

# *Optical Microscopy*

Philip D. Rack  
Assistant Professor  
University of Tennessee  
603 Dougherty Hall  
[prack@utk.edu](mailto:prack@utk.edu)

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## **Optical Microscopy**

### **1.0 Introduction and History**

- 1.1 Brief Review of Light Physics
- 1.2 Characteristic Information

### **2.0 Basic Principles**

- 2.1 Ray Optics of the Optical Microscope
- 2.2 Summary

### **3.0 Instrumentation**

- 3.1 Sample Prep
- 3.2 Measurement Systems and Types

### **4.0 Examples**

### **5.0 Correct Presentation of Results**

- 5.1 Publication
- 5.2 Presentation

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## 1.0 Introduction and History



- The story of the first "compound" (more than 1 lens) microscope is an interesting one. Much is unknown, yet many things are known.
- Credit for the first microscope is usually given to Zacharias Jansen, in Middleburg, Holland, around the year 1595. Since Zacharias was very young at that time, it's possible that his father Hans made the first one, but young Zach took over the production.
- Details about these first Jansen microscopes are not clear, but there is some evidence which allows us to make some good guesses about them.
- The above early microscope found in Middleburg, Holland, corresponds to our expectations of the Jansen microscopes.

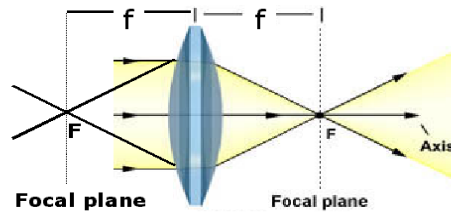
## 1.2 Characteristic Information

- Morphology, Size
- Transparency or Opacity
- Color (reflected and transmitted)
- Refractive Indices
- Dispersion of Refractive Indices
- Pleochroism
- Crystal System
- Birefringence
- Extinction Angle
- Fluorescence (UV, V, IR)
- Melting Point, Polymorphism, Eutectics

Extinction angle: The angle between the vibration direction of the light inside the specimen and some prominent crystal face. Birefringence: The numerical difference between the principal refractive indices. Pleochroism: Change in color or hue relative to the orientation of polarized light.

## 2.1 Ray Optics

### Converging (Convex) Lens



The simplest magnifying lens

$f \propto$  curvature angle and lens materials (N)  
the larger N, the shorter f

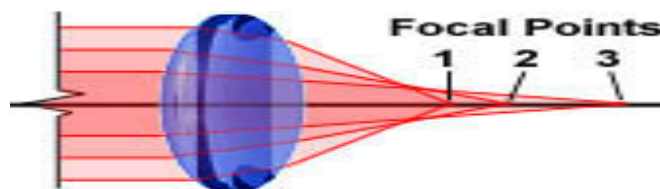
	lucite	glass	diamond
N:	1.47	1.51	2.42

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## Defects in Lenses

- **Spherical Aberration – Peripheral rays and axial rays have different focal points**
  - This causes the image to appear hazy or blurred and slightly out of focus.
  - This is very important in terms of the resolution of the lens because it affects the coincident imaging of points along the optical axis and degrades the performance of the lens

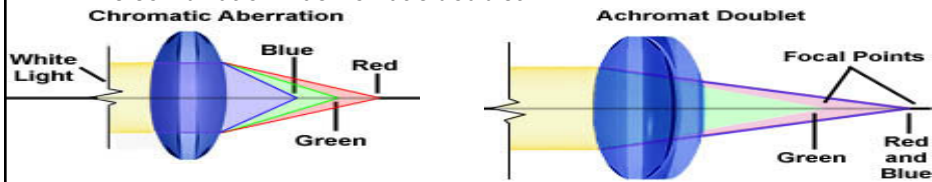
### Spherical Aberration (Monochromatic Light)



# Defects in Lenses

## ■ Chromatic Aberration

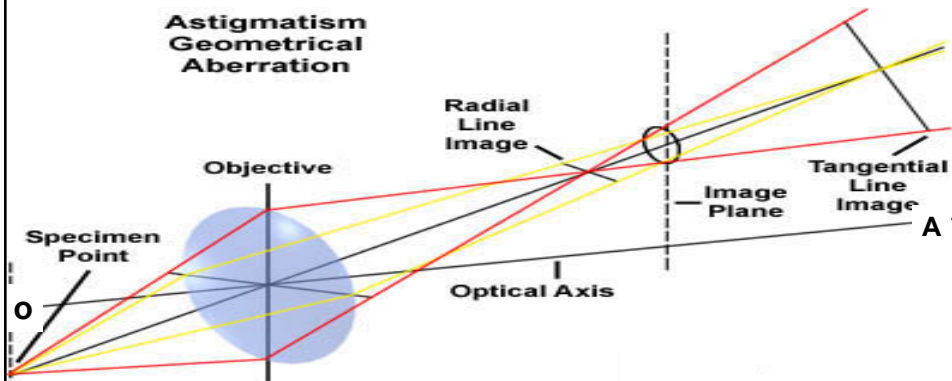
- Axial - Blue light is refracted to the greatest extent followed by green and red light, a phenomenon commonly referred to as dispersion
- Lateral - *chromatic difference of magnification*: the blue image of a detail was slightly larger than the green image or the red image in white light, thus causing color ringing of specimen details at the outer regions of the field of view
- A converging lens can be combined with a weaker diverging lens, so that the chromatic aberrations cancel for certain wavelengths:  
The combination – achromatic doublet



