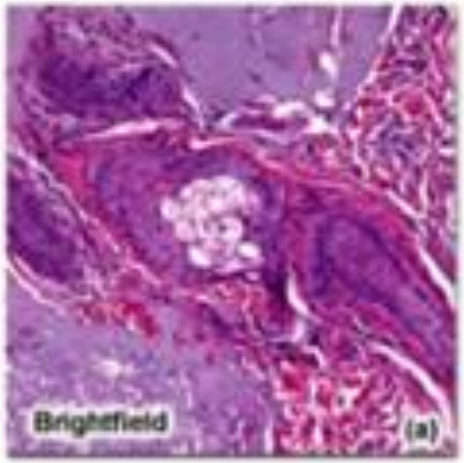


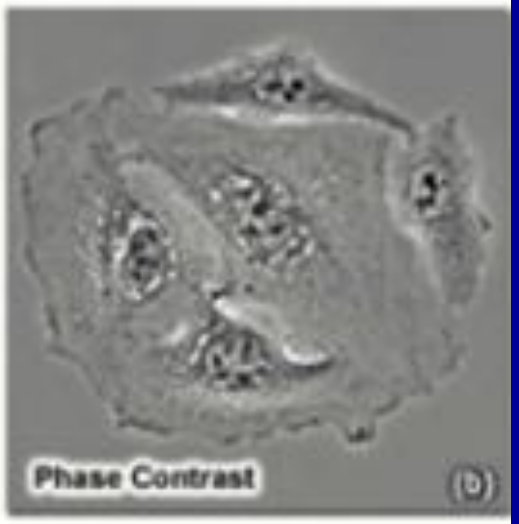
Brightfield microscopy

Dark Field microscopy

+++ +++

Phase Contrast Microscopy





Staining needed

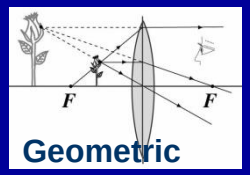
No staining needed

No staining needed

Light Microscope Theory

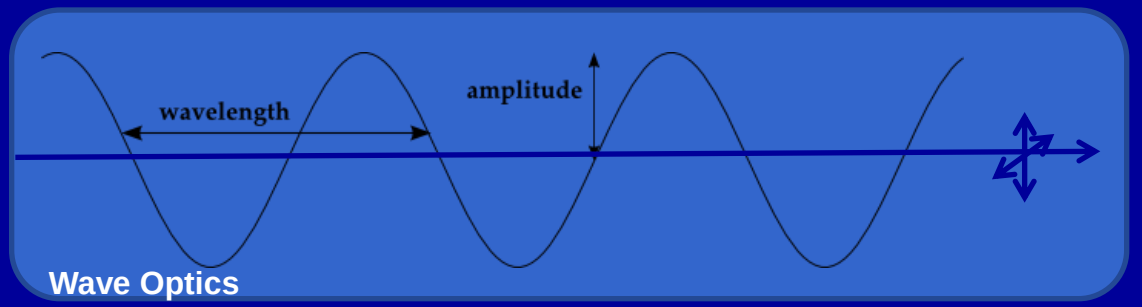
Properties of Light

- Geometric Optics
  - image location
  - Magnification / Res
- Wave Optics
  - direction
  - amplitude
  - wavelength
  - phase
  - vibrational plane
  - velocity
- Quantum Optics
  - formation & destruction
  - fluorescence



Geometric

Microscopies



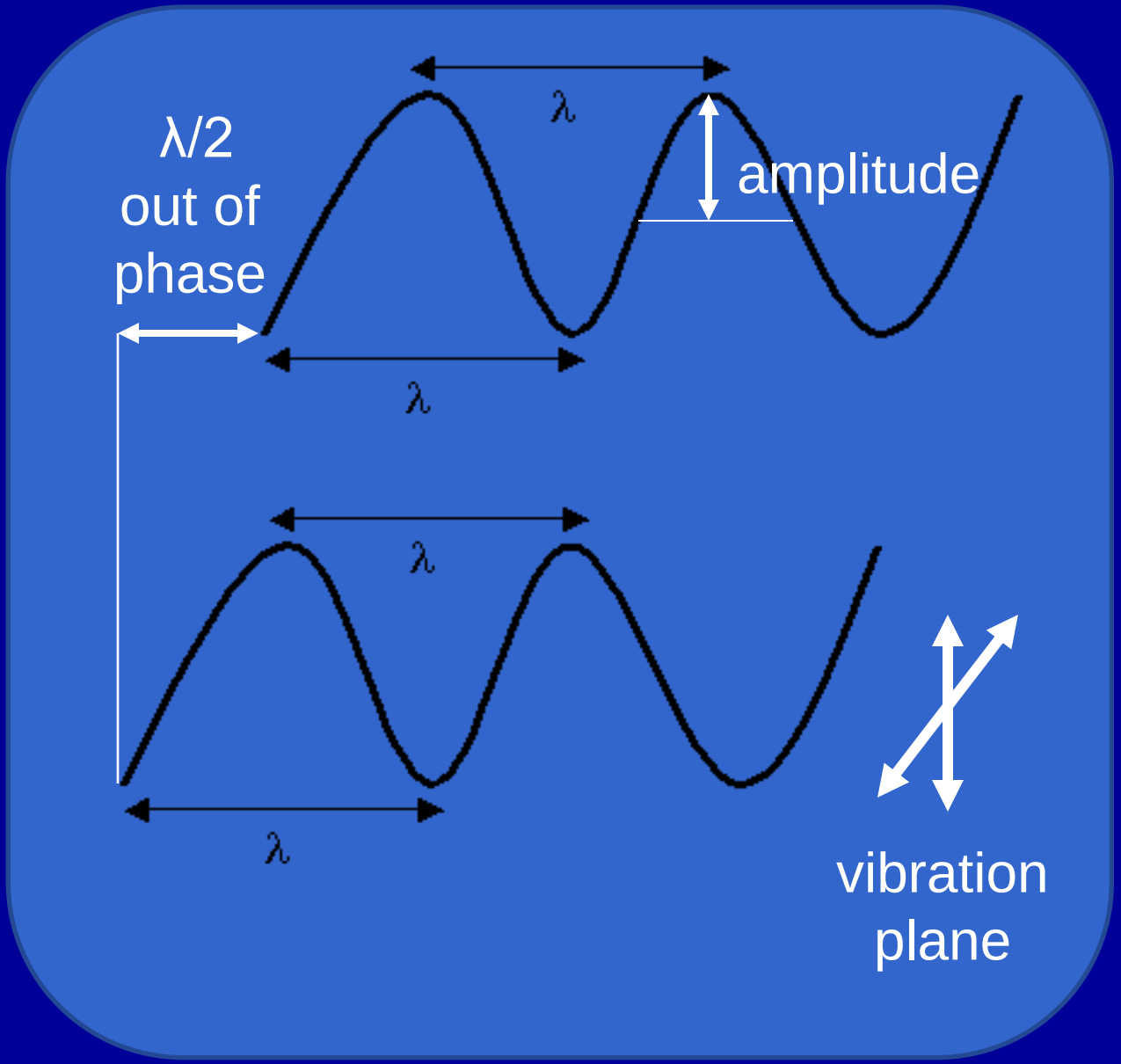
Wave Optics

- reflection,refraction,diffraction / BF / DF
- absorption / BF
- colour
- phase contrast, interference
- polarisation
- refraction / oil immersion

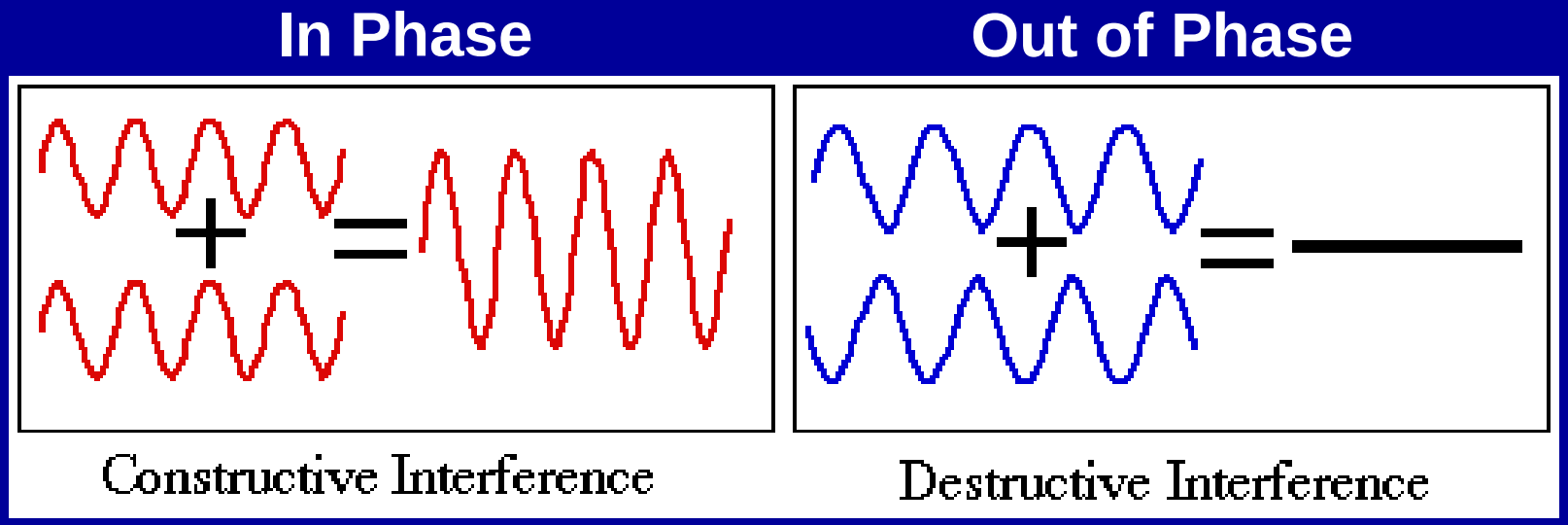

Quantum

Properties of Light

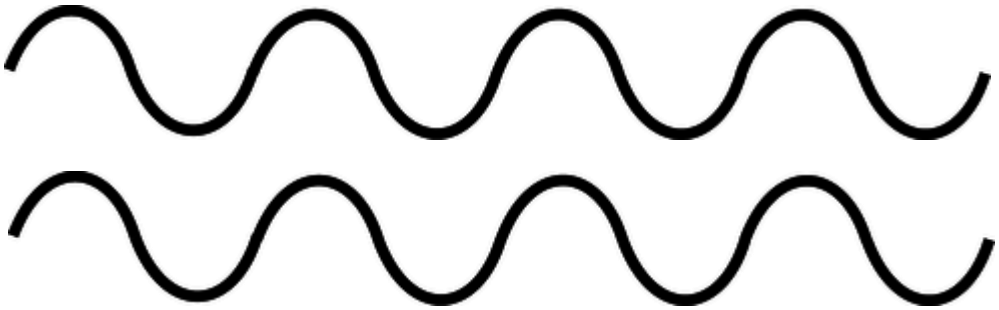
- amplitude
- vibration plane
- phase



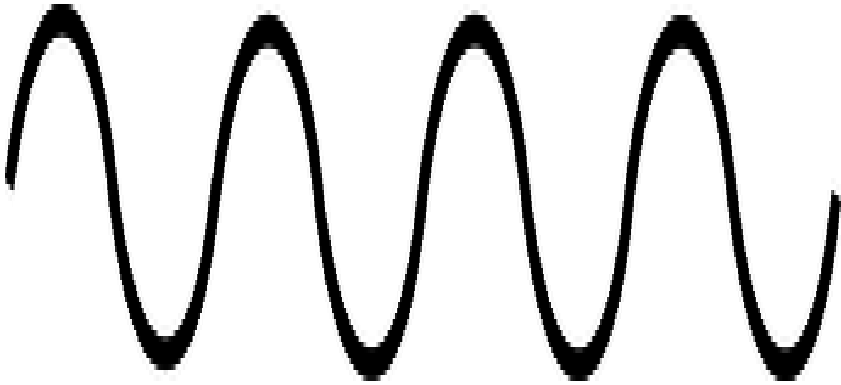
Phase contrast microscope 2



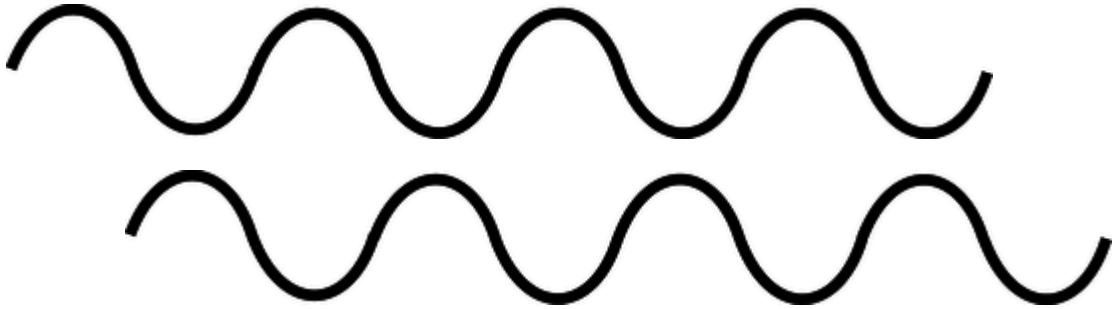
- Waves in phase reinforce each other
- Waves λ/2 out of phase destructively interfere



Waves in phase



Constructive Interference

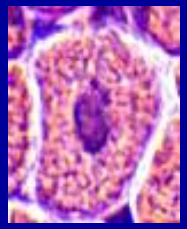


Destructive Interference

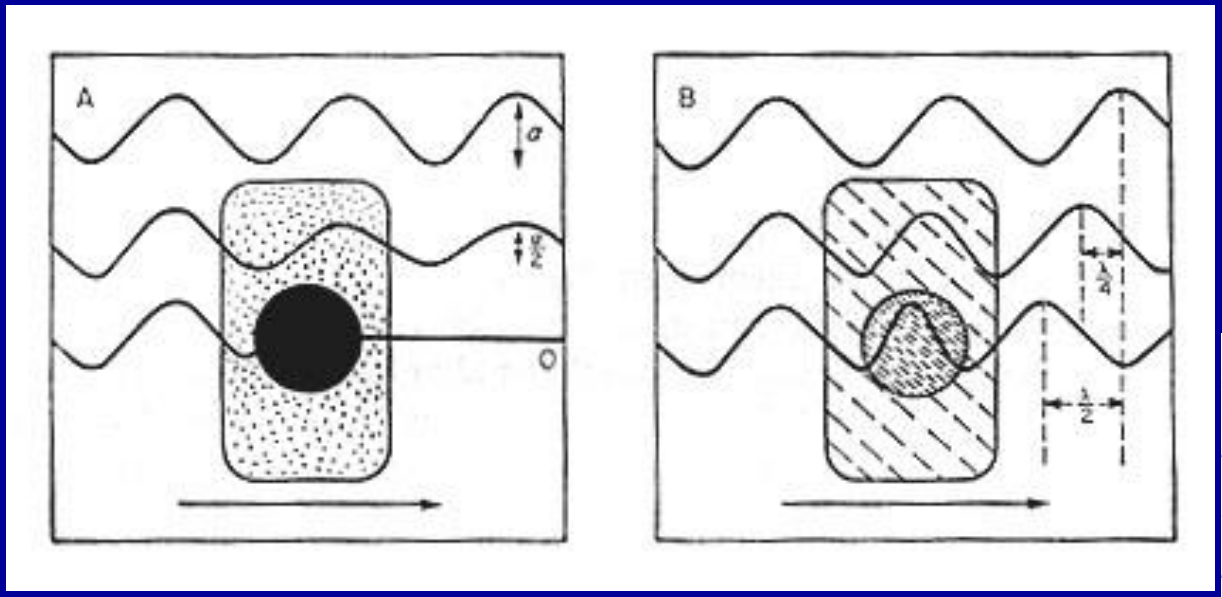
Waves out of phase by ½ λ (180^{o})

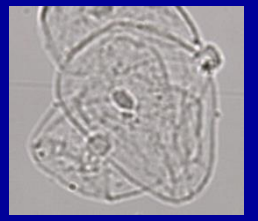
Waves out of phase by ¼ λ (90^{o}) = hardly noticeable change in amplitude !

