Light Microscopy

Lenses and the Bending of Light

- When a ray of light passes from one medium to another, **refraction occurs—that is, the ray is bent at the interface.**
- The **refractive index is a measure of how greatly a substance** slows the velocity of light, and the direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface.
- When light passes from air into glass, a medium with a greater refractive index, it is slowed and bent toward the normal, a line perpendicular to the surface (**figure 2.1).**

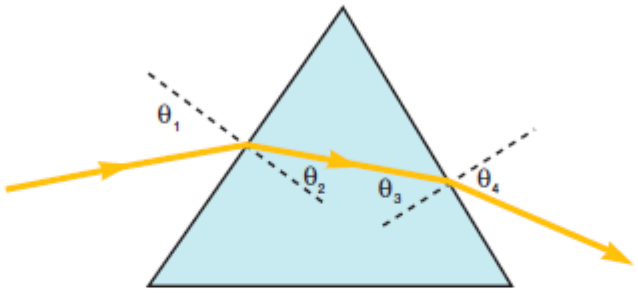


Figure 2.1 The Bending of Light by a Prism. Normals (lines perpendicular to the surface of the prism) are indicated by dashed lines. As light enters the glass, it is bent toward the first normal (angle θ_2 is less than θ_1). When light leaves the glass and returns to air, it is bent away from the second normal (θ_4 is greater than θ_3). As a result the prism bends light passing through it.

Focal Length

- Our eyes cannot focus on objects nearer than about 25 cm or 10 inches**.**
- **This limitation may be overcome by using** a convex lens as a simple magnifier (or microscope) and holding it close to an object.
- Lens strength is related to focal length; a lens with a short focal length will magnify an object more than a weaker lens having a longer focal length.