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# Defects in Lenses

- Astigmatism - The off-axis image of a specimen point appears as a disc or blurred lines instead of a point.
- Depending on the angle of the off-axis rays entering the lens, the line image may be oriented either tangentially or radially



# Resolution

- Maximum resolution:

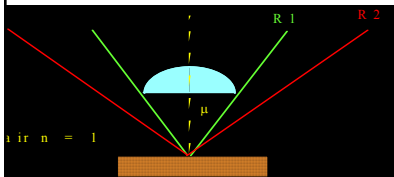
$$R = \frac{(0.61 \cdot \lambda)}{N.A.}$$

where: 0.61 is a geometrical term, based on the average 20-20 eye,  $\lambda$  = wavelength of illumination, N.A. = Numerical Aperture

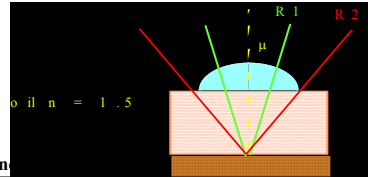
The N.A. is a measure of the light gathering capabilities of an objective lens.

N.A. =  $n \sin \alpha$  where:

$n$  = index of refraction of medium,  $\alpha$  =  $\angle$  subtended by the lens



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## Factors Affecting Resolution

- Resolution ( $d_{\min}$ ) improves (smaller  $d_{\min}$ ) if  $\lambda \downarrow$  or  $n \uparrow$  or  $\alpha \uparrow$
- Assuming that  $\sin \alpha = 0.95$  ( $\alpha = 71.8^\circ$ )

Wavelength	Air (n= 1)	Oil (n = 1.515)
Red 650 nm	0.42 $\mu\text{m}$	0.28 $\mu\text{m}$
Yellow 600 nm	0.39 $\mu\text{m}$	0.25 $\mu\text{m}$
Green 550 nm	0.35 $\mu\text{m}$	0.23 $\mu\text{m}$
Blue 475 nm	0.31 $\mu\text{m}$	0.20 $\mu\text{m}$
Violet 400 nm	0.27 $\mu\text{m}$	0.17 $\mu\text{m}$

Resolution<sub>air</sub>

Resolution<sub>oil</sub>

- (The eye is more sensitive to blue than violet)

## Magnification

- The overall magnification is given as the product of the lenses and the distance over which the image is projected:

$$M = \frac{D \cdot M_1 \cdot M_2}{250mm}$$

where:

D = projection (tube) length (usually = 250 mm);

M<sub>1</sub>, M<sub>2</sub> = magnification of objective and ocular.

250 mm = minimum distance of distinct vision for 20/20 eyes.

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## Depth of Focus

- We also need to consider the depth of focus (vertical resolution). This is the ability to produce a sharp image from a non-flat surface.

$$DOF \approx \frac{\lambda}{N.A.}$$

- Depth of Focus is increased by inserting the objective aperture (just an iris that cuts down on light entering the objective lens). However, this decreases resolution.

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## 2.2 Summary

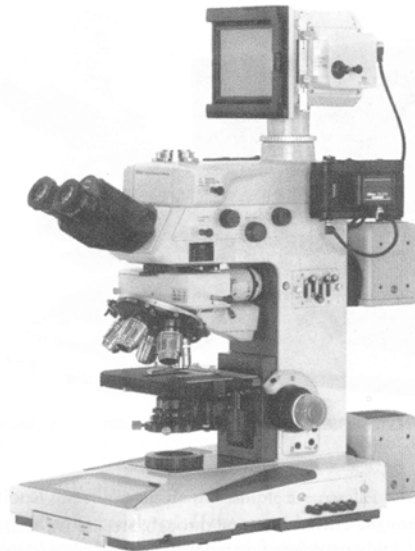
1. All microscopes are similar in the way lenses work and they all suffer from the same limitations and problems.
2. Magnification is a function of the number of lenses. Resolution is a function of the ability of a lens to gather light.
3. Apertures can be used to affect resolution and depth of field if you know how they affect the light that enters the lens.

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## 3.0 Instrumentation

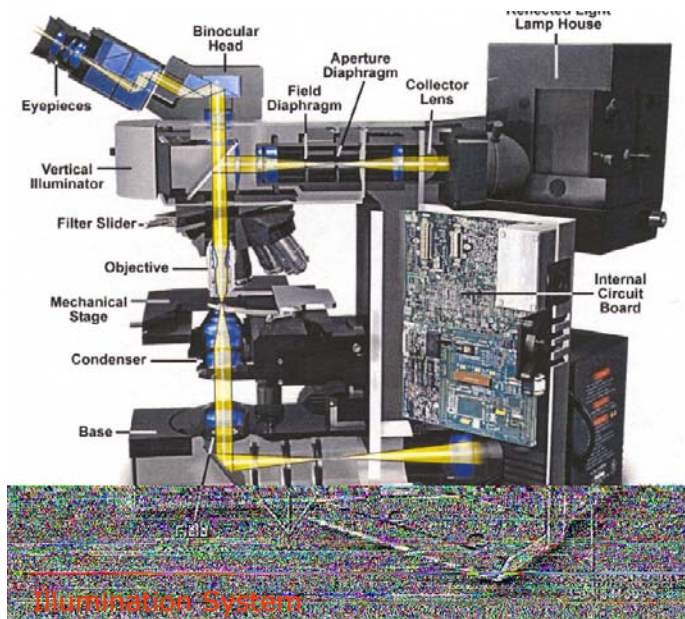
Several important features are visible:

- Lenses
- Eyepieces (oculars)
- Light source
- Camera



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## Anatomy of a modern LM



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## Contrast and Illumination

- **Brightness contrast** arises from different degrees of absorption at different points in the specimen.
- **Color contrast** can also arise from absorption when the degree of absorption depends on the wavelength and varies from point to point in the specimen.
- **Phase contrast** arises from a shift in the phase of the light as a result of interaction with the specimen.
- **Polarization-dependent phase contrast** arises when the phase shift depends on the plane of polarization of the incident light.
- **Fluorescence contrast** arises when the incident light is absorbed and partially reemitted at a different wavelength.

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# **Bright Field Microscopy**

## **Principle**

- **Light from an incandescent source is aimed toward a lens beneath the stage called the condenser, through the specimen, through an objective lens, and to the eye through a second magnifying lens, the ocular or eyepiece.**
- **The condenser is used to focus light on the specimen through an opening in the stage.**
- **After passing through the specimen, the light is displayed to the eye with an apparent field that is much larger than the area illuminated.**
- **Typically used on thinly sectioned materials**

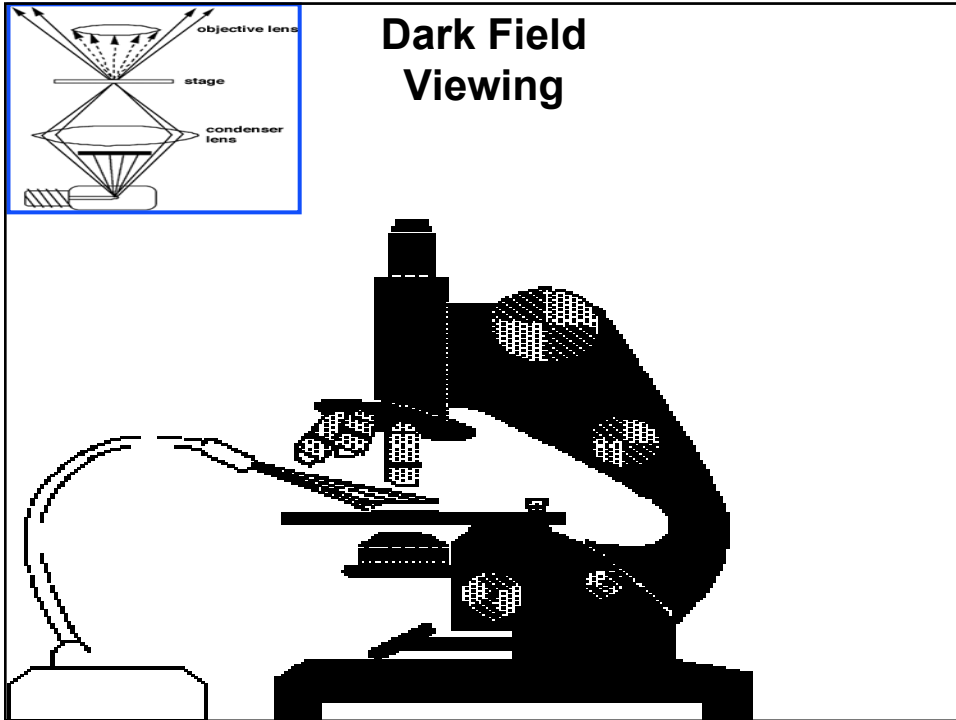
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# **Dark Field Viewing**

## **Principle**

- **To view a specimen in dark field, an opaque disc is placed underneath the condenser lens, so that only light that is scattered by objects on the slide can reach the eye.**
- **Instead of coming up through the specimen, the light is reflected by particles on the slide.**
- **Everything is visible regardless of color, usually bright white against a dark background.**

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### Specialized LM Techniques

- **Enhancement of Contrast**  
**Bright & Dark field Microscopy**  
**Phase contrast microscopy, Differential interference contrast microscopy: Convert phase differences to amplitude differences**  
**Fluorescence microscopy-mainly organic materials**
- **Confocal scanning optical microscopy (new)**  
**Three-Dimensional Optical Microscopy**  
**inspect and measure submicrometer features in semiconductors and other materials**
- **Hot- and cold-stage microscopy**  
**melting, freezing points and eutectics, polymorphs, twin and domain dynamics, phase diagram**
- **In situ microscopy**  
**E-field, stress, etc.**
- **Special environmental stages-vacuum or gases**

## 3.1 Sample Preparation



- *Before performing an experiment, always consider the information that you want to obtain and the method(s) by which to obtain ALL of it.*
- Sample preparation methods vary widely.
- Depends to some degree on the next phase of characterization.
- **Particulate:** It needs to be mounted in a refractive index liquid for determination of the optical properties. OR Mounted on tape for size and shape analysis.

