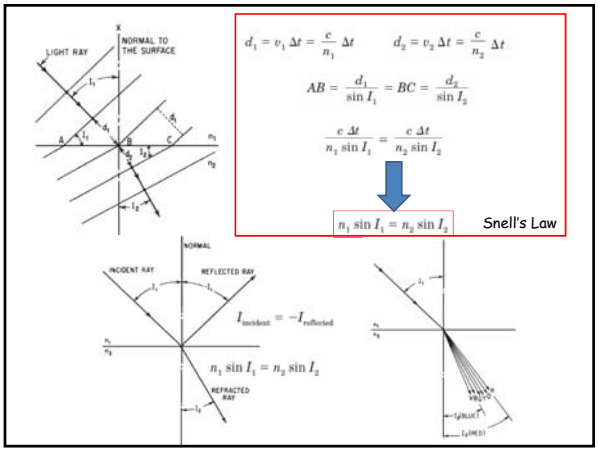
Geometrical Optics and Aberrations

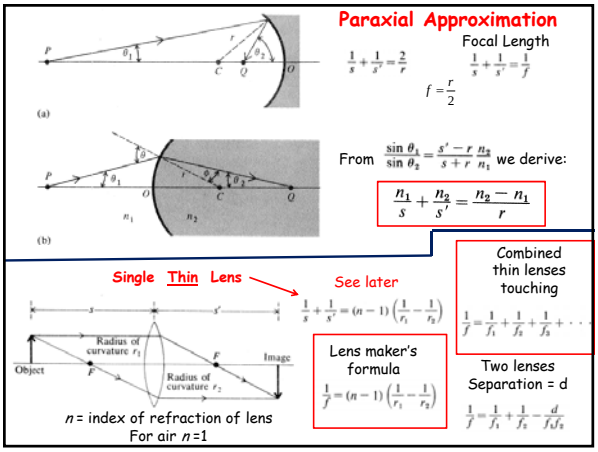
Some basics of geometrical optics and aberrations

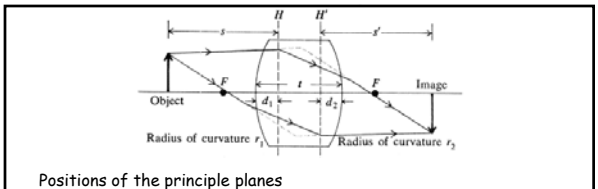
We will be switching now and then during the lecture to the following web site.

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

http://www.micro.magnet.fsu.edu/primer/lightandcolor/lenseshome.html







Positions of the principle planes

$d_1 = ft\left(\frac{1-n}{r_2}\right)$

$d_2 = ft\left(\frac{1-n}{r_1}\right)$

Focal length of the thick lens

$\frac{1}{f} = (n-1)\left[\frac{1}{r_1} + \frac{1}{r_2} - \frac{(n-1)t}{nr_1r_2}\right]$

The **object and image positions** are measured from the **principle planes**, H and H'

$$\frac{1}{s} + \frac{1}{s'} = \frac{1}{f}$$

n = index of refraction of lens
For air n =1

Aberrations

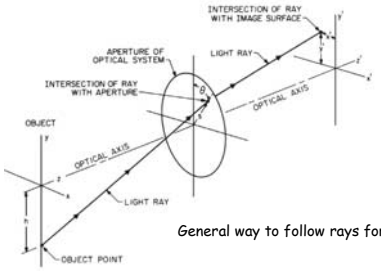
Things are not perfect; But they can be corrected. $$$$$

See also

http://www.cartage.org.lb/en/themes/sciences/physics/optics/Optical/Lens/Lens.htm

http://micro.magnet.fsu.edu/primer/anatomy/aberrationhome.html

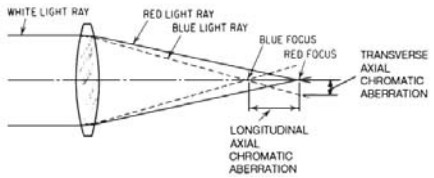
Aberrations



General way to follow rays for Aberrations

A ray from the point *y=h, (x=0) in the object passes through the optical* system aperture at a point defined by its polar coordinates, (*s*, θ *), and intersects* the image surface at *x', y'. Assume an axially symmetric object.*

Chromatic aberration



The under corrected longitudinal chromatic aberration of a simple lens is due to the blue rays undergoing a greater refraction than the red rays.

The image of an axial point in the presence of chromatic aberration is a central bright dot surrounded by a halo. The rays of light which are in focus, and those which are nearly in focus, form the bright dot, or pincushion, distortion. The out-of-focus rays form the halo. Thus, in an undercorrected visual instrument, the image would have a yellowish dot (formed by the orange, yellow, and green rays) and a purplish halo (due to the red and blue rays). If the screen on which the image is formed is moved toward the lens, the central dot will become blue; if it is moved away, the central dot will become red.

Chromatic Aberration Correction

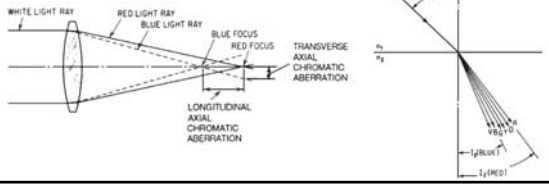
Lens combination (with n_1 and n_2) made of two different types of glass

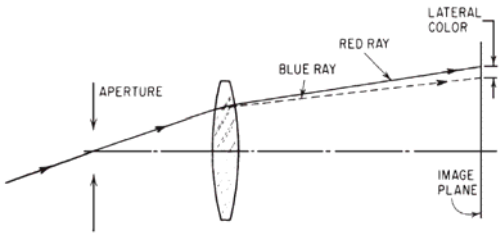
$$f_1 = f\left(1 - \frac{\delta_1}{\delta_2}\right) \qquad f_2 = f\left(1 - \frac{\delta_2}{\delta_1}\right)$$

$$\delta_1 = \frac{1}{n_1 - 1}\frac{dn_1}{d\lambda} \qquad \delta_2 = \frac{1}{n_2 - 1}\frac{dn_2}{d\lambda}$$

Since $dn/d\lambda$ also varies with wavelength, a lens can be achromatized over a limited wavelength interval only.

With just two lenses you can only correct over certain wavelength range.



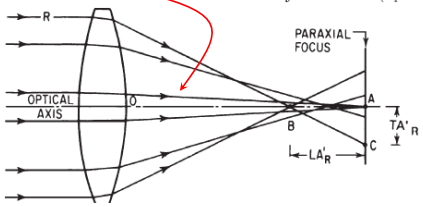


Lateral color, or chromatic difference of magnification, results in different-sized images for different wavelengths.

Spherical aberration

Remember, for paraxial rays

$$\frac{1}{f} = (n-1)\left(\frac{1}{r_1} - \frac{1}{r_2}\right)$$



A simple converging lens with under corrected spherical aberration. The rays farther from the axis are brought to a focus nearer the lens.

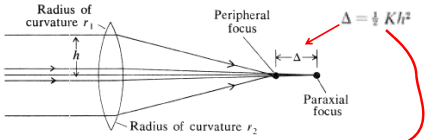
The image of a point formed by a lens with spherical aberration is usually a bright dot surrounded by a halo of light; the effect of spherical on an extended image is to soften the contrast of the image and to blur its details.

Spherical Aberration

for paraxial rays

$$\frac{1}{f} = (n-1)\left(\frac{1}{r_1} - \frac{1}{r_2}\right)$$

For a simple lens



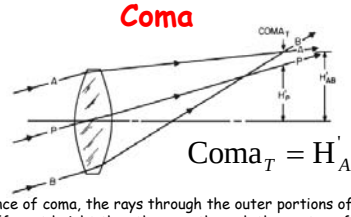
$$\Delta = \tfrac{1}{2} K h^2$$

$$K = \frac{f^2(n-1)}{n^2}\left[\frac{1}{r_1^3} + \left(\frac{1}{f} + \frac{1}{r_2}\right)\left(\frac{n+1}{f} + \frac{1}{r_2}\right)\right]$$

K is a minimum when $\dfrac{r_1}{r_2} = \dfrac{n+4-2n^2}{n+2n^2}$

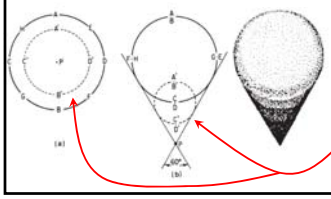
In real optical instruments (cameras, microscopes) the spherical aberration is corrected with multiple lenses With different curvatures and different lens separations.

Coma



$$\text{Coma}_T = \text{H}'_{AB} - H'_P$$

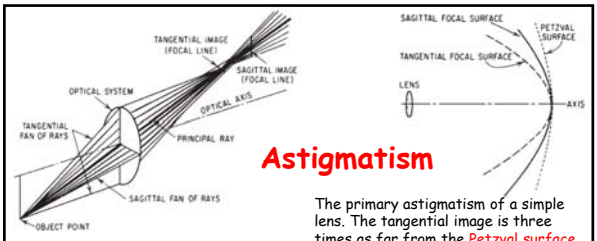
In the presence of coma, the rays through the outer portions of the lens focus at a different height than the rays through the center of the lens.



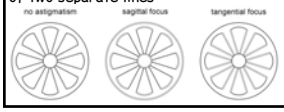
The coma patch. The image of a point source is spread out into a comet-shaped flare.

The relationship between the position of a ray in the lens aperture and its position in the coma patch.

Astigmatism



Astigmatism occurs when the tangential and sagittal (sometimes called radial) images do not coincide. In the presence of astigmatism, the image of a point source is not a point, but takes the form of two separate lines

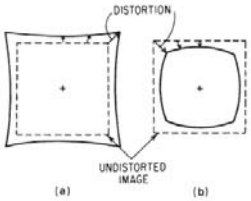


The primary astigmatism of a simple lens. The tangential image is three times as far from the Petzval surface as the sagittal image. Note that the figure is to scale.

Petzval surface = A paraboloidal surface on which point images of point objects are formed by a doublet lens whose separation is such that astigmatism is eliminated.

When the tangential image is to the left of the sagittal image (and both are to the left of the Petzval surface) the astigmatism is called negative, under corrected, or inward-(toward the lens) curving. When the order is reversed, the astigmatism is overcorrected, or backward curving.

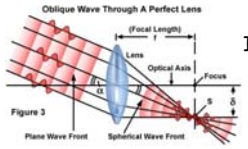
Distortion



Distortion. (a) Positive, or pincushion, distortion. (b) Negative, or barrel, distortion. The sides of the image are curved because the **amount of distortion** varies as **the cube of the distance from the axis**. Thus, in the case of a square, the corners are distorted 2sqrt(2) as much as the center of the sides.

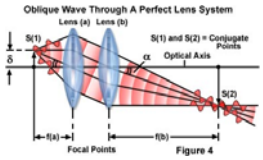
A system which produces distortion of one sign will produce distortion of the opposite sign when object and image are interchanged. Thus a camera lens with barrel distortion will have pincushion distortion if used as a projection lens (i.e., when the film is replaced by a slide). Obviously if the same lens is used both to photograph and to project the slide, the projected image will be rectilinear (free of distortion) since the distortion in the slide will be canceled out upon projection.

A few more things:



It goes both ways

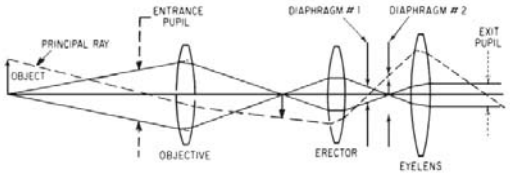
http://www.microscopyu.com/tutorials/java/components/perfectlens/index.html



http://www.microscopyu.com/tutorials/java/components/twolenssystem/index.html

pupils, stops, and fields

Relationships between pupils, stops, and fields.



Diaphragm #1 is the aperture of the system which limits the size of the axial cone of energy from the object. All of the other elements of the system are large enough to accept a bigger cone. Thus, diaphragm #1 is the aperture stop of the system.

The oblique ray through the center of the aperture stop is called the *principal, or chief, ray, and is shown in the figure as a dashed line. The entrance and exit pupils of the system are the images of the aperture* stop in object and image space, respectively.

The initial and final intersections of the dashed principal ray with the axis locate the pupils. The diameter of the axial cone of rays at the pupils indicates the pupil diameters. For any point on the object, the amount of radiation accepted by, and emitted from, the system is determined by the size and location of the pupils.

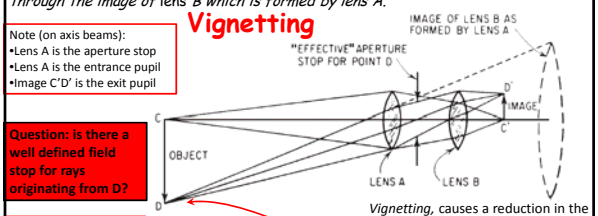
In most systems the aperture stop is located at or very near the objective lens. This location gives the smallest possible diameter for the objective, and since the objective is usually the most expensive component (per inch of diameter), minimizing its diameter makes good economic sense.
Field stops could be placed at both internal images to further reduce stray radiation.

Vignetting in a system of separated components. **The cone of rays from point *D* is limited by the lower rim of lens *A* and the upper rim of *B*, and is smaller than the cone accepted from point *C*.** *Note that the upper ray from D just passes through the image of* lens *B which is formed by lens A.*

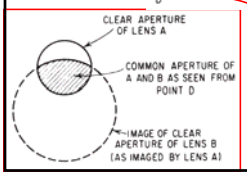
Vignetting

Note (on axis beams):
- Lens A is the aperture stop
- Lens A is the entrance pupil
- Image C'D' is the exit pupil

Question: is there a well defined field stop for rays originating from D?



Vignetting, causes a reduction in the illumination at the image point *D'*.



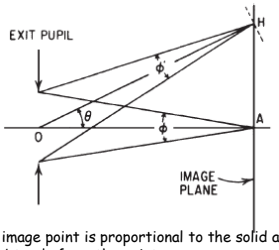
The **apertures** of an optical system as seen from point *D*.

$Cos^4\theta$ "law" of illumination (energy per area)

For off-axis image points, even when there is no vignetting, the illumination is usually lower than for the image point on the axis.

The illumination at *H* is $cos^4\theta$ *times* that at *A*.

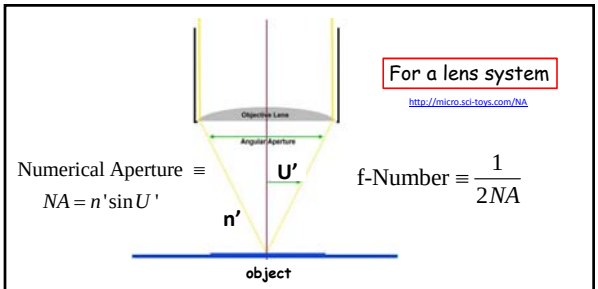
OH is greater than OA by a factor equal to 1/cos θ , so the illumination is decreased by a factor of $\cos^2\theta$. The projected area of the exit pupil at H is reduced by a factor which is *approximately* $cos^3\theta$. *Plane of illumination at H is oblique to the plane of recording – another reducing factor of* $cos^3\theta$



The illumination at an image point is proportional to the solid angle which the exit pupil subtends from the point.