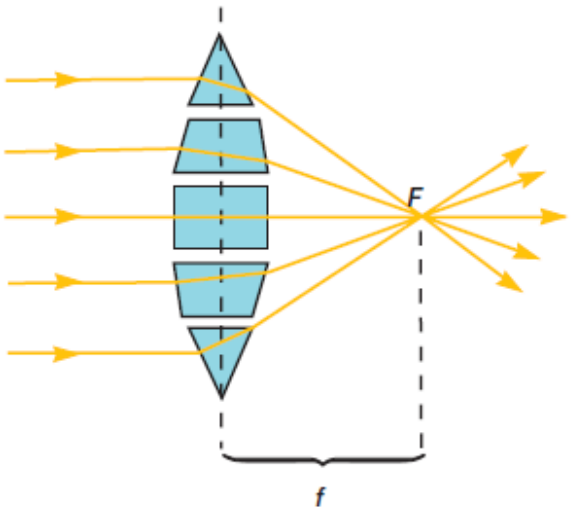


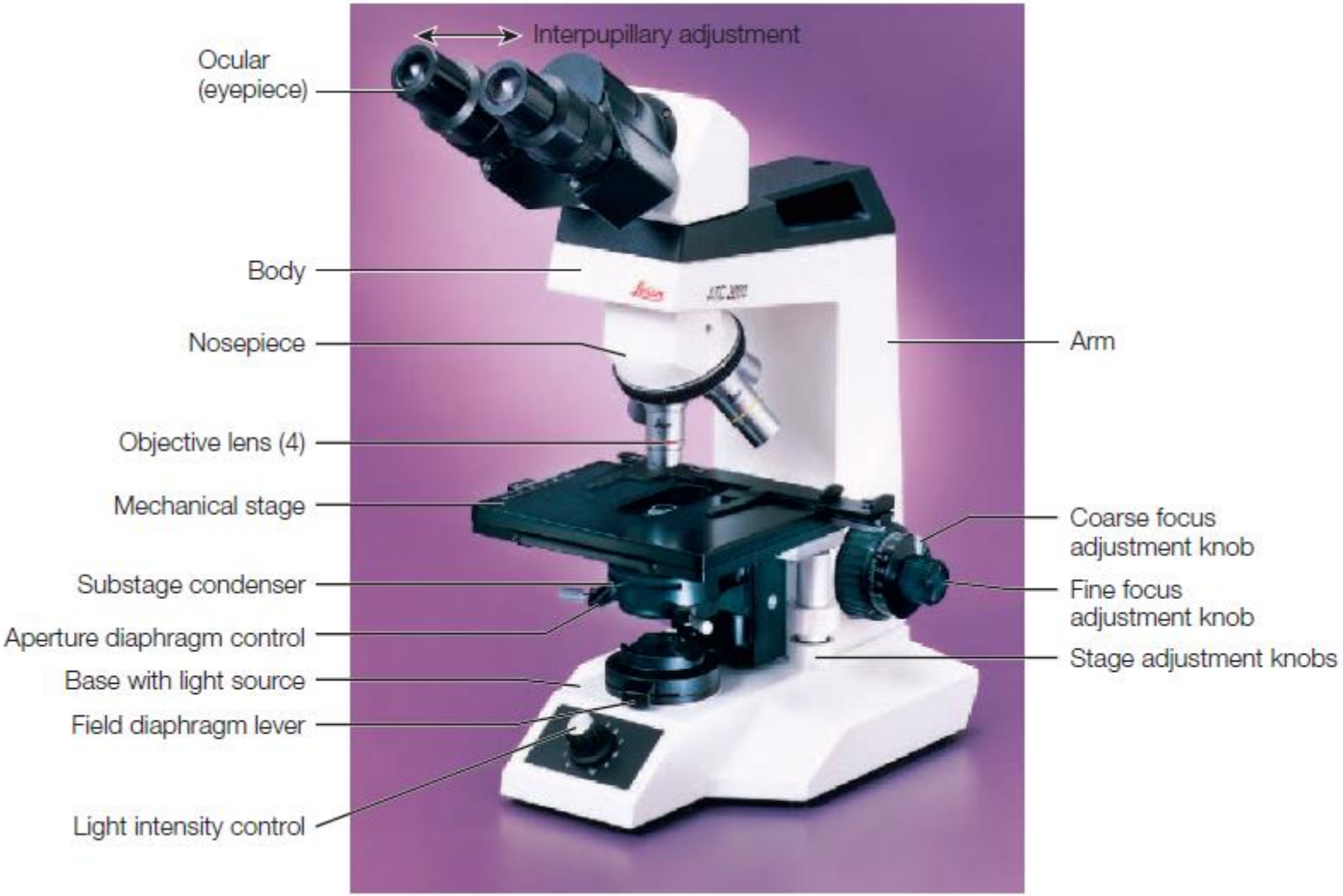
Figure 2.2 Lens Function. A lens functions somewhat like a collection of prisms. Light rays from a distant source are focused at the focal point *F.* The focal point lies a distance *f,* the focal length, from the lens center.

The Light Microscope

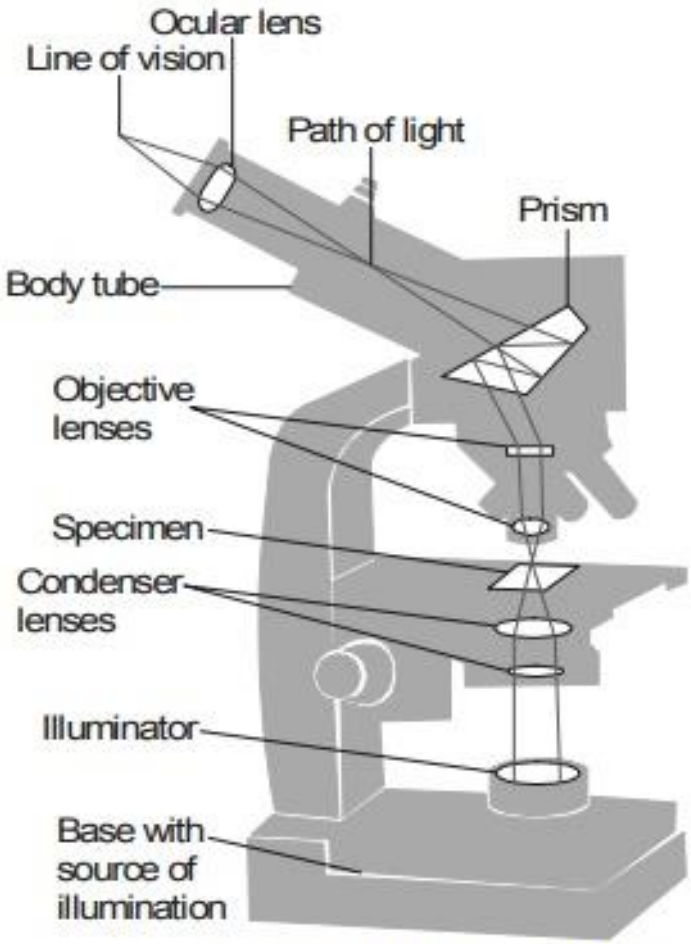
- Modern microscopes are all compound microscopes.
- That is, the magnified image formed by the objective lens is further enlarged by one or more additional lenses.

