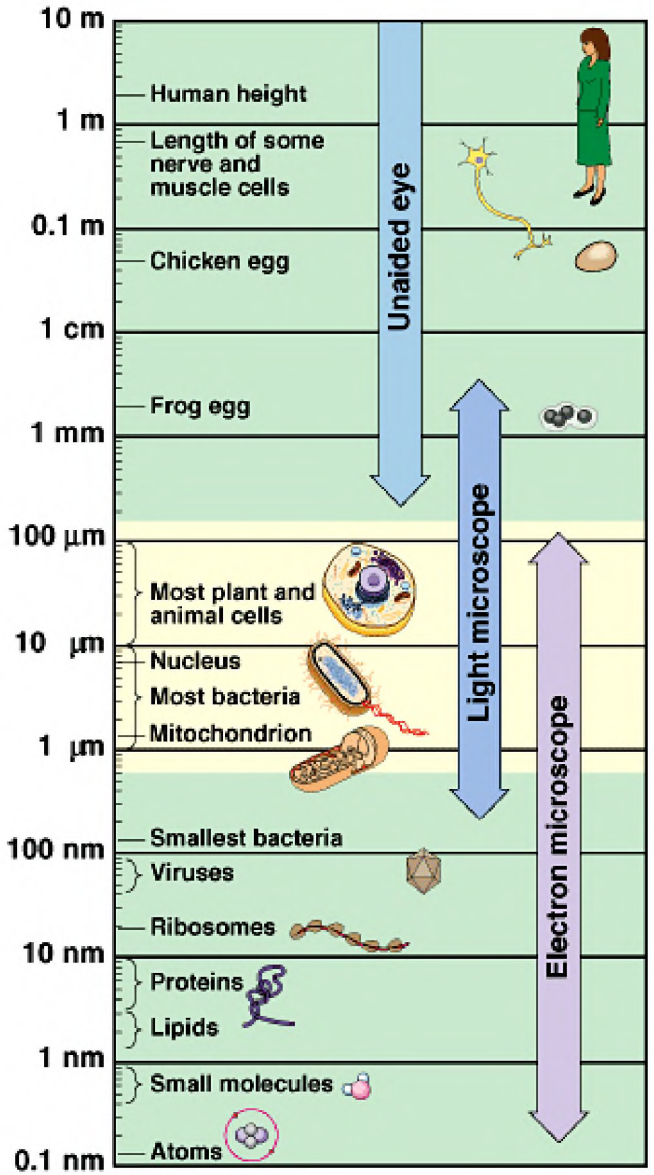
Microscopy

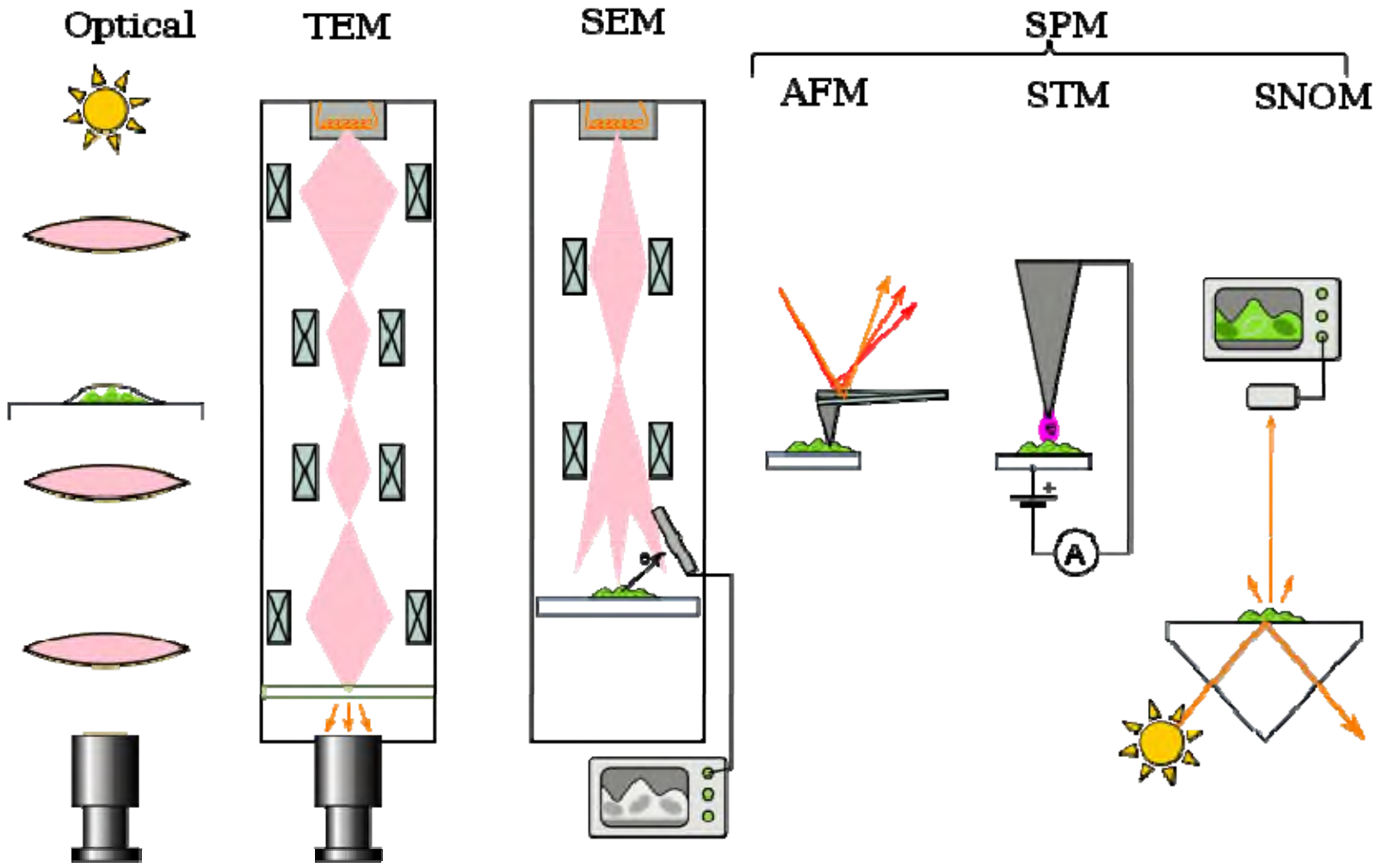
Brightfield, Darkfield, Polarized, Phase, DIC, Fluorescence

Introduction

- **How do we "see" small things?**
  - How small are "things"?
- **Why optical microscopic imaging?**
  - Potential for very high resolution (current limit: 80 nm)
  - Potential for no or minimal effects on sample
  - Can be performed using entirely endogenous sources of contrast or non-toxic exogenous chromophores
  - Can be adapted to sample/problem specifications
- **Applications:**
  - Basic molecular and cell biology studies
  - Understanding disease processes
  - Drug development screening and efficacy
  - Human disease diagnostics (cancer, diabetes, atherosclerosis)
  - Human therapeutics (Dosimetry, Response monitoring)

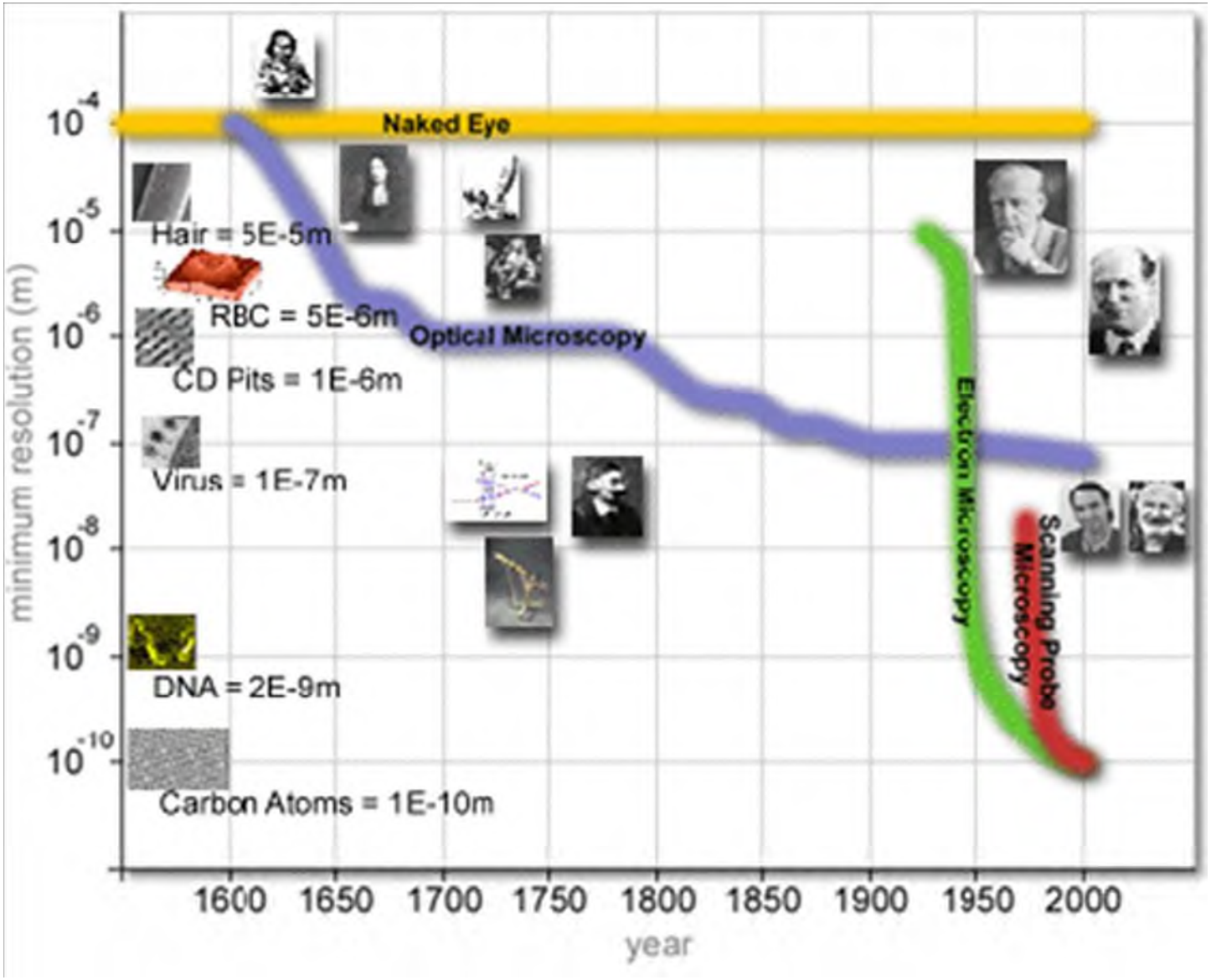


Microscopes



Microscope: Micro = Gk. *"small"* + skopien = Gk. *"to look at"*

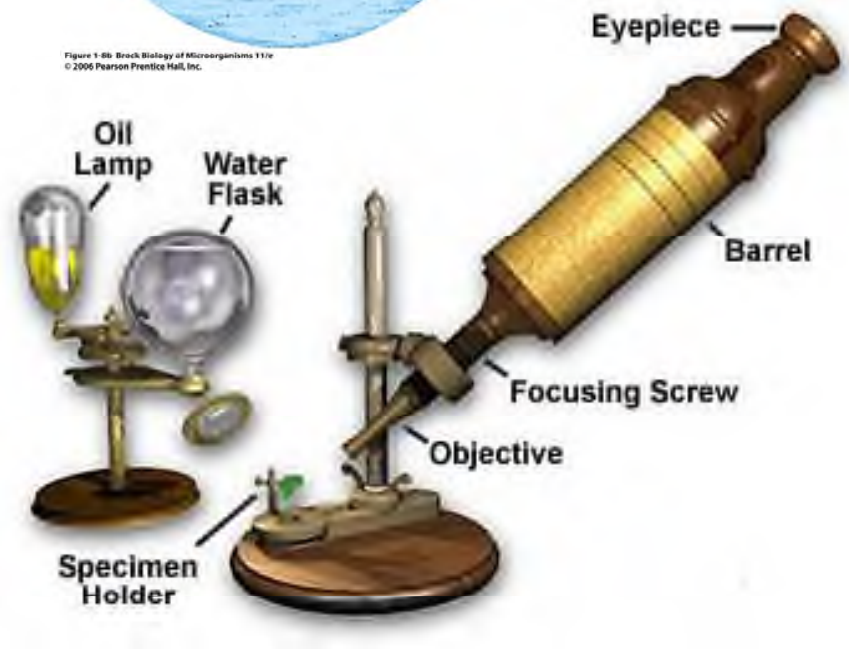
Microscopes



"Microscope" was first coined by members of the first "Academia dei Lincei" a scientific society which included Galileo

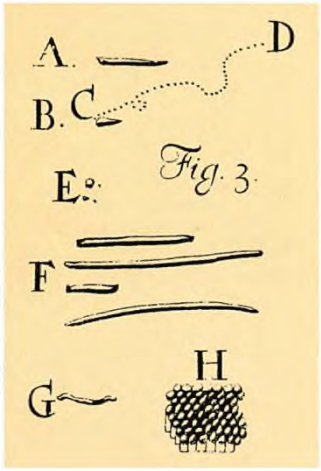
History: The First Description of Microorganisms

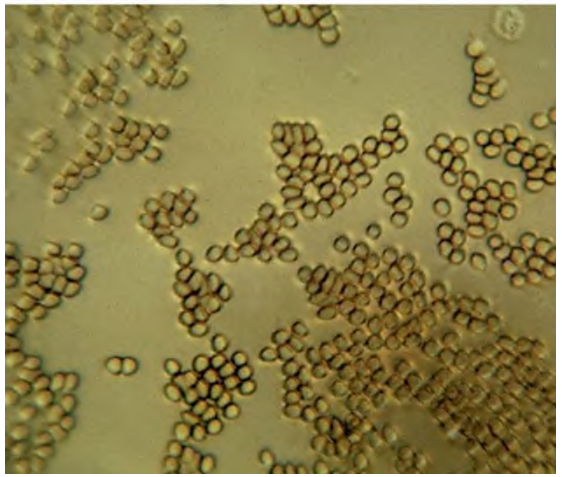
- **Robert Hooke**
  - Observed fruiting structures of molds in 1665 and was the first to describe microorganisms
  - Compound microscopes were mostly of poor quality and could only magnify up to 20-30 times.
    - Chromatic aberrations
    - Chester More Hall, a barrister, 1730s
      - Observed that flint glass (newly made glass) dispersed colors much more than “crown glass” (older glass)
      - Designed a system that used a concave lens next to a convex lens which could realign all the colors → the first achromatic lens.
  - Hooke claimed they were too difficult to use - his eyesight was poor.



History: The First Description of Microorganisms

- **Antoni van Leeuwenhoek**
  - He is incorrectly called "the inventor of the microscope"
  - Created a “simple” microscope with one ground lens that could magnify to about 275x
  - Published drawings of microorganisms in 1676
  - The field of microbiology was unable to develop until Leeuwenhoek constructed microscopes that allowed scientists to see organisms too small to be seen with the naked eye.



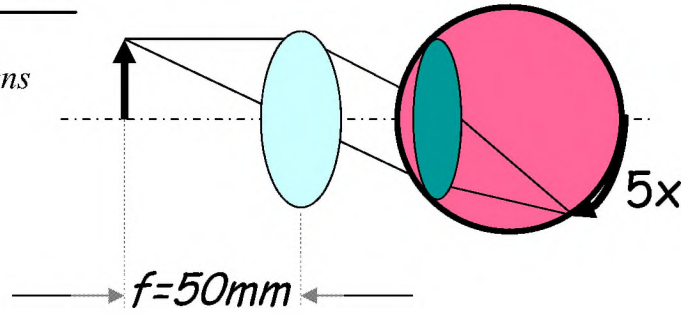


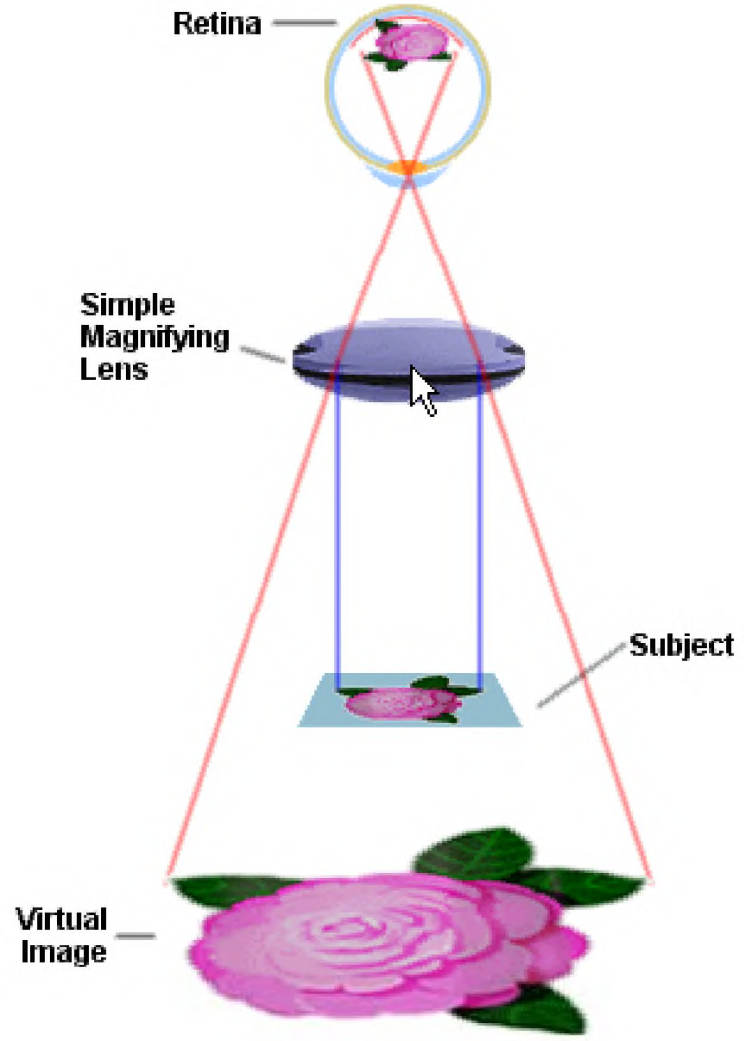
Simplest Microscope

- **Magnifying Glass**
  - Single lens magnifier makes the image appear larger.
  - Our brain processes the light as though coming in a straight line so the image appears larger

Example:

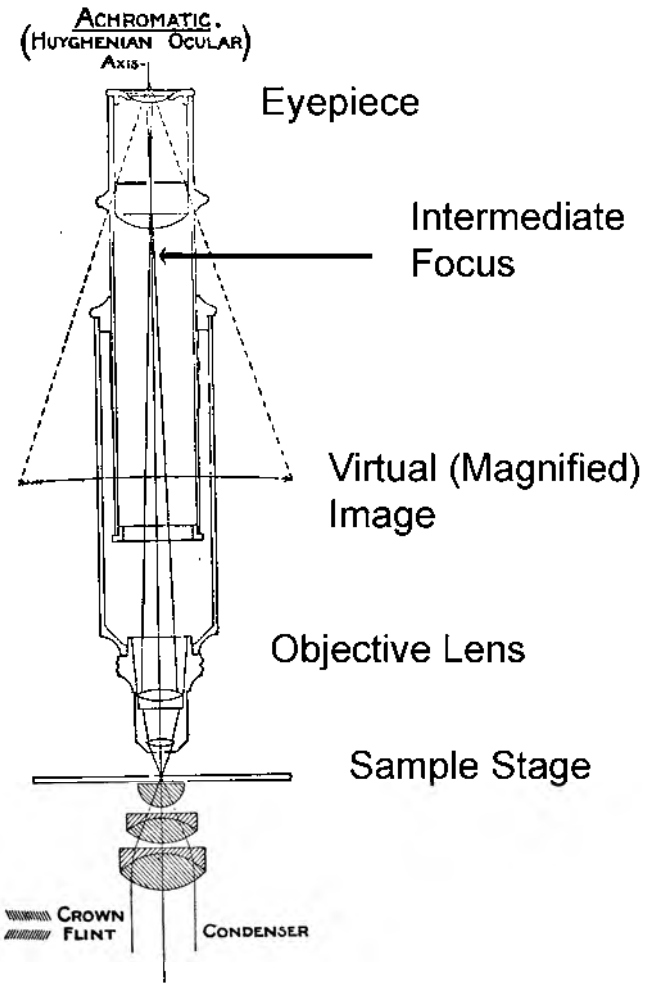
$$M = \frac{250mm}{f_{Lens}}$$





Compound Microscope

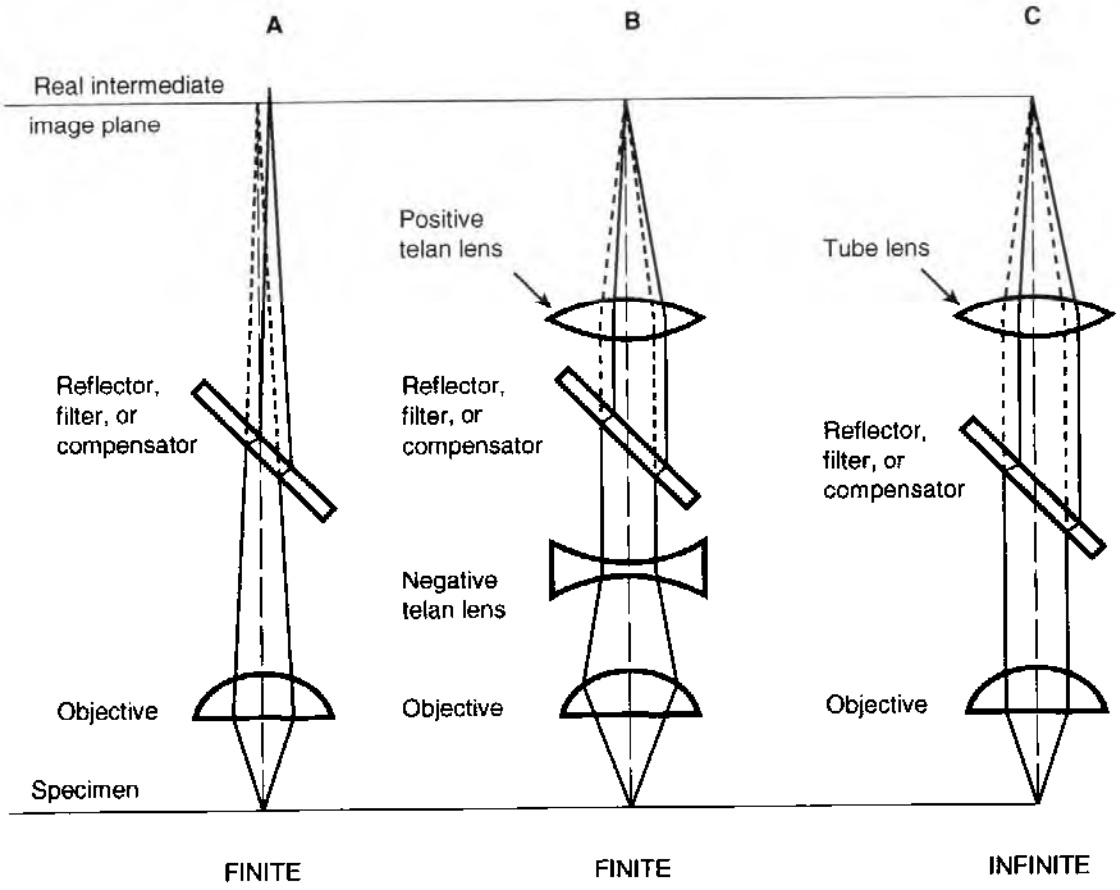
- **The compound microscope uses at least two lens systems**
  - The condenser
    - Provide illumination
    - Increase the resolution
  - The objective
    - Forms an intermediate real image of the sample at the objective tube length
    - Modern Objective lens
      - Multi-element lens
      - The number of lenses in a modern microscope can easily exceed 20.
  - The eyepiece
    - Forms a virtual image of that intermediate image to the retina of the eye
    - For a photodetector, use a projection lens to form a real image from the intermediate image



