MICRO-561

Biomicroscopy I

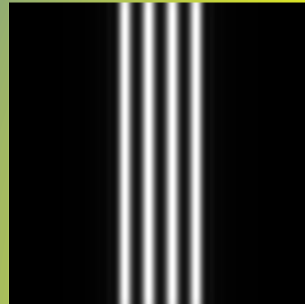
Syllabus (tentative)

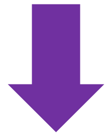
Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3 & Resolution -1
Lecture 7	Resolution-2
Lecture 8	Resolution-3
Lecture 9	Contrast
Lecture 10	Fluorescence-1
Lecture 11	Fluorescence-2
Lecture 12	Fluorescence-3, Sources, Filters
Lecture 13	Detectors
Lecture 14	Bio-application Examples

Important aspects for microscopy

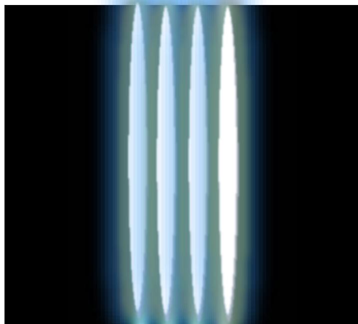
Magnification

ideal image

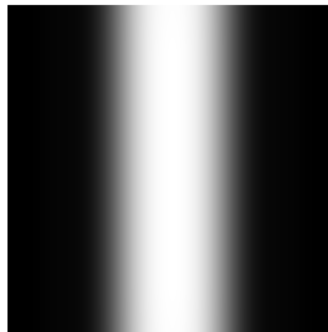




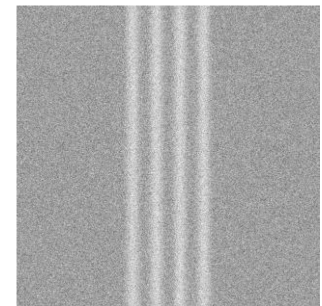
Aberrations – image quality



Resolution



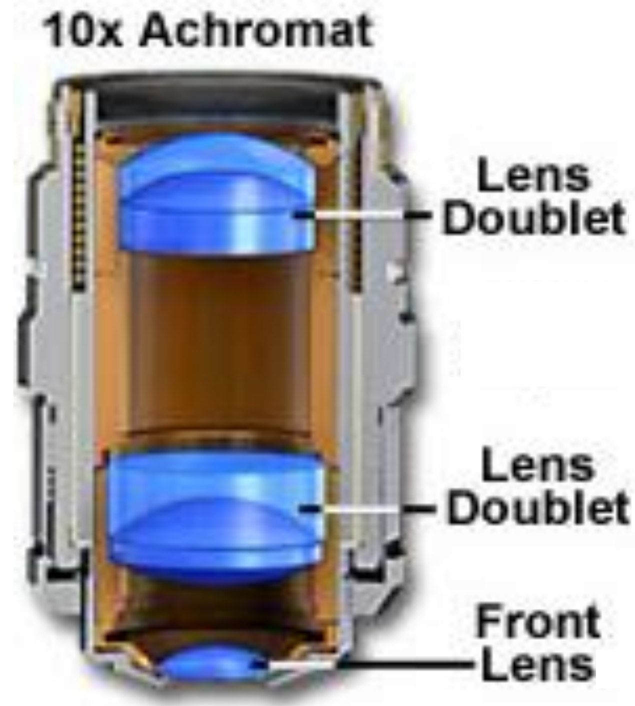
Contrast



Major Optical Aberrations in Microscopy & Objective Lenses

- The major six aberrations are:
  - Spherical aberration
  - Chromatic aberration
  - Coma
  - Astigmatism
  - Curvature of field
  - Distortion
- Lenses are associated with many of these intrinsic optical faults that can distort the image quality.
- **Aberrations can be corrected by measures** such as using compound lens designs, using glass elements with different refractive indexes and color dispersion, incorporating aspherical lens curvatures …
- The tube lens or eyepieces sometimes help in removing residual aberrations of the objective lenses.
- **Objective lenses** are designed to correct for aberrations, but they can never completely remove them.
- It is common that a solution for correcting one fault worsens other faults, so the user must prioritize the goals for optical performance and then work toward the best compromise in correcting other aberrations depending on the sample/application. For these reasons, objectives vary considerably in their design, optical performance, and cost.

Achromat: a commonly used "corrected objective" lens

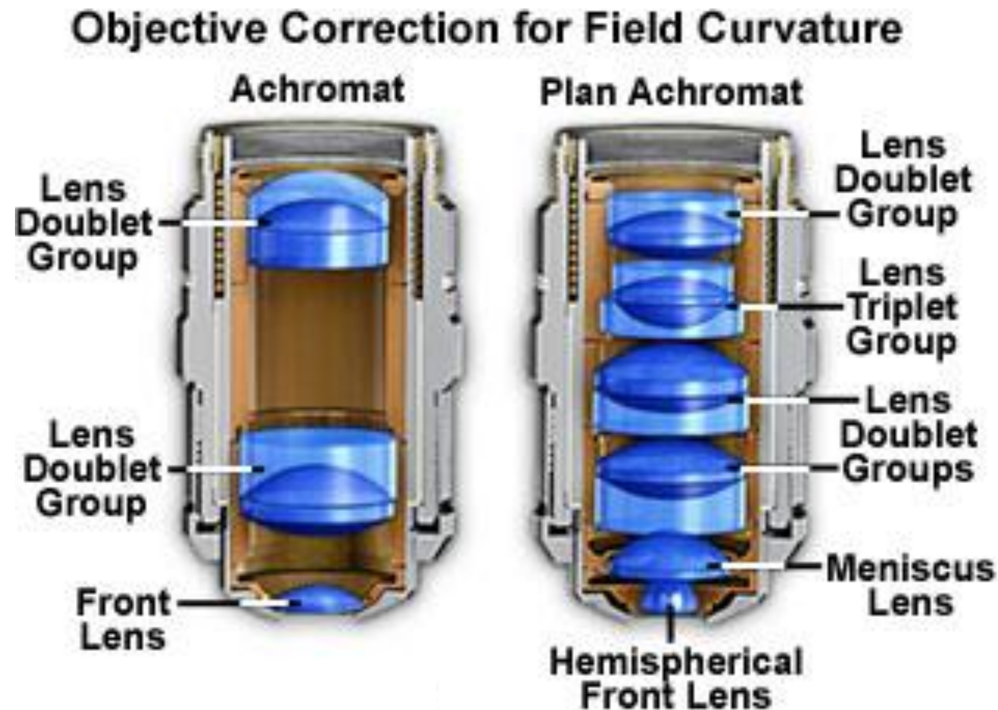


- *Achromats* are red-blue color corrected (meaning for wavelengths at 656 and 486 nm).
- Spherical correction is for mid-spectrum yellow-green light at 540 nm.

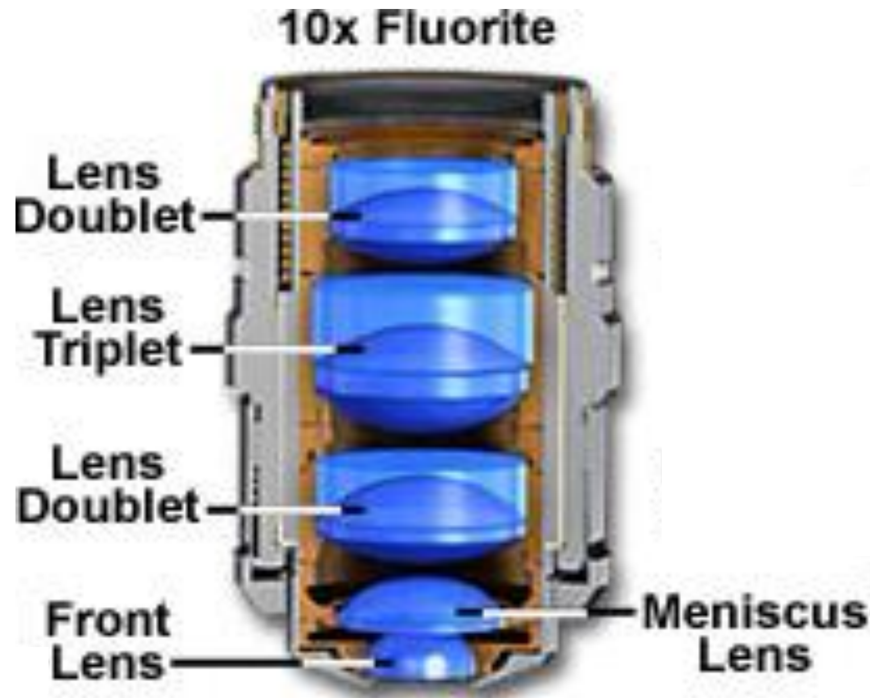
Objective Lens Correction for Field Curvature

- Correction for field curvature adds a considerable number of lens elements to the objective as illustrated below example with a simple achromat.

- In this example, **uncorrected achromat on the left** contains two lens doublets, in addition to a simple thin-lens front element.
- In contrast, **the corrected plan achromat on the right** contains three lens doublets, a central lens triplet group, and a meniscus lens positioned behind the hemispherical front lens. Plan correction, in this instance, has led to the addition of multiple elements bundled into more sophisticated lens groupings, which dramatically increases the optical complexity of the final objective lens.

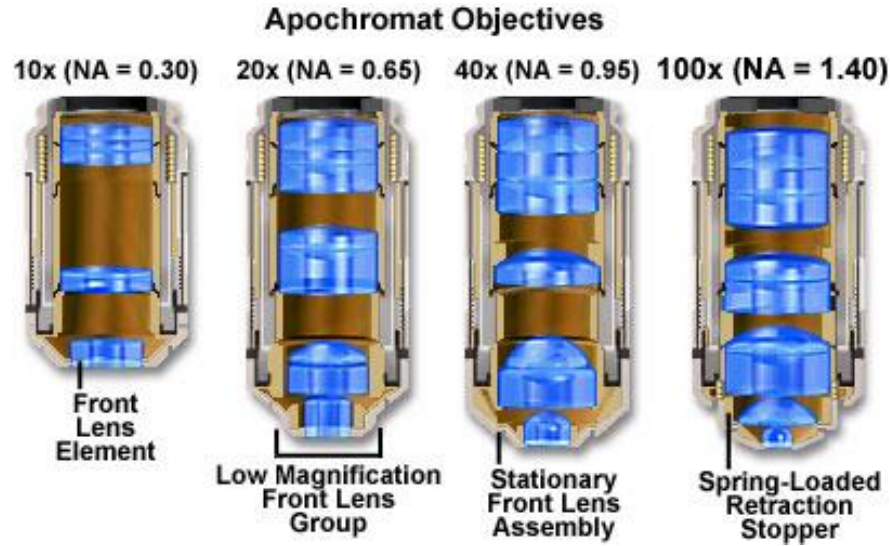


Some common "corrected objectives": Fluorite



- *Fluorite* or *semiapochromat* objectives contain elements made of fluorite (CaF_2) or synthetic lanthanum fluorite. These materials provide very high transmission and low color dispersion.