The Bright-Field Microscope

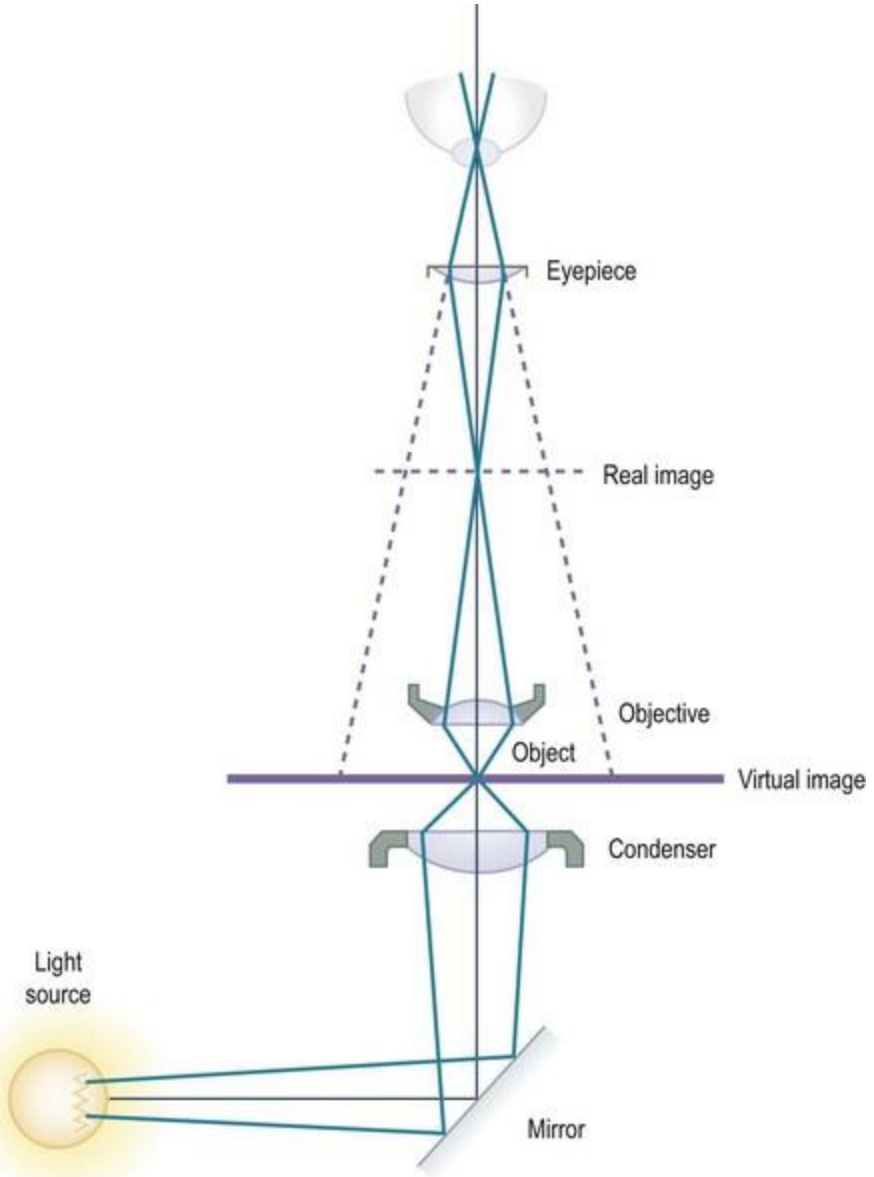
- The ordinary microscope is called a **bright-field microscope because** it forms a dark image against a brighter background.
- Ideally a microscope should be **parfocal—that is, the image should remain in focus when objectives** are changed.