Illumination at *H* = $(\cos^{-4}\theta)$ x *(illumination at A)*

$\cos^4 30° = 0.56$, $\cos^4 45° = 0.25$, and $\cos^4 60° = 0.06$

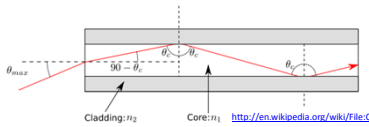


For a lens system

http://micro.sci-toys.com/NA

$$\text{Numerical Aperture} \equiv NA = n'\sin U'$$

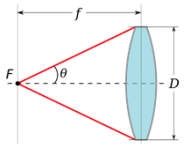
$$\text{f-Number} \equiv \frac{1}{2NA}$$



For a fiber optic

http://en.wikipedia.org/wiki/File:Optic_fibre-numerical_aperture_diagram.svg

Numerical Aperture vs f-number



$\theta \equiv U'$

$$(\text{f-number}) = f / D$$

$$NA = n\sin\theta = n\sin\left(\tan^{-1}\frac{D}{2f}\right) \approx n\frac{D}{2f}$$

$$So \quad (\text{f-number}) \approx \frac{1}{2NA}$$

n is the index of refraction **of the medium in which the lens is immersed** (1.0 for air, 1.33 for pure water, and up to 1.56 for oils)

The ratio of the focal length to the clear aperture of a lens system is called the relative aperture, *f-number, or "speed" of the system, and* (other factors being equal), the illumination in an image is inversely proportional to the square of this ratio.

$$f\text{-number} = \text{efl/clear aperture}$$

Why is this useful?

When a lens forms the image of an extended object, the amount of energy collected from a small area of the object is **1) directly proportional** to the area of the **clear aperture**, or entrance pupil, of the lens. **2) At the image** the illumination (power per unit area) **is inversely proportional** to the **image area** over which this object is spread. The aperture area is proportional to the square of the pupil diameter, and the image area is proportional to the square of the image distance, or square of the focal length..

Another way to look at it: $\text{Numerical aperture} = NA = n'\sin U'$

U' is the sine of the half angle of the cone of illumination, n' is index of refraction

NA is used for microscopes; f-# is usually used for cameras

$$f\text{-number} = \frac{1}{2NA}$$

http://micro.magnet.fsu.edu/primer/java/imagebrightness/index.html

http://micro.magnet.fsu.edu/primer/java/imageformation/airyna/index.html

$$f\text{-number} = \frac{1}{2NA}$$

The above relationship is only true for aplanatic systems (i.e., systems corrected for coma and spherical aberration) with infinite object distances.

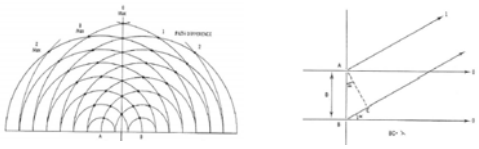
The terms "fast" and "slow" are often applied to the *f-number of an* optical system to describe its "speed." A lens with a large aperture (and thus a small *f-number) is said to be "fast," or to have a high "speed." A* smaller aperture lens is described as "slow." This terminology derives from photographic usage, where a larger aperture allows a shorter (or faster) exposure time to get the same quantity of energy on the film and may allow a rapidly moving object to be photographed without blurring.

http://micro.magnet.fsu.edu/primer/java/kohler/condenseraperture/index.html

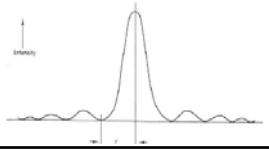
A system working at finite conjugates will have an **object-side numerical aperture** as well as an **image-side numerical aperture** and that the ratio NA/NA' (object-side NA)/(image-side NA) must equal the absolute value of the magnification.

Diffraction

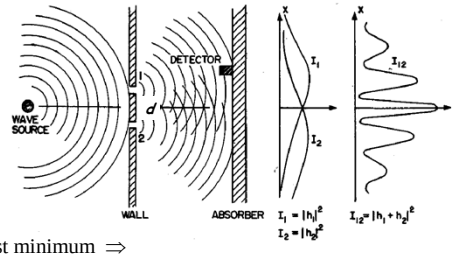
Diffraction of two slits or two holes



Diffraction through one hole



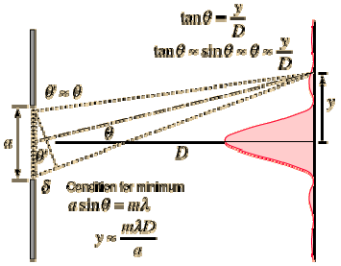
Diffraction of two slits



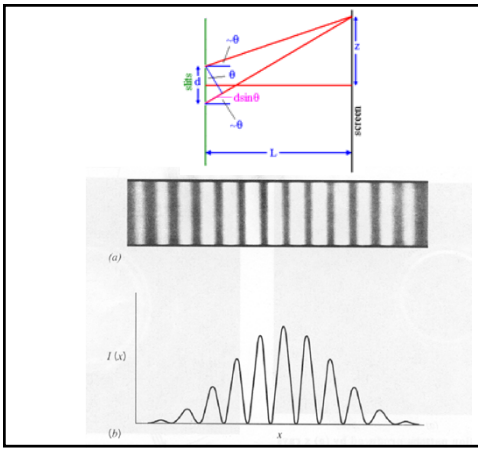
first minimum $\Rightarrow$

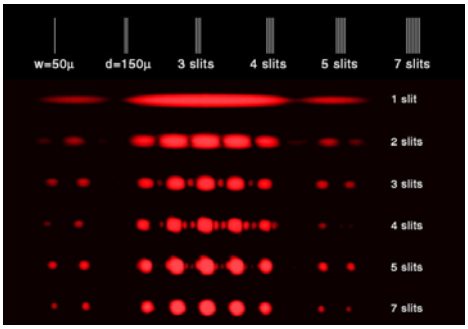
$\sin\theta_{\min_1} = (\lambda/2)/d$ $\qquad$ $d\sin\theta_{\min_n} = (\lambda/2)(n+1)$

Single Slit diffraction



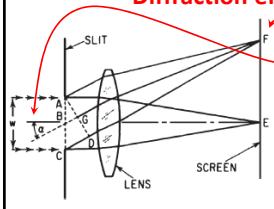
http://hyperphysics.phy-astr.gsu.edu/hbase/phyopt/sinslit.html





http://electron9.phys.utk.edu/phys136d/modules/m9/diff.htm

Diffraction effects of apertures



there is a dark zone at *F when*

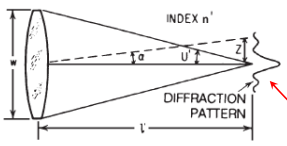
$$\sin\alpha = \frac{\pm N\lambda}{w}$$

N is an integer; λ is wavelength; w is the width of the slit

For a rectangular aperture, the illumination on the screen is given by

$$I = I_0 \frac{\sin^2 m_1}{m_1^2} \cdot \frac{\sin^2 m_2}{m_2^2}$$

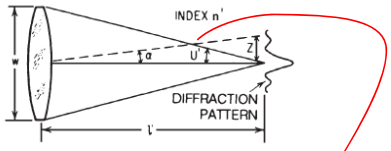
$$m_i = \frac{\pi w_i \sin\alpha_i}{\lambda} \qquad i = 1,2$$



For a circular aperture:

$$I = I_0\left[\frac{2J_1(m)}{m}\right]^2$$

Diffraction effects of apertures



$$l' = \frac{-w}{2 \sin U'}$$

When α is small: $$Z = \frac{l'\alpha}{n'} = \frac{-\alpha w}{2n' \sin U'}$$

84 percent of the energy in the pattern is contained in the central spot. The illumination in the central spot is almost 60 times that in the first bright ring

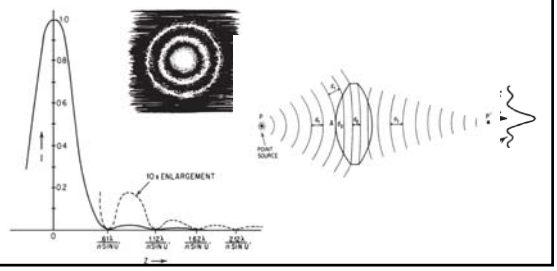
Size and distribution of energy in the diffraction pattern at the focus of a perfect lens.

	Circular Aperture			Slit Aperture	
Ring (or band)	Z	Peak Illumination	Energy in Ring	Z	Peak Illumination
Central maximum	0	1.0	83.9%	0	1.0
1st dark ring	0.61 λ/n' sin U'	0.0		0.5 λ/n' sin U'	0.0
1st bright ring	0.82 λ/n' sin U'	0.017	7.1%	0.72 λ/n' sin U'	0.047
2d dark ring	1.12 λ/n' sin U'	0.0		1.0 λ/n' sin U'	0.0
2d bright ring	1.33 λ/n' sin U'	0.0041	2.8%	1.23 λ/n' sin U'	0.017
3rd dark ring	1.62 λ/n' sin U'	0.0		1.5 λ/n' sin U'	0.0
3rd bright ring	1.85 λ/n' sin U'	0.0016	1.5%	1.74 λ/n' sin U'	0.0083
4th dark ring	2.12 λ/n' sin U'	0.0		2.0 λ/n' sin U'	0.0
4th bright ring	2.36 λ/n' sin U'	0.00078	1.0%	2.24 λ/n' sin U'	0.0050
5th dark ring	2.62 λ/n' sin U'			2.5 λ/n' sin U'	0.0

Resolution of Optical Systems

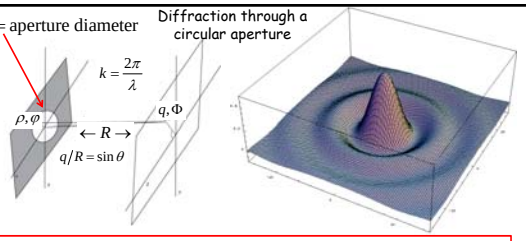
When a bright **point source of light** is imaged, an Airy disk with a perfect lens, encircling rings appear in the image plane. The distance from the center of the Airy disk to the first dark ring is "Z":

$$Z = \frac{0.61\lambda}{n' \sin U'} = \frac{0.61\lambda}{NA} = 1.22\lambda\,(f/\#)$$



Diffraction through a circular aperture

a = aperture diameter



See http://cnx.org/content/m13097/latest/ for some details of the derivation

The first zero occurs at $J_1(r)/r=0$ *which is at* $r=kaq/R = 3.83$ *for the first ring.*

$$I = I_0\left[\frac{2J_1(kaq/R)}{(kaq/R)}\right]^2 = I_0\left[\frac{2J_1(r)}{(r)}\right]^2 \quad ; r=kaq/R$$

$$I(\theta) = I(0)\left[\frac{2J_1(ka\sin\theta)}{(ka\sin\theta)}\right]^2 \quad ; \sin\theta = q/R$$

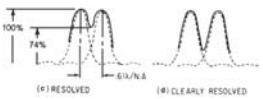
In the image the first zero is found at $\Rightarrow q_1 = \frac{1.22R\lambda}{2a}$

see http://www.matter.org.uk/tem/diffraction_at_aperture.htm

This is for EM but is the same idea

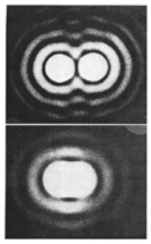
Two bright spots

NA=$n \sin U$



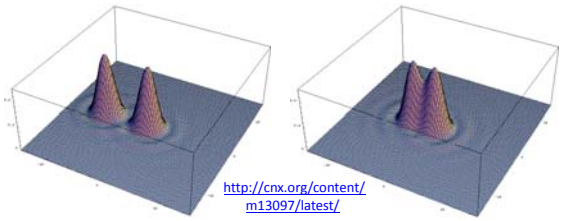
The dashed lines represent the diffraction patterns of two point images at various separations. The solid line indicates the combined diffraction pattern. Case (b) is the Sparrow criterion for resolution. Case (c) is the Rayleigh criterion.

Rayleigh's criterion, the smallest separation between two object points that will allow them to be resolved



Lord Rayleigh's criterion for resolution $$Z = \frac{0.61\lambda}{NA}$$

Diffraction limits for resolution of two sources



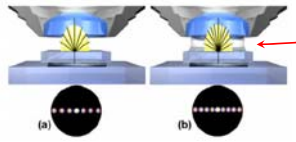
http://cnx.org/content/m13097/latest/

Light from any point in a sample that passes through a circular aperture is diffracted and this diffraction distribution in the image sets the limit of resolution on an optical device such as a microscope or telescope. The limit of resolution between two sources is when the central spot of one Airy disk is on the zero of the other Airy disk. This is known as the Raleigh criterion. This is the most used criterion for resolution. The limits are:

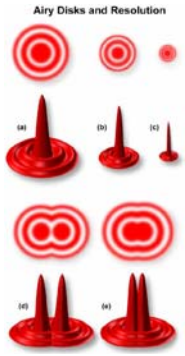
$$\Delta q = 1.22R\lambda/D \;;\; \Delta\theta = 1.22\lambda/D \;;\; \Delta\theta = \Delta q/R$$

$D \equiv 2a$ = diameter of the aperture ; R = distance from object to the image

(oil) Index of refraction *n'*

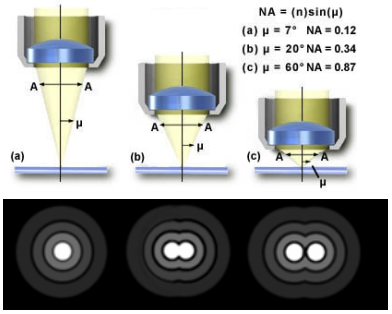


Effect of imaging medium refractive index on diffracted orders captured by the objective. (a) Conoscopic image of objective back focal plane diffraction spectra when air is the medium between the cover slip and the objective front lens. (b) Diffraction spectra when immersion oil of refractive index similar to glass is used in the space between the cover slip and the objective front lens.