Brightfield Microscopy: Effect of objects on light waves

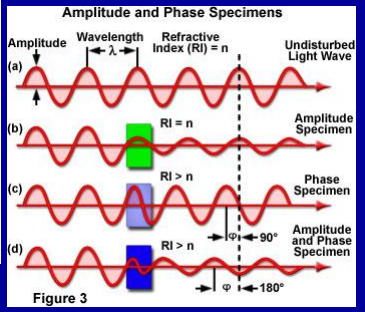


stained cell





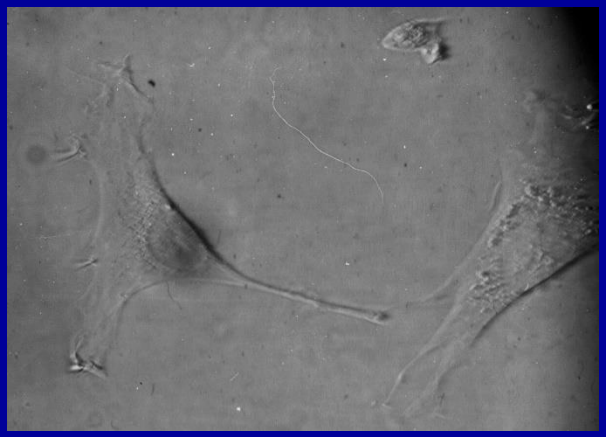
unstained cell

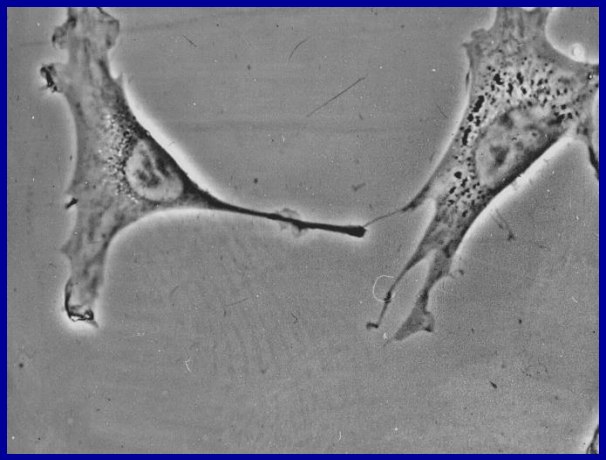


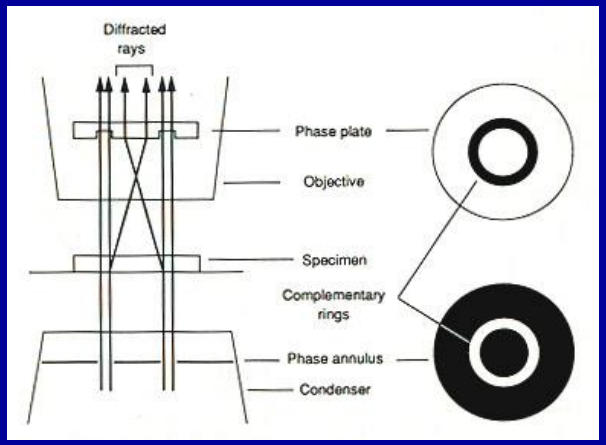
- stained cell reduces amplitude of wave
- unstained cell does not affect amplitude of wave
- unstained cell retards wave due to difference in refractive index
- retardation λ/4 for cytoplasm in this case
- (Overall Phase Shift = OPD = RI x Thickness !)

Phase contrast microscope 1

- invented by Zernike 1935
  developed by Zeiss 1942
- converts differences in phase to differences in amplitude
- simple to set up but requires:
  - phase annulus in condenser
  - phase plate in objective