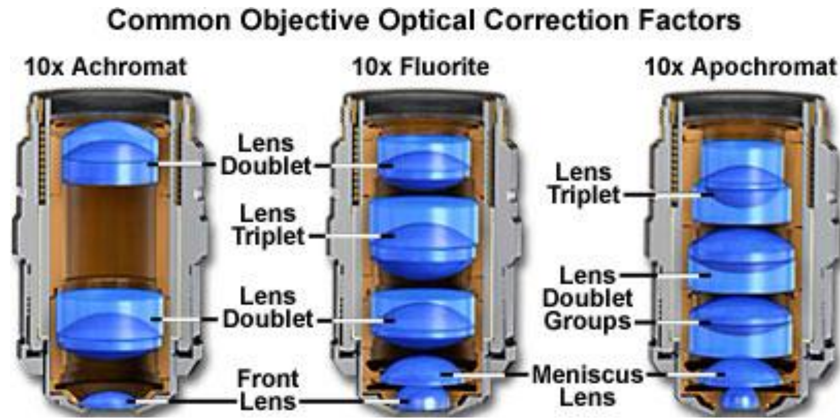
Some common "corrected objectives": Apochromats



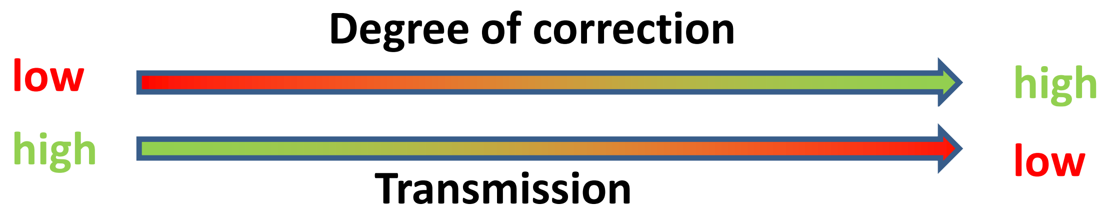
- *Apochromats* are red, green, blue, and dark blue color corrected, and also corrected for spherical aberration at green and blue wavelengths.
- **Highly color-corrected designs suitable for color photography using white light**.
- They are expensive.

Aberration correction

There is more to know than just the magnification of the Objective!

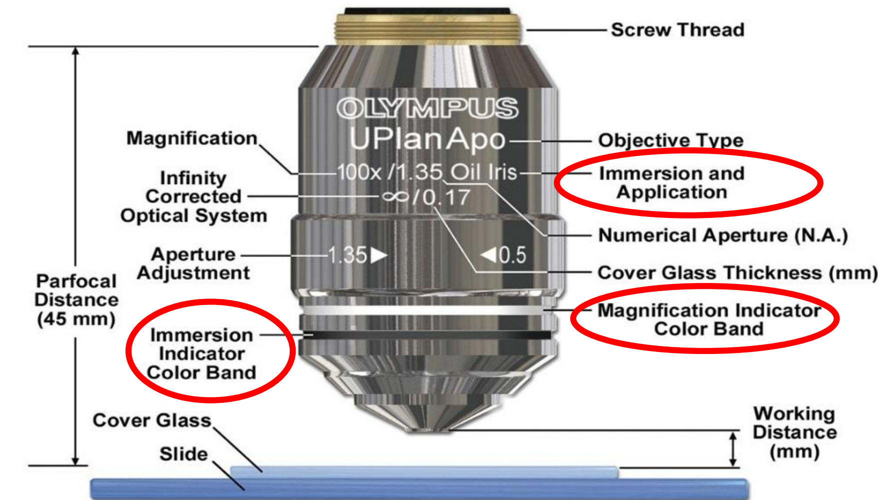


TEST BEFORE BUYING!!



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

Markings on the decorative barrel of an objective:

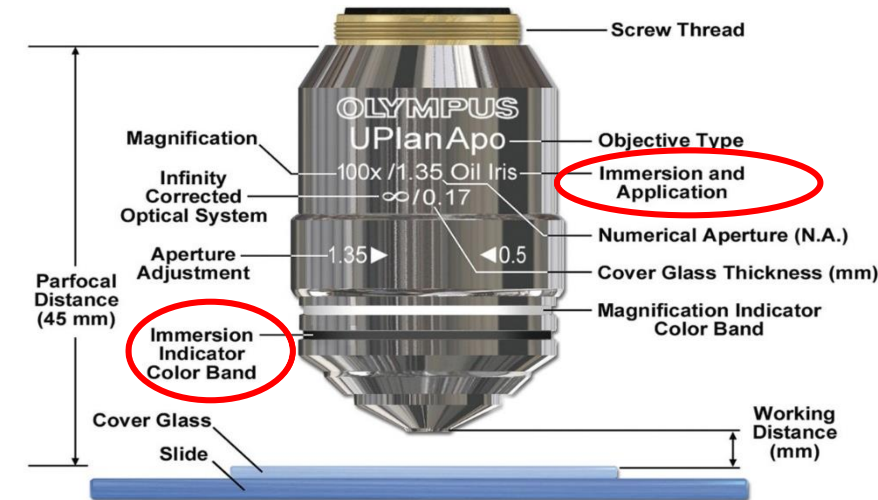


Mag.	1×	2×	4×	10×	20×	40×	50×	60×	100×
Code	Black	Gray	Red	Yellow	Green	Light Blue		Dark Blue	White
Imm. Med.	Oil		Water		Glycerin		Oil/Water/Glycerin		
Code	Black		White		Orange		Red		

Nosepiece

- Markings indicate the type of lenses and correction, initial magnification, immersion medium, numerical aperture, lens–image distance, and required coverslip thickness.
- For quick reference, the color - coded ring, farthest from the thread, denotes the type of immersion medium (black - immersion oil, white - water, orange - glycerin, yellow - methylene iodide, and red - multi - immersion).

Markings on the decorative barrel of an objective:

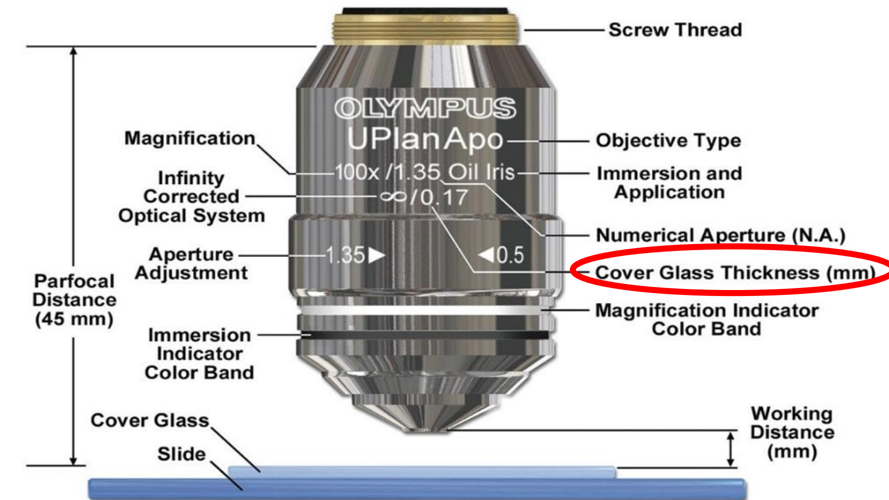


Mag.	1×	2×	4×	10×	20×	40×	50×	60×	100×
Code	Black	Gray	Red	Yellow	Green	Light Blue		Dark Blue	White
Imm. Med.	Oil		Water		Glycerin		Oil/Water/Glycerin		
Code	Black		White		Orange		Red		

Nosepiece

- Multi-immersion objectives are used in cases where it is necessary to image specimen details that are several micrometers away from the coverslip surface and where the refractive index of the tissue and/or the mounting medium causes blurring.

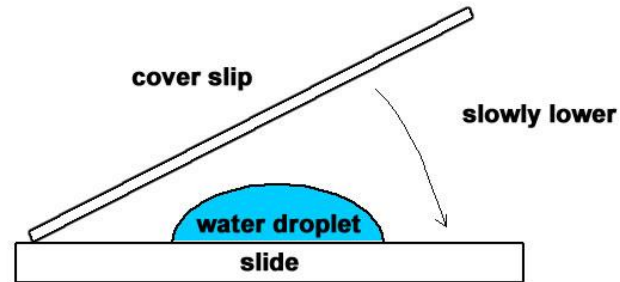
Markings on the decorative barrel of an objective:

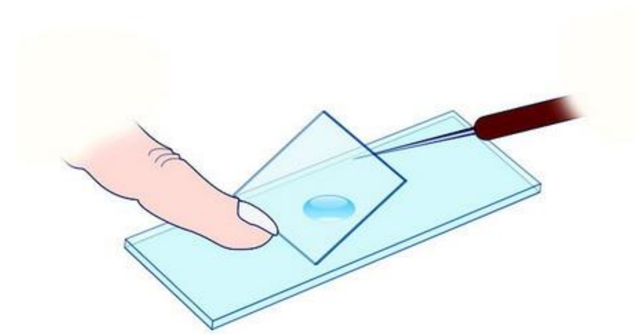


Mag.	1×	2×	4×	10×	20×	40×	50×	60×	100×
Code	Black	Gray	Red	Yellow	Green	Light Blue		Dark Blue	White
Imm. Med.	Oil		Water		Glycerin		Oil/Water/Glycerin		
Code	Black		White		Orange		Red		

- Markings indicate the type of lenses and correction, initial magnification, immersion medium, numerical aperture, lens–image distance, and required coverslip thickness.

Cover slips and glasses in bio-microscopy

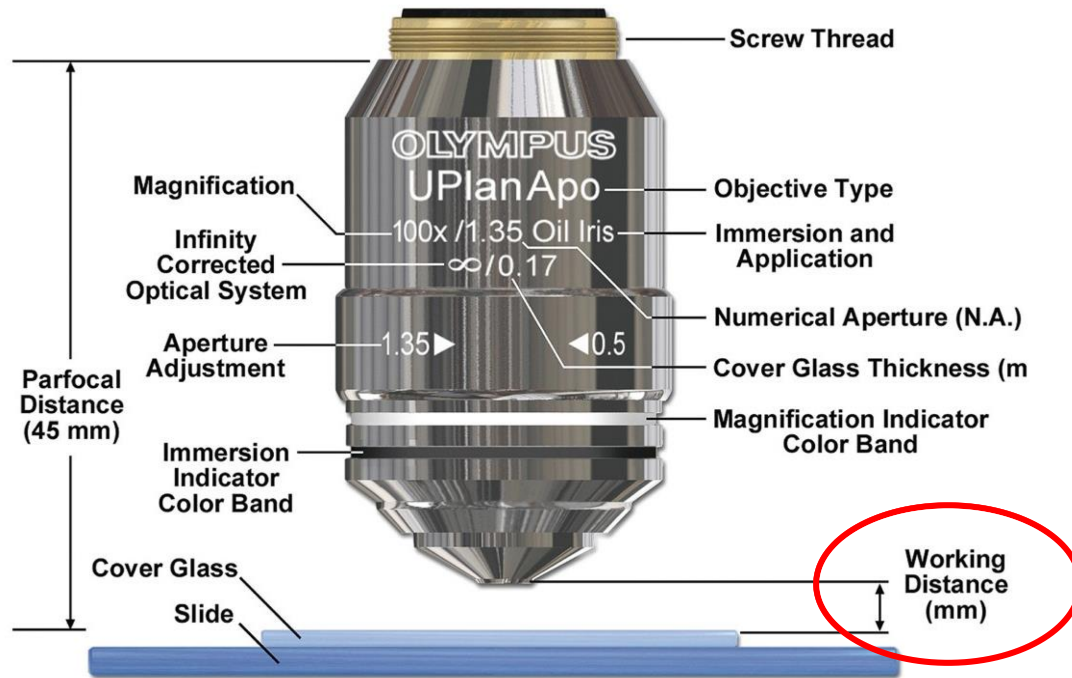




- Many objectives are designed to be used with standard (1.1 - mm thick) glass slides and coverslips of a certain thickness, usually 0.17 mm, which corresponds to thickness grade # 1.5
- Other coverslip thicknesses induce spherical aberration and give poorer performance, especially when used with high,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

Grade Number	Thickness (μm)
0	83–130
1	130–160
1.5	160–190 (standard)
2	190–250

Working distance of objectives



- ***Working distance*** is the distance between the surface of the front lens element of the objective and the surface of the coverslip nearest to the objective.