$d = 0.61\ (\lambda / NA)$

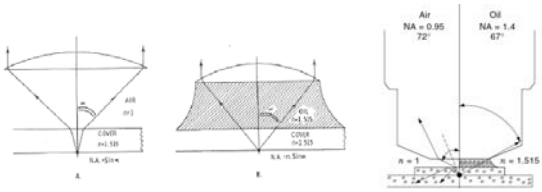
$NA = n' \sin U'$



http://www.vanosta.be/microscopy.htm

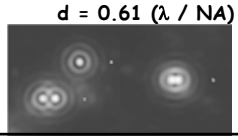
see http://www.matter.org.uk/tem/resolution.htm This is for EM but is the same idea

Lateral resolution with and without oil



Illustrating the difference in the angle collected with air and with oil. The larger angle will result in better lateral resolution

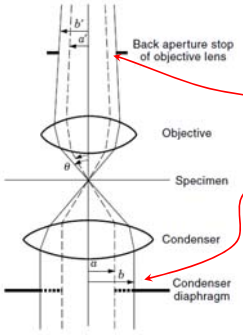
$d = 0.61\ (\lambda / NA)$ $NA = n' \sin U'$



You need a high NA to get the best lateral resolution

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

Lateral resolution

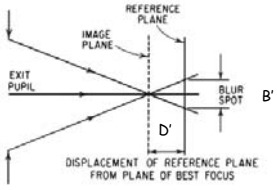


the back aperture of the objective is no longer filled at the reduced setting

Adjusting the condenser aperture directly affects spatial resolution in the microscope. Since a large aperture angle is required for maximum resolution, **the front aperture of the condenser must be fully illuminated**.

$NA = n' \sin U'$

Depth of Focus



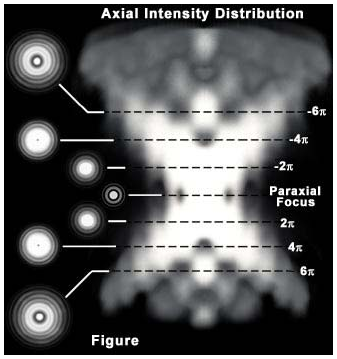
$$D_{fo} = \frac{M^2\ R.P.}{N.A.}$$

Resolution (or strictly resolving power RP) is defined as the closest spacing of two points which can be resolved by the microscope to be separate entities.

The ***depth of focus*** *is the amount by which the* ***image*** *may be shifted* longitudinally with respect to some reference plane (e.g., film, reticle) and which will introduce no more than the acceptable blur.

The ***depth of field*** *is the amount by which the* ***object*** *may be shifted before* the acceptable blur is produced.

Linear angular blur: $\beta = \frac{B}{D} = \frac{B'}{D'}$ (for the object and image side)



The **depth of field** depends on the **resolution** of the individual microscope.

The **depth of focus** depends on the **magnification**.

The depth of focus can be improved by increasing your magnification. Both resolution and magnification depend on the NA. By using a high-quality lens and increasing magnification, the focus depth is greatly improved.

http://micro.magnet.fsu.edu/primer/anatomy/focusdepth.html

depth of field for a system with a clear aperture *A*

Table 9 Depth of Field and Image Depth *

Magnification	Numerical Aperture	Depth of Field (μ)	Image Depth (mm)
4x	0.10	55.5	0.13
10x	0.25	8.5	0.80
20x	0.40	5.8	3.8
40x	0.65	1.0	12.8
60x	0.85	0.40	29.8
100x	0.95	0.19	80.0

* Source: Nikon

For diffraction-limited optics, the wave-optical value of *Z* is

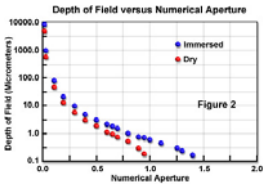
$$Z = \sqrt{n^2 - NA^2}\,\lambda / NA^2 \cong n\lambda / NA^2$$

The **depth of field** is determined by the **distance** from the **nearest object plane** in focus to that of the **farthest plane** also simultaneously in focus.

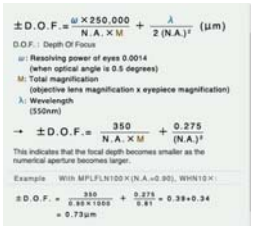
The pinhole camera has an infinitely small NA and an infinite depth of field—all objects, both near and far, are simultaneously in focus in such a camera.

http://www.microscopyu.com/articles/formulas/formulasfielddepth.html

Putting them together – rough guide



http://www.microscopyu.com/articles/formulas/formulasfielddepth.html



http://www.olympus-ims.com/en/microscope/terms/focal_depth/

For a video camera, the focal depth will vary according to number of pixels of CCD, optical magnification, and numerical aperture.

Gaussian laser beam focusing

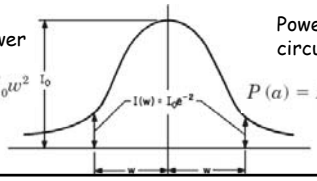
$$I(r) = I_o e^{-2r^2/w^2}$$

http://www.cvimellesgriot.com/products/Documents/TechnicalGuide/Gaussian-Beam-Optics.pdf

$I(r)$ = the beam intensity at a distance r from the beam axis
I_0 = the intensity on axis
r = the radial distance
e = 2.718....
w = the radial distance at which the intensity falls to I_0/e^2, i.e., to 13.5 percent of its central value. This is usually referred to as the beam width, although it is a semi-diameter. It encompasses 86.5 percent of the beam power.

Total power

$$P_{tot} = {}^1/_2\,\pi I_0 w^2$$



Power through a circular aperture

$$P(a) = P_{tot}(1 - e^{-2a^2/w^2})$$

The spreading of a Gaussian Beam (most lasers are Gaussian)

As the laser beam (wavelength λ) spreads it progresses along z as:

$$w_z^2 = w_0^2\left[1 + \left(\frac{\lambda z}{\pi w_0^2}\right)^2\right]$$

The angular spread of the beam is:

$$\frac{\alpha}{2} = \frac{w_z}{z}_{\text{as } z\to\infty} = \frac{\lambda}{\pi w_0}$$

$$\alpha = \frac{4\lambda}{2\pi w_0} = \frac{1.27\lambda}{\text{diameter of the beam}}$$

Note this is almost the same as the diffraction of a parallel beam of equal intensity passing through a hole of diameter D:

$$\alpha_c = \frac{1.22\lambda}{D}$$

So, now let's look at a microscope

http://www.microscopyu.com/articles/formulas/index.html

Useful links we will use throughout the lecture

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

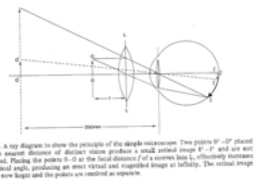
http://www.microscopyu.com/articles/formulas/formulasconjugate.html

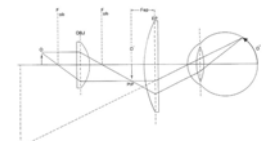
http://www.microscopyu.com/articles/dic/index.html

http://micro.magnet.fsu.edu/primer/techniques/dic/dicconfiguration.html

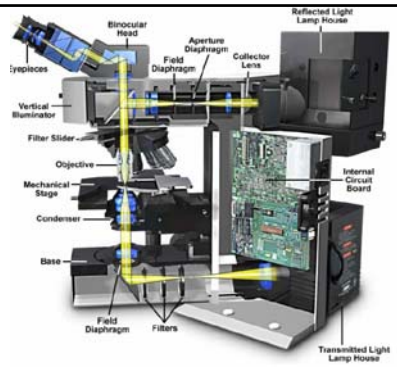
http://www.olympusmicro.com/primer/techniques/dic/dichome.html

Basic Design of the optical system for an optical microscope



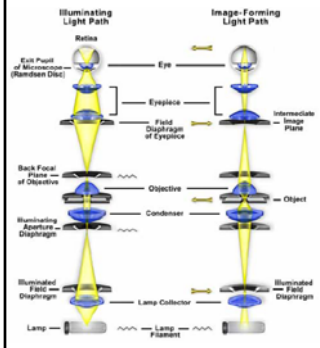


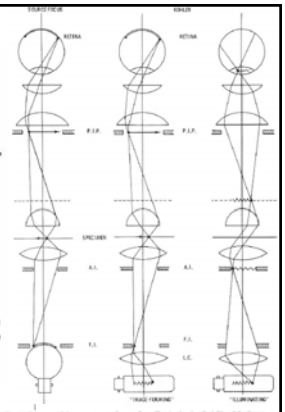
An Introduction to the optical microscope
Savile Bradbury



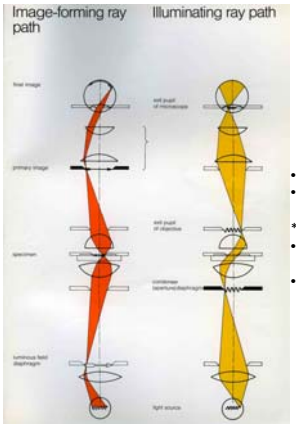
Components of a modern microscope configured for both transmitted and reflected light. This cutaway diagram reveals the ray traces and lens components of the microscope's optical trains.

Köhler Illumination





http://www.microscopyu.com/tutorials/java/kohler/index.html



Benefits for Köhler (Koehler) Illumination

- Evenly illuminated image
- Minimized reflection or glare or effect of dust in the system
- * Minimum heating of specimen
- Field diaphragm becomes a focus reference for easy focusing of specimen
- Microscope is "all set" for employing contrasting techniques (Brightfield, Oblique, Phase, DIC, etc.)

Picture: Carl Zeiss

Conjugate Planes

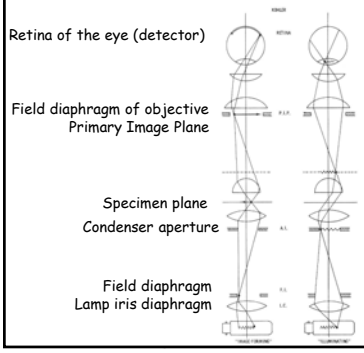
Planes in the optical system that are equivalent

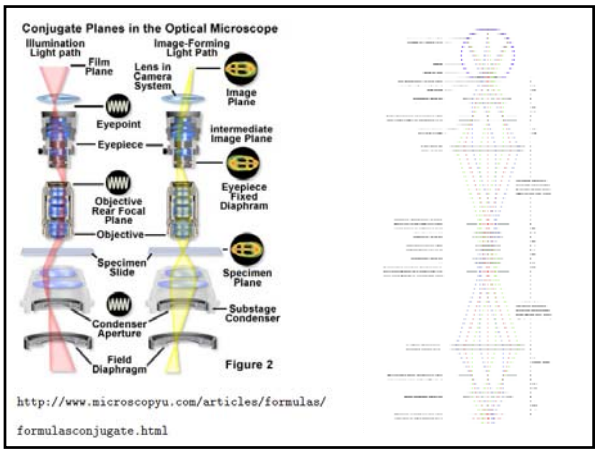
Illuminating	Image forming
Lamp filament	Lamp iris diaphragm (field iris)
Front focal plane of the condenser (aperture iris)	Specimen plane
Back focal plane of objective	Primary image plane
Exit pupil of eyepiece	Retina of the eye (detector)

In the illuminating ray path (shown in **red**), the spherical wave fronts converge and are brought into focus onto the aperture planes, while in the imaging ray path (shown in **black**), the spherical waves converge into focused rays in the field planes.

Microscope Alignment for Köhler Illumination

http://www.microscopyu.com/tutorials/java/kohler/index.html





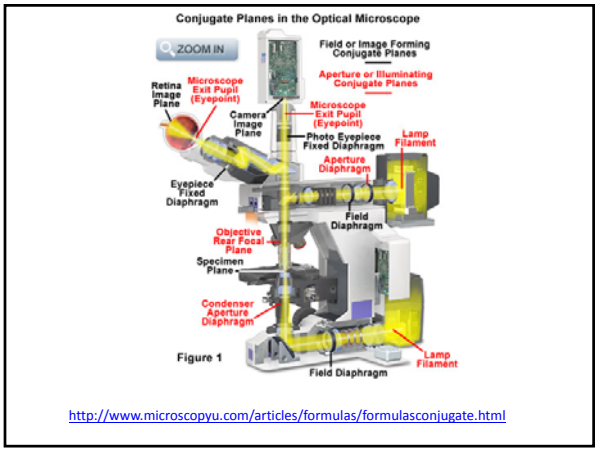
Class questions

1)Where can you place an reticule?

2)Where do you place the phase plates?

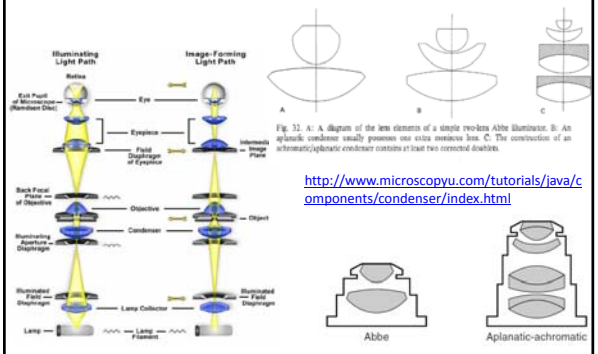
answer after discussing a phase plate

http://www.microscopyu.com/tutorials/java/conjugateplanes/index.html

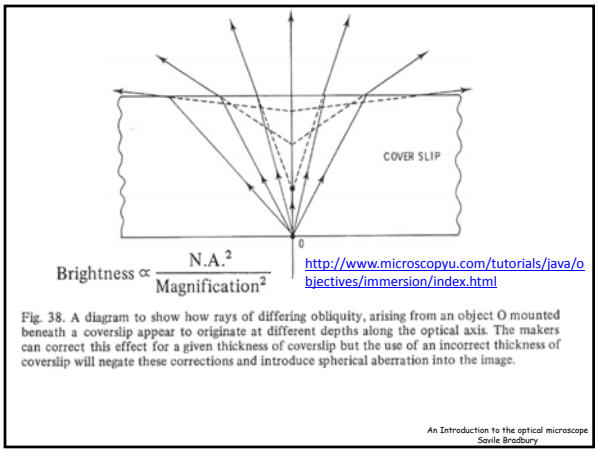


http://www.microscopyu.com/articles/formulas/formulasconjugate.html

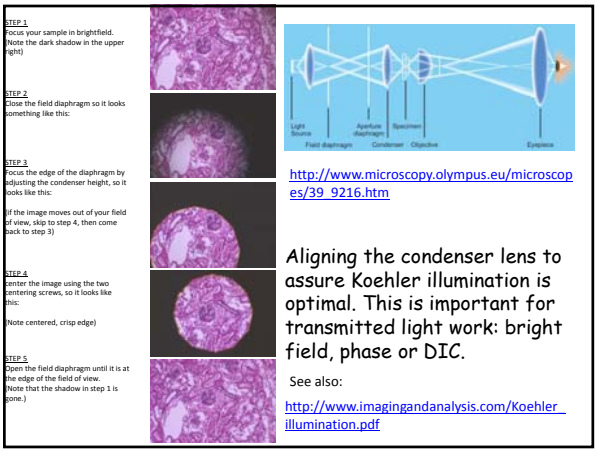
Condensers



http://www.microscopyu.com/tutorials/java/components/condenser/index.html



http://www.microscopyu.com/tutorials/java/objectives/immersion/index.html



http://www.microscopy.olympus.eu/microscopes/39_9216.htm

Aligning the condenser lens to assure Koehler illumination is optimal. This is important for transmitted light work: bright field, phase or DIC.