Interpupillary adjustment
Ocular (eyepiece)
Body
Nosepiece
Objective lens (4)
Mechanical stage
Substage condenser
Aperture diaphragm control
Base with light source
Field diaphragm lever
Light intensity control
Arm
Coarse focus adjustment knob
Fine focus adjustment knob
Stage adjustment knobs

- The path of light through a bright-field microscope is shown in figure 2.4.
- The objective lens forms an enlarged real image within the microscope, and the eyepiece lens further magnifies this primary image.
- When one looks into a microscope, the enlarged specimen image, called the virtual image, appears to lie just beyond the stage about 25 cm away.
- The total magnification is calculated by multiplying the objective and eyepiece magnifications together. For example, if a 45 objective is used with a 10 eyepiece, the overall magnification of the specimen will be 450.



The path of light (bottom to top)



Microscope Resolution

- The most important part of the microscope is the objective, which must produce a clear image, not just a magnified one.
- **Resolution is the ability of a lens** to separate or distinguish between small objects that are close together.
- The minimum distance (d) between two objects that reveals them as separate entities is given by the Abbe equation, in which lambda (λ) is the wavelength of light used to illuminate the specimen and n sin θ is the numerical aperture (NA). (Theta is defined as 1/2 the angle of the cone of light entering an objective)

$$d = \frac{0.5\lambda}{n \sin \theta}$$

- As d becomes smaller, the resolution increases, and finer detail can be discerned in a specimen.
- The wavelength must be shorter than the distance between two objects or they will not be seen clearly. Thus the greatest resolution is obtained with light of the shortest wavelength, light at the blue end of the visible spectrum (in the range of 450 to 500 nm).

Numerical aperture

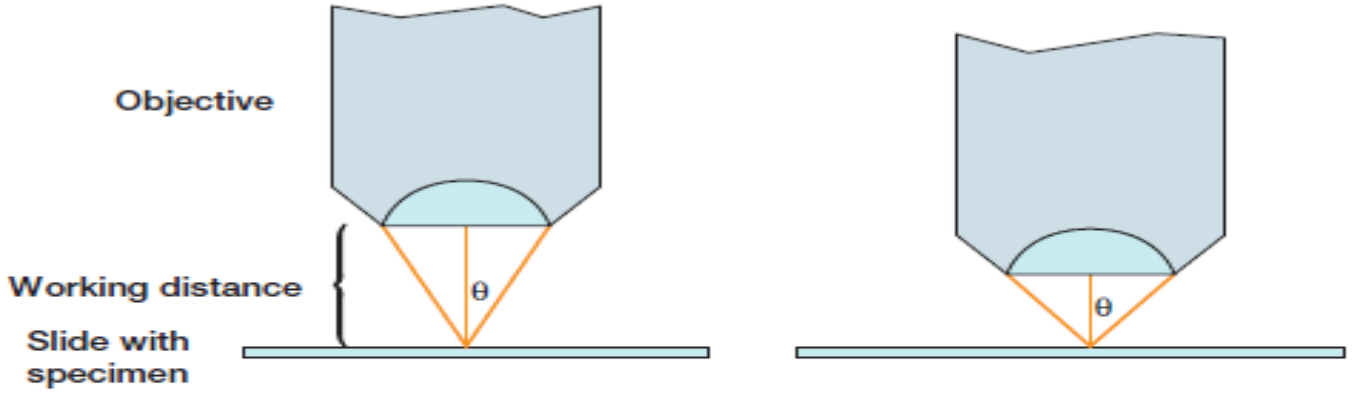


Figure 2.5 Numerical Aperture in Microscopy. The angular aperture θ is $\frac{1}{2}$ the angle of the cone of light that enters a lens from a specimen, and the numerical aperture is n sin θ. In the right-hand illustration the lens has larger angular and numerical apertures; its resolution is greater and its working distance smaller.