Adapted from Spitta, 3rd Edition

Compound Microscope

- **Current microscope objectives tend to be infinity corrected**
  - Parallel rays out of the objective
  - Infinite tube length
  - Require an additional lens in the tube to form the intermediate image
- **Advantages**
  - Objectives are simpler
  - Optical path is parallel through the microscope body
  - Elements inserted in the path do not affect the image



Benefits of infinity correction.
(A) Insertion of reflector or filter causes lateral and axial shift.
(B) Two telan lenses generate infinity space to eliminate the shift
(C) Objective directly provides infinity space

Compound Microscope

Optical image formation: Basic concepts

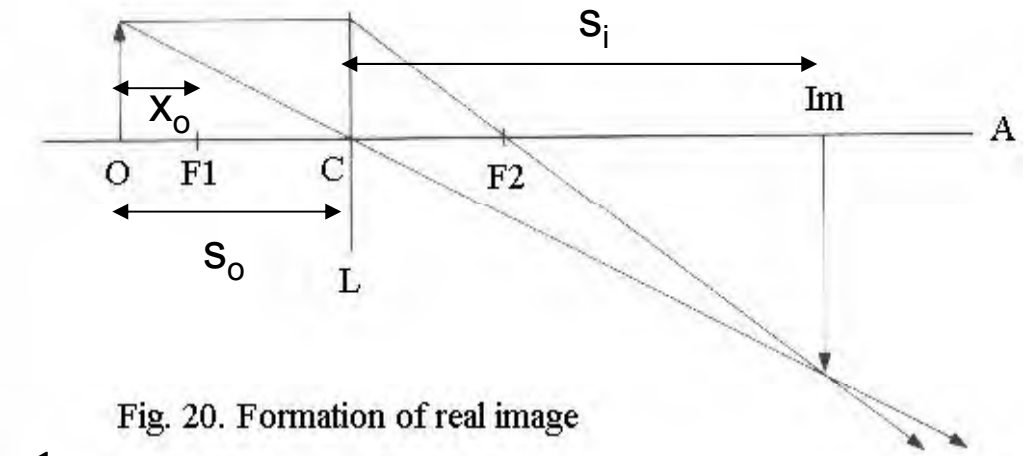


Fig. 20. Formation of real image

$$\frac{1}{f} = \frac{1}{s_o} + \frac{1}{s_i}$$

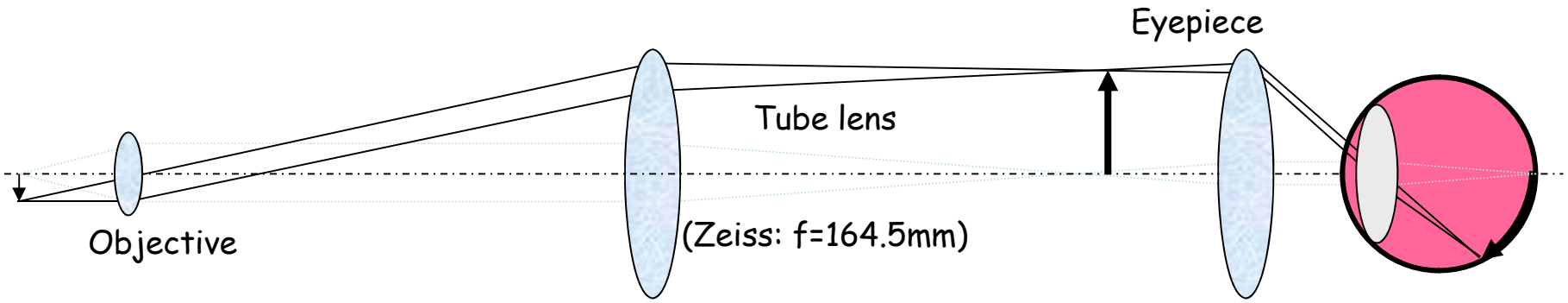
F=focal length
S_o=distance of object from principal plane of lens
S_i=distance of image from principal plane of lens

$$M = -\frac{s_i}{s_o} = -\frac{f}{x_0}$$

M=magnification
X_0=distance of object from focal back focal plane of lens
- sign because of inverted image

$$\frac{f}{s_0 - f} = \frac{|y_i|}{y_o} = \frac{f}{s_i - f}$$

Compound Microscope

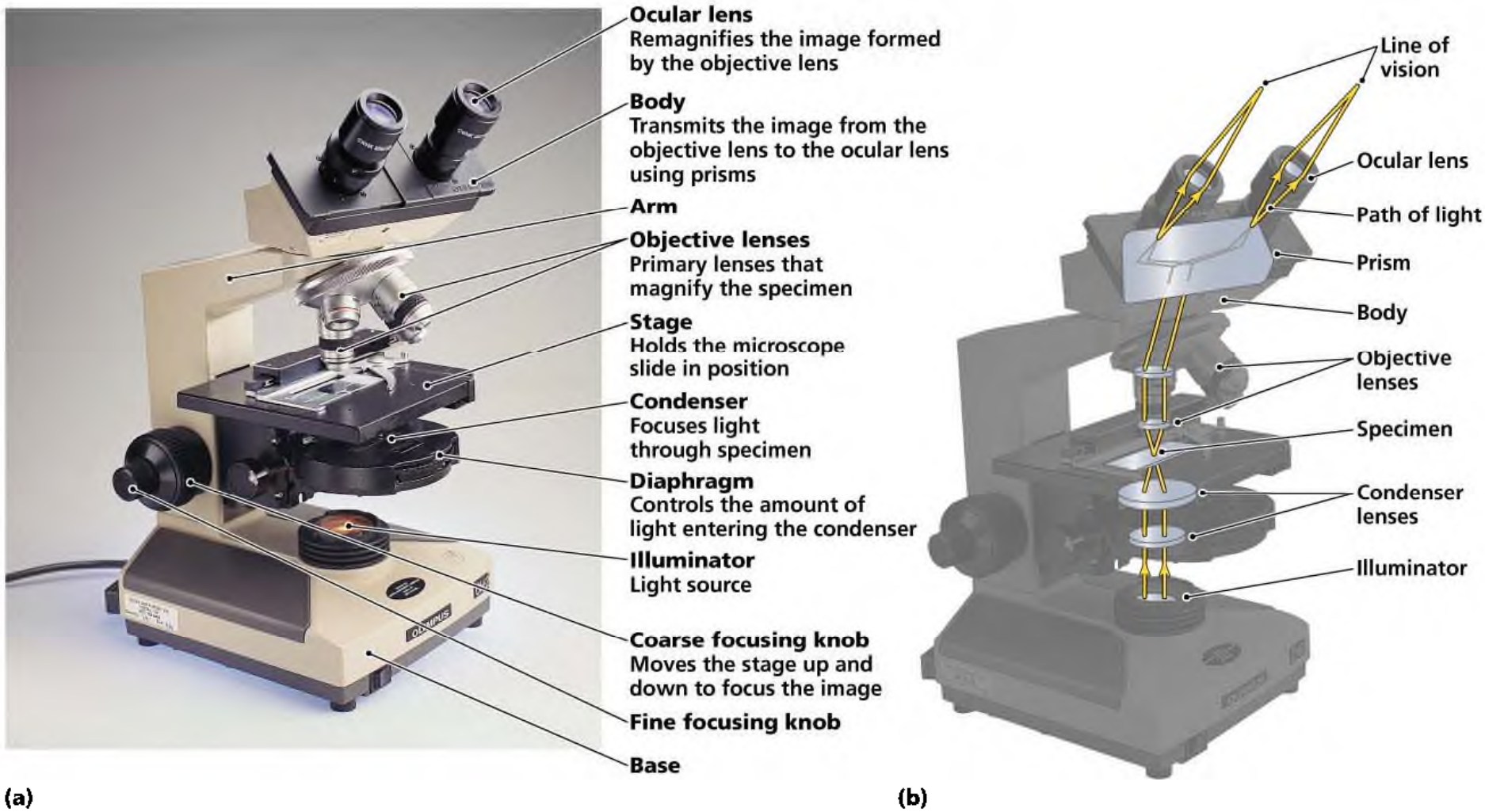


$$M = \frac{\cancel{250mm}}{f_{Objective}} \times \frac{f_{Tube}}{\cancel{250mm}} \times \frac{250mm}{f_{Eyepiece}}$$

$$M = \frac{f_{Tube}}{f_{Objective}} \times \frac{250mm}{f_{Eyepiece}}$$

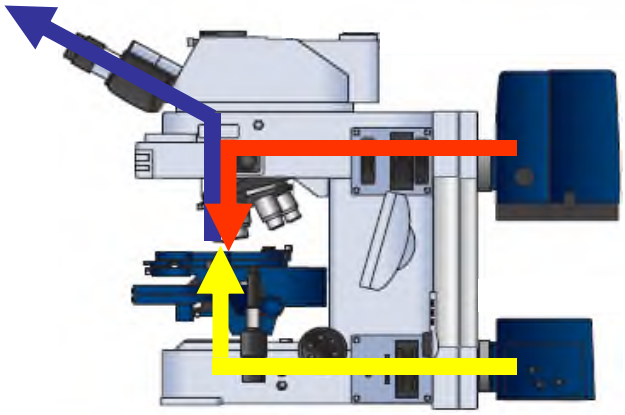
$$M_{Compound\ Microscope} = M_{Objective} \times M_{Eyepiece}$$

Compound Microscope

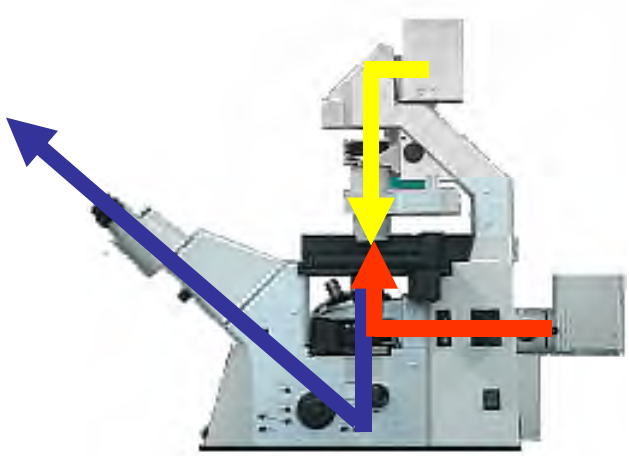


http://academic.pgcc.edu/~kroberts/Lecture/Chapter%204/04-04_CompoundLM_L.jpg

Compound Microscope



Upright microscope

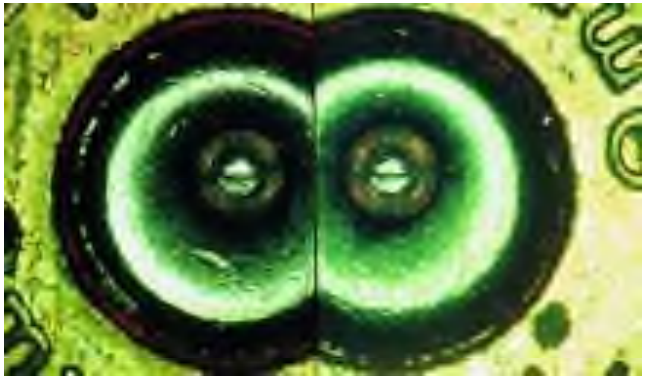


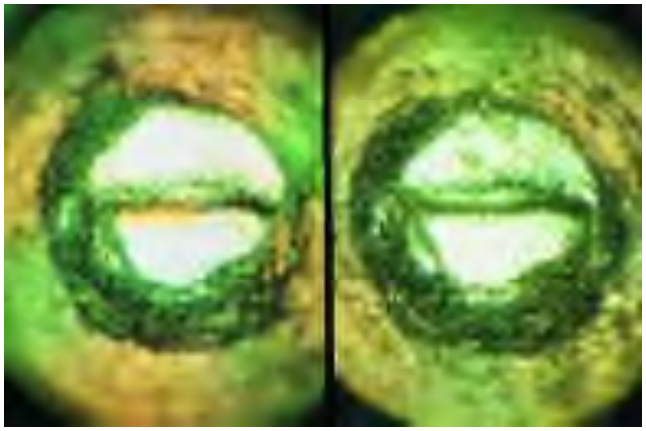
Inverted microscope

Stereo microscope

Compound Microscope

Comparison ("CSI") microscope

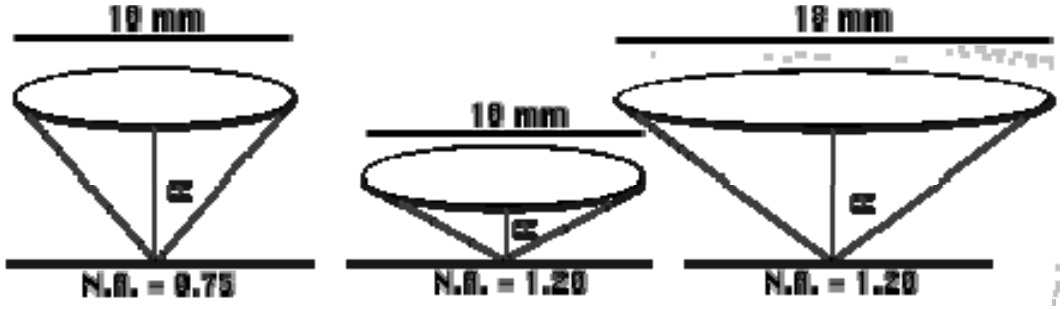


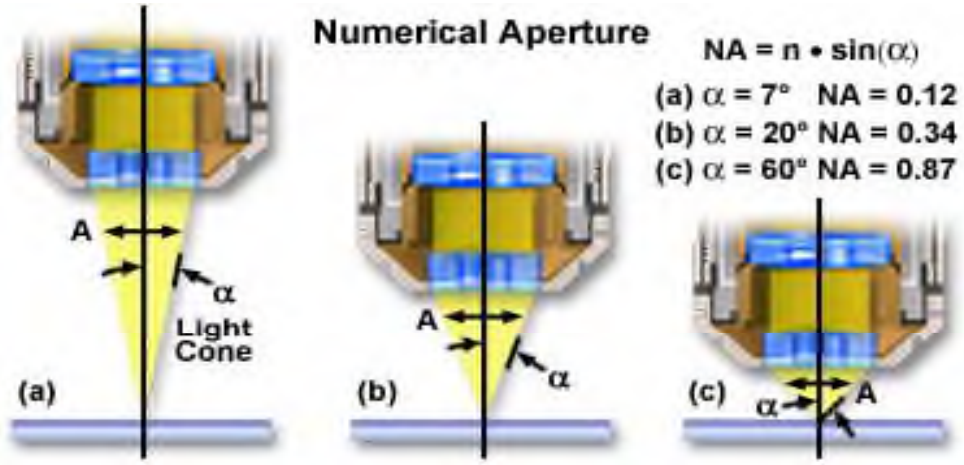


Split-image comparison of firing pin imprints in coaxial incident light

Optical Resolution

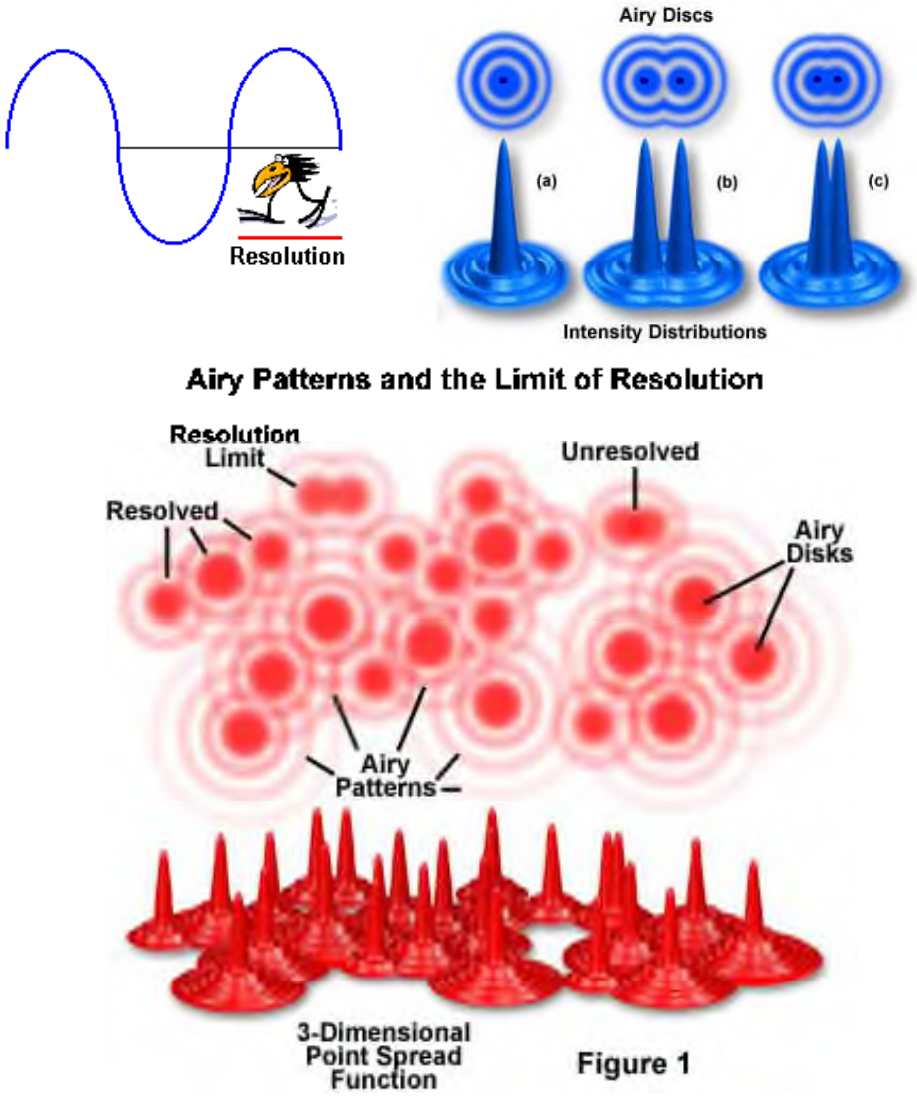
- **Numerical aperture (NA) of a lens**
  - A measure of its ability to gather light and resolve fine specimen detail at a fixed object distance
  - NA=n*sina
    - n=refractive index of medium
    - a=half angle of light collection cone
  - For fixed diameter → as magnification increases, the working distance, i.e. the distance from the edge of the objective to the sample, decreases





Optical Resolution

- **Resolution of an optical microscope**
  - The shortest distance between two points on a specimen that can still be distinguished by the observer or the camera as separate entities
- **Resolution of an ideal optical system**
  - Theoretically d = λ/2
  - Limited by the process of diffraction → formation of an Airy disk pattern when a beam of light is focused onto a spot



http://www.olympusfluoview.com/java/resolution3d/index.html

http://www.microscopyu.com/tutorials/java/imageformation/airyna/index.html

Optical Resolution

- **Point Spread Function**
  - Diffracted rays interfere
    - Either in a constructive or In a destructive way → interference rings.
  - The mathematical representation of this phenomenon is called the Point Spread Function (PSF)
  - The diffraction pattern of a point source of light.
    - Intensity profile of a diffraction spot
    - Central spot and surrounding rings
    - The separation distance between The center of the spot and the first minimum depends on the angular aperture of the lens.