See also:

http://www.imagingandanalysis.com/Koehler_illumination.pdf

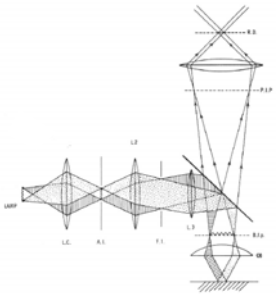


Fig. 15. A ray diagram of Köhler illumination with an incident light system. L.C. = lamp collector lens; A.I. = aperture-iris diaphragm; F.I. = field-iris diaphragm; L2, L3 = relay lenses in the illuminating system; OB = objective; B.f.p. = back focal plane of the objective; P.I.P. = primary image plane; R.D. = exit pupil of the eyepiece or the 'Ramsden disc'. Note: the angles of the rays are much exaggerated for clarity; the crossing of four rays at a single point on the mirror is coincidental, and arranged thus for ease in drawing.

The Microscope Optical Train

http://www.microscopyu.com/articles/optics/components.html

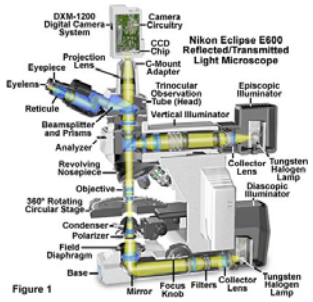


Figure 1

Two types of optical trains used in microscopes

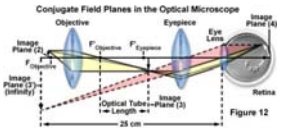


Figure 12

http://www.microscopyu.com/tutorials/java/components/fixedtubelength/index.html

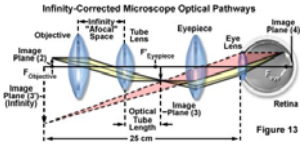


Figure 13

http://www.microscopyu.com/tutorials/java/components/infinitymicroscope/index.html

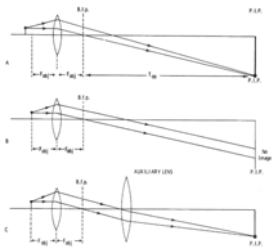


Fig. 39. A: The geometrical ray diagram for a conventional objective forming a real image in its primary image plane when the object is just outside the focal plane. B: An infinity-corrected objective cannot form a real primary image. C: The addition of an auxiliary lens to such an objective now forms the real primary image needed by the eyepiece. There are now no restrictions on the mechanical tube length. B.f.p. = back focal plane of objective; F_{obj} = focal length of objective; P.I.P. = primary image plane; T_{op} = optical tube length.

Objectives

Objective Correction for Optical Aberration

Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

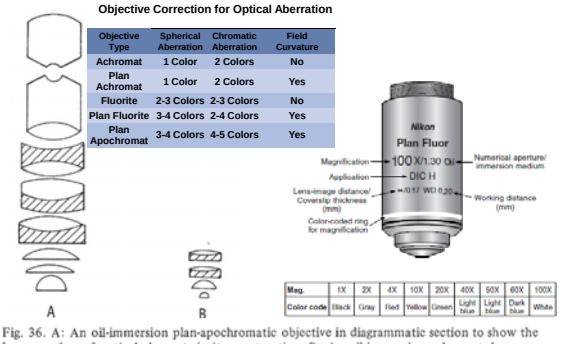


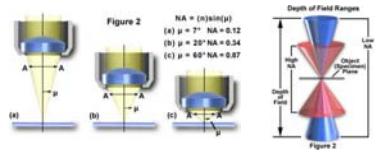
Fig. 36. A: An oil-immersion plan-apochromatic objective in diagrammatic section to show the large number of optical elements in its construction. B: An oil-immersion achromat, by comparison, is relatively simple in optical construction.

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

Table 1. *Some typical data for objectives*

Objective type	Initial mag.	Focal length (mm)	N.A.	Working distance (mm)	Approx. resoln. (μm)
Plano	X 1	33	0.04	30	7
Plano	X 2.5	56	0.08	11	3.4
Achromat	X 4	32	0.12	24	2.5
Planachromat	X 10	16	0.25	0.6	1.1
Planachromat	X 40	4	0.65	0.15	0.42
Planapo	X 40	4	0.75	0.44	0.37
Apochromat	X 40	4.6	0.95	0.09	0.30
Achromat*	X 63	2.9	0.85	0.24	0.32
Planachromat*	X 100	1.7	1.30	0.26	0.21
Planapo*	X 100	2.4	1.32	0.24	0.20

*Signifies oil-immersion objective.

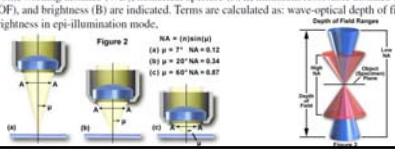


An Introduction to the optical microscope
Savile Bradbury

Characteristics of Selected Objective Lenses[a]

M	Type	Medium (n)	WD (mm)	NA	d_{min} (μm)	DOF (μm)	B
5	Achromat	1	9.9	0.12	2.80	38.19	0.1
10	Achromat	1	4.4	0.25	1.34	8.80	0.4
20	Achromat	1	0.53	0.45	0.75	2.72	1.0
25	Fluorite	1.515	0.21	0.8	0.42	1.30	6.6
40	Fluorite	1	0.5	0.75	0.45	0.98	2.0
40	Fluorite	1.515	0.2	1.3	0.26	0.49	17.9
60	Apochromat	1	0.15	0.95	0.35	0.61	2.3
60	Apochromat	1.515	0.09	1.4	0.24	0.43	10.7
100	Apochromat	1.515	0.09	1.4	0.24	0.43	3.8

[a]The magnification (M), type of lens design, refractive index (n) of the intervening medium (air or immersion oil), working distance (WD), numerical aperture (NA), minimum resolvable distance (d), depth of field (DOF), and brightness (B) are indicated. Terms are calculated as: wave-optical depth of field, $nλ/NA^2$; brightness in epi-illumination mode.



Microscope Slides and Coverslips

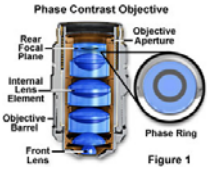
Many objectives are designed to be used with standard (1.1 mm thick) glass slides and coverslips of a certain thickness, usually 0.17 mm, which corresponds to thickness grade 1.5. Other coverslip thicknesses induce spherical aberration and give poorer performance, especially when used with high, dry lenses above 40. For lenses with an NA 0.4, coverslip thickness is not particularly important.

Grade Number	Thickness (mm)
0	0.083–0.13
1	0.13–0.16
1.5	0.16–0.19 (standard)
2	0.19–0.25

Information on Objectives

http://www.molecularexpressions.com/search/index.asp

"click on the URL, and then enter "objectives"

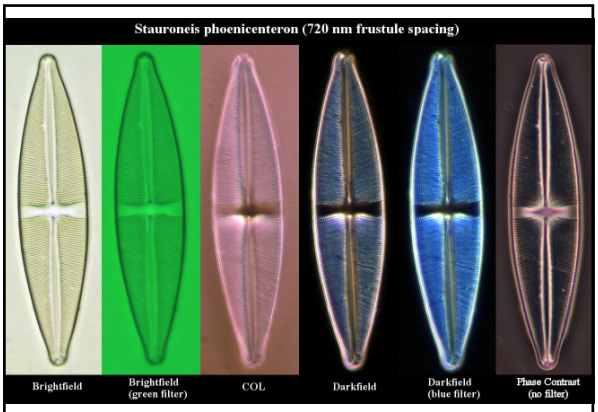


Different microscope imaging modes

Type	Feature	Appearance	Uses
Bright-field	Visible light	Colored/clear specimen – light background	Stained specimen/live ones with sufficient color contrast
Dark-field	Visible light	Bright specimen on dark background	Unstained or difficult to stain specimen
Phase-Contrast	Visible light / Phase shifting plate in objective with special condenser	Different degrees of brightness and darkness	Observe internal structure of specimen
Differential Interference contrast	Visible light out of phase	Nearly 3-D image	Observe finer detail of internal structure of unstained specimen
Fluorescence	UV-light excites molecules	Bright, fluorescent specimen	Detection of organisms or antibodies in clinical specimens
Transmission Electron microscope	Electron beam/magnetic lens	Highly magnified	Internal structures; viruses
Scanning Electron microscope	Electron beam	Three-dimensional view of surfaces	External or internal surfaces of cells
Scanning Tunneling	Wire probe over surfaces	Three-dimensional view of surfaces	Observation of external surfaces of atoms or molecules

Bright field And Dark field Illumination

http://www.molecularexpressions.com/primer/anatomy/illuminationhome.html

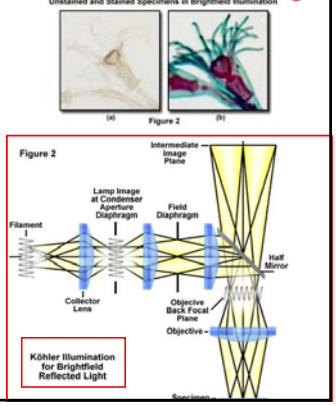


http://www.microscopy-uk.org.uk/mag/artmar06/go-phase.html

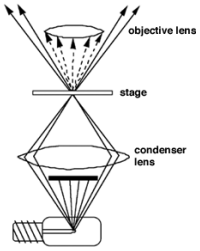
Bright Field Image – no staining

An amoeba with the numerous crystals that are found in this species.
We will use this example throughout this lecture to compare methods

http://www.microscopy-uk.org.uk/index.html?http://www.microscopy-uk.org.uk/intro/index.html

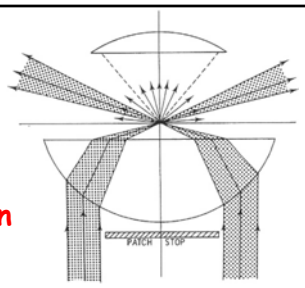


Dark field illumination



solid lines = illumination
dashed lines = scattering or fluorescence

Dark Field illumination



objective

Condenser lens

The illumination light is occluded from the central optical axis of the condenser, and this light is focused onto the sample. After passing through the sample the light that is not diffracted passes outside of the acceptance angle of the objective. The objective gathers light (and an image) of the diffracted, scattered or fluorescence light that is within the acceptance angle of the objective. The image is in "reverse contrast". **This works well for lower NA objectives, but is more difficult for higher NAA objectives.**

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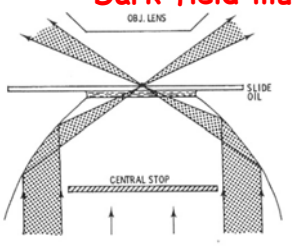
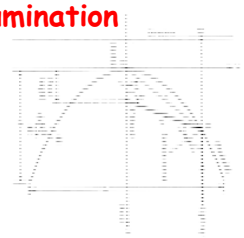
Savile Bradbury

Dark field illumination



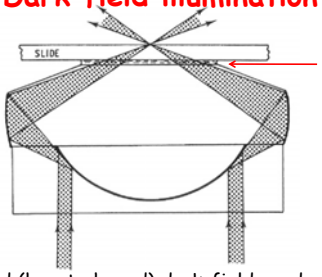


Focus of a parabola

A paraboloid dark-field condenser. The condenser is a solid piece of glass, in the shape of a parabola (flat and polished on top). Direct parallel light is occluded from the center. The glass sides are totally reflect the light, and focus at the sample. In order to focus correctly, oil must be used between the slide and the top of the condenser. **This condenser is useful for very high power objectives.**

An Introduction to the optical microscope
Savile Bradbury

Dark field illumination



Cardioid (heart-shaped) dark-field condenser.

The cone of rays is reflected from a spherical surface, and then from the cardiod outer surface of the upper block. This is also **useful for use with a high NA objective**; although, sometimes the angle of acceptance (NA) of the objective must be reduced to get a good dark field..

http://www.olympusmicro.com/primer/java/darkfield/cardioid/index.html

Dark field illumination

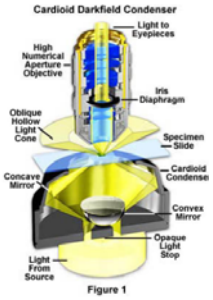


Figure 1

(a) http://micro.magnet.fsu.edu/primer/techniques/darkfield.html

Image in dark field

This photograph shows an amoeba with the numerous crystals that are found in this species showing up against a dark background. The photograph is taken with a Leitz x25 water immersion bright field objective with a numerical aperture of 0.60, while the condenser was a Leitz D 0.80 dry condenser.

http://www.microscopy-uk.org.uk/index.html?http://www.microscopy-uk.org.uk/intro/index.html

First a look at Abbe's theory of image formation Fourier Optics

Nice elementary description in:
Fundamentals of Light Microscopy and Electronic Imaging
D.B. Murphy
Or see more advanced in:
Optical Physics
S.G. Lipson, H. Lipson and D.S. Tannhauser

See also:

An Elementary Theoretical Approach to the Abbe Theory of Optical Image Formation

ANTHONY GERRARD
Bristol College of Science and Technology, Ashley Down, Bristol, England
(Received 19 December 1962)

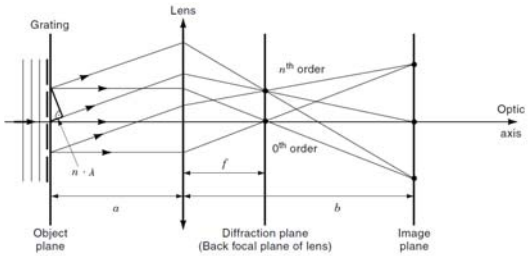


Figure 5-14

Abbe's theory for image formation in a light microscope. An objective lens focused on a grating ($2f > a > f$) in the object plane produces a magnified real image of the grating in the image plane. The diffraction plane is located at $1f$ in the back aperture of the lens. An incident planar wavefront is shown. Diffracted nth-order and nondiffracted 0th-order rays are separated in the diffraction plane, but are combined in the image plane.

D.B. Murphy

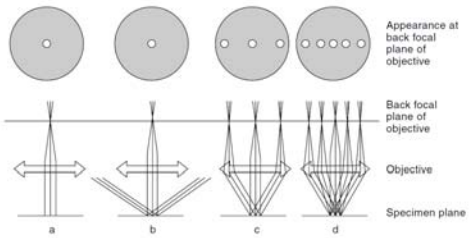


Figure 5-15

Generation of an image by interference requires collection of two adjacent orders of diffracted light by the objective lens. If diffraction at the specimen does not occur (a), or diffracted rays are not collected by the objective (b), no image is formed. In (c), a minimum of two adjacent diffraction orders (0th and 1st) are collected, and an image is formed. In (d), multiple diffracted orders are collected, leading to a high degree of definition in the image.