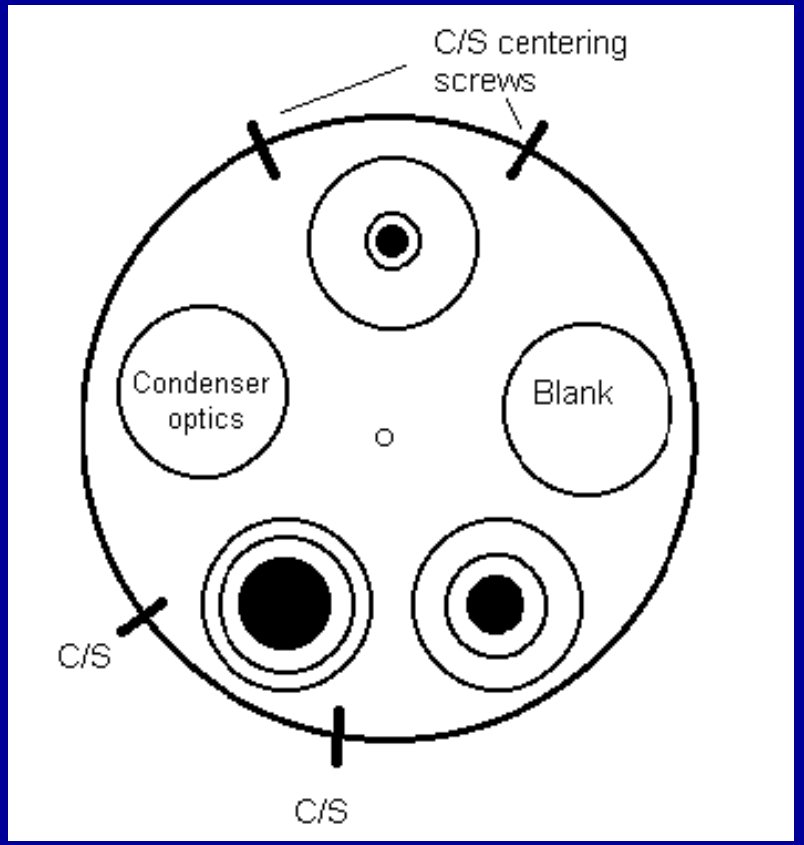




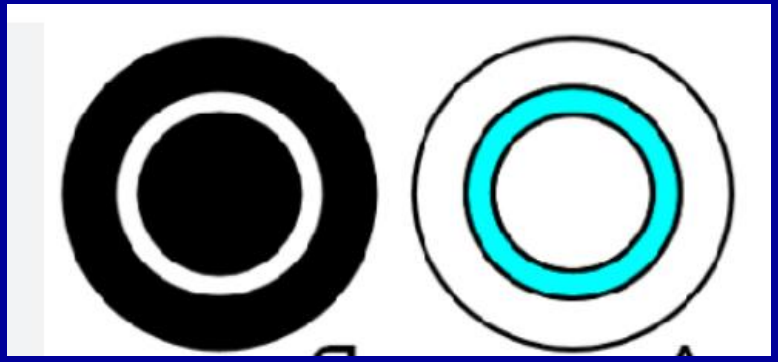

CHB
CHB2

Phase Condensers

Or …



Phase Objectives

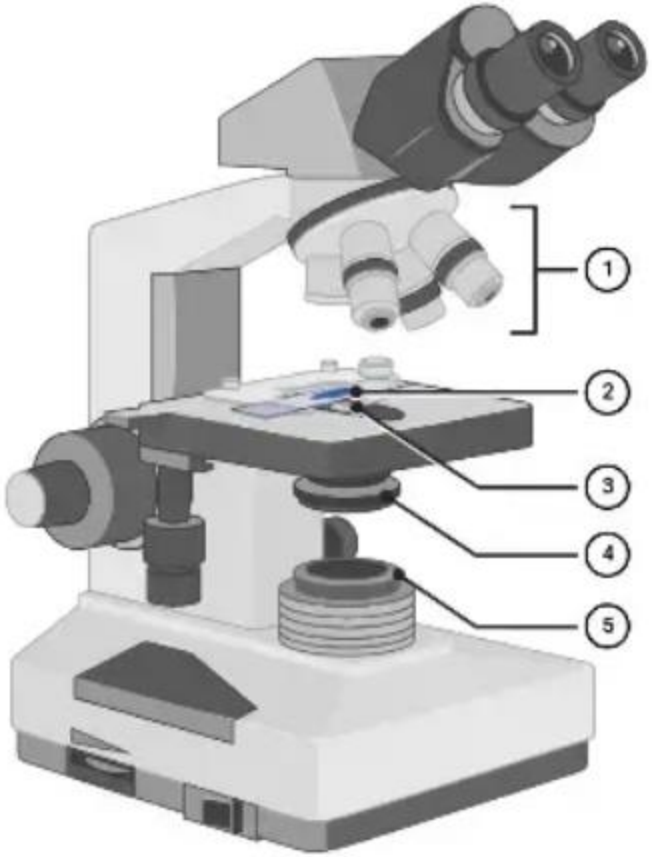


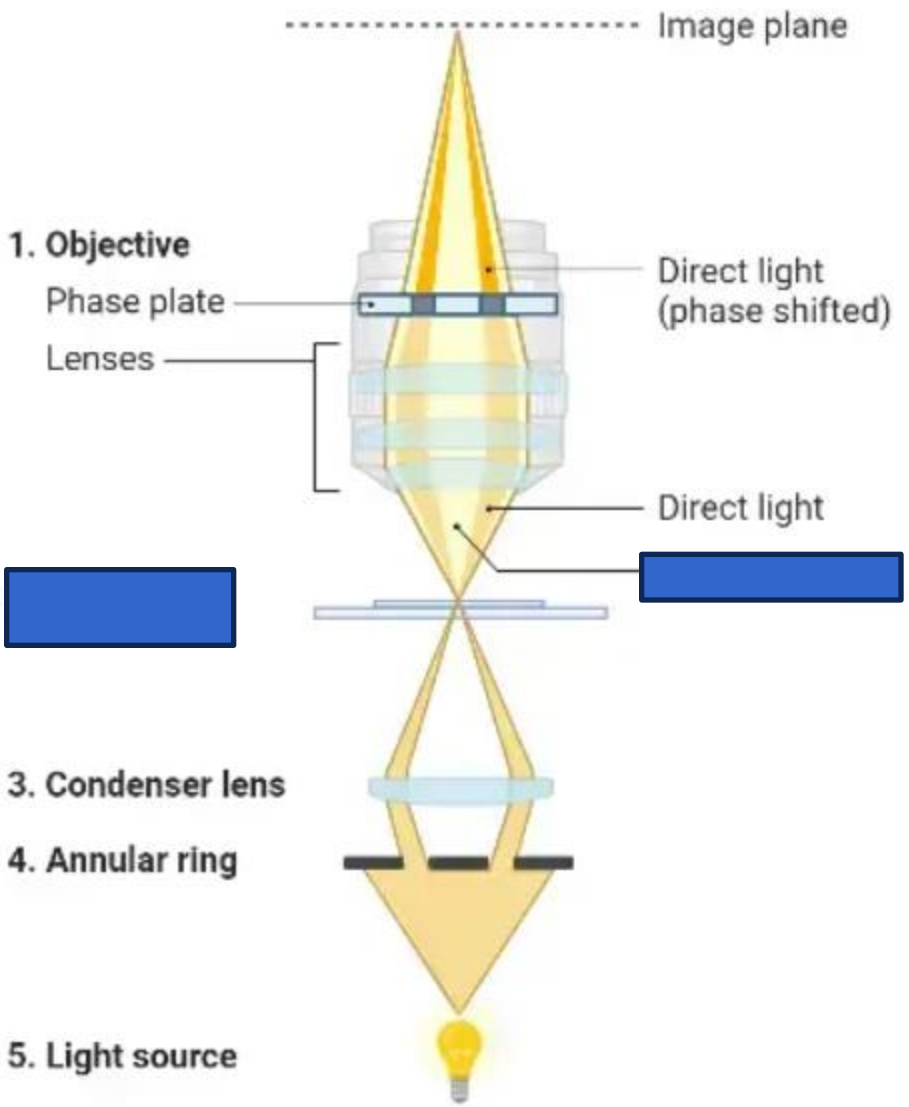
Condenser Annulus

Objective Phase Plate

Phase Contrast Microscopy 2

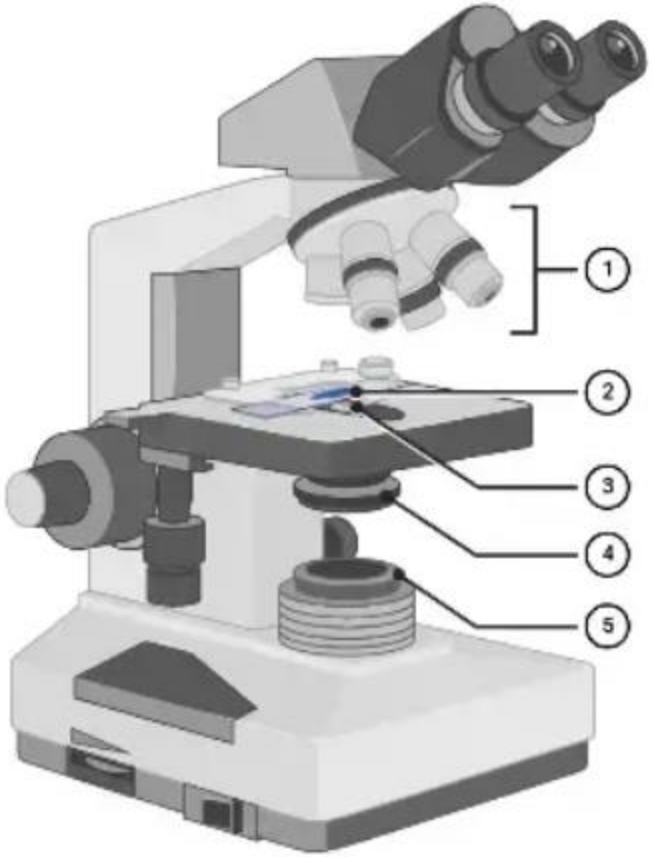
MN

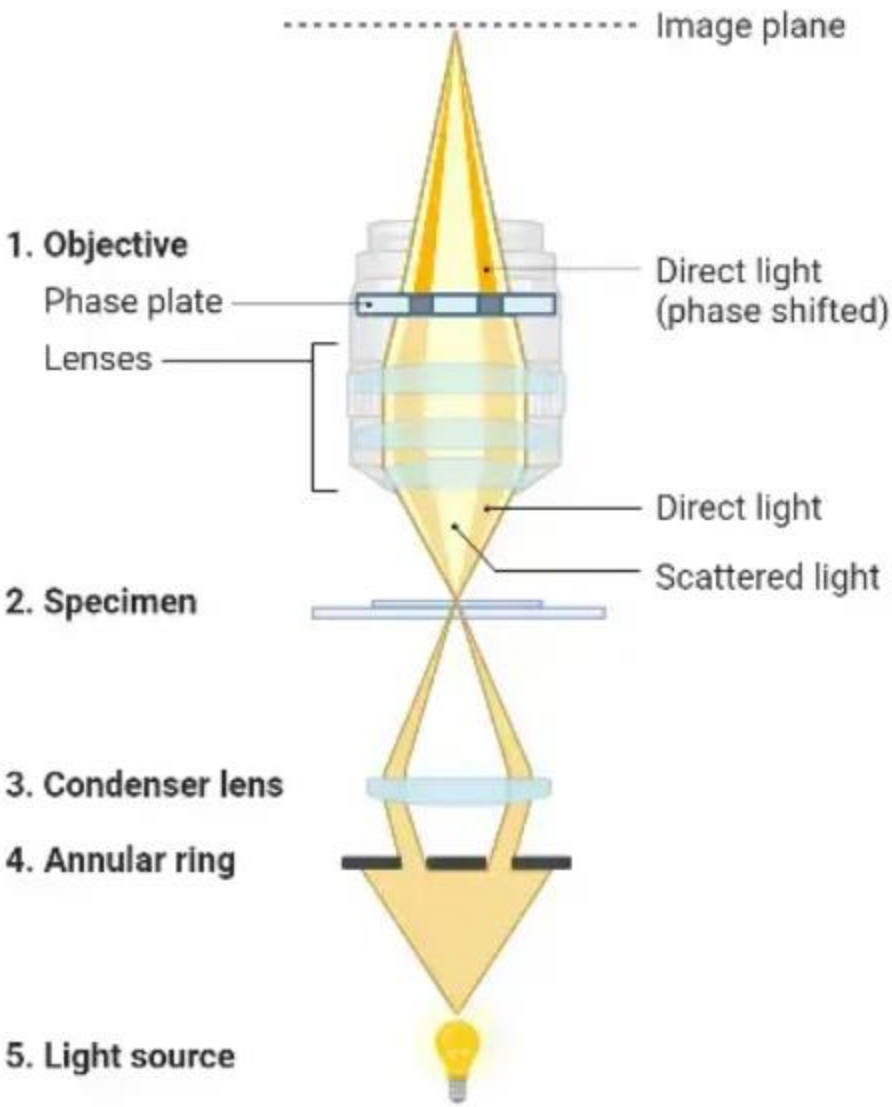




Phase Contrast Microscopy 2

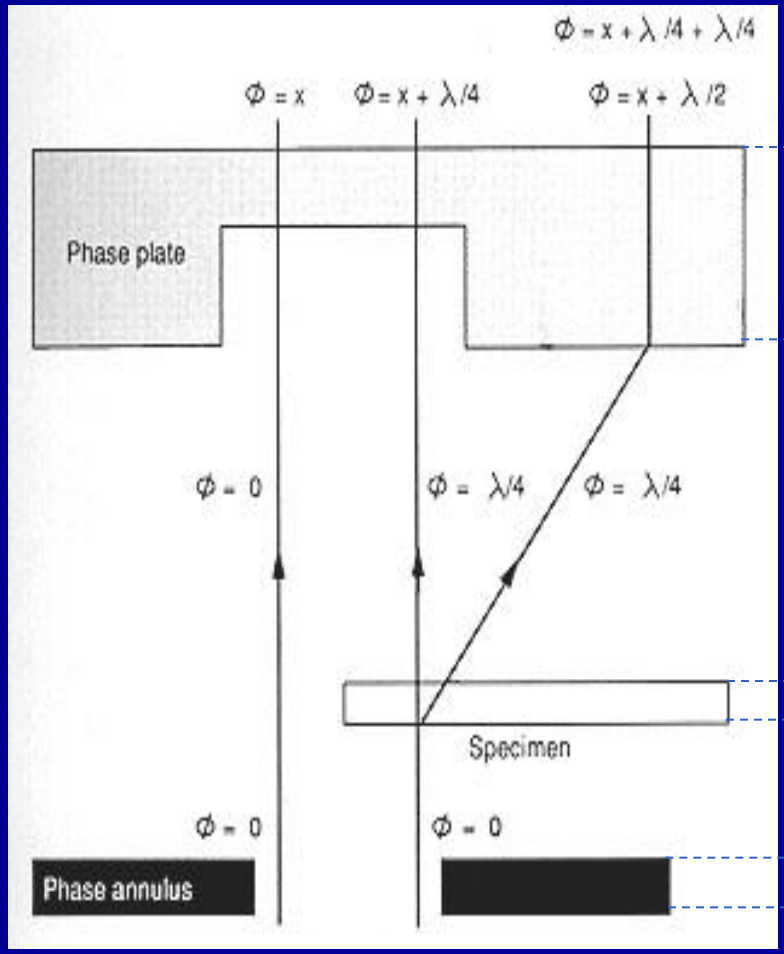
MN





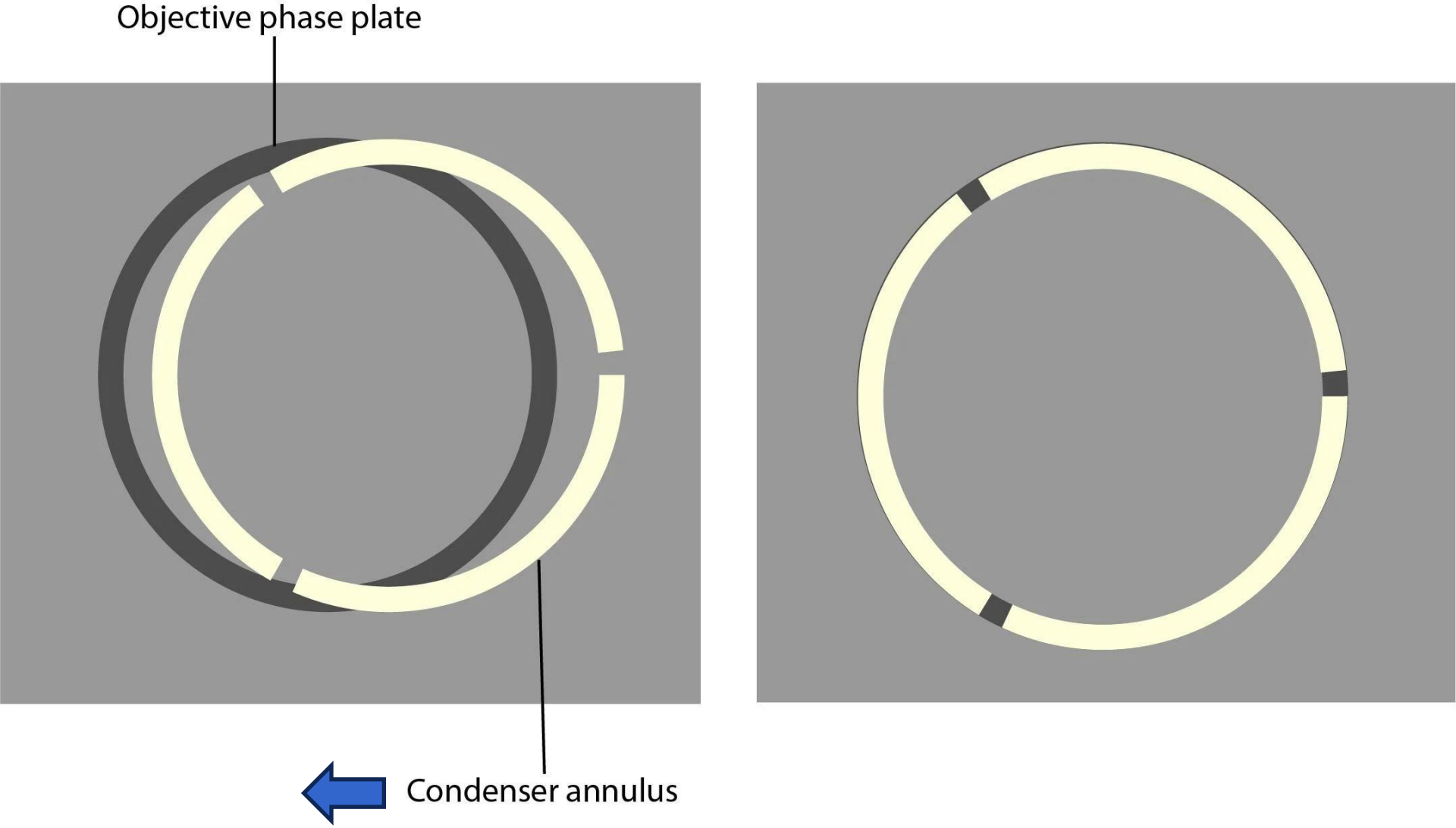
Phase contrast microscope 3

- specimen introduces a retardation of λ/4
- phase plate introduces a retardation of λ/4 for diffracted rays
- together there is a retardation of λ/2
- destructive interference will change amplitude of wave and allow specimen to be seen

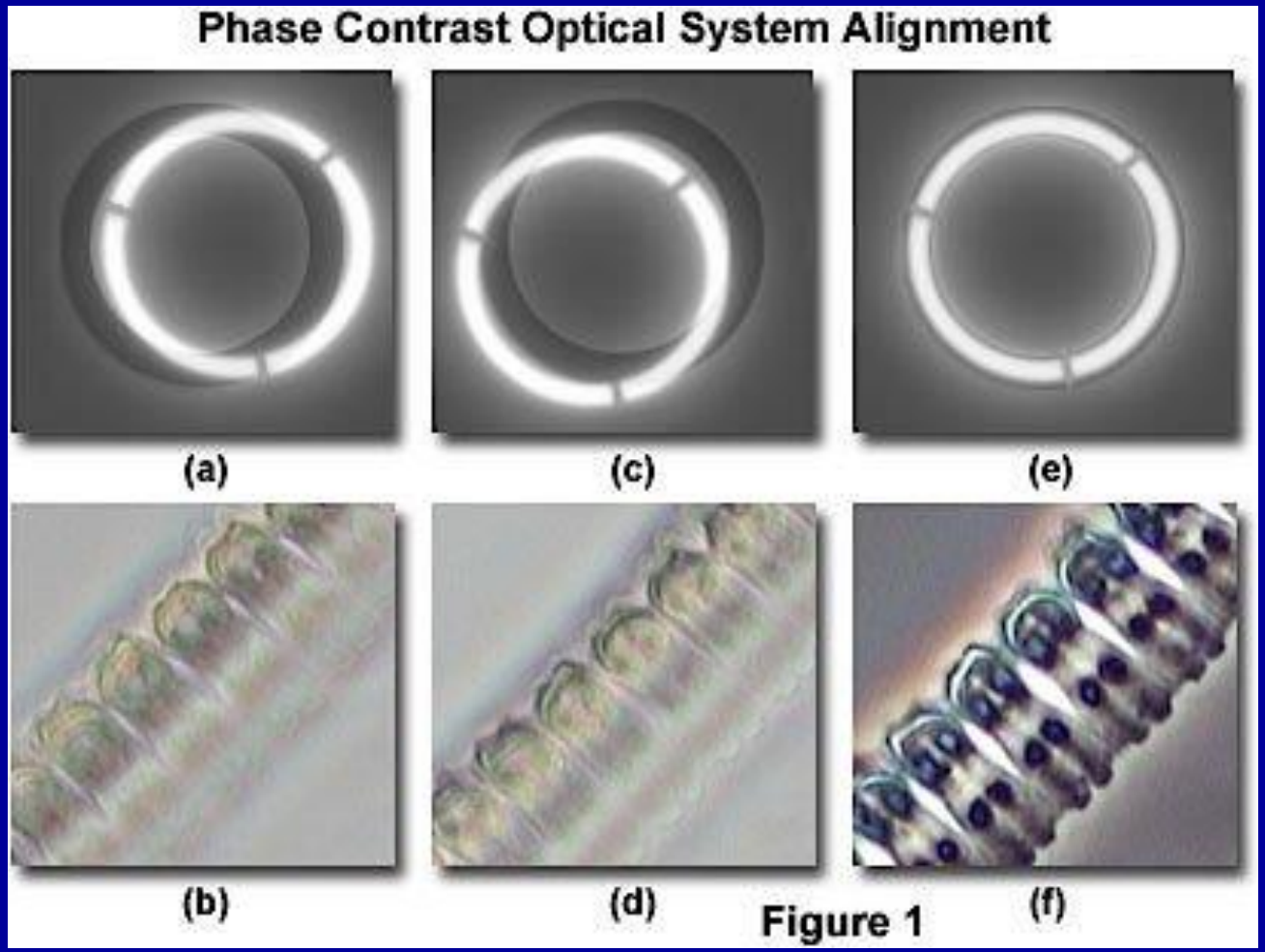


Left half only shown here …

Align Condenser Annulus



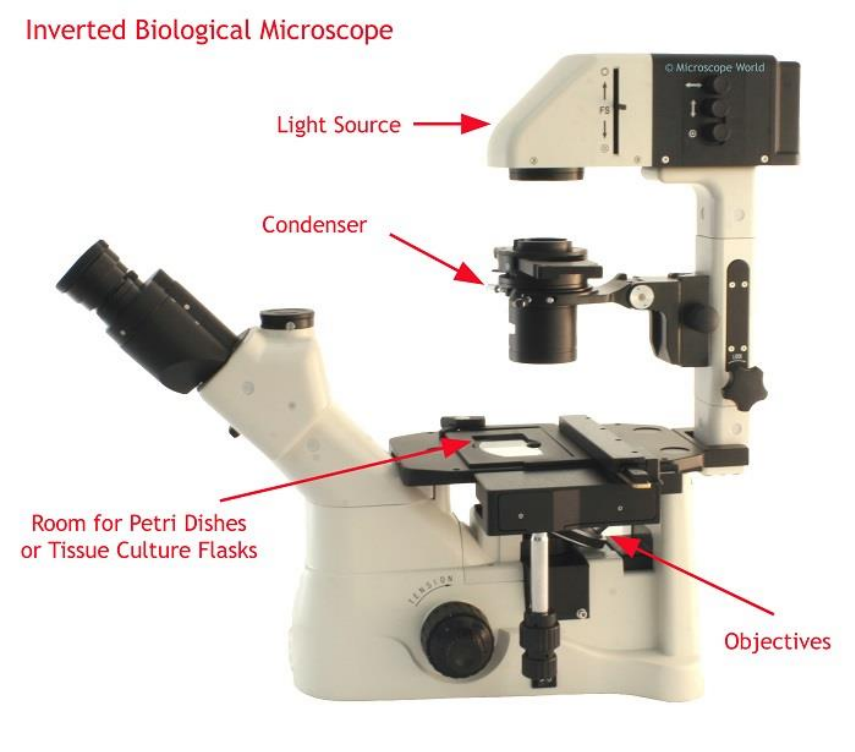
Fully Aligned !

