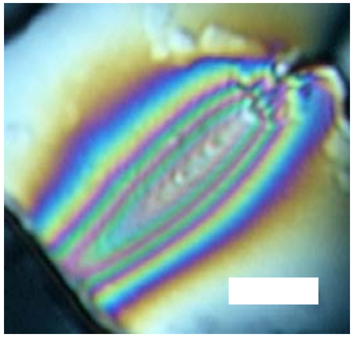
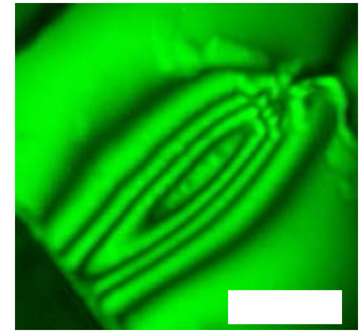
Microscopic Methods:

New Stuff in Old Places





Mohan Srinivasarao
School of Materials Science and Engineering
School of Chemistry and Biochemistry
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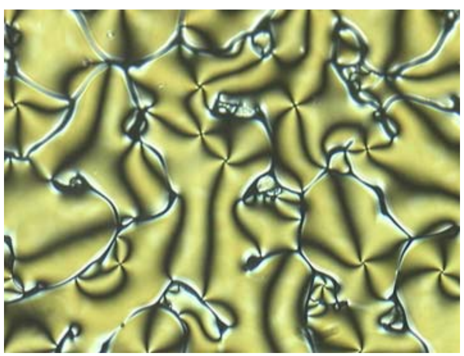
Center for Non-Linear Sciences (CNS)
Georgia Institute of Technology, Atlanta, GA



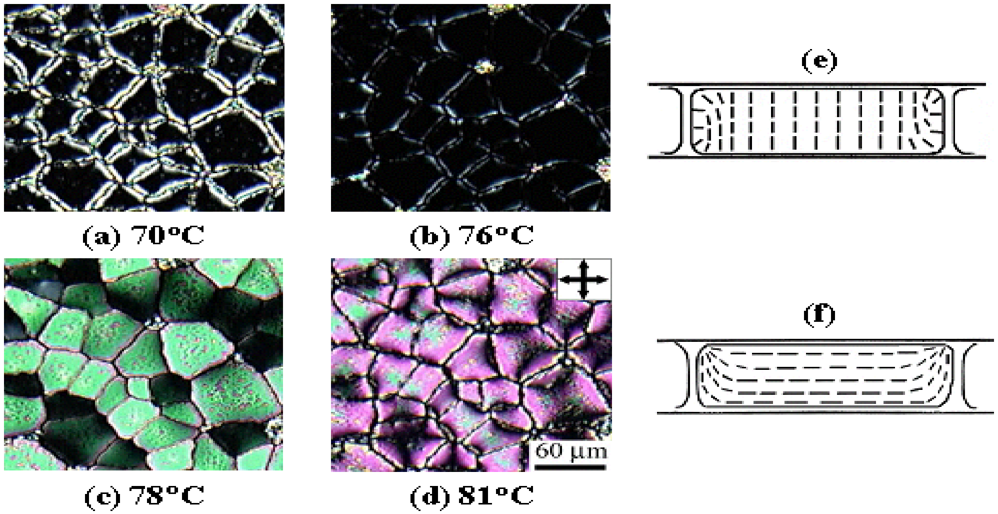
Dr. Jian Zhou
Dr. Jung Ok Park
Dr. David M. Collard

$$$$ NSF (CAREER)
DMR-0096240
DMR-03-12792

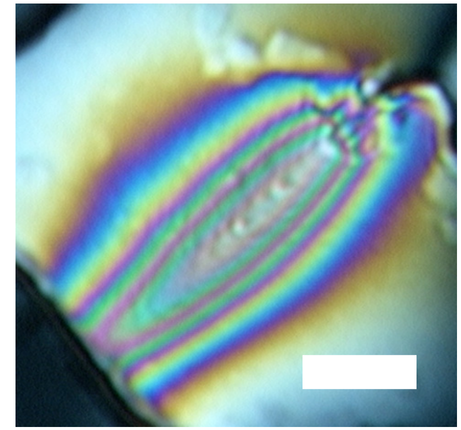
# of Centers X IQ = Constant

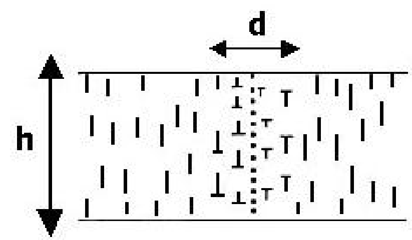
Topics for the day (Perhaps)

Anchoring Transitions

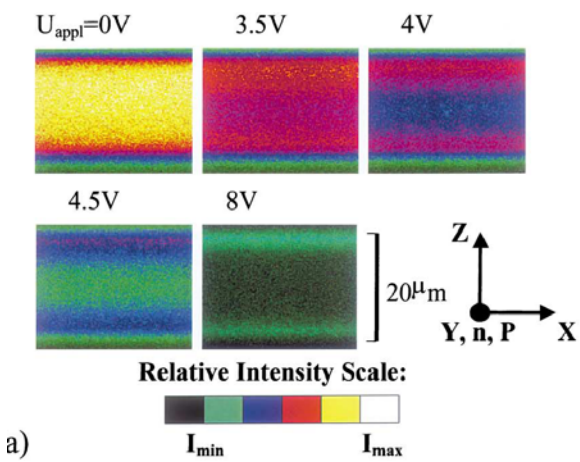


Wall Defects



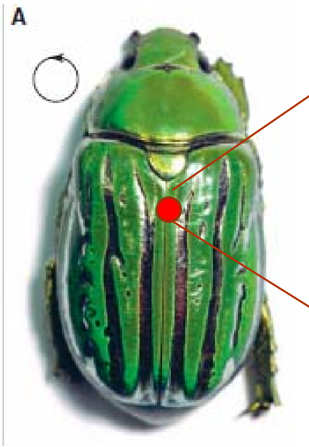


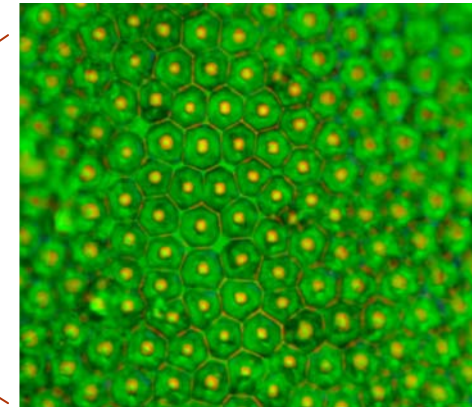
Freedericksz Transitions



Structural color in the biological world

- **Interference**



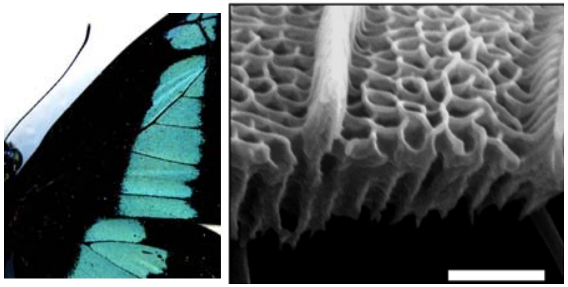


- **Selective reflection**

Structural color in biology: *Nano-optics in the biological world: Beetles, butterflies, birds and moths:* Srinivasarao, M. (1999). Chemical Reviews **99**(7): 1935-1961.

Photonic Structures in Biology, Pete Vukusic and Roy Sambles, Nature, **424**, 852 (2003).

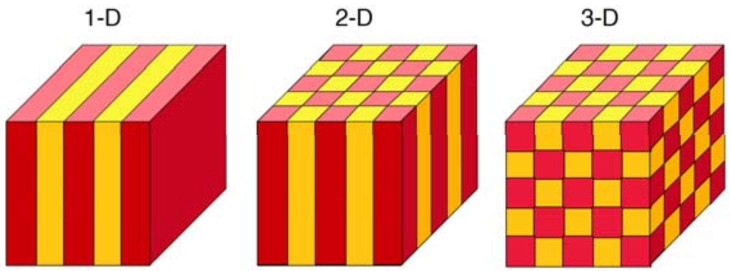
Structural color in the biological world-2

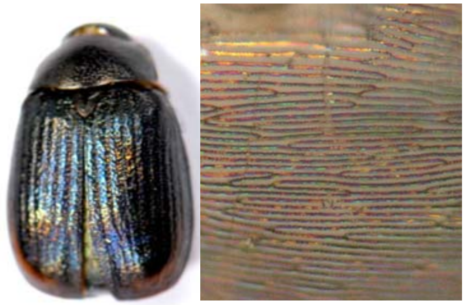


Papilio nireus

2D Photonic Crystal

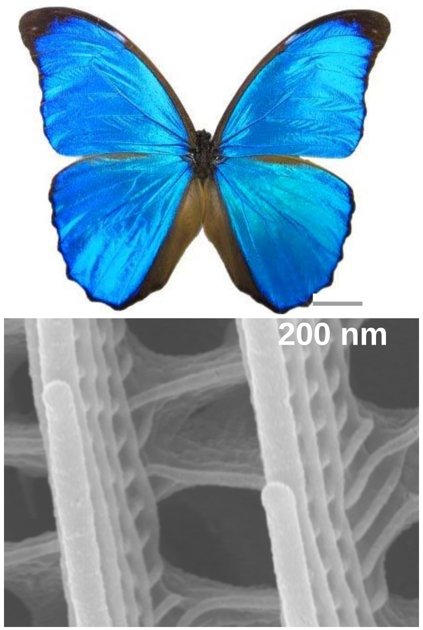
P. Vukusic & I. Hooper, Science 310, 1151 (2005)





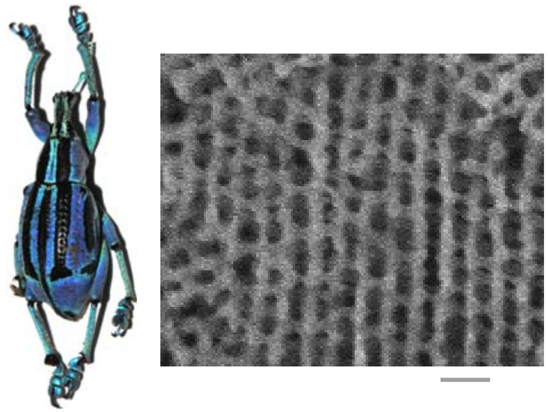
Serica tristis

Diffraction grating



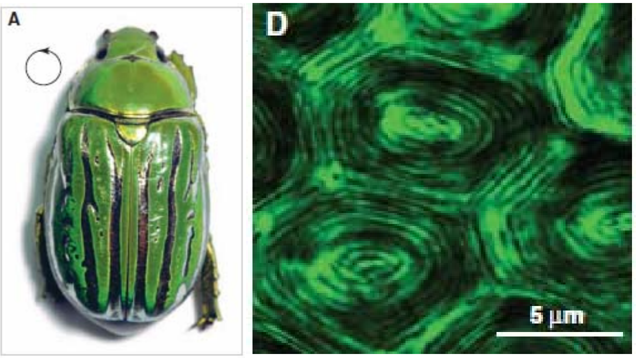
Morpho Rhetenor

Multilayer, 1D Photonic Crystal



Eupholus Benneti

3D Photonic Crystal



Chrysina gloriosa

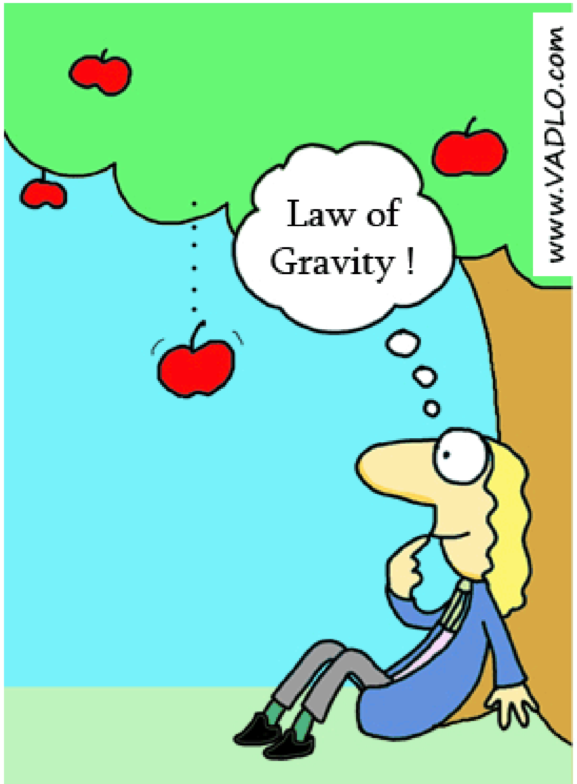
Cholesteric Liquid Crystal

V. Sharma et. al, Science 325, 449 (2009)

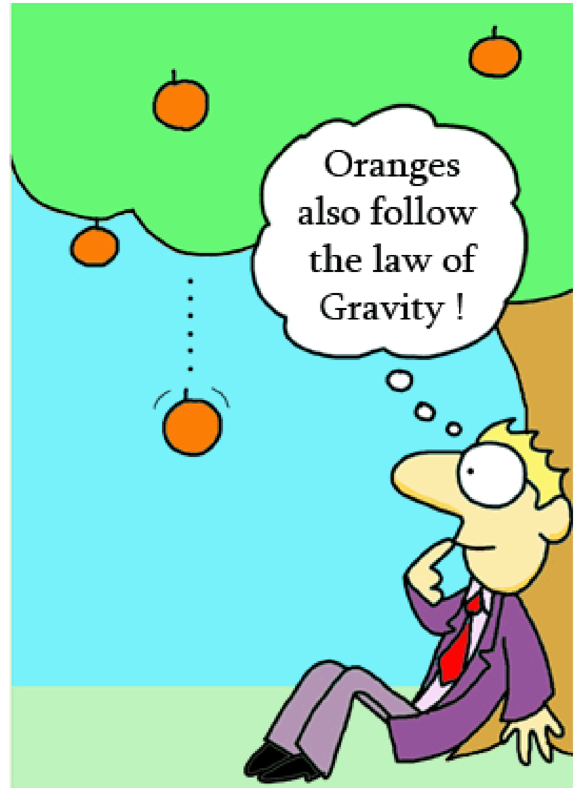
Disciplines: DEFINITIONS

If it moves, it's Biology
If it stinks, it's Chemistry
If it doesn't work, it's Physics

If it moves and stinks, it's Biochemistry
If it fails to move, it's Biophysics

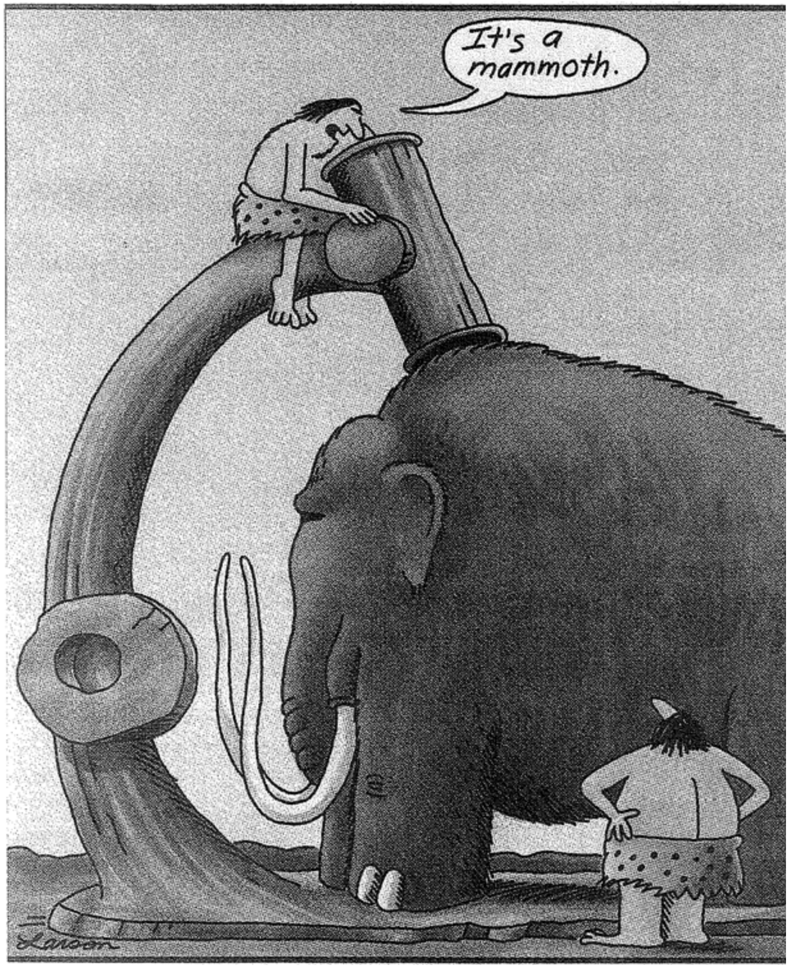


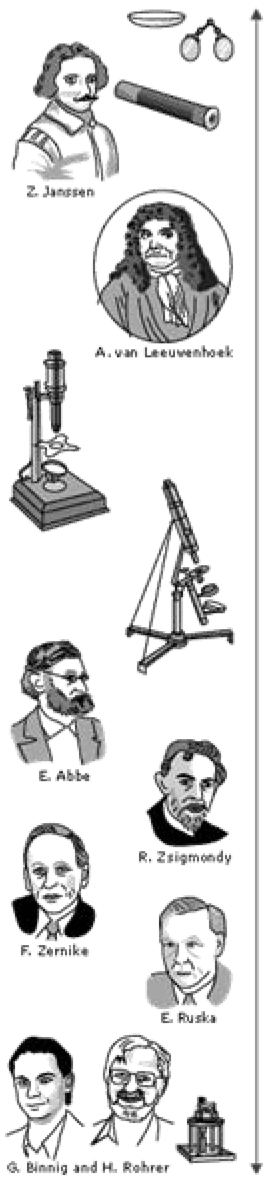
High Impact Paper



Low Impact Paper

Early Microscopists: Way of Finding Things out!





14th century – The art of grinding lenses is developed in Italy and spectacles are made to improve eyesight.

1590 – Dutch lens grinders Hans and Zacharias Janssen make the first microscope by placing two lenses in a tube.

1667 – Robert Hooke studies various object with his microscope and publishes his results in Micrographia. Among his work were a description of cork and its ability to float in water.

1675 – Anton van Leeuwenhoek uses a simple microscope with only one lens to look at blood, insects and many other objects. He was first to describe cells and bacteria, seen through his very small microscopes with, for his time, extremely good lenses.

18th century – Several technical innovations make microscopes better and easier to handle, which leads to microscopy becoming more and more popular among scientists. An important discovery is that lenses combining two types of glass could reduce the chromatic effect, with its disturbing halos resulting from differences in refraction of light.

1830 – Joseph Jackson Lister reduces the problem with spherical aberration by showing that several weak lenses used together at certain distances gave good magnification without blurring the image.

1878 – Ernst Abbe formulates a mathematical theory correlating resolution to the wavelength of light. Abbe's formula make calculations of maximum resolution in microscopes possible.

1903 – Richard Zsigmondy develops the ultramicroscope and is able to study objects below the wavelength of light.

1932 – Frits Zernike invents the phase-contrast microscope that allows the study of colorless and transparent biological materials.

Microscopes

- Upright
- Inverted
- Köhler Illumination
- Fluorescence Illumination

"Microscope" was first coined by members of the first "Academia dei Lincei" (Academy of the Lynx} scientific society which included Galileo. It was not Galileo tho' who came up with the word, it was Johannes Faber, an entomologist and member of the same society that gave the magnifying instrument the name "microscope"

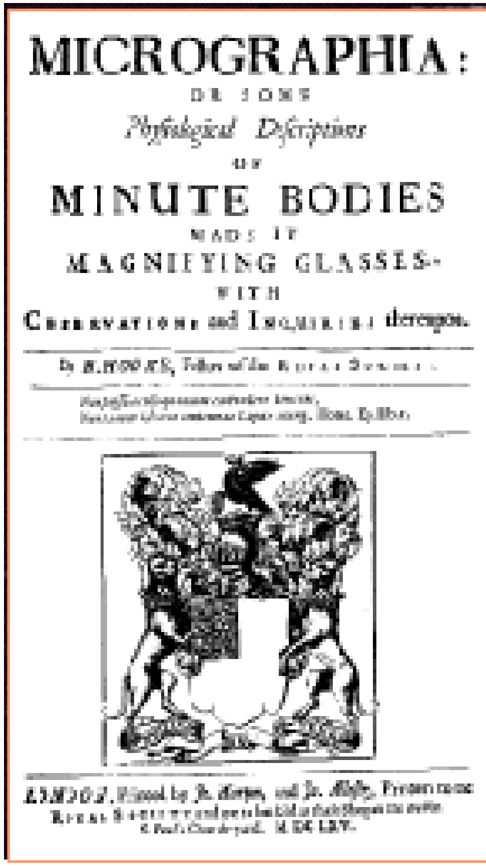
Earliest Microscopes

- 1590 - Hans & Zacharias Janssen of Middleburg, Holland manufactured the first compound microscopes
- 1590 – 1609 - Galileo – one of the earliest microscopists (naming of term “microscope”
- **1660** - Marcello Malpighi **circa 1660,** was one of the first great microscopists, considered the father embryology and early histology - observed capillaries in 1660
- 1665 - Robert Hooke (1635-1703)- book *Micrographia*, published in 1665, devised the compound microscope most famous “microscopical” observation was his study of thin slices of cork. He wrote:

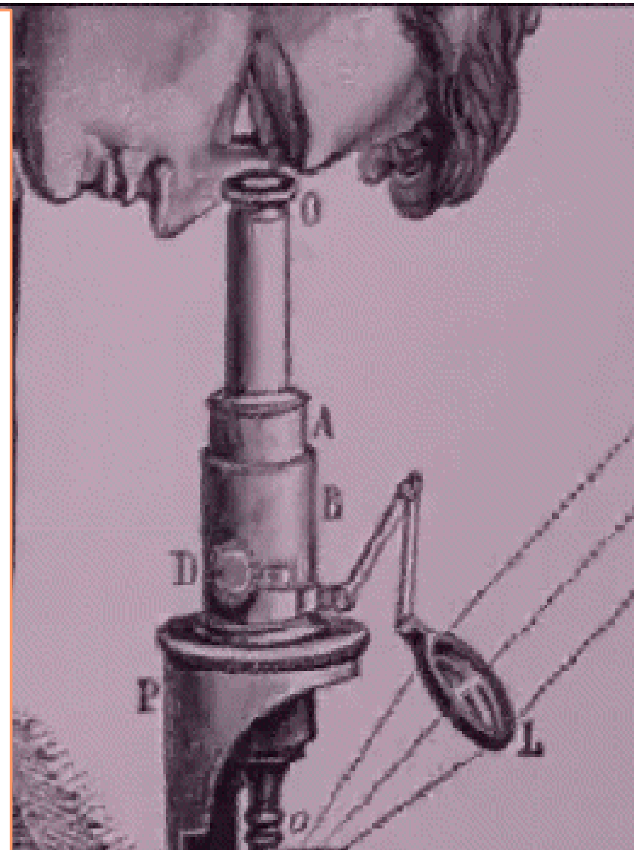
“. . . I could exceedingly plainly perceive it to be all perforated and porous. . . these pores, or cells, . . . were indeed the first microscopical pores I ever saw, and perhaps, that were ever seen, for I had not met with any Writer or Person, that had made any mention of them before this.”