D.B. Murphy

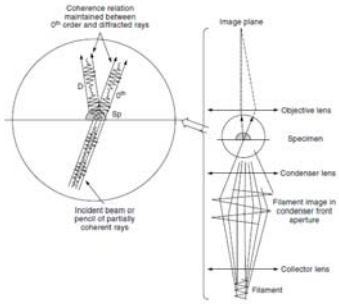
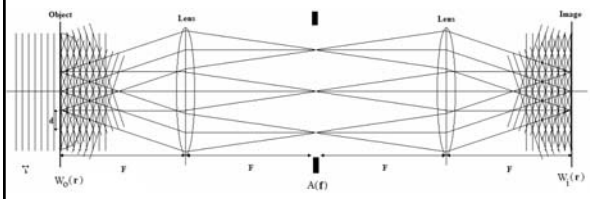


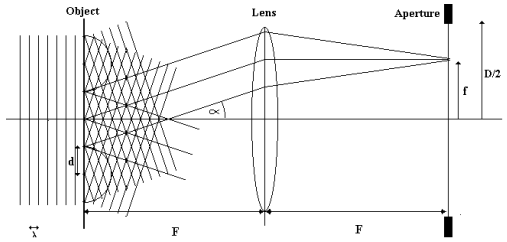
Figure 5-17
Partially coherent wave bundles in the light microscope. Small domains in the lamp filament emit partially coherent wave bundles that reform the image of the filament in the front aperture of the condenser. Rays (small beams or "pencils" of partially coherent photons) incident on a small particle in the specimen plane are broken up into diffracted and undeviated (0th-order) waves that maintain their coherence relationship. A myriad of ray pairs go on through the objective lens and combine incoherently with other rays in the image plane to form the image. The coherence relationship between undeviated and diffracted rays is essential for image formation in all forms of interference microscopy (phase contrast, polarization, differential interference) described in the following chapters.

D.B. Murphy



Imaging devices perform a "double" Fourier transform. This leaves space for manipulation of the data in Fourier space by modifications of the diffracted waves in the back-focal plane.

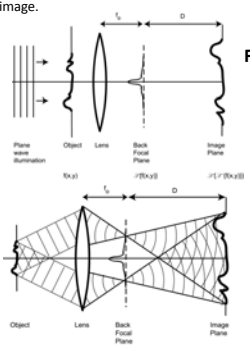
Principles of Phase Contrast (Electron) Microscopy
Marin van Heel Imperial College, London



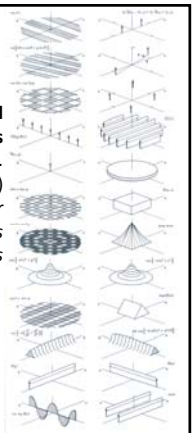
The limiting aperture (with diameter "D") in the back focal plane of the imaging instrument prevents the fine detail image information to reach the image and it limits the resolution achievable by the imaging system.

Principles of Phase Contrast (Electron) Microscopy
Marin van Heel Imperial College, London

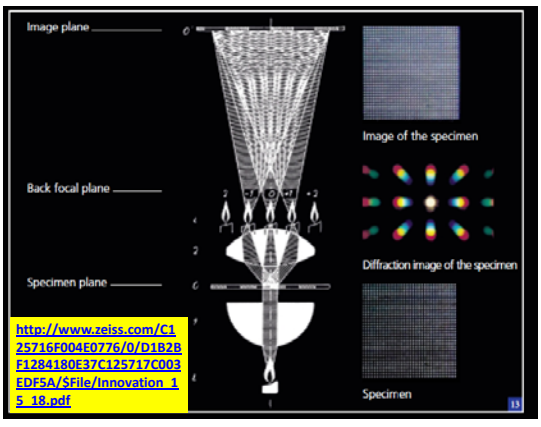
Abbe's theory of image formation. Plane waves emanating from the image at a particular angle will converge in the back focal plane at points corresponding to diffraction peaks. Spherical wave will be emitted from these points to form the image.



2 dimensional Fourier transforms
Bracewell, R.N. (1978)
The Fourier transform and its applications



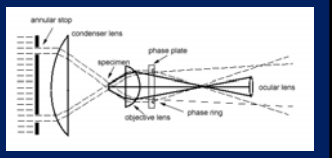
From Advanced Optics: Unit 5 Abbe's Theory of Image Formation



Phase Contrast and Differential Interference Contrast (DIC) Microscopy

http://www.jove.com/video/844/phase-contrast-and-differential-interference-contrast-dic-microscopy

Phase Contrast



"The microscope image is the interference effect of a diffraction phenomenon." Abbe

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

PHASE CONTRAST,
A NEW METHOD FOR THE MICROSCOPIC
OBSERVATION OF TRANSPARENT OBJECTS

PART II *)

by F. ZERNIKE, Groningen

Physica IX, no 10 December 1942

Fig. 19–20. Salivary chromosomes of *Drosophila*, 0,65 objective, enl. 2200 × ; fig. 19 bright ground with narrow illuminating cone, fig. 20 positive phase contrast.

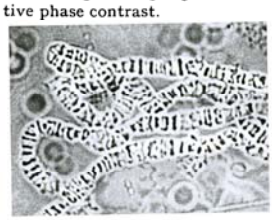


Fig. 19

Fig. 20

How I Discovered Phase Contrast

F. Zernike

Department of Physics, University of Groningen, Netherlands

Science (1955) 121, 345

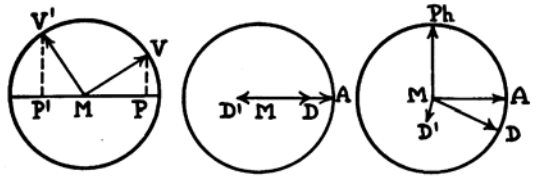


Fig. 1. Vector diagrams showing the interplay of phases.

PHASE CONTRAST,
A NEW METHOD FOR THE MICROSCOPIC
OBSERVATION OF TRANSPARENT OBJECTS

PART II *)

by F. ZERNIKE, Groningen

Physica IX, no 10 December 1942

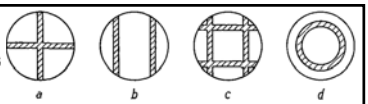


Fig. 9. Various forms of phase strips suggested.

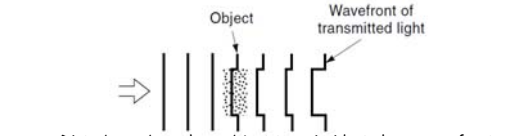
How I Discovered Phase Contrast

F. Zernike

Department of Physics, University of Groningen, Netherlands

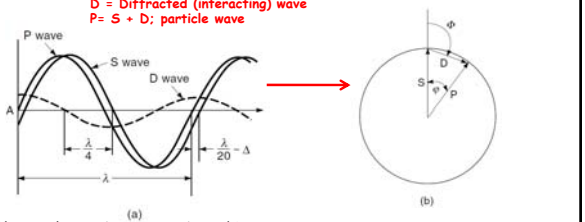
Science (1955) 121, 345

On looking back to this event, I am impressed by the great limitations of the human mind. How quick we are to learn—that is, to imitate what others have done or thought before—and how slow to understand —that is, to see the deeper connections. Slowest of all, however, are we in inventing new connections or even in applying old ideas in a new field.



Disturbance by a phase object to an incident planar wave front.

S = undeviated (non-interacting) source (surround) wave
D = Diffracted (interacting) wave
P= S + D; particle wave



Phase relations between S, D, and P waves in bright-field microscopy. P=S+D

Fundamentals of Light Microscopy and Electronic Imaging
Douglas B Murphy

Double refraction or birefringence

optical path difference Δ $\Delta = (n_2 - n_1)t$

t = thickness of the object

Relative retardation and birefringence are related by $\Gamma = (n_E - n_O)t$

optical phase difference $= \delta = 2\pi \frac{t}{\lambda_0}(n_2 - n_1) = 2\pi \frac{t}{\lambda_0}(n_E - n_O) = 2\pi \frac{\Delta}{\lambda_0}$

Phase-Contrast Microscopy

By C. R. Burch, B.A., Ph.D., F.Inst.P., University of Bristol and J. P. P. Stock, M.D. (Hon. Physician, Burslem, Tunstall and Haywood War Memorial Hospital, Hon. Medical Registrar, North Staffordshire Royal Infirmary)

[MS. first received 10th November 1941 and in final form 8th December 1941]

VOL. 19, MAY 1942 [71]

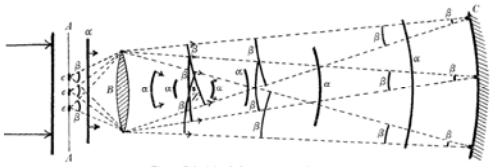
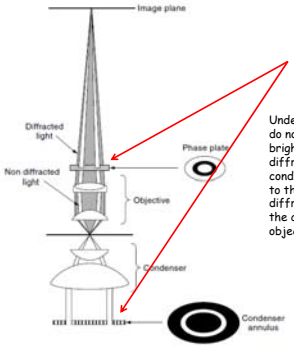


Fig. 1. Principle of phase-contrast microscopy

The Optical Design of the Phase Contrast Microscope



The image of the annulus is in sharp focus in the phase plate plane because it is conjugate to the front aperture plane of the condenser. Diffracted specimen rays fill the shaded region of the illumination path.

Under conditions of Koehler illumination, S waves that do not interact with the specimen are focused as a bright ring in the back focal plane of the objective (the diffraction plane). Remember that under these conditions the objective's back focal plane is conjugate to the condenser's front aperture plane, so non-diffracted (0th-order) waves form a bright image of the condenser annulus at the back aperture of the objective.

The condenser annulus replaces the variable diaphragm in the front aperture of the condenser.

Phase Contrast Microscope Configuration

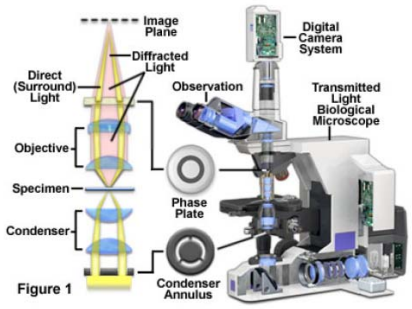
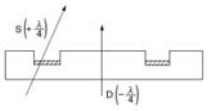


Figure 1

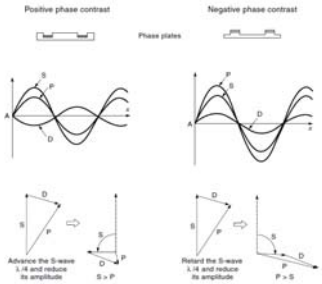
http://www.microscopyu.com/articles/

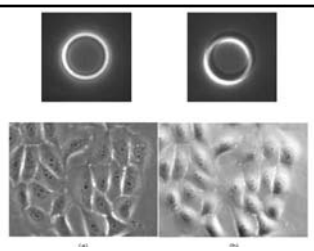
phasecontrast/phasemicroscopy.html



The action of a phase plate at the rear surface of the objective lens. **Surround or background rays (S)** are advanced in phase relative to the D wave by λ/4 at the phase plate. Relative phase advancement is created by etching a ring in the plate that reduces the physical path taken by the S waves through the high-refractive-index plate. Since **diffracted object rays (D)** are retarded (approximately) by λ/4 at the specimen, the optical path difference between D and S waves upon emergence from the phase plate is λ/2, allowing destructive interference in the image plane. The recessed ring in the phase plate is made semitransparent so that the amplitude of the S wave is reduced by 70–75% to optimize contrast in the image plane.

http://www.microscopyu.com/tutorials/java/phasecontrast/opticaltrain/index.html





Alignment of condenser and objective annuli. An eyepiece telescope or Bertrand lens is used to examine the back aperture of the objective lens. (a) The dark ring of the phase plate must be perfectly centered with the bright ring of light from the condenser annulus. The adjustment is made using two condenser plate-centering screws. These screws are distinct from the condenser centration screws, which are used to center the condenser lens with respect to the optic axis of the microscope. (b) Notice the low-contrast shaded image resulting from a misaligned annulus.

http://www.microscopyu.com/tutorials/java/phasecontrast/microscopealignment/index.html

Phase ring and condenser annulus not adjusted

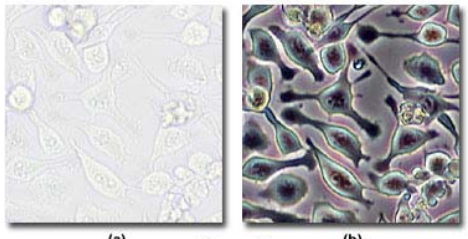
Eye piece telescope lens to focus on the phase ring

Phase ring and condenser annulus well adjusted

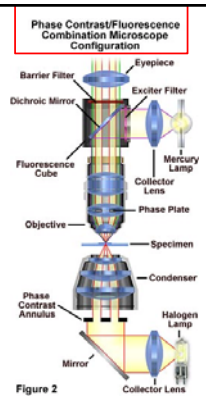
Transparent image – no phase ring

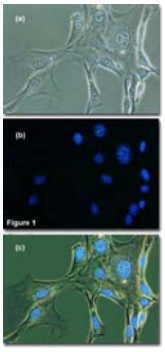
Phase contrast image

Living Cells in Brightfield and Phase Contrast

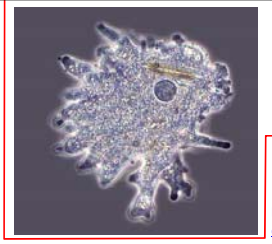


(a) Figure 2 (b)





http://www.olympusmicro.com/primer/techniques/fluorescence/fluorophase.html



http://www.microscopy-uk.org.uk/index.html?http://www.microscopy-uk.org.uk/intro/index.html

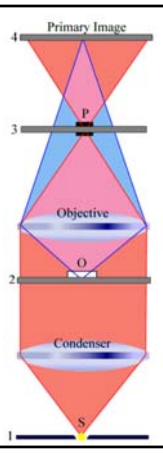
An amoeba with the numerous crystals that are found in this species.

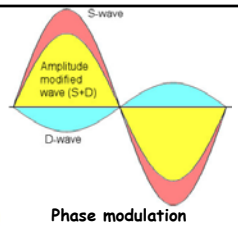
http://www.microscopyu.com/articles/phascontrast/index.html

http://www.olympusmicro.com/primer/techniques/phasecontrast/phaseindex.html

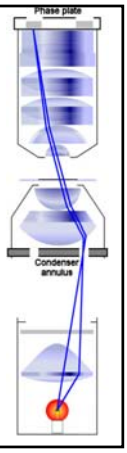
There are some limitations of phase contrast microscopy:

- **Phase images are usually surrounded by halos around the outlines of details.** Such halos are optical artifacts, which sometimes obscure the boundaries of details.
- The phase annuli **do limit the working numerical aperture** of the optical system to a certain degree, thus reducing resolution.
- Phase contrast **does not work well with thick specimens** because of shifts in phase occur from areas slightly below or slightly above the plane that is in focus. Such phase shifts confuse the image and distort image detail.
- Phase images appear gray if white light is used and green if a green filter is used. In the past, many microscopists restricted their film to black and white when performing photomicrography on phase specimens. Today, many color films reproduce black, white, and grayscales very effectively.