THE CARE AND CLEANING OF OPTICS

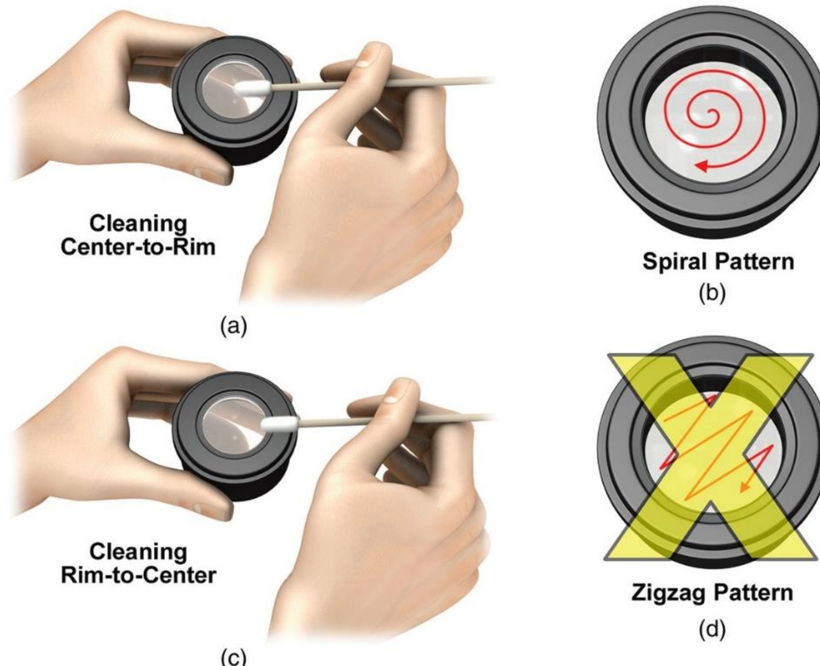
- Maintenance and care are required to protect an expensive optical instrument and to guarantee that optimal high-contrast images can be obtained from it.

- Neglect, such as not removing immersion oil, forgetting to cover open ports and apertures, or accidental twisting or dropping of an objective can ruin its optical performance.

- Even if the microscope is left unused but unprotected on the lab bench, image quality can deteriorate rapidly due to the accumulation of dust from the air.

Care and cleaning of optics -1: dust

- Keep the microscope protected with a plastic or cloth cover.
- Wipe dust off the microscope body and stage with a damp cloth.
- Keep the objective turret spotless and free of dust, immersion oil, spilled culture medium, and salt solutions.



(a) Cleaning is achieved using a spiral motion of a moistened cotton swab from the center to the rim.
(b) Correct spiral pattern used to clean lens surface.
(c) Avoid cleaning the lens rim - to - center.
(d) Never wipe using zigzag movements as this will only spread the dirt.

Care and cleaning of optics -2: Oil Immersion

- Immersion oil is a slow-acting solvent that can weaken the cementing compounds that act as a seal between the front lens element and the metal lens cap of the objective.

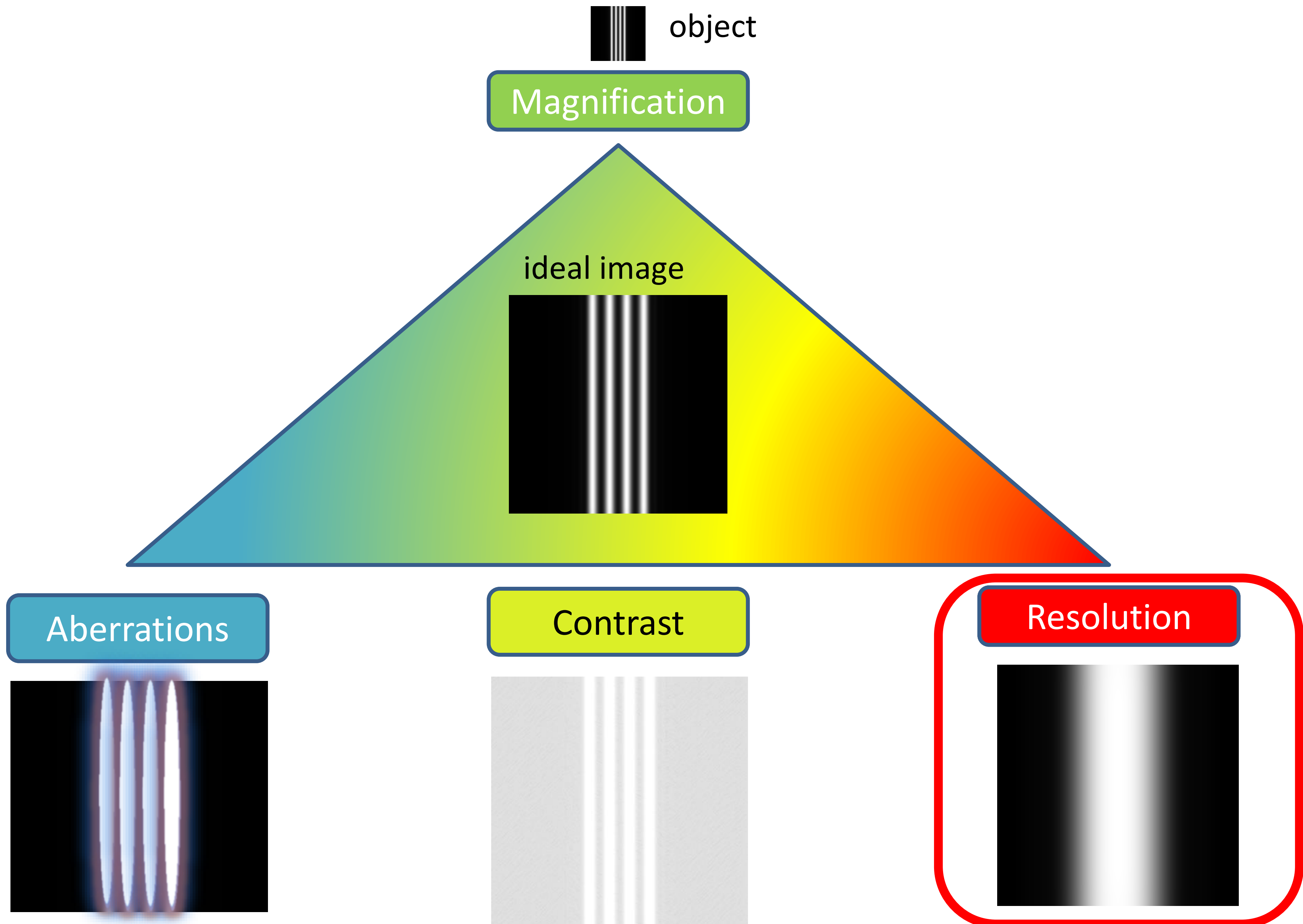
- Residual oil should be removed with a lens tissue wetted with a mild lens cleaner.

- Gently wipe off and clean away excess oil with a high-quality lens tissue and then clean the lens surface with an agent designed for cleaning microscope optics.

Care and cleaning of optics -3: Scratches and Abrasions

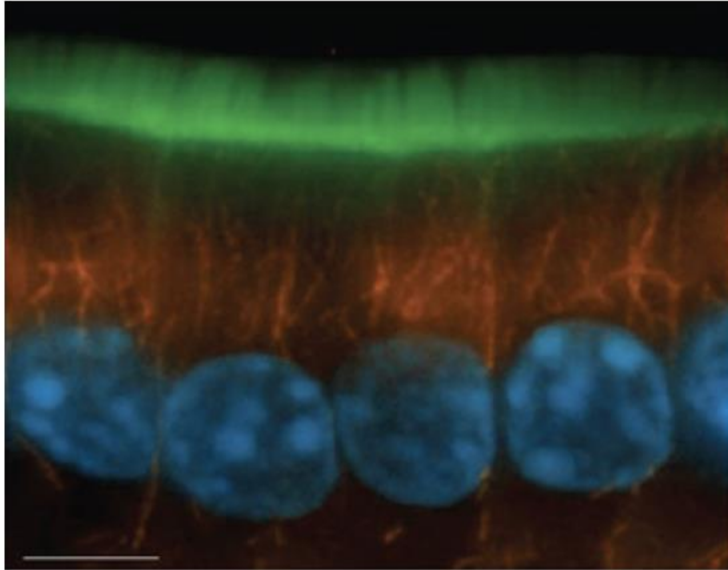
- Never wipe the surfaces of objectives with papers or cloths that are not certified to be free of microscopic abrasives.
- All objectives contain an exposed optical surface that must be protected from abrasion.
- Strands of wood fibers in coarse paper, or the stick end of a cotton swab applicator, are strong enough to place permanent scratch marks (sleeks) on the front lens element with a single swipe.
- Once present, scratches cannot be removed, and their effect (even if hardly visible) is to scatter light and permanently reduce image contrast.
- Further, most lenses contain an antireflection coating composed of layers of a dielectric material; each layer is just a few atoms thick.
- Although antireflection surfaces are protected with a layer of silicon monoxide, you should use only high-quality lens tissue and apply only a minimum of force to wipe off drops of excess oil.