Early Microscopes (Hooke)

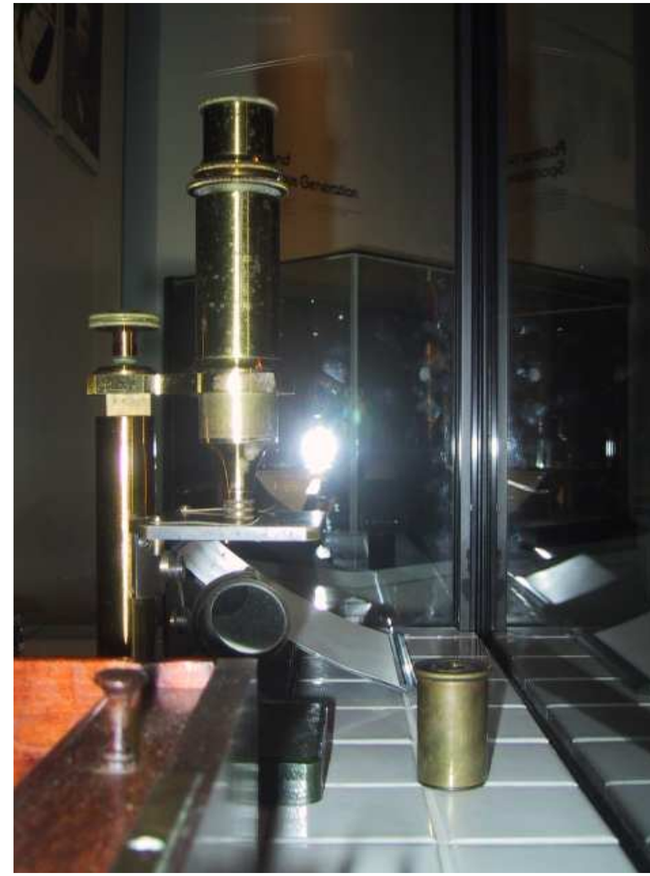
1665



MICROGRAPHIA:
OR SOME
Physiological Descriptions
OF
MINUTE BODIES
MADE BY
MAGNIFYING GLASSES.
WITH
OBSERVATIONS and INQUIRIES thereupon.



Pasteur - 1860



Louis Pasteur – his microscope was made in Paris by Nachet in about 1860 and was brass

Abbe & Zeiss

- **Ernst Abbe** together with **Carl Zeiss** published a paper in 1877 defining the physical laws that determined resolving distance of an objective. Known as Abbe’s Law

$$d = \frac{\lambda}{2\, n \sin \theta}$$

“minimum resolving distance (d) is related to the wavelength of light (lambda) divided by the Numeric Aperture, which is proportional to the angle of the light cone (theta) formed by a point on the object, to the

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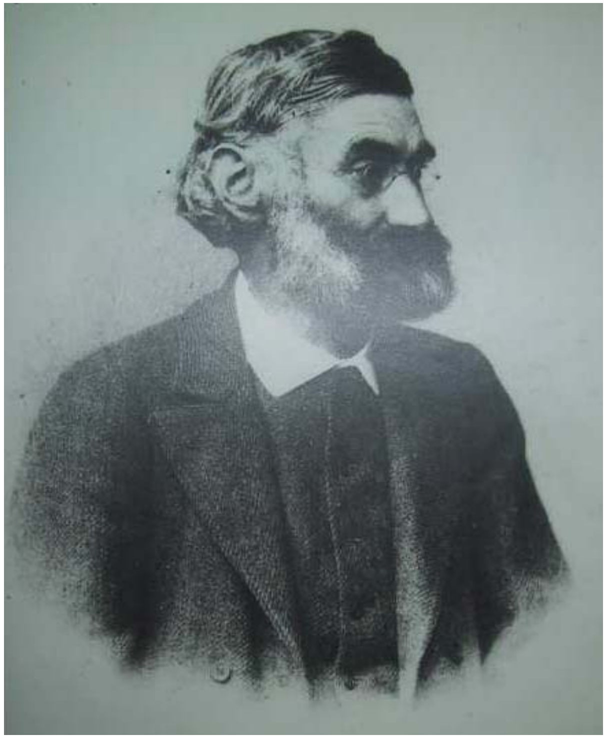

Abbe & Zeiss

- **Abbe and Zeiss** developed oil immersion systems by making oils that matched the refractive index of glass. Thus they were able to make the a Numeric Aperture (N.A.) to the maximum of 1.4 allowing light microscopes to resolve two points distanced only 0.2 microns apart (the theoretical maximum resolution of visible light microscopes). **Leitz** was also making microscope at this time.

Zeiss student microscope 1880

Abbe, Zeiss & Schott

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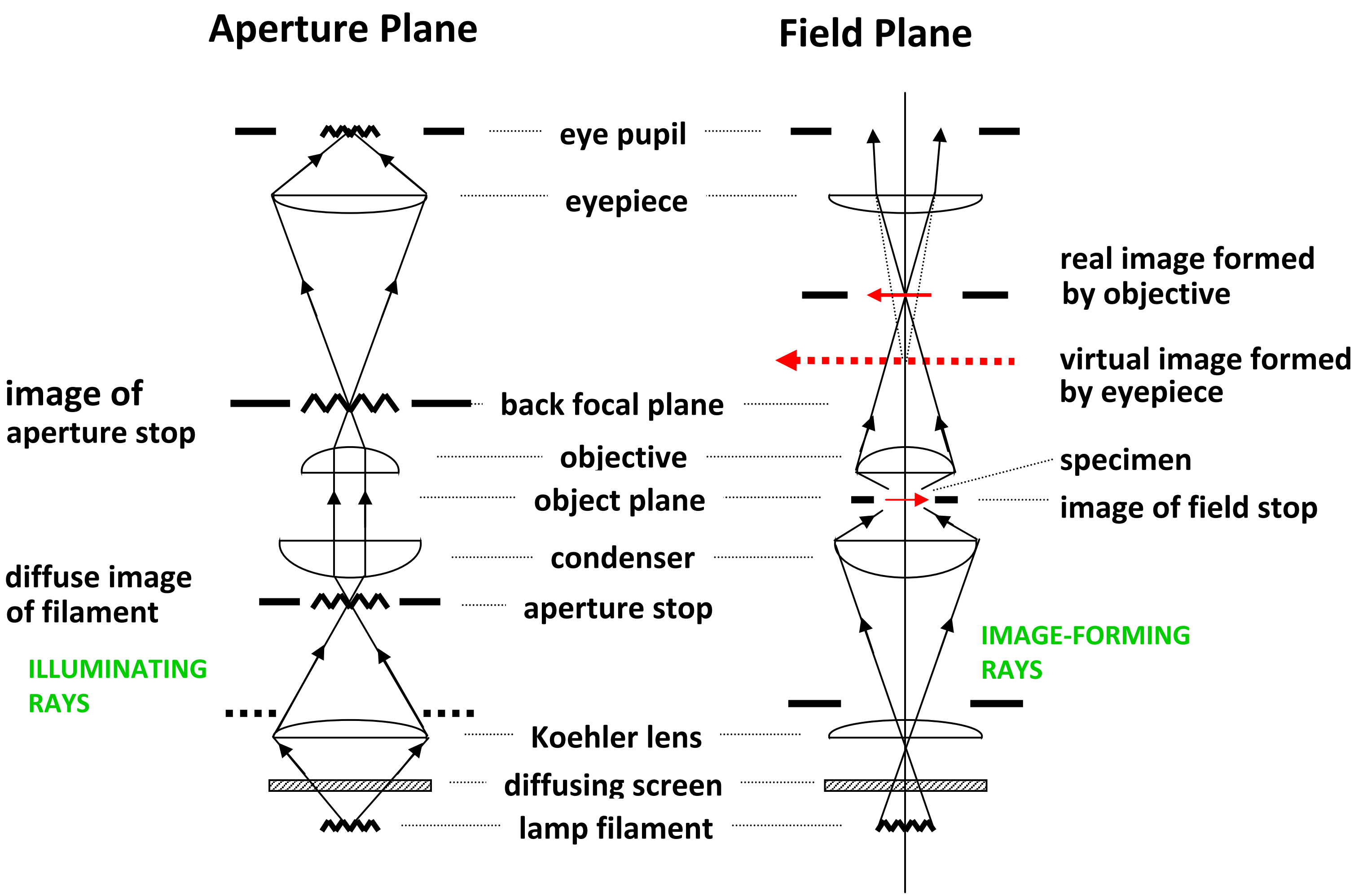
- Dr Otto Schott formulated glass lenses that color-corrected objectives and produced the first “apochromatic” objectives in 1886.

Modern Microscopes

- Early 20th Century Professor Köhler developed the method of illumination still called "Köhler Illumination"
- Köhler recognized that using shorter wavelength light (UV) could improve resolution

Koehler Illumination



Contrast Generation

- **Refractive index**
- **Spectral information**
- **Optical “tricks”**
- **Optical sectioning**
- **Multiphoton microscopy**

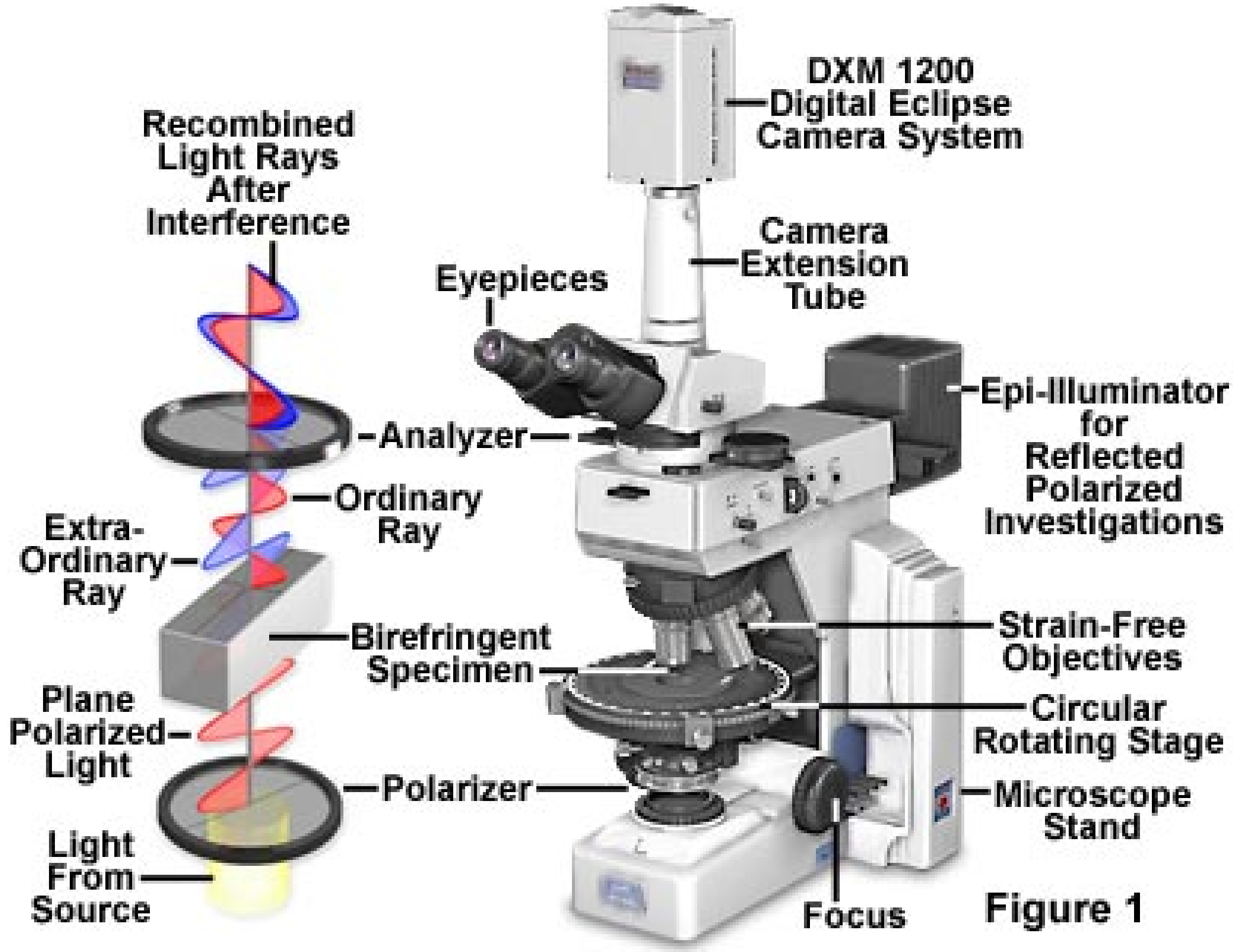
Such Methods Enable

- **Nondestructive analysis of materials**
- **Observation of nanoscale heterogeneity**
- **Combination of imaging with any method possible on an optical table**
- **Fabrication of 3-D nanostructures**

Components of the Optical Train in a Light Microscope

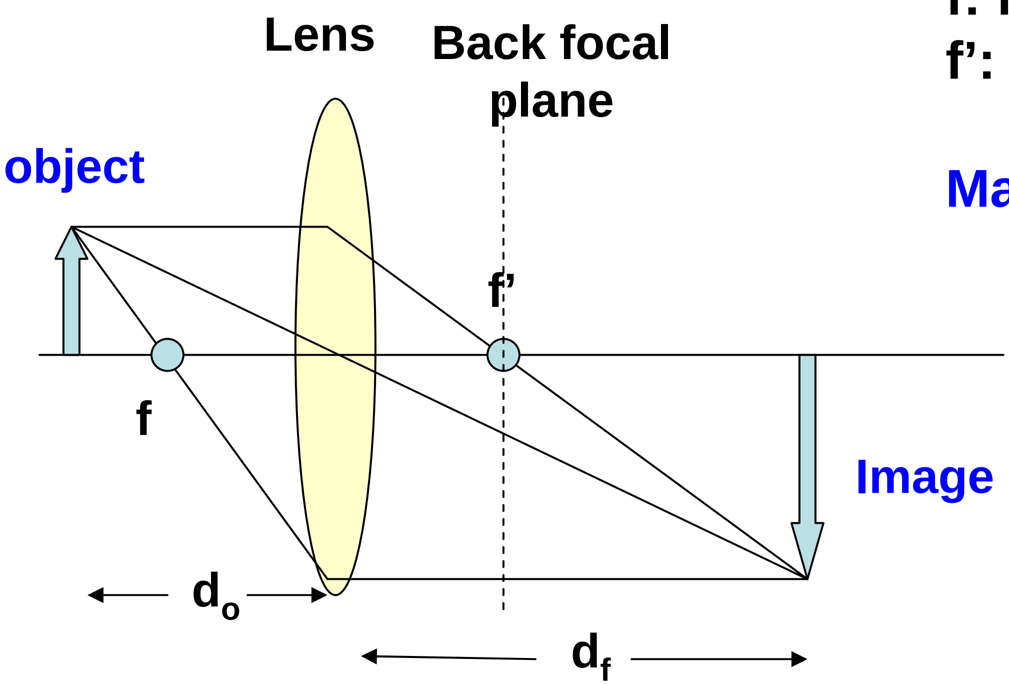
ILLUMINATOR	**Light source, Collector, Field diaphragm**
↓	
CONDITIONER	**Condenser iris, Phase annulus, Polarizer, Dark-field annulus, Normarski prism, Fluorescence excitation filter**
↓	
CONDENSER	**Focal length, Numerical aperture, Aberration, Transmission**
↓	
SPECIMEN	**Slide thickness/immersion, Coverslip thickness/immersion, Absorb, diffract, fluoresce, retard, etc., Azimuth**
↓	
OBJECTIVE LENS	**Magnification/focus, Numerical aperture, Aberration, Transmission, OTF**
↓	
IMAGE FILTER	**Nomarski prism, Objective iris, Compensator, Phase plate, Analyzer, SSEE filter, Fluorescent barrier filter**
↓	
OCULAR	**Magnification, Aberration, Field size, Eye point**
↓	
RECEIVER	**Eye, Photographic emulsion, Photomultiplier, Photodiode array, Video camera**

Polarized Light Microscope Configuration



http://www.microscopyu.com/articles/polarized/polarizedintro.html

Basics: Magnification



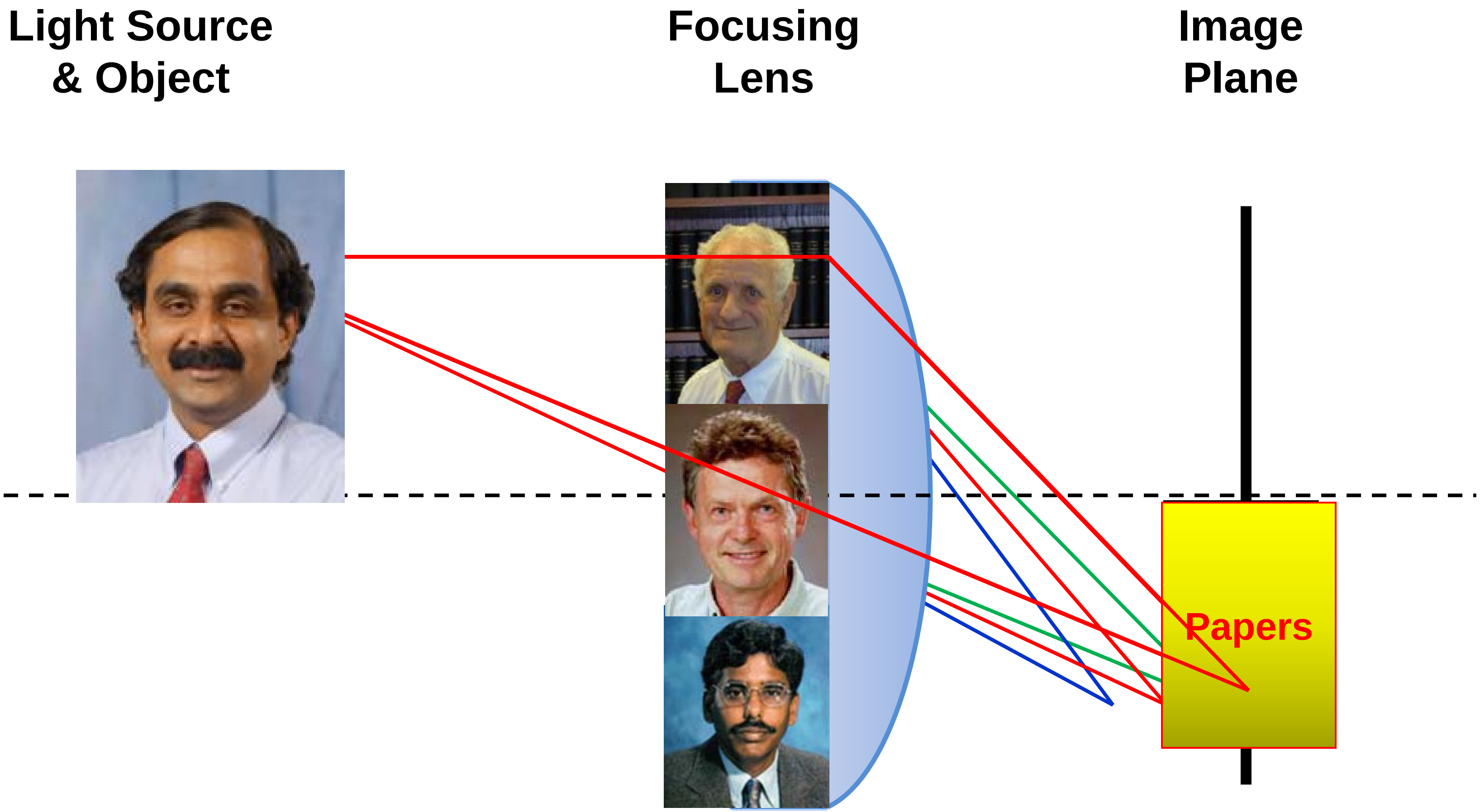
f: front focal point
f': back focal point

Magnification: d_f/d_o

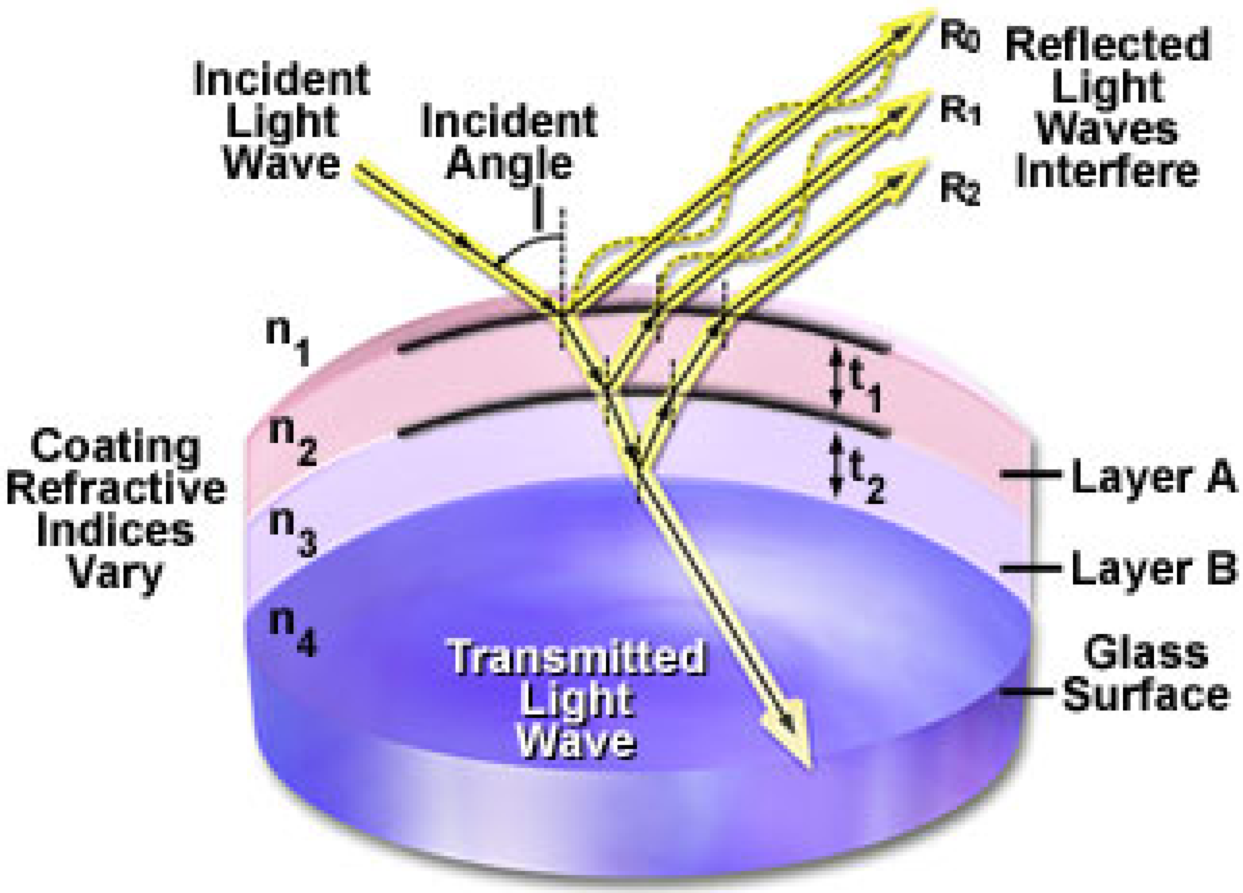
Simple biconvex lenses form and magnify images (real, inverted)