Phase modulation transformed into Amplitude contrast



Recap for Phase Contrast from:

http://www.microscopy-uk.org.uk/mag/artmar06/go-phase.html

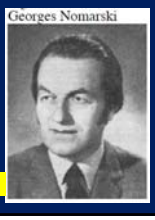
Unlike *differential interference contrast* (see next slides) and **Hoffman modulation contrast**, the circular geometry of **phase contrast** illumination and detection enables specimen observation **without orientation-dependent artifacts**. Phase contrast is also insensitive to polarization and birefringence effects, which is a major advantage when examining living cells growing in plastic tissue culture vessels.

http://www.olympusmicro.com/primer/techniques/hoffman.html

DIC

Differential interference contrast (Nomarski) imaging is a modification of phase microscopy.

Nomarski imaging is a phase imaging technique that gives good rejection of out-of-focus interference. It provides an image of the gradient (i.e. spatial rate of change) of refractive index inhomogeneities in the sample.



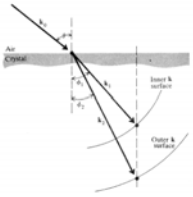
http://www.microscopyu.com/articles/dic/index.html

http://microscopy.berkeley.edu/Resources/instruction/DIC.html

Light propagation in crystals

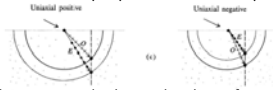
Propagation of light in crystals

When light propagates down the optical axis, the index of refraction does not depend on the polarization direction

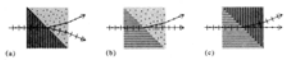


Wave vectors for **double refraction** at the boundary of a crystal

O = ordinary ray, E = extraordinary ray



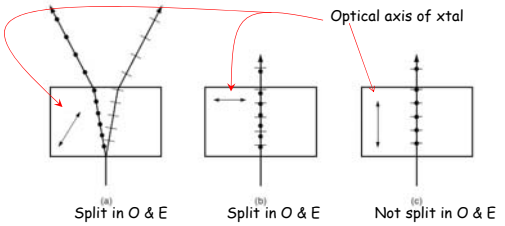
Optical axis perpendicular to the plane of incidence (the plane of incidence in this case is the page).



Three ways to separate unpolarized light into two divergent rays of orthoganol polarization. b = Wollaston prism.

Splitting of an incident ray into O- and E-ray components by a birefringent crystal.

When light propagates down the optical axis, the index of refraction does not depend on the polarization direction



(a) Optic axis at oblique angle to incoming light ray
(b) An incident ray whose propagation axis is perpendicular to the optic axis
(c) An incident ray whose propagation axis is parallel to the optic axis is not split

Different indices of refraction and therefore different speeds, $n_O \sin \phi_O = \sin \theta$, $n_E \sin \phi_E = \sin \theta$

Double refraction or birefringence

optical path difference Δ $\quad \Delta = (n_2 - n_1)t$

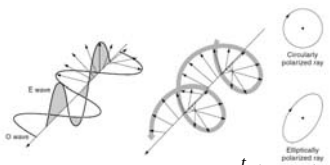
t = thickness of the object

Relative retardation and birefringence are related by $\Gamma = (n_E - n_O)t$

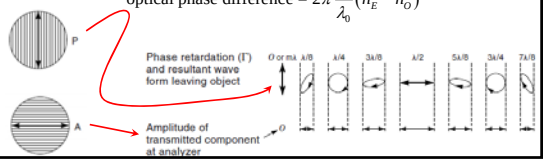
optical phase difference $= \delta = 2\pi \frac{t}{\lambda_0}(n_2 - n_1) = 2\pi \frac{t}{\lambda_0}(n_E - n_O) = 2\pi \frac{\Delta}{\lambda_0}$

E & O wave travelling through a birefringent object

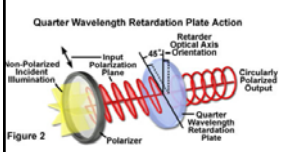
Waves resulting from the combination of superimposed O and E rays have **elliptical, spherical, or planar waveforms,** depending on the amount of relative phase shift between the two rays.

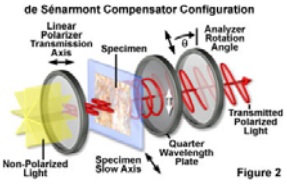


$$\text{optical phase difference} = 2\pi \frac{t}{\lambda_0}(n_E - n_O)$$



http://www.molecularexpressions.com/primer/techniques/polarized/compensatorshome.html

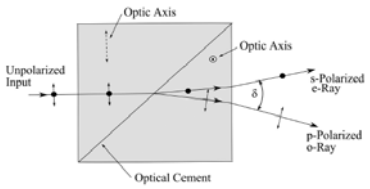




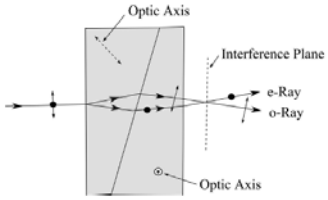
http://www.molecularexpressions.com/primer/techniques/polarized/quarterwavelengthplate.html

http://www.molecularexpressions.com/primer/techniques/polarized/desenarmontcompensator.html

The principle behind the de Sénarmont compensation technique rests on the fact that the elliptically (or circularly) polarized light electric vector emerging from the specimen is superimposed upon the circular polarization vector introduced by the quarter wavelength plate to yield plane (linearly) polarized light having a vibration azimuth different from that of the polarizer (see Figure 2). The effect occurs because the quarter wavelength plate produces linearly polarized light from elliptically or circular polarized light that is incident on the crystal surface. The azimuth of the linear polarized light emerging from the compensator is a direct function of the optical path difference induced by the specimen. By rotating the analyzer until the specimen is extinct (dark), the azimuth of the vibration produced by the de Sénarmont compensator can be ascertained along with the optical path difference introduced by the specimen.

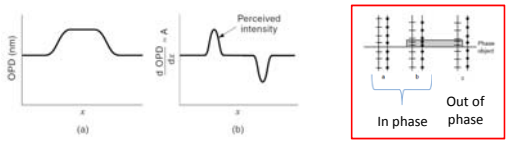


Wollaston polarizing prism
It usually consists of two calcite right-angle prisms optically cemented together at the hypotenuse. The optic axis of each section is orthogonal to that of the other section. **An unpolarized ray traversing the first prism section is not split, but the o-ray is retarded with respect to the e-ray.** The o-ray vibrates parallel to the optic axis and the e-ray perpendicular to the optic axis. Upon entering the second section, the o-ray becomes the e-ray, and vice versa. The o-ray, now slower, is bent toward the interface normal, and the e-ray is bent away from the interface normal. Prisms with a deviation angle from about 5 to 45 deg between the exit beams can be obtained, depending on the right-angle prism base angles. For very high-power applications, the prisms may not be cemented together, resulting in a reduction of transmission.

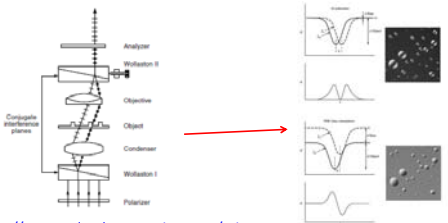


Nomarski polarizing prism
The optic axis of the first right-angle calcite prism is skewed as shown, while the optic axis of the second prism is oriented the same as for the Wollaston prism. This angled optic axis causes the ordinary and extraordinary rays to intersect outside the prism, forming an interference plane. The resulting phase shifts can be detected by an analyzer. The exact distance of this interference plane from the prism is determined by the angle of the skewed optic axis and is set by the manufacturer. Normarski prisms are used in differential interference contrast (DIC) microscopes.

Implementation in DIC microscopy



Gradients in optical path length yield differences in amplitude.



http://www.molecularexpressions.com/primer/techniques/dic/dichome.html

Fundamentals of Light Microscopy and Electronic Imaging
Douglas B Murphy

Math digression

$f(x,y)$ is the E&M amplitude, and $I(x,y)$ is the intensity

We are measuring incoherent light (the intensity)

$$I(x,y) = \left| f(x,y) - f(x+\delta x) \right|^2 \otimes p(x,y) \; ; \; p(x,y) = \text{point spread function}$$

$$f(x+\delta x, y) \approx f(x,y) + \delta x \frac{\partial f}{\partial x}$$

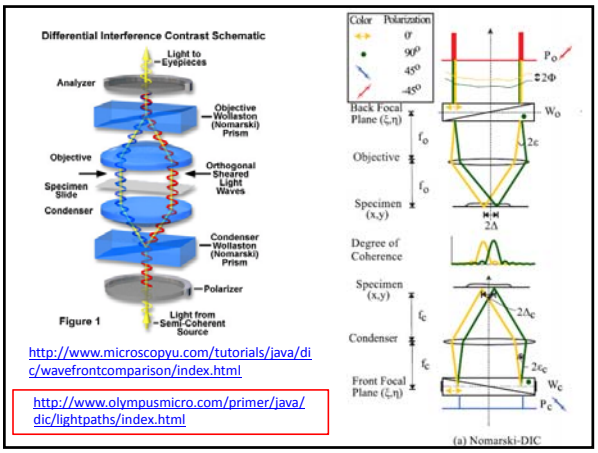
$$I(x,y) = \delta x^2 \left| \frac{\partial f}{\partial x} \right|^2 \otimes p(x,y)$$

$$f = |f| \exp(i\phi(x,y))$$

$$\frac{\partial f}{\partial x} = if \frac{\partial \phi}{\partial x} + \exp(i\phi(x,y)) \frac{\partial |f|}{\partial x}$$

If the object is dominated by phase changes, and not amplitude changes, then:

$$\boxed{I(x,y) = \delta x^2 |f|^2 \left| \frac{\partial \phi}{\partial x} \right|^2 \otimes p(x,y)}$$

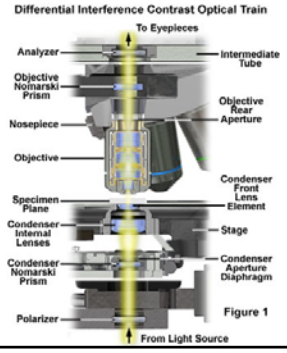


http://www.microscopyu.com/tutorials/java/dic/wavefrontcomparison/index.html

http://www.olympusmicro.com/primer/java/dic/lightpaths/index.html

DIC alignment

http://www.molecularexpressions.com/primer/techniques/dic/dicconfiguration.html



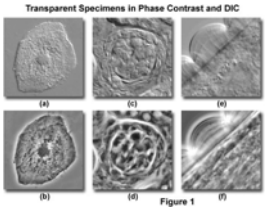
Example of DIC

An amoeba with the numerous crystals that are found in this species.
We will use this example throughout this lecture to compare methods

http://www.microscopy-uk.org.uk/index.html?http://www.microscopy-uk.org.uk/intro/index.html

Comparison of DIC and Phase contrast

http://www.molecularexpressions.com/primer/techniques/index.html



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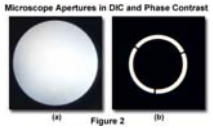
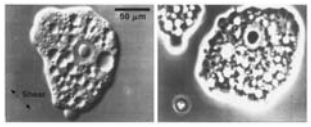


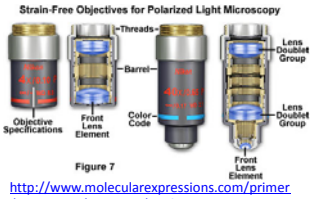


Fundamentals of Light Microscopy and Electronic Imaging
Douglas B Murphy

Polarized Light Microscopy

Polarized Light Microscopy

http://www.molecularexpressions.com/primer/techniques/polarized/polarizedhome.html

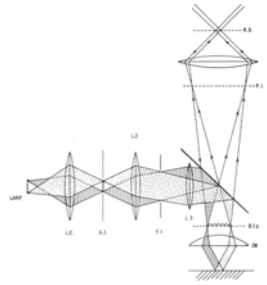


http://www.molecularexpressions.com/primer/techniques/polarized/configuration.html

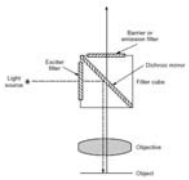
Example

http://www.molecularexpressions.com/primer/virtual/polarizing/index.html

Köhler Illumination For Fluorescence Microscopy Or Reflected Light

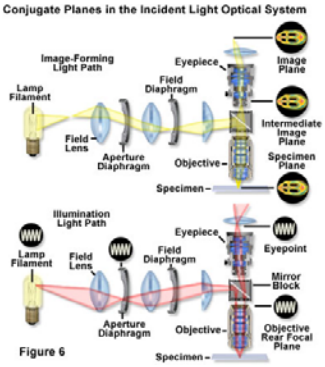


Dichroic



A ray diagram of Köhler illumination with an incident light system. L.C. = lamp collector lens; A.I. = aperture-iris diaphragm; F.I. = field-iris diaphragm; L2, L3 = relay lenses in the illuminating system; OB = objective; B.f.p. = back focal plane of the objective; P.I.P. = primary image plane; R.D. = exit pupil of the eyepiece or the 'Ramsden disc'. Note: the angles of the rays are much exaggerated for clarity; the crossing of four rays at a single point on the mirror is coincidental, and arranged thus for ease in drawing.

An Introduction to the optical microscope
Savile Bradbury



http://www.microscopyu.com/articles/formulas/formulasconjugate.html

Confocal Microscopy

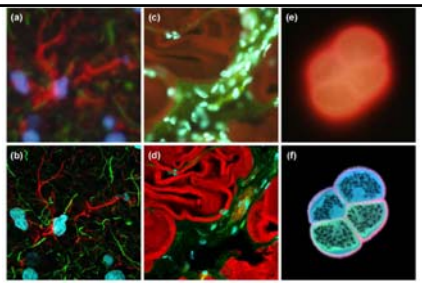
http://www.microscopyu.com/tutorials/java/virtual/confocal/index.html

http://www.microscopyu.com/tutorials/flash/resonantscanning/confocalresonantscanning/index.html

http://www.nature.com/milestones/milelight/index.html

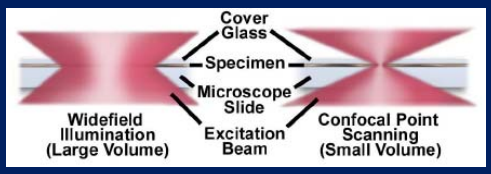
See (for many references):
Methods in Enzymology
Volume 307, Pages 3-663 (1999)
Confocal Microscopy
Edited by: P. Michael Conn

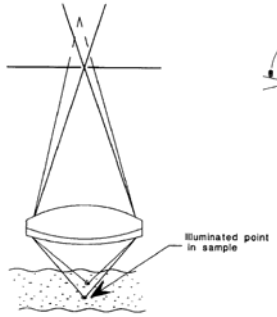
Confocal optical microscopy Robert H Webb
Rep. Prog. Phys. **59 (1996) 427-471**



Comparison of widefield (upper row) and laser scanning confocal fluorescence microscopy images (lower row). Note the significant amount of signal in the widefield images from fluorescent structures located outside of the focal plane. (a) and (b) Mouse brain hippocampus thick section treated with primary antibodies to glial fibrillary acidic protein (GFAP; red), neurofilaments H (green), and counterstained with Hoechst 33342 (blue) to highlight nuclei. (c) and (d) Thick section of rat smooth muscle stained with phalloidin conjugated to Alexa Fluor 568 (targeting actin; red), wheat germ agglutinin conjugated to Oregon Green 488 (glycoproteins; green), and counterstained with DRAQ5 (nuclei; blue). (e) and (f) Sunflower pollen grain tetrad autofluorescence.