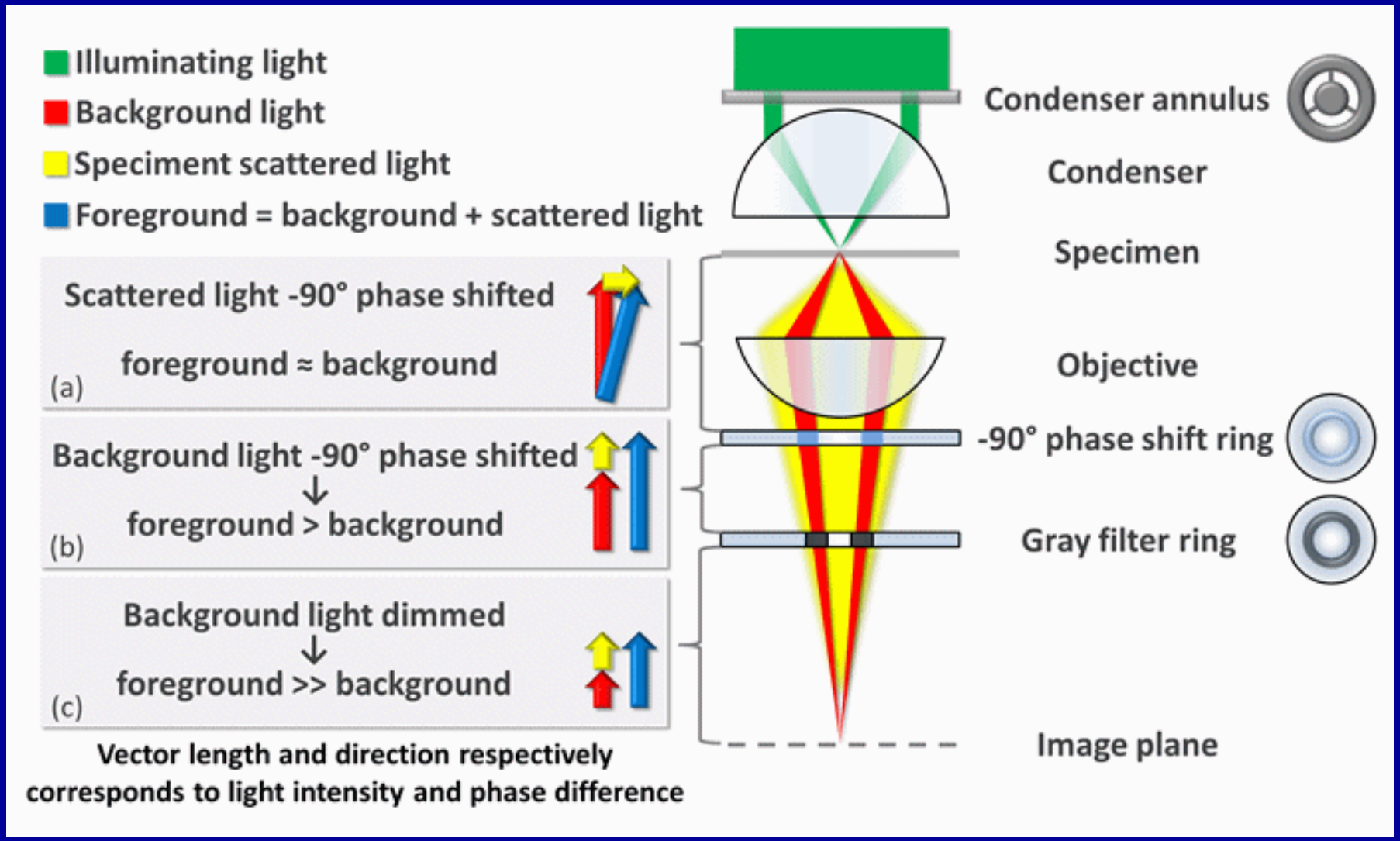


Phase Telescope
or
Bertrand Lens

Inverted Microscope

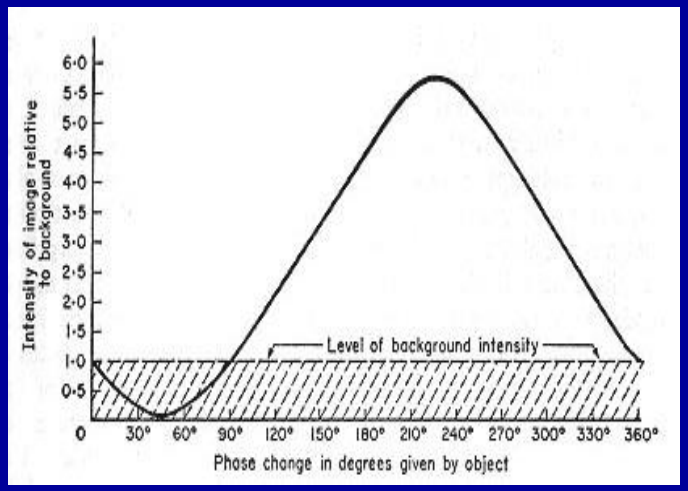
View live cells in petri dish etc …

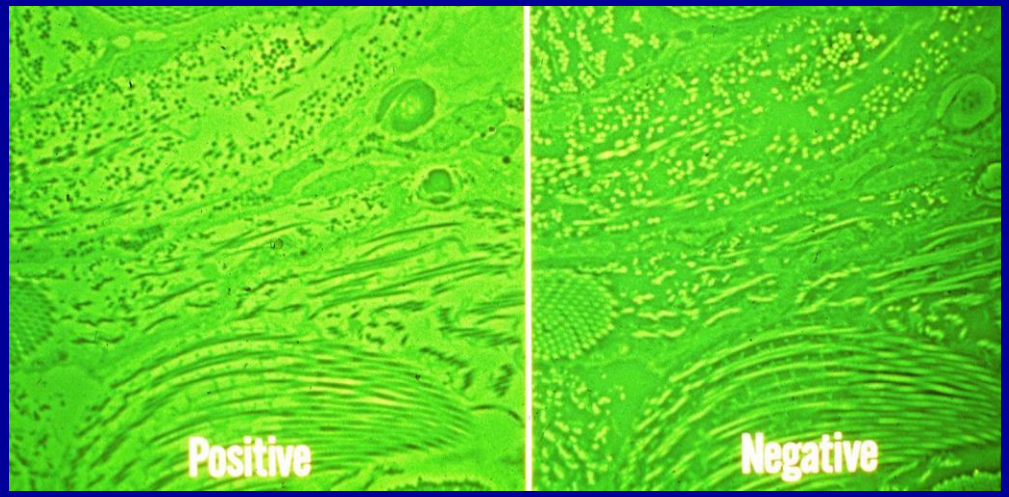



Illuminating light
Background light
Speciment scattered light
Foreground = background + scattered light
Scattered light -90° phase shifted
foreground ≈ background
(a)
Background light -90° phase shifted
↓
foreground > background
(b)
Background light dimmed
↓
foreground >> background
(c)
Vector length and direction respectively corresponds to light intensity and phase difference
Condenser annulus
Condenser
Specimen
Objective
-90° phase shift ring
Gray filter ring
Image plane

Wikipedia

Phase contrast microscope 5





- small phase changes – objects darker than background
- larger phase changes – objects brighter than background
- possibility of negative phase contrast microscopes

Phase Contrast (Positive / Negative)

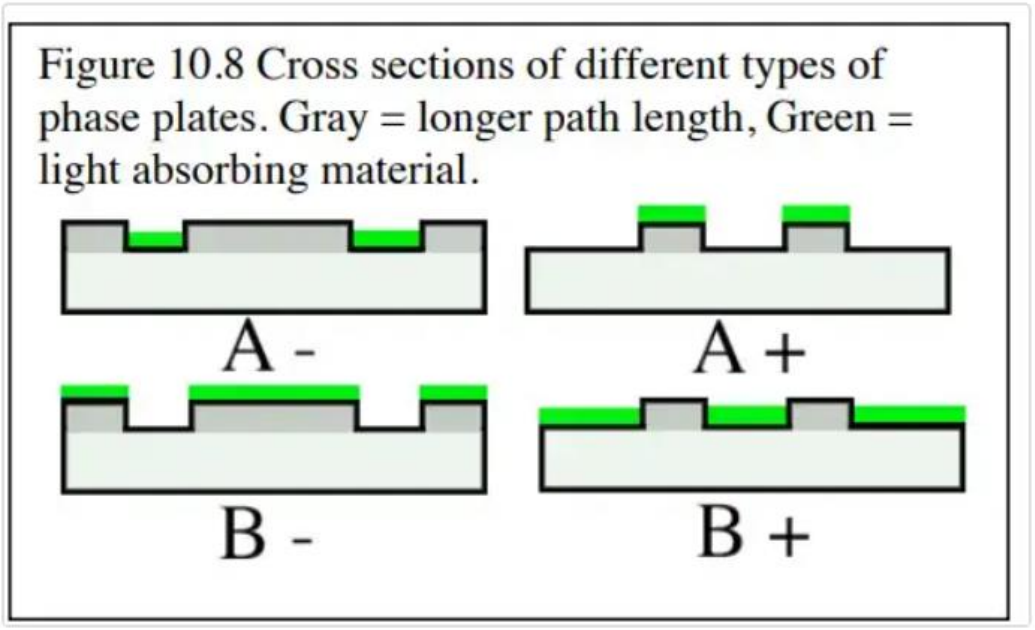
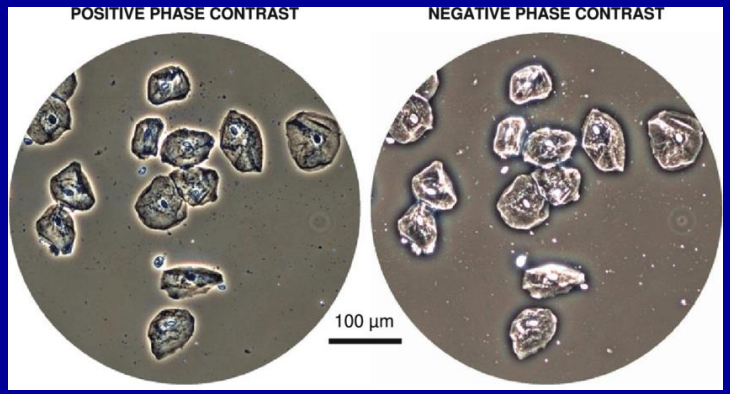


Fig: Cross sections of different types of phase plates. Gray = longer path length, Green = light abs orbing material.



Positive PC

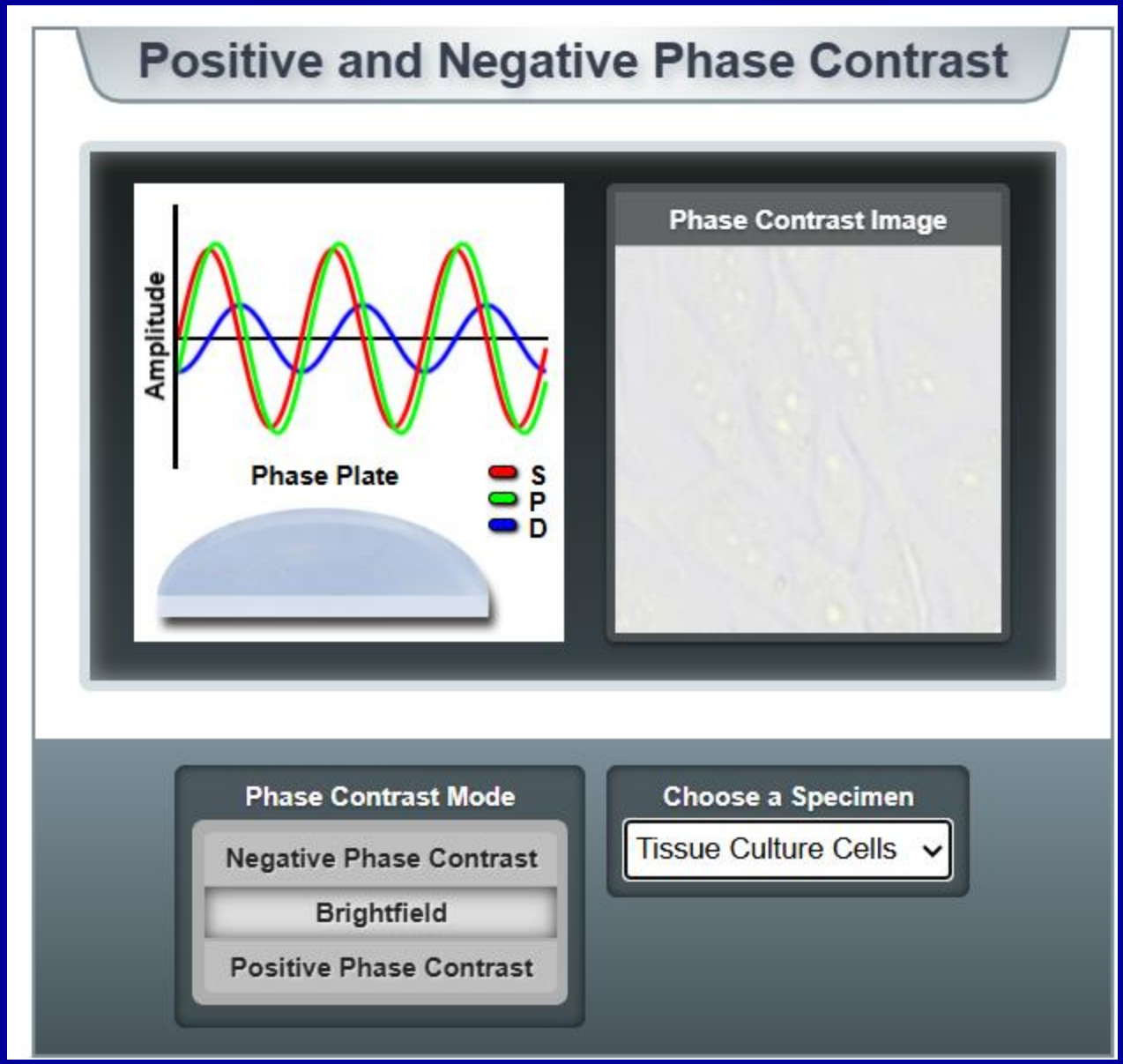
Negative PC

1. **In "positive" phase contrast,** the phase of the direct light is advanced by 1/4 wave (- type). This results in destructive interference and dark details on a bright background. The most prevalent form of phase contrast.
2. **In "negative" phase contrast,** direct light is slowed by a quarter-wave in phase (+ type). This results in luminous details on a dark background due to constructive interference.
3. **In positive or negative phase contrast,** the phase plate might be one of two varieties. Either the straight light (type A) or the diffracted light (type B) can be absorbed (B type). A – is the most prevalent type. Both A and B type plates are assessed by the transmission percentage of the ring area, with 20 percent being the norm.