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## 3.1 Sample Preparation



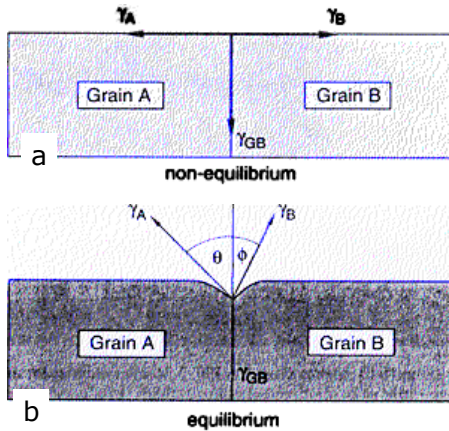
- If the sample is metal, embed in a polymer, section and polish.
- Organic samples may be sectioned / processed with a cryomicrotome, among other types to reduce sample prep damage.

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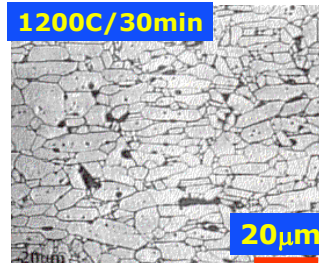
# 4.0 Examples

## Grain Size Examination

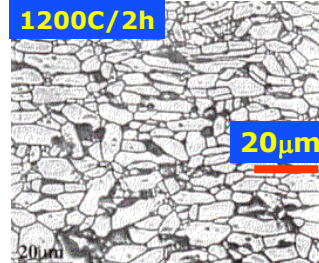
### Thermal Etching



1200C/30min



1200C/2h



A grain boundary intersecting a polished surface is not in equilibrium (a). At elevated temperatures (b), surface diffusion forms a grain-boundary groove in order to balance the surface tension forces.

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## Contrast

Contrast is defined as the difference in light intensity between the specimen and the adjacent background relative to the overall background intensity.

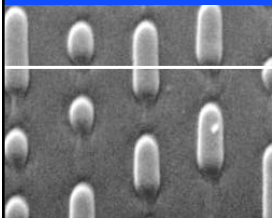
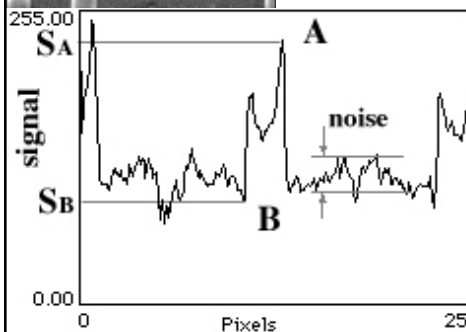


Image contrast,  $C$  is defined by

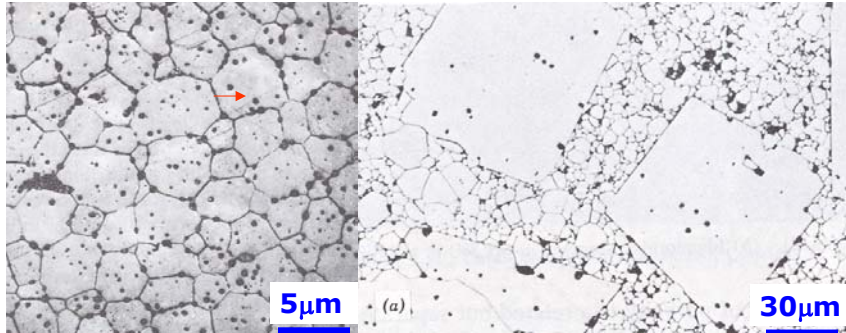
$$C = \frac{(S_{specimen} - S_{background})}{S_{background}}$$



$S_{specimen}$  and  $S_{background}$  are intensities measured from the specimen and background, e.g., A and B, in the scanned area.

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## Grain Growth - Reflected OM



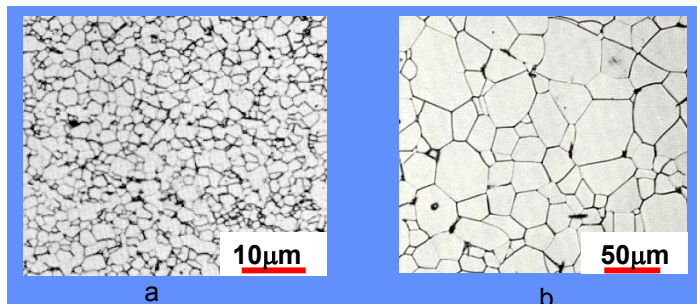
Polycrystalline  $\text{CaF}_2$  illustrating normal grain growth. Better grain size distribution.

Large grains in polycrystalline spinel ( $\text{MgAl}_2\text{O}_4$ ) growing by secondary recrystallization from a fine-grained matrix.

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## Effect of Microstructure on Mechanical Property

$$\sigma_f \propto d^{-1/2} \quad d\text{-grain size}$$



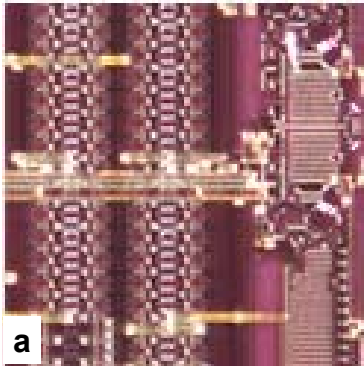
OM images of two polycrystalline samples.