How to observe only a very small focal volume without interference of the out of focus fluorescence





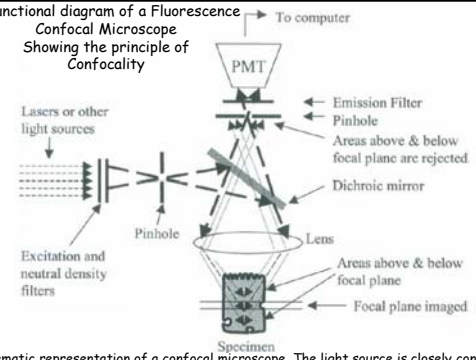
Confocal optical microscopy, R. Webb Rep. Prog. Phys. **59 (1996) 427–471**

The relation of the pinhole to the position in the sample. The pinhole selects mainly the fluorescence from the focal point of the excitation beam.

Theoretical Basis of Confocal Microscopy

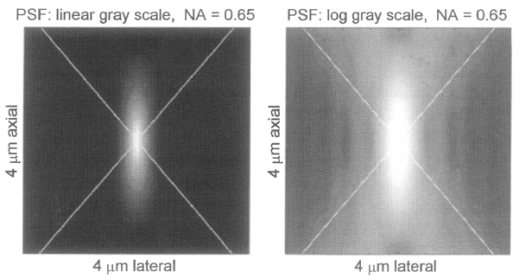
By Robert H. Webb Methods in Enzymology, Vol. 307

A functional diagram of a Fluorescence Confocal Microscope Showing the principle of Confocality



Schematic representation of a confocal microscope. The light source is closely controlled by various filters, pinholes, and dichroic mirrors before reaching the lens. Signals return from the specimen through the dichroic mirror and those outside the focal plane are rejected by the pinhole. The emission filter restricts the bandwidth in which the signals are received and the photomultiplier (PMT) enhances the signal as necessary.

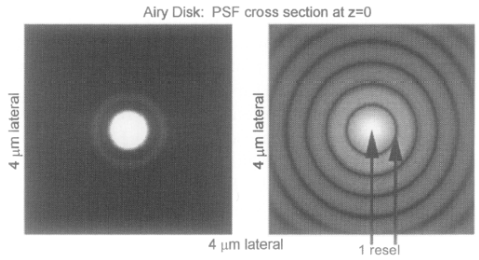
Point Spread Functions



The point-spread function close to the focus. These are the patterns of the previous slide, magnified 10 times.

Theoretical Basis of Confocal Microscopy

By Robert H. Webb Methods in Enzymology, Vol. 307



The point-spread function in the focal plane: the pattern of Fig. 6 rotated about the z axis at z = 0. *Left: Linear gray scale. Right." Logarithmic gray scale. The central part of the pattern is called the Airy disk.*

Theoretical Basis of Confocal Microscopy
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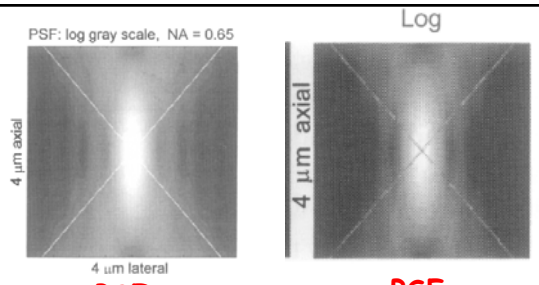
Point spread function of illumination (from a point source).

Important: The **point spread functions** discussed above are due to the **objective lens**. The complex geometric form of the point spread function (PSF) is due to **diffraction** from the **circular lens (diffraction from a circular aperture)**, **using a point source of light**. This is because of the wave nature of light. The smaller the lens aperture, the wider the pattern, and the lower the resolution. A lens with a large aperture has a much greater resolution, simply because the diffraction pattern is smaller (remember the experiment in the optical lab). The **central part of the diffraction pattern** is called the **Airy Disk**, and the size of the Airy disk sets the **spatial resolution**. The radius of this disc sets a measure of the spatial resolution.

The **confocal point spread function (PSF_{CF})** is a combination of the **PSF_S of the illumination** (light source) and the **PSF_P of the pinhole**.

$$PSF_{CF} = PSF_S \times PSF_P$$

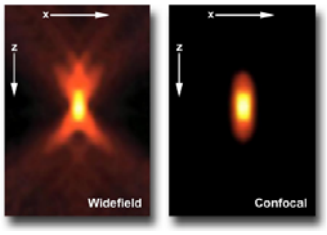
The objective lens images every point of the sample fluorescence as a PSF at the image plane. That is, the PSF from every point of the sample is summed up at the image plane. This ends up as a **convolution of all the point spread functions from the different points at one point of the image** (see later discussion of deconvolution). But we can make the same calculation by imagining the PSF_p of the pinhole imaged on the sample. This is because we are only interested in the light passing through the pinhole. These two PSF calculations, which are the distribution of the intensity of light – passing from the sample through the pinhole, or through the pinhole to the sample - are identical.



PSF_S **PSF_{CF}**

Comparison of the PSFs of the wide field case (PSF_S) and the PSF of the confocal case (PSF_{CF})

The wing peaks of the PSF that were 0.01 of the maximum are now only 0.0001 of the maximum



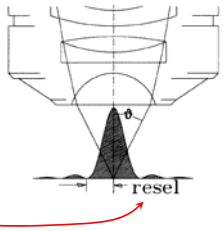
Comparison of axial (x-z) point spread functions for widefield (left) and confocal (right) microscopy.

$r_{lateral} = 1.22\ \lambda / (2 \cdot NA) = 0.6\ \lambda / NA$

$r_{lateral} = 0.4\ \lambda / NA$

$r_{axial} = 1.4\ \lambda \cdot \eta / NA^2$

Resel



The resolution element due to a lens of NA = n sin θ is called a **resel**: the radius of the first dark fringe in the diffraction pattern, or half the diameter of the Airy disc.

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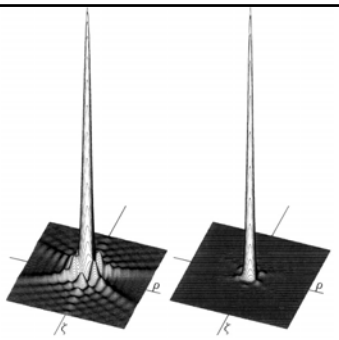

The function $p_{conf}(\zeta, \rho)$= $p(\zeta, \rho)$ x $p(\zeta, \rho)$ for the confocal case is shown on the right. The left is just $p(\zeta, \rho)$.

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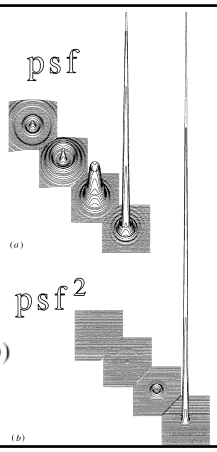
One way to win resolution with confocality

psf

$P(\zeta, \rho)$ for the focal plane and planes parallel to it: in (a) this is for the conventional diffraction pattern, and in (b) it is for the confocal case.



$$p_{\text{conf}}(\zeta, \rho) = p(\zeta, \rho) \times p(\zeta, \rho)$$

psf^2

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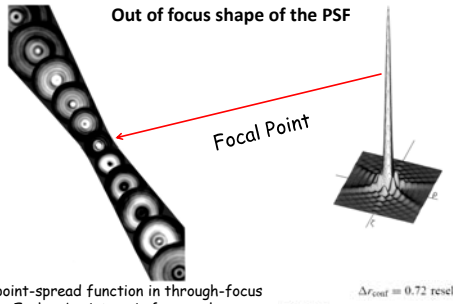
Out of focus shape of the PSF



The point-spread function in through-focus series. Each sub-picture is from a plane parallel to the focal plane. These are actual photographs as a microscope is stepped through focus. Intensity has been manipulated, since the centre of the in-focus panel is really 100 times brighter than any of the others.

which is

$$\Delta r_{\text{conf}} = 0.72 \text{ resel} = 0.44\lambda/n \sin\vartheta,$$

$$\Delta r_{\text{conf}} = 0.88\lambda' f/\#$$

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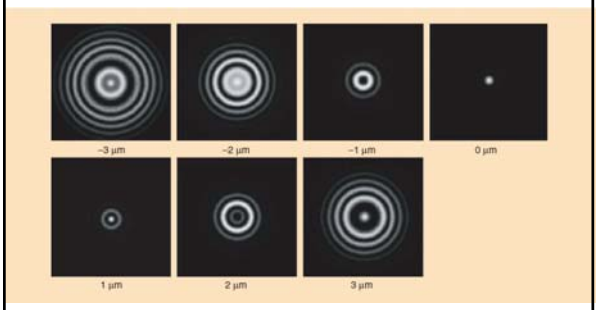
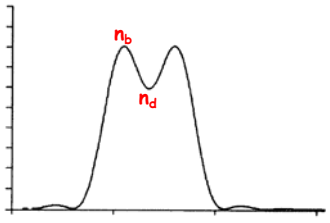


Numerically computed 2-D PSF images on the focal planes from −3.0 *μm above to 3.0 μm below the plane of reference at 0 μm*

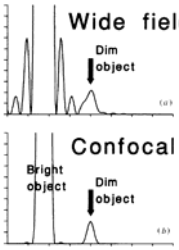
C = contrast **Visibility**

$$C = (n_b - n_d)/2n_{avg} \qquad 2n_{avg} = n_b + n_d$$



Two equal points separated by one **resel** illustrate the Rayleigh criterion for resolution.

Separation of weak fluorescing object from the wings of a neighboring object with a strong fluorescence PSF



Confocal imaging has better detection of weakly fluorescent objects

Two points of very different (200:1) remission intensity, are well resolved (4.5 resels). In **(a) the conventional view** leaves the dimmer point obscured, but in **(b) the confocal contrast enhancement** allows its display. Arrows indicate the weaker remitter.

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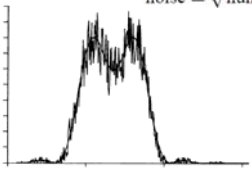
Rep. Prog. Phys. **59 (1996) 427–471**

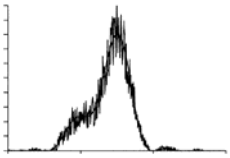
But remember there is still "photon" noise in the acquisition

It is not really contrast, but *noise that determines visibility; this is* due to the random arrival times of the photons—the so-called *photon noise or quantum noise*

$$\text{noise} = \sqrt{\text{number of photons}}$$



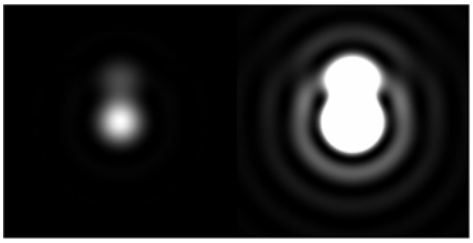
Two noisy equal points are still resolved according to the Rayleigh criterion, but we may not be able to see the dip.



Two points separated by one resel, but of different brightness and obscured by noise. These may not be resolvable at this one resel separation.

$$\text{SNR} = (n_b - n_d)/\sqrt{n_{avg}} = C\sqrt{n_{avg}} \qquad N \propto \sqrt{n_{avg}} \qquad C = (n_b - n_d)/n_{avg}$$

But very often it is the background noise (not the sample directly) that causes most of the noise



The two points have unequal intensity, and the secondary diffraction maxima are visible only in the very overexposed frame, on the right.

Integration of the point spread function in a full field image. Integrating when focusing at different planes.

$$\int_{\text{Plane}} \text{PSF} = \int_{\text{Plane}} \text{light} = \text{constant}$$

Integration of the point spread function of a confocal image

$$\int_{\text{Focal plane}} \text{PSF}_{CF} \gg \int_{\text{Any other plane}} \text{PSF}_{CF}$$

and

$$\int_{\text{Focal plane}} \text{PSF}_{CF} \gg \iint_{\text{All other planes}} \text{PSF}_{CF}$$

For the confocal point-spread function the integral is not of "light," it is of "**light that reached a sample point *and got back* through the pinhole.**"

Magnification and the size of the pinhole:

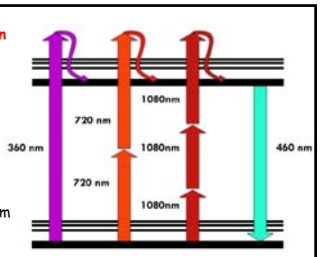
Every lens has two point spread functions: one on each side. If the lens magnifies by a factor of 60, then the point-spread function on one side is small and that on the other side is roughly 60 times as big (roughly, because the diffraction peak details are not exactly images of each other and magnification is a concept from geometric optics). A 50× objective lens with NA = 0.85 forms a resel at the sample that is 0.4 μ across, but at the image plane of the microscope, the NA is 0.014 and the resel is 24 μ. **So there is no need to make submicron pinholes; use one that is comfortably in the 50- to 100-μ range.**

Note: If you use an new objective with low magnification but high NA, it is important to adjust pinhole size to suit the objective lens.

2-photon excitation scanning microscopy

P_{ave} is the average power of the illumination beam, **δ_2 is the two-photon cross section** of the fluorescent molecule, and λ is the excitation wavelength. **1 GM (Göppert-Mayer) = 10^{-58} [m^4 s] per photon.**

For a δ_2 of approximately 10 GM, focusing through an objective of NA >1, an average incident laser power of ≈ 1–50 mW, operating at a wavelength ranging from 680 to 1100 nm with 80–150 fs pulse width and 80–100 MHz repetition rate, one should get fluorescence without saturation.



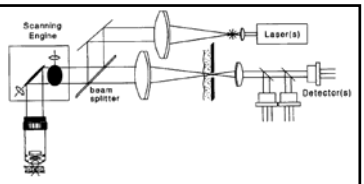
n_a, is the probability that a certain fluorophore simultaneously absorbs two photons during a single pulse; in the paraxial approximation this is given by

$$n_a \propto \frac{\delta_2 \cdot P_{ave}^2}{\tau_p f_p^2} \cdot \left(\frac{NA^2}{2hc\lambda} \right)^2$$

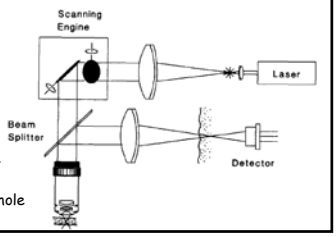
Pulse duration τ_p and f_p repetition rate

*BioMedical Engineering OnLine 2006, **5:36***
Diaspro et al; June 2006

A generic confocal microscope.