Key concepts: object plane, focal plane, image plane

Image Formation and Human Abberation

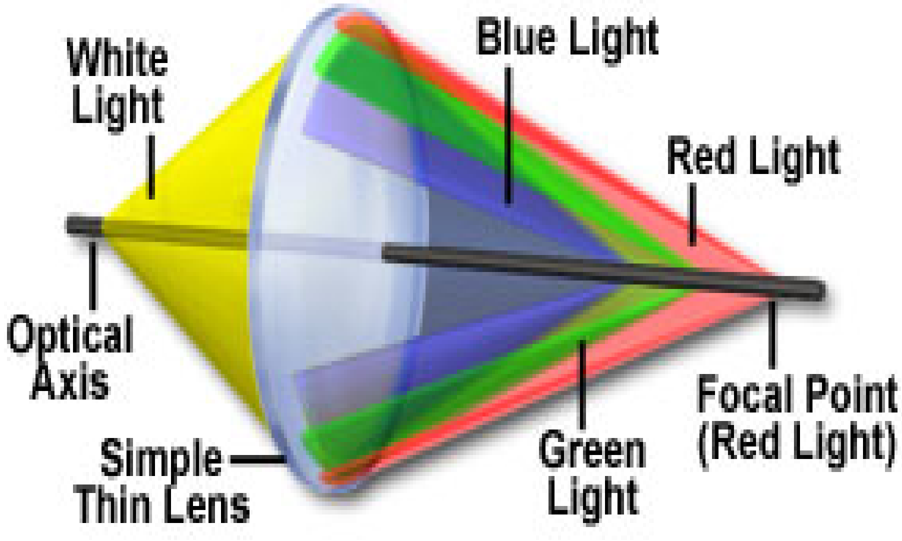


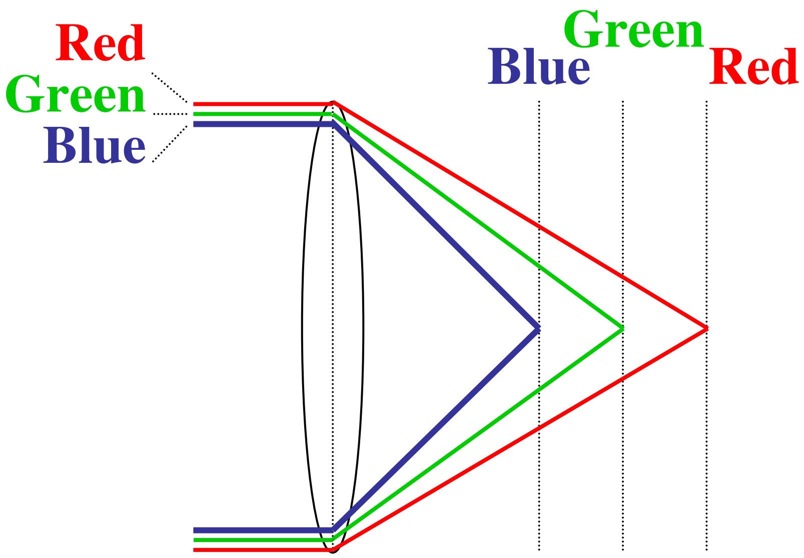
Antireflective Coatings



http://www.olympusmicro.com/primer/anatomy/objectives.html

Chromatic Aberration

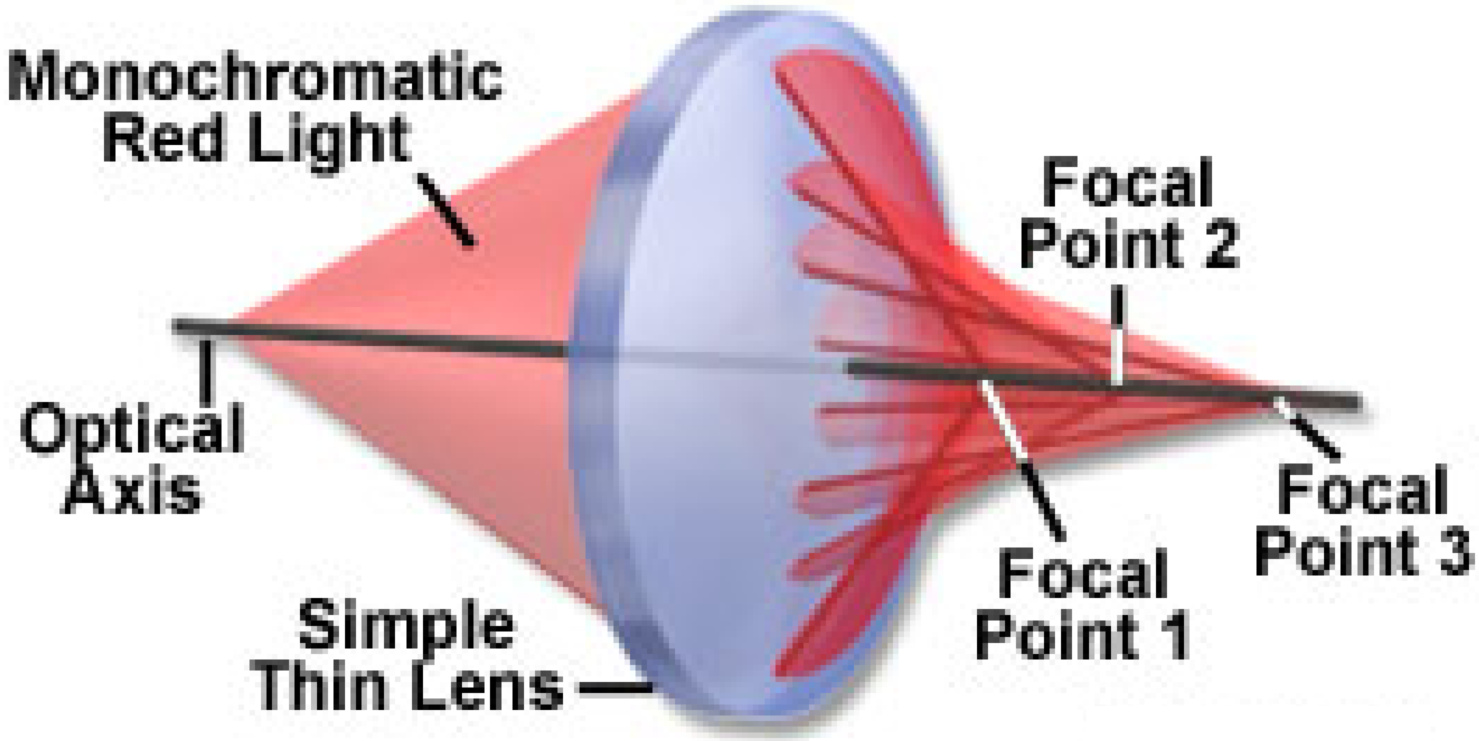




http://www.mic-d.com/curriculum/lightandcolor/aberrations.html

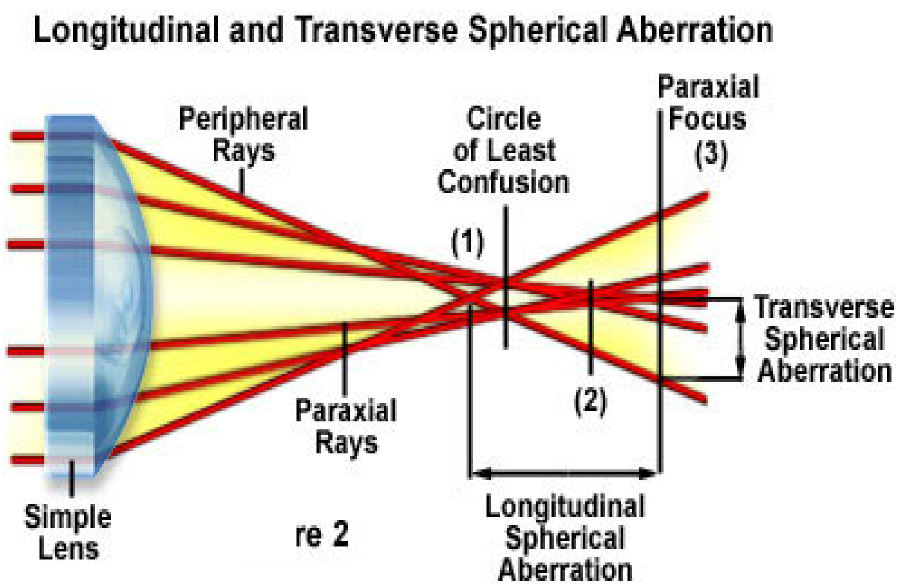
Light of a shorter wavelength is refracted more, therefore converges at a point closer to the lens.
Focal distance is different for different wavelengths of light.

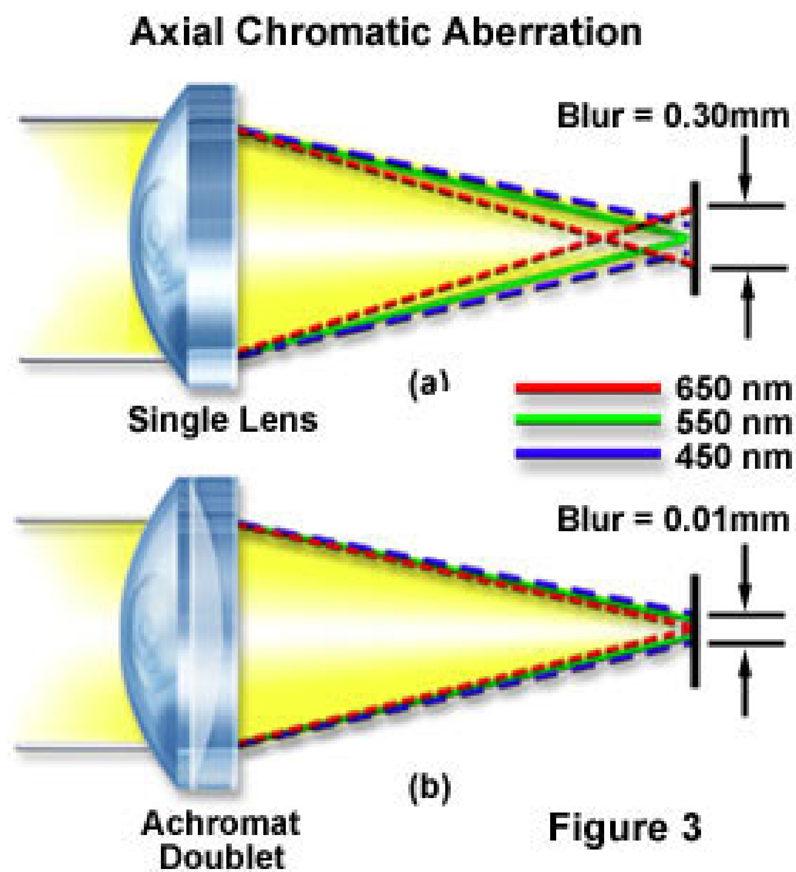
Spherical Aberration



http://www.mic-d.com/curriculum/lightandcolor/aberrations.html

Optical Aberrations





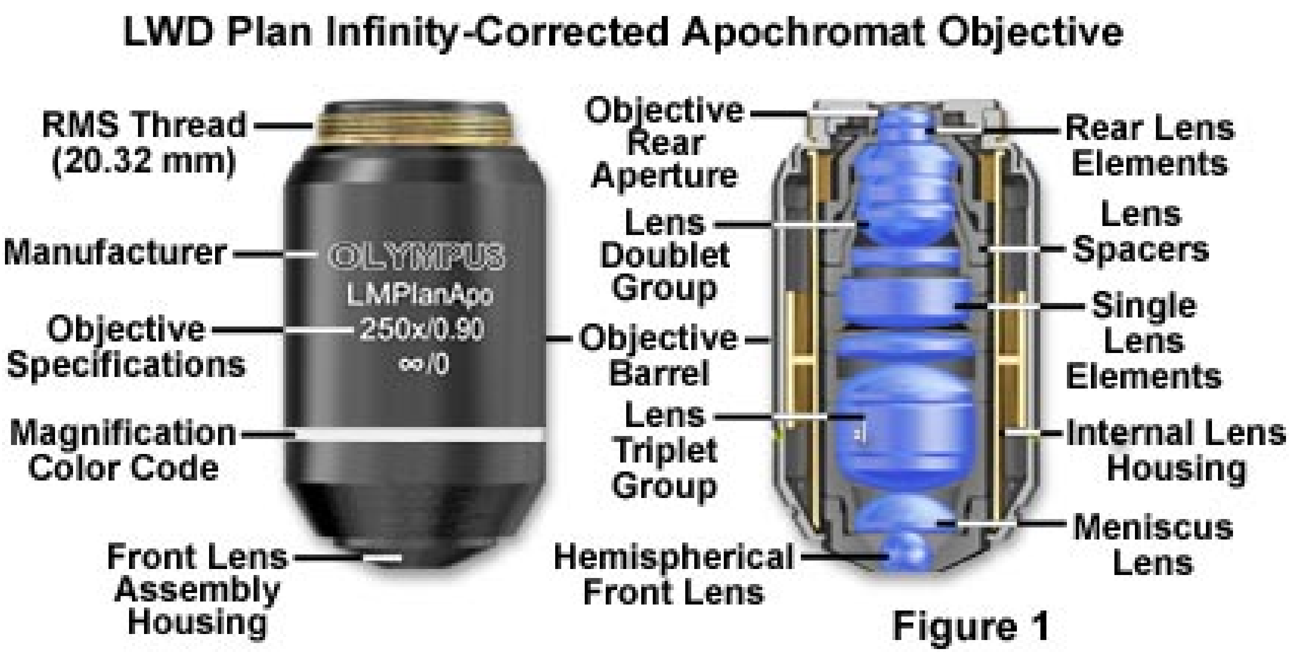
- **Spherical and chromatic aberrations are readily corrected in modern objectives**
- **Spherical aberrations (and astigmatism, among others) can be introduced through improper use or alignment**

- **Aberrations quickly degrade image quality**

Images from: http://www.microscopy.fsu.edu/primer/anatomy/aberrations.html

Microscope Objectives

- ***Higher power objectives have more optical elements***
- ***Only became possible with antireflection coating technology***
- ***Most important and expensive element of microscope***
- ***Somewhat compatible with other microscope manufacturers***



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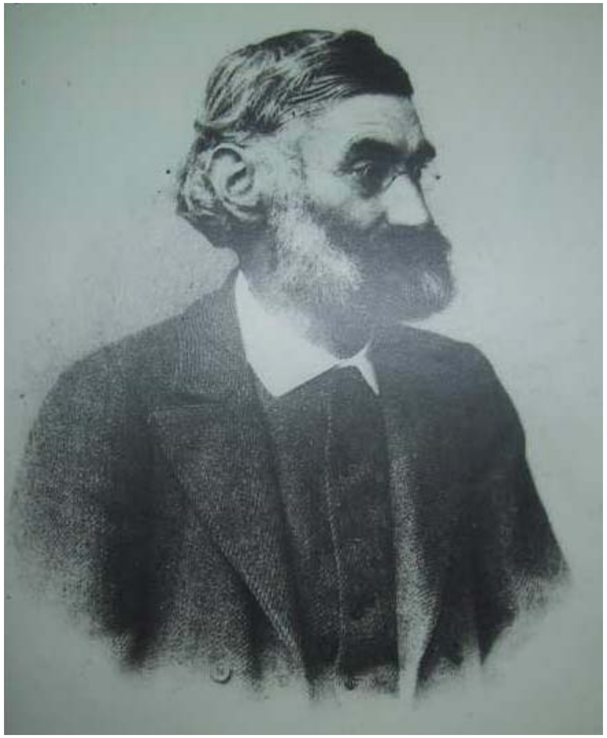
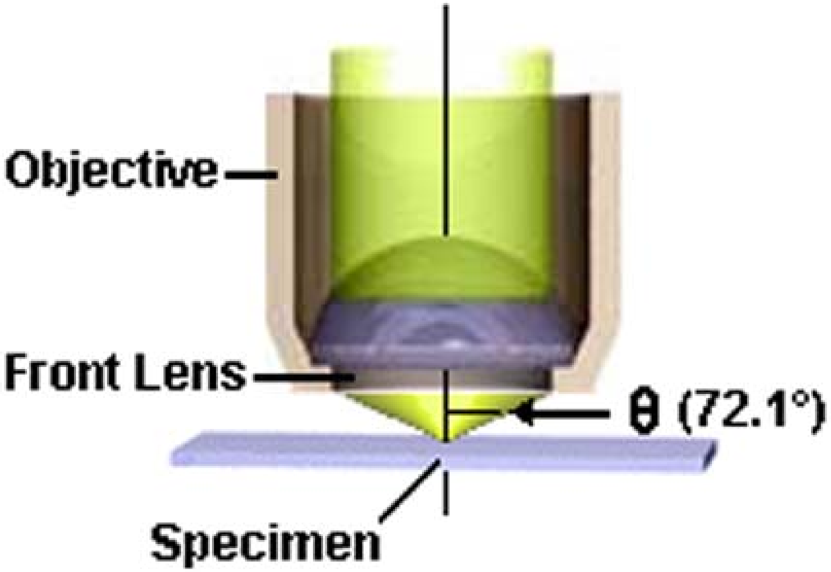
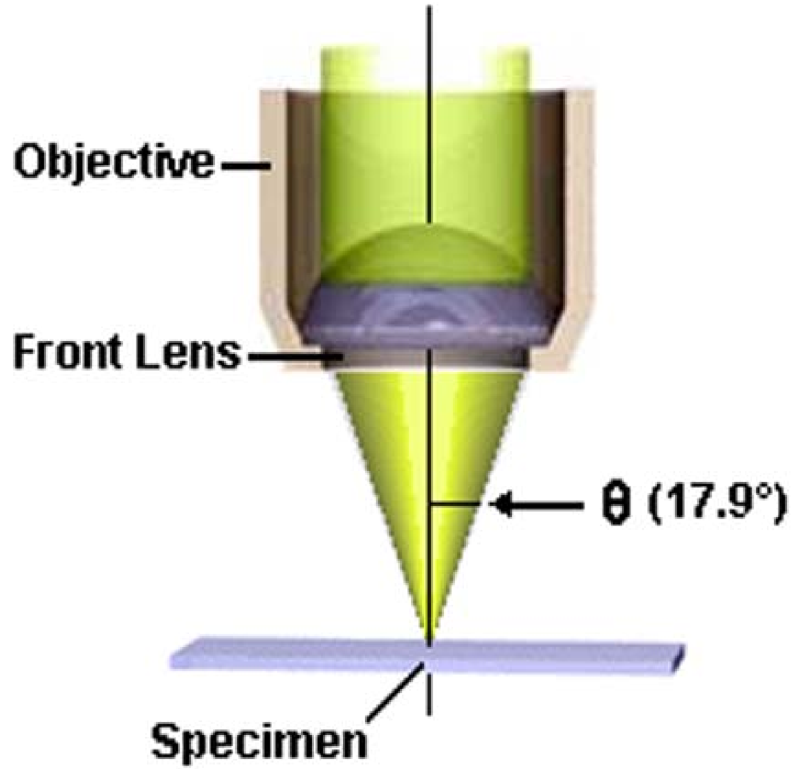


Numerical Aperature Light Cones



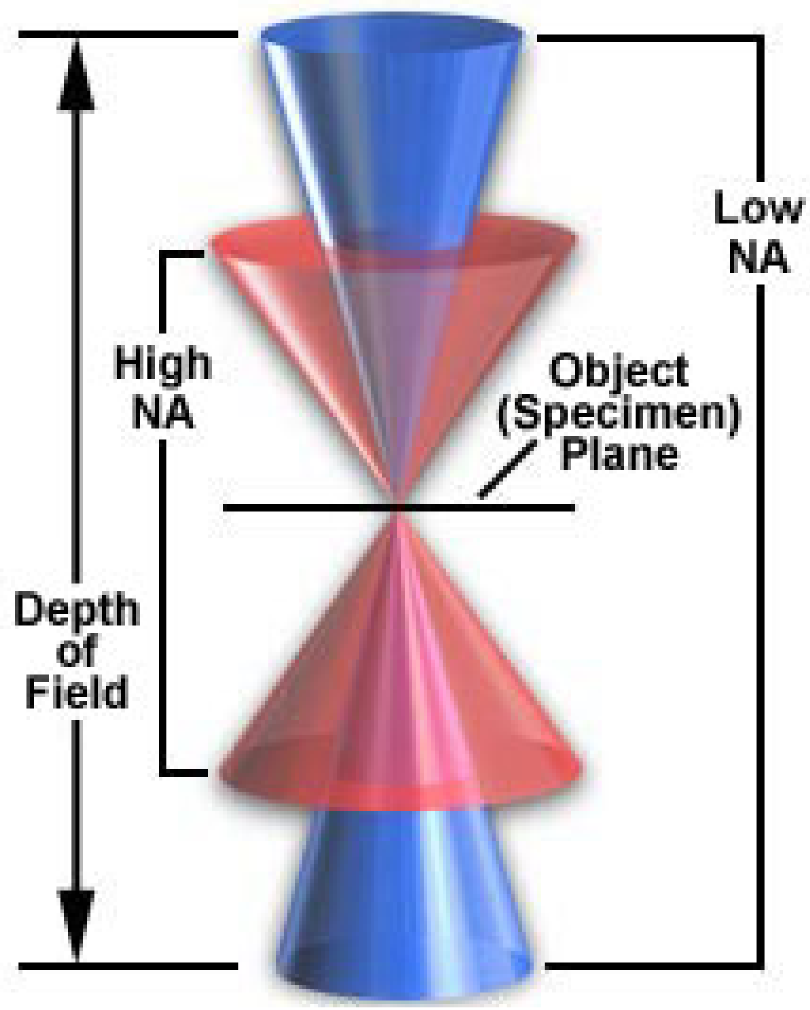


$$NA = n \cdot \sin(\theta)$$

- **To increase light collection, increase collection angle or change refractive index, n**
- **Resolution scales as λ/(2NA)**

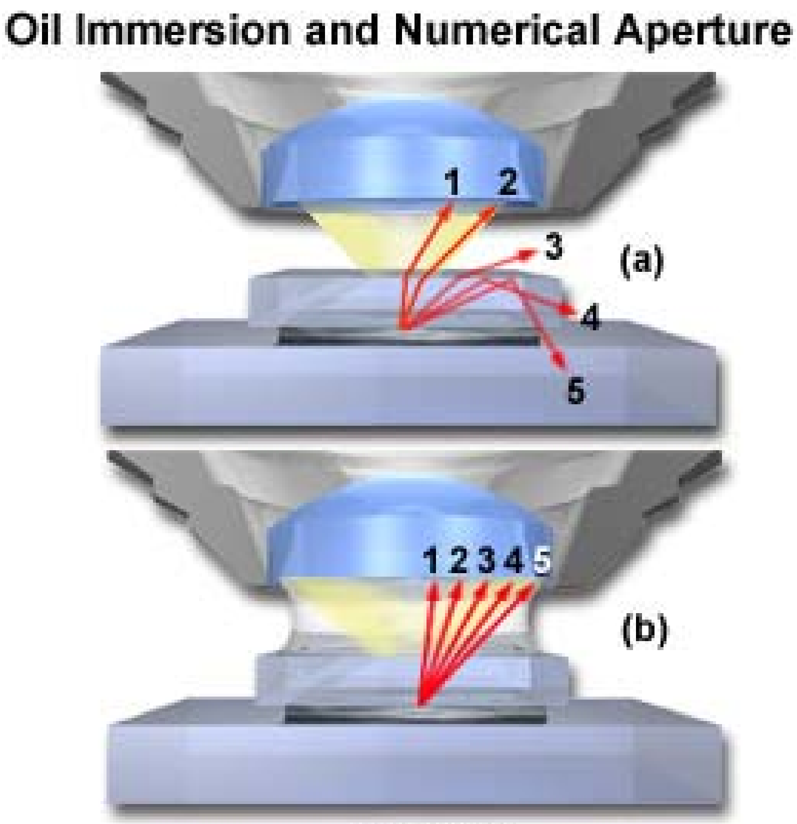
Images from: http://www.microscopy.fsu.edu/primer/java/nuaperture/index.html