Mechanical test:  $\sigma_{fa} > \sigma_{fb}$  Mechanical property

Microscopic analysis:  $d_a < d_b$  Microstructure

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## Polarized Optical Microscopy (POM)



a

Reflected POM



b

Transmitted POM

- (a) Surface features of a microprocessor integrated circuit
- (b) Apollo 14 Moon rock

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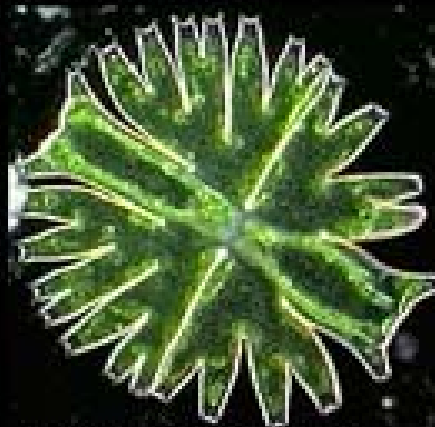
## Contrast Enhancement

bright field



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darkfield

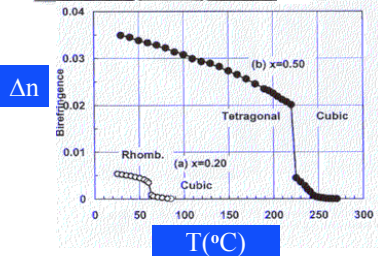
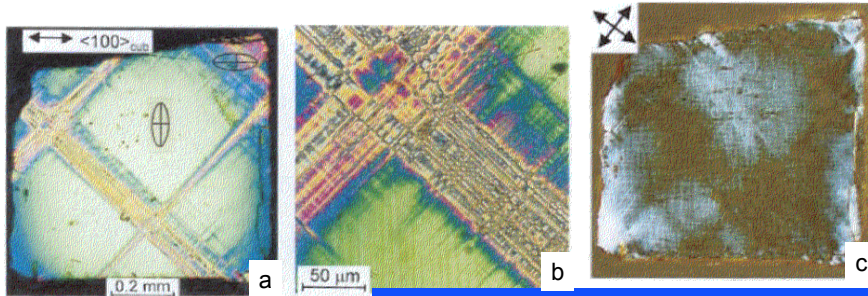


www.cellsalive.com

OM images of the green alga *Micrasterias*

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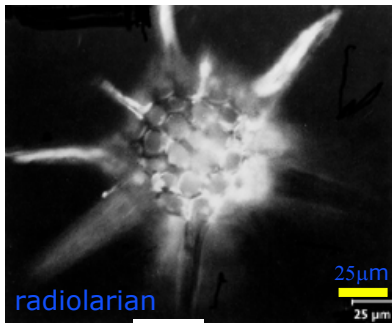
## Hot-stage POM - Phase Transformations in $\text{Pb}(\text{Mg}_{1/3}\text{Nb}_{2/3})\text{O}_3\text{-PbTiO}_3$ Crystals



(a) and (b) at 20°C, strongly birefringent domains with extinction directions along  $\langle 100 \rangle_{\text{cube}}$ , indicating a tetragonal symmetry; (c) at 240°C, phase transition from the tetragonal into cubic phase with increasing isotropic areas at the expense of vanishing strip domains.

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## Optical Microscopy vs Scanning Electron Microscopy



OM

Small depth of field  
Low resolution



SEM

Large depth of field  
High resolution

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## 5.0 Correct Presentation of Results



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## Publication and Presentation Responsibilities of a Scientist

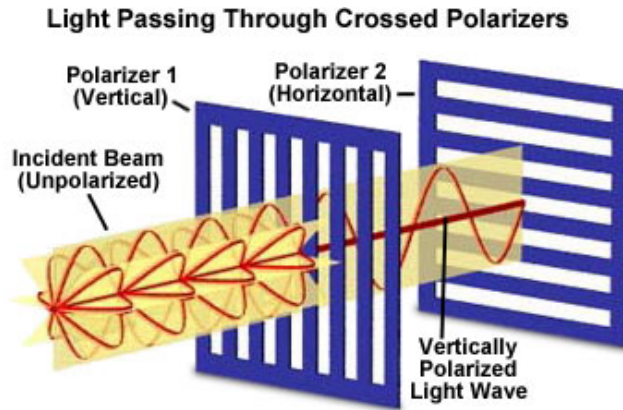
- Understand the technique you are discussing, presenting at the required level.
- Have supporting characterization if possible.
- How much information are you drawing off of in terms of numerical analysis ?
- Is the data supportive of measurements you are quoting ?
- How clear is the image / features that you are specifying in your talk or paper ?
- Are the magnification bars and text properly labeled and displayed ?