+Coatings -75%
+Apodized ND filters

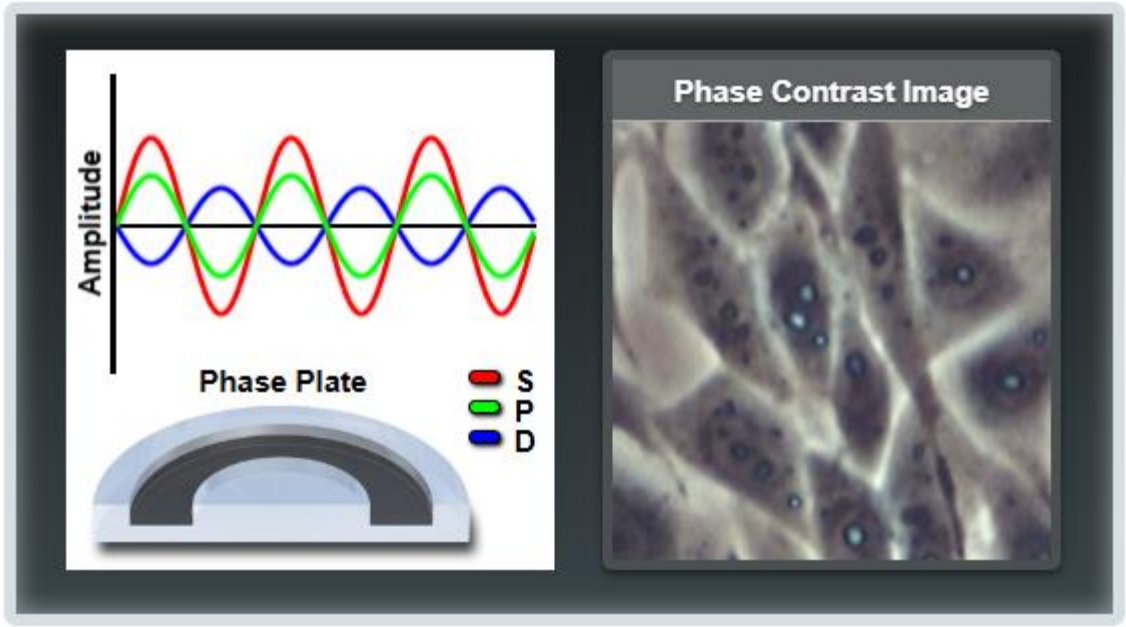
Phase Contrast Microscopy: definition, parts, uses, working principle. - Biology Notes Online

Online Interactive Tutorials



Nikon MicroscopyU

Positive and Negative Phase Contrast



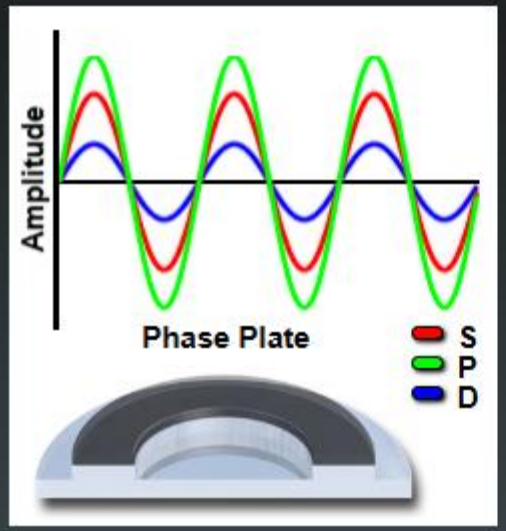
Phase Contrast Mode

- Negative Phase Contrast
- Brightfield
- Positive Phase Contrast

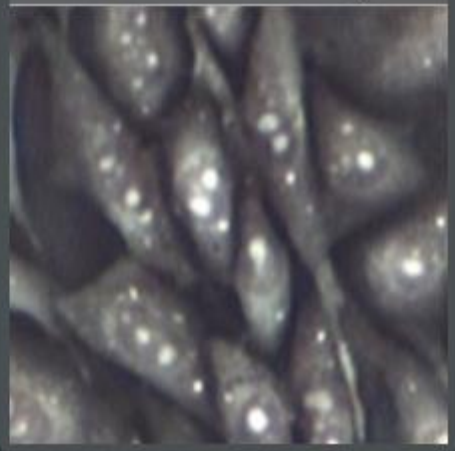
Choose a Specimen

Tissue Culture Cells

Positive and Negative Phase Contrast



Phase Contrast Image



Phase Contrast Mode

- Negative Phase Contrast
- Brightfield
- Positive Phase Contrast

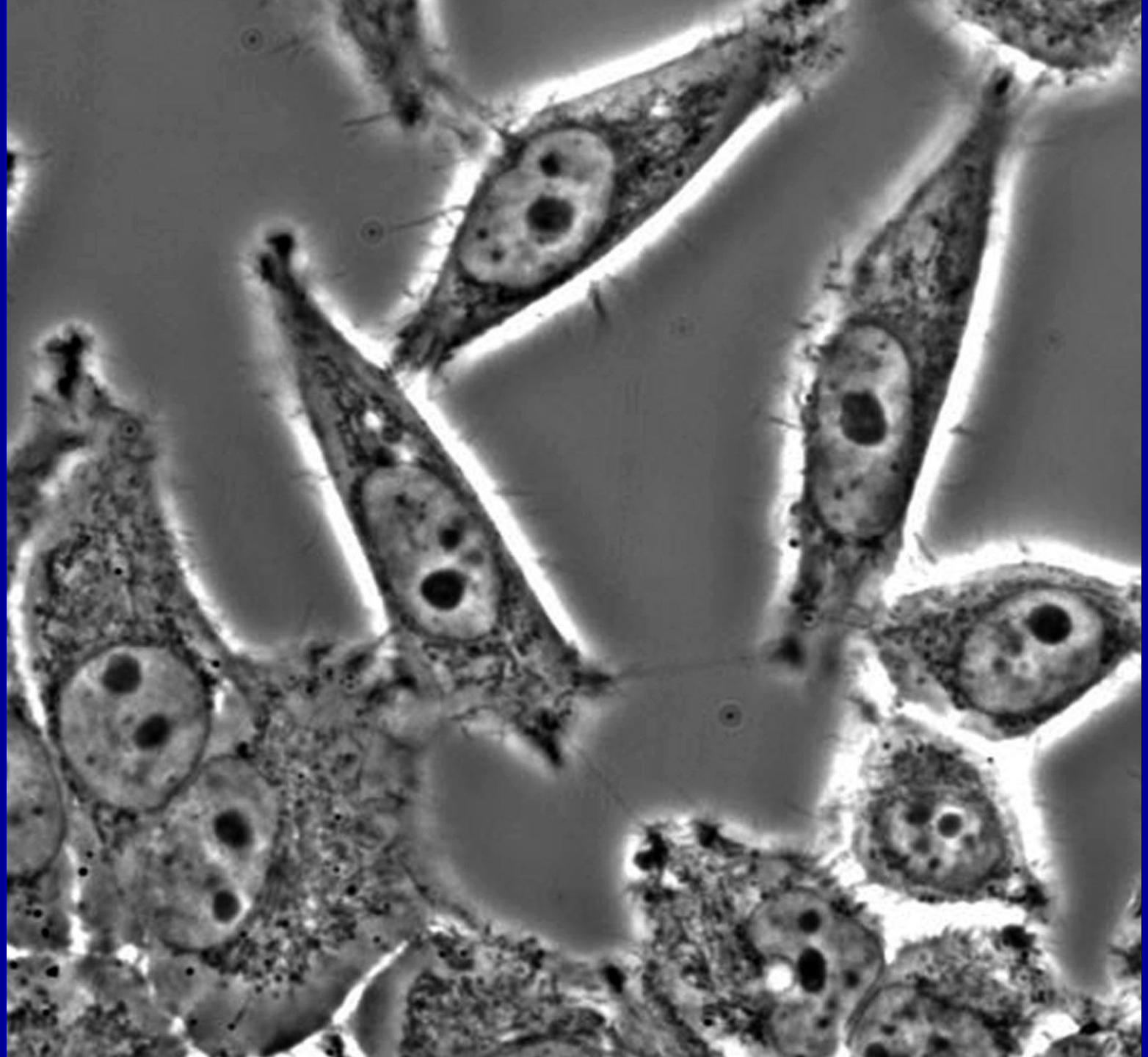
Choose a Specimen

Tissue Culture Cells

Nikon MicroscopyU

▲Fibroblast (Human embryo). PC D 20XPL, NFK 2.5XLD.

▲PC D 20XPLL, NFK 2.5XLD.



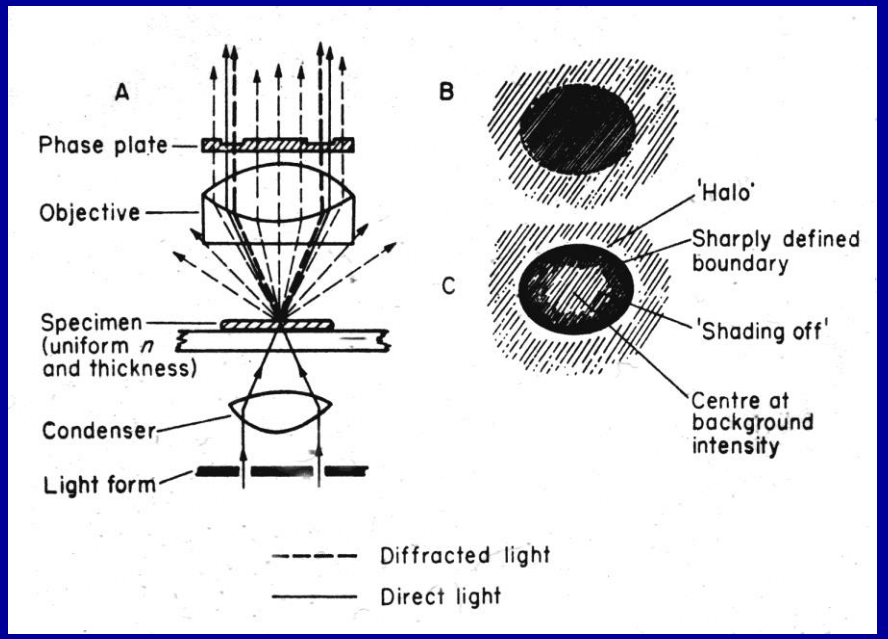
Phase contrast microscope 6

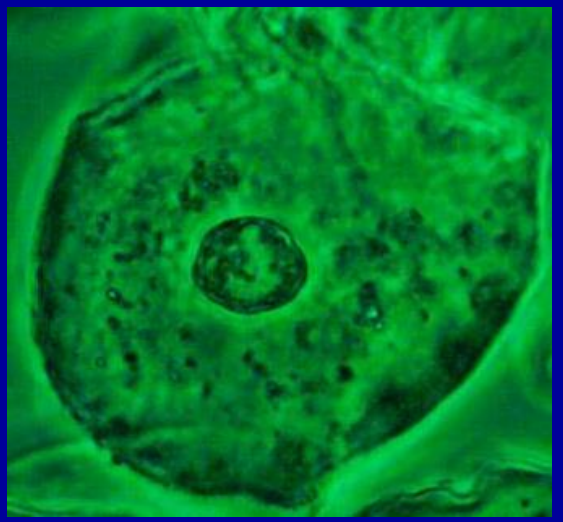
Problems/Artefacts:

due to incomplete separation of rays

- halos
- shading off
- contrast inversion (OPD)
- colours
- Stained = ‘muddy’

- **Use <u>Thin</u>, <u>Unstained</u>, + <u>Green</u> (550nm) filter to increase contrast**