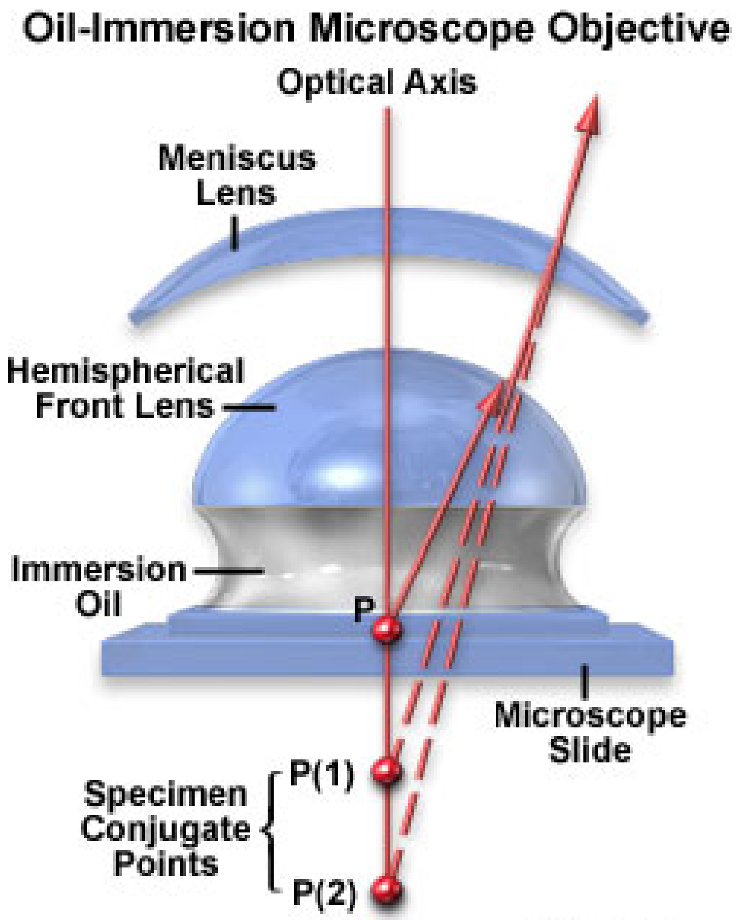
Depth of Field Ranges



http://www.olympusmicro.com/primer/anatomy/objectives.html

Oil Immersion





Immersion fluid (oil, n = 1.516) is matched to glass and increases light collection

Images from: http://www.microscopy.fsu.edu/primer/anatomy/immersion.html and http://www.microscopy.fsu.edu/primer/java/microscopy/immersion/index.html

Objectives

∝ - Infinity corrected

PLAN-APO-40X 1.30 N.A. 160/0.22

Flat field

Apochromat

Magnification Factor

Numerical Aperture

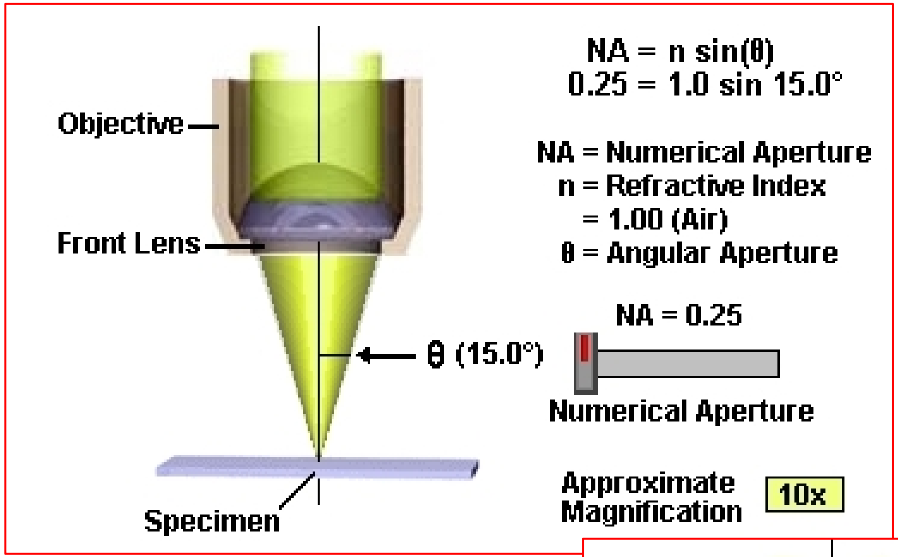
Tube Length

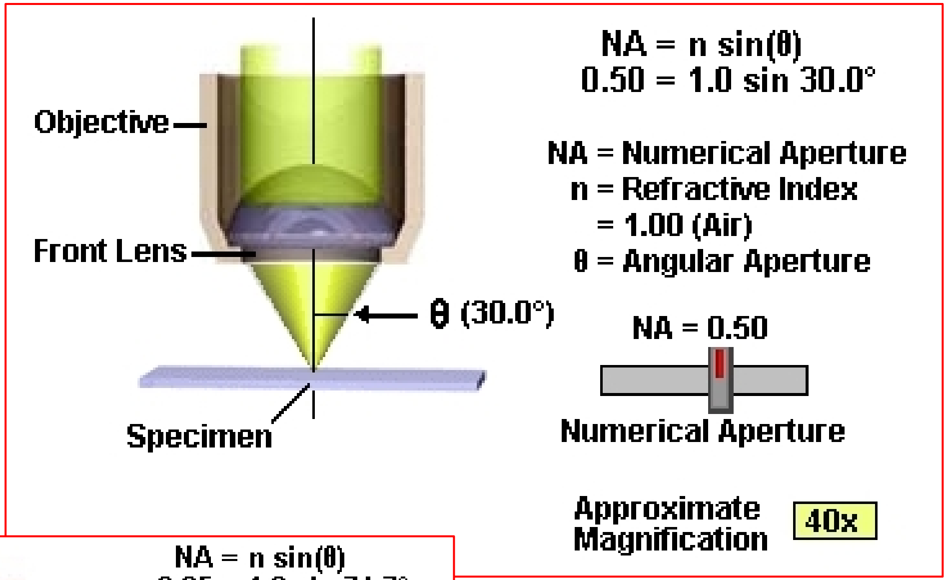
Coverglass Thickness

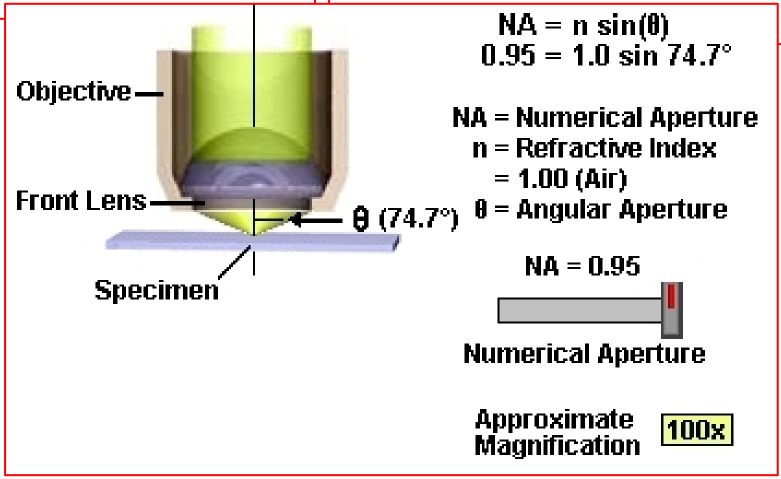
http://www.cyto.purdue.edu/

Numerical Aperture

- The wider the angle the lens is capable of receiving light at, the greater its resolving power
- The higher the NA, the shorter the working distance







Images reproduced from:

http://micro.magnet.fsu.edu/

Numerical Aperture

- For a narrow light beam (i.e. closed illumination aperture diaphragm) the finest resolution is (at the brightest point of the visible spectrum i.e. 530 nm)…(closed condenser).

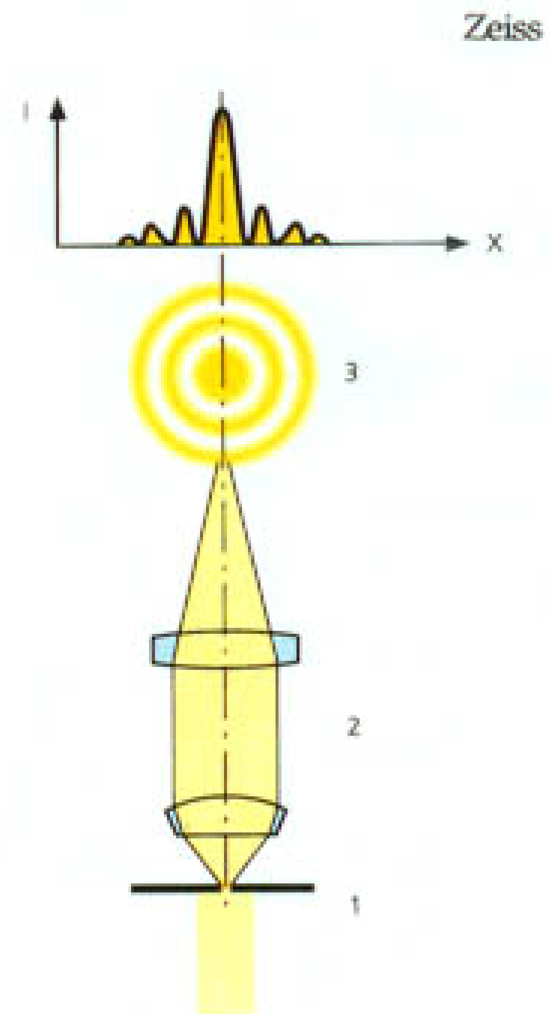
$$\frac{\lambda}{NA} = \frac{.00053}{1.00} = 0.53\ \mu m$$

- With a cone of light filling the entire aperture the theoretical resolution is…(fully open condenser)..

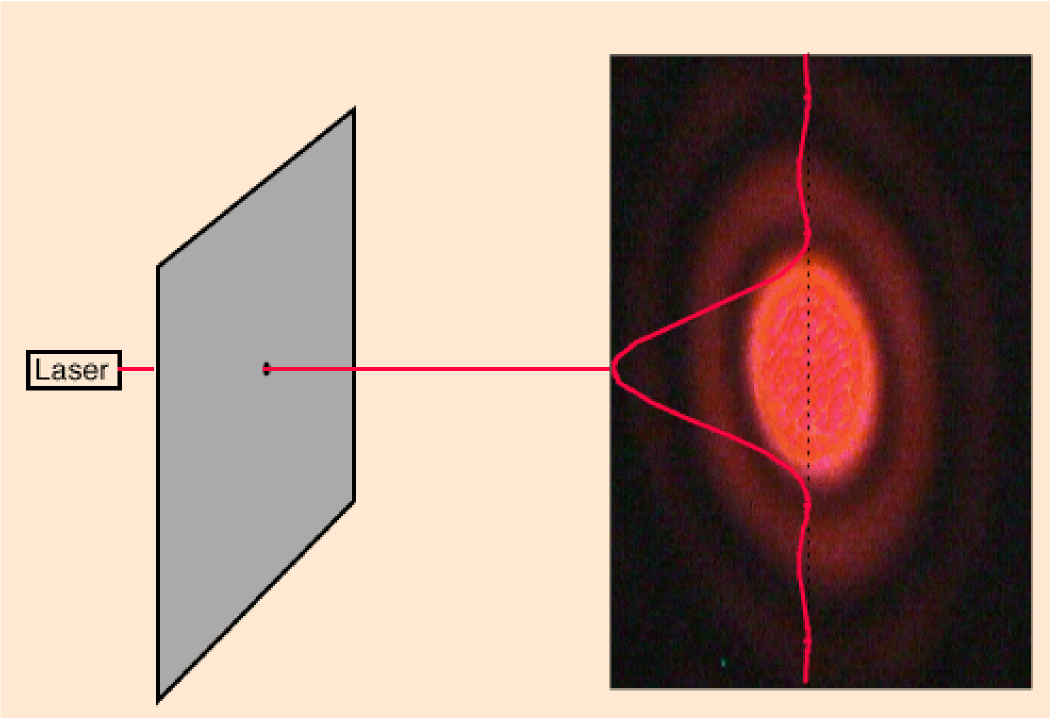
$$\frac{\lambda}{2 \times NA} = \frac{.00053}{2 \times 1.00} = 0.265\ \mu m$$

Airy Disk Formation by Finite Objective Aperture

The width of
central maximum
prop. to λ and
inversly prop. to
objective aperature



Diffraction by a circular aperture (lens ...)

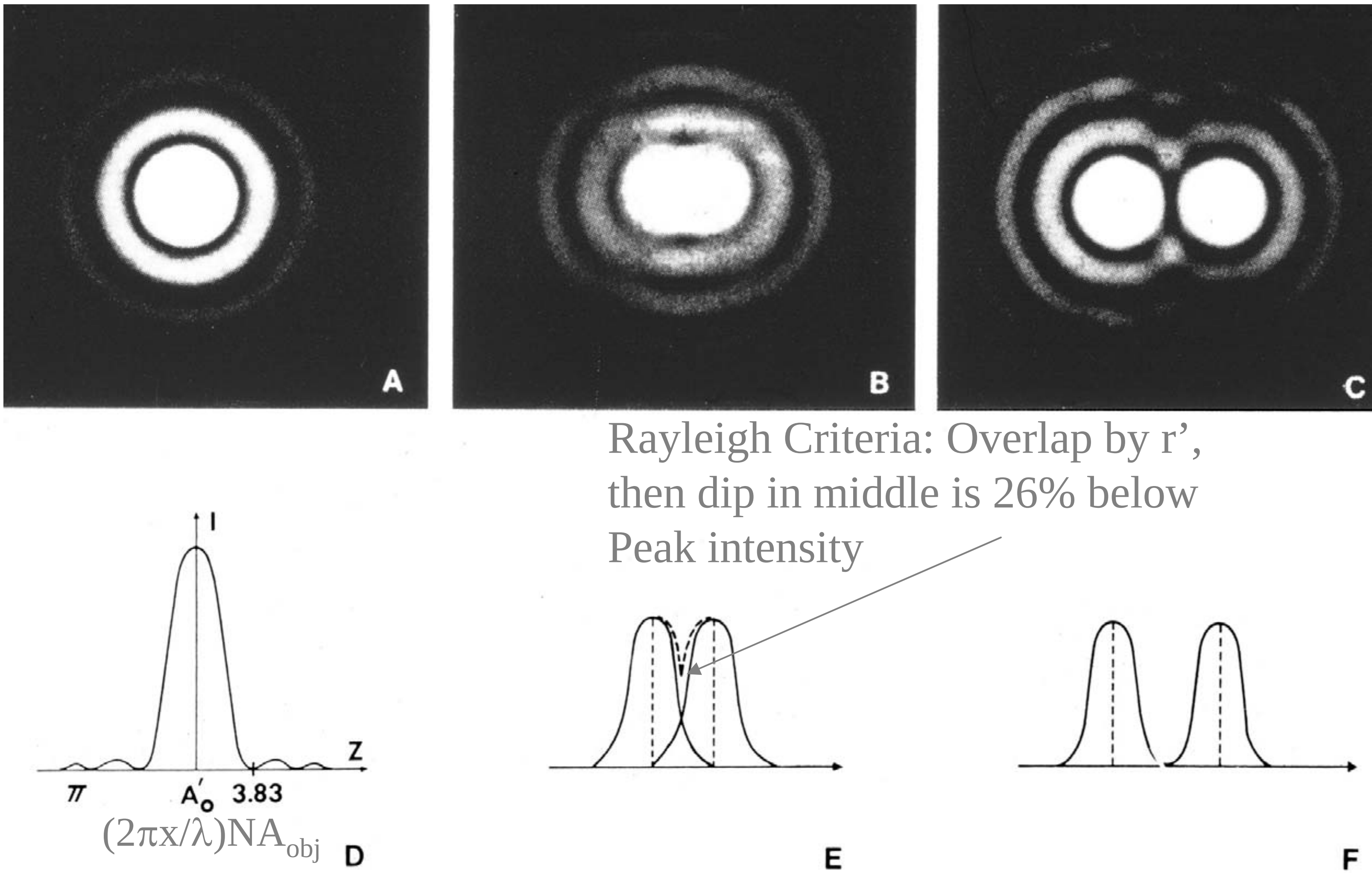


When light from a point source passes through a small circular aperture, it does not produce a bright dot as an image, but rather a diffuse circular disc known as Airy's disc surrounded by much fainter concentric circular rings. This example of diffraction is of great importance because the eye and many optical instruments have circular apertures.

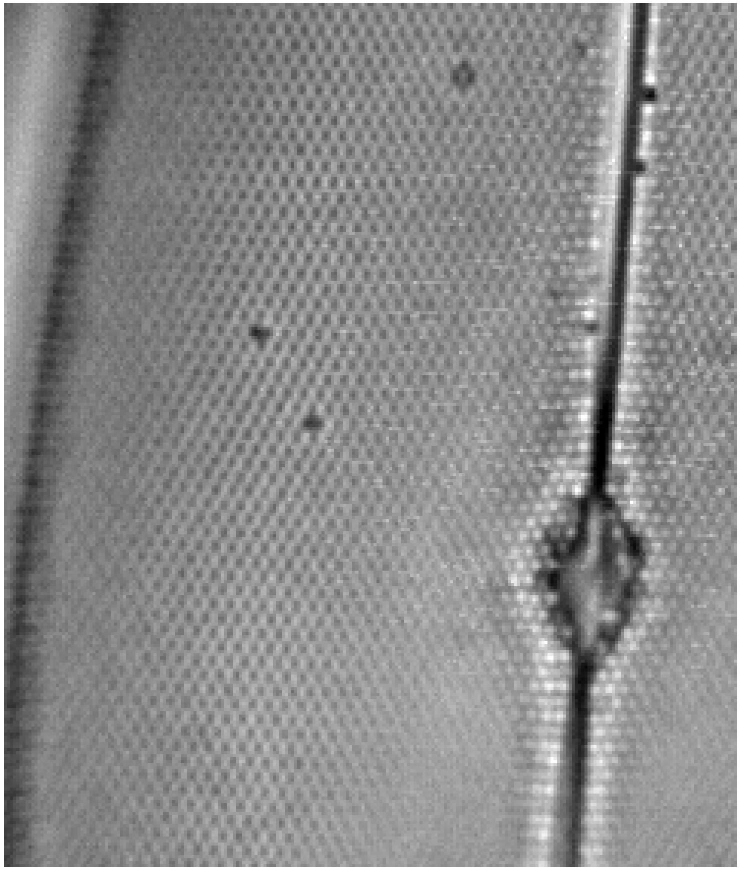
A complex analysis leads to $$\sin\theta = 1.22\frac{\lambda}{d}$$ First minimum

Compare to the relation for a single slit $$\sin\theta = \frac{\lambda}{d}$$

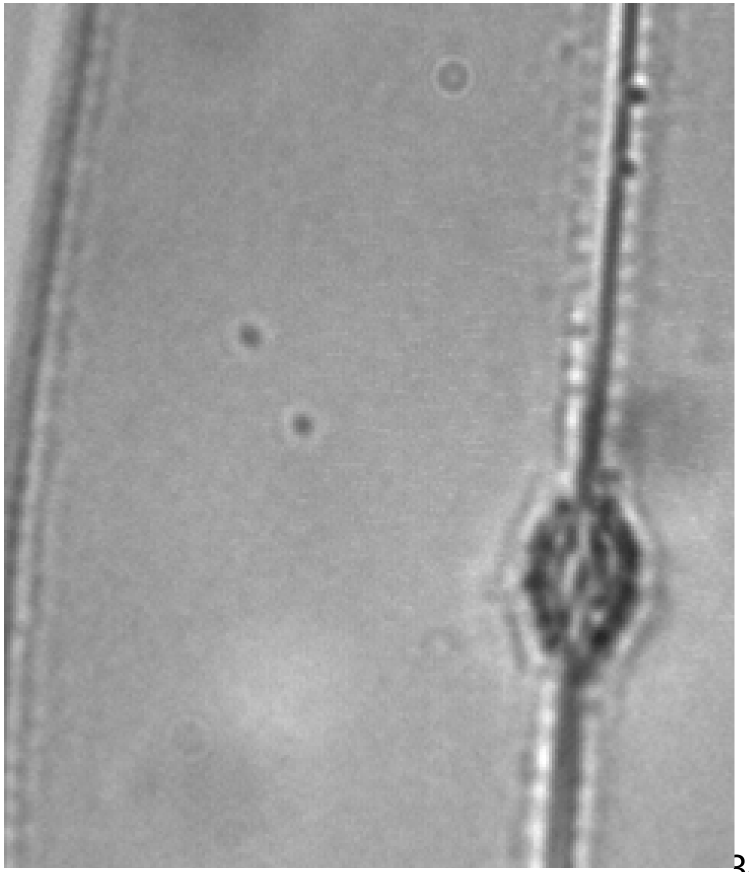
Lateral Resolution in Fluorescence Depends on Resolving Overlapping "Airy Disks"



Resolution is better at shorter wavelengths: higher objective NA and/or higher condenser NA



High NA and/or shorter λ



Low NA and/or longer λ

Periodic Diffraction Gratings

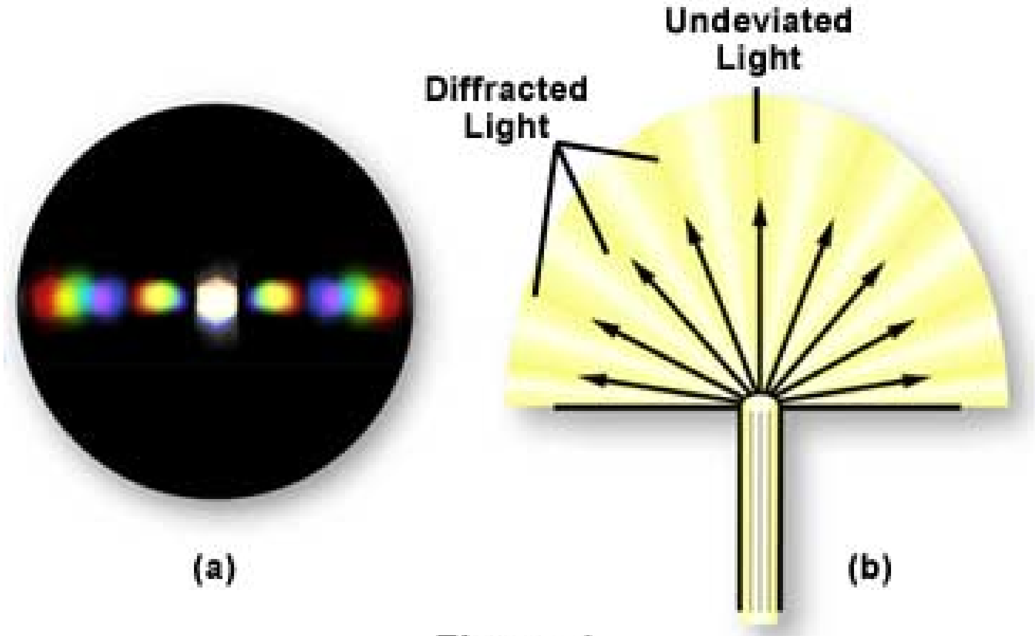
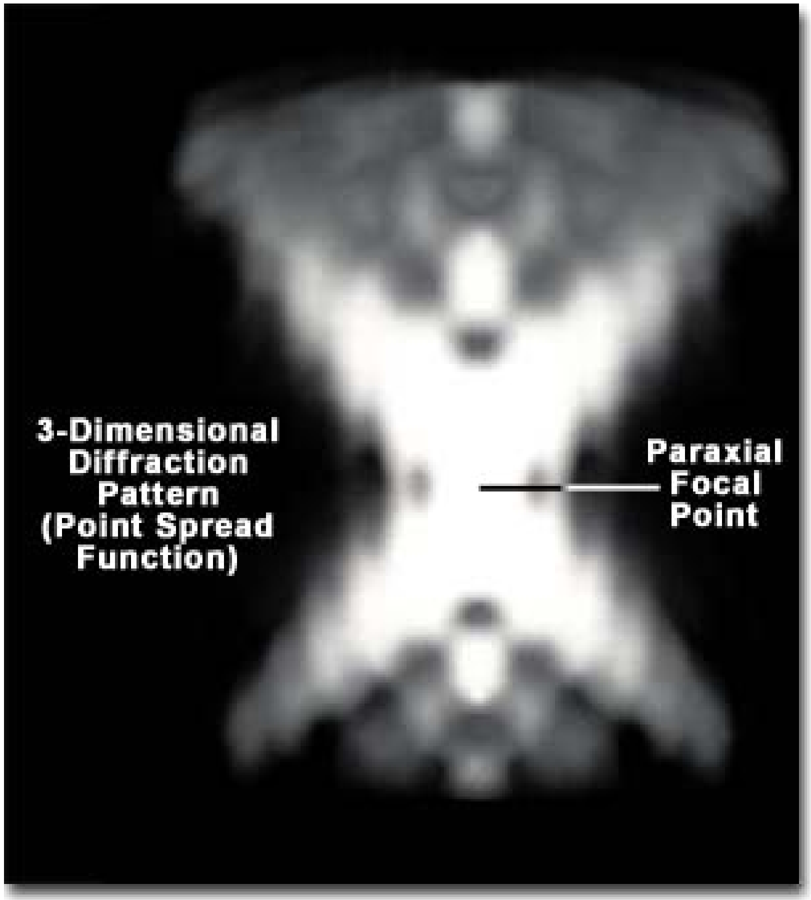


Figure 4

- **Imaging a diffraction grating provides clear demonstration of Abbe's principle of image formation**
- **Different diffracted orders yield well-defined spatial frequencies in the back focal plane and are re-diffracted to yield an image**
- **Image is object convoluted with optical response (PSF)**

Image from: http://www.microscopy.fsu.edu/primer/anatomy/image.html

Axial Resolution



Simulated 3-dimensional diffraction pattern near the paraxial focal plane of a point source of light focused by a lens with uniform circular aperture.