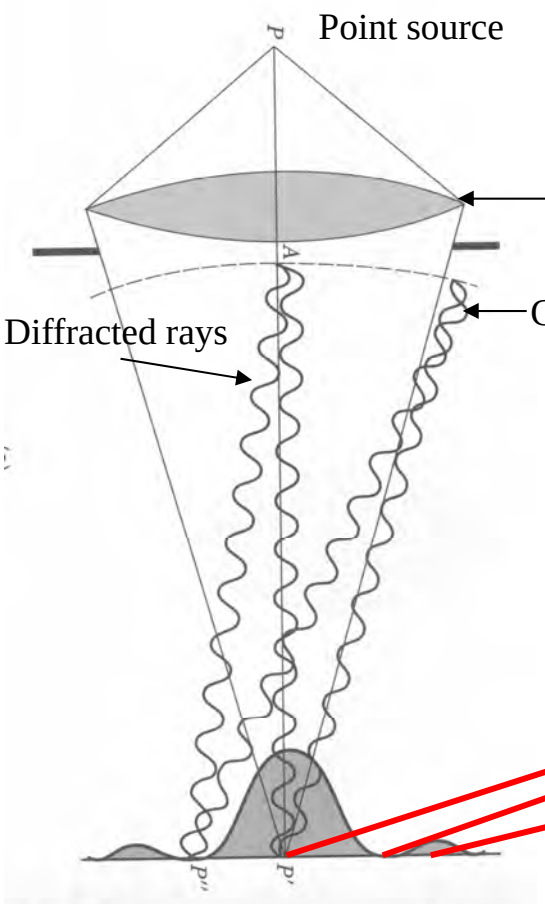
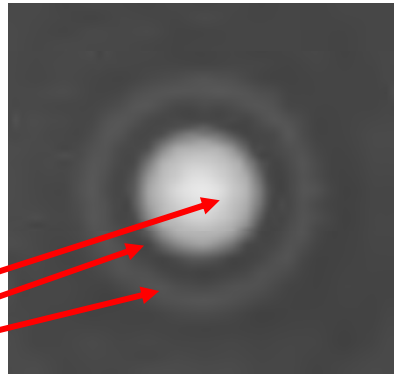
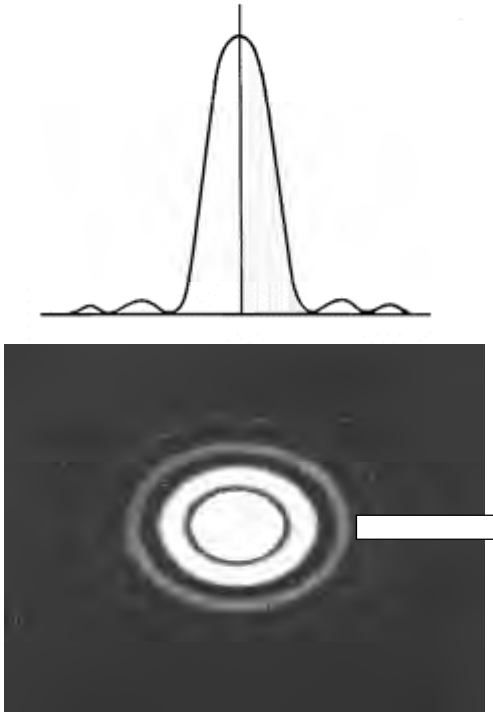


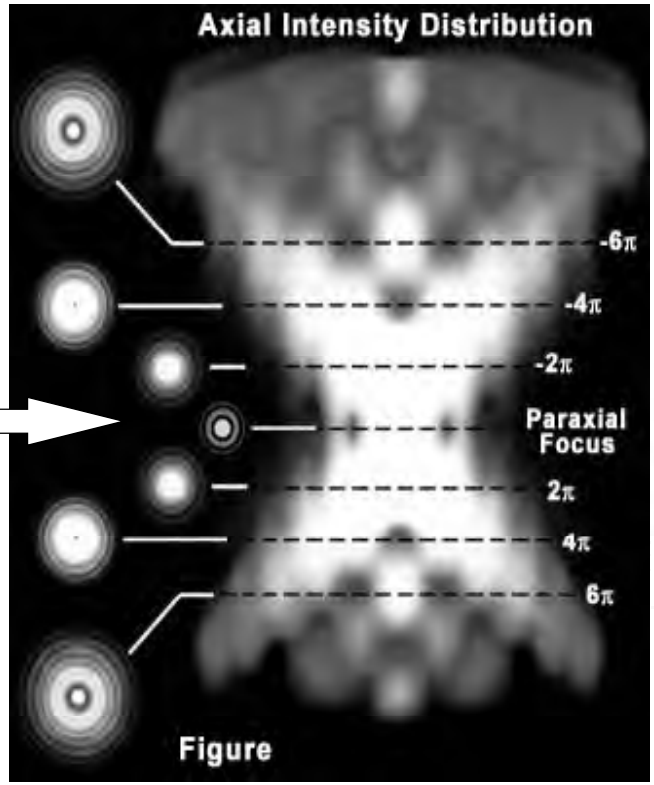
Image formation by an objective lens

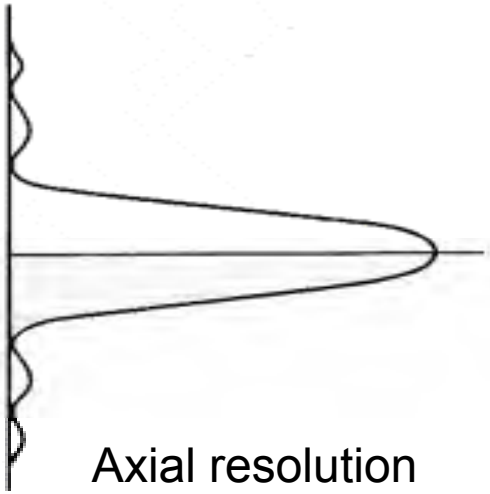


Optical Resolution

- **Airy disk in 2D and 3D**
  - Axial resolution is inversely proportional to the squared NA







Lateral resolution

$$d_{lat} = 1.22\frac{\lambda}{NA}$$

Axial resolution

$$d_{ax} = 4\frac{\lambda n}{(NA)^2}$$

Optical Resolution

- **Resolution**
  - The shorter the wavelength and the higher the NA the better the resolution
  - For standard light microscopy, diffraction limited resolution is on the order of 200 nm
- **How to improve?**
  - Larger NA (lenses, immersion fluid)
  - Shorter λ
- **Add a condensor**
  - D = 1.22 λ / (NAobj. + NAcond.)
  - So, for a 1.3 NA lens and condensor, D drops to ~250 nm
- **Examples**
  - 10 x, 0.3 NA
  - 60 x, 1.3 N.A.

$$d_{lat} = 1.22\frac{\lambda}{NA_{obj} + NA_{cond}} \quad d_{ax} = 4\frac{\lambda n}{\left(NA_{obj} + NA_{cond}\right)^2}$$

10 x, 0.3 NA objective at 530 nm light , 10x Eyepiece

$$M = M_{obj}M_{eyep} = 10x10 = 100$$

$$d_{lat} = 1.22\frac{530nm}{0.3+0.3} = 1.08\mu m$$

$$d_{ax} = 4\frac{530nm}{(0.3+0.3)^2} = 5.89\mu m$$

60 x, 1.3 N.A. objective at 530 nm light, 10x Eyepiece

$$M = M_{obj}M_{eyep} = 60x10 = 600$$

$$d_{lat} = 1.22\frac{530nm}{1.3+1.3} = 248.7nm$$

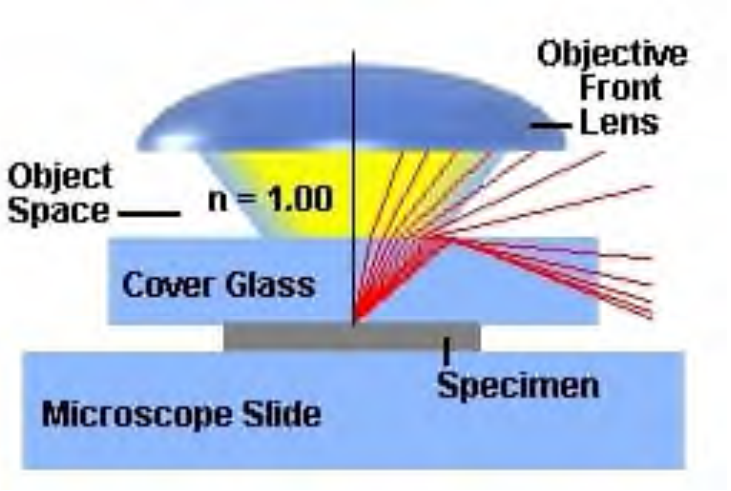
$$d_{ax} = 4\frac{530nm}{(1.3+1.3)^2} = 313.6nm$$

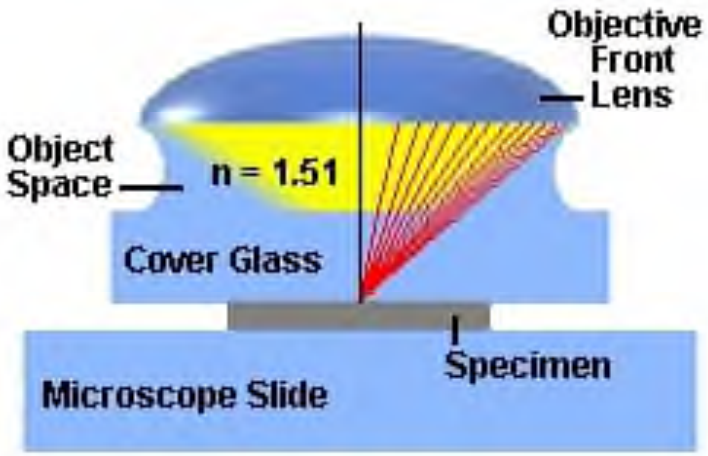
Objective Specifications

Figure 1

Objective Specifications

- **Oil immersion**
  - Required for large NA





Images reproduced from: http://micro.magnet.fsu.edu/
Please go to this site and do the tutorials

Objective Specifications

- **Aberrations**
  - Spherical aberration
    - Most severe
    - Immersion fluid
  - Field curvature
  - Chromatic aberration
  - Astigmatism, coma
  - http://micro.magnet.fsu.edu/primer/lightandcolor/opticalaberrations.html

Achromats	Most common Lowest price Poorly corrected, bad for demanding applications.
Fluorites or Semi Plan Apochromats	Mid-grade lenses, better correction, flat field.
Plan Apochromats	Best grade, most expensive (>$3,000 for some) , very well corrected.

Major Light Paths

- **Illumination**
  - Major goal: provide uniform sample illumination
- **Imaging**
  - Major goal: reproduce magnified sample image with minimal distortion, high light collection efficiency, and high resolution

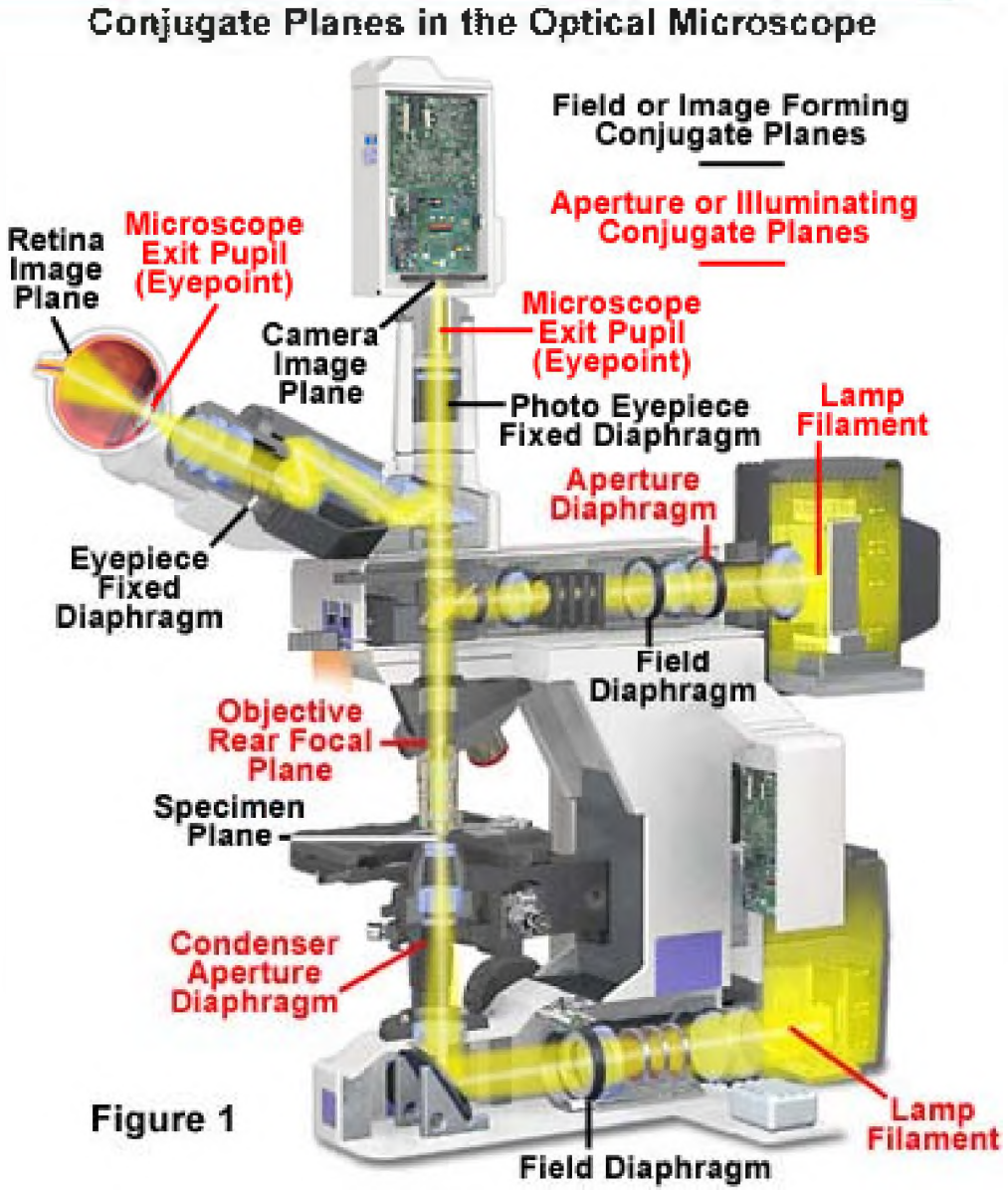
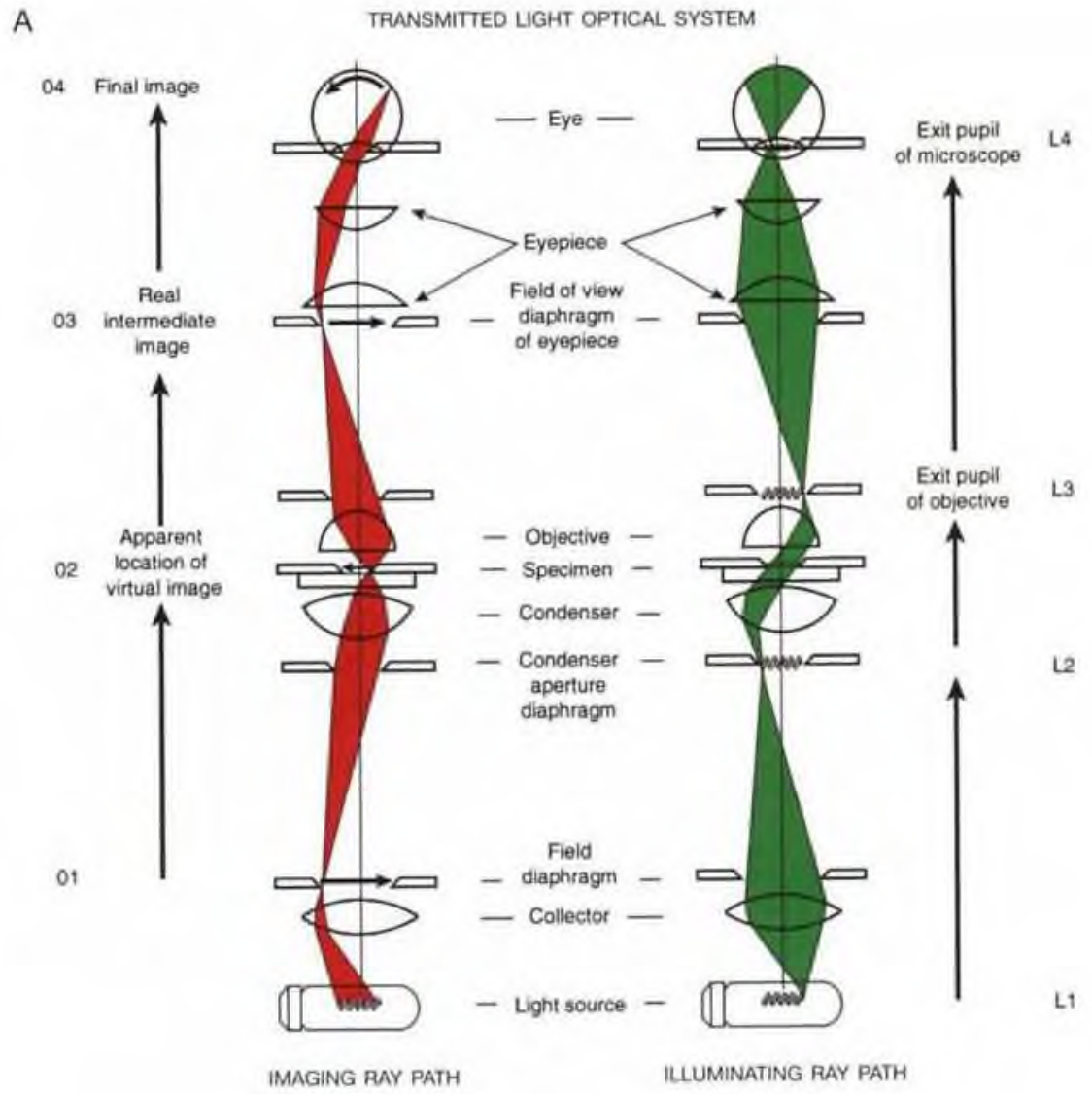


Figure 1

Major Light Paths

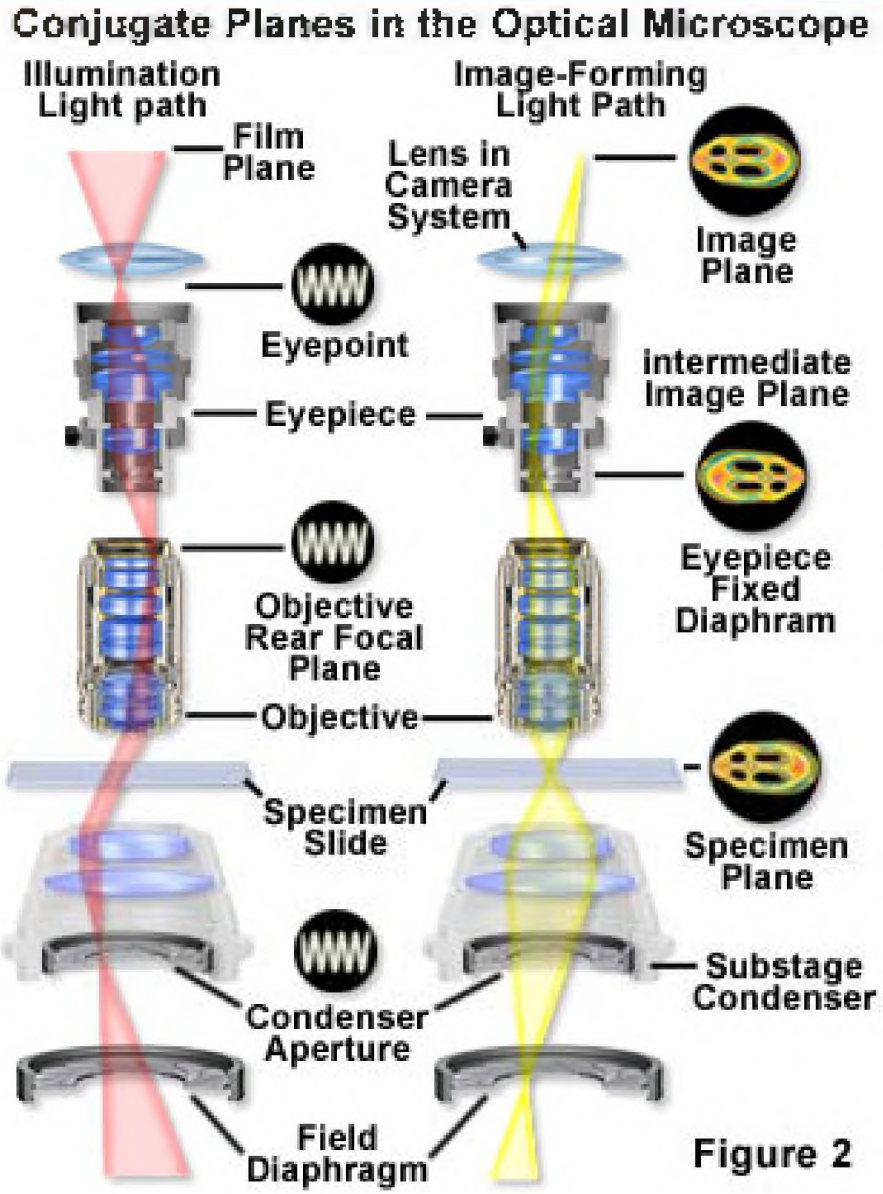
- **Köhler illumination**
  - Creates an evenly illuminated field of view while illuminating the specimen with a very wide cone of light
  - Two conjugate image planes are formed
    - One contains an image of the specimen
    - The other the filament from the light



Major Light Paths

- **Transillumination**

- **Condenser aperture**: will affect the numerical aperture of the condenser
- **Field diaphragm**: will affect size of field that is illuminated at the focal plane



Major Light Paths

- **Epi-illumination**

  - **Aperture diaphragm**: will affect the numerical aperture of the objective for illumination
  - **Field diaphragm**: will affect size of field that is illuminated at the focal plane