Important aspects for microscopy



Importance of Resolution in Microscopy

Normal resolution



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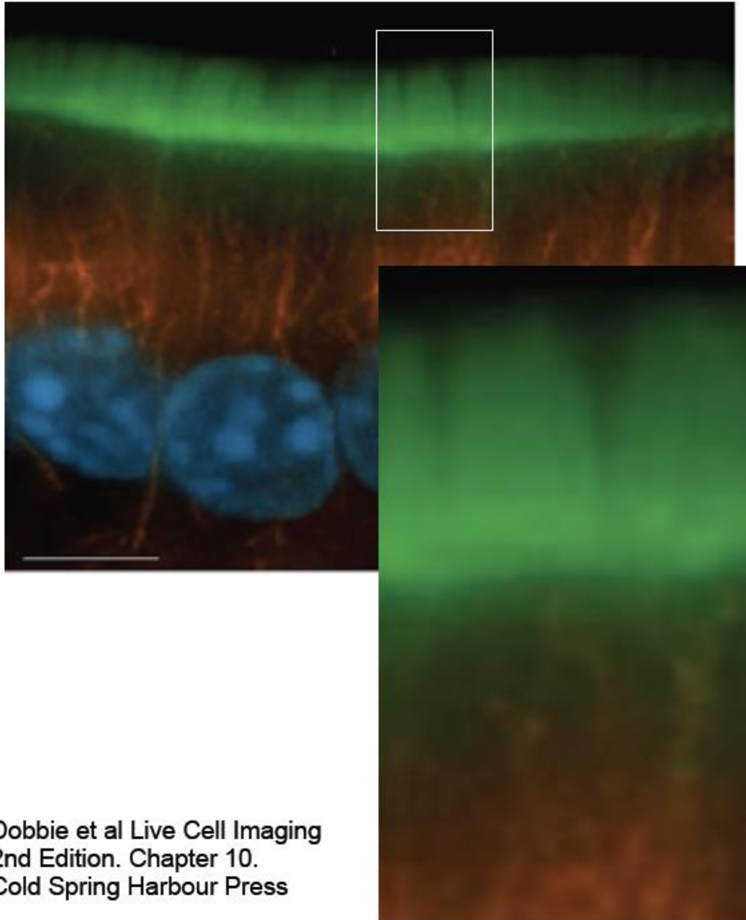
Importance of Resolution in Microscopy



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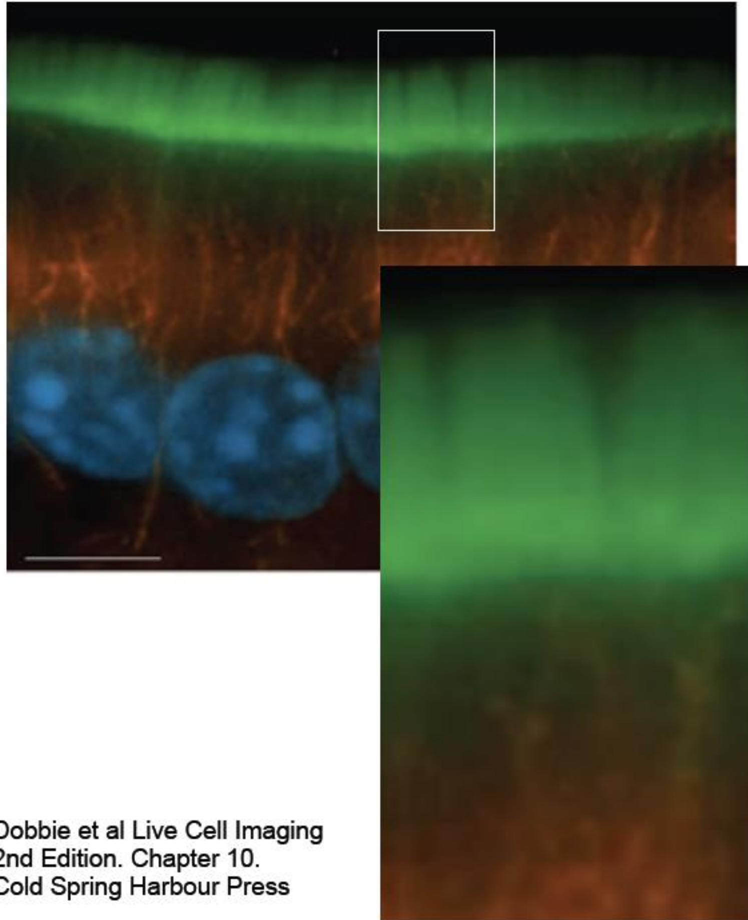
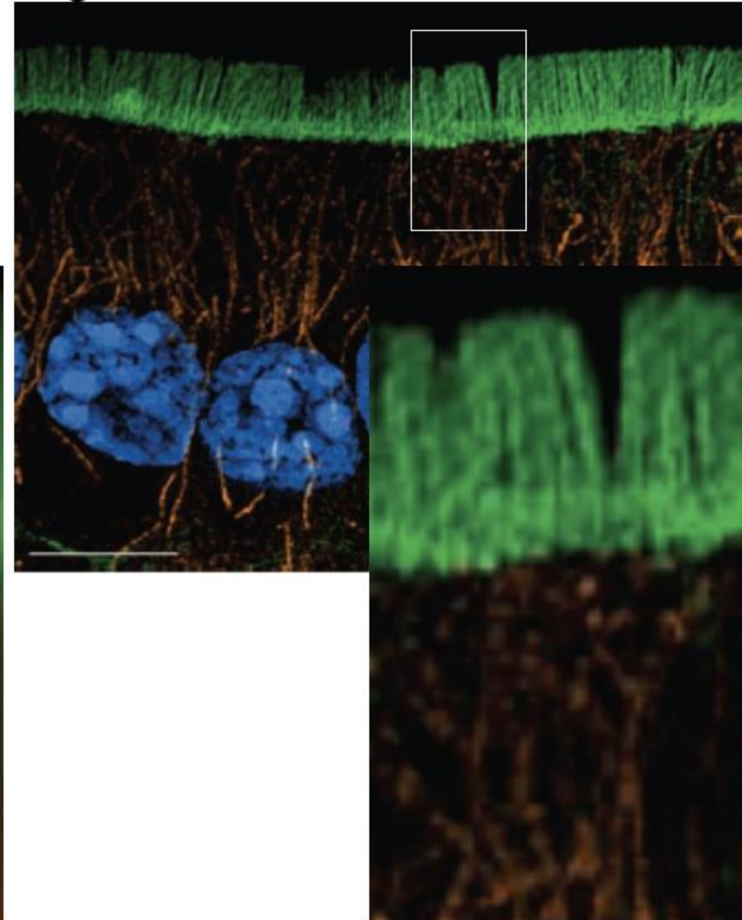
- without a proper resolution, increasing magnification will yield to no new information

Importance of Resolution in Microscopy





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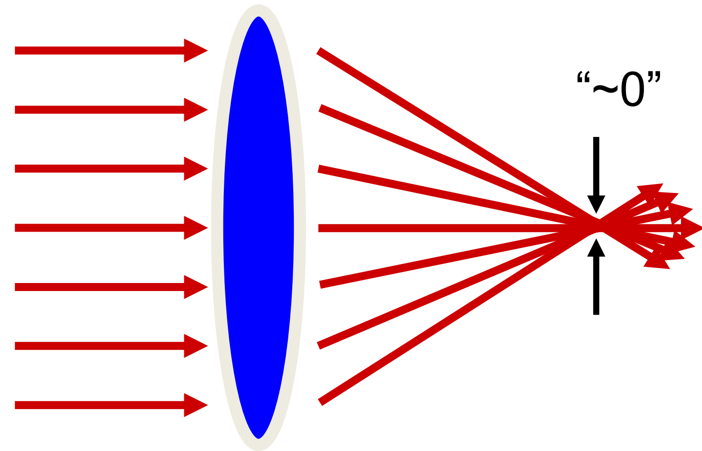
Geometrical optics

Ray (geometrical) optics imply that we can focus a beam to an ideal "point" with zero size.



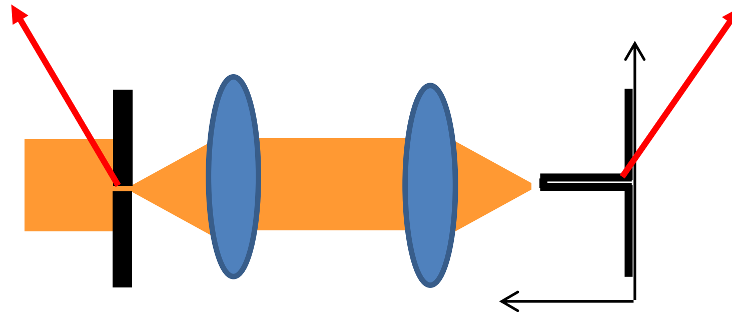
What does this imply for microscopy?

Resolution in microscopy

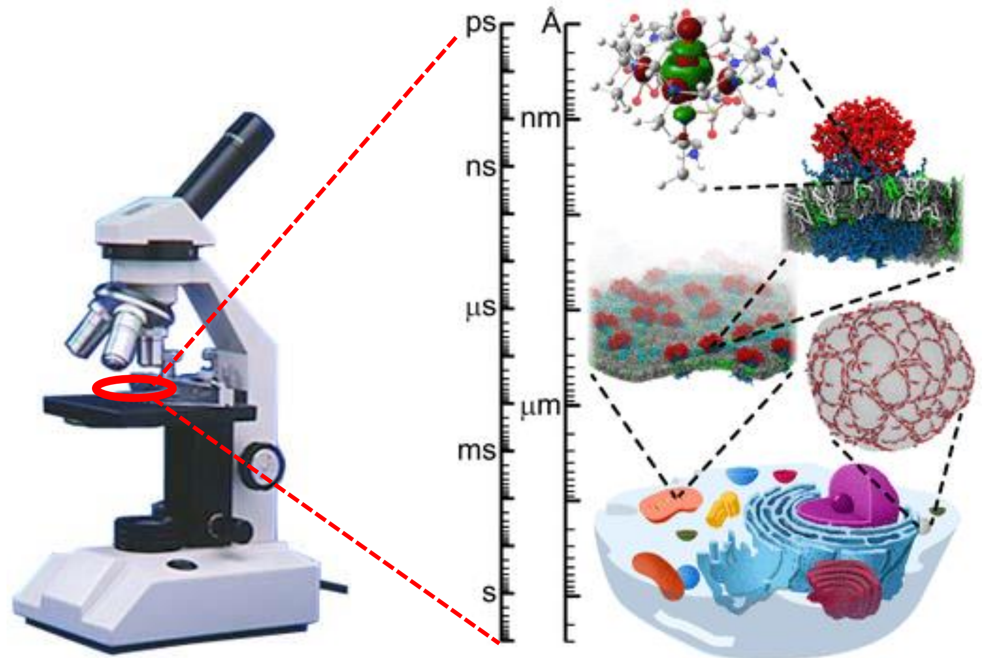
Object/Specimen:
If object is a pin-hole
(which represents a point source)

Image:

Ideally the image will also a point source

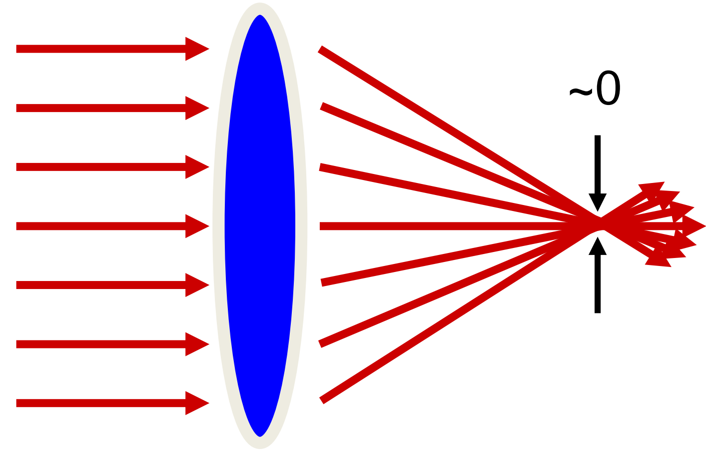


Ability to image an exact copy of a point source (with ~zero dimension) implies that a microscope should able to see **ANY** object, even those with extremely small dimensions such as: proteins, DNA, atoms …