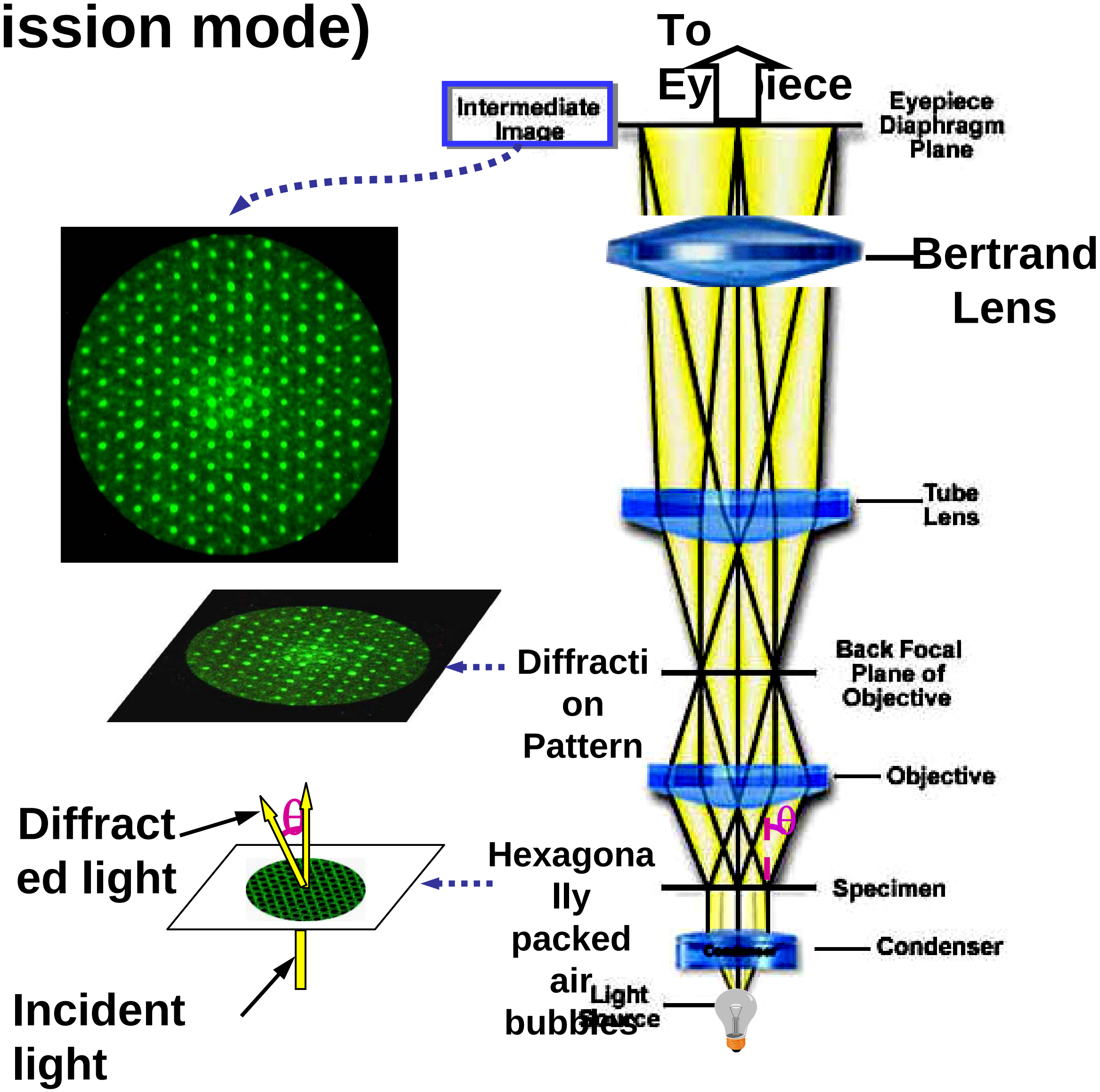
- Incorporate many spatial frequencies in x and y – good resolution
- Poor resolution in z – very few spatial frequencies.
- Light from different planes is out of focus, but still has high intensity.
- Confocal and multiphoton microscopy incorporate more axial spatial frequencies to generate z-resolution.

D ~ $\lambda n/NA^2$, depth of field

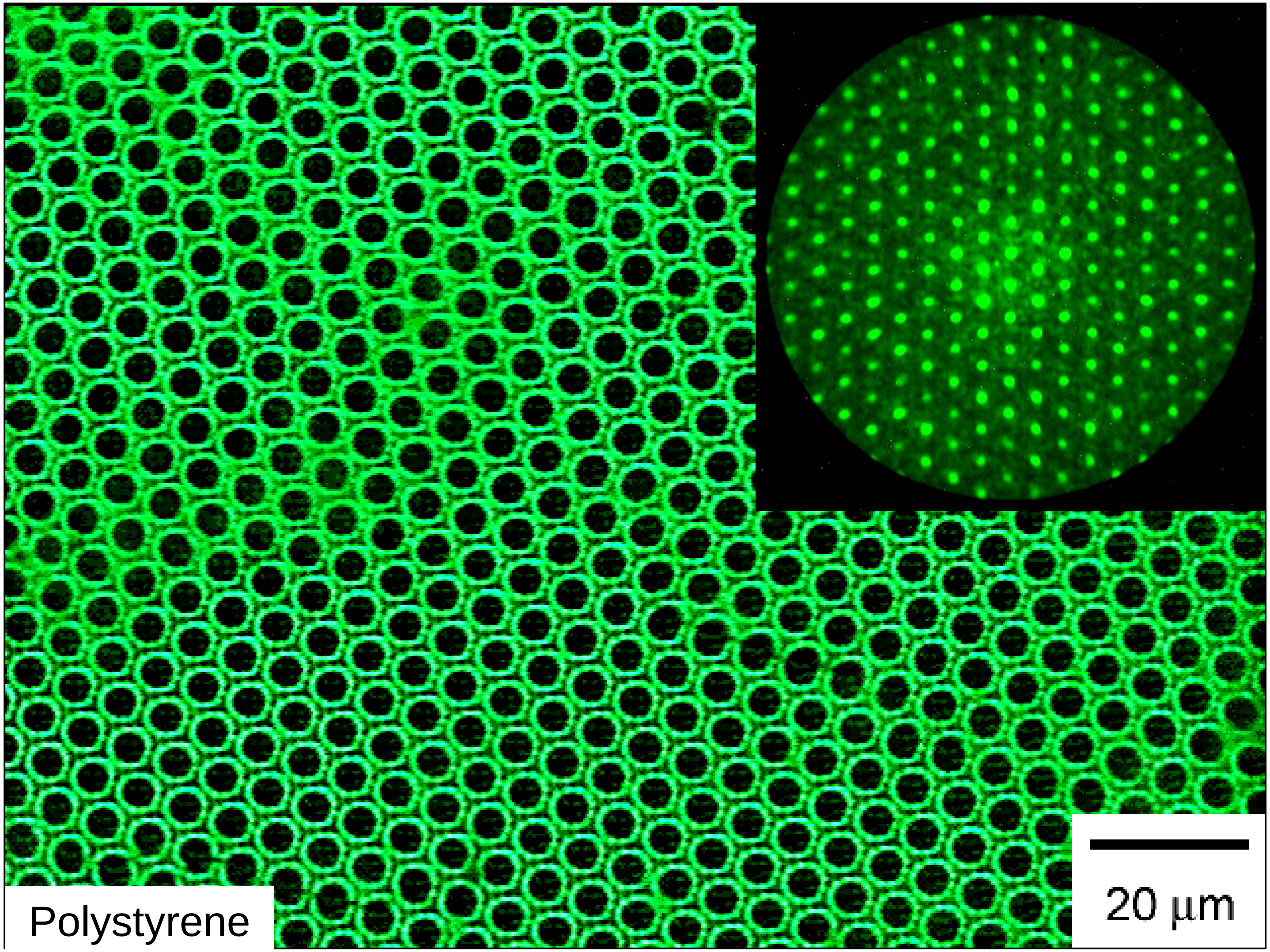
Image from:
http://www.microscopy.fsu.edu/primer/java/imageformation/depthoffield/index.html

Image Formation

(Transmission mode)



On Volatile Fluid Sufaces: Hexagonal Order



Desire to Increase the Resolution - ~1927, 28

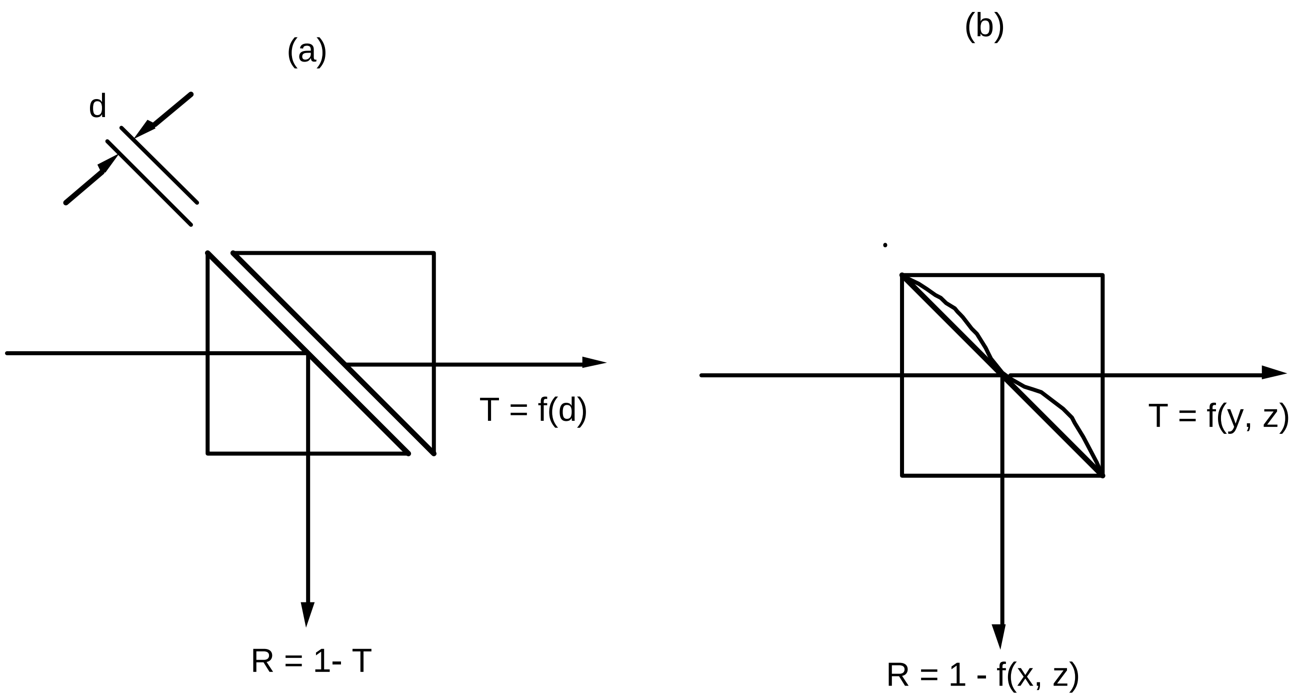
I T is generally accepted as an axiom of microscopy that the only way to extend resolving-power lies in the employment of light of smaller wave-lengths. Practical difficulties, however, rapidly accumulate as light of increasingly small wave-length is brought into service, and probably little hope is entertained of arriving at a resolution much beyond 0.1μ, with perhaps, 0.05μ as an extreme limit.

Yet a method offers itself which lies a little outside the beaten track of microscopic work and raises various technical problems of a new kind, but which makes the **attainment of a resolution of 0.01μ**, and even beyond, dependent upon a technical accomplishment which does not seem impracticable at present. The idea of the method is exceedingly simple, and it has been **suggested to me by a distinguished physicist** that it would be of advantage to give it publicity, even though I was unable to develop it in more than an abstract way.

Mr. E.H. Synge on a Method, Phil Mag. 6, 358 (1928)

Beam Splitter based on FTIR

(FTIR: frustration of total internal reflection)



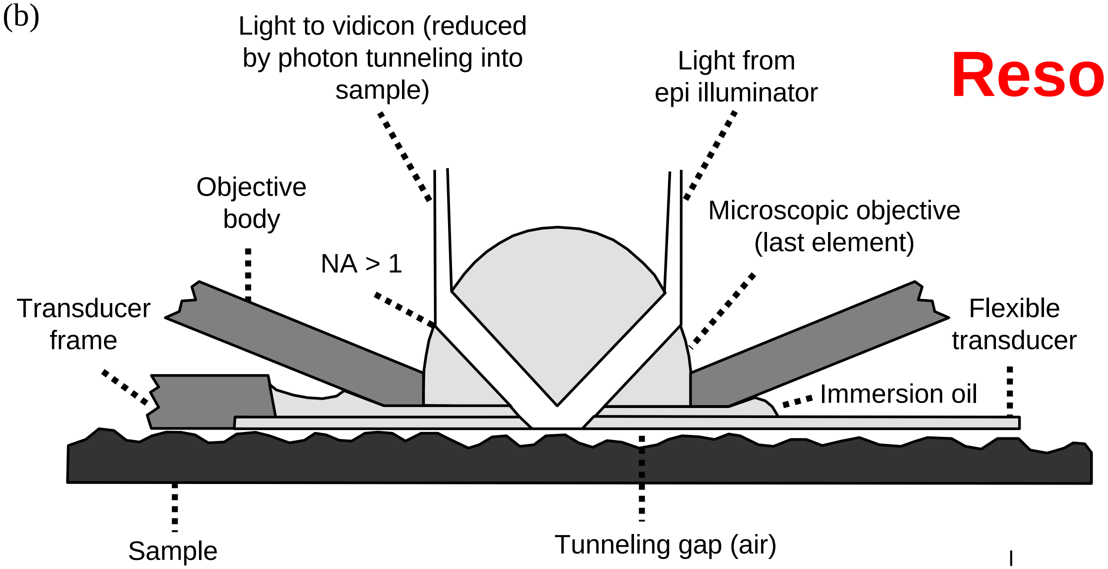
$$\mathbf{E_{evanescent} = E_o \exp (-z/d_p)}$$

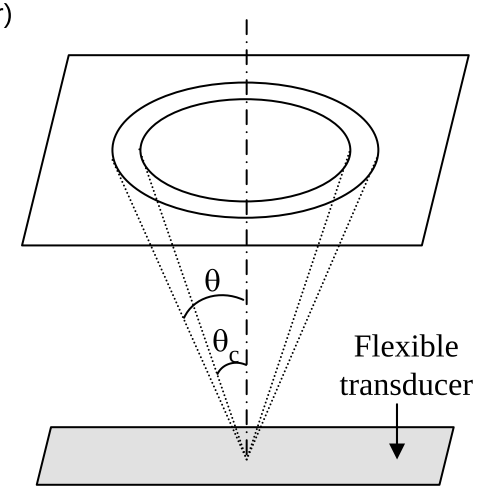
$$d_p = \frac{\lambda_1}{2\pi(\sin^2\theta - n_{21}^2)^{1/2}}$$

Photon Tunneling Microscopy

Resolution ~ λ/6

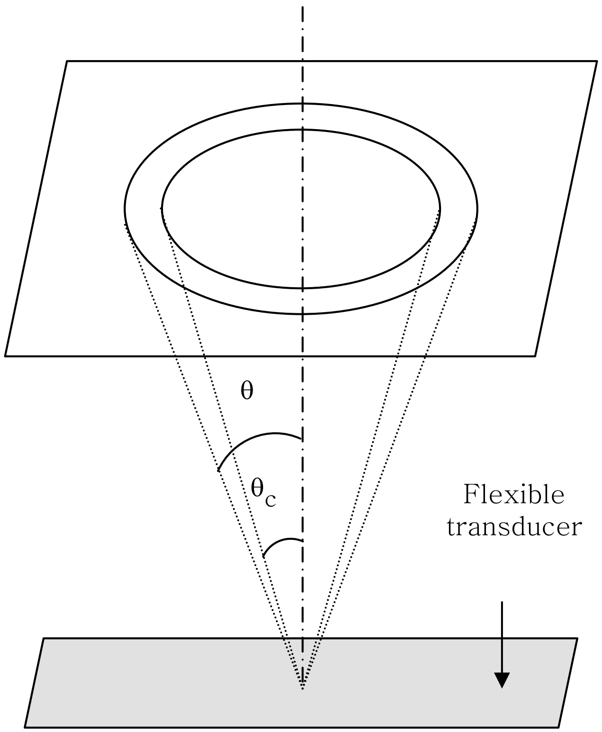
(b)

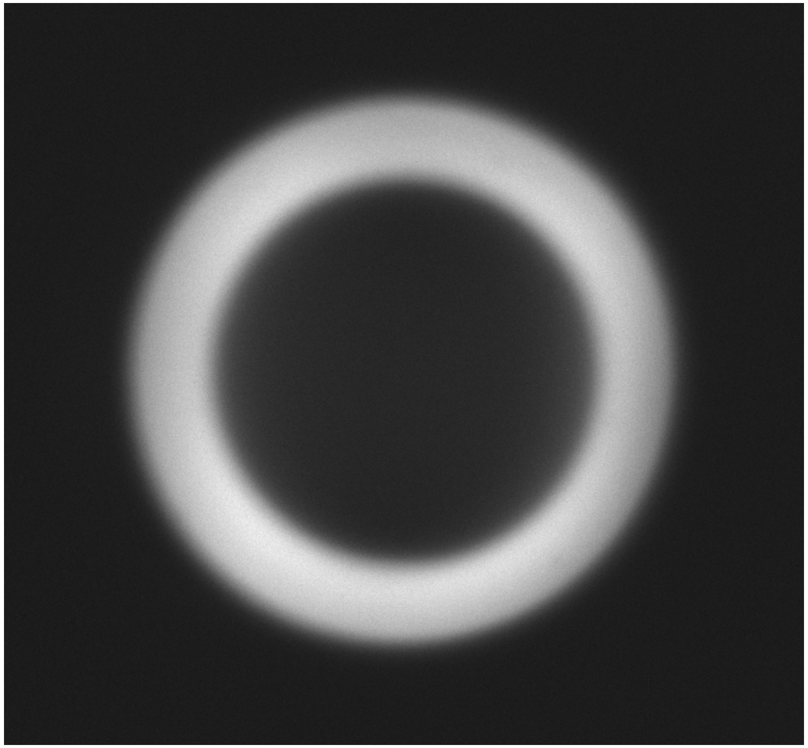




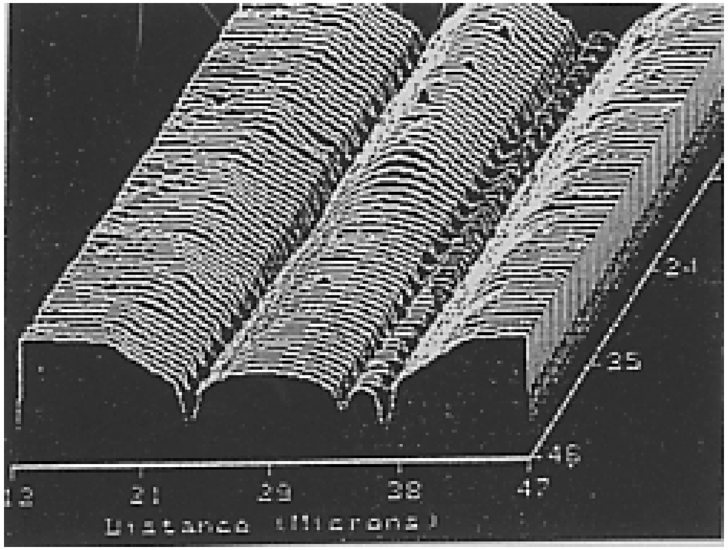
Guerra, Srinivasarao, Stein, ***Science*****, 1993**