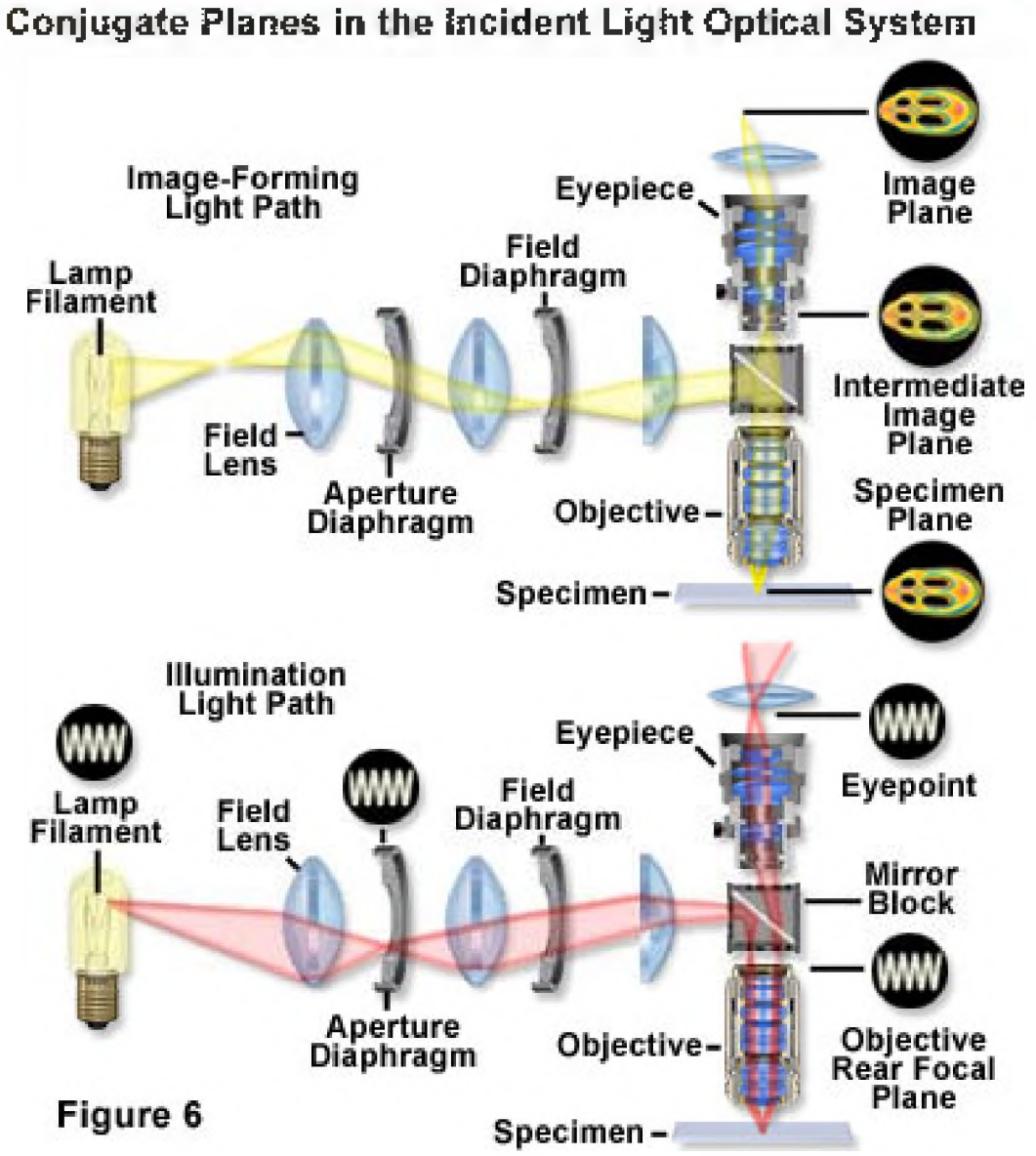


Figure 6

0 Units

50 Units

100 Units

50

50

50

CONTRAST

$$C = \frac{\text{Brightness of Specimen-Brightness of Background}}{\max(\text{Brightness of Specimen,Brightness of Background})}$$

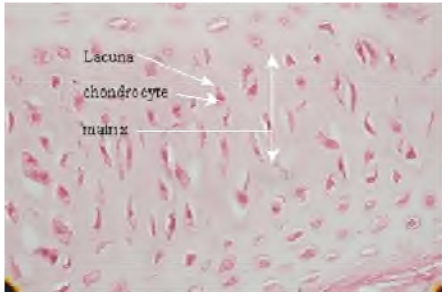
50 - 0 / 50 = 1

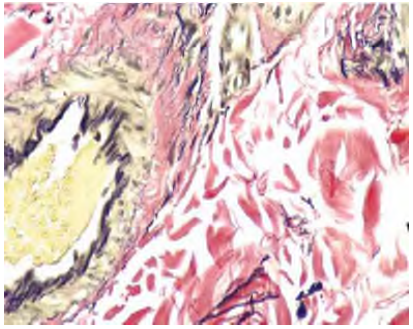
50 - 50 / 50 = 0

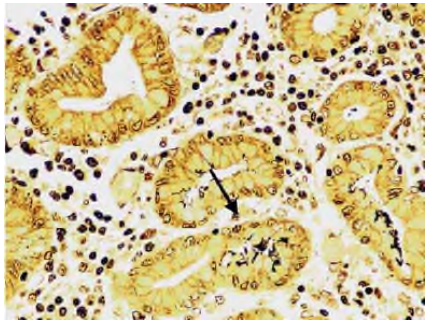
50 - 100 / 100 = -0.50

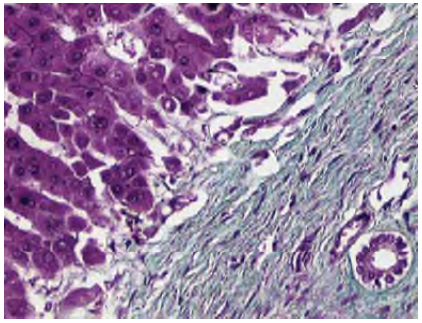
Contrast

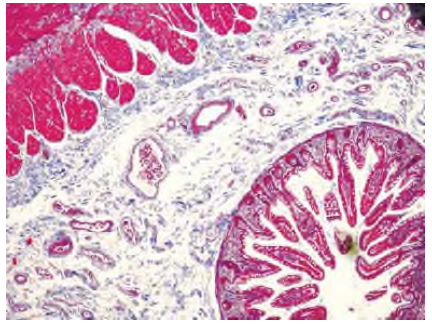
- **Ability to tell the difference between objects and background**
  - Specimen properties that produce
    - changes in brightness
    - color differences
  - Arise from
    - Light absorption, reflection, scattering, diffraction
    - Spatial variation in refractive index
    - Birefringence
    - Fluorescence and similar optical phenomena.
- **Can be improved using stains**
  - The sample has to be sacrificed, fixed, sectioned, and stained
  - Variety of stains → stain different cells/cellular components with different colors
  - Immunohistochemistry → antibody based staining









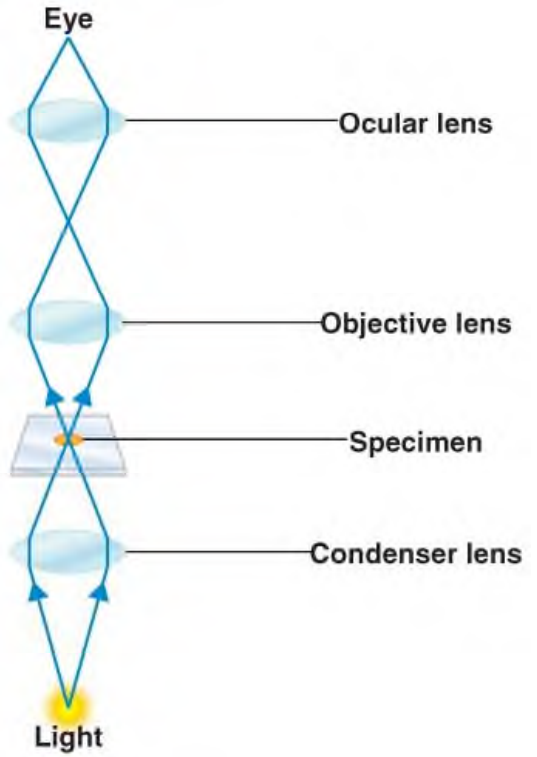


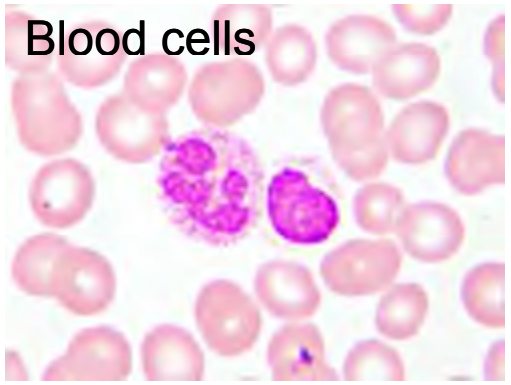
Contrast

- **Contrast can be enhanced by different illumination/imaging techniques**
  - Brightfield
  - Darkfield
  - Phase Contrast
  - Polarized Light
  - DIC (Differential Interference Contrast)
  - Fluorescence (and related techniques)

Brightfield Microscopy

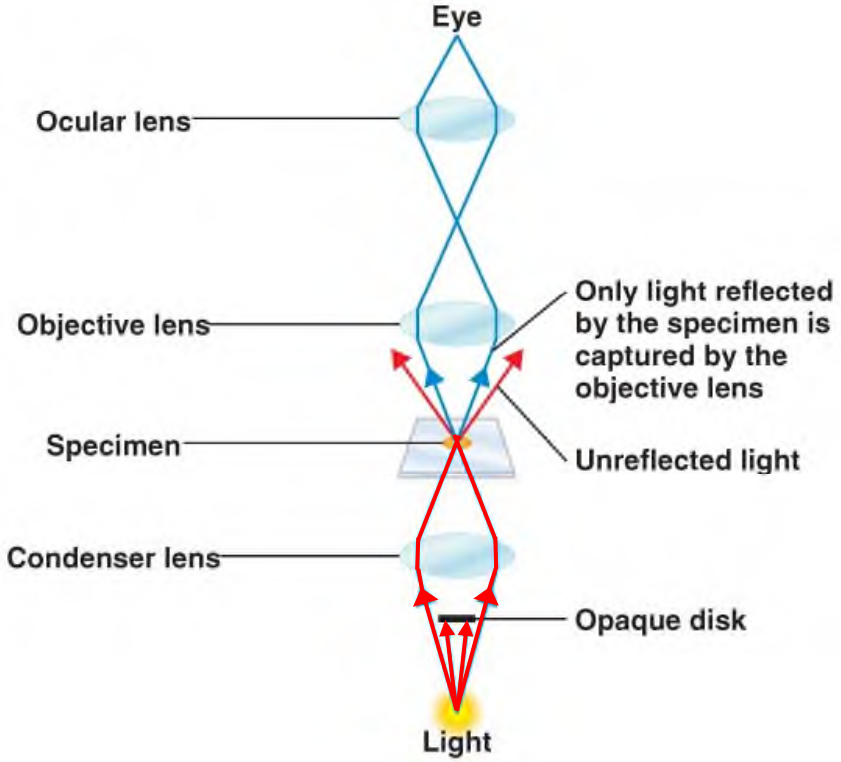
- **Simplest type of microscopy**
  - Light floods the objective, making the field bright
  - Objects absorb or deflect light out of the field, making the objects dark against the bright background.
- **Contrast provided mainly by absorption**
  - Biological specimens are not highly absorbing naturally
    - Use stains, which typically require fixation, i.e. cells no longer alive
- **Used routinely in histopathology and hematology and basic science studies for which looking at live specimen is not crucial**

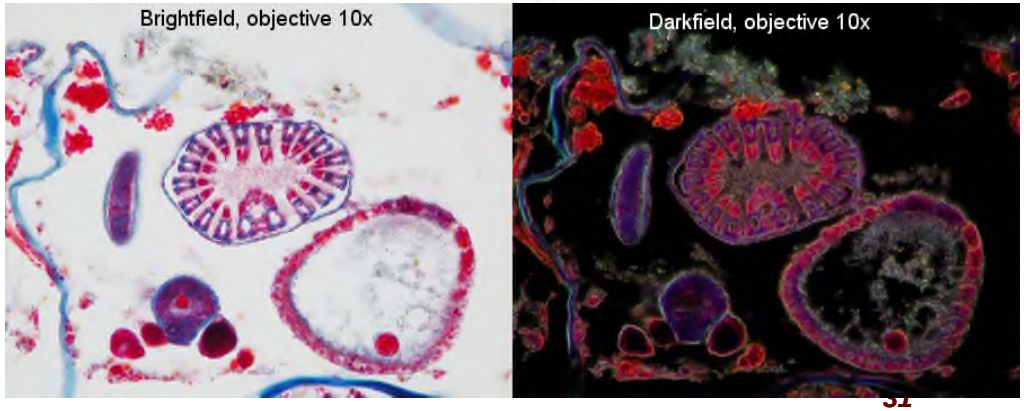




Darkfield Microscopy

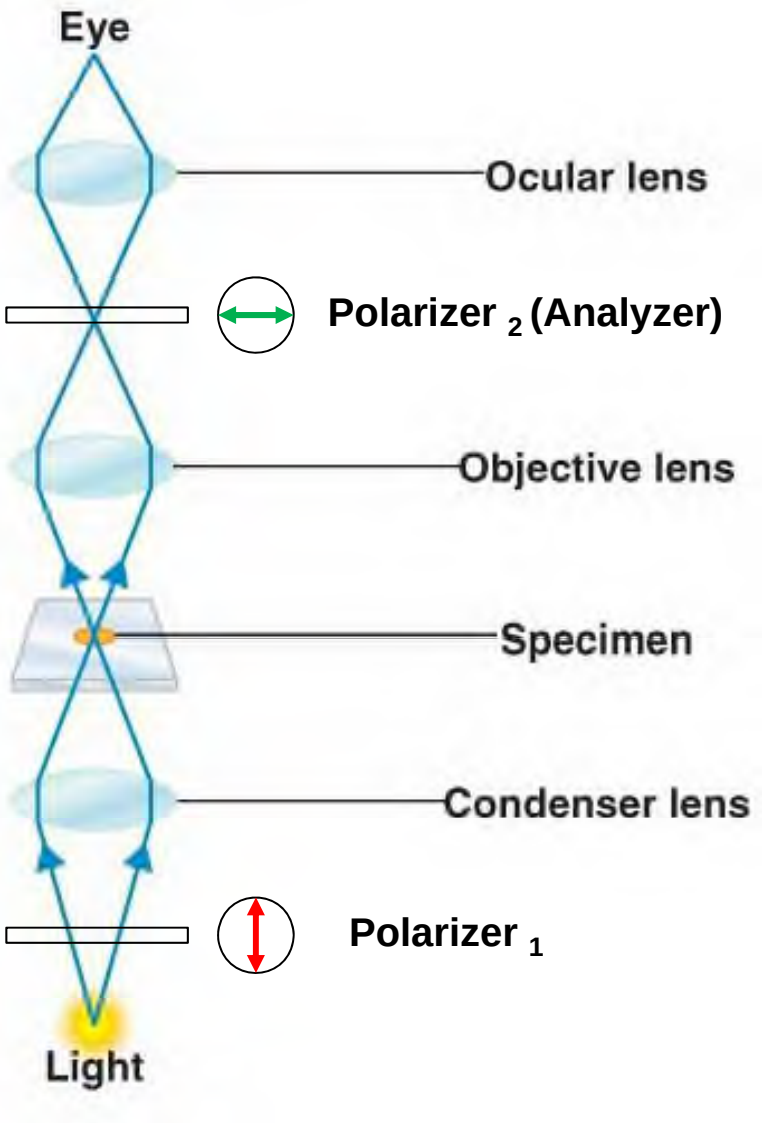
- **In Darkfield Illumination**
  - Light from outside the Field, does not normally enter the objective, making the field dark.
  - Light striking objects is displaced into the objective.
  - Objects appear bright against dark background → easier to see
- **Useful for examining**
  - Live organisms
  - Microorganisms which cannot be stained by standard methods
    - Treponema pallidum, the causative agent of syphilis





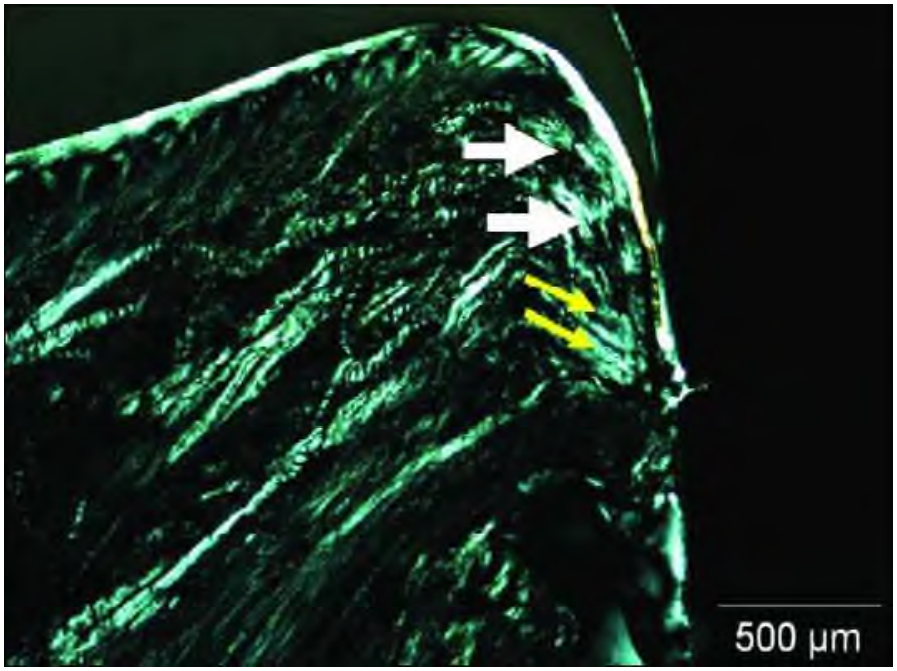
Polarized Light Microscopy

- **Specimen is placed between 2 crossed polarizers.**
  - When Polarizers are crossed, only items that rotate the plane of polarization reach the detector.
  - Only light produced by birefringent particles (e.g. crystals) or coming from the edges of particles (“edge birefringence”) is visible.
  - Looks sometimes like Darkfield
  - Wave plate adds color
- **Orientation-specific (linear Polarization)**



Polarized Light Microscopy

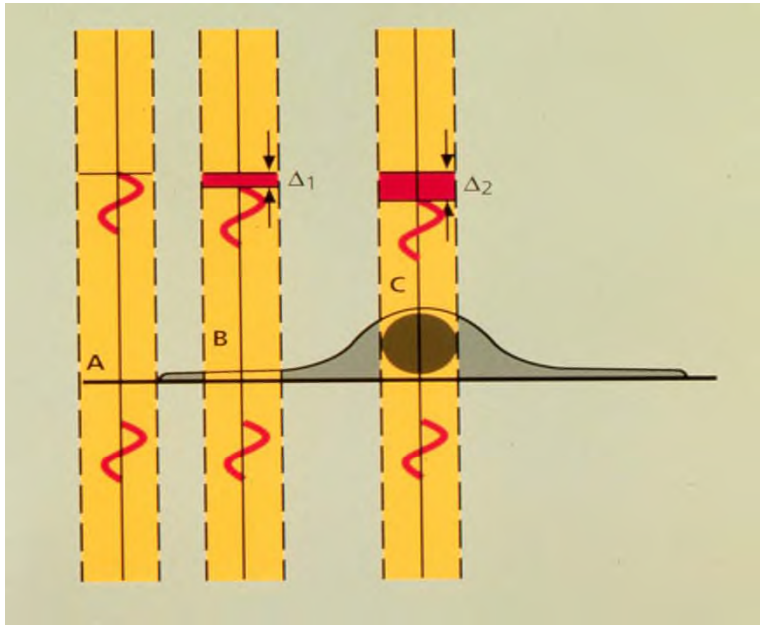
Color of sample and background modified by wave plate

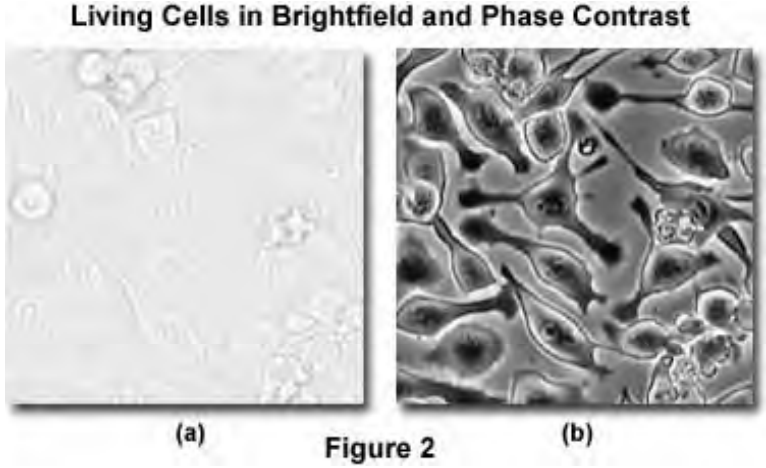


Photomicrografy under polarized microscopy. Parallel collagen fibers between the implant surface (white arrows). Oblique fibers in direction to bone crest (yellow arrows). Bar - 500µm

Phase Contrast Microscopy

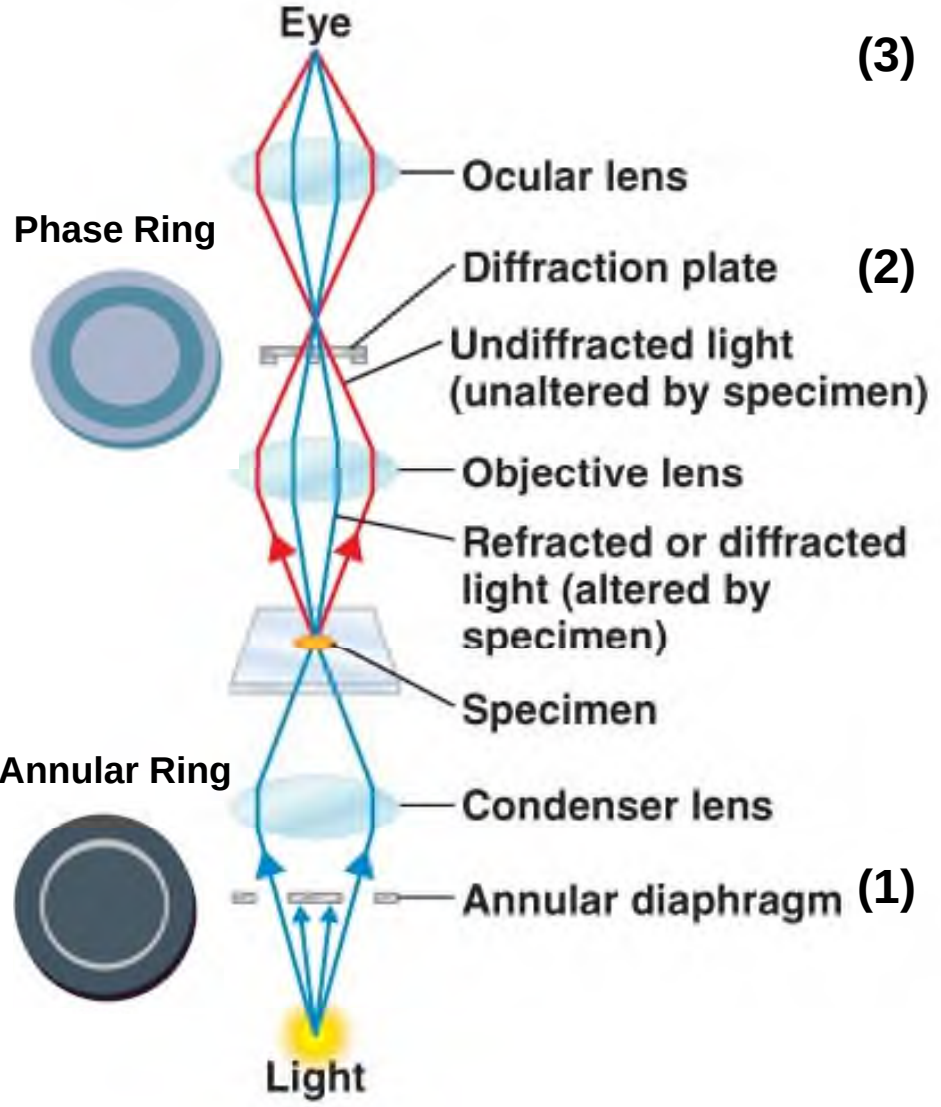
- **First microscopic method which allowed visualization of live cells in action**
  - Nobel prize in physics was awarded to Frits Zernike in 1953 for its discovery
  - It enhances contrast in transparent and colorless objects by influencing the optical path of light
  - It uses the fact that light passing through the specimen travels slower than the undisturbed light beam, i.e. its phase is shifted