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## **The Polarizing Microscope**

- **Light from an incandescent source is passed through a polarizer, so that all of the light getting through must vibrate in a single plane.**
- **The beam is then passed through a prism that separates it into components that are separated by a very small distance - equal to the resolution of the objective lens. The beams pass through the condenser, then the specimen.**
- **In any part of the specimen in which adjacent regions differ in refractive index the two beams are delayed or refracted differently.**
- **When they are recombined by a second prism in the objective lens there are differences in brightness corresponding to differences in refractive index or thickness in the specimen.**

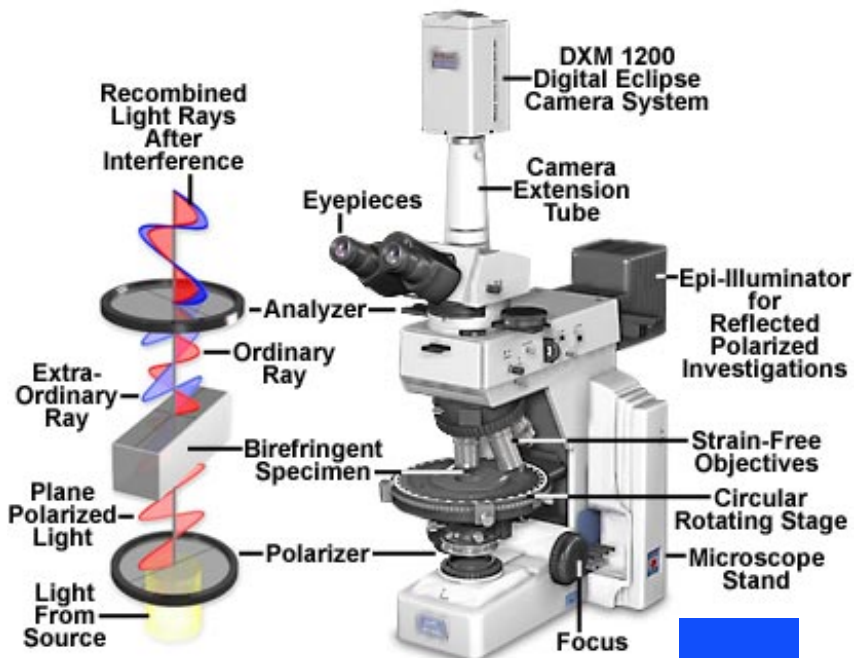
# Polarization of Light



When the electric field vectors of light are restricted to a single plane by filtration, then the light is said to be polarized with respect to the direction of propagation and all waves vibrate in the same plane.

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# Polarized OM Configuration



## **Phase Contrast Microscopy**

**(Zernike, Nomarski DIC, Hoffman Modulation Contrast)**

- **If the sample is colorless, transparent, and isotropic, and is embedded in a matrix with similar properties, it will be difficult to image.**
- **This is due to the fact that our eyes are sensitive to amplitude and wavelength differences, but not to phase differences.**

## **Phase Contrast Microscopy**

- **Phase contrast - Introduced in the 1930's by Zernike, converts phase differences into amplitude differences.**
- **Differential interference microscopy (DIC) requires several optical components, therefore it can be very expensive to set up.**

Phase contrast microscopy works by introducing an additional phase shift between  $R_0$  and  $R_1$ , creating a difference in amplitude of light at  $C$  and  $D$ . That is, contrast is created. This phase shift is introduced by placing a phase plate in the back focal plane of the objective lens. In positive phase contrast the first-order diffracted beam is shifted an additional  $90^\circ$  relative to the zeroth-order (direct) beam. The result is that  $R_1$  is directed opposite to  $R_0$  so that they subtract at image point  $C$  and give a minimum of intensity. At image point  $D$ ,  $R_1$  is further retarded by  $90^\circ$  with respect to  $R_0$  and is therefore at right

**Phase Contrast Microscopy** - Phase contrast microscopy, first described in 1934 by Dutch physicist Frits Zernike, is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens such as living cells, microorganisms, thin tissue slices, lithographic patterns, and sub-cellular particles (such as nuclei and other organelles). In effect, the phase contrast technique employs an optical mechanism to translate minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast. One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without being killed, fixed, and stained. As a result, the dynamics of ongoing biological processes in live cells can be observed and recorded in high contrast with sharp clarity of minute specimen detail.

<http://www.microscopyu.com/tutorials/java/phasecontrast/microscopealignment/index.html>  
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### 33.5 PHASE CONTRAST MICROSCOPY

Optical phase contrast microscopy is used with essentially transparent specimens and is probably most widely used with biological materials. It is important to have some degree of understanding of phase contrast microscopy with respect to materials characterization, not only for its direct use but also because of the analogy to phase contrast effects in electron microscopy. The subject is complicated and a full treatment is beyond the scope of this book. An attempt is made to present the basic ideas in enough detail to give a physical understanding of the origin of the enhanced contrast and the techniques for achieving such contrast. The treatment here is an abbreviated version of the simplified vector model given by Goldstein (Goldstein, 1982).

An incident beam of parallel light is shown illuminating a specimen in Figure 33.4. According to Huygens' construction each point of the specimen can be considered to emit a spherical wave and the resulting waves added to give the result of scattering of light by the specimen (Born and Wolf, 1975). The Abbe theory of image formation considers the behavior of a grating in the specimen to treat the formation of an image and its resolution and contrast. The figure shows a grating made up of transmitting portions (slots) and opaque portions (bars). A spherical wave is emitted from the center of each slot. The envelope of these waves gives the zeroth-order diffraction maximum, which is the direct beam. The drawing also shows the formation of the first-order diffraction maximum by interference of light at a distance one wavelength from the first slot with light at a distance two wavelengths from the second slot, and so on. This is the same effect as one-dimensional Bragg diffraction of X-rays or electrons.