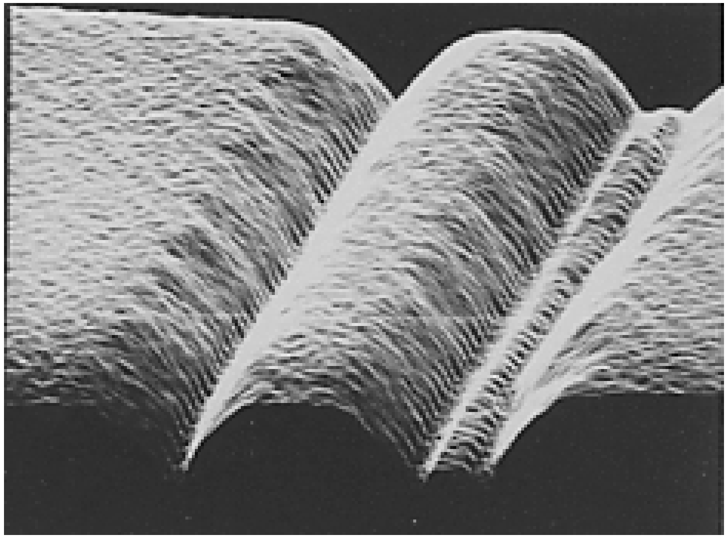
Photon Tunneling Microscopy-Contd

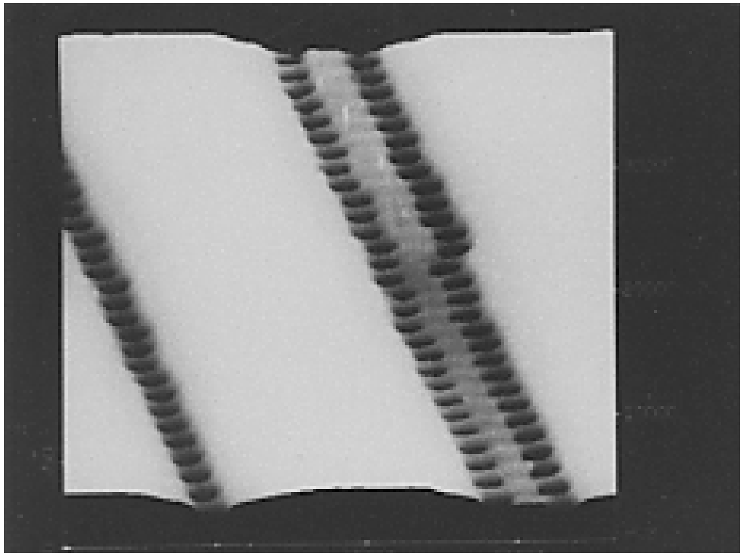


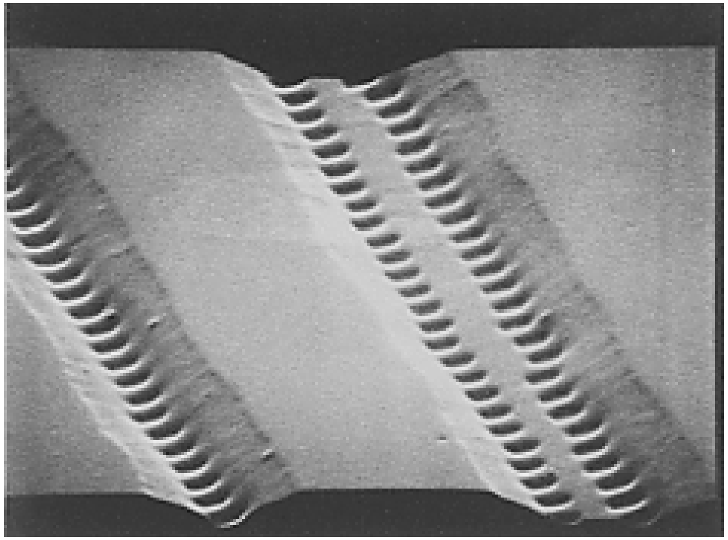


Photon Tunneling Microscopy-Contd



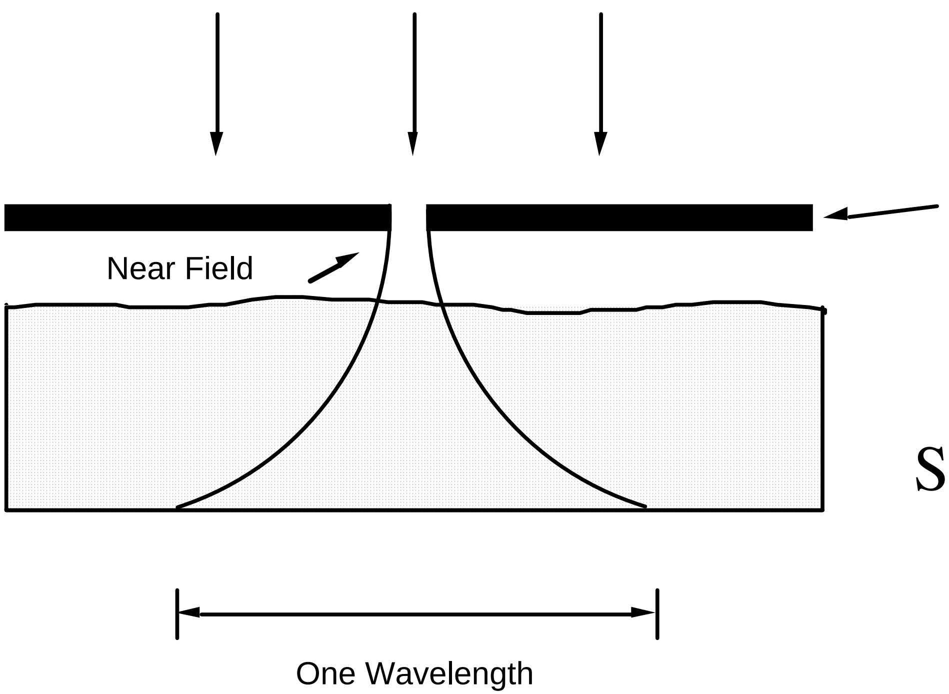






Subwavelength Aperture

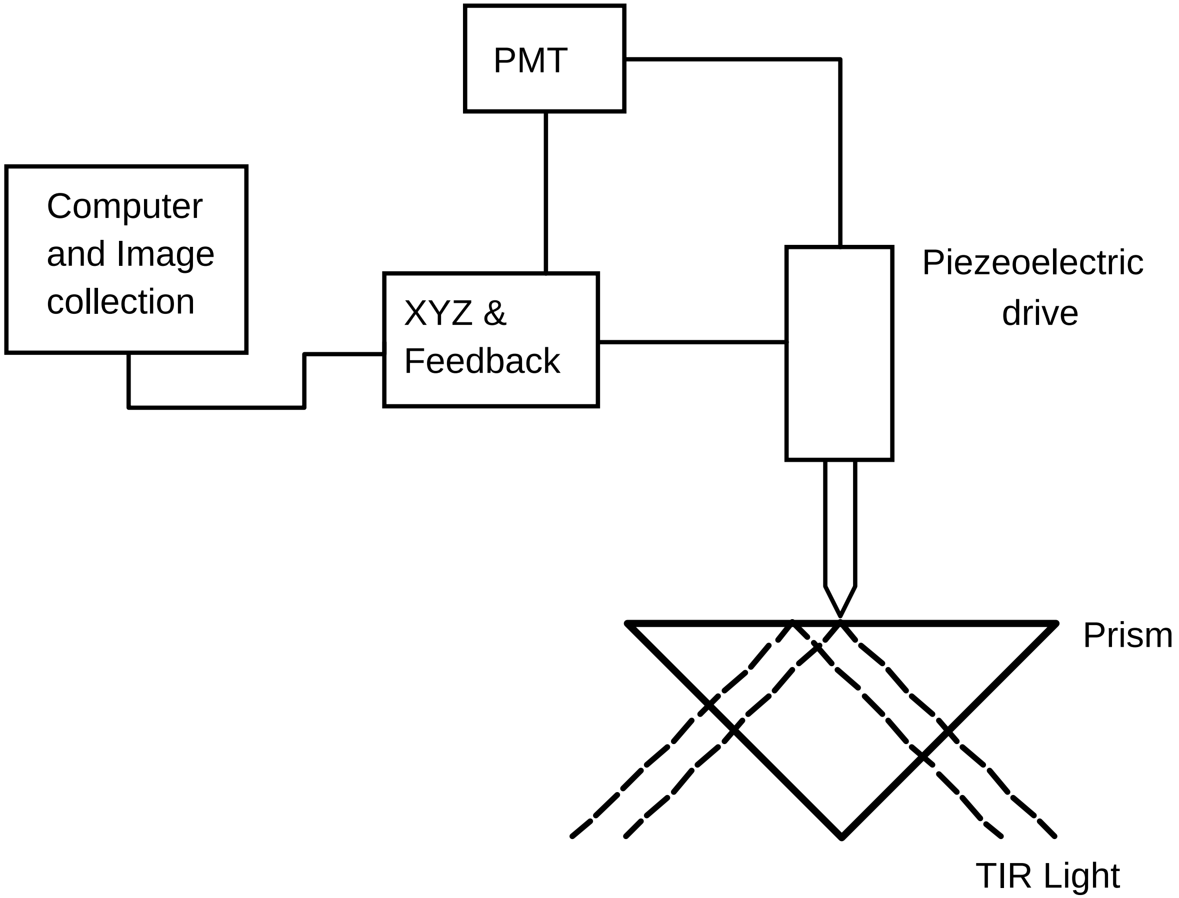
Resolution ~ $\lambda/43$



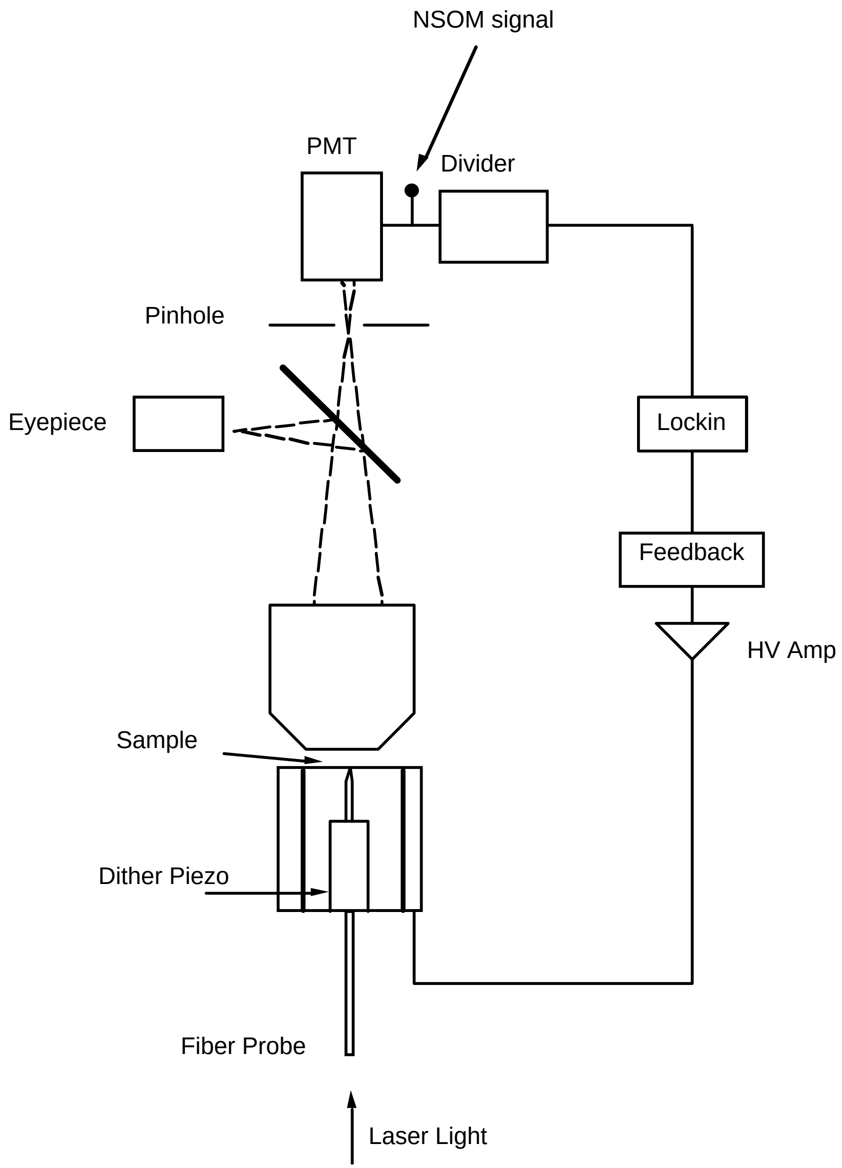
Synge, 1927, 1928

Betzig and Trautman, *Science*, 1991,1992

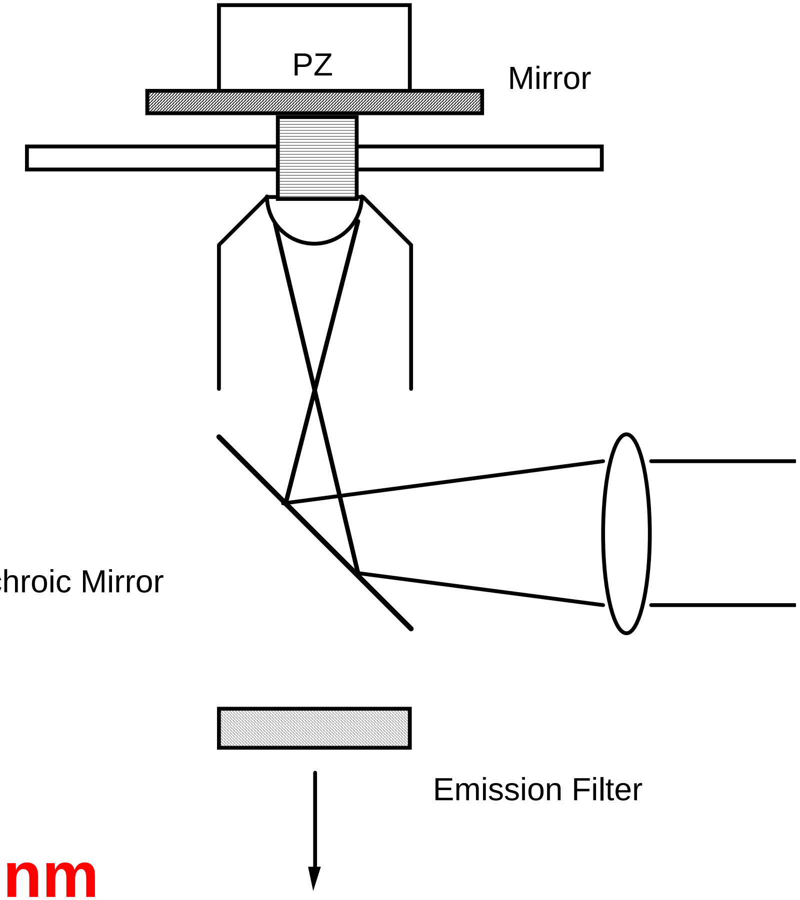
Photon Scanning Tunneling Microscopy



Near-field Scanning Optical Microscope



Reflected-Beam SWFM



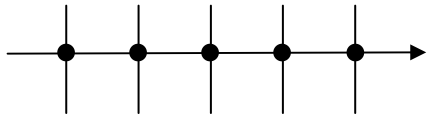
Axial Resolution ~ 35nm

SWFM: Standing Wave Fluorescence Microscopy

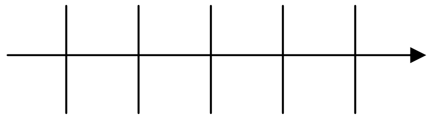
Lanni, 1993

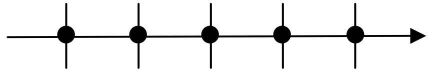
Polarized Light

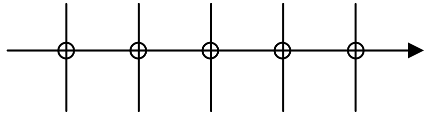
Representation of states of plane-polarization

(a)  **Unpolarized Light**

(b)  **Polarized perpendicular to plane of page**

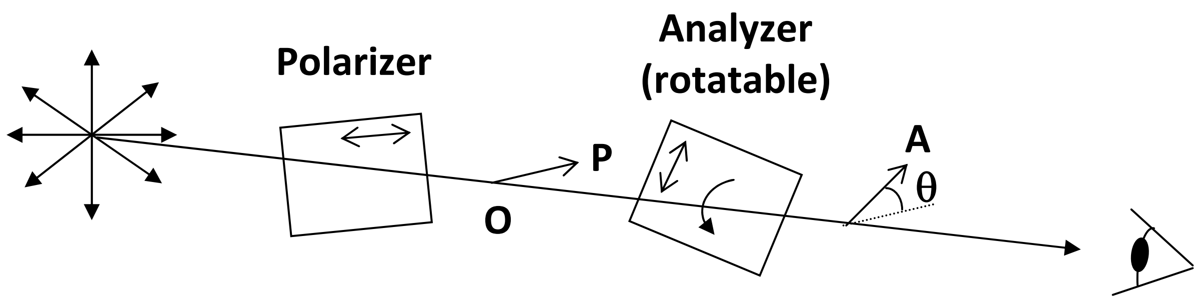
(c)  **Polarized in plane of page**

(d)  **Partially polarized perpendicular to plane of page**

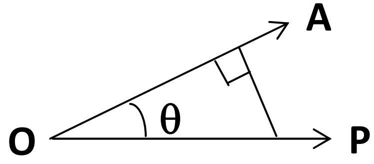
(e)  **Partially polarized in plane of page**

Ref. Optical Methods in Biology, John Wiley & Sons, 1970

Polarizer and analyzer relationship



*** Vectorial Projection for Ideal Polarizers:**



$$OA = OP \cos\theta$$

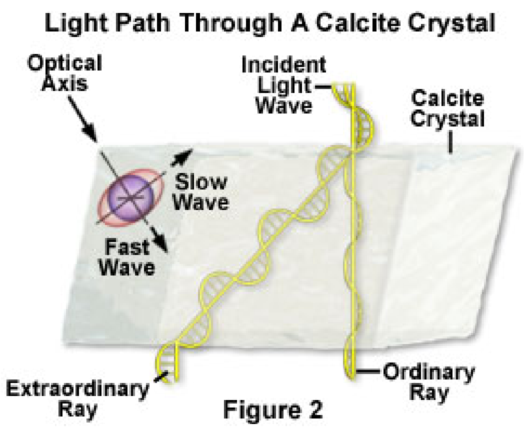
$$I_a = OA^2 = OP^2 \cos^2\theta$$

$$I_a = I_p \cos^2\theta$$

Law of Malus (1775-1812)

Birefringence (Double Refraction) in Calcites

Crystals of the mineral Iceland spar (a transparent, colorless variety of calcite) produces a double image when objects are viewed through the crystals in transmitted light (Erasmus Bartholin ~ 1669).



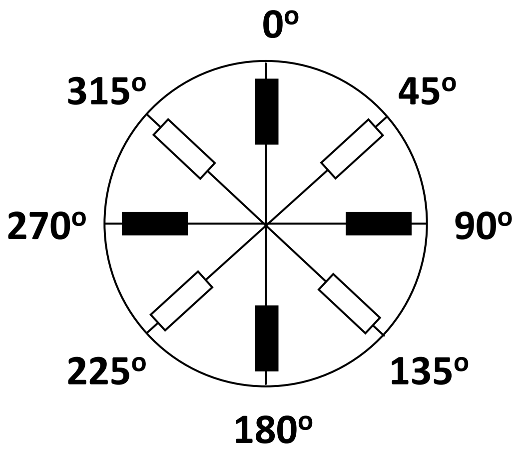
When the Iceland spar crystal is rotated, one of the images remains stationary, while the other precesses around the first. This is due to the strong birefringence in calcite, which separates the two refracted light rays by a wide margin. One of the light waves, termed the ordinary ray, travels straight through the crystal (its image remains stationary), while the other ray is refracted to a significant degree. The refracted ray is termed the extraordinary ray, and is the one that precesses around the ordinary ray.

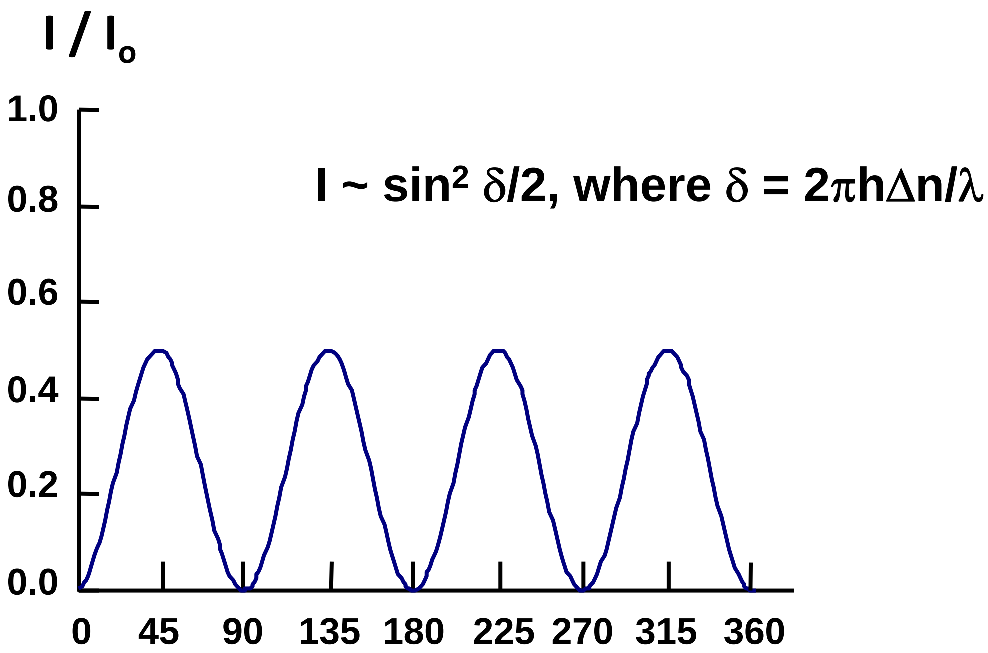
Birefringence (B) = $|n_e - n_o|$

The ordinary and extraordinary light rays, when passed through a polarizer, have permitted electric vibration directions that are mutually perpendicular. The extraordinary ray always vibrates in a plane that joins it and the ordinary ray, while the ordinary ray always vibrates at right angles to this plane. The speed with which the light rays pass through the crystal also varies. Calcite has negative birefringence so the ordinary ray travels slower than the extraordinary ray.

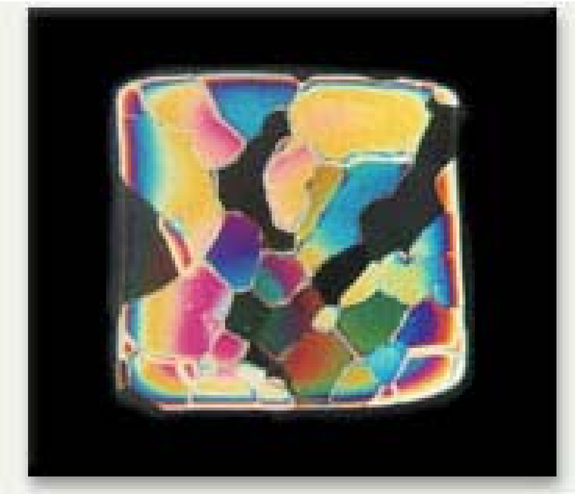
http://www.olympusmicro.com/primer/java/polarizedlight/icelandspar/index.html

Angular Dependence of the Intensity



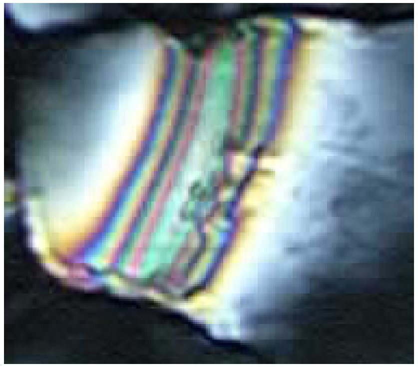


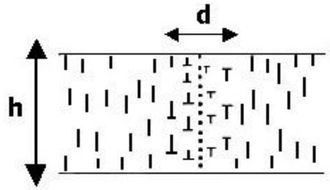
COLORS DUE TO INTERFERENCE



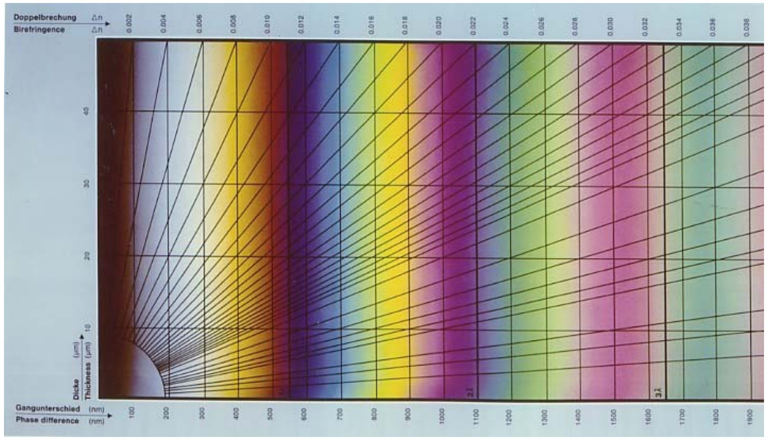
Interference colors in a thinned ice cube, 3 cm across (between crossed polarizers).