- The angle of the cone of light that can enter a lens depends on the refractive index (*n) of the medium in which the* lens works, as well as upon the objective itself. The refractive index for air is 1.00. Since sin θ cannot be greater than 1 (the maximum is 90° and sin 90° is 1.00), no lens working in air can have a NA greater than 1.00.
- The only practical way to raise the numerical aperture above 1.00, and therefore achieve higher resolution, is to increase the refractive index with immersion oil, a colorless liquid with the same refractive index as glass (**table 2.2).**

… Numerical aperture

- **If air is replaced with immersion** oil, many light rays that did not enter the objective due to reflection and refraction at the surfaces of the objective lens and slide will now do so **.**
- **An increase in numerical** aperture and resolution results (Fig 2.6).

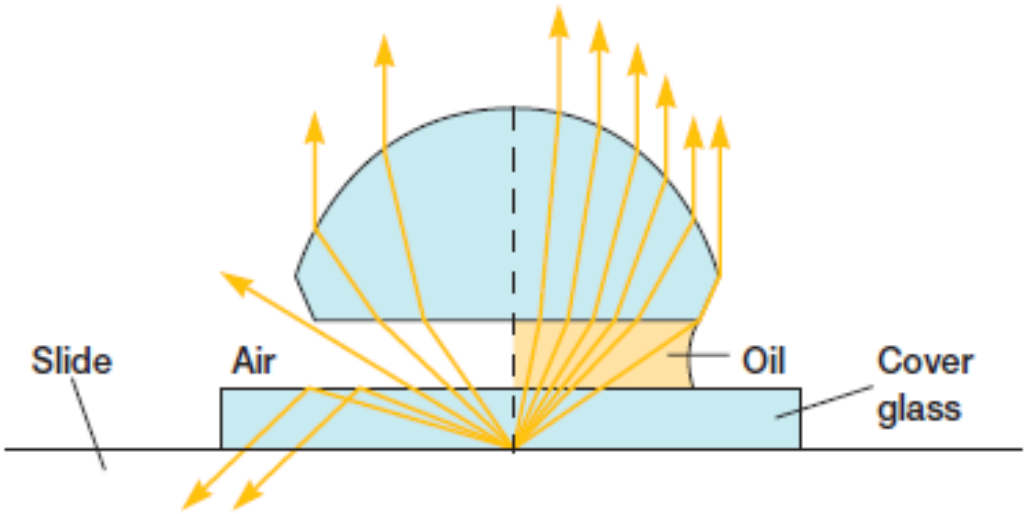


Figure 2.6 **The Oil Immersion Objective.** An oil immersion objective operating in air and with immersion oil.

… Numerical aperture

- The resolution of a microscope depends upon the numerical aperture of its condenser as well as that of the objective.
- This is evident from the equation describing the resolution of the complete microscope.

$$d_{\text{microscope}} = \frac{\lambda}{(\text{NA}_{\text{objective}} + \text{NA}_{\text{condenser}})}$$

- Most microscopes have a condenser with a numerical aperture between 1.2 and 1.4. However, the condenser numerical aperture will not be much above about 0.9 unless the top of the condenser is oiled to the bottom of the slide.
- During routine microscope operation, the condenser usually is not oiled and this limits the overall resolution, even with an oil immersion objective.

… Numerical aperture

- The limits set on the resolution of a light microscope can be calculated using the Abbe equation.
- The maximum theoretical resolving power of a microscope with an oil immersion objective (numerical aperture of 1.25) and blue-green light is approximately 0.2 m.

$$d = \frac{(0.5)(530\ \text{nm})}{1.25} = 212\ \text{nm or } 0.2\ \mu\text{m}$$

- At best, a bright-field microscope can distinguish between two dots around 0.2 m apart (the same size as a very small bacterium).