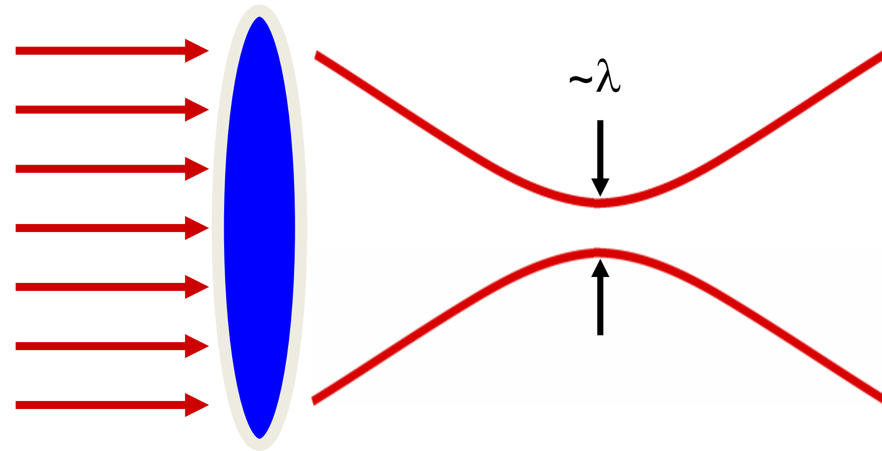


Is geometrical optics the whole story?

Ray optics imply that we could focus a beam to an ideal "point" with zero size.



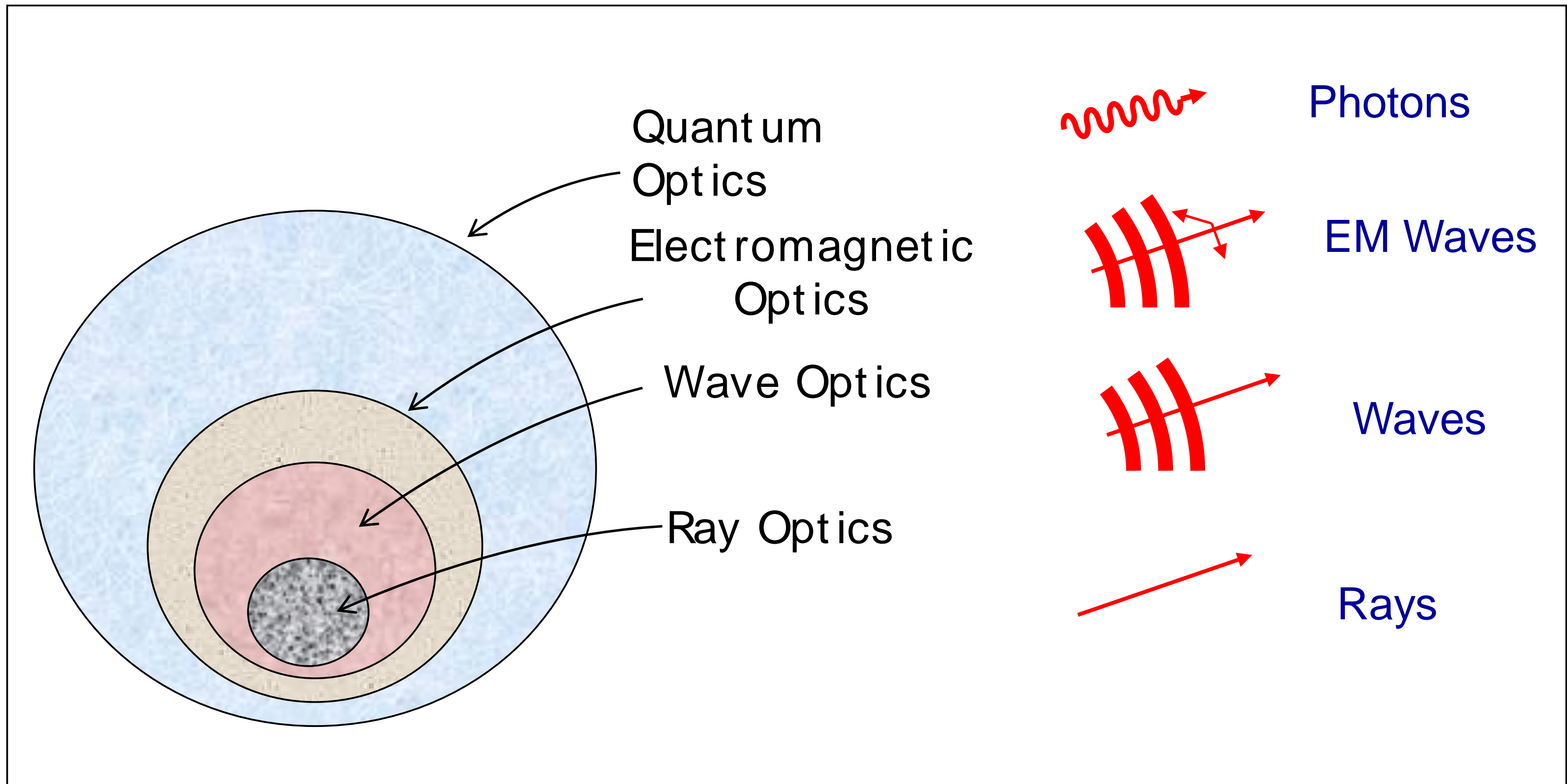
But, this does not hold in reality….



- The minimum possible "spot size" is about one wavelength, λ.
- The finite spot size is due to diffraction, which is not described by ray optics.
- This minimum spot size (i.e. $\sim\lambda$) also gives the best spatial resolution one can achieve with a microscope.
- In practice, this implies an object having a dimension smaller than λ, will appear at best having a size of λ.

Hierarchy of Theories in Optics

Ray optics cannot explain diffraction → **we need to consider wave optics**



Outline

- **Wave optics**
  - **Wave equation**
  - **Wavelength, period, amplitude & phase of a wave**
  - **Monochromatic waves**
  - **Real waves and complex waves**
  - **Elementary waves: plane wave and spherical wave**

Waves & Optics

An optical wave is described mathematically by the wavefunction $u(\mathbf{r}; t)$, at position $\mathbf{r} = (x, y, z)$ and time t.

Wavefunction satisfies the wave equation:

$$\nabla^2 u - (1/v^2)\partial^2 u/\partial t^2 = 0$$

$$\nabla^2 = \partial^2/\partial x^2 + \partial^2/\partial y^2 + \partial^2/\partial z^2$$

- " v" is the speed of the wave.
- For E&M waves we use "c", for the speed of light in free space.

- Wave equation is general.
- It can be applied to E&M radiation at different spectrum (i.e. light waves, microwaves, radio waves) and also other waves such as water, acoustic, seismic..

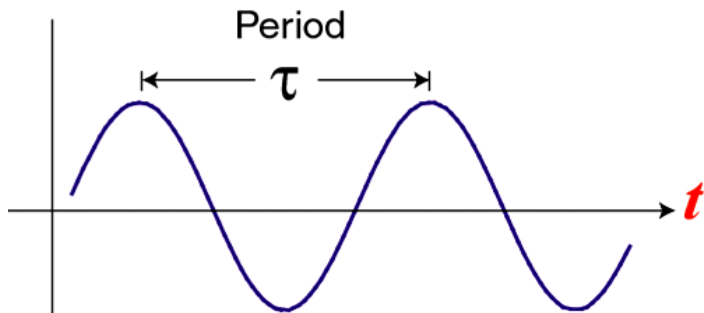
Monochromatic waves

- A monochromatic wave is one of the possible solutions to wave equation.
- It has a harmonic dependence in time and space.

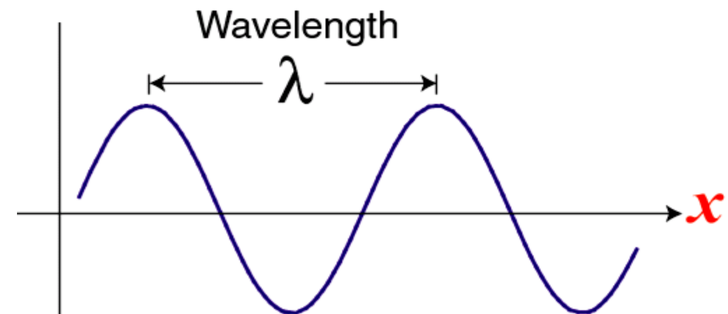
$$u(x,t) = A\cos[(kx - \omega t) - \theta]$$

- Monochromatic wave function oscillates periodically in space & time.
- "One" wavelength defines the monochromatic wave (i.e. it is a "mono/single" color wave)

Temporal quantities:



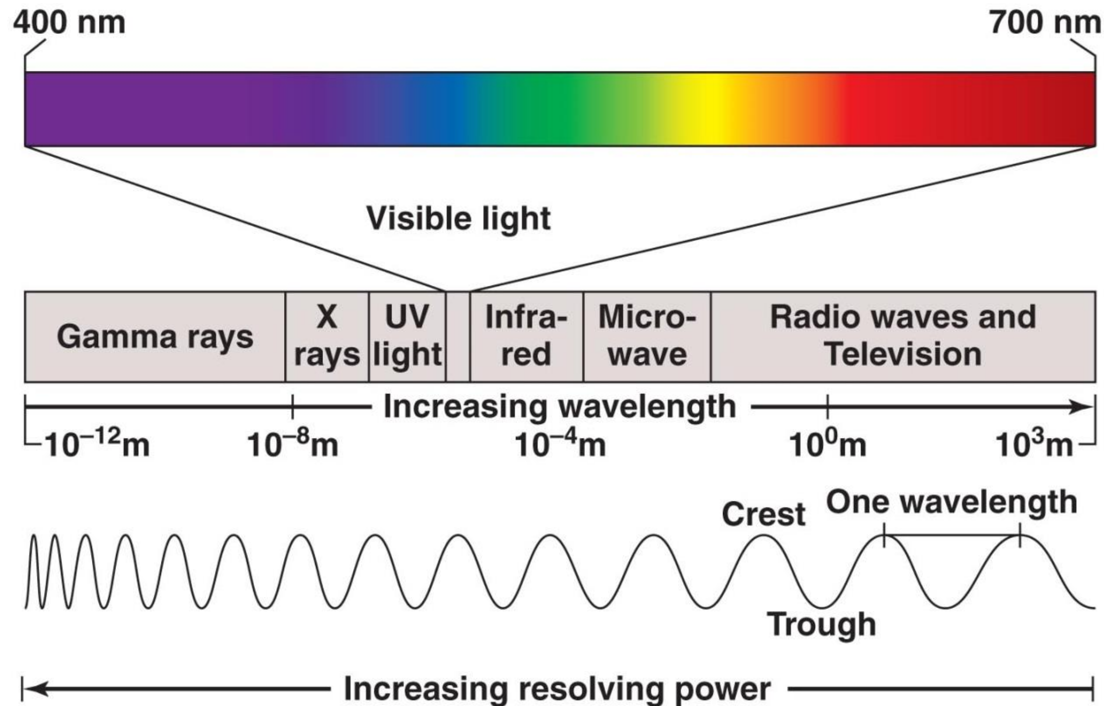
Spatial quantities:



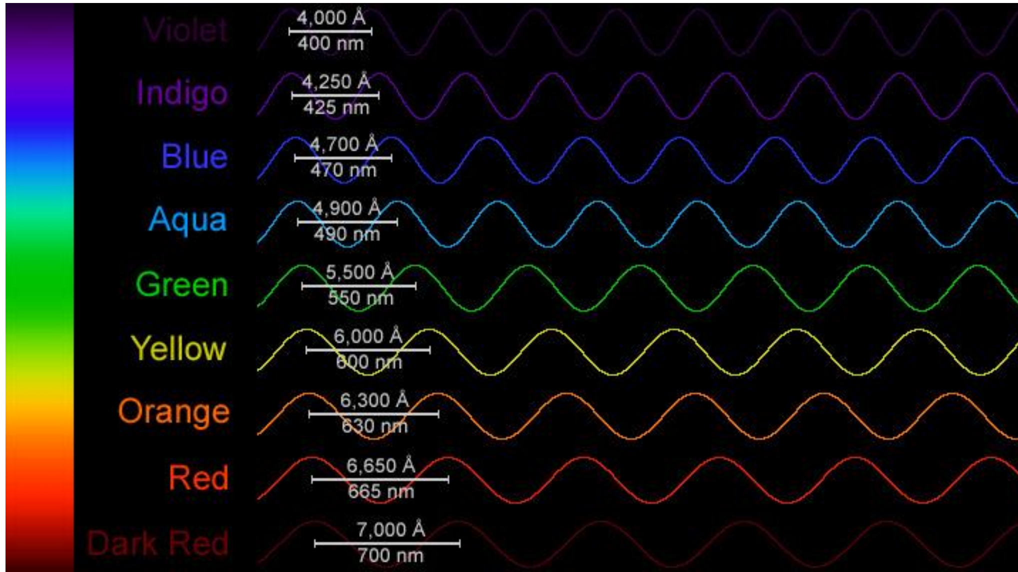
Wavelength of Radiation

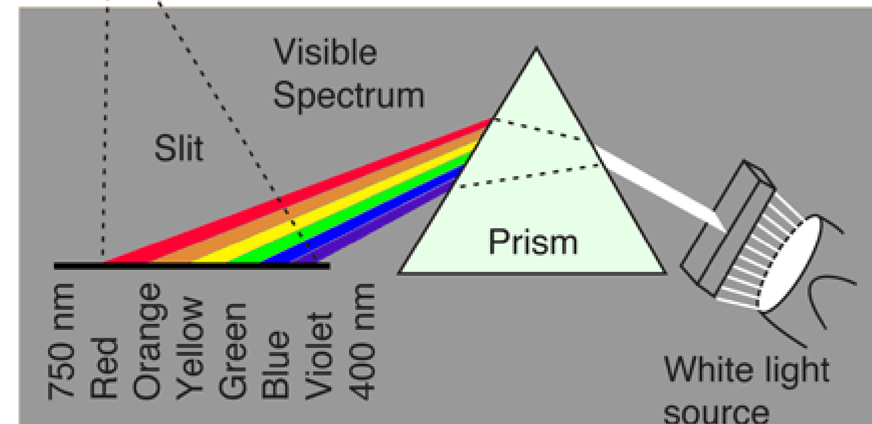
Wave radiation differs in **wavelength**

The electromagnetic spectrum



Visible spectrum

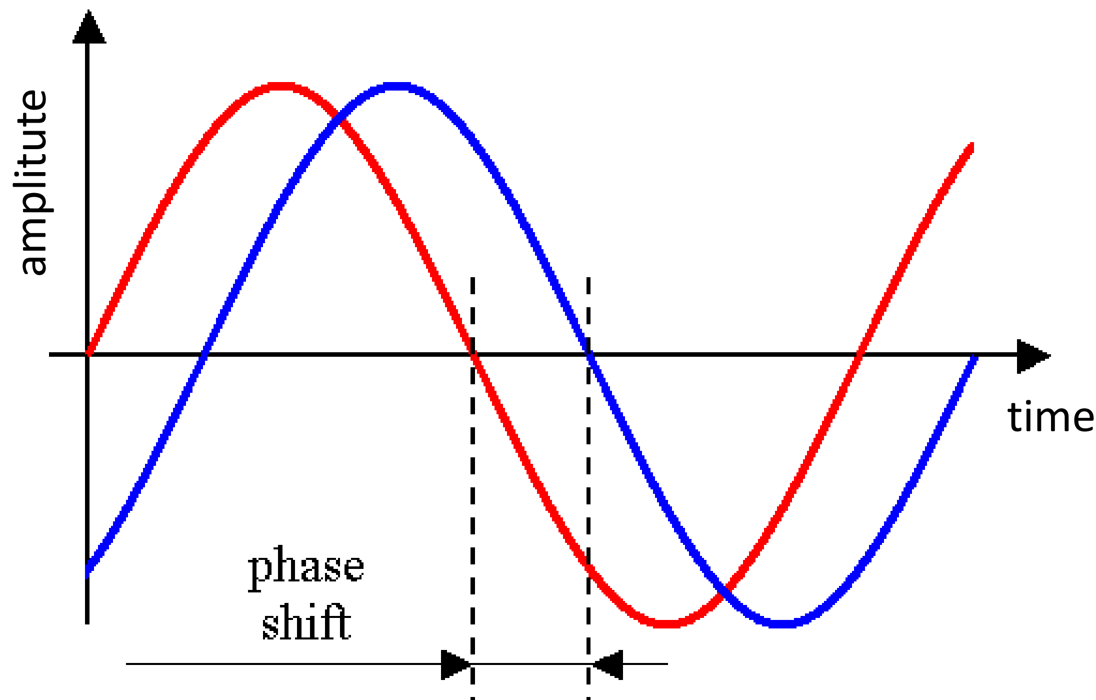




White light is a combination of all wavelengths (colors, frequency ..)

Characteristics of a wave - PHASE

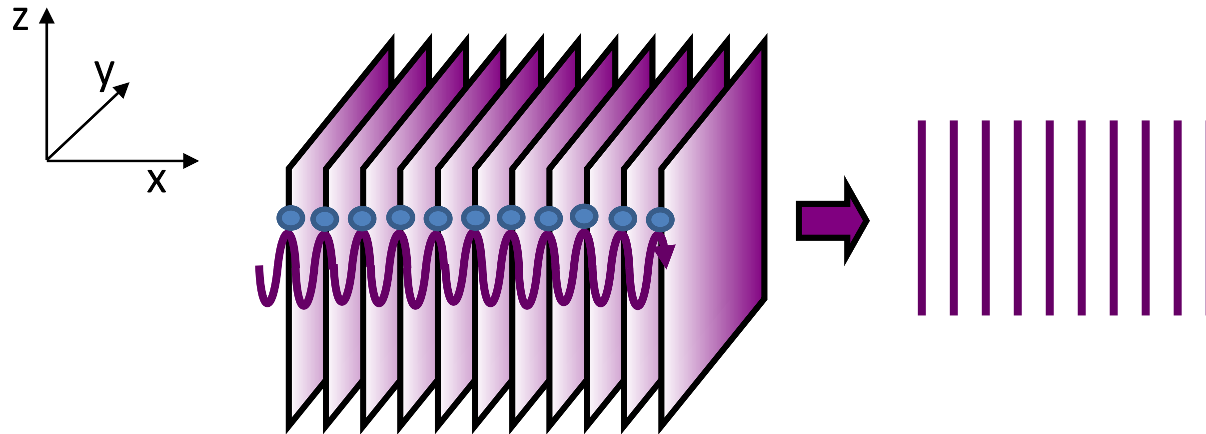
Below figure shows two waves with same amplitude & frequency (i.e. wavelength, period) but they differ in **PHASE**:



- Phase concept is introduced in wave optics.
- It does not exist in ray optics.

Monochromatic "plane wave" function

This equation represents a monochromatic **"plane"** wave: $u(x,t) = A\cos[(\omega t - kx) - \theta]$



Contours of maximum field, called wave-fronts or phase-fronts, are planes.

Usually we just draw lines

- They extend over all space (y-z).
- Wave-fronts are equally spaced: one wavelength apart.
- They're perpendicular to the propagation direction (x).

Monochromatic wave function

This equation represents a monochromatic **"plane"** wave: $u(x,t) = A\cos[(\omega t - kx) - \theta]$

If we generalize this equation to "**any**" monochromatic wave (planar, spherical, etc…), then the expression becomes:

$$u(r,t) = a(r)\cos\left[\omega t + \varphi(r)\right]$$

This represents a real wavefunction for a monochromatic wave with harmonic time dependence.

Complex representation of a monochromatic wave function

In optics (and E&M), it is convenient to work with **complex wave** formalism

Real wave function is: $u(r,t) = a(r)\cos\left[\omega t + \varphi(r)\right]$

Complex wave function representation of u(r,t) is: $U(r,t) = a(r)e^{i\varphi(r)}e^{i\omega t}$

$$U(r,t) = U(r)e^{i\omega t}$$

Here, U(r) is called complex amplitude.

Mathematically, U(r,t) describes the wave completely, and the wavefunction u(r,t) is its real part:

$$u(r,t) = \mathrm{Re}\left\{U(r,t)\right\} = \frac{1}{2}\left[U(r,t) + U^*(r,t)\right] = \frac{1}{2}[U(r)e^{i\omega t} + U^*(r)e^{-i\omega t}]$$

Here, sign * signifies complex conjugation.

Complex wave function also satisfies the wave equation: $\nabla^2 U - \frac{1}{c^2}\frac{\partial^2 U}{\partial t^2} = 0$

Amplitude, intensity and phase of a monochromatic wave

$$u(r,t) = a(r)\cos\left[\omega t + \varphi(r)\right]$$

← this defines the real monochromatic wave, u(r,t)

$$U(r,t) = a(r)e^{i\varphi(r)}e^{i\omega t}$$

← This is the complex wave function representation of u(r,t)

$$U(r,t) = U(r)e^{i\omega t}$$

← Here, U(r) is called complex amplitude.

Real part of U(r) gives the **amplitude** of the wave**:**

$$|U(r)| = a(r)$$

Intensity of the wave is the square of its amplitude**:**

$$I(r) = |U(r)|^2 = a(r)^2$$

Imaginary part of U(r) gives the **PHASE** of the wave**:**

$$\arg\{U(r)\} = \varphi(r)$$

- "Phase" is introduced in wave optics. It does not exist in ray optics.
- "Phase" explains the "**interference & diffraction**" phenomena.