$I \sim \sin^2 \delta/2$, where $d = 2\pi h \Delta n/\lambda$

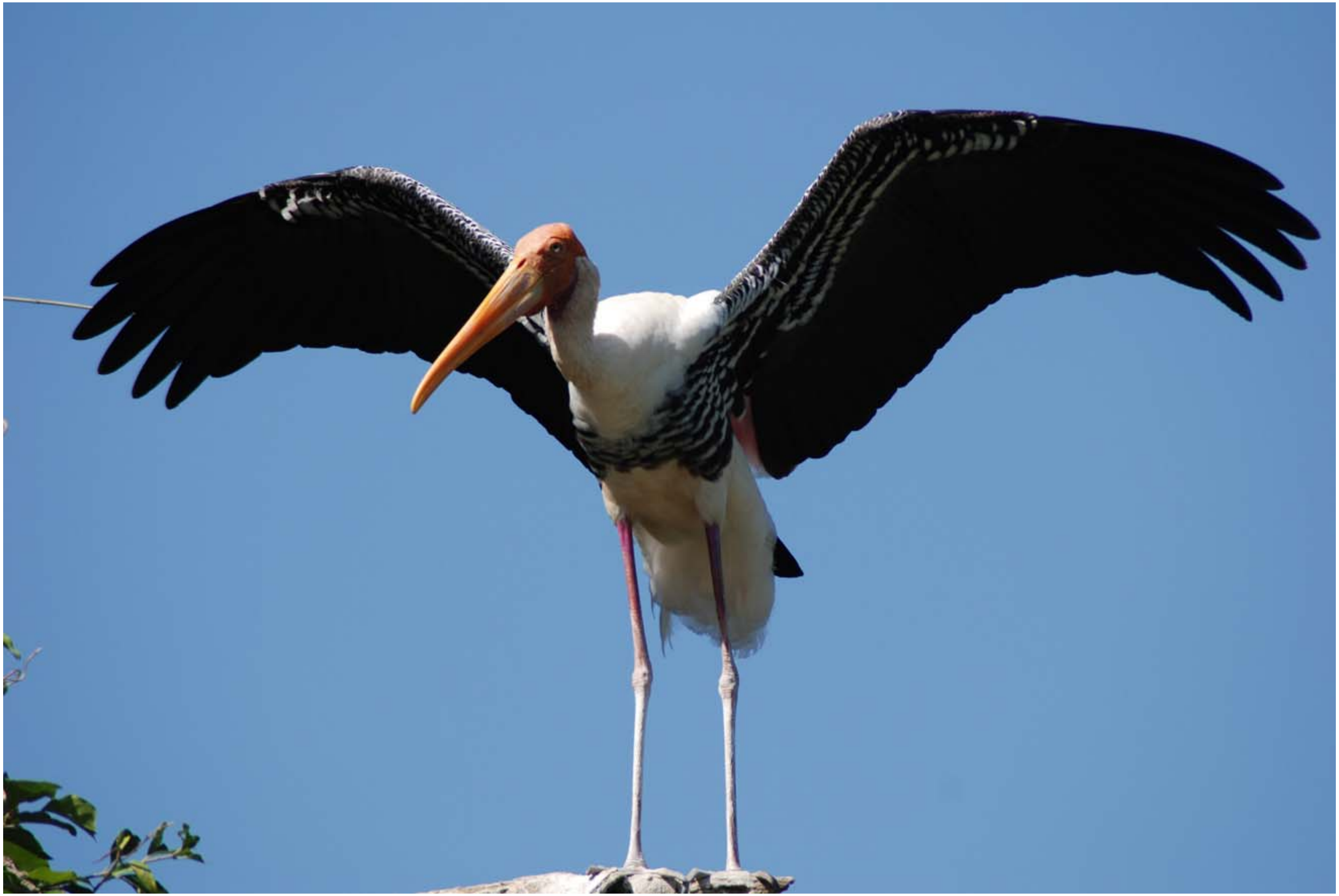




Wall Defects

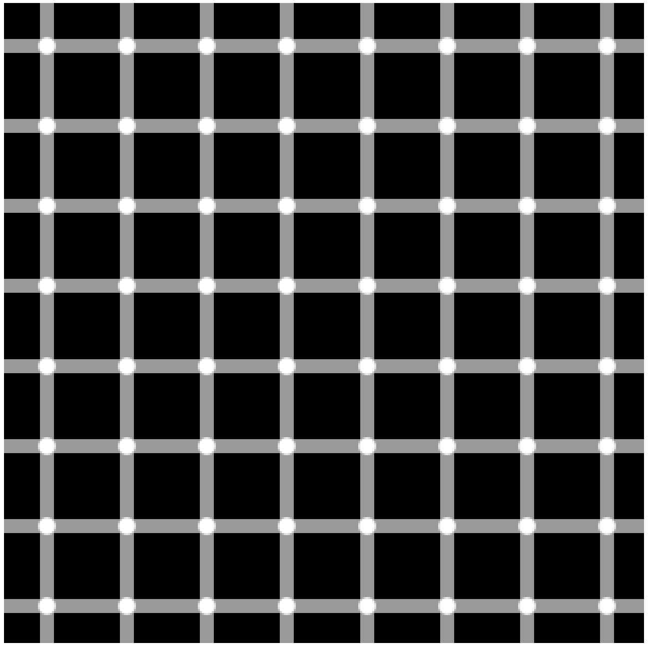


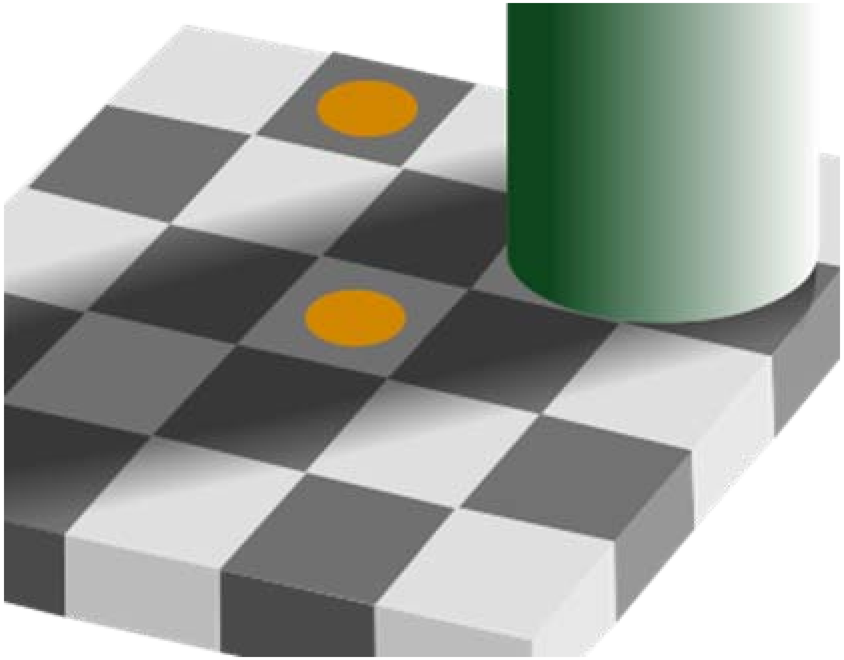
Michel-Levy Chart



Illusions – Tricks that the Brian Plays

Seeing is Believing: Are you sure?

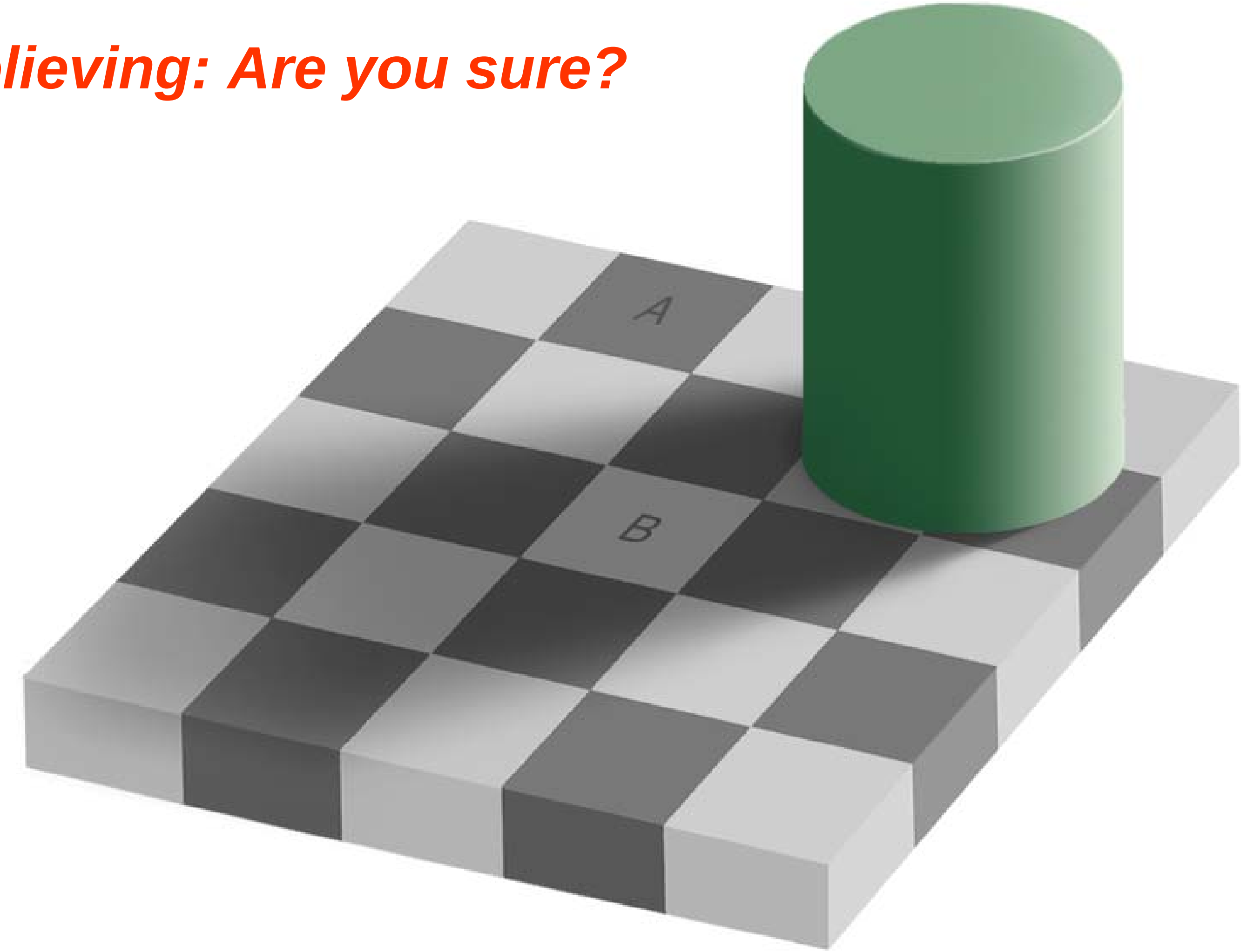




How many of you think the colored object has the same Reflectance as the other one?
(Difference in Context)

Illusions-2 – Tricks that the Brian Plays

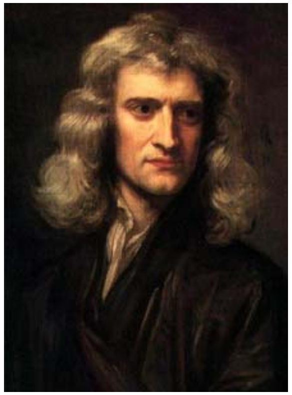
Seeing is Believing: Are you sure?



Do A and B have the same reflectance?

Color – Time Line

Aristotle 384-322 BC



Newton – Identified Light as the source of Color (~1707)

Goethe – Theory of Colors - ~1810

Thomas Young – Trichromatic Theory of Color - ~1801

Color – Time Line (Cont'd)

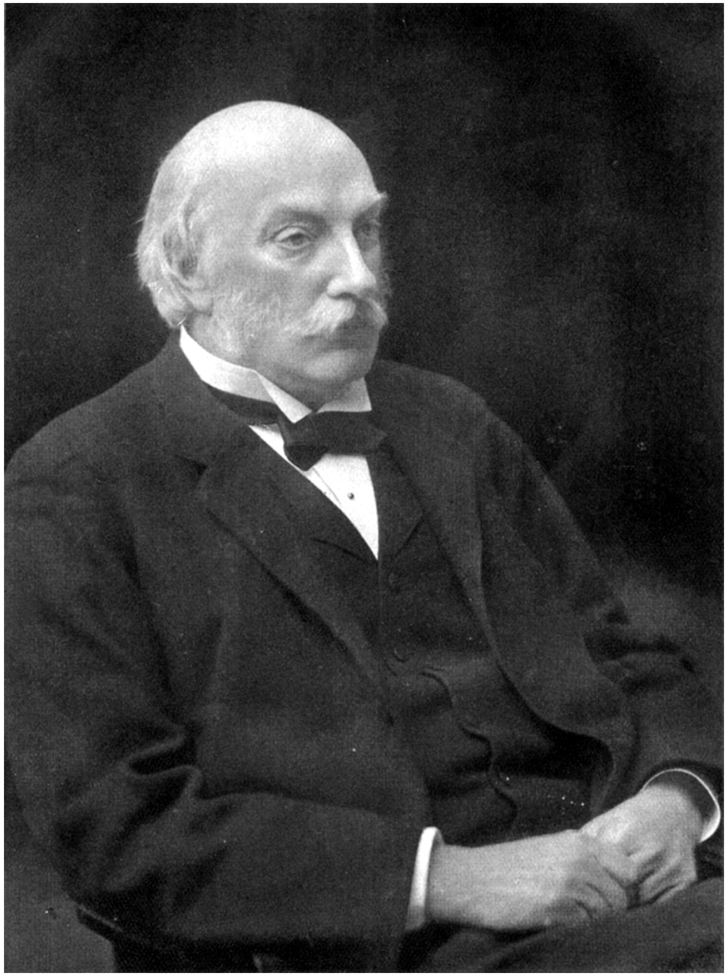
Hermann von Helmholtz - ~ 1850's
Sensory Physiology

James Clerk Maxwell - ~ 1852

"Color Science is a mental Science"
Representation of color as a triangle

Awarded Rumford Medal: 1860
"For his researches on the composition of colours, and other optical papers"

Breath Figures (Lord Rayleigh, Physics Prize 1904)



- **Rayleigh Scattering – 1871, 1899**

  Blue of the Sky **$I \sim 1/\lambda^4$**

  – **Discovery of Argon** (**Physics Prize, 1904**)

- **Breath Figures**: Arguments with Aitken (1911)

  – **Rayleigh Instability**

  – **Various Contributions to Color science**

Charles Thomas Rees(‘CTR’) Wilson and The Cloud Chamber (Physics Prize, 1927)

CTR Wilson in 1889. (Plate 8 of Wilson (1960),

***Sir Lawrence Bragg* on the importance of the Wilson Cloud Chamber:**

The cloud chamber has become the vital means for studying the ultimate particles of matter, and is responsible for a major part of modern physics. (Bragg, 1968)

…

*It can tell us the history of a single one of these minute particles, which leaves a trail in the cloud chamber like that left in the upper air by an aeroplane under certain meteorological conditions. (*Bragg, 1969)

LANGAUGE OF COLOR SCIENCE

DEFINITION OF COLOR:

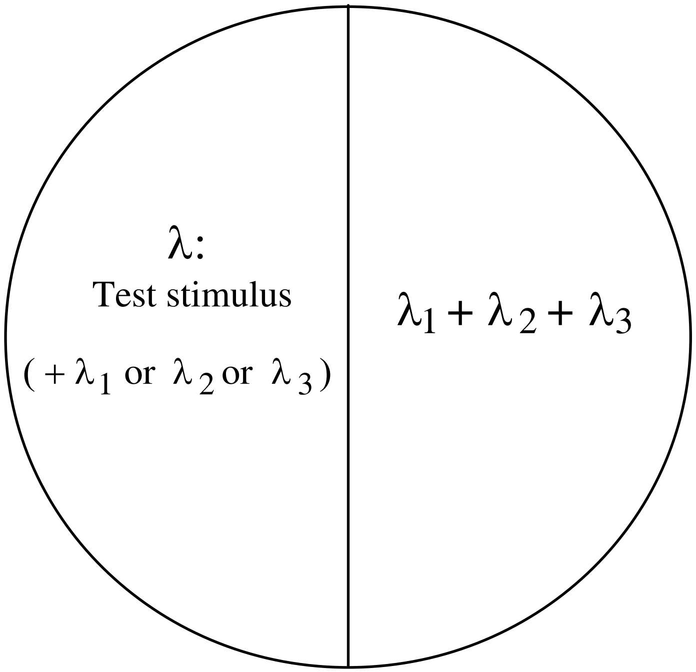
“The particular color of a body depends upon the molecular constitution of its surface, as determining the character and number of light vibrations it reflects. Subjectively, colour may be viewed as the particular sensation produced by the stimulation of the optic nerve by particular light vibrations….. This sensation can be produced by other means, such as pressure on the eye-back or an electric current”

Hue: Attribute of visual sensation which has given rise to color names such as: Blue, Green, Yellow, Red etc;

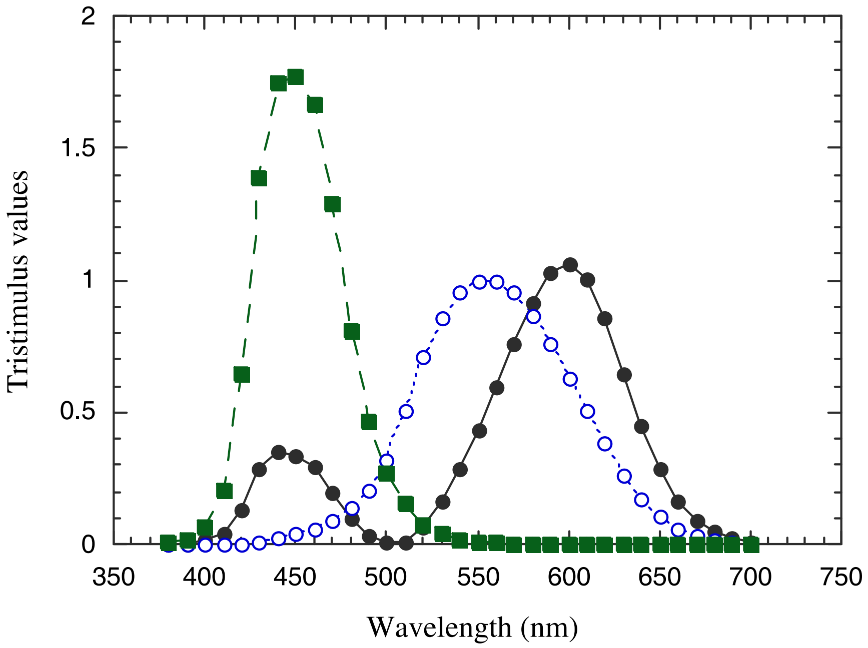
Saturation: Attribute of a visual sensation which permits a judgment to be made of the proportion of pure chromatic color in the total sensation.