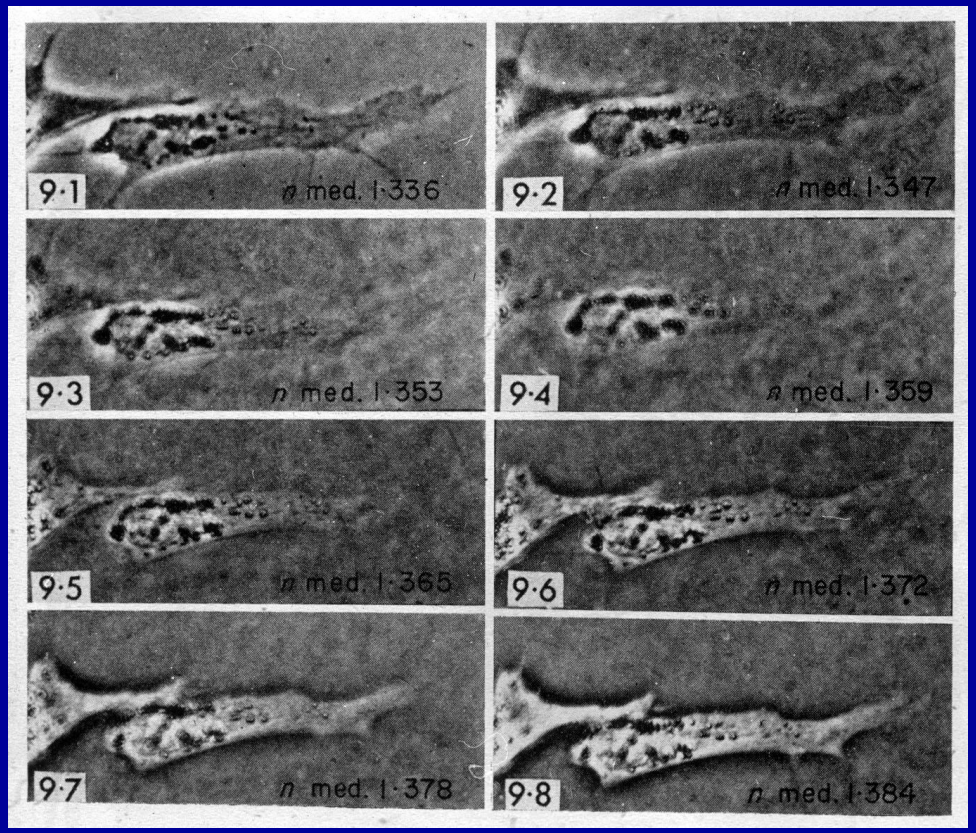


Quantitative Microscopy

Measure alterations in light …	Specimen	Microscope	Quantitation
Direction	Opaque material	Reflectance SEM	Count silver grains Autoradiography
Amplitude Amplitude (λ)	Dyes > contrast Dyes > colour	TEM BF LM	Morphometry Stereology Eyescales,Photometry Microdensitometry Microspectrophotometry
Wavelength	Fluorescent X-ray emission	Fluorescence Electron probe microanalysis	Fluorometry Photometry
Plane of Vibration	Anisotropy Birefringence	Polarising	Macromol arrangement Refractive index
Phase change	Living cells R.I. & Diffraction	Phase contrast	Immersion refractometry
Phase change	R.I. & Thickness	Interference	Thickness, Mass, Weight

See Quantitation Lecture …

Phase contrast microscope 4

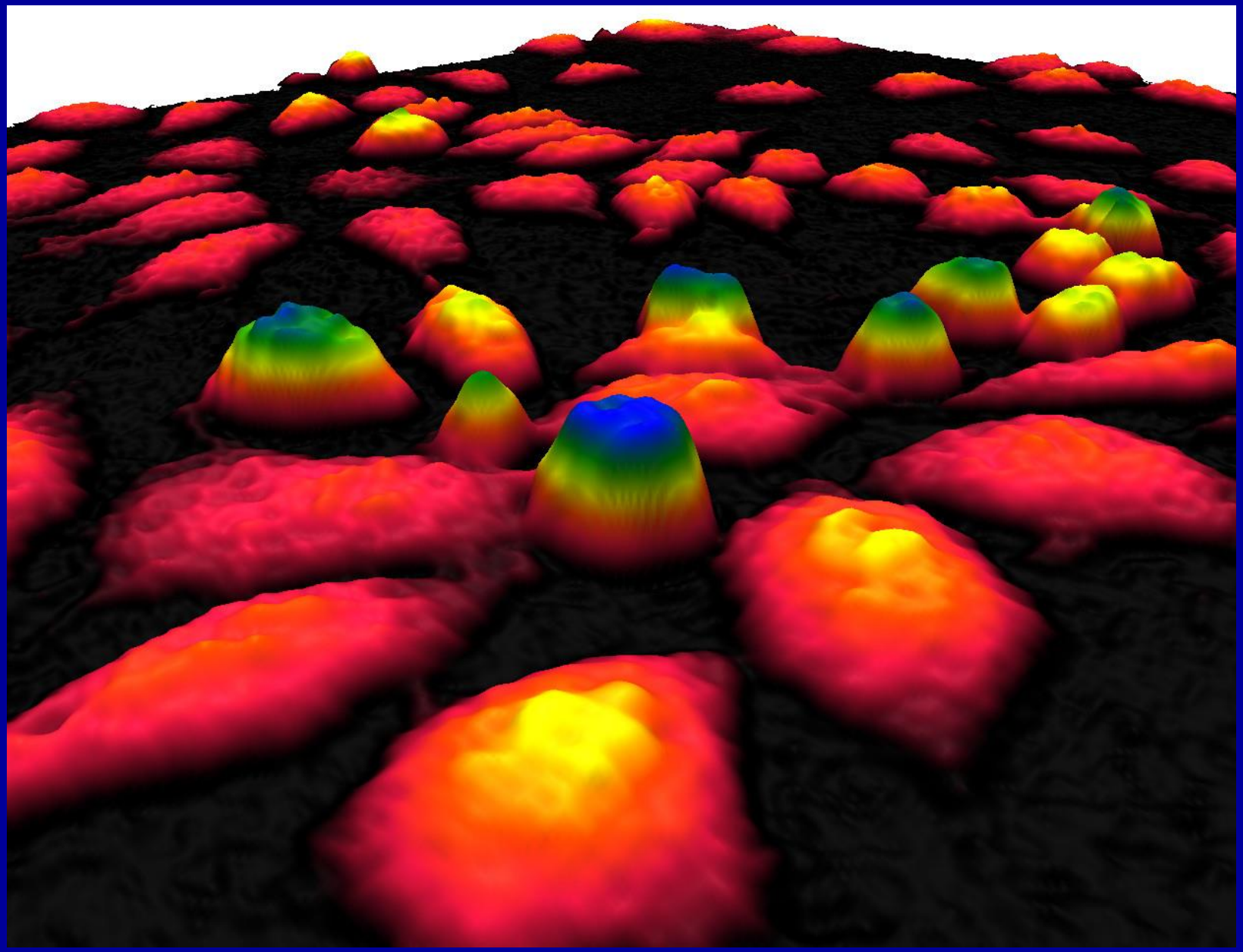


Immersion Refractometry

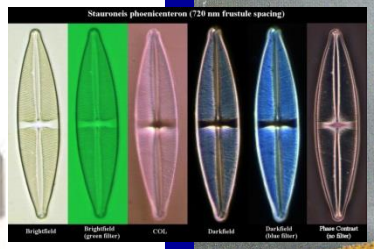
- refractive index of mounting medium is important
- changing it changes contrast
- **Barer (1950s)** first used it to quantify density, mass

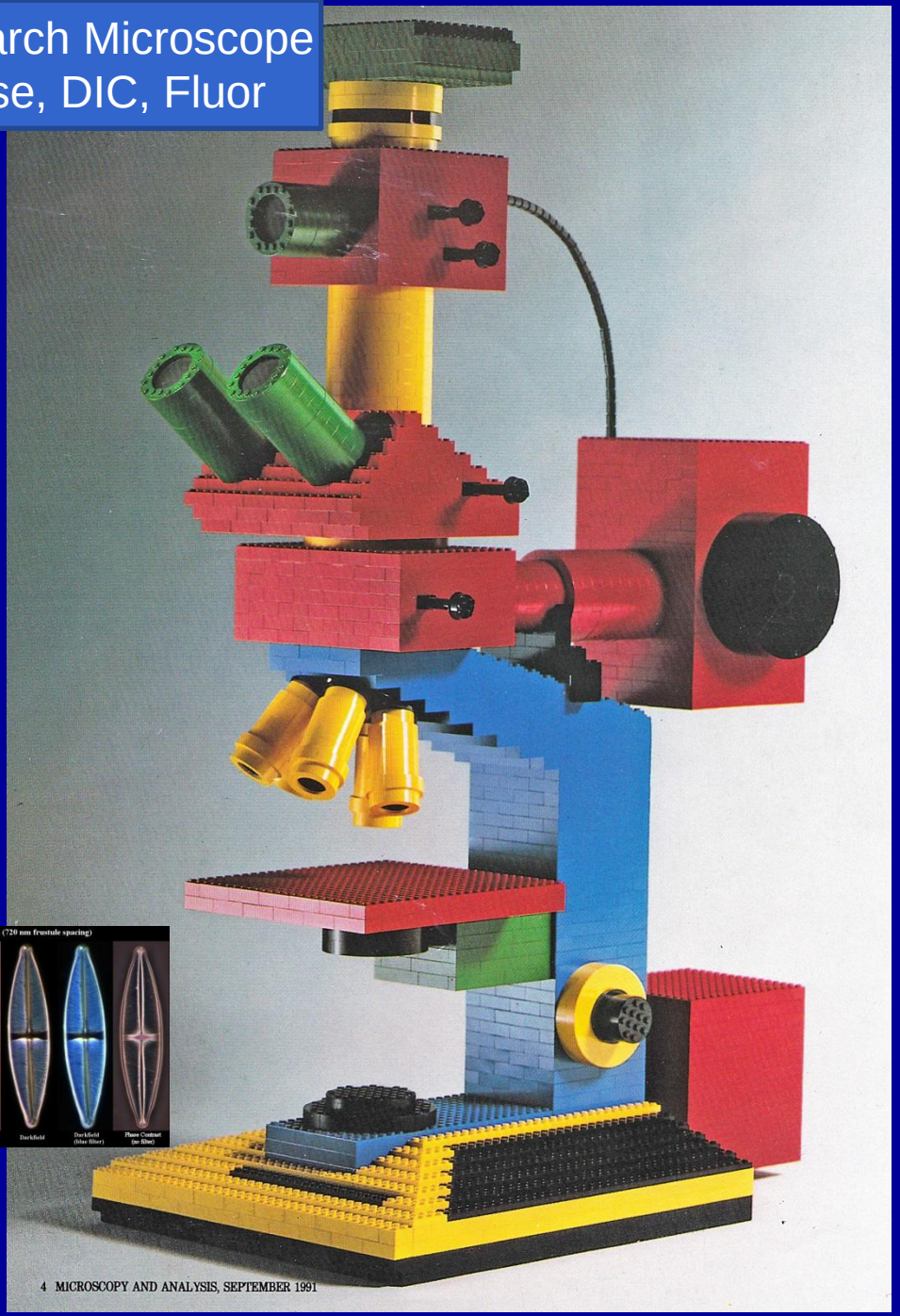
D=M / V M=D x V (weigh cells & organelles !!)

‘Quantitative’ Phase Contrast – Optical thickness is colour coded (Wikipedia)