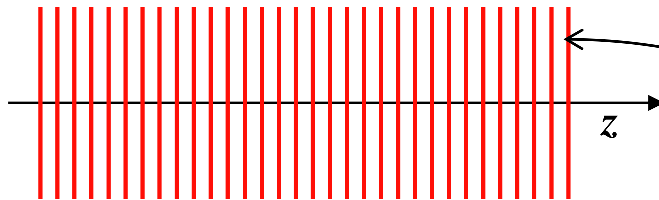
1st example for "elementary wave": Plane wave

- Plane wave traveling in z-direction

$$u(\mathbf{r};t) = a\cos[\omega t - kz + \varphi_o]$$

$$U(\mathbf{r}) = a\exp(i\varphi_o)\exp(-ikz)$$

$$U(\mathbf{r}) = A\exp(-ikz)$$



Wavefronts $\varphi_o - kz = 2\pi m$

$kz = 2\pi m + \text{constant}$

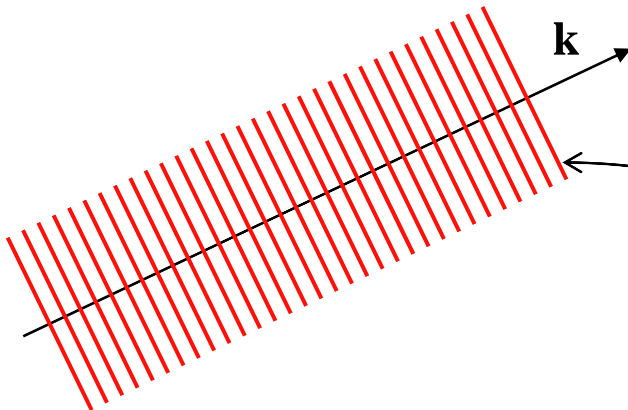
$$U(r) = Ae^{\pm ikz}$$

Propagation direction (left or right)

- Plane wave traveling in arbitrary direction

$$U(\mathbf{r}) = A\exp(-i\mathbf{k}.\mathbf{r})$$

$$= A\exp[-i(k_x x + k_y y + k_z z)]$$

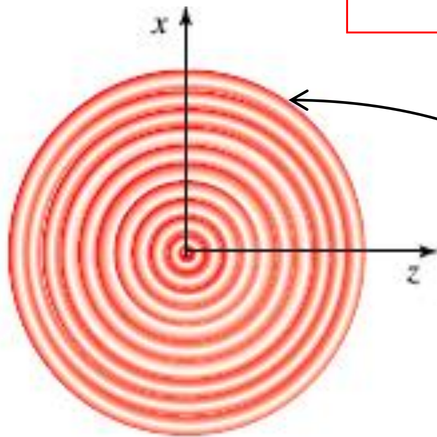


Wavefronts $k_x x + k_y y + k_z z = 2\pi m + \text{constant}$

2nd example for "elementary wave": spherical wave

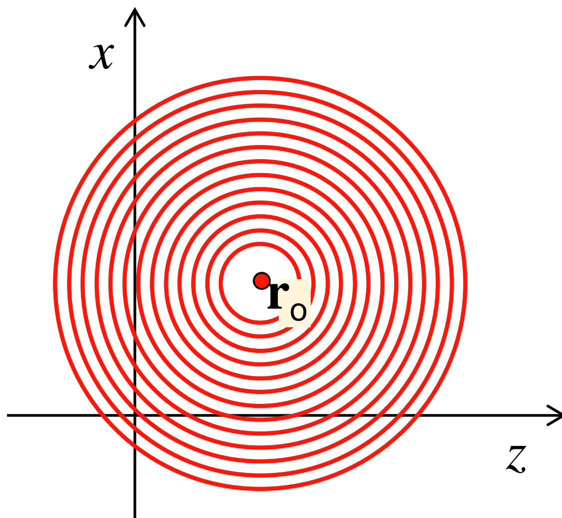
$$U(r) = \frac{A}{r} e^{\pm ikr}$$

Propagation direction (inward or outwards)



Wavefronts $kr = 2\pi m + \text{constant}$

$$I(\mathbf{r}) = |A|^2 / r^2$$

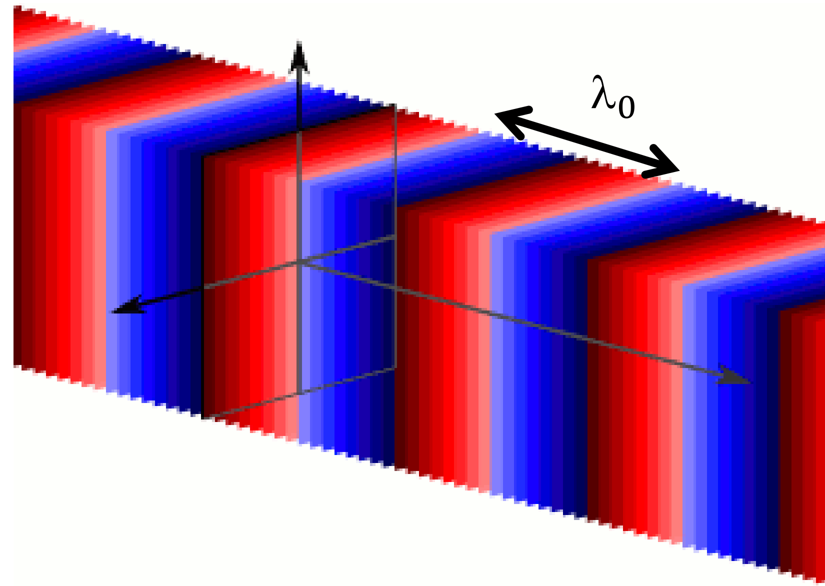


$$U(r) = \left(\frac{A}{|r - r_o|}\right) e^{-ik|r - r_o|}$$

Spherical wave centered at $\mathbf{r}_o$

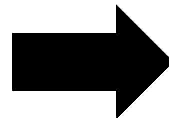
Wave Propagation

Example:
Propagating plane wave in free space



- In free space, one wavelength (λ_0) is the distance that light travels over one period in time (τ) at a speed of *c:*

Distance = Speed x Time



$$\lambda_0 = c \times \tau$$

- Velocity in vacuum (i.e. "*c*") = 2.99792458 • 10^8 m/sec

Wave Propagation in Free Space

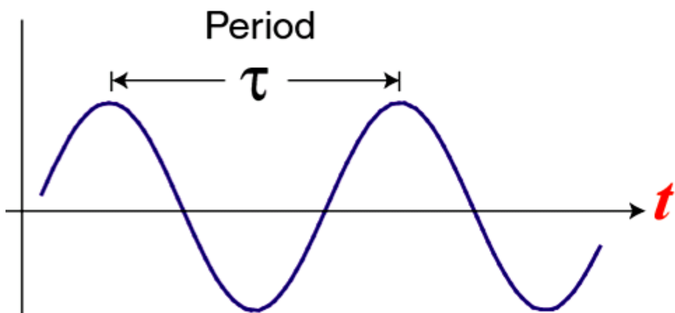
- In free space, one wavelength (λ_o) is the distance that light travels over one period in time (τ) at a speed of *c:*

$$\lambda_0 = c \times \tau$$

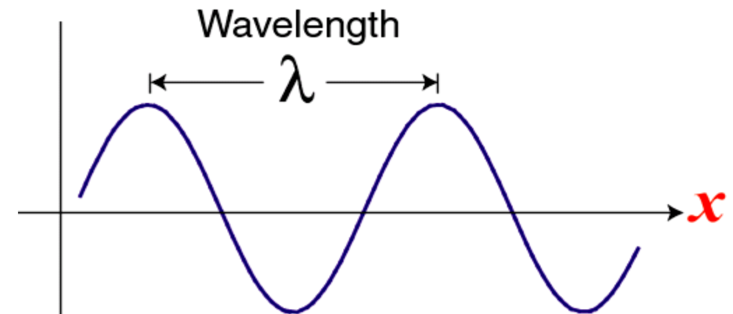
- Velocity in vacuum (i.e. "*c*") = 2.99792458 • 10^8 m/sec
- Recall the definition of frequency & period in time: $\upsilon = 1/\tau$
- Thus, velocity and wavelength of light are linked to each other as:

$$c = \upsilon \lambda_0$$

Temporal quantities:



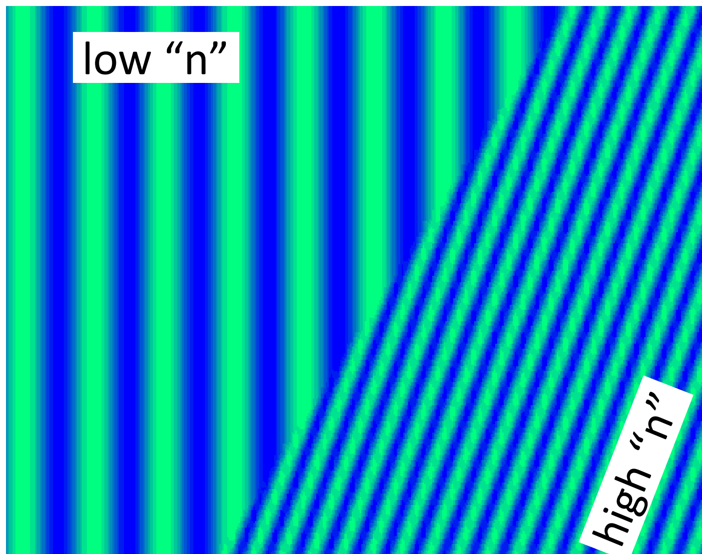
Spatial quantities:



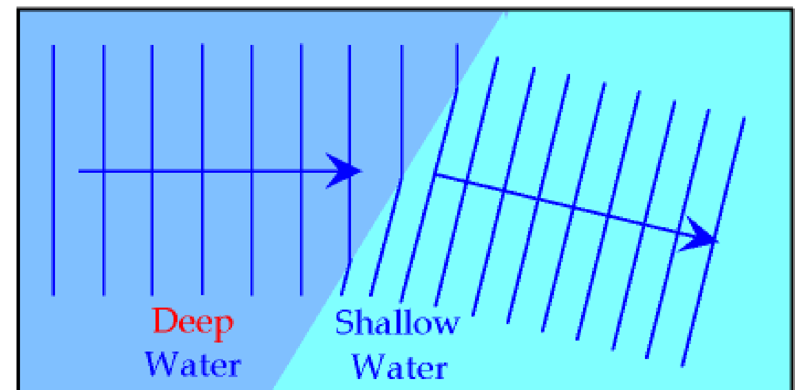
Wave Propagation in Medium

- In free scape: $c = \upsilon \lambda_0$
- When light enters into a medium (with index n), its frequency (which is linked to energy) remains constant but its wavelength and speed (v) change as follows: $v = \upsilon \lambda$
- Both wavelength and speed of light scale down linearly with the refractive index n:

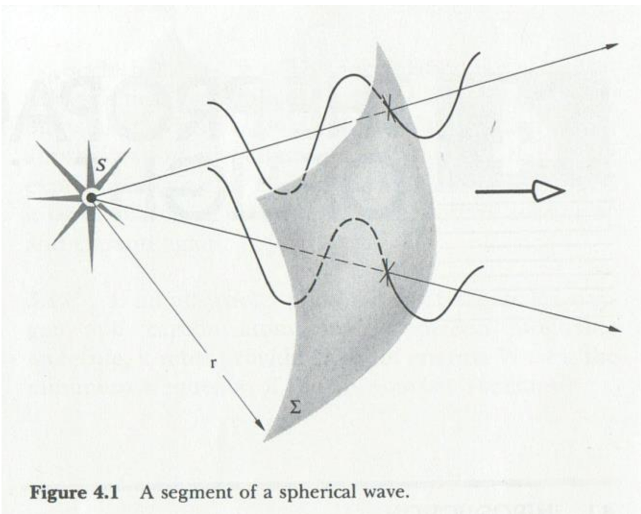
$$v = c/n \qquad \lambda = \lambda_0 /n$$

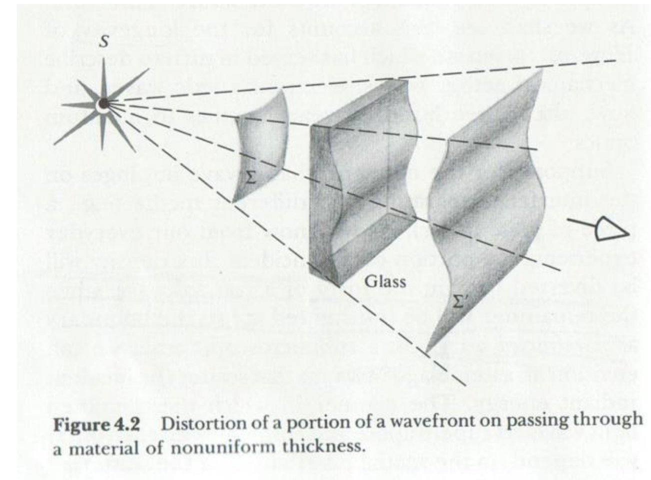


Analogy with “water” waves

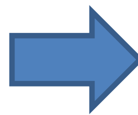


Wave Propagation Through Objects



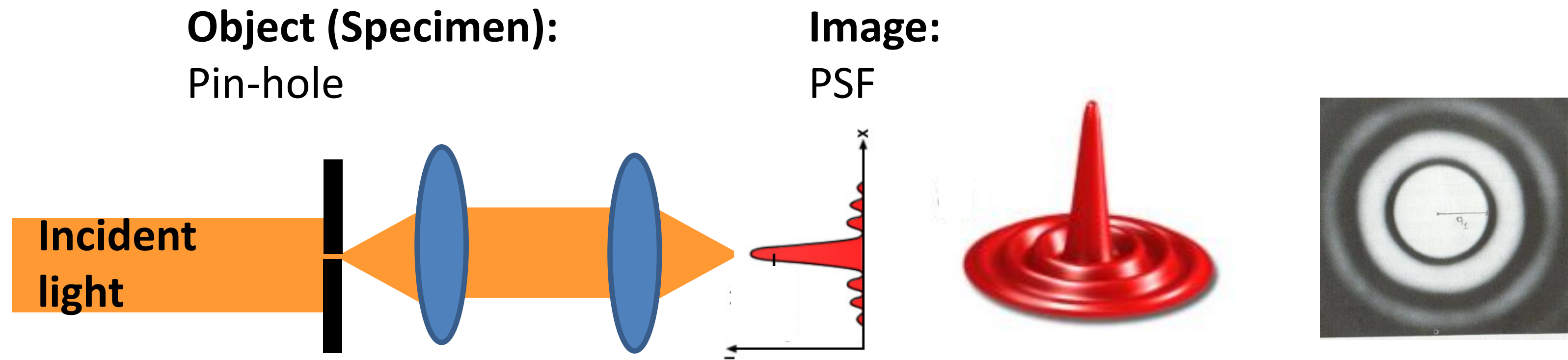


consider a spherical weave interacts with an object (such as a lens, aperture, slit)



its wave fronts gets distorted with this interaction

Wave phenomena & microscopy



In a microscope:

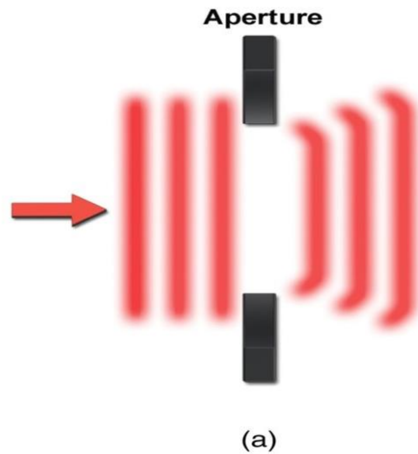
1) Light from the illuminator is **diffracted** (i.e. spread) by the specimen (**object**)

2) Then, diffracted light is **collected** by the **objective lens**

3) And **focused** by the following optical components in the image plane where the propagating light waves **constructively & destructively interfere** to form the **image**

Wave phenomena : diffraction

Depending on the specimen type, diffracted light can be perceived in different ways



Example-1 (a): When a beam of light is directed to an aperture, light appears to **bend around the edges**. The aperture could be the "stops" in the microscope (or the edges of the lenses)

Wave phenomena : diffraction

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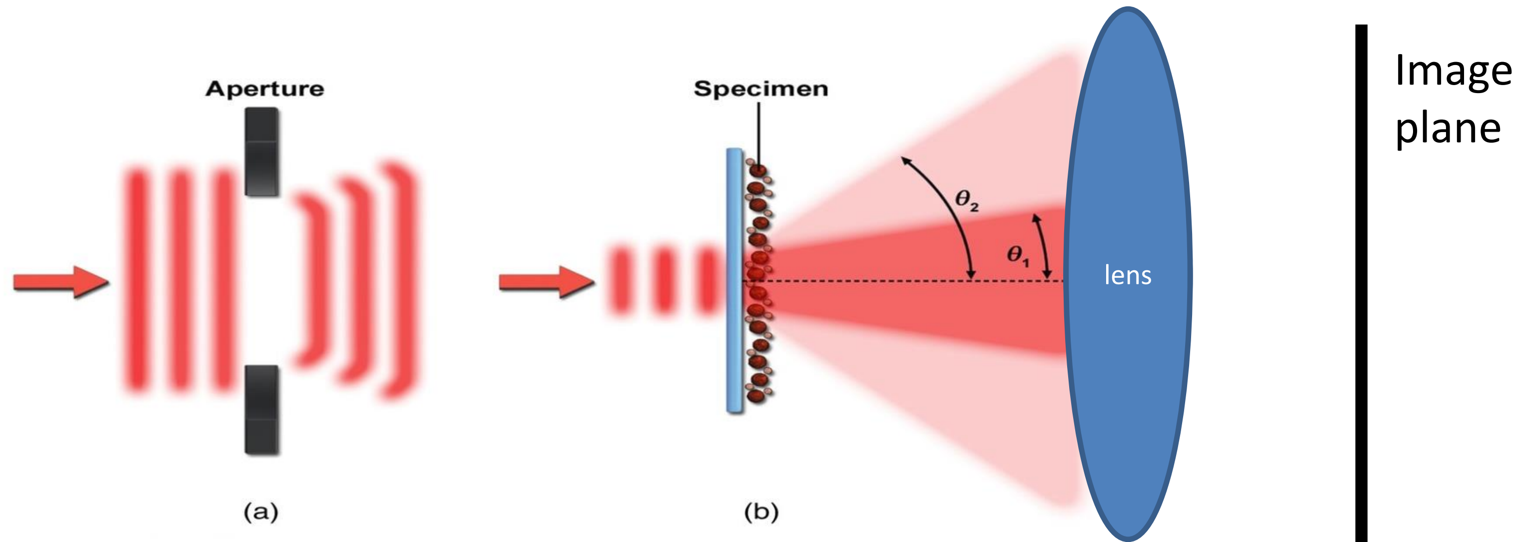


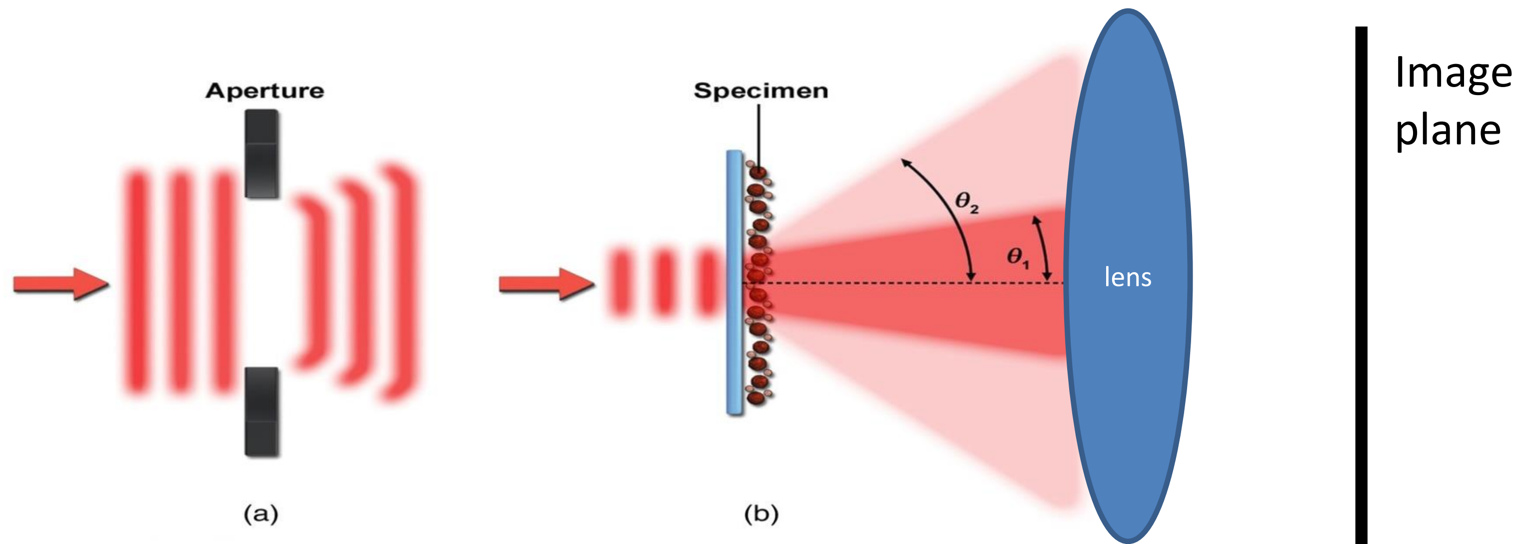
Example-1 (a): When a beam of light is directed to an aperture, light appears to **bend around the edges**. The aperture could be the "stops" in the microscope (or edges of the lenses)

Example-2 (b): Diffraction also occurs when light illuminates a microscope slide covered with **small particles**. The amount of light **scattering** & the angle of spreading depend on the size & density of the diffracting particles on the slide.

In the right drawing, let's assume a mixture of 0.2 um & 2 um diameter particles as specimen.

The angle of spreading is inversely proportional to the particle size.
➔ Larger angle (θ_2) corresponds to light diffraction by the smaller particles

Wave phenomena : diffraction & interference



Diffraction - In microscopy, there are two primary sites for diffraction:

1- the specimen itself

2- the most limiting aperture of the microscope system

Interference can be seen as the combination of diffracted waves.

➔ This is also the process responsible for creating images.