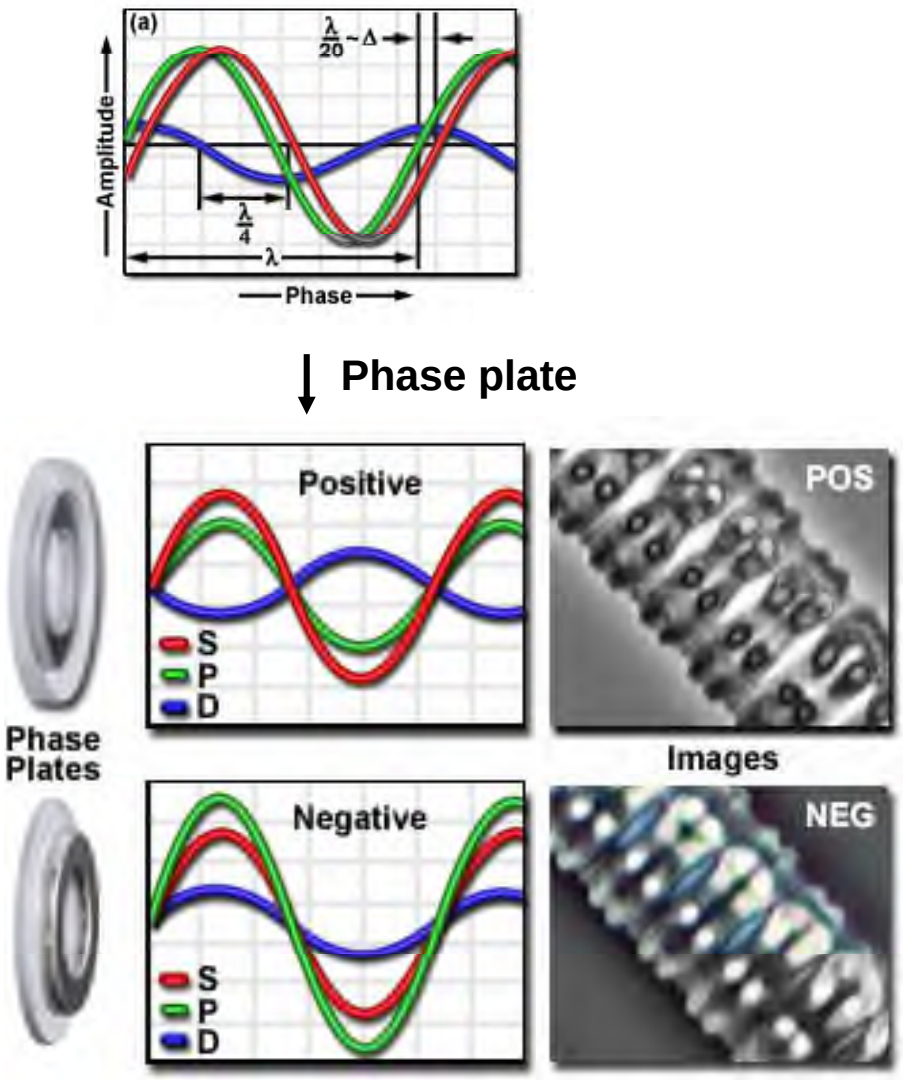
Phase Contrast Microscopy

- **Illumination from Condenser Phase Ring (“0” Order) → meets phase ring of objective (1)**
- **Objective Phase Ring (2)**
  - a) attenuates the non-diffracted 0th Order (red)
  - b) shifts it ¼ wave forward
- **Affected rays from specimen (blue)**
  - Expressed by the higher diffraction orders
  - Do not pass through phase ring of objective >¼ wave retarded
- **Non-diffracted and diffracted light are focused via tube lens into intermediate image (3)**
  - Interfere with each other
  - ¼+¼= ½ wave shift
  - Causes destructive interference i.e. specimen detail appears dark



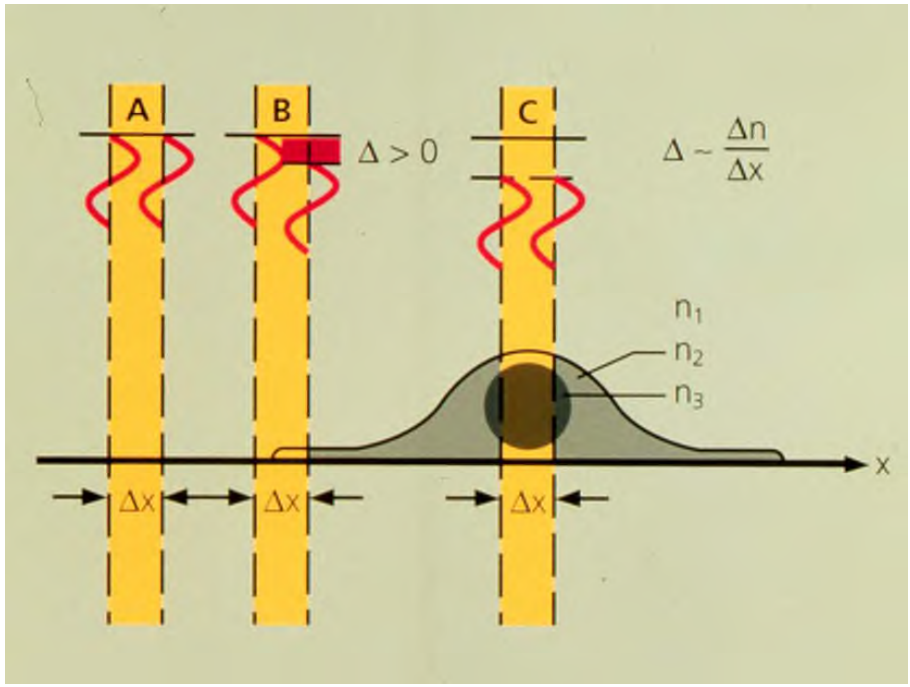
Phase Contrast Microscopy

- **S (red) be light passing through medium surrounding sample**
- **D (blue) light interacting with specimen.**
- **S and D typically interfere to yield P (green)**
  - What we can usually detect.
- **P will be phase shifted compared to S**
  - Our eyes cannot detect phase shifts.
- **Phase contrast microscopy effectively converts this phase shift into an intensity difference we can detect**



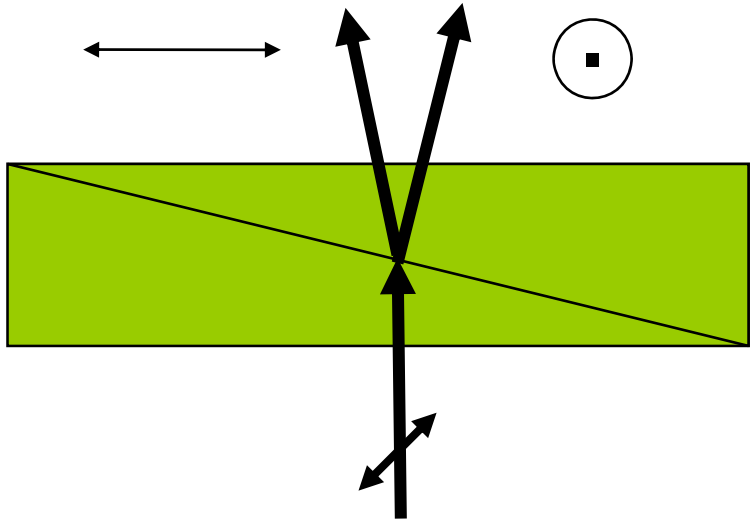
Differential Interference Contrast (DIC)

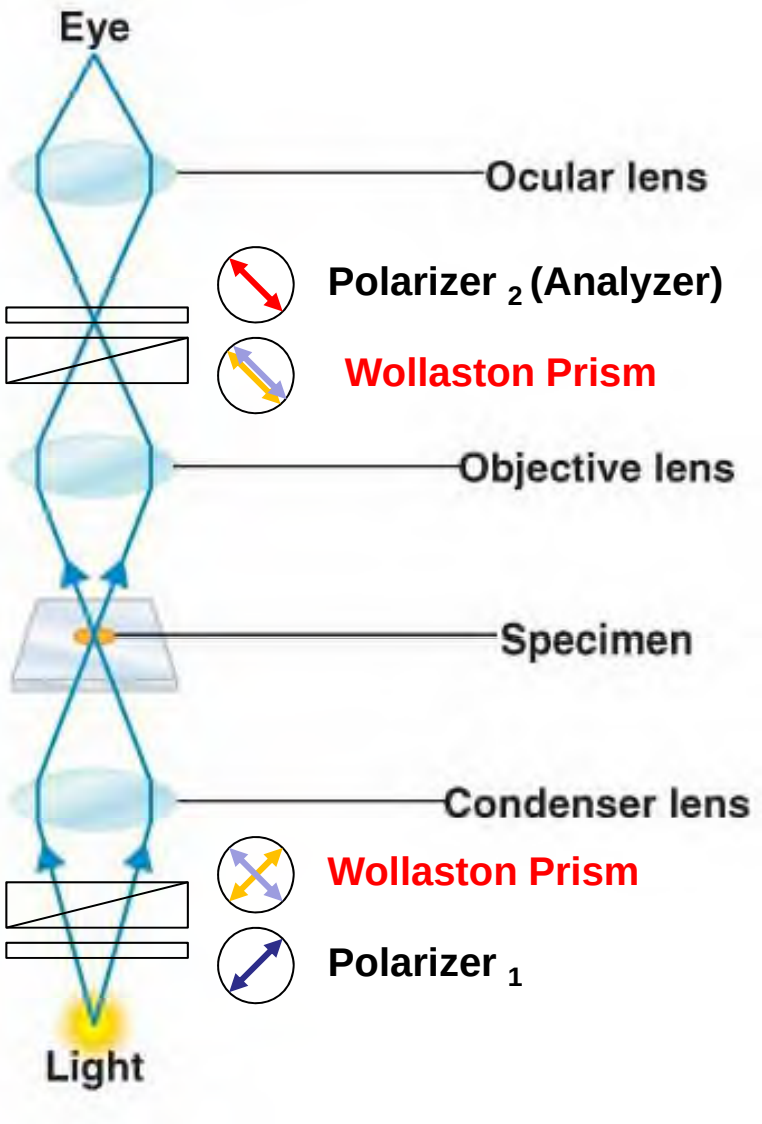
- **Changes phase GRADIENTS across different parts of a specimen into brightness differences**
  - 3-D Image appearance
  - Color DIC by adding a wave plate
  - Orientation-specific > orient fine detail perpendicular to DIC prism
- **Live, unstained speciments**
- **High Contrast and high resolution**
  - Doesn't suffer from some artifacts seen in phase contrast
  - Uses full NA of objective



Differential Interference Contrast (DIC)

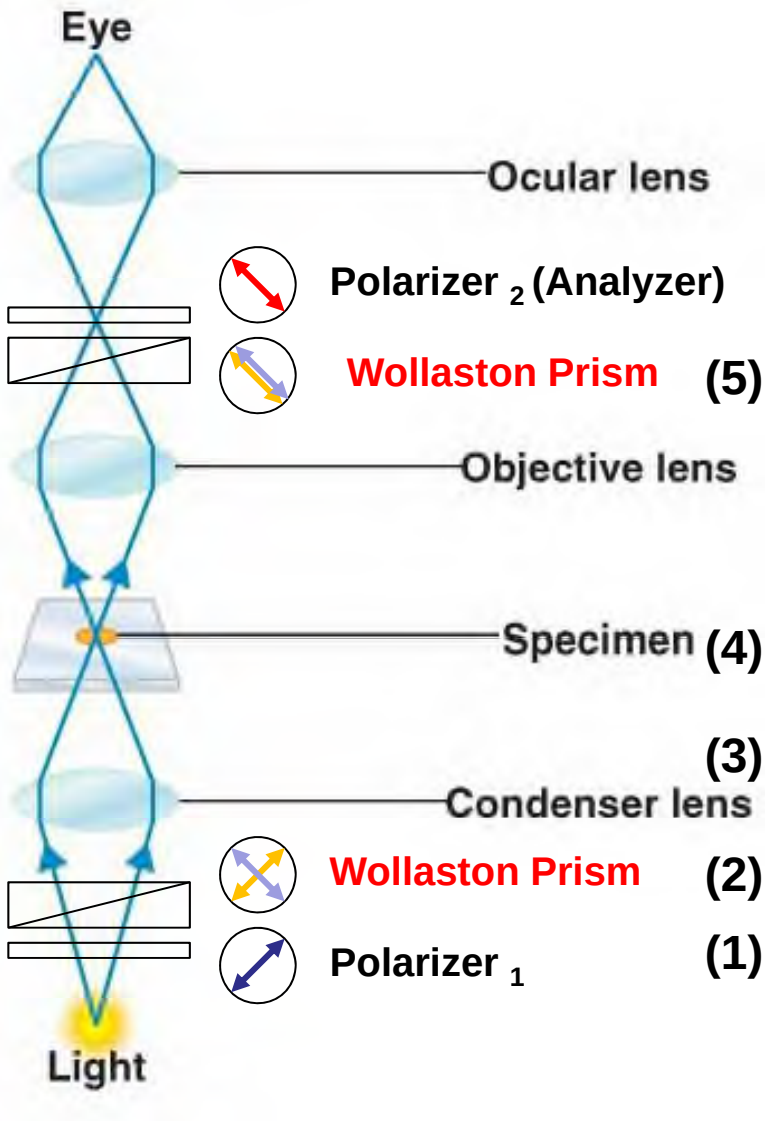
- **Nomarski-modified Wollaston prism**
  - Polarized beam, under 45˚ to prism, gets split into “ordinary” and “extraordinary” beam





Differential Interference Contrast (DIC)

- **Light Path**
  1. Unpolarised light → polarised at 45°.
  2. First prism → separated into two rays polarised at 90° to each other
  3. Condenser → focuse with a separation of around 0.2 µm apart (similar to the resolution of the microscope)
  4. Through adjacent areas of the sample → different optical path lengths where the areas differ in refractive index or thickness → change in relative phase
     - Many pairs of rays → an image of the sample carried by both the 0° and 90° polarised light
     - Like bright field images of the sample, slightly offset from each other
  5. Second prism → rays recombined into one polarised at 135° → interference → brightening or darkening the image at that point according to the optical path difference.
     - Can adjust so that 0 phase difference cancels
     - Wave plate adds color



Differential Interference Contrast (DIC)

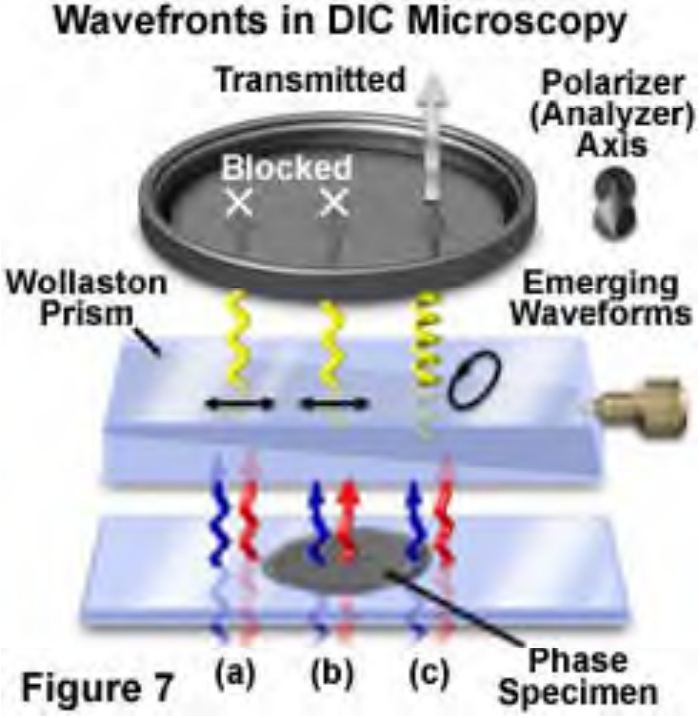


Figure 7

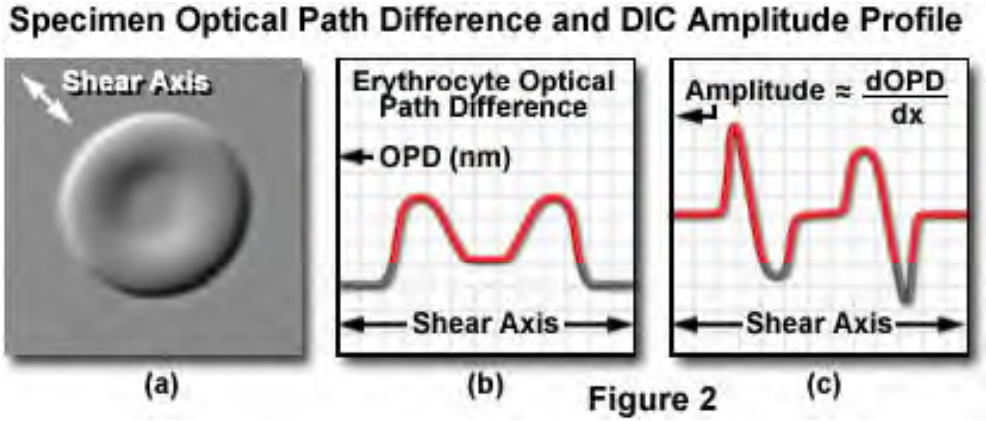
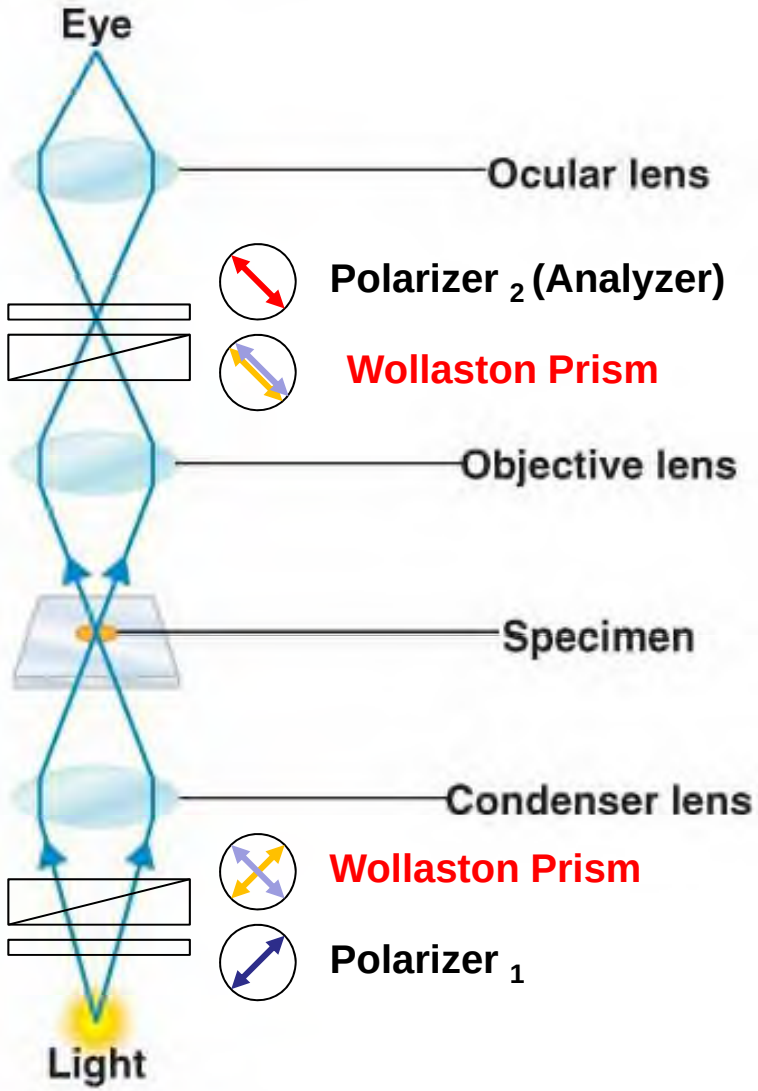


Figure 2



Differential Interference Contrast (DIC)

The 3T3 cell line is an important fibroblast culture, widely utilized in laboratory research, which was established from disaggregated tissue of an albino Swiss mouse. The fact that 3T3 cells could apparently grow indefinitely, while being unable to instigate tumor growth, helped scientists delineate for the first time the differences between cell mortality and a cell's ability to undergo oncogenic transformation.

http://www.microscopyu.com/moviegallery/livecellimaging/3t3/index.html

Phase-Contrast and DIC Comparison

Phase-Contrast

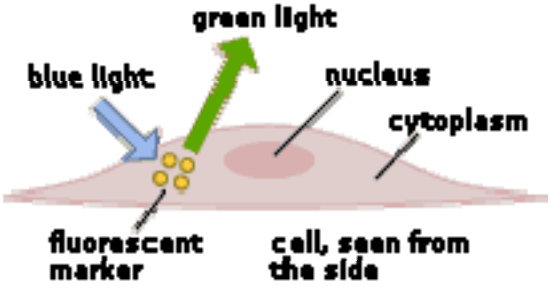
- Uses wave nature of light
- One set of light rays are direct and one set are reflected
- Makes detailed images of internal structure of living microorganisms possible
- Image in greyscale