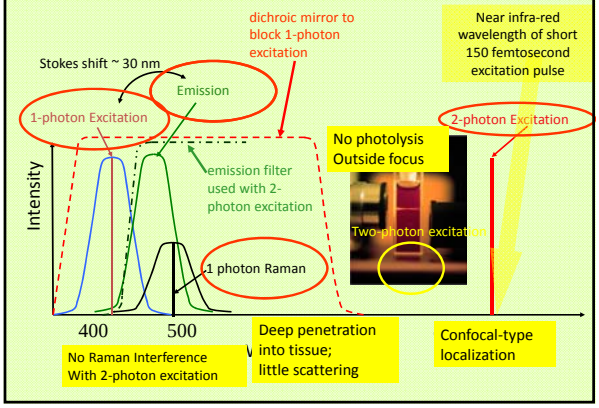


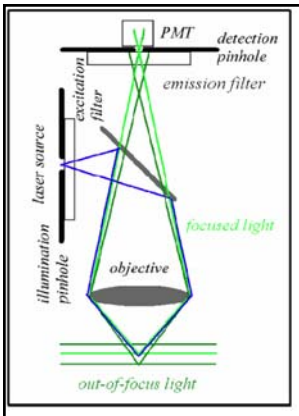
The similarities of the one-photon confocal and two-photon microscopes



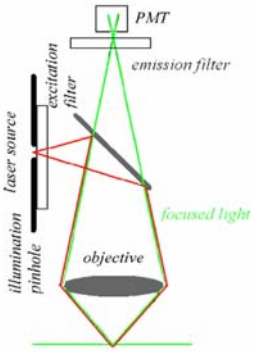
A multiphoton microscope. The laser delivers all its energy in a very short pulse, so two photons can reach tile sample nearly simultaneously. No pinhole is needed.

Two photon excitation: Separation of Excitation and Emission





With pinhole



Without pinhole

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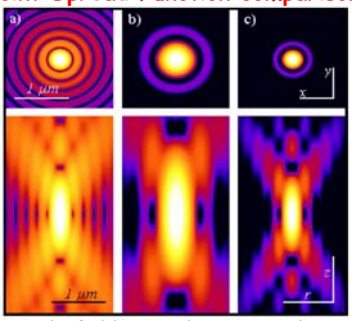
Point Spread Function comparisons



Wide field **2-photon non-confocal** **1-photon confocal**

Optical conditions: excitation wavelengths are 488 nm and 900 nm for 1PE and 2PE, respectively; emission wavelength is 520 nm; numerical aperture is 1.3 for an oil immersion objective with oil refractive index value set at 1.515. *BioMedical Engineering OnLine 2006, **5:36** Diaspro et al; June 2006*

Deconvolution Microscopy

Convolution of the object with the PSF

The general relationship between input and output

$$i(x,y,z) = N(\iiint o(x',y',z')\, h(x,y,z,x',y',z')\, dx'\, dy'\, dz')$$

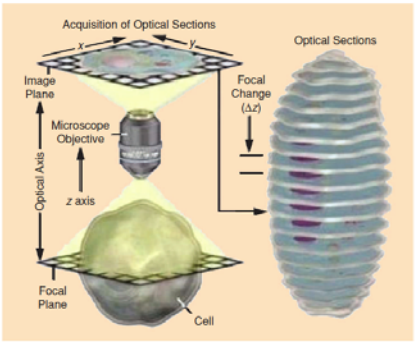
i is the acquired image, o the object acquired, h the PSF and N the noise.

Assuming linearity and shift invariance in the image formation process

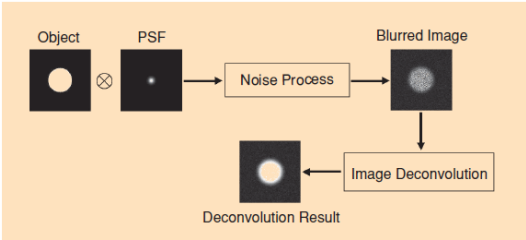
$$i(x,y,z) = N(\iiint o(x',y',z')\, h(x-x',y-y',z-z')\, dx'\, dy'\, dz')$$

This is a standard 3D convolution integral

$$i(x,y,z) = N(h(x,y,z) \otimes o(x,y,z))$$



An example of the acquired 3-D image of a cell, captured by a fluorescence microscope.



Schematic of a general deconvolution procedure

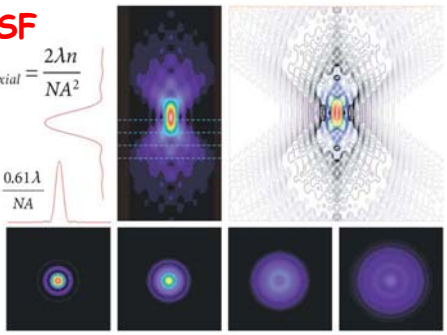
In order to correct for artifacts, you have to be able to measure or calculate or measure the correct PSF

Ideal PSF

$$r_{axial} = \frac{2\lambda n}{NA^2}$$

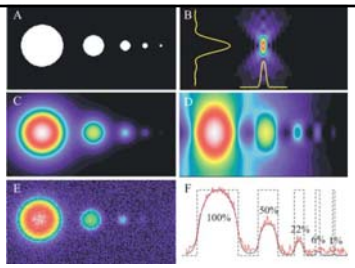
Rayleigh criterion:

$$r_{lateral} = \frac{0.61\lambda}{n\sin\alpha} = \frac{0.61\lambda}{NA}$$

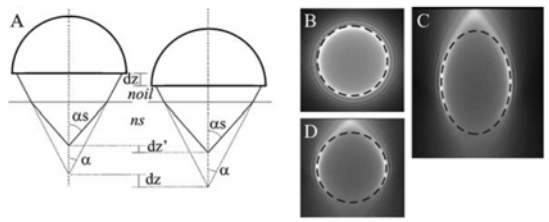


Diffraction pattern of an ideal, aberration-free objective, in one, two and three dimensions

Blur from PSF and Noise

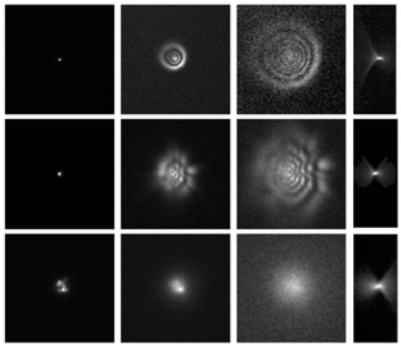


Three-dimensional simulation of an object observed through a microscope, in the presence of optical blur and noise. The object consists of five spheres with different diameters but the same fluorescence density: **A initial object; B point spread function (PSF) of the** microscope; **C, D lateral and axial cross sections of the object after convolution with the microscope's PSF; E lateral cross section of the object after blurring and the addition of noise; F intensity profiles and percentage of the object's maximum intensity of original** (*dashed), blurred (black) and blurred+noisy (red) data. Following blurring, the smaller the* object, the weaker its maximum intensity is likely to be. Noise reduces the likelihood of detecting small and highly attenuated objects

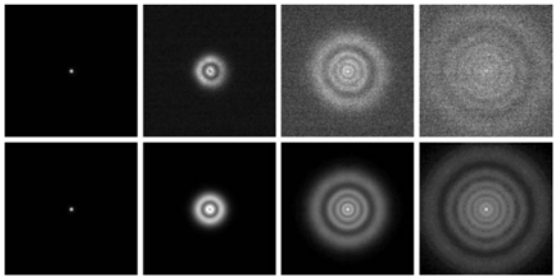


A Axial distance mismatch due to the presence of layers with different refractive indices along the optical path. **B Lateral section of the bead. C,D Observation of a 6 µm fluorescent** calibration bead without (C) and with (D) distance calibration

Examples of artifacts: optical problem (*top), oil mixing (middle) and vibrations (bottom)*

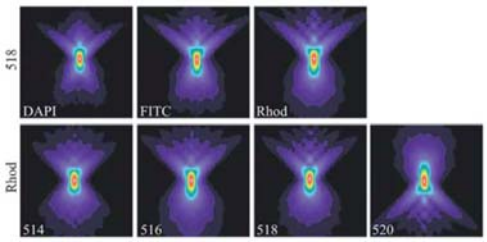


The signal-to-noise ratio can be increased significantly by applying circular averaging with respect to the optical axis



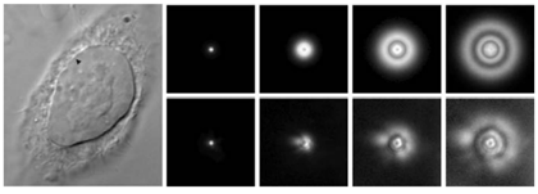
Experimental PSF extraction: raw data (*top), and data after bead averaging* and circular symmetry processing (*bottom)*

PSF is a function of emission wavelength, and the objective must be characterized with different color beads



PSF for Dapi, GFP and rhodamine wavelengths, using an oil with a refractive index of 1.518. *Bottom: PSF for the rhodamine wavelength,using oils with refractive indices* of 1.514, 1.516, 1.518 and 1.520

Ideally,PSF should be measured under the same conditions as the specimen itself.



Distortion of a PSF due to the natural fluctuation of local refractive indices. Fluorescent beads were injected into a cell before fixation. ***Top:** Experimental PSF determined in* the absence of a cell. ***Bottom:** Distorted PSF determined from a bead within a cell. Left: DIC* image of the cell, showing local refractive index variations; the *black arrow indicates the* location of the bead

Convolution & Deconvolution

The general relationship between input and output

$$i(x,y,z) = N(\iiint o(x',y',z')\, h(x,y,z,x',y',z')\, dx'\, dy'\, dz')$$

i is the acquired image, o the object acquired, h the PSF and N the noise.

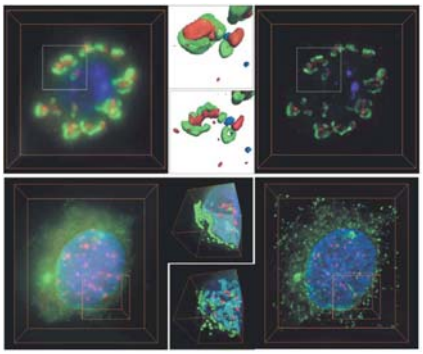
$$i(x,y,z) = N(\iiint o(x',y',z')\, h(x-x',y-y',z-z')\, dx'\, dy'\, dz')$$

$$i(x,y,z) = N(h(x,y,z) \otimes o(x,y,z))$$

The Fourier transformation of the PSF, *H, is called the optical transfer function* (OTF).

$$FFF\{h(x,y,z) \otimes o(x,y,z)\} = H(u,v,w)\, O(u,v,w)$$

Deconvolving full field images with software



Before After

[TABLE 2] GENERAL CHARACTERISTICS OF DECONVOLUTION METHODS OF 3-D FLUORESCENCE MICROSCOPY IMAGES.

TYPE	METHOD	REFERENCES	NOISE MODEL	COMMENTS
1) NO-NEIGHBORS		[6], [15]	NOT ASSUMED	SIMPLE AND FAST; REDUCE SNR AND INTRODUCE STRUCTURAL ARTIFACTS IN THE RESTORED IMAGE.
2) NEIGHBORING	NEAREST-NEIGHBORS METHODS	[1]		
	MULTI-NEIGHBORS METHODS			
3) LINEAR	INVERSE FILTERING	[6]	NOT ASSUMED	SIMPLE AND FAST; AMPLIFY NOISE; INTRODUCE RINGING ARTIFACTS AND SUPPRESS HIGH FREQUENCY COMPONENTS IN THE RESTORED IMAGE.
	WIENER FILTERING	[16]-[17]	ADDITIVE GAUSSIAN	
	LLS	[18]		
	TIKHONOV FILTERING	[5]		
4) NONLINEAR, ITERATIVE	JVC	[1]	NOT ASSUMED	ENFORCE NONNEGATIVITY CONSTRAINT IN AN AD HOC MANNER; INCREASE RESOLUTION IN THE ESTIMATED IMAGE BUT NOT EFFICIENT IN REMOVING NOISE.
	NLS	[16]-[19]	NOT ASSUMED	
	ICTM	[4], [8], [20], [21]	ADDITIVE GAUSSIAN	INCREASE COMPUTATION; DECONVOLUTION RESULT DEPENDS ON REGULARIZATION PARAMETER AND REGULARIZATION MATRIX.
	CARRINGTON	[4], [20], [21]		
5) STATISTICAL, ITERATIVE	ML/EM	[11], [23]-[25]	POISSON	RESTORE HIGH NOISE LEVEL; EM HAS SLOW RATE OF CONVERGENCE; ML CONVERGES TO NOISE ASYMPTOTICALLY, REQUIRING REGULARIZATION AT THE COST OF INCREASED COMPUTATIONAL LOAD.
	PARAMETRIC ML	[9]	ADDITIVE GAUSSIAN, POISSON	APPLICABLE WHEN BOTH THE SPECIMEN AND PSF ARE IN PARAMETRIC FORM.
	MAP	[26]-[28]	ADDITIVE GAUSSIAN, POISSON	GAUSSIAN OR ENTROPY PRIOR DISTRIBUTION OF THE OBJECT IS ASSUMED AND HENCE GIVES BETTER PERFORMANCE; EMPLOY REGULARIZATION AND THE CHOICE OF REGULARIZATION PARAMETER DETERMINES THE TRADE-OFF BETWEEN AMOUNT OF SMOOTHNESS AND FITTING ACCURACY.
6) BLIND DECONVOLUTION	BD/RL	[7], [24], [29]-[33]	POISSON	LARGE NUMBER OF PARAMETERS ARE INVOLVED, HENCE COMPUTATIONALLY INTENSIVE.

IEEE SIGNAL PROCESSING MAGAZINE Pinaki Sarder and Arye Nehorai , Deconvolution Methods for 3-D Fluorescence Microscopy Images pp. 32-45, MAY 2006

"IMAGING SYSTEMS ALWAYS DISTORT OBJECTS. IMAGE DECONVOLUTION RESTORES OBJECTS TYPICALLY BY CHARACTERIZING THIS DISTORTION.

A limitation of most algorithms is the underlying assumption of the shift-invariance property of the microscope system, which is not true in practice."

IEEE SIGNAL PROCESSING MAGAZINE Pinaki Sarder and Arye Nehorai , Deconvolution Methods for 3-D Fluorescence Microscopy Images pp. 32-45, MAY 2006