Brightness: Attribute of a visual sensation according to which an area appears to emit more or less light

COLOR MATCHING FUNCTIONS



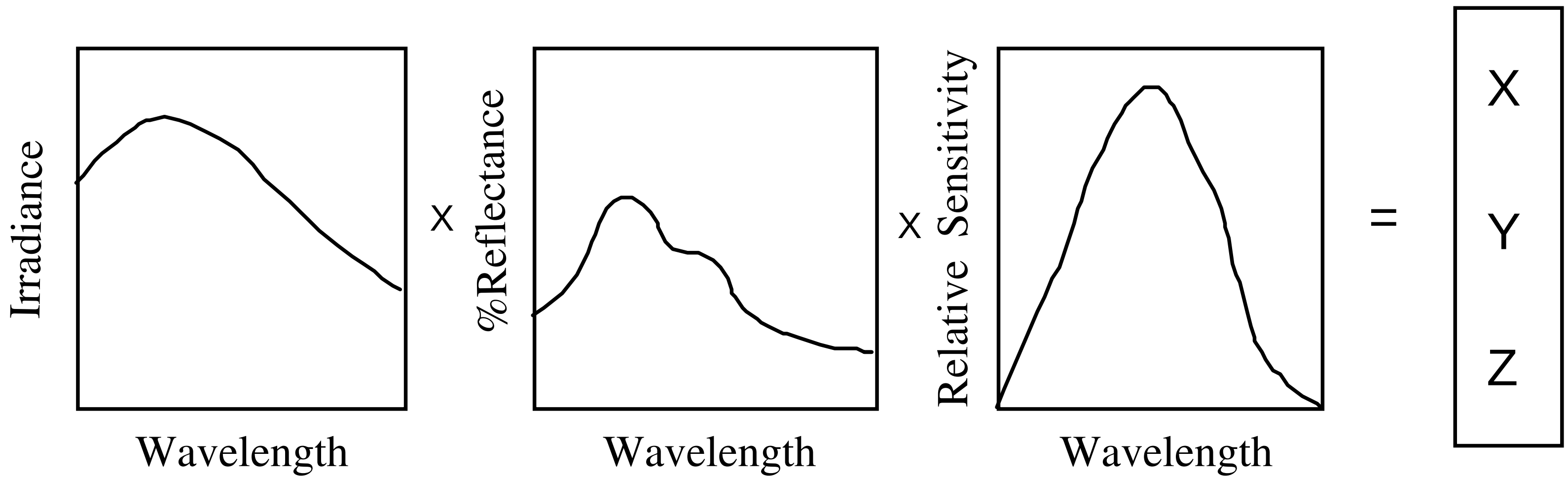
1931 CIE Standard Observer



Necessary items for color:

- A light source
- An object that it illuminates
- The eye (and Brain!) to perceive the colour

PROCEDURE FOR CALCULATING TRISTIMULUS VALUES



Spectrum of Light Source | Reflectivity of the object | Standard Observer Functions | Tristimulus Values

$$X = k \int R(\lambda) P(\lambda) \bar{x}(\lambda)\ d\lambda$$

$$Y = k \int R(\lambda) P(\lambda) \bar{y}(\lambda)\ d\lambda$$

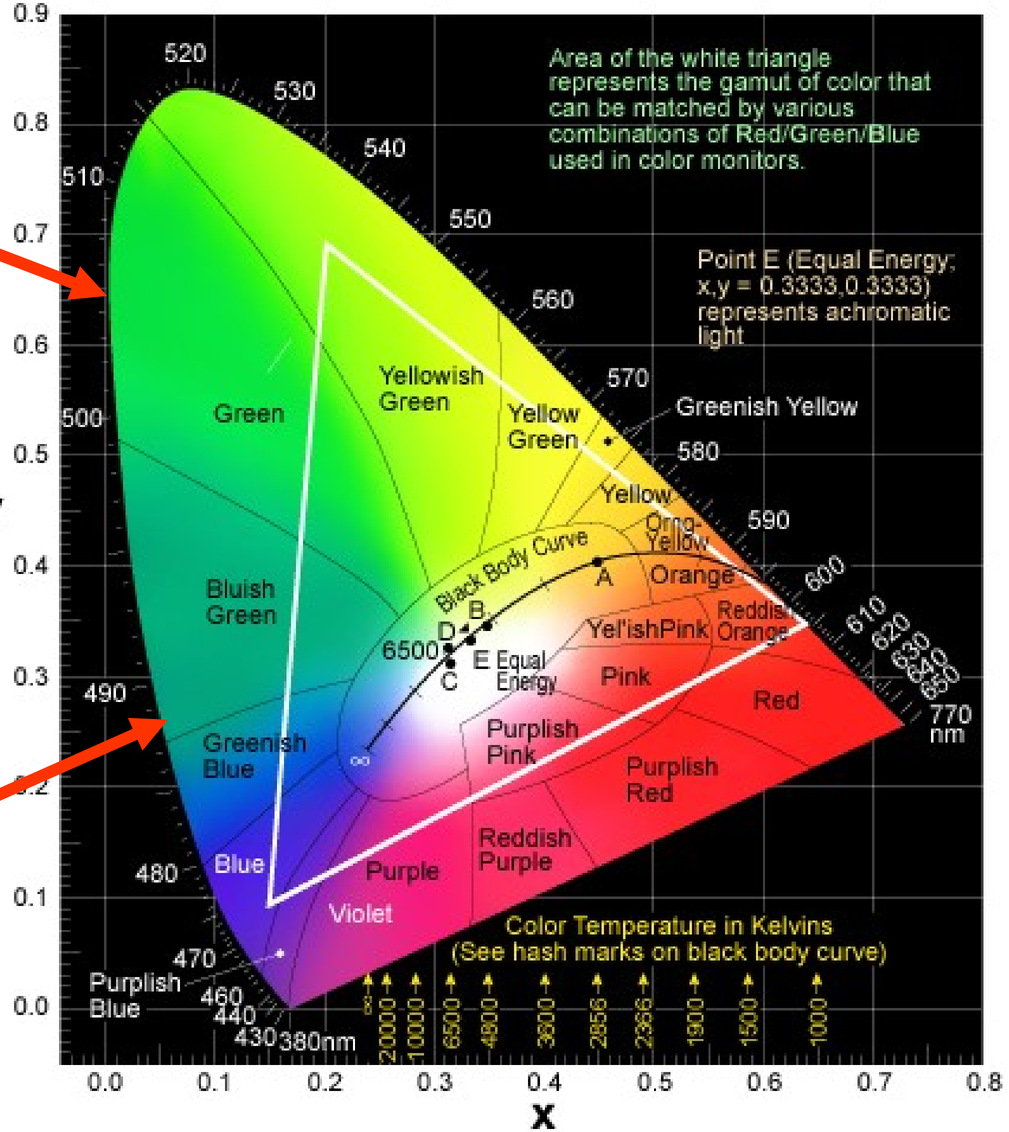
$$Z = k \int R(\lambda) P(\lambda) \bar{z}(\lambda)\ d\lambda$$

$$x = X/(X+Y+Z)$$

$$y = Y/(X+Y+Z)$$

CHROMATICITY DIAGRAM

Pure colors



SPECTRUM LOCUS

Maxwell Colour Triangle

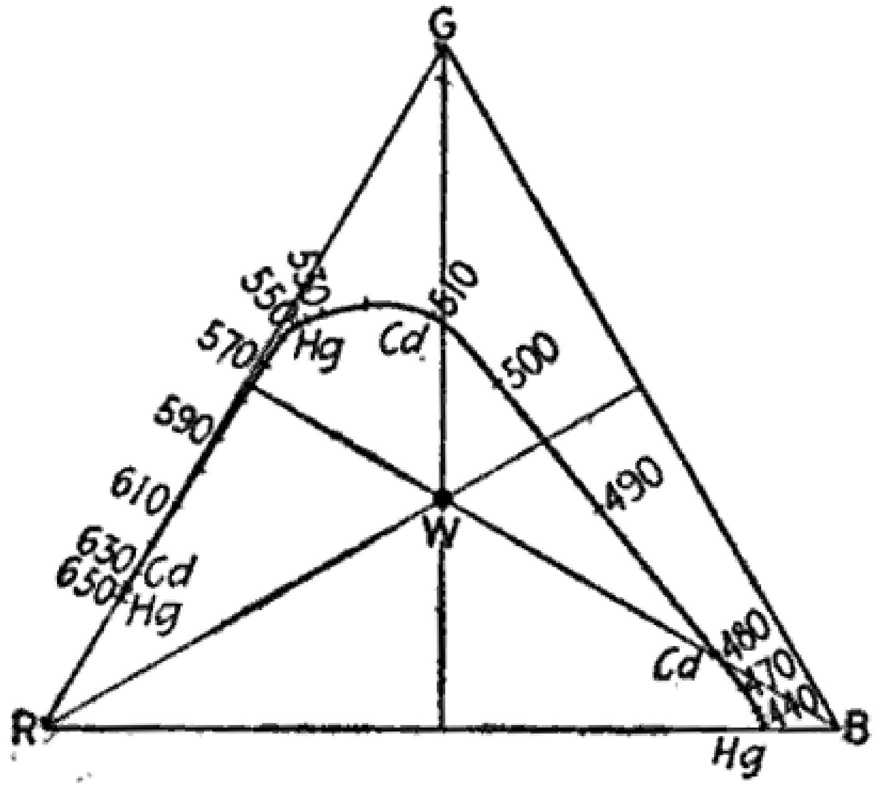
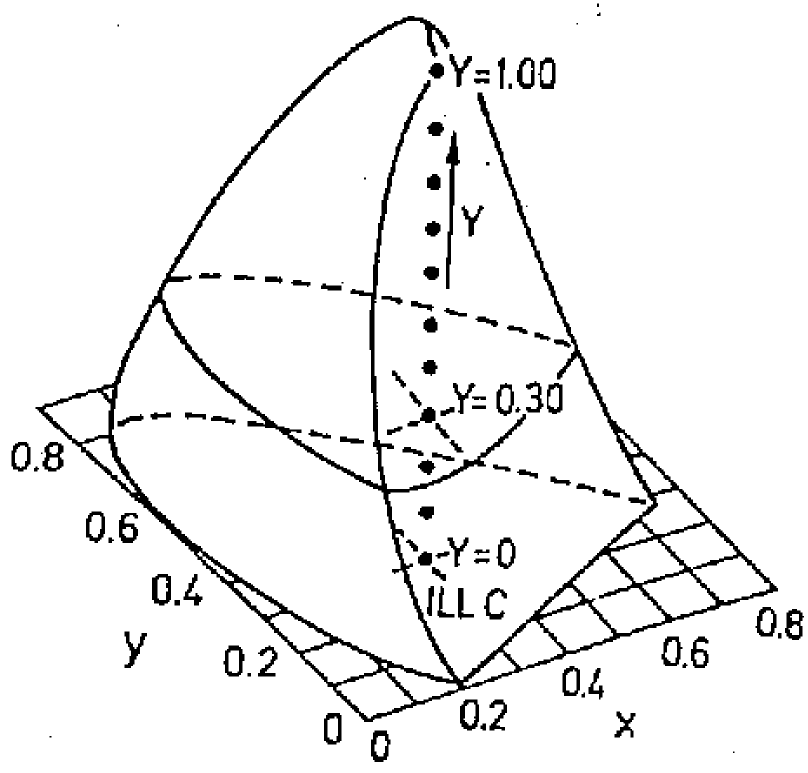
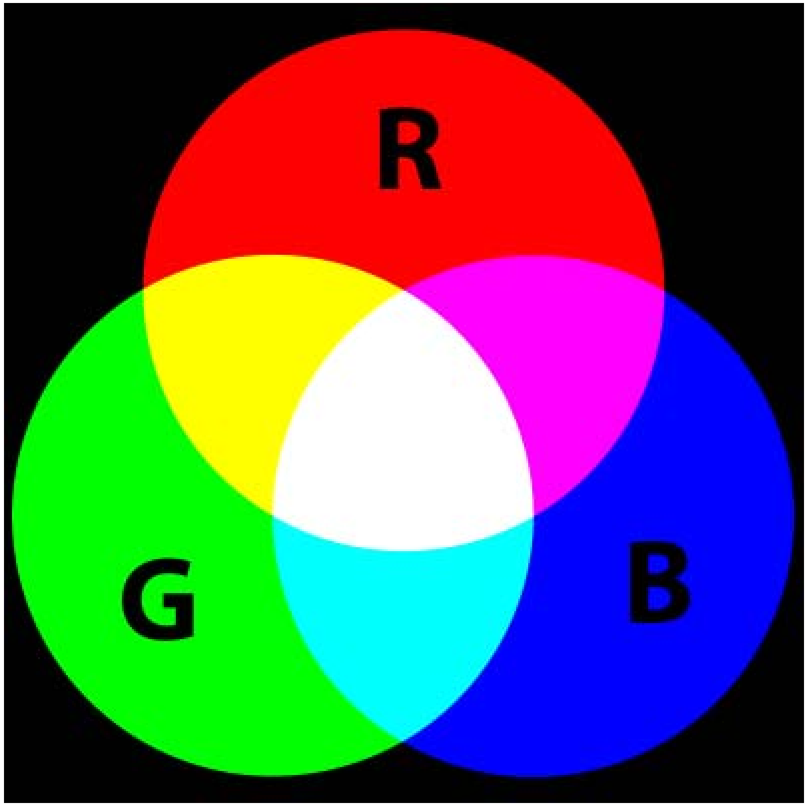


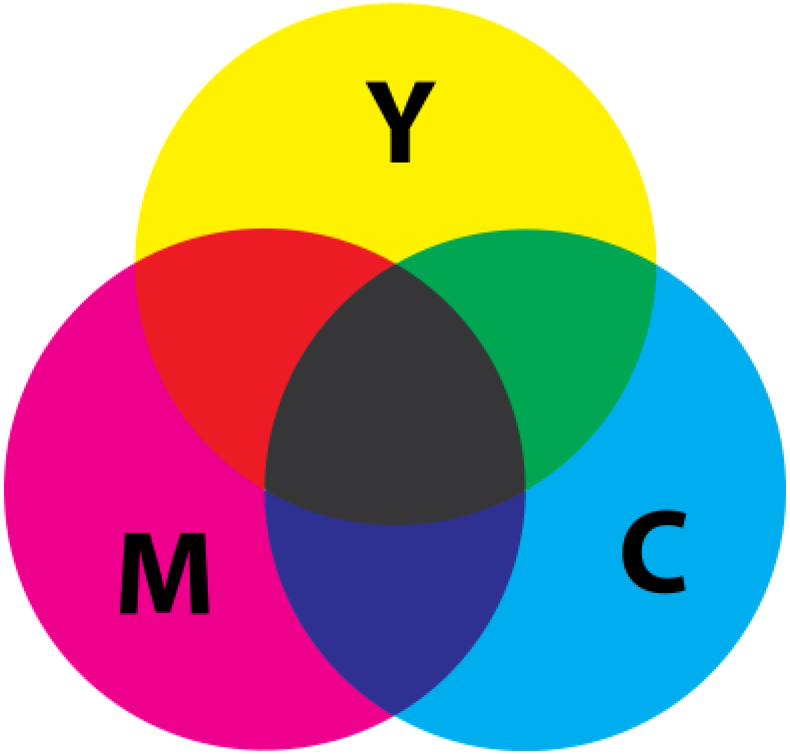
Fig. 31.—The Maxwell color-triangle.

THREE DIMENSIONAL NATURE OF COLOR

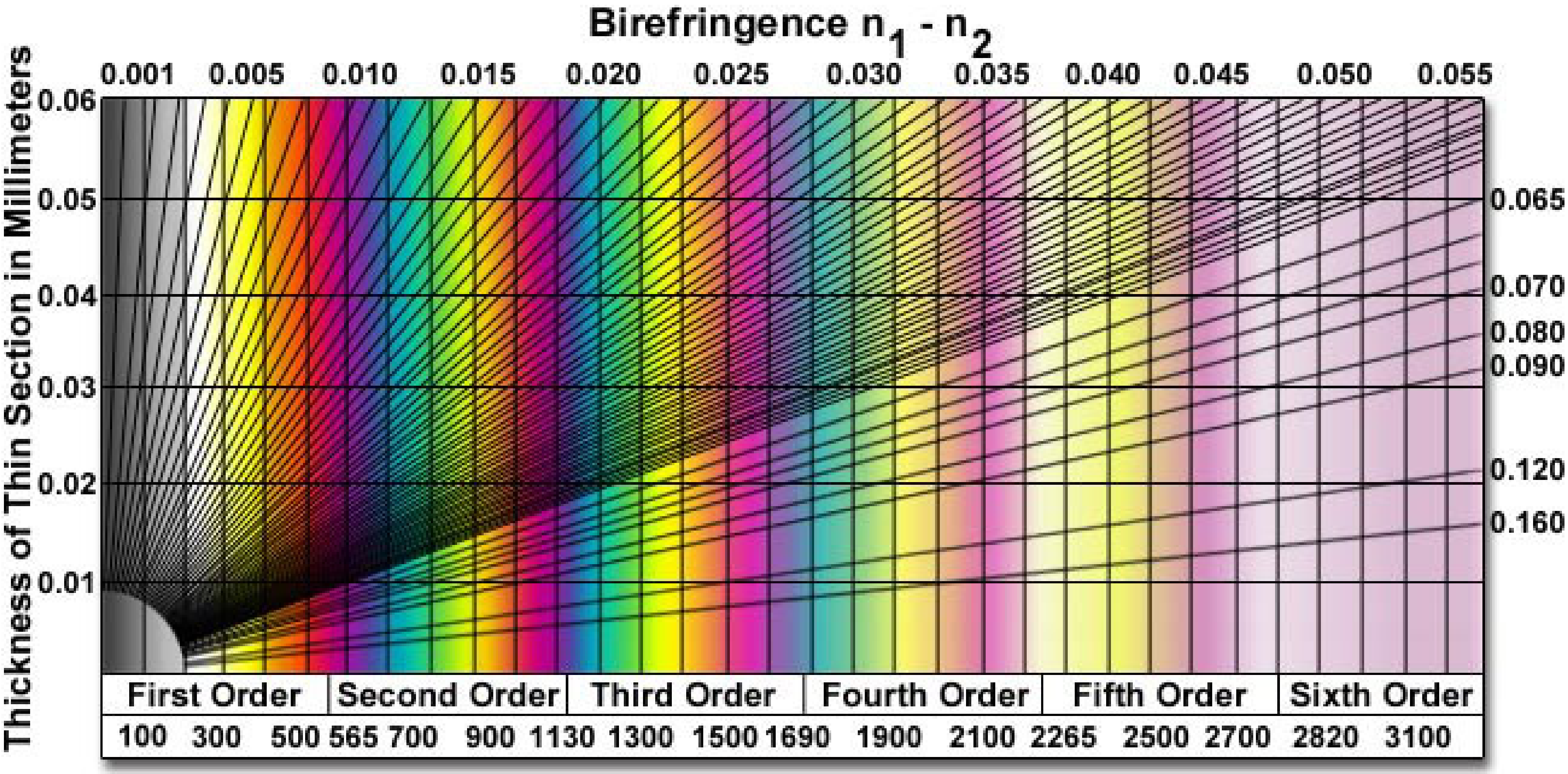


Color Mixing – Additive and Subtractive



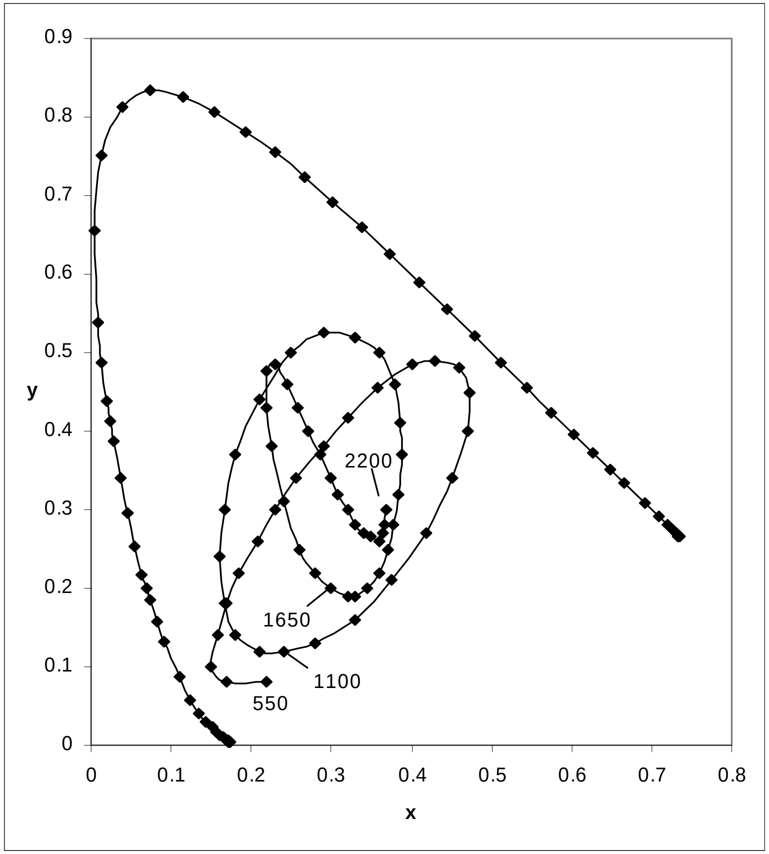


Michel-Levy Chart: Progression of Colors

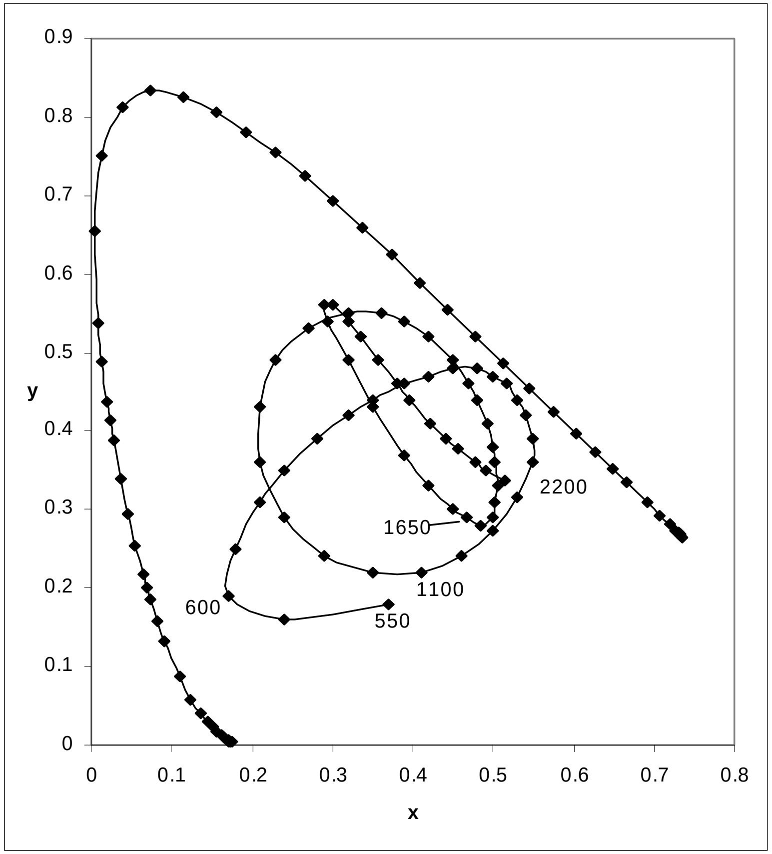


COLORS DUE TO INTERFERENCE

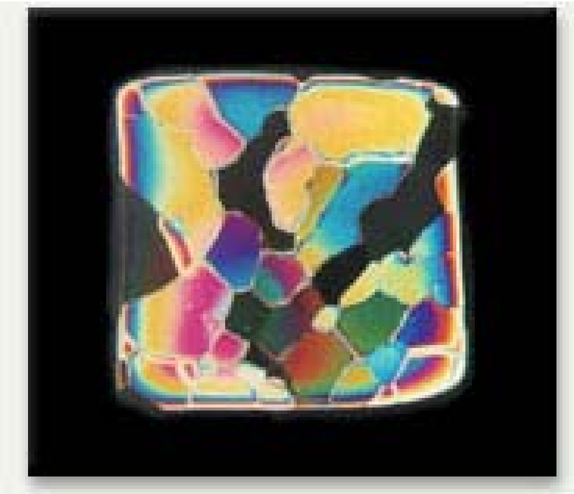
Polarization colors for Ill C



Polarization colors for Halogen

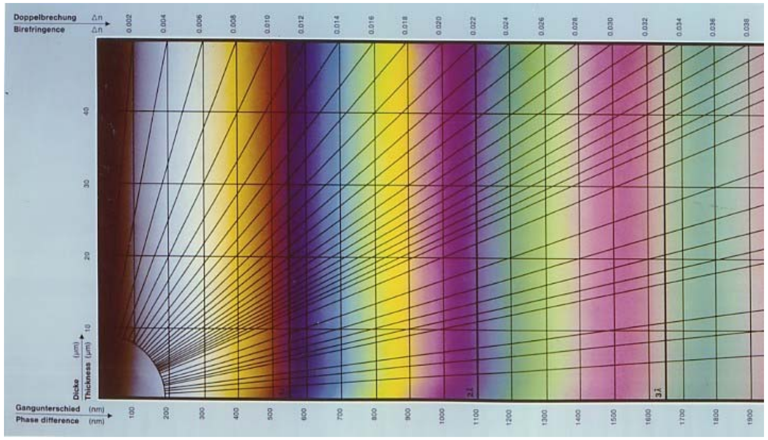


COLORS DUE TO INTERFERENCE

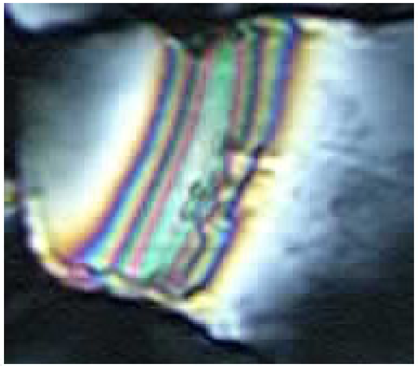


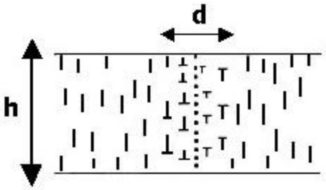
Interference colors in a thinned ice cube, 3 cm across (between crossed polarizers).

$I \sim \sin^2 \delta/2$, where $d = 2\pi h\Delta n/\lambda$



Michel-Levy Chart





Wall Defects