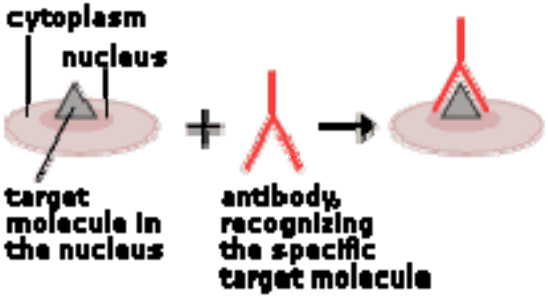
DIC

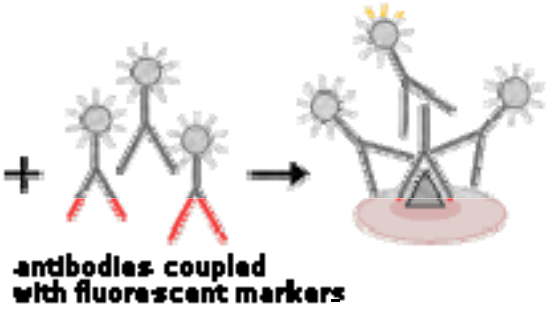
- Uses differences in refractive indices
- Uses 2 beams of light
- Resolution higher
- Brightly colored image
- Image appears nearly three-dimensional

Fluorescence Microscopy

- **Advantages of fluorescence**
  - Highly sensitive method
  - Simple implementation
  - Highly sophisticated fluorescent probes (fluorophores)
- **Fluorophores**
  - Fluorescent dyes that accumulate in different cellular compartments or are sensitive to pH, ion gradients
  - Fluorescently tagged antibodies to specific cell features
  - Endogenously expressed fluorescent proteins
    - Really endogenous
      - NADH/FAD: enzymes involved in ATP production
      - structural proteins: collagen/elastin
      - amino-acids: tryptophan/tyrosine
    - After gene modification
      - Green fluorescent protein and variants
- **Methods**
  - All fluorescence methods can be done (FLIM, FRAP, FRET, TIRF, etc)

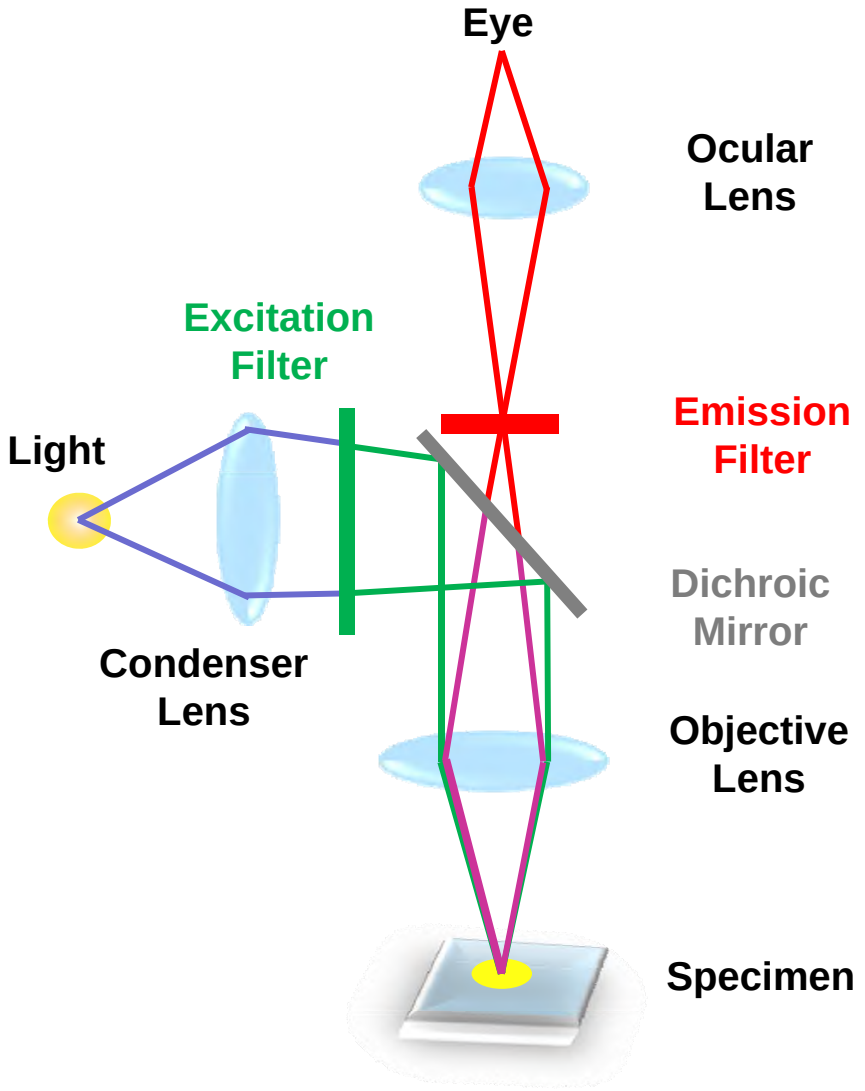






Fluorescence Microscopy

- **Light Path**
  - Light source through condenser passes through an excitation filter → narrow excitation band
  - Dichroic mirror directs excitation to the sample
  - Reflection and fluorescence from the sample
  - Dichroic mirror only passes fluorescence
  - Emission Filter → emission band



Fluorescence Microscopy

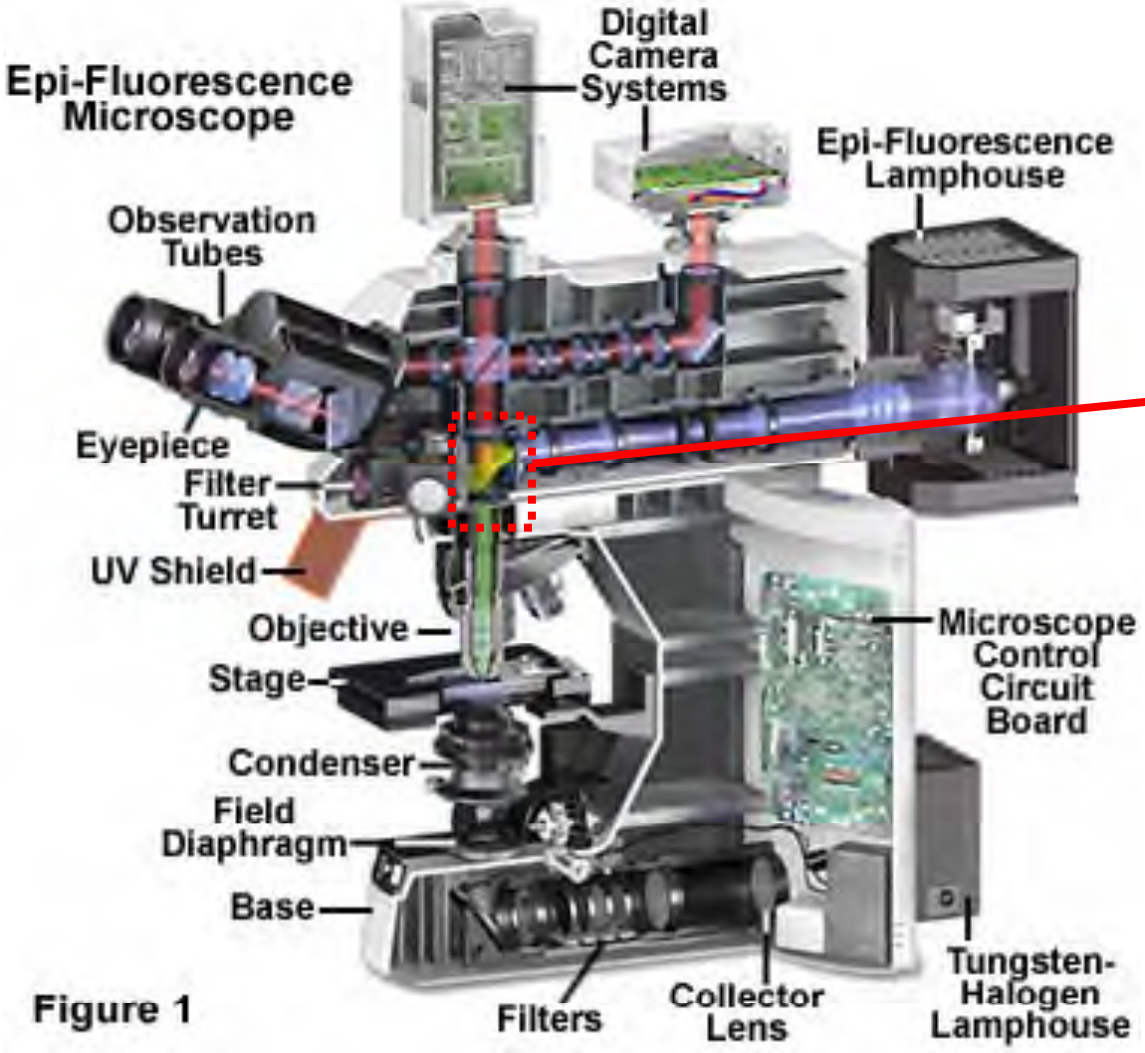


Figure 1

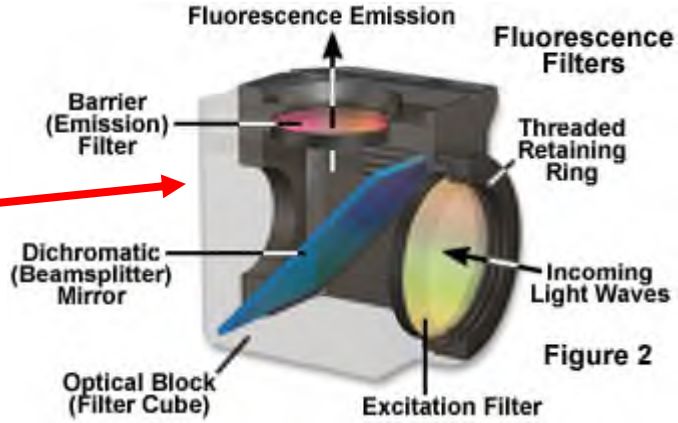


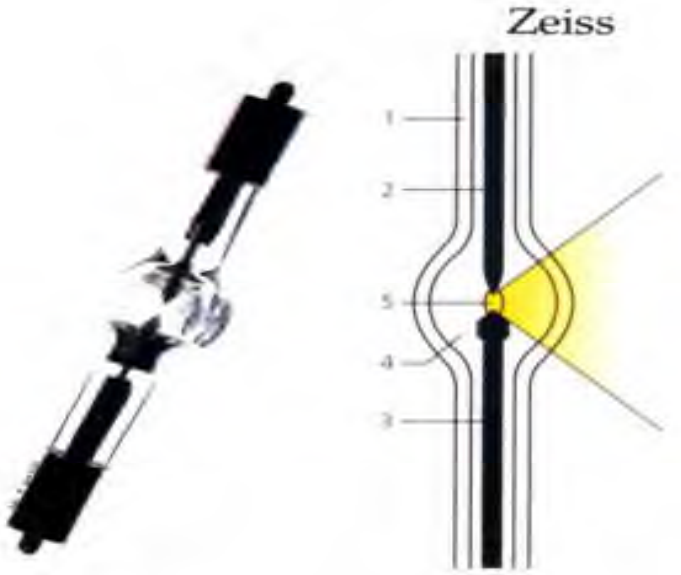
Figure 2

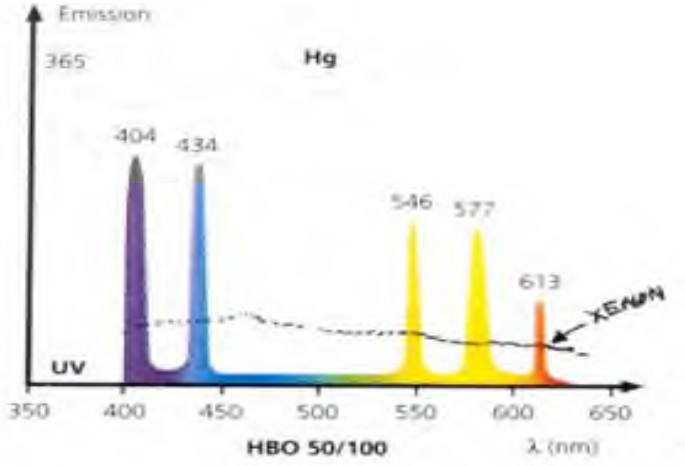
Dichroic filter: reflects excitation and transmits fluorescence

http://www.microscopyu.com/articles/fluorescence/fluorescenceintro.html

Fluorescence Microscopy

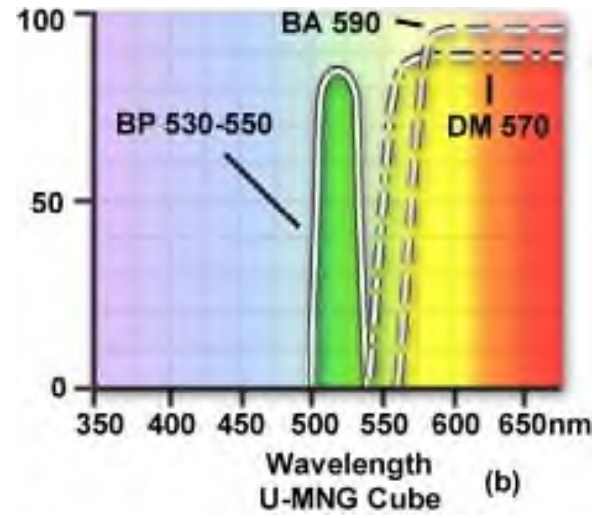
- **Common non-laser light sources**
  - Arc lamps (Mercury and Xenon)
- **Aligning the light source**
  - The epi fluorescence microscope is a reflected light microscope
    - The arc of the lamp imaged at the back focal plane of the objective
    - Ideally just filling the back aperature (Koehler illumination)

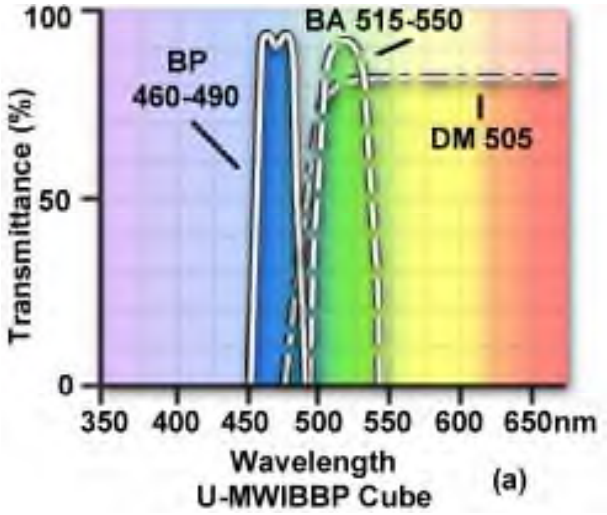




Fluorescence Microscopy

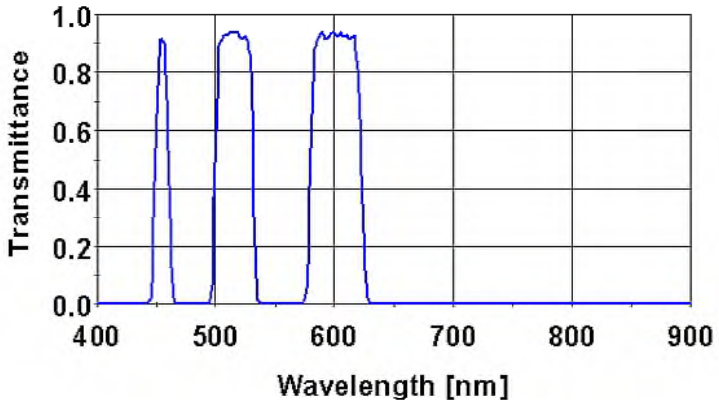
- **Dichroic Mirror**
  - Reflects Excitation Band
  - Passes Emission Band
  - Sharp cut-off required
- **Filter selection**
  - **Broadband filters** → more excitation, less contrast [more autofluorescence may be excited]
  - **Narrowband filters** →less signal, more contrast
  - Note: eye responds to contrast while detectors respond to signal

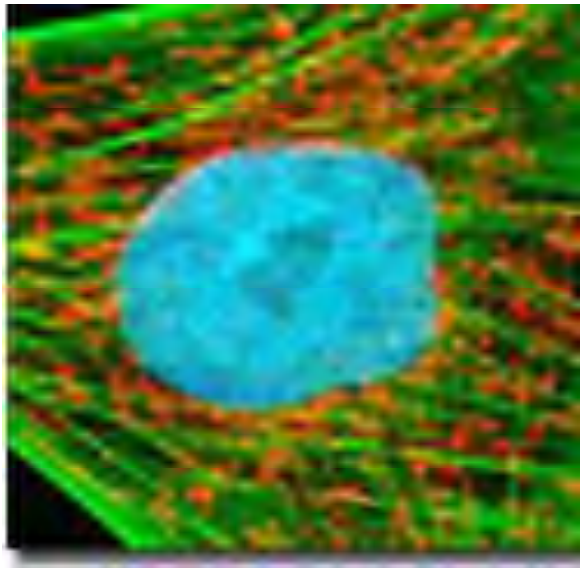




Fluorescence Microscopy

- **Multi-band Imaging**
  - Image (simultaneously or sequentially) the same sample at different excitation emission wavelengths
  - Look at different cell components
- **Example**
  - Cell nucleus stained with blue Hoechst dye
  - Mitochondria stained with Mitotracker red
  - Actin cytoskeleton stained with phalloidin derivative conjugated to Alexa 488 (green)





Limitations of Fluorescence Microscopy

- **Photobleaching**
  - Often limits the number of exposures or the exposure time

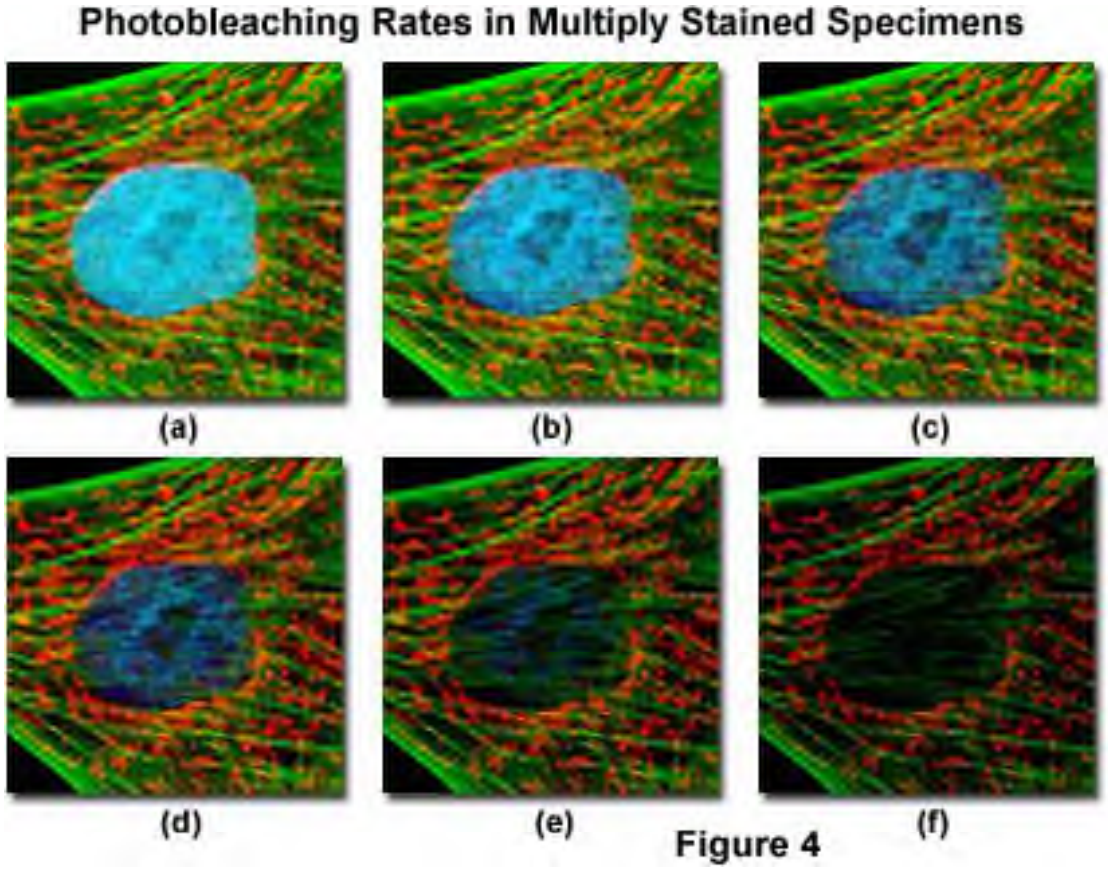
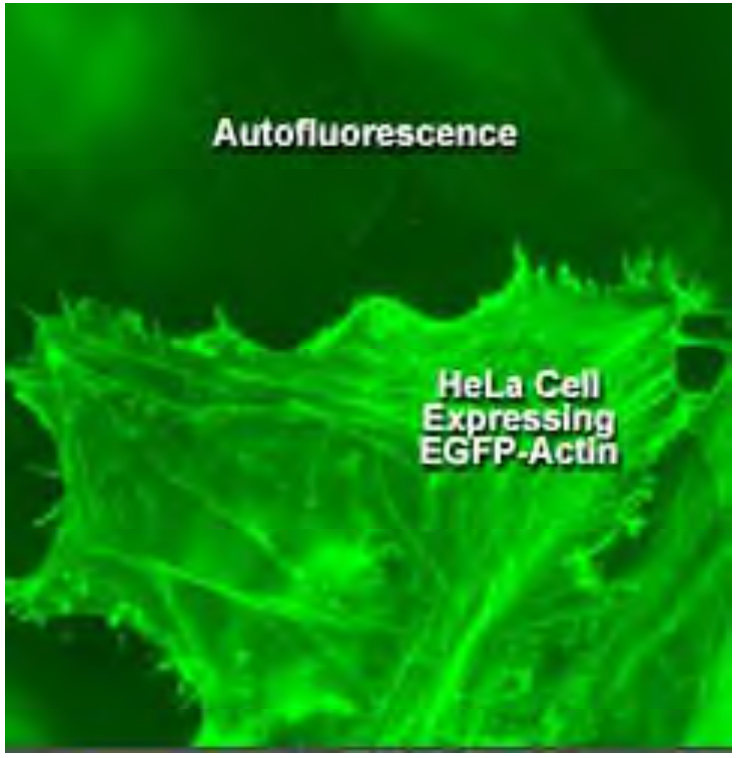


Figure 4

Photobleaching is the irreversible photochemical destruction of the fluorescent chromophores

Limitations of Fluorescence Microscopy

- **Autofluorescence**
  - Autofluorescence can be present in the images
  - Image at narrow band or use NIR excitation to minimize this effect (NIR exogenous fluorophores)