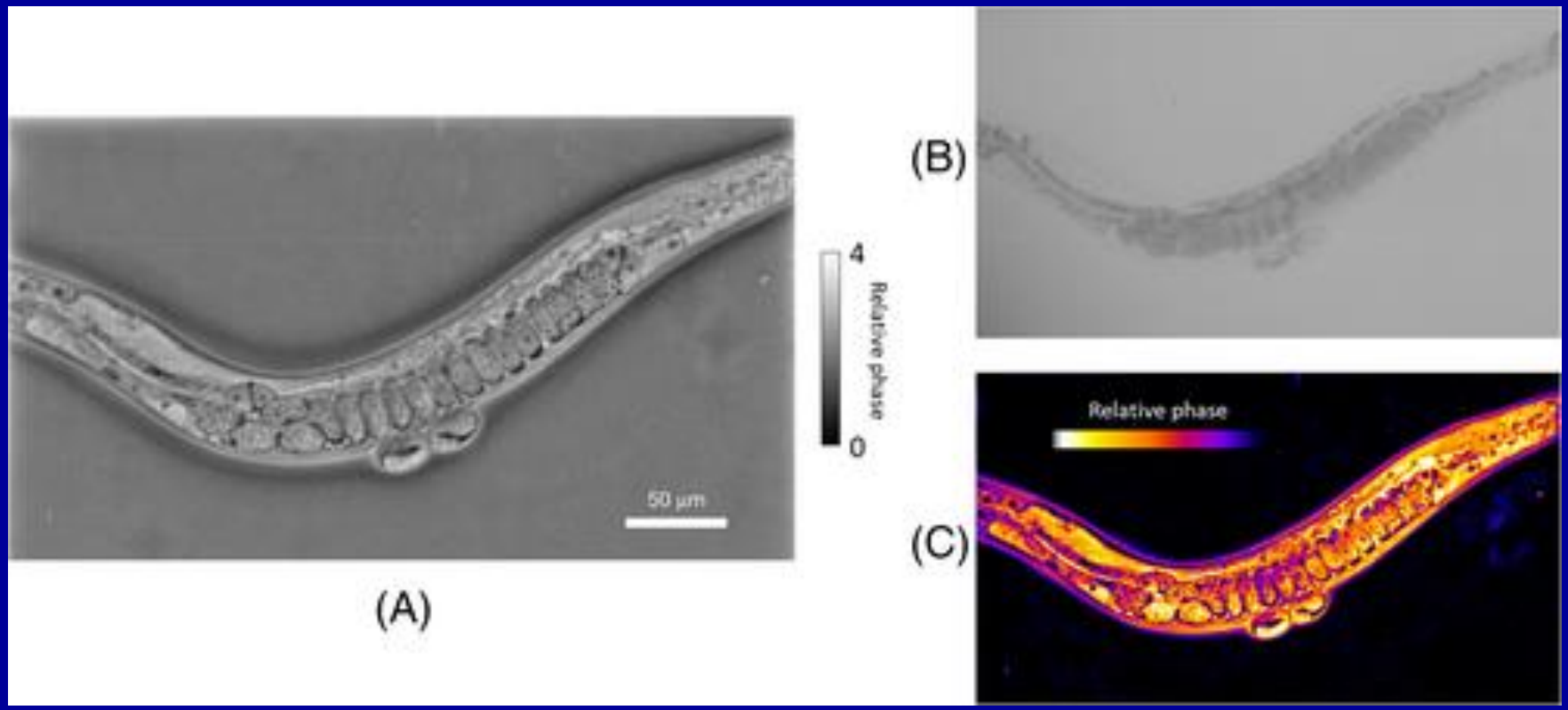


Modular Research Microscope
BF, DF, Phase, DIC, Fluor

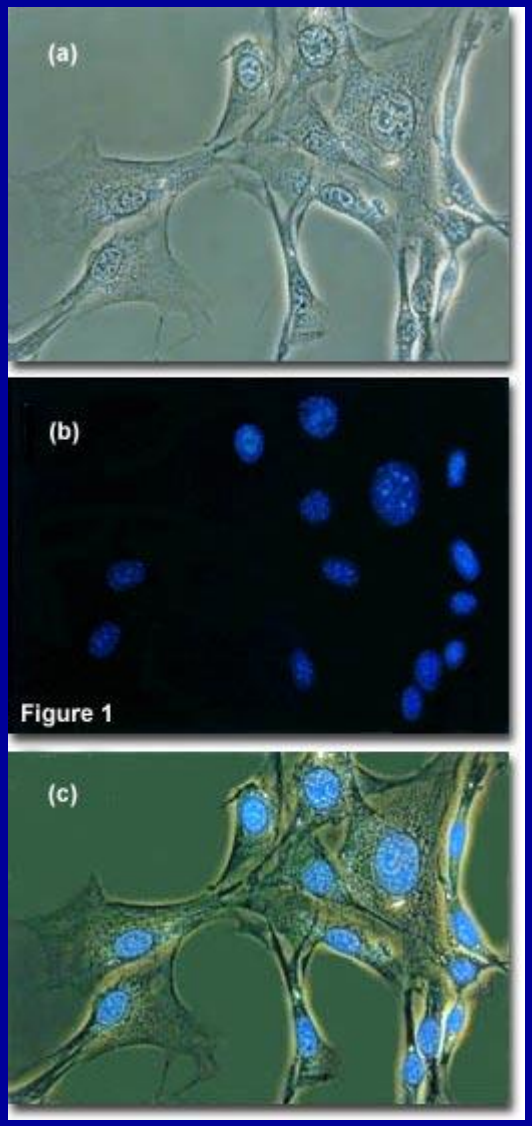


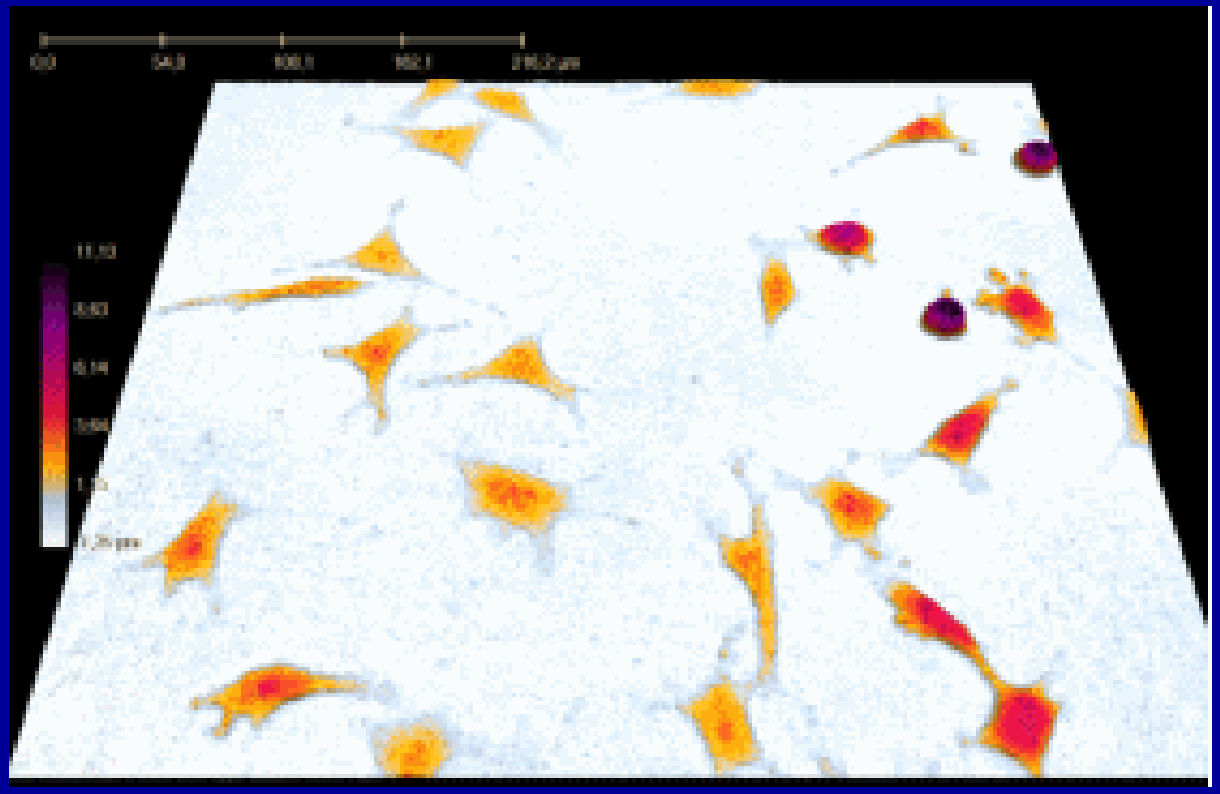


Phase plus DIC



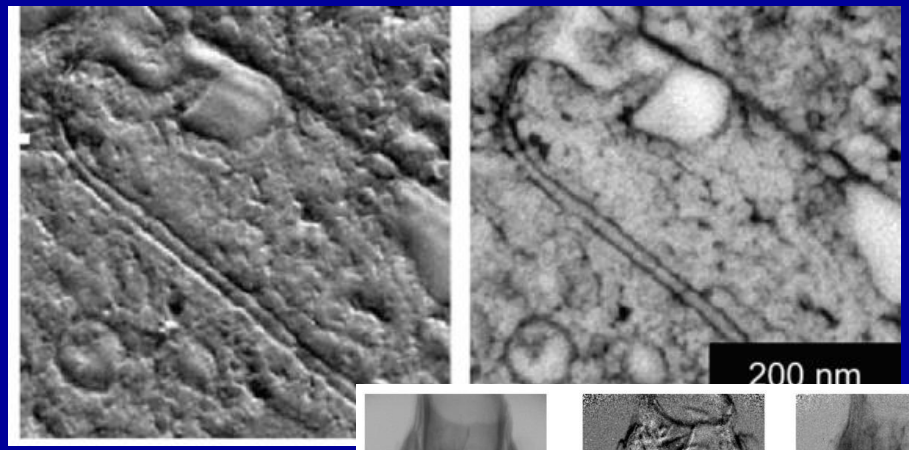
Phase + Fluorescence



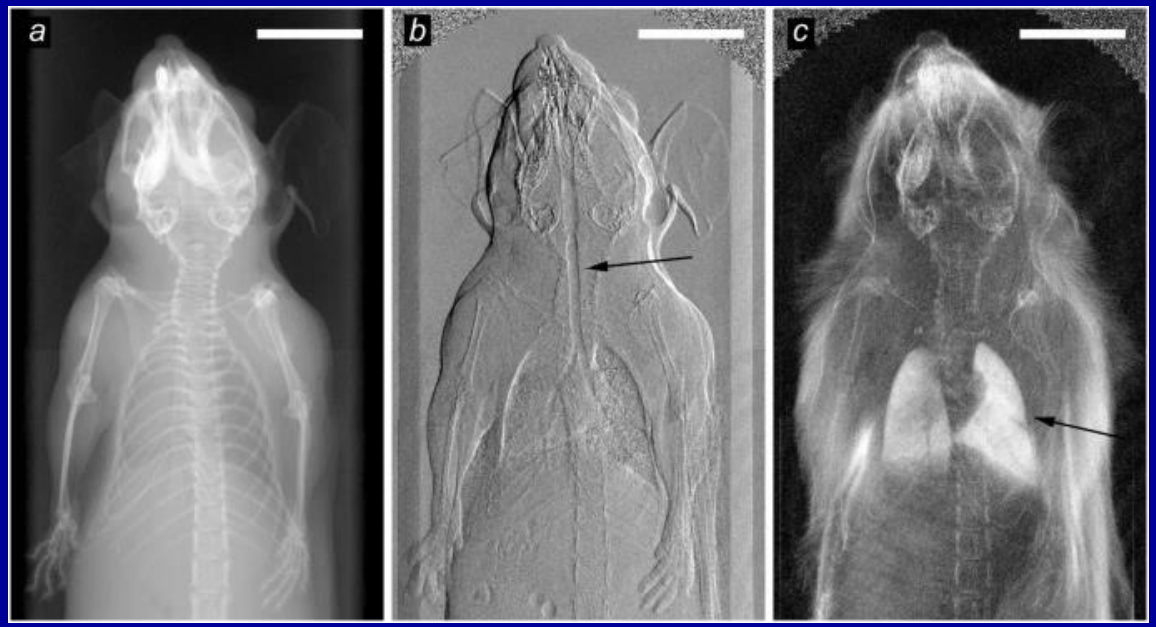


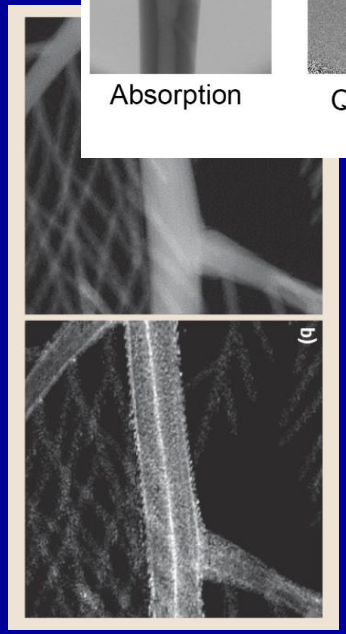
Recent …

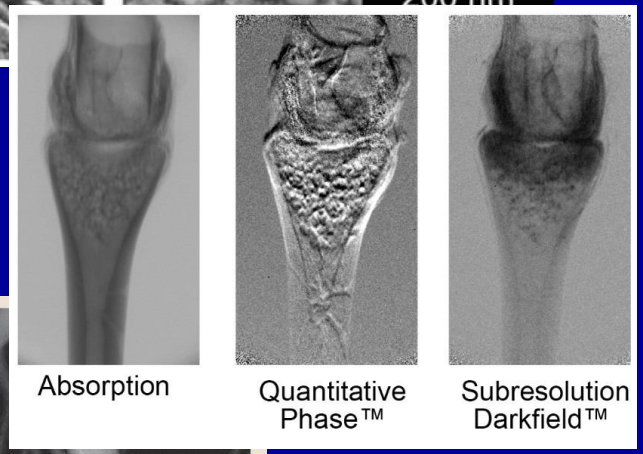
Phase contrast electron microscopy



Phase X-ray microscopy







SUMMARY: Contrast Enhancement

- Staining (dyes, metals, fluorophores), media
- Setup Δ – out of focus (Becke), condenser position
- Oblique illumination
- Dark Field
- Rheinberg, Spikeberg, VAC
- Dispersion ‘Staining’
- Polarising
- **Phase Contrast, Leitz Heine PC Condenser**
- Hoffman Modulation Contrast, L IMC, NAHC, O RC, Z Varel
- Interference Contrast, DIC
- Colour Filters, Photoshop !!

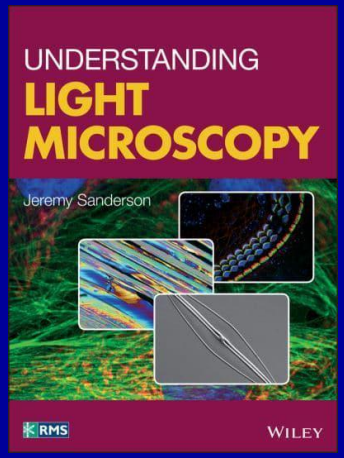
References

Phase-contrast microscopy - Wikipedia

BW OPTICS

Introduction to Phase Contrast Microscopy | Nikon’s MicroscopyU

Phase Contrast Microscopy - Introduction | Olympus LS



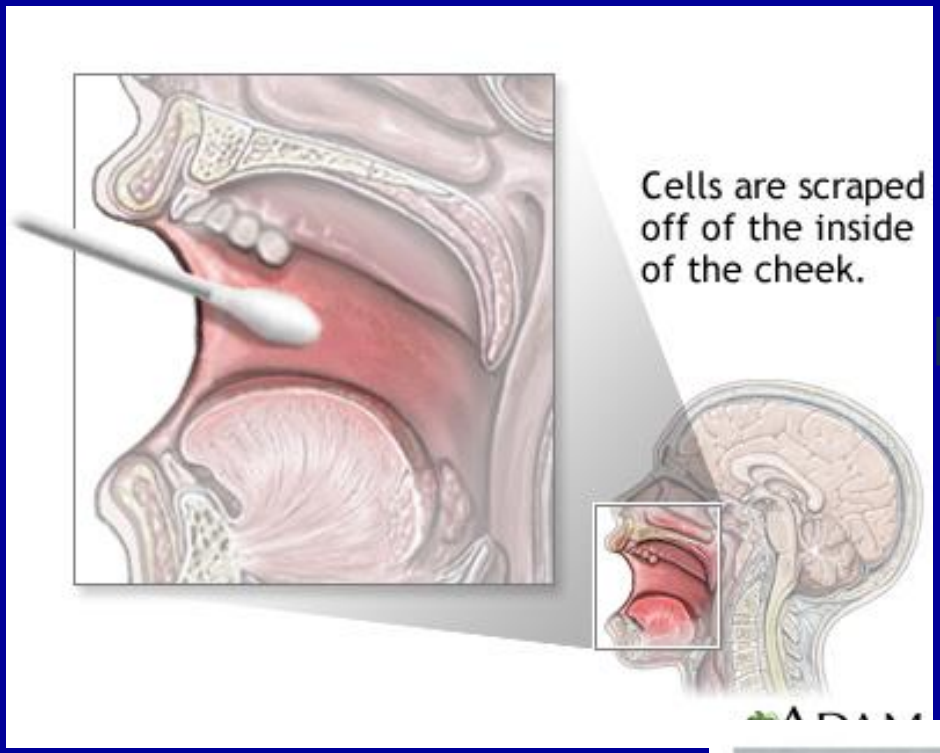
Practical

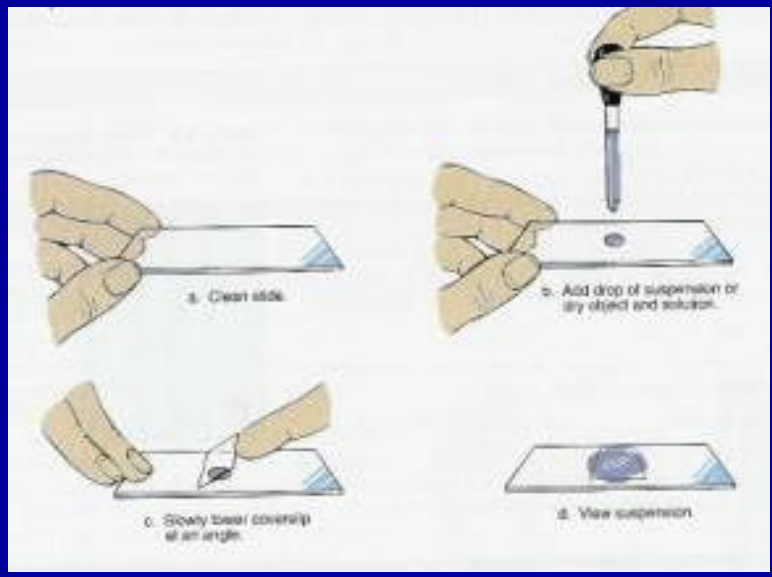
- **View Phase Contrast microscopes**
  - Olympus
  - Lomo