Figure 33.5 shows the formation of a diffraction pattern and an image from the diffracted beams. Only the zeroth- and first-order diffracted beams are shown, but these are sufficient to illustrate the principles involved. Each diffracted beam consists of parallel light, and is brought into focus in the back focal plane of the objective lens. The position of focus in the back focal plane will depend on the angle that the diffracted beam makes with the optic axis of the microscope. Thus the direct beam is focused into a

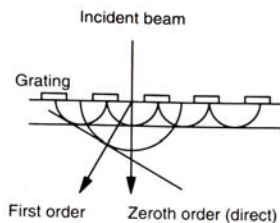


Figure 33.4 Diffraction from a grating

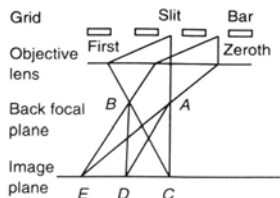


Figure 33.5 Diffraction pattern and image

diffracted spot at point *A* on the optic axis, while the first-order diffracted beam is focused into a diffraction spot at point *B* off the optic axis. The rays of light continue to form an image of the grating in the image plane. Points *A* and *B* are equidistant from point *C* (the back focal "plane" is approximately, but not strictly, a plane), and the rays from *A* and *B* constructively interfere to form an intensity maximum at *C* and produce an image of a slit. At point *D* the waves are completely out of phase and destructively interfere, forming an image of a bar of the grating. At point *E* the waves are again in phase and interfere constructively to form the image of another slit of the grating.

To understand the principle of phase contrast microscopy one considers the situation of bars and slits that are transparent but that have different indexes of refraction. If the indexes of refraction were the same, each bar would form a spherical wave centered on the bar, as do the slits in Figure 33.4. For the zeroth-order beam all the waves are in phase, giving a strong direct beam. For the first-order beam, the waves from the bars are exactly out of phase with the waves from the slits so that the intensity of the first-order diffracted beam is zero. The Huygens' construction and diffraction picture thus correctly describes the transmission of light through a specimen that is transparent and has a uniform index of refraction.

The phase relations are shown in more detail in Figure 33.6. The arrows show amplitude (length of arrow) and phase (angle of arrow with retardation taken counterclockwise). At location *A* of Figure 33.5 (the central diffraction spot in the back focal plane of the objective lens), the phase is taken as zero and the arrow is labeled  $B_0$  for bar, zeroth-order. Here, following Goldstein, a transparent bar has been taken to be on the optic axis and the slit has been taken for the moment to be opaque. At location *B*, the first-order diffraction from the bar  $B_1$  is of the same magnitude and phase as  $B_0$ . Point *C* is located an integral number of wavelengths from both *A* and *B* so that  $B_0$  and  $B_1$  are parallel and equal in size. Point *E* is located a half-wavelength farther from *A* than *C*, and a half-wavelength closer to *B* than *C*. At *E* the waves will again constructively interfere. Point *D* is located one-quarter wavelength farther from *A* than *C* and one-quarter wavelength closer to *B* than *C*. Comparing the arrows at *D* with those at *C* it is apparent that  $B_0$  has been rotated  $90^\circ$  clockwise (one-quarter wavelength retardation), and  $B_1$  has been rotated  $90^\circ$  counterclockwise (one-quarter wavelength advance in phase). Vectors  $B_0$  and  $B_1$  cancel, giving zero intensity at *D*.

In the second portion of Figure 33.6 both bar and slit are taken to be transparent and to have equal index of refraction. At *A* the amplitudes from the bar  $B_0$  and from the slit  $S_0$  are of equal amplitude and in phase. At *B* the phase of  $S_1$  is retarded by  $180^\circ$ , so that there is zero total first-order diffracted amplitude. At *C* the contributions are in phase and

add. At *D* both are retarded by one-quarter wavelength and still add. The result is uniform brightness.

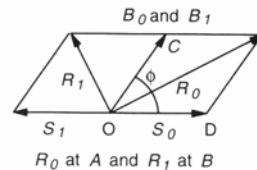
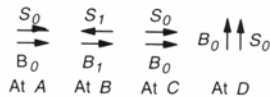
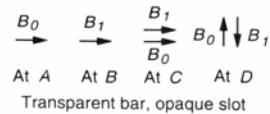
In the third portion of Figure 33.6 the bars are taken to have a higher index of refraction than the slits. This index difference causes both  $B_0$  and  $B_1$  to be retarded in phase by an angle  $\phi$ . At point *A*,  $S_0$  and  $B_0$  add to a resultant  $R_0$  as shown. At point *B*,  $S_1$  and  $B_1$  add to a resultant  $R_1$ . The magnitudes of all vectors are equal and the direction of  $S_1$  is opposite to that of  $S_0$ . Using boldface for vectors and taking the dot product gives

$$\mathbf{R}_0 \cdot \mathbf{R}_1 = (\mathbf{S}_0 + \mathbf{B}_0) \cdot (-\mathbf{S}_0 + \mathbf{B}_0) = 0 \quad (33.9)$$

Thus  $R_0$  and  $R_1$  are perpendicular; i.e., the waves are  $\pi/4$  out of phase.