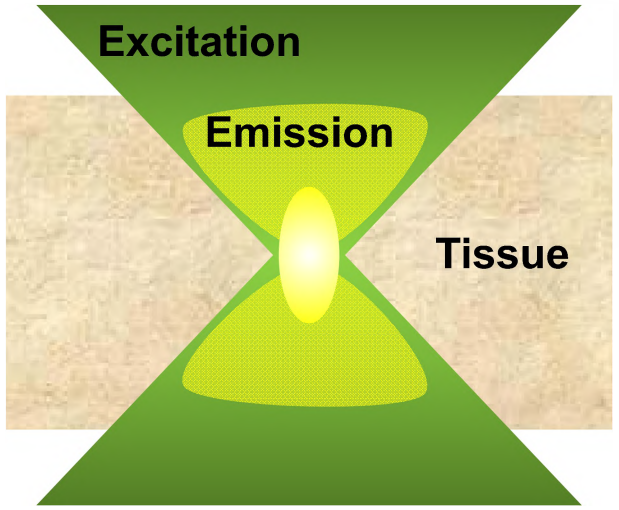


Endogenous Fluorophores

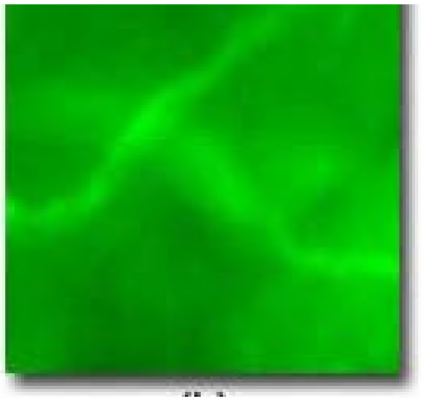
- amino acids
- structural proteins
- enzymes and co-enzymes
- vitamins
- lipids
- porphyrins

Limitations of Fluorescence Microscopy

- **Resolution is limited in thick specimens**
  - Detection of out-of-focus fluorescence
  - The excitation beam illuminates uniformly a wide field of the sample.
  - If the sample is thick, fluorescence will be excited within the focal plane, but also within planes above and below the focus.
  - Some of this fluorescence will be imaged onto the detector and will result in a defocused-looking image



Human medulla



Rabbit Muscle Fibers

Pollen Grain

Limitations of Fluorescence Microscopy

- **Reject out-of-focus light → Optical "sectioning" (next lecture)**
  - Create 3d images
  - Confocal Microscopy, Two Photon Microscopy, SIM

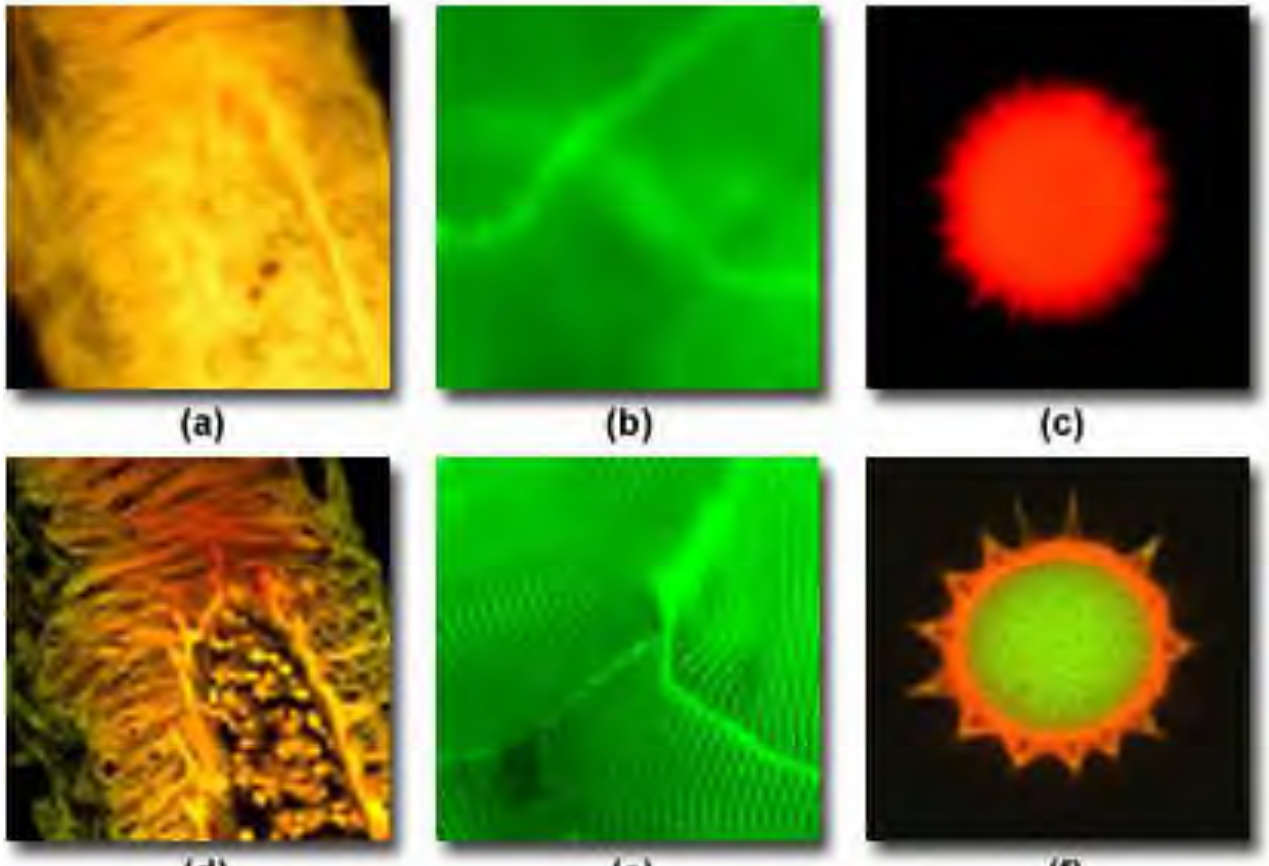
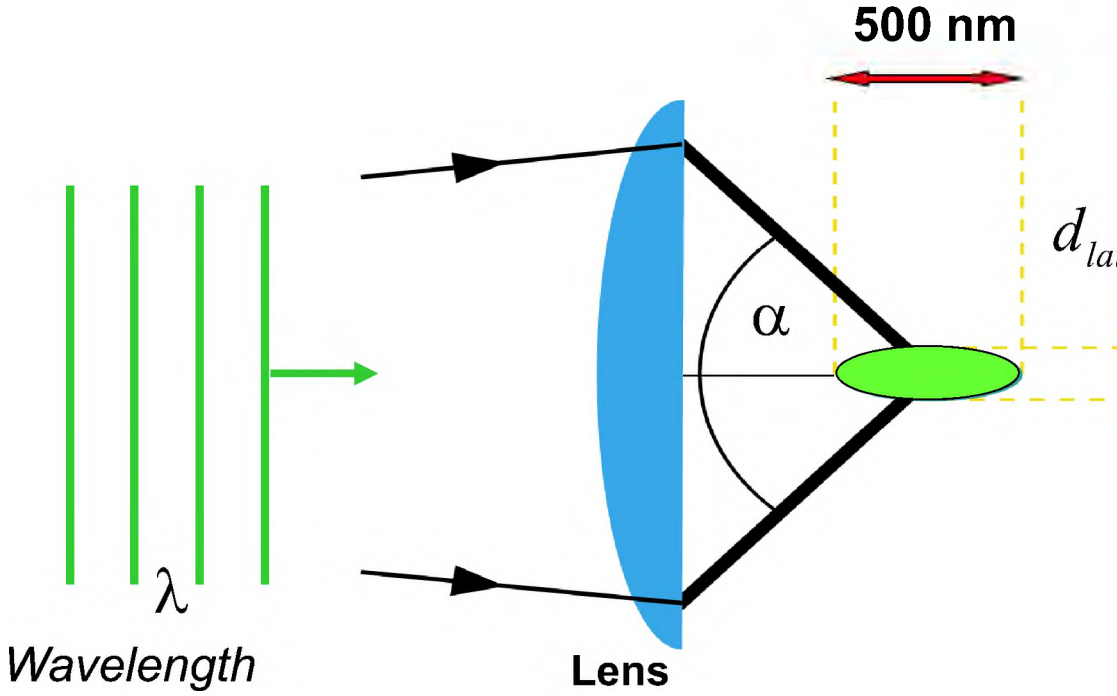


Figure 1

Limitations of Fluorescence Microscopy

- **Resolution is limited by Abbe's law**
  - Resolution limit (~ 200 nm)
  - Can you break it?

$$d_{ax} = 4\frac{\lambda n}{\left(NA_{obj} + NA_{cond}\right)^2}$$

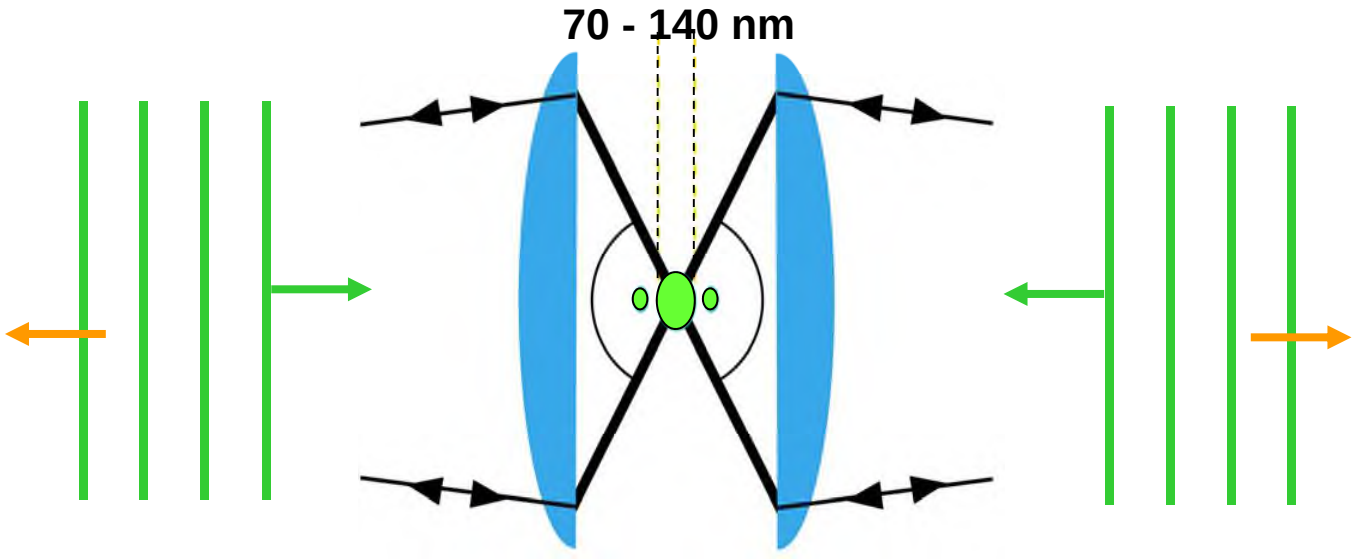


$$d_{lat} = 1.22\frac{\lambda}{NA_{obj} + NA_{cond}}$$

200 nm

4Pi- Microscopy

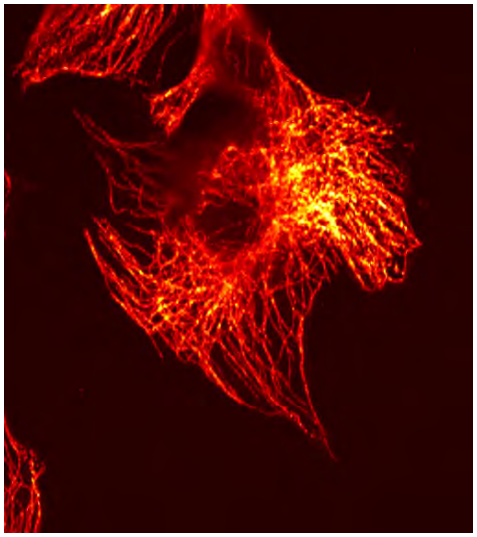
- **Basic Principles**
  - Image from two directions → approaching 4pi solid angle
  - Coherent illumination and/or fluorescence detection
  - Interference results in smaller spot size



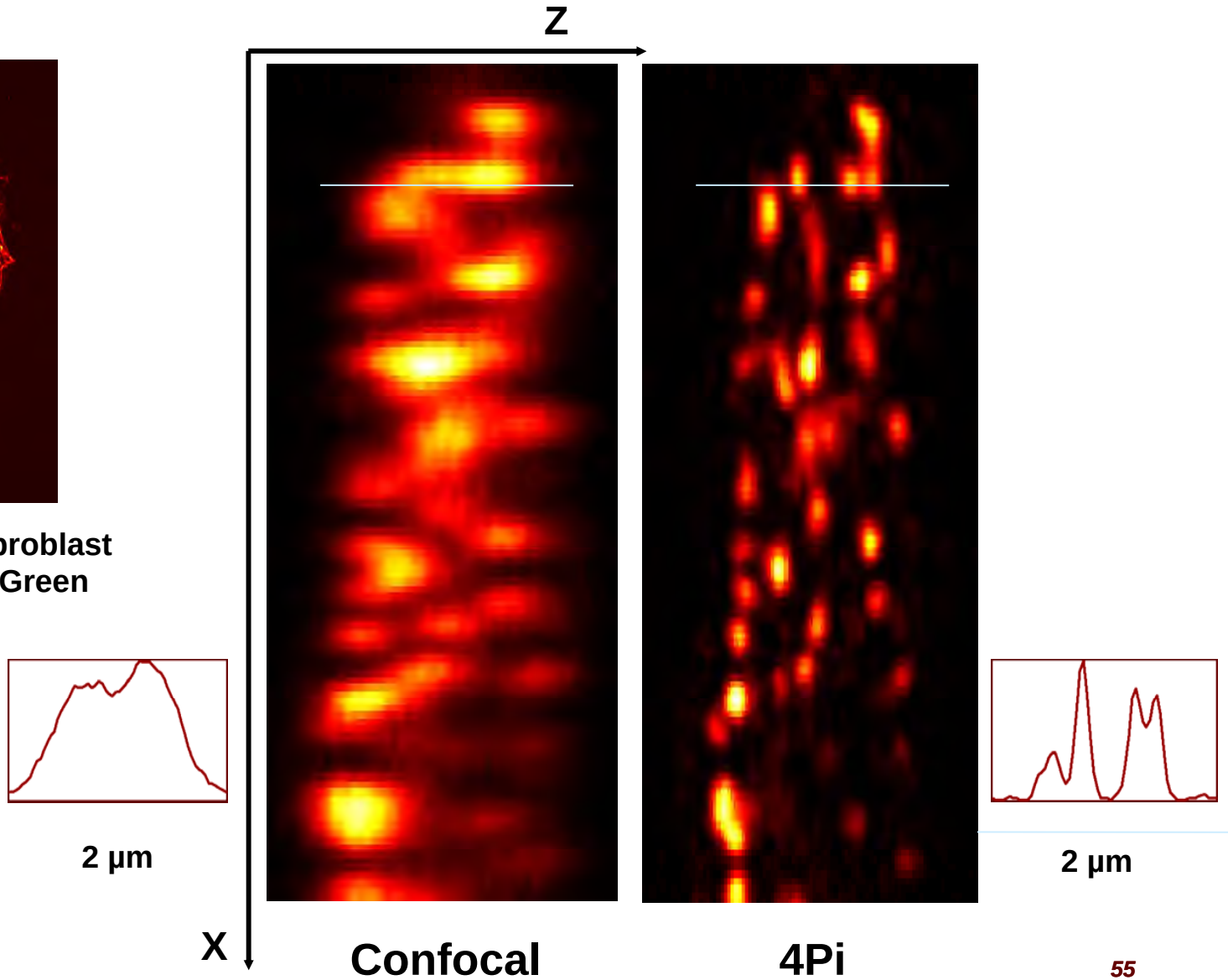
$$\vec{E}^{4Pi}(r,z,\varphi) = \vec{E}_1(r,z,\varphi) + \vec{E}_2(r,-z,\varphi)$$

S.W. Hell, et al. (1992), Opt. Commun. 93, 277

4Pi- Microscopy

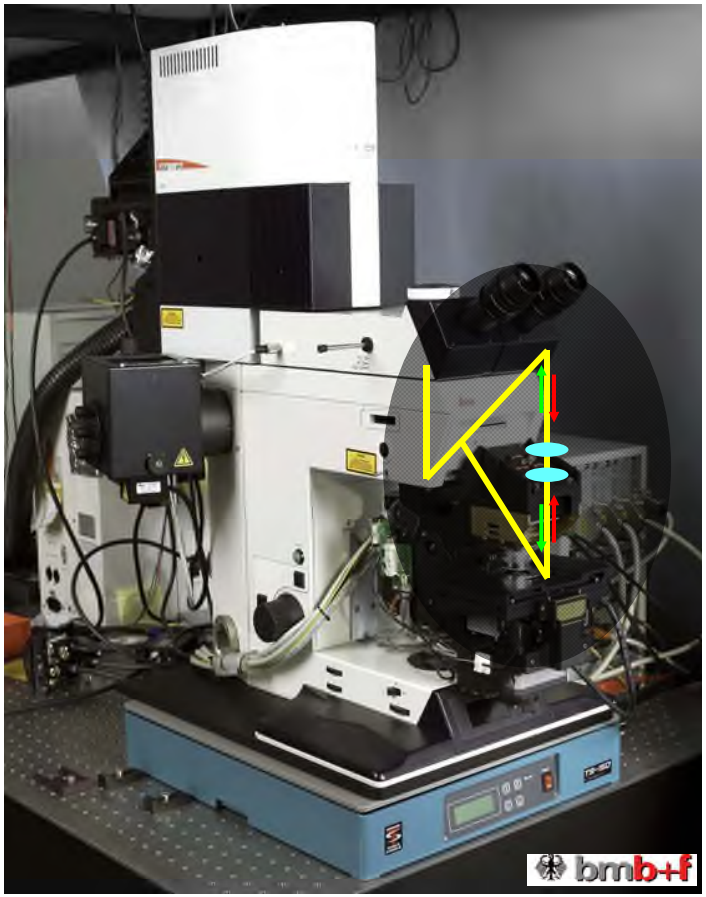


Microtubules, mouse fibroblast
Immunofluor, Oregon Green



4Pi- Microscopy

- **Commercial 4Pi-microscope**

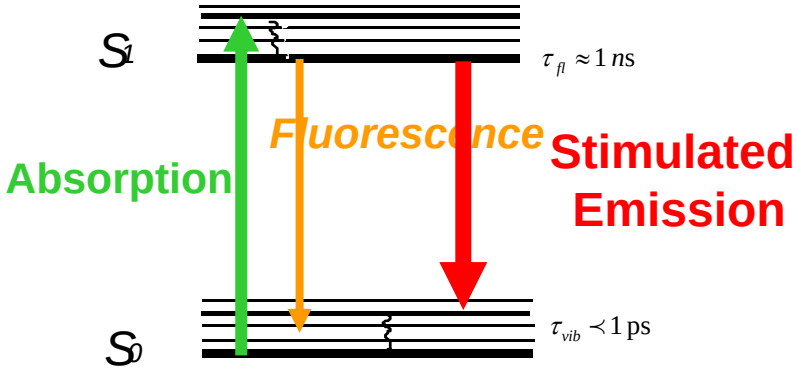


Z- resol < 90 nm (Live cells / aqueous cond.)

H. Gugel, et al. (2004), Biophys J 87, 4146.

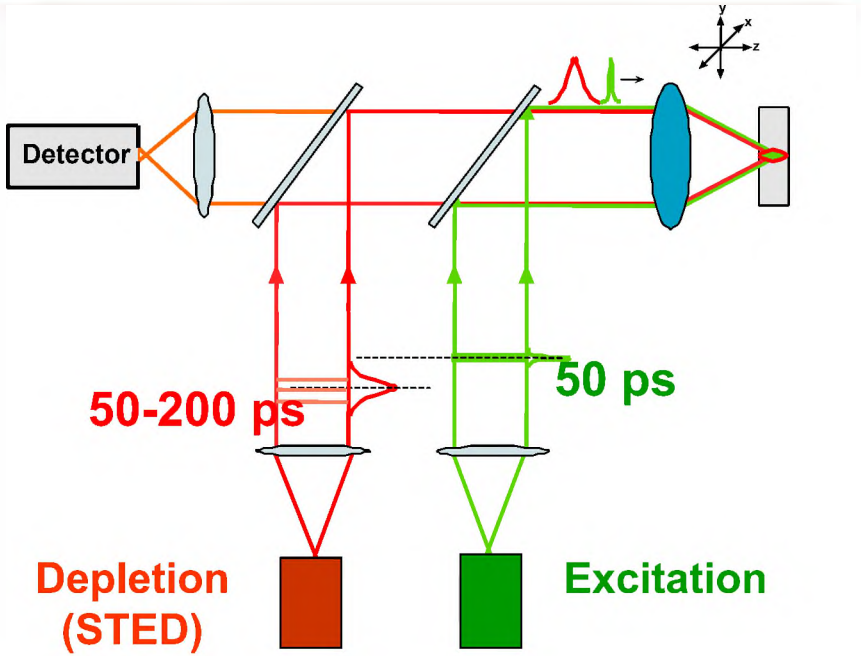
STED Microscopy

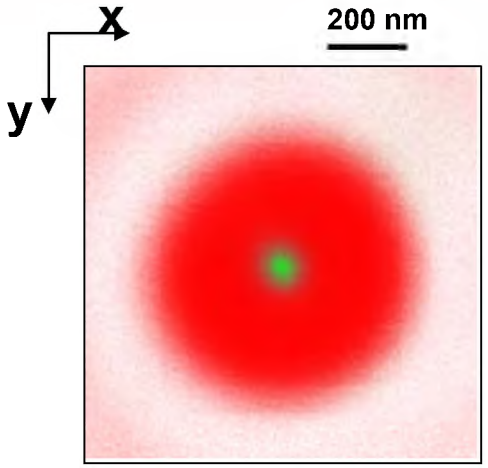
- **1st physical concept to break the diffraction barrier in far-field fluorescence microscopy**
- **Basic Principles**
  - Stimulate the fluorescent dye
  - Cause stimulated emission to de-excite part of the excitation focal volume (doughnut shape)
  - Measure fluorescence from the remaining excited volume



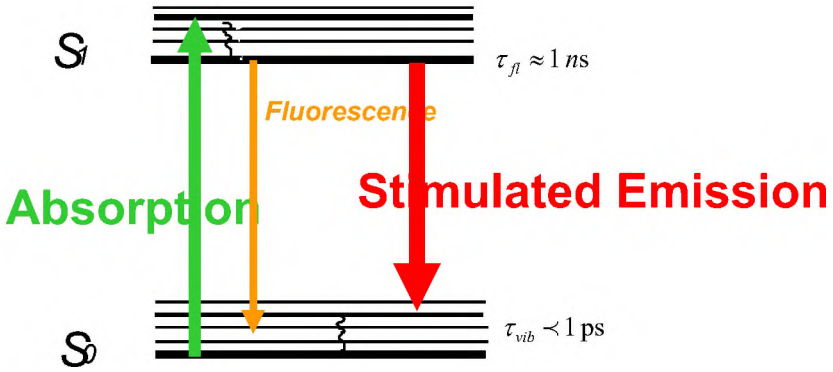
S.W. Hell & J. Wichmann (1994), Opt. Lett. 19, 780.