- **Prepared slides**
  - Cheek cells
  - Pond water organisms
  - Diatoms
  - …

- **Prepare own cheek smears & view with Phase Contrast**

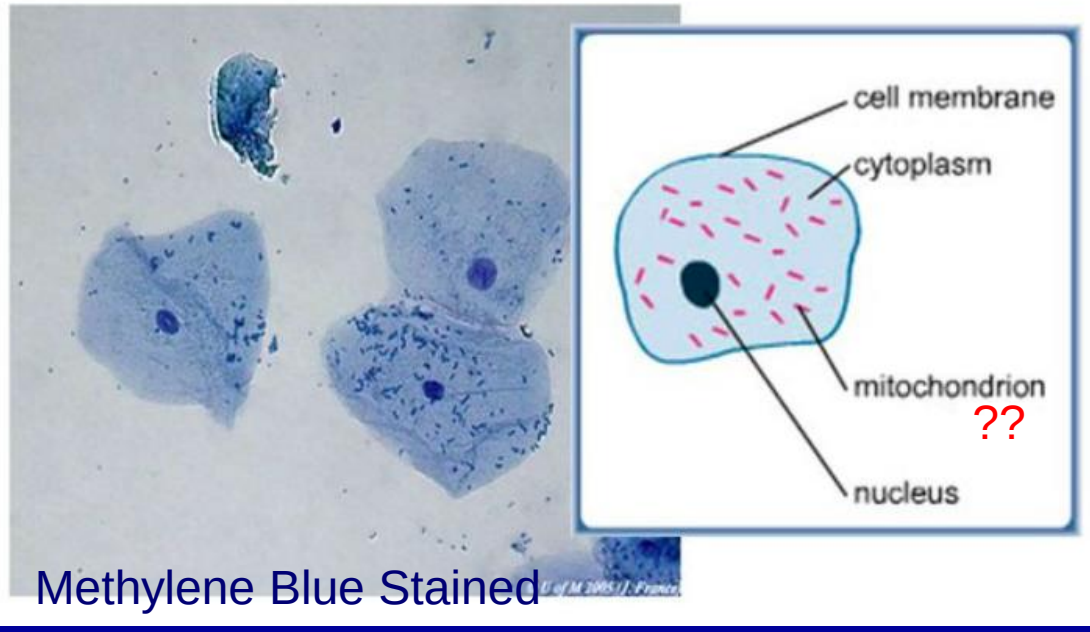
- **At home**
  - view links to videos & references
  - Prepare check smears – stain with methylene blue



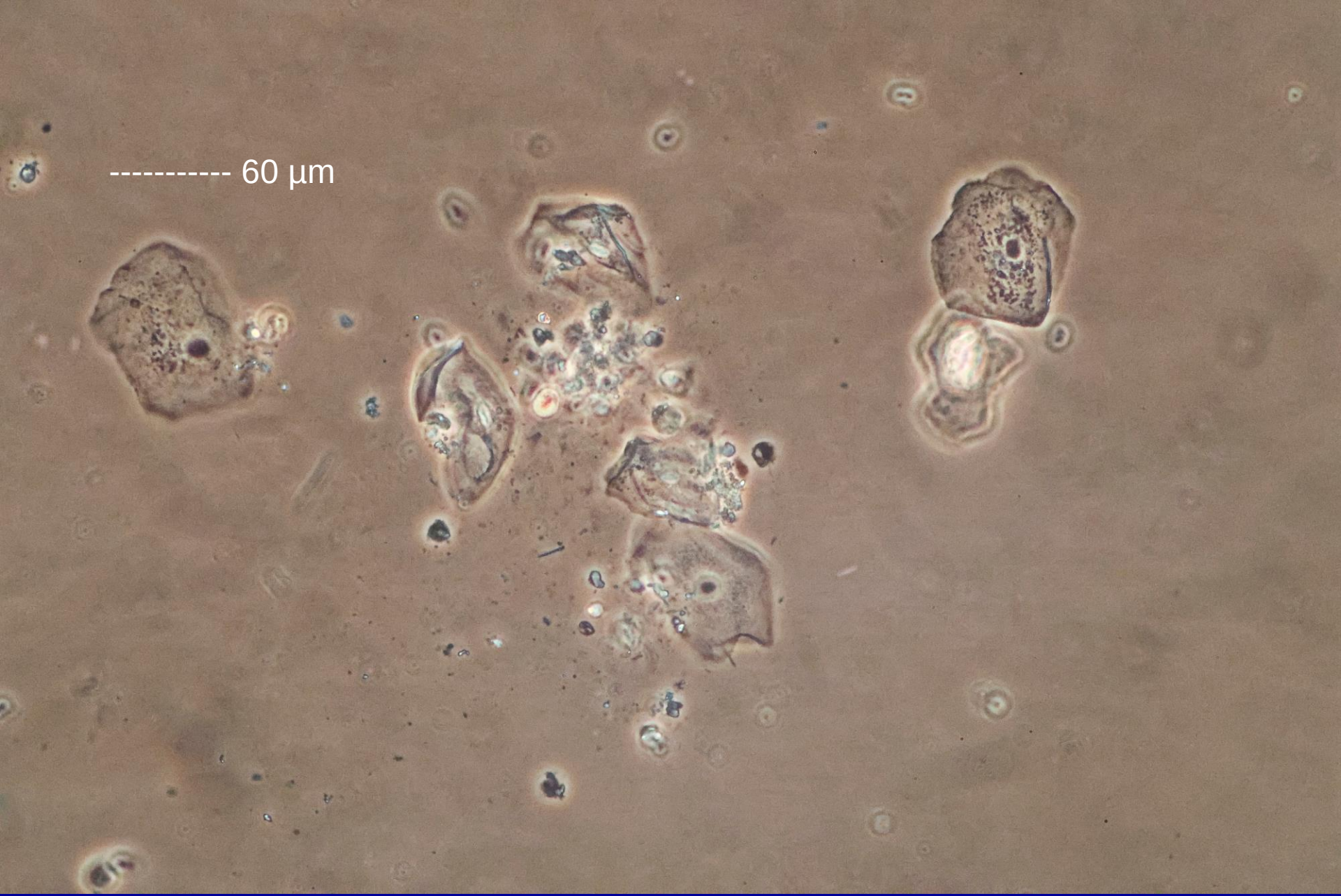


Try and get as close to this appearance **without** fixing/staining

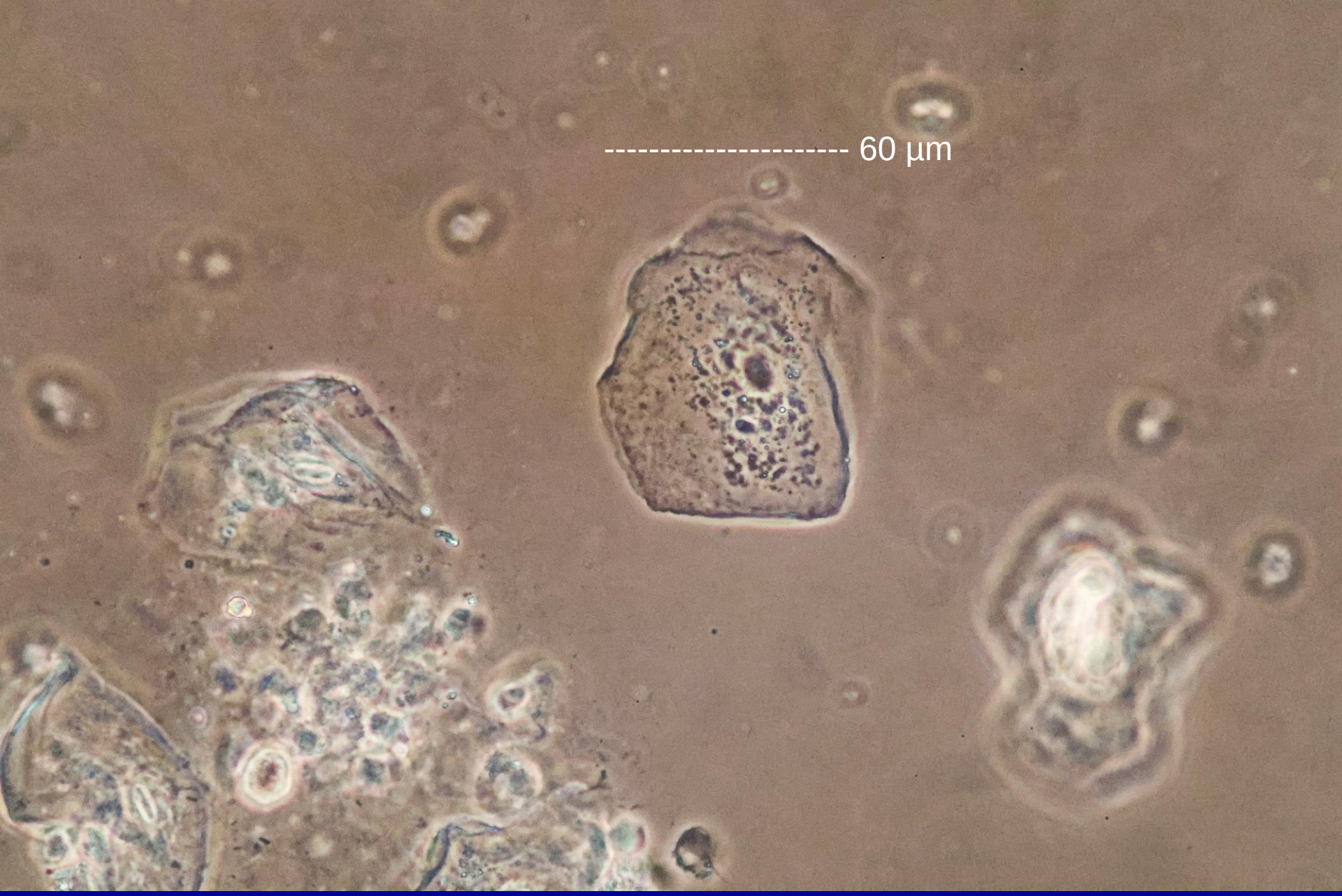
>



Methylene Blue Stained

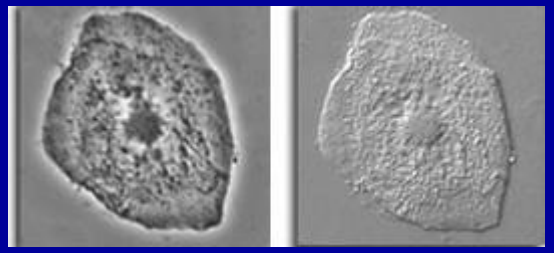


MM cheek cells – Phase Contrast x20

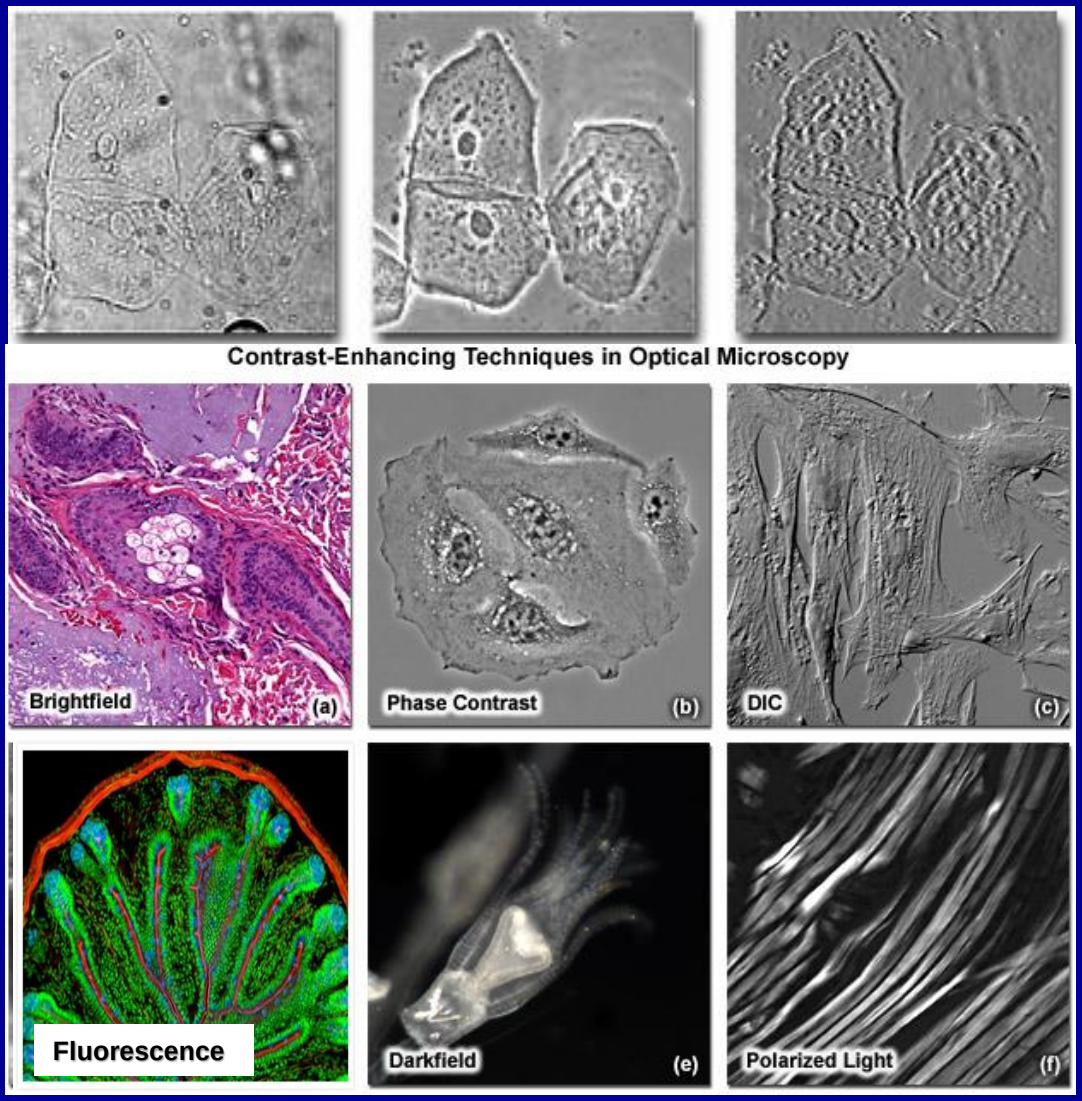


MM cheek cells – Phase Contrast x40

Types of Microscopy



- Bright field
- Dark Field/Refl
- Polarising
- **Phase Contrast**
- Interference
- Fluoresecence



Microscopy on Postage Stamps