The image at *C* will be formed by interference between  $R_0$  and  $R_1$  with no further change of phase and so will be twice  $OC$  in amplitude and phase. The image at *D* will be formed by  $R_0$  and  $R_1$  after a further phase change of  $\lambda/4$  and so will be twice  $OD$ . The eye responds to intensity, which is the square of the amplitude, so that the same intensity will be seen at *C* and *D* and there will be no contrast in the image despite the difference in phase of the light at *C* and *D*.

Phase contrast microscopy works by introducing an additional phase shift between  $R_0$  and  $R_1$ , creating a difference in amplitude of light at *C* and *D*. That is, contrast is created. This phase shift is introduced by placing a phase plate in the back focal plane of the objective lens. In positive phase contrast the first-order diffracted beam is shifted an additional  $90^\circ$  relative to the zeroth-order (direct) beam. The result is that  $R_1$  is directed opposite to  $R_0$  so that they subtract at image point *C* and give a minimum of intensity. At image point *D*,  $R_1$  is further retarded by  $90^\circ$  with respect to  $R_0$  and is therefore at right



Transparent bar, transparent slot, unequal index

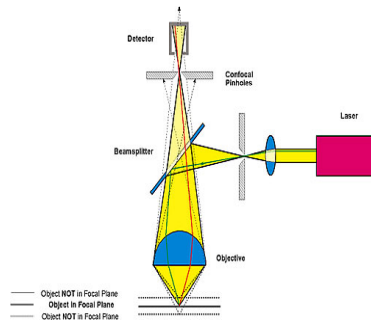
Figure 33.6 Vector model of phase contrast

# The Confocal Microscope

- In the confocal microscope all structures out of focus are suppressed at image formation.

- This is obtained by an arrangement of diaphragms which, at optically conjugated points of the path of rays, act as a point of source and as a point detector respectively, Rays from out-of-focus are suppressed by the detection pinhole.

- The depth of the focal plane is is, besides the wavelength of light, determined in particular by the numerical aperture of the objective used and the diameter of the diaphragm.



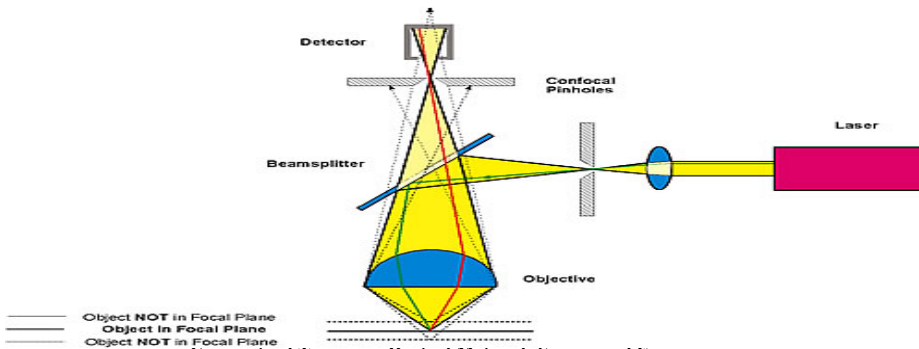
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# The Confocal Microscope II

- At a wider detection pinhole the confocal effect can be reduced.

- To obtain a full image, the image point is moved across the specimen by mirror scanners.

- The emitted/reflected light passing through the detector pinhole is transformed into electrical signals by a photomultiplier and displayed on a computer monitor screen.



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## The Confocal Microscope III

Major improvements offered by a confocal microscope over the performance of a conventional microscope may be summarized as follows:

1. Light rays from outside the focal plane will not be recorded.
2. Defocusing does not create blurring, but gradually cuts out parts of the object as they move away from the focal plane. Thus, these parts become darker and eventually disappear. This feature is called optical sectioning.
3. True, three-dimensional data sets can be recorded.
4. Scanning the object in x/y-direction as well as in z-direction (along the optical axis) allows viewing the objects from all sides.
5. Due to the small dimension of the illuminating light spot in the focal plane, stray light is minimized.
6. By image processing, many slices can be superimposed, giving an extended focus image which can only be achieved in conventional microscopy by reduction of the aperture and thus sacrificing resolution.

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## *Crystals Growth-Interference Contrast Microscopy*

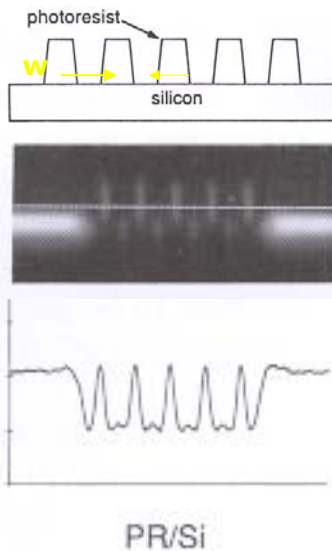


**Growth spiral on  
cadmium iodide  
crystals growing  
From water  
solution (1025x).**

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## Confocal Scanning Optical Microscopy

### Three-Dimensional Optical Microscopy



#### Critical dimension measurements in semiconductor metrology

Cross-sectional image with line scan at PR/Si interface of a sample containing  $0.6\mu\text{m}$ -wide lines and  $1.0\mu\text{m}$ -thick photoresist on silicon.

The bottom width,  $w$ , determining the area of the circuit that is protected from further processing, can be measured accurately by using CSOP.

Measurement of the patterned photoresist is important because it allows the process engineer to simultaneously monitor for defects, misalignment, or other artifacts that may affect the manufacturing line.