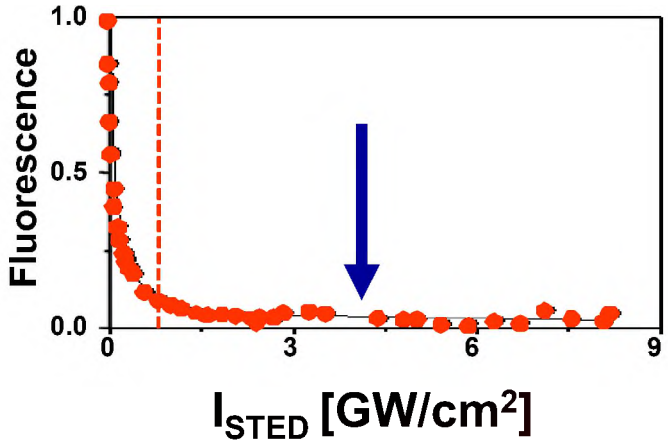
STED Microscopy





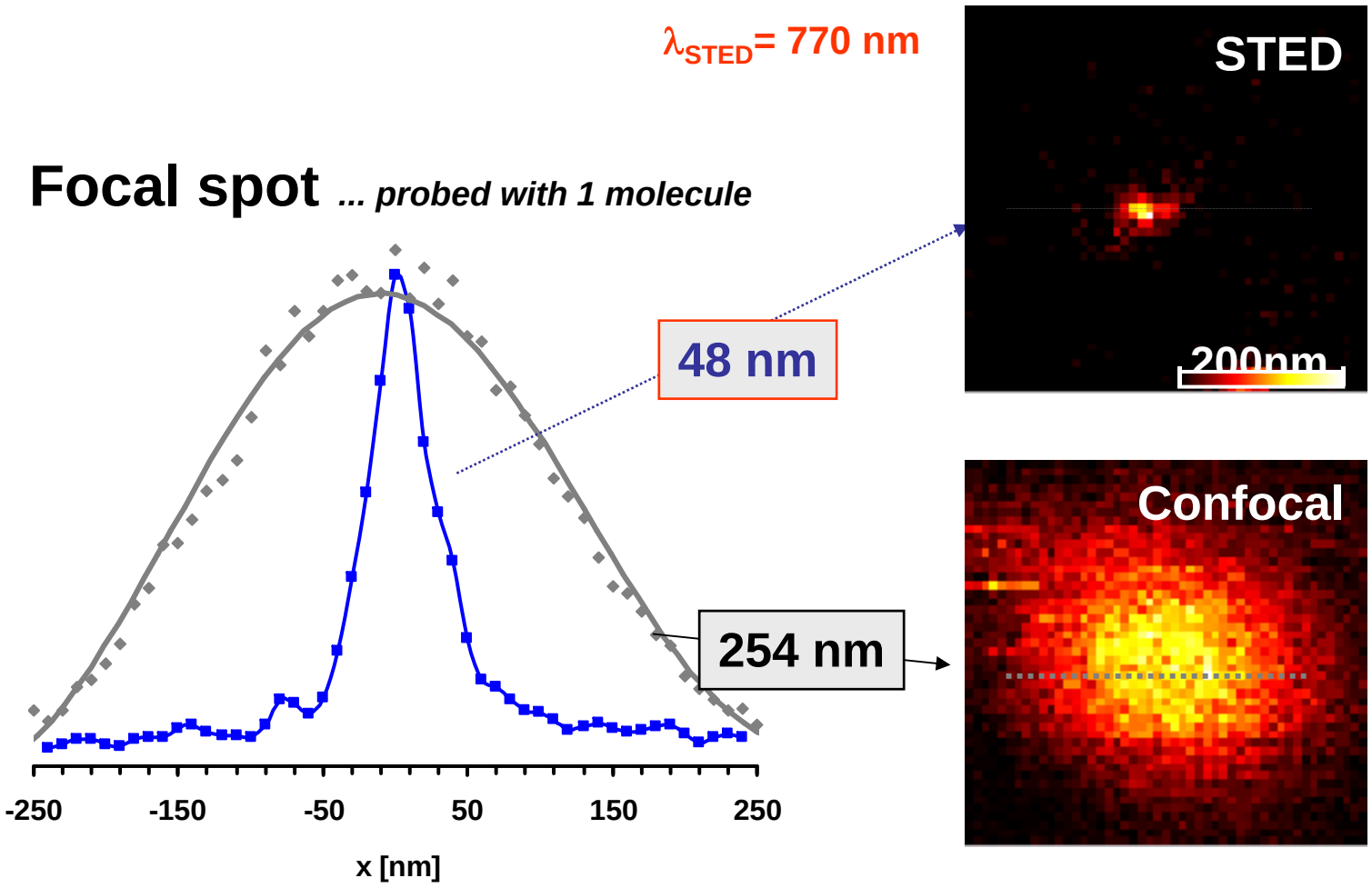
The stronger the STED beam the narrower the fluorescent spot!





S.W. Hell & J. Wichmann (1994), Opt. Lett. 19, 780.

STED Microscopy

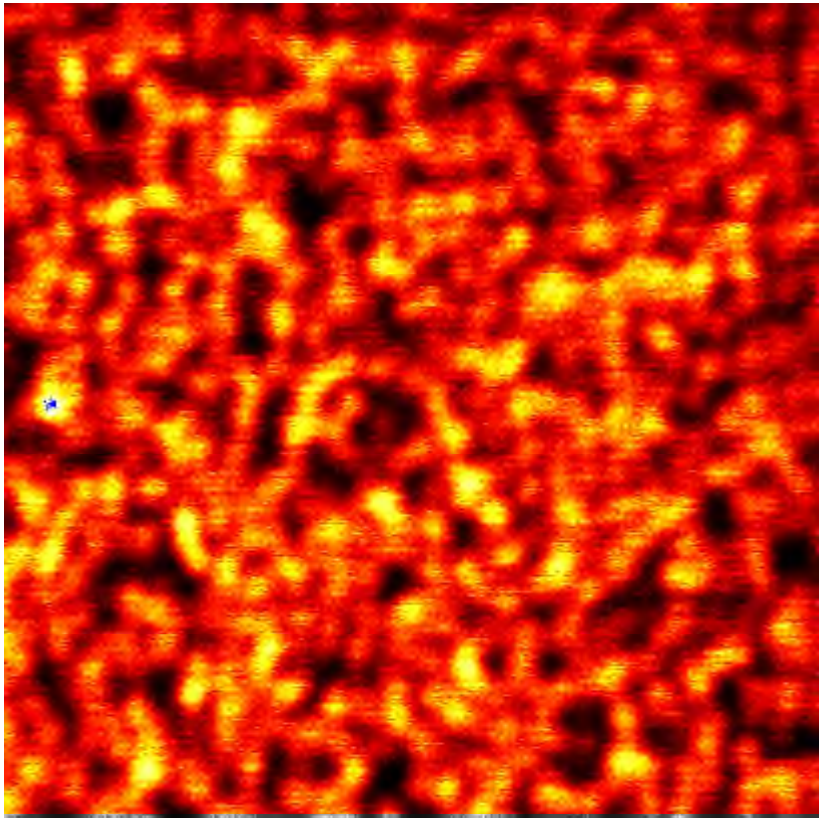


V. Westphal & S.W. Hell (2005), Phys. Rev. Lett. 94, 143903.

STED Microscopy

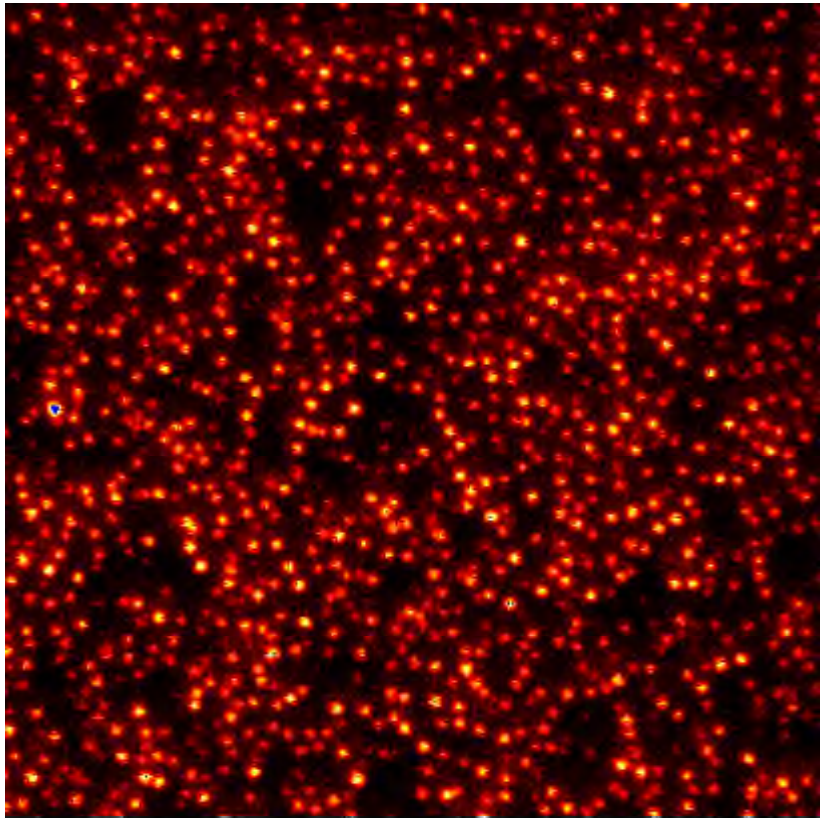
Imaging 40 nm fluorescence beads:

Confocal



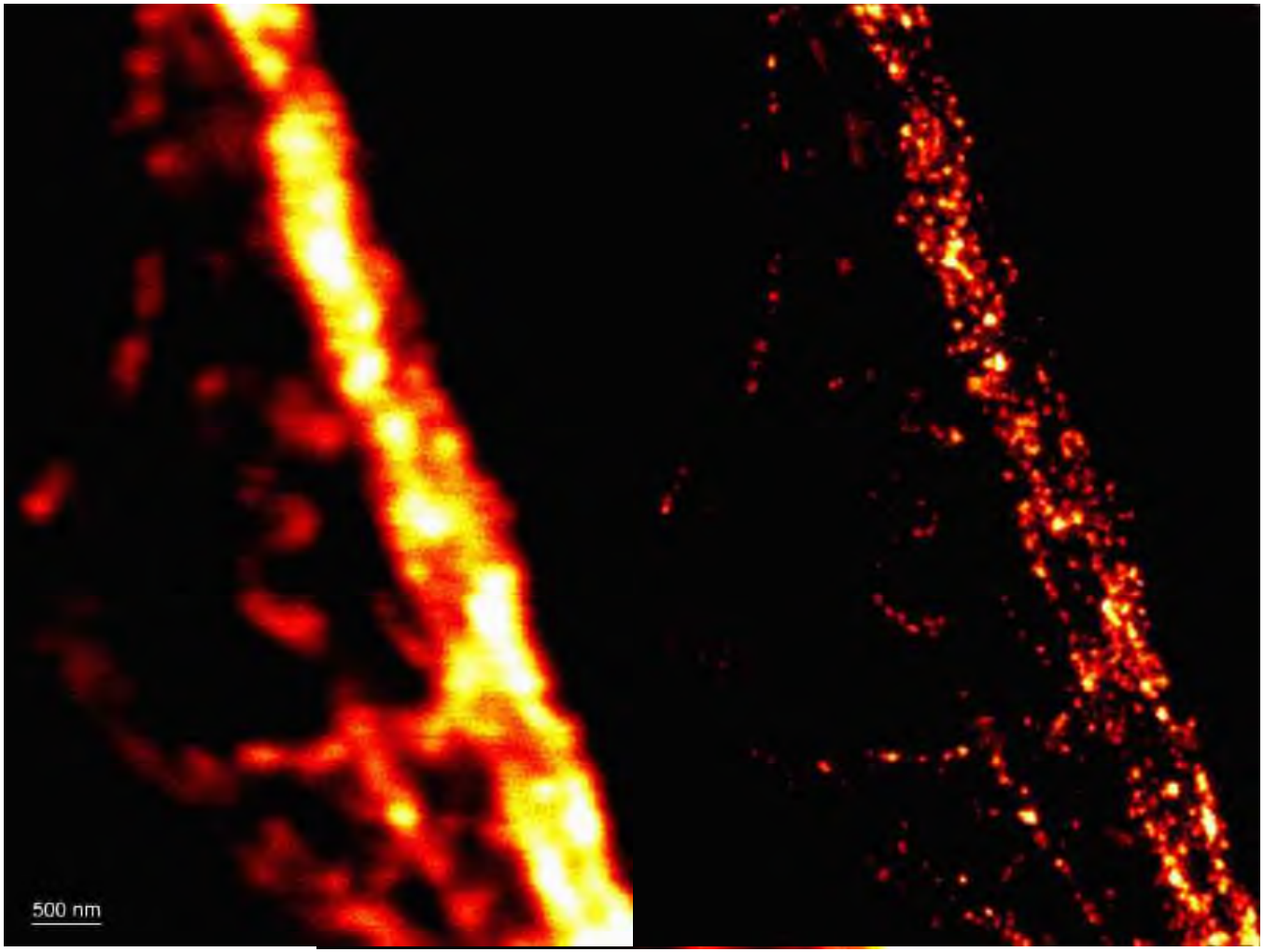
10 counts/0,3ms 204

STED



5 counts/0,3ms 89

STED Microscopy

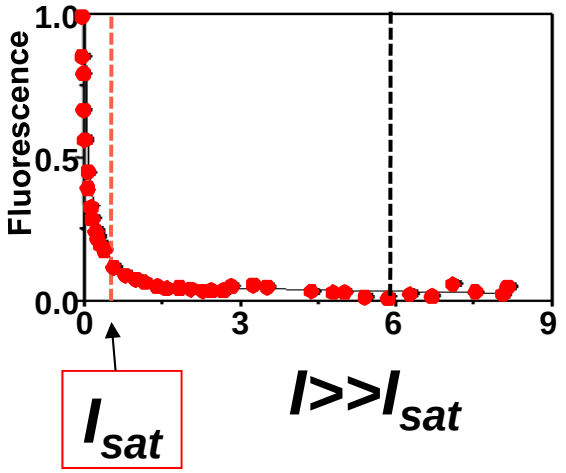


***Heavy subunit of neurofilaments* in neuroblastoma**

G. Donnert, et al. (2006), PNAS 103, 11440.

STED Microscopy

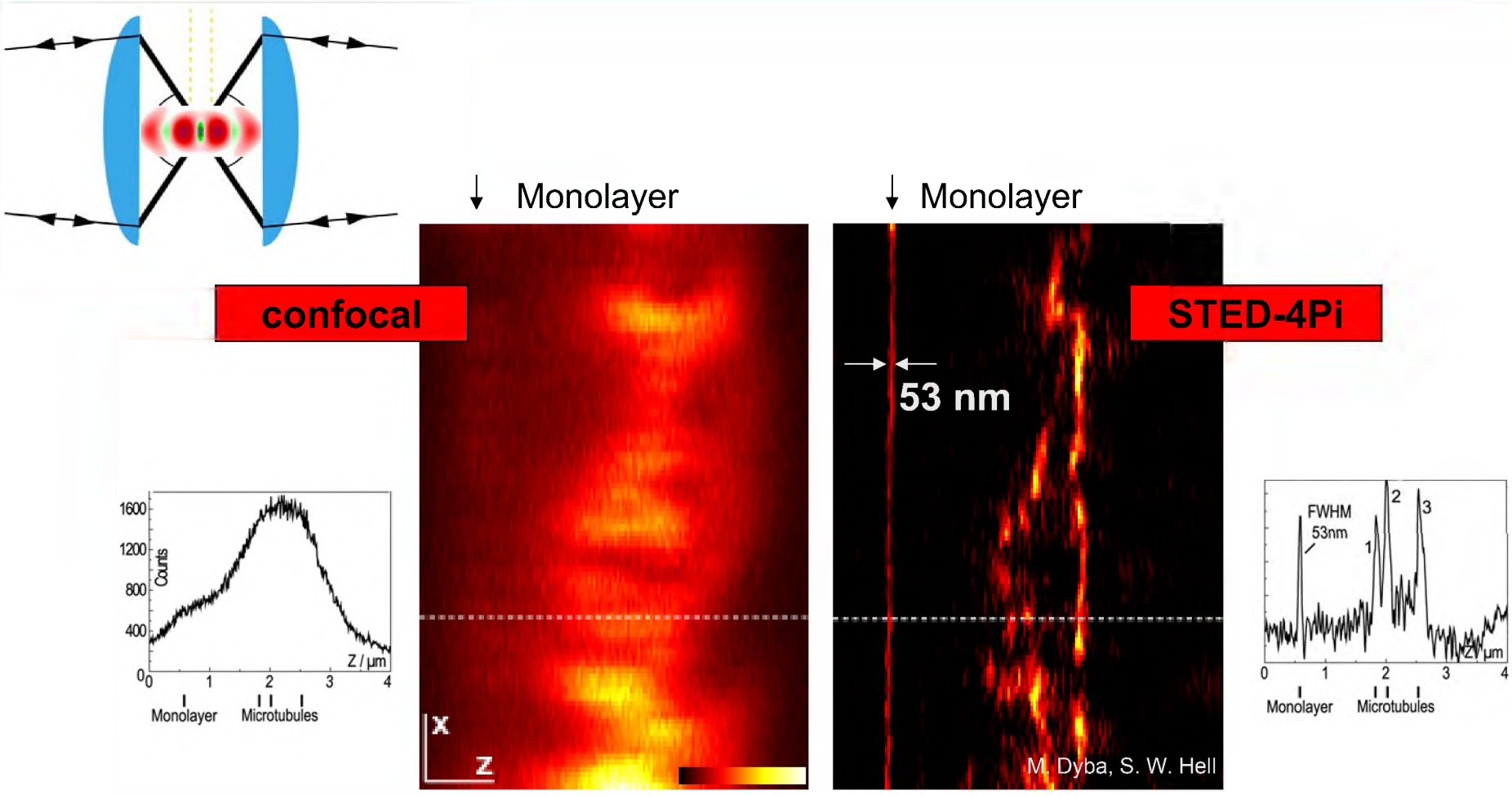
- **Resolution is not limited by the wavelength of light!**
  - Resolution just depends on the level of fluorescence depletion.
- **Resolution at the molecular scale is possible with visible light and regular lenses!**
- **Resolution follows a new law; a <u>modification of Abbe's law</u>**



$$\Delta x \approx \frac{\lambda}{2n \sin\alpha \sqrt{1 + I/I_{sat}}}$$

S.W. Hell (2003), Nature Biotech. 21, 1347.

The combination: STED-4Pi-Microscopy

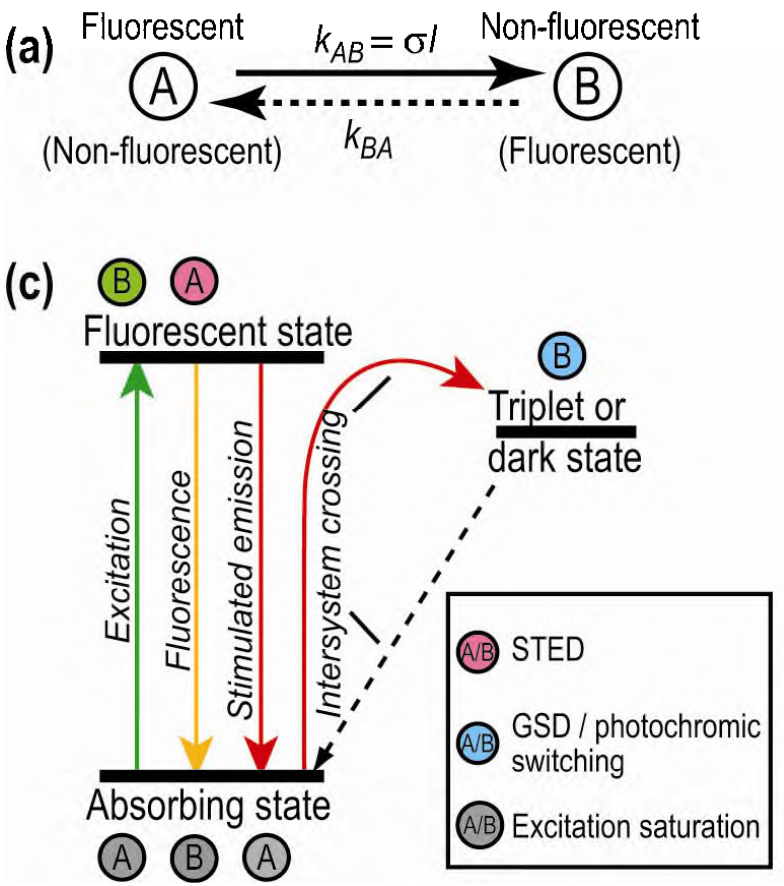


Fluorescently tagged microtubuli
with an axial resolution of 50-70 nm

M. Dyba, S. Jakobs, S.W. Hell (2003), Nature Biotechnol. 21, 1303.

RESOLFT Microscopy

- **Reversible Saturable (Switchable) Linear Fluorescence Transition (RESOLFT) microscopy is the generalized principle of STED microscopy**
- **Same concept as STED but dyes are made to "dark state" by other mechanisms:**
  - switch to triplet state
  - switch to ground state
  - use reversibly photoswitchable dyes
- **Advantages:**
  - less powerful lasers need to be used (100 W/cm^2)
  - this leads to many more dyes and even fluorescent proteins being used



S.W. Hell (2003), Nature Biotechnol. 21, 1347.